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Omega-3 Fatty Acid Supplementation Prevents Hepatic Steatosis in a Murine Model of Nonalcoholic Fatty Liver Disease

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ABSTRACT

Prolonged use of total parenteral nutrition can lead to nonalcoholic fatty liver disease, ranging from hepatic steatosis to cirrhosis and liver failure. It has been demonstrated that omega-3 fatty acids are negative regulators of hepatic lipogenesis and that they can also modulate the inflammatory response in mice. Furthermore, they may attenuate hepatic steatosis even in leptin-deficient *ob/ob* mice. We hypothesized that omega-3 fatty acid supplementation may protect the liver against hepatic steatosis in a murine model of parenteral nutrition in which all animals develop steatosis and liver enzyme disturbances. For testing this hypothesis, groups of mice received a fat-free, high-carbohydrate liquid diet *ad libitum* for 19 d with enteral or i.v. supplementation of an omega-3 fatty acid emulsion or a standard i.v. lipid emulsion. Control mice received food alone or the fat-free, high-carbohydrate diet without lipid supplementation. Mice that received the fat-free, high-carbohydrate diet only or supplemented with a standard i.v. lipid emulsion developed severe liver damage as determined by histology and magnetic resonance spectroscopy as well as elevation of serum liver function tests. Animals that received an i.v. omega-3 fatty acid emulsion, however, showed only mild deposits of fat in the liver, whereas enteral omega-3 fatty acids prevented hepatic pathology and led to normalization of liver function tests. In conclusion, whereas standard i.v. lipid emulsions fail to improve dietary-induced steatotic injury to the liver, i.v. supplementation of omega-3 fatty acids partially and enteral supplementation completely protects the liver against such injury. **(*Pediatr Res* 57: 445–452, 2005)**

Abbreviations

HCD, fat free, high carbohydrate diet
MRS, magnetic resonance spectroscopy
O3FA, omega-3 fatty acid

Nonalcoholic fatty liver disease consists of a variety of pathologic states ranging from the simple buildup of fat in the liver (hepatic steatosis) to nonalcoholic steatohepatitis, cirrhosis, and ultimately liver failure (1–4). Nonalcoholic fatty liver disease is being increasingly appreciated as a major cause of liver-related morbidity and mortality. A recent survey indicated that nonalcoholic fatty liver disease may account for ~80% of individuals with elevated serum liver enzymes (5) and further that up to 30% of the Western population may have nonalcoholic fatty liver disease (6). Furthermore, nonalcoholic fatty liver disease is associated with metabolic disorders such as obesity (7–9) and diabetes (10), as well as with prolonged chemotherapy (11,12) and total parenteral nutrition (13–17). In fact, liver injury related to total parenteral nutrition is one of the major causes of liver injury in premature children (18,19).

Several hypotheses have been proposed to explain the pathogenesis of nonalcoholic fatty liver disease, although none have been conclusive. The most accepted theory is the "two hit" hypothesis, in which the first hit involves the development of hepatic steatosis, rendering the liver more susceptible to a second, as yet undefined, hit, resulting in more severe liver damage. The development of hepatic steatosis results from an imbalance in the rates of entry, synthesis, or clearance of fat from the liver (20). More specific, the hepatic uptake of fatty acids and triglycerides and their rates of synthesis, the secretion of these compounds *via* plasma or bile, or the hydrolysis of triglycerides or oxidation of fatty acids may be altered (21). These mechanisms may also be important in the development of hepatic steatosis as a result of the prolonged use of total

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parenteral nutrition, as some investigators have proposed that deficiencies or toxicities of the parenteral nutrition solution may lead to hepatic injury (22–24).

Since the mid-1980s, a renewed interest in omega-3 fatty acids (O3FAs) led to consideration of their role in the cause and management of diverse diseases such as psychiatric (25–27) and cardiovascular disorders (28,29), inflammatory bowel disease (30), and cystic fibrosis (31). The O3FAs, present in fish oil, interfere with the arachidonic acid pathway of inflammation (32) and can also modulate the response of macrophages to endotoxin by inhibition of TNF-α production *in vitro* (33,34). Dietary O3FAs can decrease IL-6 and other proinflammatory cytokine levels as well, both *in vitro* and *in vivo* (35–37). Furthermore, O3FAs have been shown to accelerate the clearance of chylomicron triglycerides in humans, thus effectively reducing triacylglycerol concentrations in serum *in vivo* (38). Moreover, it has been demonstrated that i.v. administration of fish oil reduces parenteral nutrition–induced cholestasis in newborn piglets (39) and rats (40,41) and that dietary omega-3 and omega-6 polyunsaturated fatty acids can regulate hepatic lipogenesis by reducing sterol regulatory element-binding protein-1 in the liver (42). Finally, a recent clinical study that compared 19 patients who had nonalcoholic fatty liver disease with 11 normal control subjects revealed that patients with nonalcoholic fatty liver disease had a higher ratio of long-chain polyunsaturated fatty acids omega-6/omega-3 in their livers than did control subjects (43).

These findings provide the rationale for undertaking the experiments to test the hypothesis that supplementation of O3FAs could attenuate fatty liver changes in a novel murine model of hepatic steatosis that mimics the effects of total parenteral nutrition. Furthermore, we were interested in comparing O3FA supplementation with a standard lipid emulsion that is commonly administered with parenteral nutrition to provide additional calories and prevent essential fatty acid deficiency. Other investigators have shown that the lipid emulsions used in parenteral nutrition may in fact be associated with the development of hepatic steatosis (44). In this model, mice that have exclusive *ad libitum* access to a fat-free liquid diet that contains 20% dextrose develop hepatic steatosis within 19 d in conjunction with essential fatty acid deficiency (45). Hepatic changes are characterized by the development of diffuse macro- and microvesicular steatosis seen predominantly in the periportal and midzone hepatocytes by histology and elevated serum alanine aminotransferase and aspartate aminotransferase levels. In this study, we demonstrate that i.v. and enteral supplementation of O3FAs can indeed prevent dietary-induced hepatic steatosis in mice, whereas i.v. supplementation of a conventional lipid emulsion that is commonly used in total parenteral nutrition fails to improve hepatic steatosis.

METHODS

Animal model. Experiments were performed on 6- to 8-wk-old C57BL6 mice (Taconic, Germantown, NY). The animals, in groups of five, were housed in a barrier room and were acclimated to their environment for at least 72 h before the initiation of each experiment. Animal protocols complied with the National Institutes of Health Animal Research Advisory Committee guidelines and were approved by the Children's Hospital Boston Animal Care and Use Committee. The animals were weighed every third day, and at the time when the animals were killed, each group of mice consisted of five animals.

Diet and experimental groups. Experimental mice had exclusive *ad libitum* access to a liquid fat-free, high-carbohydrate diet (HCD) identical to the parenteral nutrition solution used at Children's Hospital, Boston. This solution contains 20% dextrose, a commercial mixture of 2% essential and nonessential amino acids (TrophAmine; B. Braun Medical, Irvine, CA), 0.2% pediatric trace elements (American Regent, Shirley, NY), 0.5% pediatric multivitamins (MVI Pediatric; aaiPharma, Wilmington, NC), 30 mEq of sodium, 20 mEq of potassium, 15 mEq of calcium (as gluconate), 10 mEq of magnesium, 10 mM of phosphate, 5 mEq of acetate, and 30 mEq of chloride per liter.

The control animals received standard rodent diet and water *ad libitum*. All animals in the experimental groups were fed the experimental HCD *ad libitum* placed in one bottle per cage. No additional sources of nutrition or hydration were provided for these animals. The bottles with the HCD were replaced daily to minimize bacterial contamination.

One group of animals received HCD without other supplements (HCD-only) for 19 d. A second group of animals additionally received O3FAs as a commercial lipid emulsion (Omegaven; Fresenius Kabi Deutschland GmbH, Neufahrn, Germany) *via* orogastric gavage at 600 μL every other day (HCD+O3FA-oral). This dose (2.4 g of fat/kg body weight of fish oil) contains 7.5–16.9 mg of omega-3 eicosapentaenoic acid and 8.6–18.5 mg of omega-3 docosahexaenoic acid. A third group of animals were similar to HCD+O3FA-oral but received the same dose of O3FA i.v. (HCD+O3FA-iv). A final group of animals (HCD+LIP-iv) received HCD for 19 d supplemented by 600 μL every other day (4.8 g of fat/kg body weight) of a standard i.v. lipid emulsion (20% Intralipid; Baxter Healthcare/Fresenius Kabi Clayton LP, Clayton, NC).

Specimen collection. At 19 d, mice were anesthetized with 300 μL of 2.5% Avertin (Tribromoethanol; Sigma Chemical Co.-Aldrich Corp., St. Louis, MO) by i.p. injection. Approximately 400 μL of blood was collected from each mouse *via* retro-orbital puncture. The specimens then were placed into serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 4°C at 8000 rpm for 10 min to collect serum. Serum was frozen at −80°C and delivered to the Clinical Laboratory at Children's Hospital for measurement of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total and direct bilirubin levels. A fatty acid profile was also obtained from these samples as described below.

Animals underwent a midline laparotomy to observe, excise, and weigh the liver. Approximately one half of the liver was fixed in 10% formalin overnight, washed with PBS, and then embedded in paraffin. After 5-μm sections were cut, slides were stained at the Harvard Rodent Pathology Facility and the Department of Pathology, Children's Hospital Boston with hematoxylin and eosin to examine cellular architecture and lipid accumulation and with periodic acid-Schiff (PAS) to identify the presence of glycogen.

Another portion of the liver was collected as frozen sections, placed in embedding medium (Optimal Cutting Temperature OCT; Sakura Fenetek, Torrance, CA), and promptly immersed in liquid nitrogen. A last portion was immediately snap-frozen in liquid nitrogen and placed on dry ice for future fatty acid analysis. The samples were stored at −80°C. Sections were stained at the Harvard Rodent Pathology Facility and the Department of Pathology, Children's Hospital Boston with Oil Red "O" to visualize hepatic fat.

Magnetic resonance imaging. The remaining liver was snap-frozen and stored at −80°C for evaluation by magnetic resonance spectroscopy (MRS) to determine percentage of liver fat content. MR imaging and MRS were performed on a Bruker 8.5 T magnet. The liver samples were thawed at room temperature 1 h before the MR analyses. Spin-lattice relaxation time T1 measurements were made with the saturation recovery approach using spin echo images with a TE of 6.4 ms and 8 TRs ranging from 0.05 to 4000 ms. Three 2-mm-thick slices were imaged for each sample, and the saturation recovery curves were generated from signal intensities measured in identically sized regions of interest within a given slice. Care was taken to exclude macroscopic fat from the selected region of interest. Free induction decays with 1024 time points and a 5-kHz bandwidth were also acquired from each sample using a hard 90° radiofrequency pulse with 16 signal averages, a 10-s TR, and a flip angle of 90°. Spectra were obtained following Fourier transformation and phasing, and percentage of fat content was determined relative to water by numerical integration of the areas under the lipid and water peaks by an independent, blinded reviewer.

Fatty acid analysis. Each serum sample (45 μL) was diluted to 0.5 mL in PBS before lipids were extracted with 6 volumes of chloroform-methanol (2:1, vol/vol) and centrifuged at 800 $\times$ *g* for 3 min, and the resulting lower phase was aspirated. Heptadecanoic acid was added to all samples as an internal standard in the form of triheptadecanoyl glycerol and diheptadecanoyl phosphatidylcholine [30 μg of each, from chloroform:methanol (1:1, vol/vol) stock solutions; Nu-Chek Prep, Elysian, MN] before extraction. Lipid extracts from the different sample preparations were fractionated into triglycerides and

phospholipids by solid-phase chromatography using an aminopropyl column, as described elsewhere (46). The resulting fractions were evaporated to dryness under nitrogen. Fatty acids were transmethylated by alkaline methanolysis using the BF_3 reagent kit (Supelco, Bellefonte, PA). Dry fractions were resuspended in 0.5 mL of methanolic-base and incubated at 100°C for 3 min, followed by addition of boron trifluoride-methanol (0.5 mL), incubation at 100°C for 1 min, addition of hexane (0.5 mL), incubation at 100°C for 1 min, and addition of 6.5 mL of saturated NaCl. Samples were centrifuged at 800 × *g* for 4 min. The hexane upper layer was transferred to a new glass tube, and an aliquot was injected in a Hewlett Packard 5890A gas chromatograph. A Supelcowax column of 30-m length and 0.5-mm internal diameter was used. Initial temperature was 150°C, and final temperature was 260°C. FID temperature was 300°C, and the total running time was 27 min. Fatty acid methyl ester peaks were identified by comparison of retention times of standard mixtures (Nu-Chek-Prep) and quantified in comparison with the internal standard (methylheptadecanoate) detector response. Mouse livers were homogenized in PBS by sonication, and the lipid fraction was extracted by addition of 6 volumes of chloroform-methanol (2:1, vol/vol). The subsequent processing for fatty acid profiling was performed as for blood serum.

Statistical analysis. Comparisons of means between two experimental groups were made using two-tailed, independent *t* tests. Comparisons between multiple experimental groups were performed using a one-tailed, ANOVA test. $p < 0.05$ was considered statistically significant. All statistical tests were performed using SigmaStat (SPSS, Chicago, IL). Values are listed as mean ± SEM.

RESULTS

Animal and macroscopic liver findings. All animals survived the protocol, and no animal had any signs of morbidity. All experimental groups gained weight on the HCD after 19 d (data not shown). There were no statistically significant differences in animal weights among experimental groups.

When the animals were killed, livers from the groups that were supplemented with O3FAs (i.v. and oral) had a similar macroscopic appearance as those of the control animals. Livers from HCD-only and HCD+LIP-iv groups, however, were pale yellow, suggesting fatty liver changes. There were no significant differences in liver weights between groups (data not shown).

Histology. A pathologist who was blinded to the groups performed histologic evaluation of the livers. There were no signs of steatohepatitis, as we did not observe any acute inflammatory changes in the experimental groups on hematoxylin and eosin sections.

Figure 1 presents histologic results from all groups. Control mice (standard diet fed) showed normal hepatic architecture (Fig. 1*A*) and glycogen storage patterns (Fig. 1*B*), without evidence of hepatic steatosis (Fig. 1*A–C*). In contrast, livers from HCD-only mice had diffuse macro- and microvesicular steatosis. These changes were most marked (black arrow) in the periportal and midzone hepatocytes (Fig. 1*D–F*). Central vein hepatocytes were spared of steatosis for a two- to three-cell layer; cells outside this perimeter, however, showed an abrupt change to steatosis (red arrow). HCD-only livers revealed minimal PAS positivity in these sections, indicating minor glycogen storage (Fig. 1*E*). Most of the PAS-positive cells were present in a two- to three-cell layer around the central vein (green arrow). HCD+O3FA-oral livers had well-preserved hepatic architecture with only rare microvacuoles in the cytoplasm of midzone hepatocytes (Fig. 1*G* and *I*). PAS staining was strongly positive, finely granular, and diffuse but most prominent within hepatocytes around the portal and central vein regions and less prominent in the midzone (Fig. 1*H*). HCD+O3FA-iv livers had minimal microvesicular steatosis in midzone hepatocytes (Fig. 1*J–L*), and there was positive PAS staining (Fig. 1*K*), albeit to a lesser extent than HCD+O3FA-oral livers. HCD+LIP-iv livers had severe macro- and microvesicular steatosis (Fig. 1*M–O*) that, in contrast to HCD-only livers, included the central vein hepatocytes with minimal glycogen storage.

Serum liver function tests. Serum liver function tests were obtained as an additional marker of liver injury. The results for these tests are summarized in Table 1. Values obtained from control standard diet–fed mice were considered to be within the normal range. The levels of aspartate aminotransferase, alanine aminotransferase, and total bilirubin were significantly higher in the HCD-only animals (109.0 ± 6.4 U/L, 71.6 ± 8.2 U/L, and 0.20 ± 0.00 mg/dL, respectively) compared with controls [72.2 ± 5.5 U/L ($p \leq 0.01$); 48.2 ± 3.6 U/L ($p \leq 0.05$); and 0.10 ± 0.02 mg/dL ($p \leq 0.05$), respectively]. It is interesting that both aspartate aminotransferase (53.0 ± 5.8 U/L) and alanine aminotransferase (23.0 ± 2.6 U/L) values in the HCD+O3FA-oral–treated animals were significantly lower than in control animals ($p \leq 0.05$ and $p \leq 0.01$, respectively) and HCD-only animals ($p \leq 0.01$). In addition, alkaline phosphatase levels were significantly decreased in the HCD+O3FA-oral (81.8 ± 7.2 U/L) mice compared with control animals (113.2 ± 4.2 U/L; $p \leq 0.01$) and HCD-only animals (126.0 ± 5.9 U/L; $p \leq 0.01$). The serum liver function tests of the HCD+O3FA-iv or HCD+LIP-iv animals were not different from control animals or HCD-only animals except for total bilirubin levels of HCD+O3FA-iv animals (0.12 ± 0.02 mg/dL). These were significantly lower than in HCD-only mice ($p \leq 0.05$).

Radiologic fat measurements. To quantify changes in hepatic fat content, we studied livers with MRS. Livers of control mice that received only standard diet were used as a baseline with which all groups were compared. There was a close correlation between the percentage of fat calculated from the MRS data and degree of T1 shortening determined from the T1 relaxation curves (data not shown). Liver fat content values for all experimental groups are demonstrated in Fig. 2.

The liver fat content for control mice was 3.4 ± 0.6%. Animals that were fed HCD-only showed a significant increase in liver fat content to 24.1 ± 1.7% ($p \leq 0.001$). HCD+O3FA-oral and HCD+O3FA-iv mice had a liver fat content of 7.2 ± 0.4 and 9.2 ± 0.6%, respectively. These values all are significantly lower than in HCD-only animals ($p \leq 0.01$) but significantly higher than in control mice ($p \leq 0.05$ and $p \leq 0.001$, respectively). Livers from HCD+LIP-iv mice had a fat content of 21.0 ± 2.5%; this was not different from HCD-only mice but was significantly higher than in control animals ($p \leq 0.05$) and O3FA-oral– and O3FA-iv–supplemented animals ($p \leq 0.01$).

Fatty acid analysis. A comprehensive analysis of fatty acid composition of serum and livers was performed; the results are summarized in Table 2. As expected, animals that were fed HCD-only were essential fatty acid deficient, whereas all other animals, including HCD+LIP-iv, were not. Essential fatty acid deficiency is generally characterized as a serum Mead acid (20:3 n-9) to arachidonic acid (20:4 n-6) serum triglyceride

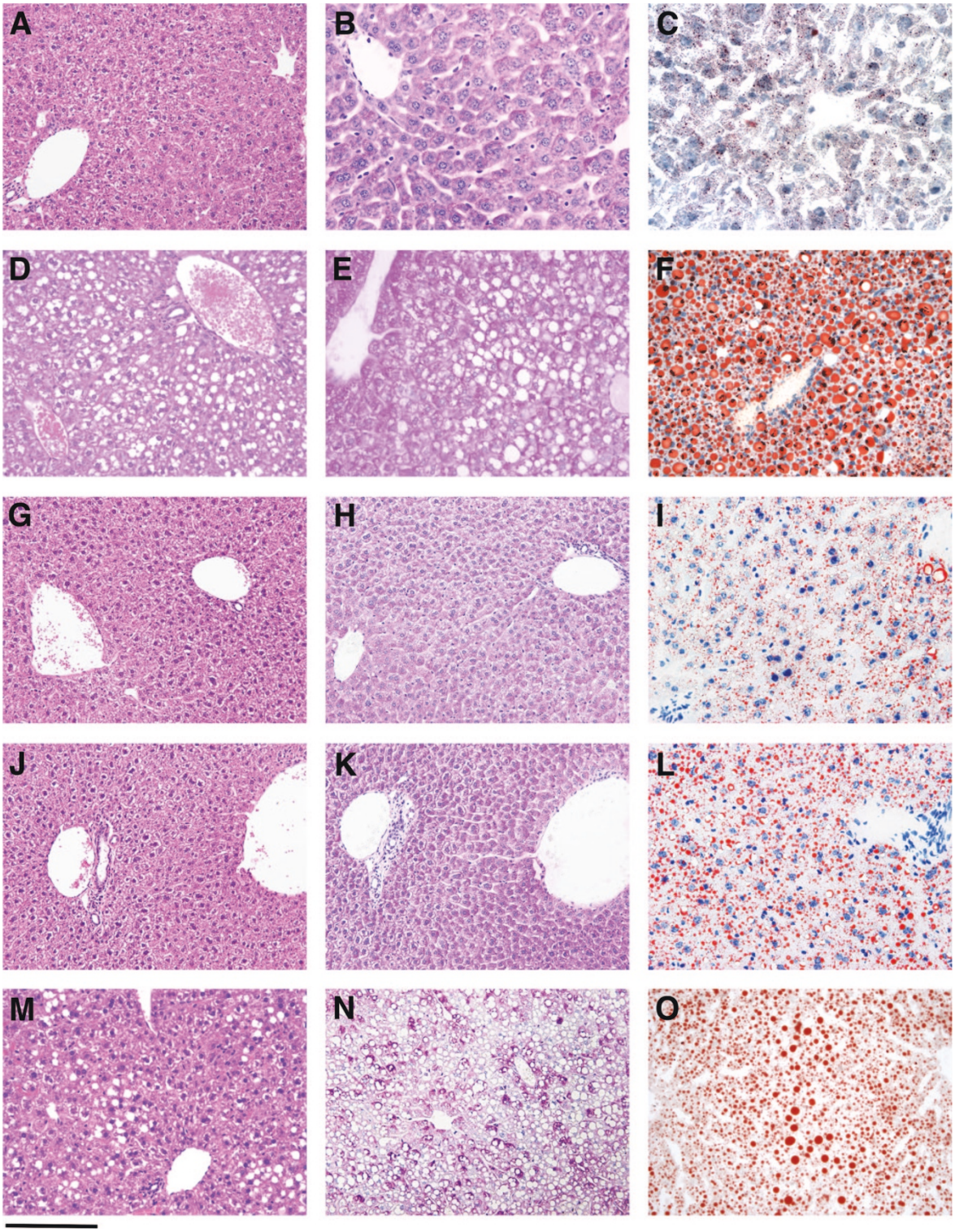

Figure 1. Liver histology. Left column H&E, middle column PAS, and right column Oil Red O staining as described in methods section. All sections are at 400× magnification (Bar = 100 μm). Control mice show normal hepatic architecture (*A*) and glycogen storage patterns (*B*) without evidence of hepatic steatosis (*A, C*). Livers from HCD-only mice had diffuse macro- and micro-vesicular stcatosis (black, red arrow, *D–F*) with minimal glycogen storage (green arrow, *E*). HCD+O3FA-oral livers had well-preserved hepatic architecture with only rare microvacuoles in the cytoplasm of midzone hepatocytes (*G, I*). HCD+O3FA-iv livers had minimal microvesicular steatosis in midzone hepatocytes (*J, L*), and appeared to have less glycogen than HCD+O3FA-oral livers (*K*). HCD+LIP-iv livers had severe macro- and micro-vesicular steatosis (*M, O*) with minimal glycogen storage (*N*).

ratio (MA:AA) >0.2. The MA:AA for HCD-only was 0.43 ± 0.06, whereas the ratios for the animals that received standard diet, HCD+O3FA-oral, HCD+O3FA-iv, and HCD+LIP-iv were 0.01 ± 0.00, 0.02 ± 0.01, and 0.10 ± 0.02, respectively. These ratios were also demonstrated in the liver tissue fat of the studied animals (Table 2). Animals that received HCD-only also demonstrated a significant amount of *de novo* lipogenesis as indicated by high absolute amounts of liver palmitate (16:0),

Table 1. *Serum liver function tests*

	Aspartate aminotransferase	Alanine aminotransferase	Alkaline phosphatase	Total bilirubin	Direct bilirubin
Control	72.2 ± 5.5	48.2 ± 3.6	113.2 ± 4.2	0.10 ± 0.02	0.08 ± 0.02
HCD-only	109.0 ± 6.4*	71.6 ± 8.2†	126.0 ± 5.9	0.20 ± 0.00†	0.10 ± 0.00
HCD+O3FA-oral	53.0 ± 5.8†‡	23.0 ± 2.6*‡	81.8 ± 7.2*‡	0.13 ± 0.03	0.10 ± 0.00
HCD+O3FA-iv	120.0 ± 25.5	73.6 ± 30.8	129.6 ± 10.5	0.12 ± 0.02§	0.06 ± 0.02
HCD+LIP-iv	172.4 ± 66.4	93.4 ± 34.7	88.8 ± 9.4	0.13 ± 0.03	0.10 ± 0.00

Data are means ± SEM; $n = 5$ for each group.
* $p \leq 0.01$, statistical significance compared with control animals.
† $p \leq 0.05$, statistical significance compared with control animals.
‡ $p \leq 0.01$, statistical significance compared with HCD-only animals.
§ $p \leq 0.05$, statistical significance compared with HCD-only animals.

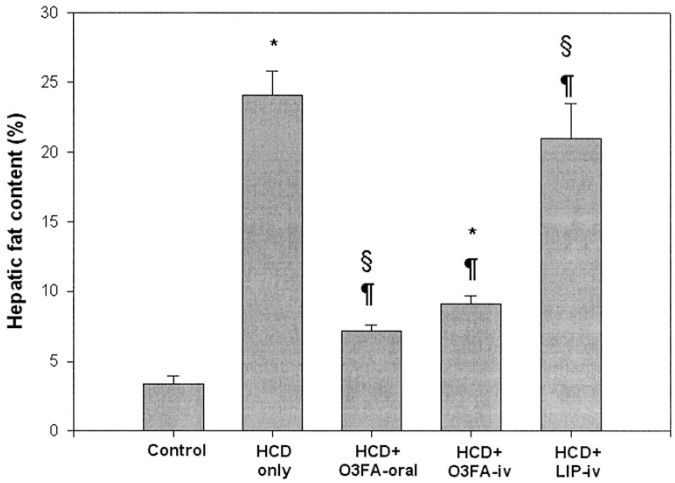

Figure 2. Hepatic fat content (%) as measured by MR-spectroscopy. The liver fat content for control mice was 3.4 ± 0.6%. Animals fed HCD-only showed an increase in liver fat content to 24.1 ± 1.7% (*$p \leq 0.001$). HCD+O3FA-oral and HCD+O3FA-iv mice had a liver fat content of 7.2 ± 0.4 and 9.1 ± 0.6%, respectively, both lower than HCD-only animals (¶$p \leq 0.01$), but higher than control mice (§$p \leq 0.05$ and *$p \leq 0.001$, respectively). Hepatic fat content from HCD+LIP-iv mice was 21.0 ± 2.5%; this was not different from HCD-only mice, but higher than control (§), O3FA-oral- and O3FA-iv-supplemented animals. Results are expressed as mean ± SE ($n = 5$ per group). (¶)

palmitoleate (16:1 n-7), and oleate (18:1 n-9), compared with control animals (36.3 ± 2.1, 12.7 ± 0.5, and 85.8 ± 4.3 nmol/μL *versus* 4.6 ± 0.6, 0.9 ± 0.1, and 6.1 ± 0.7 nmol/μL, respectively; $p \leq 0.001$). The total liver fat content of HCD-only–fed livers as determined by fatty acid analysis was also significantly higher than in control animals (137.9 ± 5.5 *versus* 16.7 ± 2.1 nmol/mg; $p \leq 0.001$). As expected, the molar percentage of the O3FA eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid (22:6 n-3) in the livers of control (0.7 ± 0.1 and 4.2 ± 0.3, respectively), HCD+O3FA-oral (0.6 ± 0.1 and 3.3 ± 0.4, respectively), and HCD+O3FA-iv (0.5 ± 0.1 and 3.9 ± 0.4, respectively) animals are similar, whereas the percentages in the HCD-only (0.0 ± 0.0 and 0.2 ± 0.0, respectively) and HCD-LIP-iv (0.0 ± 0.0 and 0.9 ± 0.4, respectively) are significantly lower ($p \leq 0.05$). It is interesting that the relative amounts of serum arachidonic acid in these groups were significantly higher than in the control and O3FA-supplemented animals ($p \leq 0.05$) suggesting a potential for a greater degree of inflammation.

DISCUSSION

We show that supplementation of O3FAs can prevent the occurrence of dietary-induced hepatic steatosis in a murine model of total parenteral nutrition. This model results in severe steatotic damage to the liver within 19 d and is accompanied by essential fatty acid deficiency and enhanced *de novo* lipogenesis (45). The liver injury that is observed in this model is essentially identical to that seen in conventional rodent models that use i.v. parenteral nutrition. However, this model is unique in that it is easy to manage and, because there are no i.v. catheters present, interpretation of data is not confounded by catheter sepsis seen in other models that are dependent on i.v. access where parenteral nutrition can be administered reliably for only 7 d (47,48). Furthermore, this model enables us to study specifically the roles of lipid solutions in parenteral nutrition–induced hepatic steatosis and allows for potential use of genetically modified animals to examine specific molecular mechanisms of such injury. The caloric intake of mice on this regimen is sufficient to satisfy established dietary energy needs. We emphasize that the amount of lipid that was supplemented, either as O3FAs or as Intralipid, did not affect satiety in these animals as the daily per-animal intake of the HCD averaged 15 mL for all experimental groups; this corresponded to ~450 kcal $\cdot$ kg^{-1} $\cdot$ d^{-1}, a caloric load similar to the established dietary energy needs of the mouse (49). Furthermore, because all animals gained appropriate weight compared with control animals, it seems that the supplemented lipids did not contribute substantially to the total caloric intake in these animals.

Our results demonstrate that O3FA supplementation, both orally and intravenously, but not i.v. supplementation of a standard lipid emulsion can attenuate hepatic steatosis in mice. It has been well described that essential fatty acid deficiency alone can cause fatty changes of the liver (50) and that reversing essential fatty acid deficiency can prevent hepatic steatosis (51). Although our model produces essential fatty acid deficiency, we have shown that the beneficial effect that O3FA supplementation has on hepatic steatosis is not solely due to the reversal of essential fatty acid deficiency in these mice, which both routes of administration of O3FAs were able to accomplish through their small but important content of arachidonic acid. In this study, we demonstrated that animals that received the HCD supplemented with a standard i.v. lipid emulsion (HCD+LIP-iv group) developed severe hepatic steatosis, similar to HCD-only as demonstrated by histology, magnetic resonance imaging liver fat content data, and liver

Table 2. *Fatty acid (triglycerides) composition of serum and liver*

Mole %	Control	HCD-only	HCD+O3FA-oral	HCD+O3FA-iv	HCD+LIP-iv
Serum					
16:0	12.7 ± 1.1	7.8 ± 0.5	11.9 ± 0.8	11.7 ± 0.7	13.5 ± 0.7
16:1 n-7	4.6 ± 0.1	14.1 ± 0.9	16.9 ± .05	13.4 ± 1.1	9.7 ± 0.6
18:0	3.0 ± 0.2	2.4 ± 0.3	4.0 ± 0.8	3.7 ± 0.4	3.0 ± 0.3
18:1 n-9	20.0 ± 1.5	22.9 ± 0.5	26.0 ± 0.6	26.9 ± 1.1	26.1 ± 0.6
18:2 n-6	36.8 ± 1.2	9.3 ± 0.3	7.0 ± 0.5	9.2 ± 1.3	15.9 ± 0.2
18:3 n-3	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.1
20:3 n-9	0.2 ± 0.0	10.1 ± 0.5	0.8 ± 0.2	0.9 ± 0.2	0.8 ± 0.1
20:3 n-6	0.7 ± 0.0	1.3 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	1.9 ± 0.1
20:4 n-6	13.0 ± 1.2	24.7 ± 1.9*	7.8 ± 0.3	9.9 ± 0.8	18.3 ± 0.8*
20:5 n-3	2.8 ± 0.1	0.7 ± 0.1	14.2 ± 0.8	10.7 ± 1.5	1.0 ± 0.1
22:5 n-6	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5 n-3	0.4 ± 0.0	0.0 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	1.6 ± 0.2
22:6 n-3	5.5 ± 0.3	6.4 ± 0.4	10.6 ± 0.3	12.6 ± 0.6	7.3 ± 0.6
Liver					
16:0	27.8 ± 0.1	24.4 ± 0.3	28.4 ± 0.4	26.6 ± 1.3	24.2 ± 1.1
16:1 n-7	5.4 ± 0.3	8.8 ± 0.6	12.9 ± 0.8	12.6 ± 0.8	10.3 ± 0.5
18:0	1.3 ± 0.8	0.0 ± 0.0	1.3 ± 0.1	0.0 ± 0.0	0.8 ± 0.0
18:1 n-9	36.4 ± 0.6	64.5 ± 0.6	48.6 ± 1.9	51.6 ± 2.0	60.7 ± 1.1
18:2 n-6	19.9 ± 1.3	1.4 ± 0.3	3.5 ± 0.7	3.3 ± 0.6	1.7 ± 0.3
18:3 n-3	1.0 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3 n-9	0.3 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.1
20:3 n-6	0.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
20:4 n-6	1.5 ± 0.5	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.2
20:5 n-3	0.7 ± 0.1	0.0 ± 0.0*	0.6 ± 0.1	0.5 ± 0.1	0.0 ± 0.0*
22:5 n-6	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
22:5 n-3	0.8 ± 0.1	0.0 ± 0.0	0.8 ± 0.1	0.7 ± 0.1	0.0 ± 0.0
22:6 n-3	4.2 ± 0.3	0.2 ± 0.0*	3.3 ± 0.4	3.9 ± 0.4	0.9 ± 0.4*

* $p \leq 0.05$, statistical significance compared with control, HCD+O3FA-oral, and HCD+O3FA-iv animals.

function tests. These animals, however, are not essential fatty acid deficient, because they are provided with substantial quantities of the essential fatty acid linoleic. In contrast, animals that received the HCD supplemented by i.v. O3FAs and also those that were supplemented through the orogastric route did not develop fatty changes of the liver. These data suggest that the content or perhaps the ratio of omega-6:omega-3 fatty acids, not the essential fatty acid status, is paramount in preventing hepatic steatosis in this model. It has been shown that a high polyunsaturated omega-6:omega-3 long-chain fatty acid ratio is associated with nonalcoholic fatty liver disease and may favor lipid synthesis over oxidation and secretion (43). The standard lipid emulsion that we administered had a 15-times higher polyunsaturated omega-6:omega-3 fatty acid ratio compared with the O3FA emulsion and 11 times higher than the normal diet (data not shown).

The source of the hepatic lipid was not determined in this study, but the locations are suggestive. With HCD-only, the lipid was found primarily in the portal area, suggesting that this is the primary site of *de novo* lipogenesis, because exogenous fat was not provided. With HCD+LIP-iv, both the portal area and the central vein had increases in fat, as determined by histology. Although not proved, this suggests that the fat that was found adjacent to the central vein was the fat that was administered. The source of the fat in the portal area in this group could also be the administered fat, suggesting a reduction or elimination of *de novo* lipogenesis, or, conversely, could represent the product of continued *de novo* lipogenesis that was not prevented by the i.v. administration of a high linoleic acid–containing fat, perhaps as a result of altered hepatic metabolism of these artificial chylomicra. Only further studies will distinguish between these two possibilities.

Multiple reports in the literature have recognized that supplementation of lipid emulsions may be a factor in the development of fatty livers as a result of total parenteral nutrition. In 1982, Allardyce (52) described a cohort of 35 patients who received total parenteral nutrition with conventional lipid emulsions for >3 wk and concluded that the symptoms of cholestasis in these patients recovered when the lipid supplementation was reduced. These data were corroborated by other clinical observations (44,53–55) and in animal models (56). A study by Zaman *et al.* (57) demonstrated that isolated perfusion of rat livers with total parenteral nutrition that was supplemented with Intralipid led to more steatosis and poorer liver function than livers that were perfused with parenteral nutrition only. Potential mechanisms include the accumulation of lipid metabolites in hepatic sinusoidal macrophages, thereby interfering with the clearance of bacteria and their products. Furthermore, it has been proposed that standard lipid emulsions may enhance the production of proinflammatory cytokines, because they are a relatively rich source of omega-6 fatty acids, precursors to arachidonic acid (36,58).

The notion that O3FAs may be beneficial in hepatic injury is not novel. Chen *et al.* (32,41,56) described the effects that various fatty acid compositions had on rats that received total parenteral nutrition. They demonstrated that emulsions that were derived from fish oil and were high in O3FAs led to a lower hepatic fat content in rats that received total parenteral nutrition than emulsions that were derived from olive and safflower oils. Although a mechanism for the beneficial effects

of O3FA supplementation is not provided in these studies, several hypotheses have since been put forward. O3FAs may stimulate both peroxisomal and mitochondrial β-oxidation of fatty acids, thereby decreasing the availability of nonesterified fatty acids as substrates for triacylglycerol synthesis and secretion (59–61). In addition, O3FAs have been shown to reduce the activity of acetyl CoA carboxylase and thereby inhibit *de novo* lipogenesis in rat livers (60). Other mechanisms include lowering the activity of superoxide dismutase and glutathione peroxidase (40), the property of O3FAs to act as suboptimal substrates for esterification to glycerol (62,63), and reducing the synthesis of arachidonic acid by inhibiting the activities of omega-6- and omega-5 desaturases on linoleic acid in the liver (64). It seems likely that O3FAs exert their beneficial effect in lowering hepatic fat content through a combination of these mechanisms.

In conclusion, we have demonstrated that enteral and i.v. supplementation of O3FAs can ameliorate hepatic steatosis in a murine model of parenteral nutrition as demonstrated by histology, MRS, serum liver function tests, and serum and liver fatty acid analysis. In contrast, supplementation with the standard i.v. lipid emulsion Intralipid does not improve the fatty liver changes in these animals. Because i.v. lipid supplementation is a key component of nutritional support in patients who receive total parenteral nutrition, further studies into the mechanisms of these effects and the potential substitution of standard lipid emulsions with O3FA-containing emulsions in these patients are warranted.

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Erratum

The article, "Effect of Dietary Ribonucleotides on Infant Immune Status. Part 1: Humoral Responses," by Joseph P. Schaller, Matthew J. Kuchan, Debra L. Thomas, Christopher T. Cordle, Timothy R. Winship, Rachael H. Buck, Geraldine E. Baggs, and J. Gary Wheeler (Pediatric Research 56: 883–890), contained several errors.

On page 886, right column, paragraph 1, the sentence beginning "Comparison of the RCFD " should read: Comparison of the RCFD curves revealed statistically significant distribution differences only at 12-mo (FN different from F, $p = 0.023$) using the Kolmogorov-Smirnov test (30).

On page 887, the third footnote in Table 3 should read: Different by a post hoc three-group RMA on the evaluable data subset for 6, 7, and 12 mo group X time interaction $p = 0.0285$.

On page 888, for the legend of Figure 1, the sentence beginning "Reverse cumulative distributions " should read: Reverse cumulative distributions were different at 12 mo (¶FN > F, $p = 0.023$).

On page 889, right column, paragraph 2, the sentence beginning "In this regard, " should read: In this regard, it is most interesting that significant differences in key indicators of immune function (poliovirus VN1 responses and subsets of T and NK cells) were identified not only between formula groups but also more often between breast-fed infants and those fed formula without ribonucleotides.

Table 2 had missing percentage values and is reprinted below.

Table 2. *Demographic Variables in the F, FN, and HMF Groups**

	F	FN	HMF
Subjects, *n* (%)	147	138	192
Male	62 (42)	61 (44)	104 (54)
Female	85 (58)	77 (56)	88 (46)
Ethnicity, *n* (%)			
Caucasian	126 (85.7)	119 (86.2)	170 (88.5)
African-American	14 (9.5)	14 (10.1)	12 (6.3)
Hispanic	1 (0.7)	1 (0.7)	4 (2.1)
Asian	0 (0.0)	1 (0.7)	0 (0.0)
Other	6 (4.1)	3 (2.2)	6 (3.1)
Gestational age (wk)†	39	39	39
Weight (g) at 10 d of age‡			
Male	3528 ± 66	3561 ± 67	3625 ± 51
Female	3535 ± 59	3397 ± 48	3509 ± 49
Maternal education, *n* (%)			
Some high school	11 (8)	9 (7)	5 (3)
High school	38 (27)	35 (26)	30 (16)
Some college	49 (34)	53 (39)	64 (33)
College or more	45 (31)	39 (28)	93 (48)

* F, control; FN, ribonucleotide-supplemented; HMF, fed exclusively human milk for at least 2 months followed by human milk or commercial Similac with Iron at weaning.

† Values are mean [median].

‡ Values are mean ± SEM.