Prevention of Reperfusion Injury and Microcirculatory Failure in Macrosteatotic Mouse Liver by Omega-3 Fatty Acids

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Macrovesicular hepatic steatosis has a lower tolerance to reperfusion injury than microvesicular steatosis with an abnormally high ratio of omega-6 (n-6): omega-3 (n-3) polyunsaturated fatty acids (PUFAs). We investigated the influence of PUFAs on microcirculation in steatotic livers and the potential to minimize reperfusion injury in the macrosteatotic liver by normalization of PUFAs. *Ob/ob* mice were used as a model of macrovesicular hepatic steatosis and C57/Bl6 mice fed a choline-deficient diet for microvesicular steatosis. Steatotic and lean livers were subjected to 45 minutes of ischemia and 3 hours of reperfusion. Hepatic content of omega-3 and omega-6 PUFAs was determined. Microcirculation was investigated using intravital fluorescence microscopy. A second group of *ob/ob* mice was supplemented with dietary omega-3 PUFAs and compared with the control diet–fed group. Microcirculation, AST, and Kupffer cell activity were assessed. Macrosteatotic livers had significant microcirculatory dysfunction correlating with high omega-6: omega-3 PUFA ratio. Dietary omega-3 PUFA resulted in normalization of this ratio, reduction of intrahepatic lipids, and decrease in the extent of macrosteatosis. Defective microcirculation was dramatically ameliorated with significant reduction in Kupffer cell activity and protection against hepatocellular injury both before ischemia and after reperfusion. *Conclusion:* Macrosteatotic livers disclosed an abnormal omega-6: omega-3 PUFA ratio that correlates with a microcirculatory defect that enhanced reperfusion injury. Thus, protective strategies applied during or after ischemia are unlikely to be useful. Preoperative dietary omega-3 PUFAs protect macrosteatotic livers against reperfusion injury and might represent a valuable method to expand the live liver donor pool. (HEPATOLOGY 2007;45:855-863.)

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The shortage of quality donor organs and the increasing demand for orthotopic liver transplantation contribute to increased mortality of patients in need of an organ. This growing disparity is forcing transplant surgeons to consider steatotic livers, which represent one of the most common types of organs from "marginal donors."[1] In living-donor liver transplantation, donors with steatotic livers are more at risk for poor outcome. Because a larger portion of the liver mass is made up of fat, less functional liver tissue is available. The decision not to transplant a liver is frequently based on steatotic characteristics observed during procurement of cadaveric livers or during the preoperative evaluation of a potential living liver donor.[2]

Steatosis of the liver is common in Western countries, affecting approximately 30% of donors for liver transplantation and 20% of patients undergoing liver resection.[3] Qualitatively, fat deposits in hepatocytes can be classified as microvesicular or macrovesicular steatosis.[4] The histological lesion in microsteatosis consists of fatty microvesicles, measuring less than 1 μm, filling the hepatocyte cytoplasm, whereas the nucleus remains centrally located.[5] In contrast, in macrosteatosis, hepatocytes contain one single large vacuole of fat, which displaces the nucleus to the periphery of the cell.[4,6] High degrees of

Abbreviations: EPA, eicosapentaenoic acid; FSD, functional sinusoidal density; I/R, ischemia/reperfusion; PUFA, polyunsaturated fatty acids; SPR, sinusoidal perfusion rate.

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Table 1. PUFA Composition in Control and Fish Oil–Enriched Diet

PUFA	Control Diet*	Fish Oil–Enriched Diet*
EPA + DHA	0.00013	2.28
α-Linolenic acid	0.25	0.32
Linoleic acid	2.225	2.1
Total n-3 PUFAs	0.25	2.6
Total n-6 PUFAs	2.25	2.25

*Grams of fish oil per 100 g diet.

macrosteatosis are known risk factors of primary nonfunction; grafts with severe macrosteatosis are no longer recommended to be used,[7] whereas a safe use of microsteatotic organs has been reported.[8]

Essential fatty acids include two classes, n-3 and n-6 polyunsaturated fatty acids (PUFA). They are not interconvertible, are metabolically and functionally distinct, and often have important opposing physiological functions; therefore, their balance is essential for health.[9] In Western diets, the ratio of n-6 to n-3 PUFAs ranges from 20:1 to 30:1 instead of the presumably healthy range of 1:1 to 2:1. High intake of n-6 PUFAs shifts the physiologic state to one that is prothrombotic with increases in blood viscosity, vasospasm, and vasoconstriction. In contrast, n-3 PUFAs have antiinflammatory, antithrombotic, vasodilatory, and hypolipidemic properties,[10,11] and a negative regulatory influence on hepatic lipogenesis.[12]

We hypothesize that microcirculatory failure attributable to deranged PUFA contents is implicated in the reduced tolerance of macrosteatotic livers to reperfusion injury and that pretreatment with n-3 PUFAs improves the outcome after ischemia/reperfusion.

Materials and Methods

Diets. All diets were purchased from KLIBA, Kaiseraugust, Switzerland. The choline-deficient diet was supplied with methionine.[4] The 10% fish oil–enriched diet (Table 1) is a standard laboratory chow enriched with fish oil as a source of eicosapentaenoic acid (EPA) and docosapentaenoic acid (long chain n-3 PUFAs).[13]

Animal Model. All experiments were performed on male wild-type C57/BL6 and obese *ob/ob* C57/ BL6 mice (Harlan, Netherlands). Animals were purchased at the age of 6 to 8 weeks. Two groups of wild-type mice were fed either standard laboratory chow or choline-deficient diet for 6 weeks. Three groups of *ob/ob* mice were fed either standard laboratory chow for (6 and 12 weeks, respectively) or fish oil–enriched diet (for 12 weeks). The number of animals was 5 in each group, in all experimental procedures except for PUFA assay (described later). All animals were fed the respected diets and water *ad libitum* until use and were kept under constant environmental conditions with 12-hour light/dark cycles. All procedures were performed in accordance with the Zurich University Institutional Animal Care and were approved by the local animal ethics committee.

Study Design. Two models of hepatic steatosis (predominantly macrosteatosis in *ob/ob* mice and predominantly microsteatosis in C57/ BL6 mice fed a choline-deficient diet) have been compared with lean livers. Differences in liver weight, body weight, and intrahepatic lipid contents in addition to hepatocyte injury and microcirculation before ischemia and after reperfusion were studied. Livers were investigated for n-3 and n-6 PUFA contents.

In a second set of experiments, two groups of *ob/ob* mice were provided with either standard laboratory chow (control) or fish oil–enriched diet for 12 weeks. The impact of dietary n-3 PUFA supplementation on liver and body weight, hepatic content of PUFAs, pattern of steatosis, microcirculation, hepatocyte injury, and activity of Kupffer cells was investigated.

Surgical Procedure. A model of segmental (70%) hepatic ischemia was used. Briefly, mice were anesthetized by isoflurane inhalation (Pittman-Moore, Chicago, IL). After a midline laparotomy, all structures in the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes were clamped for 45 minutes. At the end of the ischemic period, the animal was reanesthetized, the abdomen was reopened, and the clamp was removed. Thirty minutes before the end of reperfusion, the animal was reanesthetized again then placed in supine position on a heating pad to maintain body temperature at 37°C. The midline laparotomy was reopened and transversely extended. The right internal iliac artery was exposed; then a polyethylene catheter (PE-10, ID 0.28 mm, Portex, Hythe, UK) was inserted for injection of fluorescent dyes and monitoring the mean arterial pressure (Hellige, Freiburg, Germany). After a tension-free exposure, the left hepatic lobe was carefully exposed and fixed in a warm saline solution bath under a cover glass slip for microscopy. After intravital fluorescence microscopy examination, blood samples were withdrawn from the inferior vena cava, then the left lobe was harvested, and enough tissue was obtained for either preservation in 10% formalin or immediate freezing in liquid nitrogen and storage at −80°C.

In Vivo *Fluorescence Microscopy and Analysis of Microcirculation.* An epi-illumination intravital microscope (Leica DM/LM; Leica Microsystems, Wetzlar, Germany) attached to a blue (excitation 450-490/emission >520 nm) and green (excitation 530-560/emission >580 nm) filter system was used. The microscopy se-

quences were captured by a television camera (intensified charge-coupled device camera, Kappa Messtechnik, Gleichen, Germany), displayed on a television screen (Trinitron PVM-20N5E; Sony, UK) and recorded on video (Panasonic AG 7350-SVHS; Tokyo, Japan) for subsequent off-line analysis. The preparation was observed visually with water immersion objective (×20/0.50W, Leica, Germany), which resulted in total optical magnification of 800× on the video monitor. Sinusoidal perfusion was evaluated under blue-filtered light after injection of soluble sodium fluorescein (2 μmol/kg intraarterially, Fluka Chemie, Switzerland). Ten randomly chosen nonoverlapping Rappaport acini were recorded for 30 seconds each. Sinusoidal perfusion rate (SPR) was determined by the ratio of perfused sinusoids to the total number of visible sinusoids. Functional sinusoidal density (FSD) was defined as the total length of all perfused sinusoids per observation area (cm/cm^2)[14] and assessed by a computer-assisted image analysis software system (CapImage; Zeintl Software, Heidelberg, Germany).

Phagocytic activity of Kupffer cells was investigated by measuring the uptake of plain fluorescent latex particles with a diameter of 1.0 μm (Polyscience Inc., Warrington, PA) at a dosage of 1.5×10^8/kg. Immediately after the injection of the particles, 10 to 15 high-power fields per minute were recorded under blue filtered light for a total of 5 minutes. Kupffer cell activity was then determined separately for each minute of observation by the number of particles moving as a percentage of all particles visible in the acini during the observation period.

Intrahepatic Lipid Assay. Lipid extraction and measurement was performed using the method described by Van Handel.[15]

Hepatic PUFA Assay. Determination of hepatic contents of PUFAs was performed by gas chromatography with a polar column.[16] Because lean and microsteatotic livers do not contain much fat, six livers were pooled from lean, five from mice fed a choline-deficient diet for the assay. In *ob/ob* mice, PUFAs were measured in three separate livers for each group. Each fatty acid is expressed as a percentage ratio of the sum of all fatty acids (total is 100%). The results are recalculated to indicate the relative fat content given as mg/g of mouse total hepatic fat.

Histological Pattern of Steatosis. Using hematoxylin-eosin–stained liver sections, 10 randomly chosen fields were investigated per slide. Each field was photographed; the cells were then counted using three categories: no fat (normal), many small fatty inclusions in the cytoplasm without displacement of the nucleus (microsteatosis), and one single vacuole of fat, which displaces the nucleus to the periphery of the cell (macrosteatosis).

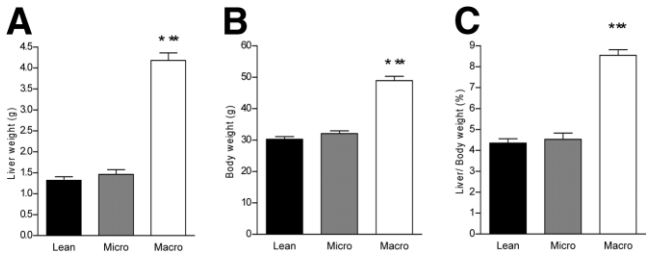

Fig. 1. *Ob/ob* mice (white bars) have significantly higher (A) liver weight, (B) body weight, and (C) liver/body weight ratio compared with lean mice (black bars) and mice with microsteatosis (gray bars). * and $**P < 0.001$ versus lean and microsteatosis, respectively.

Hepatocyte Injury. The degree of hepatic injury was assessed by serum levels of AST using a serum multiple analyzer (Johnson & Johnson, Ektachem DTSC II multianalyzer).

Statistical Analysis. Data were analyzed using GraphPad Prism, and values were expressed as means ± SD. Differences between groups in AST (first set), microcirculation, histological evaluation of macrosteatosis, and Kupffer cell activity were evaluated using the analysis of variance with Tukey's post hoc test. Data on AST, PUFA contents (second set), liver and body weight, and intrahepatic lipids were analyzed using unpaired *t* test. Differences were considered statistically significant with a *P* value of less than 0.05.

Results

Do Macro- and Microsteatosis Differ Regarding Liver or Body Weight?

Liver weight, body weight, and liver/body weight ratio were significantly higher in *ob/ob* mice (4.18 ± 0.18, 48.94 ± 1.37 g, and 8.54 ± 0.27%, respectively) compared with lean (1.32 ± 0.18, 30.26 ± 0.87 g, and 4.34 ± 0.21%, respectively) and choline-deficient diet–fed animals (1.46 ± 0.11, 32.06 ± 0.92 g, and 4.53 ± 0.30%, respectively) (Fig. 1A-C).

Do Livers with Macrosteatosis or Microsteatosis Have a Similar Tolerance to Ischemic Injury?

Before ischemia, we observed slightly higher AST levels in macrosteatotic livers (541.8 ± 171.7 U/l) compared to lean and microsteatotic livers (168.0 ± 43.8 and 200.0 ± 97.0 U/l, respectively). This difference, however, did not reach a statistical significance. After reperfusion, AST levels were significantly elevated in macrosteatotic (11,802 ± 4976 U/l) compared with lean and microsteatotic livers (3688 ± 1228 and 3788 ± 607.9 U/l, respectively) (Fig. 2).

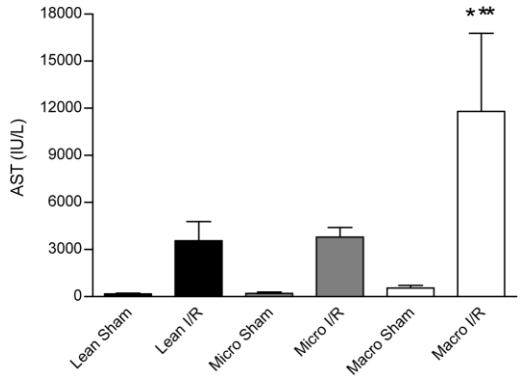

Fig. 2. No significant difference between lean (black bars), microsteatotic (gray bars), and macrosteatotic (white bars) livers in AST release after a sham operation. After 45 minutes of ischemia and 3 hours of reperfusion, AST release was significantly higher in macrosteatotic compared with lean and microsteatotic livers. * and $**P < 0.001$ versus lean and microsteatotic livers, respectively.

Do Macrosteatotic and Microsteatotic Livers Differ Regarding Sinusoidal Perfusion?

Although disturbances in microcirculation after reperfusion of ischemic fatty livers has been reported,[17,18] accurate assessment of microcirculation by intravital microscopy in models of predominant macrovesicular or microvesicular steatosis is not available. We used 2 parameters to assess sinusoidal perfusion: (1) the sinusoidal perfusion rate, which is determined by counting the number of perfused sinusoids and expressed as the percentage of total number of observed sinusoids; and (2) the functional sinusoidal density, which indicates the total length of perfused sinusoids per observation area (cm/cm^2). This is assessed by a computer-assisted image analysis. The latter parameter is preferable for the analysis of irregular arrangement of microvessels.[14]

SPR. After sham operation, SPR was comparable among lean (95.02 ± 2.33%), microsteatotic (94.38 ± 3.14%) and macrosteatotic livers (93.66 ± 3.27%). Ischemia/reperfusion (I/R) resulted in marked deterioration in SPR of macrosteatotic (38.10 ± 9.61%) compared with lean (75.12 ± 4.30%) and microsteatotic livers (75.08 ± 3.94%) (Fig. 3A).

FSD. A defective microcirculation was observed in macrosteatotic livers even before ischemia, as indicated by a significantly reduced FSD (328.0 ± 52.4 cm/cm^2) compared with lean (621.5 ± 58.5 cm/cm^2) and microsteatotic (608.5 ± 60.1 cm/cm^2) livers. After reperfusion, this impairment became more dramatic in macrosteatosis (184.4 ± 48.5 cm/cm^2) in comparison with lean livers (475.8 ± 35.28 cm/cm^2) and with those with microsteatosis (465.6 ± 69.66 cm/cm^2) (Fig. 3B,C).

Does Microcirculatory Failure in Macrosteatotic Livers Correlate with Abnormal n-6:n-3 PUFA Ratio and the Amount of Intrahepatic Lipids?

Whereas lean and microsteatotic livers had identical n-6:n-3 PUFA ratio of 4:1; macrosteatotic livers displayed a ratio of 9:1. Furthermore, macrosteatotic livers contained significantly more lipids (93 ± 20.70 μg/mg) compared with microsteatotic livers (34.53 ± 15.44 μg/mg), whereas fat was undetectable in lean livers (Table 2). Of note, the amount of both PUFA classes was reduced in fatty compared with lean livers. This could be explained by the depletion of PUFAs as a consequence of increased oxidative stress in fatty livers.[19] Together with the impaired sinusoidal perfusion, these results suggest that the pronounced microcirculatory failure in macrosteatotic

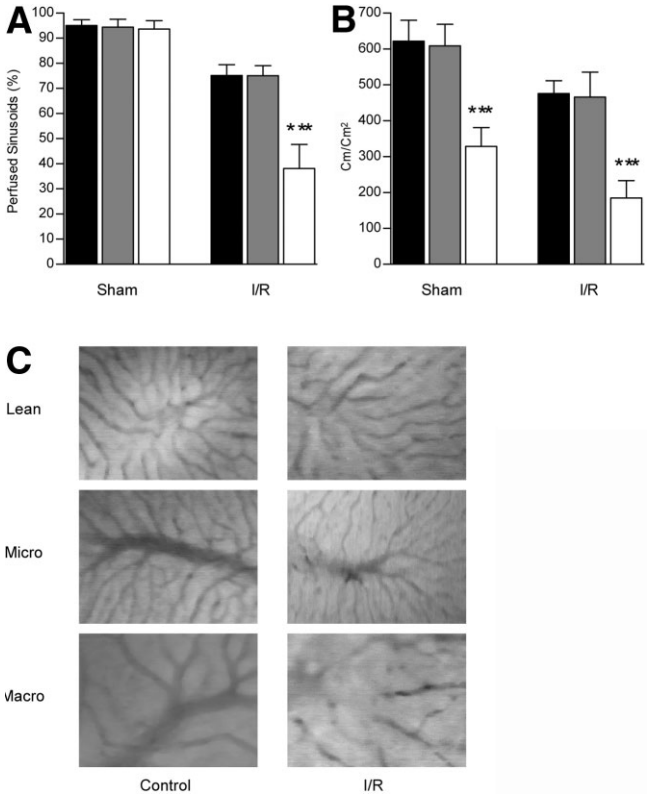

Fig. 3. (A) Sinusoidal perfusion rate: In the control group, there is no significant difference between macrosteatotic and both lean and microsteatotic livers. After I/R, macrosteatotic livers had a significantly reduced percentage of perfused sinusoids compared with lean and microsteatotic livers. (B) Functional sinusoidal density: In sham-operated animals, the total length of perfused sinusoids was significantly lower in macrosteatotic livers compared with lean and microsteatotic livers. I/R led to marked deterioration in livers with macrosteatosis in comparison with lean and microvesicular steatosis. Lean (black bars), microsteatotic (gray bars), and macrosteatotic (white bars) livers. * and $**P < 0.05$ versus lean and microsteatotic livers, respectively. (C) Representative intravital fluorescence microscopy images show markedly reduced functional sinusoidal density in macrosteatosis both in control livers (left panel) and after reperfusion (right panel). Original magnification ×800.

Table 2. Intrahepatic Lipid Content and PUFA Profile in Lean, Microsteatotic, and Macrosteatotic Livers

Liver Status	Intrahepatic Lipids*	Total n-6 PUFAs†	Total n-3 PUFAs†	n-6/n-3 PUFA Ratio
Lean	Undetectable	258.60	66.40	4:1
Microsteatosis	34.53 ± 15.44	126.70	32.30	4:1
Macrosteatosis	93 ± 20.70‡	74.07 ± 11.10	8.33 ± 5.14	9:1

*Microgram per milligram liver tissue.
†Milligram per gram liver fat.
‡$P < 0.05$ versus microsteatosis.

livers might be a consequence of abnormally high n-6:n-3 ratio.

Does Dietary Supplementation of ob/ob *Mice with n-3 PUFAs Influence Hepatic n-6:n-3 PUFA Ratio and Hepatic Lipid Contents?*

To test the hypothesis that impaired microvascular perfusion in macrosteatotic livers could be a result of abnormally high n-6:n-3 PUFA ratio; we tried to shift this ratio to normal by dietary n-3 PUFA supplementation. The dietary supplementation with n-3 PUFAs increased the hepatic contents of these fatty acids and normalized the n-6:n-3 ratio to 1.5:1. Further, dietary n-3 PUFAs significantly reduced the total amount of intrahepatic lipids to 67.9 ± 24.7 μg/mg in comparison with 161.4 ± 80.2 μg/mg in the control diet group (Table 3).

Do Dietary n-3 PUFAs Influence Liver Weight, Body Weight, or the Histological Pattern of Fat in ob/ob *Mice?*

Liver weight and liver/ body weight ratio were significantly reduced in n-3 PUFA–fed animals (4.64 ± 0.40 g and 6.99 ± 0.63 %, respectively) compared with controls (5.32 ± 0.19 g and 8.18 ± 0.45%, respectively). However, no significant difference could be detected in body weight (66.26 ± 1.98 and 64.94 ± 2.20 g, respectively) (Fig. 4). Additionally, macrosteatosis was significantly blunted in livers of n-3 PUFA–fed animals (26 ± 15 %) compared with the control group (49 ± 2%) (Fig. 5).

Does Normalization of Hepatic n-6:n-3 PUFA Ratio in ob/ob *Mice Confer Protection from Sinusoidal Perfusion Failure?*

Improvement of hepatic microcirculation was reported in fatty livers of rats supplemented with EPA.[20] However, the impact of n-3 PUFAs on I/R injury in fatty liver has not been reported. We observed that normalization of hepatic n-6:n-3 PUFA ratio correlated with amelioration of sinusoidal perfusion before ischemia and after reperfusion.

Sinusoidal Perfusion Rate. After a sham operation, both control and n-3 PUFA–fed mice showed comparable SPR (93.66 ± 3.27% and 95.40 ± 2.61%, respectively). I/R resulted in remarkable reduction in the fraction of perfused sinusoids in animals fed a control diet. In contrast, although reperfusion reduced the sinusoidal perfusion rate in n-3 PUFAs diet–fed group, the percentage of perfused sinusoids was significantly higher compared with the control diet (59.24 ± 15.63% and 32.76 ± 11.21, respectively) (Fig. 6A).

Functional Sinusoidal Density. Animals fed n-3 PUFAs had higher functional density of perfused sinusoids compared with control diet both before ischemia (471.6 ± 155.0 cm/cm^2 and 313.2 ± 50.0 cm/cm^2, respectively) and after reperfusion (309.7 ± 157.2 cm/cm^2 and 166 ± 37.3 cm/cm^2, respectively). These findings support our hypothesis that amelioration of baseline defective microcirculation is a valuable method to improve outcome after I/R in macrosteatotic livers (Fig. 6B,C).

Does Correction of Hepatic n-6:n-3 PUFA Ratio in ob/ob *Mouse Livers Protect from Hepatocyte Injury?*

Dietary provision with fish oil protects against hepatocyte injury in fatty livers.[12] However, no data are available on its influence on reperfusion injury. To further investigate the protective effects of n-3 PUFAs against ischemic injury observed during *in vivo* microscopy; we assessed AST levels. In n-3 PUFA–fed animals, AST release was significantly blunted compared with controls after sham operation (502.0 ± 64.2 and 872.0 ± 173.0 U/l, respec-

Table 3. Total Intrahepatic Lipid Content and PUFA Profile in *ob/ob* Mouse Livers, n-3 PUFA Versus Control Diet

Diet	Intrahepatic Lipids*	Total n-6 PUFAs†	Total n-3 PUFAs†	n-6/n-3 PUFA Ratio
Control diet	161.4 ± 80.15	65.30 ± 4.09	9.67 ± 1.00	7:1
N-3 PUFA diet	67.94 ± 24.73‡	67.53 ± 3.09	46.50 ± 1.75§	1.5: 1

*Microgram per milligram liver tissue.
†Milligram per gram liver fat. ‡$P < 0.05$ versus control diet.
§$P < 0.001$ versus control diet.

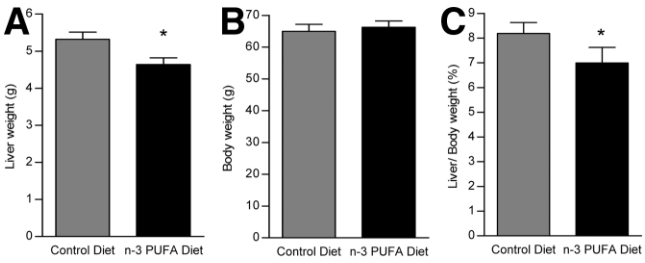

Fig. 4. In *ob/ob* mice, dietary supplementation with n-3 PUFAs (black bars) (A) significantly reduced liver weight, (B) did not affect body weight, and (C) significantly decreased liver/ body weight ratio compared to control diet (gray bars). $*P < 0.05$.

tively) and after reperfusion (1,652 ± 21 and 11,250 ± 21 U/l, respectively) (Fig. 7).

Does the Amelioration of Hepatocyte Injury by n-3 PUFAs Modulate Phagocytic Activity of Kupffer Cells?

Owing to their role in production of injurious eicosanoids that aggravate the outcome after hepatic I/R,[21] we assessed the influence of n-3 PUFA supplementation on Kupffer cell activation. Determination of kinetics of fluorescence-labeled latex particles revealed dampened activity of Kupffer cells in the n-3 PUFAs group compared with control diet. In sham-operated animals, the percentage of free latex particles was significantly higher in n-3 PUFAs compared with control diet. Despite the enhancement of Kupffer cell activity after I/R, the n-3 PUFA–fed mice disclosed significantly more free latex particles, indicating reduced Kupffer cell activity compared with control diet (Fig. 8).

Discussion

Using 2 models of predominantly hepatic macrovesicular and microvesicular steatosis,[4] we show significantly greater microcirculatory defects in the macrosteatotic liver, a finding that is exacerbated by I/R. Furthermore, microcirculatory impairment and hepatocyte injury in the macrosteatotic liver were associated with reduced hepatic n-3 PUFA content. We also demonstrate that dietary supplementation with n-3 PU-

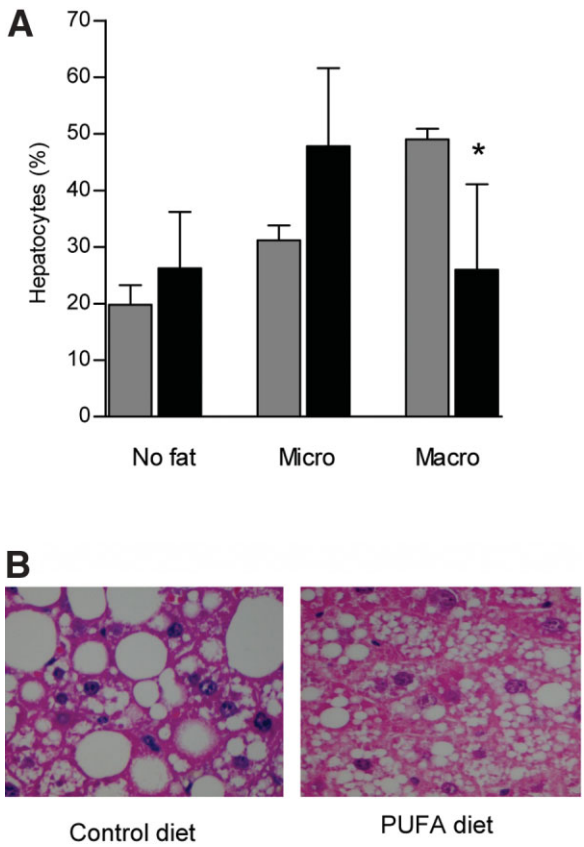

Fig. 5. (A) Extent of microsteatosis and macrosteatosis in hepatocytes in *ob/ob* mice fed n-3 PUFAs versus control diet: Percentage of hepatocytes disclosing macrosteatosis was significantly lower in *ob/ob* mice fed n-3 PUFAs (black bars) compared with control diet (gray bars). $*P < 0.05$. (B) Hematoxylin-eosin–stained sections of *ob/ob* mouse livers show a reduction in macrosteatotic hepatocytes in the n-3 PUFA group. Original magnification ×400.

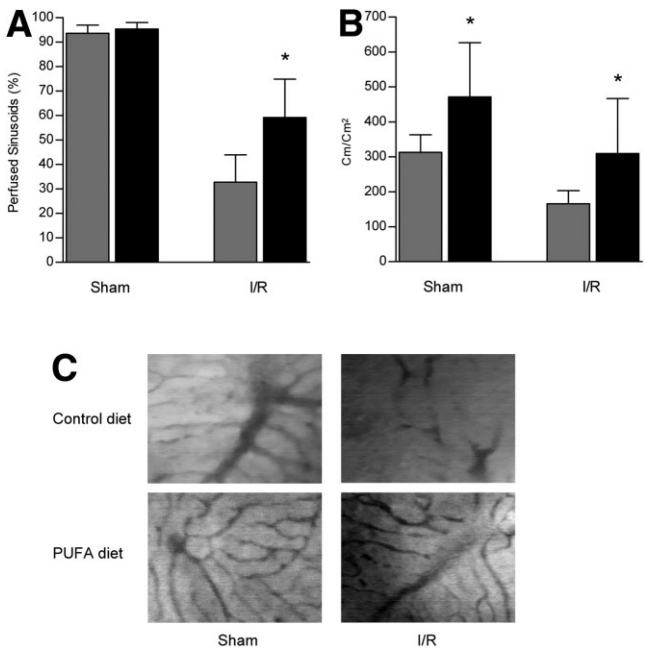

Fig. 6. (A) Sinusoidal perfusion rate: No significant difference was seen between *ob/ob* mice fed n-3 PUFA or those fed a control diet after a sham operation. After I/R, n-3 PUFA supplementation resulted in significantly higher percentage of perfused sinusoids. (B) Functional sinusoidal density: A significantly higher density before ischemia with n-3 PUFA compared with control diet is observed. Dietary n-3 PUFA resulted in significantly less deterioration in functional sinusoidal density after I/R in comparison with the control diet. Control diet (gray bars), n-3 PUFA diet (black bars). $*P < 0.001$. (C) Representative intravital fluorescence microscopy images of the liver displaying dramatically higher functional sinusoidal density with n-3 PUFA (lower panel) compared with control group (upper panel). Original magnification ×800.

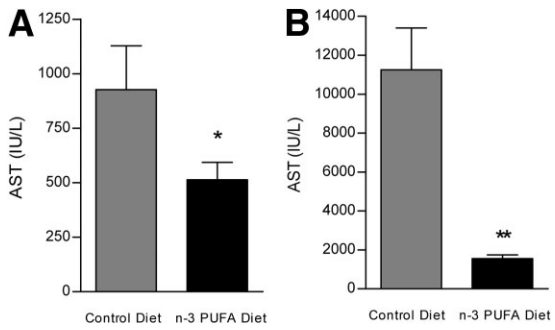

Fig. 7. AST release was significantly reduced in *ob/ob* mice fed n-3 PUFA (black bars) in comparison with those fed the control diet (gray bars) after sham operation (A) or I/R (B). $*P < 0.05$ and $**P < 0.001$.

FAs decreases the vulnerability of the macrosteatotic liver to I/R injury by improving microcirculation and that n-3 PUFAs might emerge as a useful strategy to expand the donor pool.

Impaired microcirculation has been proposed to explain the increased susceptibility of fatty organs to ischemic injury.[17,18] So far, an *in vivo* description of microcirculation changes in models of hepatic microsteatosis and macrosteatosis has not been reported. We found that macrosteatotic livers disclosed impaired sinusoidal perfusion before ischemia and after reperfusion. Evaluation of microvascular perfusion was carried out by determination of SPR and FSD. We found that SPR might be less reliable because it expresses only the percentage of perfused to all visible sinusoids without reference to the surface area served by these sinusoids. To better assess the actual density, we used a computer-assisted analysis to determine the FSD.[14] This parameter convincingly showed that the total length of perfused sinusoids for the same surface area was significantly reduced in macrosteatotic compared with lean and microsteatotic livers both before ischemia and after reperfusion. These findings demonstrated a functional defect in microvascular perfusion in *ob/ob* mice, which might contribute to the decreased tolerance of macrosteatotic livers to reperfusion injury.

Hepatic microsteatosis and macrosteatosis are usually understood as distinct entities; however, this assumption might not be true. Fatty changes refer to abnormal accumulation of fat within parenchymal cells. At the outset, lipid accumulates in a microvesicular form within the cytoplasm of liver cells; with further accumulation the small vacuoles coalesce, creating large clear vesicles.[22] Microscopic observations in 83 donor livers disclosed two types of steatotic patterns, a high-grade microsteatosis and combined macromicrosteatosis with no pure macrosteatosis.[23] Increased fat droplet size in fatty livers is related to old age,[7] increased body weight,[24] and increased total amount of lipids in hepatocytes.[25] Microsteatosis was originally described in association with conditions such as acute fatty liver of pregnancy, Reye's syndrome, and valproate toxicity. Microsteatosis has been described in a wide variety of conditions such as alcoholism, toxicity of several medications, and delta hepatitis. Therefore, maintaining the concept of microsteatosis as a unique entity is no longer justified.[5] Macrosteatosis is a feature of nonalcoholic fatty liver disease that is characterized by accumulation of triglycerides in the liver,[26] depletion in n-3 long chain PUFAs and enhancement of n-6:n-3 PUFA ratio. This is associated with changes in gene expression, with decreased fatty acid oxidation and triacylglycerol export, and enhanced lipid synthesis, leading to fat accumulation in the liver.[19,27] We believe that macrosteatosis (as a feature of non-alcoholic fatty liver disease) results, at least partially, from abnormal hepatic lipid metabolism caused by imbalanced dietary PUFAs. Inadequate dietary intake of α-linolenic compared with lenoleic acid (the parent n-3 and n-6 PUFAs, respectively) leads to defective elongation and desaturation of α-linolenic acid because both compete for the enzymes responsible for their elongation and desaturation, leading to decreased production of α-linolenic acid long-chain derivatives (EPA and docosapentaenoic acid).[11] Long-chain n-3 PUFAs upregulate peroxisomal proliferator-activated receptor-α, which increases transcription of genes responsible for fatty acid degradation, such as mitochondrial carnitine palmitoyl transferase 1 and peroxisomal acyl-CoA oxidase. Conversely, n-3 PUFAs downregulate steroid-responsive element binding protein 1-c, which increases transcription of genes responsible for fatty acid synthesis such as fatty

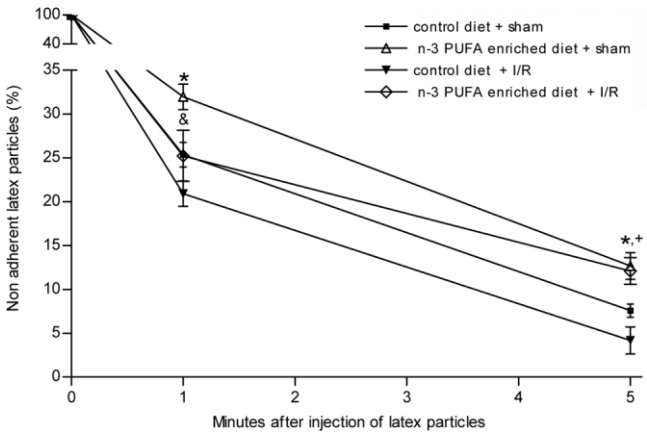

Fig. 8. The percentage of free latex particles is higher in animals fed n-3 PUFA versus those fed the control diet after sham operation or I/R, indicating n-3 PUFA has dampened the phagocytic activity of Kupffer cells. $*P < 0.05$ compared with control diet after sham operation; + indicates $P < 0.05$; & indicates $P < 0.001$ compared with control diet after I/R.

acid synthase and stearoyl Co-A desaturase.[28] Dietary provision of n-3 PUFAs via fish oil modulates the fatty acid composition of cell membrane phospholipids[29] and reduces intrahepatic lipid accumulation as well as the percentage of big fat droplets and therefore improves microcirculation.[20] In *ob/ob* mice, n-3 PUFA supplementation resulted in conversion of hepatic macrosteatosis to microsteatosis with a change of n-6:n-3 PUFA ratio to approach that of lean livers.[30] In humans, liver biopsies from diabetic patients showed a clear inverse relationship between the size of fat droplets and the hepatic content of EPA.[31]

In this study, lean and microsteatotic livers disclosed an almost identical n-6:n-3 PUFA ratio of 4:1, whereas macrosteatotic livers had a higher ratio of 9:1. These findings further support our assumption that microsteatosis might be simply a stage in the development of macrosteatosis and that enhanced reperfusion injury is, at least partially, influenced by the essential fatty acid contents rather than the size of fat droplets.

Fish oil has been reported to reduce hepatic reperfusion injury in low-flow, reflow perfusion model in rats by minimizing the rise of portal pressure and improving microcirculation.[32] Juniper berry oil, which is rich in 5,11,14-eicosatrienoic acid, a polyunsaturated fatty acid similar to one found in fish oil, ameliorated reperfusion injury in rat liver.[29] In contrast, failure of dietary provision with fish oil to attenuate warm I/R injury in rat liver also has been reported.[33]

We found remarkable amelioration of microcirculation associated with significantly lower AST release both before ischemia and after reperfusion in the n-3 PUFA–supplemented mice. Previous studies without I/R have indicated improvement of hepatic microcirculation after dietary provision with EPA.[20]

Kupffer cells are activated by reperfusion after hypoxia and produce 70% to 80% of the eicosanoids from arachidonic acid in the liver.[29] Dietary n-3 PUFAs reduce the activation of Kupffer cells in rats exposed to *Escherichia coli* lipopolysaccharide.[13] We observed an increase in the percentage of free latex particles in mice supplemented with n-3 PUFAs compared with controls before ischemia and after reperfusion, indicating a reduction in phagocytic activity of Kupffer cells.

Our study represents a successful intervention against hepatic macrosteatosis that might yield remarkable clinical relevance. The n-3 PUFAs might be a simple, inexpensive, and safe preoperative regimen that opens the potential for rendering macrosteatotic livers acceptable for transplantation and, therefore, expanding the donor pool from marginal donors.[34] This strategy also may be used before liver resection and thereby decrease the risk of surgery.

In conclusion, we demonstrated that a preexisting microcirculatory defect related to abnormal n-6:n-3 PUFA ratio enhances reperfusion injury in macrosteatotic compared with lean and microsteatotic livers. The study also introduces dietary provision with n-3 PUFA as a novel protective strategy in liver resection and transplantation.

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