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Pretreatment with Intravenous Fish Oil Reduces Hepatic Ischemia Reperfusion Injury in a Murine Model

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Abstract

Background—Ischemia reperfusion injury (IRI) is a barrier to liver surgery and transplantation, particularly for steatotic livers. The purpose of this study was to determine if pretreatment with a single dose of intravenous fish oil (IVFO) decreases hepatic IRI and improves recovery of injured livers.

Methods—Sixty adult male C57BL/6 mice received 1 g/kg IVFO (Omegaven®, Fresenius Kabi) or isovolumetric 0.9% NaCl (aline) via tail vein 1 hour prior to 30 minutes of 70% hepatic ischemia. Animals were sacrificed 4-, 8-, or 24-hours post-reperfusion and livers harvested for histologic analysis.

Results—Four hours post-reperfusion, saline-treated livers demonstrated marked ischemia diffusely around the central veins, while IVFO-treated livers demonstrated only patchy necrosis with intervening normal parenchyma. Eight hours post-reperfusion, all livers demonstrated pale areas of cell loss with surrounding regenerating hepatocytes. Ki67 staining confirmed 14.4/10 HPF (95% CI 3.2, 25.6) more regenerating hepatocytes around areas of necrosis in IVFO-treated livers. Twenty-four hours post-reperfusion, all livers demonstrated patchy areas of necrosis, with an 89% (95% CI 85, 92) decrease in the area of necrosis in IVFO-treated livers.

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Potential Conflicts of Interest: A license agreement for the use of Omegaven has been signed by Boston Children's Hospital and Fresenius Kabi, and a patent has been issued to Boston Children's Hospital on behalf of Drs. Gura and Puder. Drs. Gura and Puder serve as consultants for Pronova-BASF and Sancilio and Company. Dr. Gura is also a member of the B Braun Medical Inc. advisory board. The remaining authors report no biomedical financial interests or potential conflict of interests.

Conclusions—IVFO-treatment prior to hepatic IRI decreased ed the area of hepatic necrosis and increased hepatocyte regeneration compared to saline-treatment in a mouse model.

TOC image

IVFO before IRI increases hepatocyte regeneration and decreases necrosis in murine livers. The importance of this report is that decreasing injury and accelerating regeneration after liver surgery and transplantation could improve patient outcomes.

Introduction

Hepatic ischemia reperfusion injury (IRI) can occur after liver transplantation or major hepatic resection when the portal triad is occluded temporarily to limit blood loss. IRI is particularly problematic in steatotic livers, limiting their use as donor organs^{1,2}. Clinical evidence of IRI includes remnant liver dysfunction after resection or donation and early allograft dysfunction or primary nonfunction after liver transplantation. Primary nonfunction necessitates urgent re-transplantation in up to 10% of patients, and early allograft dysfunction which occurs in up to 25% of patients is associated with an increased incidence of rejection^{3–6}.

Fish oil lipid emulsion rich in anti-inflammatory omega-3 fatty acids (O3FA) and the antioxidant alpha-tocophero, may play a role in preventing or ameliorating IRI^{7,8}. Intravenous fish oil (IVFO) decreases inflammatory and oxidative insults in intestinal failure-associated liver disease, and long-term oral fish oil supplementation may decrease steatosis in nonalcoholic fatty liver disease^{9–13}. The role of IVFO in decreasing IRI when given before liver surgery and transplantation, however, is unknown. The purpose of this study was to determine if pretreatment with a single dose of intravenous fish oil (IVFO) can decrease hepatic IRI and improve recovery of the injured livers in a murine model.

Methods

Animal Model and Surgery

Experimental protocols were approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital. Only male mice were used to eliminate sex as a potentially confounding variable. Sixty, 6–8 week old, C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) received 1 g/kg of IVFO (n=30; Omegaven®; Fresenius Kabi, Bad Homburg, Germany) or isovolumetric 0.9% NaCl (saline) (n=30) via tail vein injection. The IVFO dose of 1 g/kg was chosen after a lesser dose (0.2 g/kg) was found to be ineffective, this dose (ASK THE AUTHORS TO EXPLAIN WHICH DOSE THE WORDS “thi dose” REFERS TO) has been used clinically for over a decade in children with a well demonstrated safety profile¹⁰. One hour later, mice were anesthetized with inhaled isoflurane for IRI procedure. After upper midline laparotomy, the portal triads to the cephalad liver lobes were dissected free to allow for suture ligation/occlusion with a 4-0 PDS (Ethicon, Somerville, NJ) weighted with a straight snap placed at a 45° angle to the mouse's long axis. Ischemia was confirmed visually as the cephalad lobes promptly turned grey. After 30 min of ischemia, suture ligatures were removed, reperfusion was confirmed visually as the previously grey lobes became red, and the abdomens were closed. The ischemia time of 30 min was chosen

after a greater time (60 min) was found to produce near-complete necrosis with no viable liver to salvage.

After 4 (n=20), 8 (n=20), or 24 (n=20) h of reperfusion, mice were euthanized by carbon dioxide inhalation. Blood was collected immediately from the inferior vena cava, and livers, spleens, and right kidneys were procured.

Serum Analysis

Whole blood was centrifuged at $2348 \times g$ at 4°C for 18 min. Serum was aspirated into a new tube and stored at -80°C . Serum levels of alanine transaminase (ALT) were measured by the Boston Children's Hospital clinical laboratory.

Liver Analysis

Livers were weighed and then placed in anatomic position for isolation and orientation of the cephalad lobes which had been exposed to IRI. In order to prevent potential bias from choosing liver segments for histologic analysis based on gross appearance, specimen collection was standardized. The lateral half of the left lateral lobe (cut diagonally) and inferior half of the right medial lobe (cut transversely) were marked medially with yellow pathology paint and inferiorly with black paint (Davidson Marking System; Bradley Products, Bloomington, MN), fixed in 10% formalin, and paraffin-embedded¹⁴.

Formalin-fixed, paraffin-embedded liver specimens were stained with hematoxylin and eosin (H&E) by the clinical pathology department for cross-sectional microscopic analysis of hepatic architecture by a blinded, board-certified pathologist. Livers procured 8 h after reperfusion were also stained with Ki67 in order to quantify proliferating hepatocytes. Starting at a distance of one high-powered field (HPF, $400\times$ magnification) from the edge of the specimen, Ki67-stained hepatocytes were counted in a blinded fashion across each specimen for every other row and reported as number of Ki67-positive hepatocytes per 10 HPFs. H&E slides for livers procured 24 h after reperfusion were assessed in a blinded fashion to determine the percent of necrosis per area using ImageJ software (NIH, Bethesda, MD). Percent necrosis was calculated for each lobe for consecutive fields at $100\times$ magnification across the middle of the specimen starting at the yellow painted edge and for the lobe as a whole at $20\times$ magnification.

Statistical Analysis

Pre-operative weights, organ weights as a percentage of pre-euthanasia body weights, and serum ALT among the 6 groups were analyzed using two-way analysis of variance (ANOVA) with factors for time of sacrifice (4, 8, or 24 h post-reperfusion), treatment group (saline or IVFO), and their interaction. Percent change in weight from pre-operative weight to pre-euthanasia weight was analyzed using analysis of covariance (ANCOVA), where outcome was regressed on the time of sacrifice, treatment group, and their interaction, and adjusted for pre-operative weight. The comparison of group means at 4, 8, and 24 h of reperfusion were adjusted for multiple comparisons using the method of Holm¹⁵.

Ki67 (Ki67-positive hepatocytes/10 HPF) and necrosis (percent of necrosis per area, both as a line across and for lobe as a whole) were analyzed at 8 and 24 h post-reperfusion, respectively, with a repeated-measures mixed model ANOVA. A compound-symmetric covariance matrix was used to model the within-mouse correlation between right- and left-lobes.

ALT, Ki67, and necrosis were right-skewed. ALT and necrosis were log-normal, while Ki67 required transformation to normal scores calculated from the ranked data as $y_i = \Phi^{-1}((r_i - 3/8)/(n+1/4))$, where Φ^{-1} is the inverse cumulative normal function, r_i is the rank of the i^{th} observation, and n is the number of observations¹⁶. Results for ALT and necrosis were back-transformed to their original scales by exponentiation. Mixed model results for Ki67 normal scores were consistent with analysis on the original scale, and only the latter are reported. All results are reported as mean with 95% confidence interval (CI). Tests of significance are two-sided with $P < 0.05$ considered statistically significant. Data were analyzed using SAS® version 9.4 (Cary, NC), and GraphPad Prism 7.0 (La Jolla, CA).

Results

Animal and Organ Weights

There were no differences in pre-operative body weight among the 6 groups ($n=10/\text{group}$, $P=0.53$, data not shown). There were, however, differences in percent change in body weight from pre-operative to pre-euthanasia weight among the 6 groups ($P=0.03$). At 4 hours after reperfusion, IVFO-treated mice experienced a mean decrease of -0.9% (95% CI $-2.3, 0.6$) body weight, while saline-treated mice experienced a mean increase of 1.6% (95% CI $0.1, 3.1$) body weight ($P=0.04$). At 8 hours after reperfusion, IVFO-treated mice lost more weight on average than saline-treated mice (IVFO -4.9% (95% CI $-6.4, -3.4$) vs. saline -1.7% (95% CI $-3.2, -0.2$), $P=0.01$). At 24 hours after reperfusion, there was no difference in weight loss between mice who received IVFO and those who received saline ($P=0.54$), with mice in both groups experiencing an average decrease of -6.7% (95% CI $-7.7, -5.7$) from their pre-operative weight. There were no differences in liver, right kidney, or spleen weights as a percentage of pre-euthanasia body weights among the 6 groups (Figure 1; $P_{\text{liver}}=0.58$, $P_{\text{kidney}}=0.11$, $P_{\text{spleen}}=0.12$).

Serum ALT

At 4 hours after reperfusion, there was no difference in serum ALT between mice who received saline and those who received IVFO prior to IRI (Figure 2). At 8 hours after reperfusion, IVFO-treated mice had lesser values of serum ALT on average than saline-treated mice (IVFO 1806 IU/L (95% CI 1398, 2335) vs. saline 5451 IU/L (95% CI 4218, 7045); $P=0.01$). There was no statistical difference in serum ALT between IVFO- and saline-treated mice at 24 hours after reperfusion.

Histologic Analysis

Livers from saline-treated mice killed 4 hours after reperfusion demonstrated marked ischemia diffusely around the central veins with some scattered cells and areas of necrosis consistent with early ischemia (Figure 3). Livers from IVFO-treated mice killed 4 hours

after reperfusion demonstrated small scattered foci of necrosis that were not limited to the areas surrounding central veins, with normal liver in between areas of necrosis. Some regenerating hepatocytes were also seen.

After 8 hours of reperfusion, both saline- and IVFO-treated livers demonstrated pale areas of cell loss with surrounding regenerating hepatocytes. It appeared there were more regenerating cells around areas of necrosis in IVFO-treated livers compared to saline-treated mice which was confirmed with Ki67 staining (Figure 4). There were on average 22.7 Ki67-positive hepatocytes per 10 HPF (95% CI 14.8, 30.7) in IVFO-treated livers compared to 8.4/10 HPF (95% CI 0.4, 16.3) in saline-treated livers (n=10/group; $P=0.01$).

After 24 hours of reperfusion, both saline- and IVFO-treated livers demonstrated patchy areas of frank necrosis and normal appearing liver without preference for central vein or periportal space. There was significantly less necrosis in the livers of mice who received IVFO prior to IRI compared to mice who received saline (Figure 5). Calculated for consecutive fields across the middle of the specimens, there was on average 1.3% (95% CI 1.0, 1.6) necrosis in IVFO-treated livers compared to 9.6% (95% CI 7.7, 11.9) necrosis in saline-treated livers (n=10/group; $P<0.0001$). Calculated for the entire specimens, there was on average 0.8% (95% CI 0.6, 1.0) necrosis in IVFO-treated livers compared to 7.8% (95% CI 6.2, 9.8) necrosis in saline-treated livers (n=10/group; $P<0.0001$).

Discussion

Despite major improvements in liver surgery and transplantation and in pre- and post-operative care, hepatic IRI remains an important complication. Steatotic livers are particularly sensitive to IRI, which increases morbidity and mortality after major hepatic resections and limits their use as donor organs^{1,2,17}. Our study demonstrates that pretreatment with a single dose of IVFO increased hepatocyte proliferation and decreased the extent of necrosis in a murine model.

Our results are consistent with the results of Raptis et al., who reported decreased necrosis at 24 hours post-IRI in mice pretreated with IVFO⁷. These findings also support the work of Linecker et al., who showed increased Ki67 positivity after major hepatectomy in steatotic mice pre-treated with IVFO⁸. Our results differ from these prior studies in that the present study describes a temporal progression of histologic findings suggesting that in IVFO-treated mice, injury still occurs but is associated with increased regeneration and decreased necrosis over time.

The underlying mechanism for the observed decrease in hepatic injury and increased cellular regeneration remains unclear, and further mechanistic explanations are pending further experimentation. The O3FA eicosapentaenoic acid and docosahexaenoic acid are precursors to anti-inflammatory prostaglandins and leukotrienes and to specialized pro-resolving mediators¹⁸. O3FA agonism of Free Fatty Acid Receptor 4 (also known as GPR120) in Kupffer cells suppresses NF κ B-mediated inflammatory pathways¹⁹. Given this knowledge, it is possible that IVFO accelerates recovery from hepatic IRI through attenuated release of inflammatory mediators⁷. O3FA-derived, pro-resolving mediators may also accelerate the

transition from injury to repair, allowing cells not directly injured to return to normal function sooner, and cells around injured cells to begin the recovery process sooner. A single bolus of IVFO given 1 hour before ischemia is unlikely to change cellular membrane composition. One study by Kasuga et al., however, demonstrated that circulating O3FA as opposed to cell membrane released O3FA accumulate quickly in inflammatory exudates²⁰. They further demonstrated that administration of the O3FA-derived resolvin RvD1 was able to decrease pulmonary neutrophil infiltration just 2 hours after hind limb IRI.

While it would be difficult to prove the difference between the effects of the anti-inflammatory and pro-resolving pathways, because they are undoubtedly intertwined, there are important clinical implications to these findings. It is not always possible to prevent injury; but if liver healing and regeneration can be accelerated, it may be possible to improve patient outcomes. IVFO could be administered before or during liver surgery when other pre-emptive measures to decrease IRI (such as dietary changes or oral O3FA supplementation to decrease steatosis) or to increase healthy remnant liver (such as portal vein embolization) are not feasible. That IVFO accelerates regeneration after IRI may help explain the findings of Zhu et al. and Zhang et al., who reported improved outcomes in patients receiving O3FA-enriched parenteral nutrition after liver transplantation and hepatic resection, respectively^{21,22}.

A limitation of this study is the attribution of the hepatoprotective effects of IVFO to O3FA rather than to the anti-oxidant alpha-tocopherol. The data on whether alpha-tocopherol is able to decrease hepatic IRI is somewhat conflicting^{7,23}, but the potential synergistic effect between alpha-tocopherol and O3FA cannot be overlooked. The production of IVFO requires alpha-tocopherol, and therefore, lipid emulsions with added alpha-tocopherol would be needed to discriminate with any confidence the effects of these two components. In addition, the effect of dose-response and duration of ischemia were not fully explored in this study. The IVFO dose of 1 g/kg was chosen, because this is used clinically in parenteral nutrition-dependent children receiving IVFO for treatment of intestinal failure-associated liver disease, however, a greater dose may have a more profound effect, and dose-response studies with associated toxicity analysis and testing of coagulation parameters will be necessary before any clinical use can be considered.

In summary, this study demonstrates that a single dose of 1 g/kg IVFO given before hepatic IRI results in increased hepatocyte regeneration and decreased necrosis in murine livers. Given the safety profile of IVFO, these findings have the potential to be translated into preventative and treatment strategies for IRI in liver surgery and transplantation. Further studies are needed to determine the mechanism of this protection.

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Abbreviations

ALT	alanine transaminase
ANCOVA	analysis of covariance
ANOVA	analysis of variance
CI	confidence interval
H&E	hematoxylin and eosin
HPF	high-powered field
IRI	ischemia reperfusion injury
IVFO	intravenous fish oil
O3FA	omega-3 fatty acids

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Figure 1.

(A) Liver, (B) right kidney, and (C) spleen weight as a percentage of pre-euthanasia body weight for mice treated with 0.9% NaCl (saline) or IVFO prior to IRI and sacrificed 4, 8, or 24 h after reperfusion (n=10/group). There were no differences among the groups (Liver $P=0.58$, Kidney $P=0.11$, Spleen $P=0.12$). Shown are mean (95% CI). P values are from ANOVA.

Figure 2. Serum ALT for mice treated with saline or IVFO prior to IRI and sacrificed 4, 8, or 24 h after reperfusion (n=10/group). Shown are mean (95% CI). *P* values are from analysis of variance, adjusted for multiple comparisons.

Figure 3.
Representative H&E images of livers after IRI at 200× magnification.

Figure 4.

Quantification of proliferating hepatocytes by Ki67 staining of livers of mice treated with saline or IVFO prior to IRI and sacrificed 8 h after reperfusion. Shown are mean (95% CI). P value is from a repeated-measures mixed model ANOVA.

Figure 5.

Percent area of necrosis in livers of mice treated with saline or IVFO prior to IRI and sacrificed 24 h after reperfusion, calculated for consecutive fields across the middle of the specimen at 100× magnification (line across) and for the entire specimen at 20× magnification (whole specimen). Shown are mean (95% CI) for the right- and left-lobes by group. *P* values are from a repeated-measures mixed model ANOVA.