The route of lipid administration affects parenteral nutrition–induced hepatic steatosis in a mouse model

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Abstract

Background: The etiology of parenteral nutrition (PN)–associated hepatic injury remains unresolved. Recent studies have suggested that the intravenous (IV) lipid emulsion administered with PN may contribute to PN-associated hepatic injury. We therefore examined whether the route of lipid administration would affect the development of PN-associated liver injury in a previously established animal model of PN-induced hepatic steatosis.

Methods: Mice were fed ad libitum PN solution as their only nutritional source for 19 days with lipid supplementation by either the enteral or the IV route. Control mice received chow alone, and a final group received enteral PN solution without lipid supplementation.

Results: All mice gained equivalent weight during the study. Mice receiving PN alone or PN with IV lipid developed severe histologic liver damage that was not seen in control mice or in mice receiving PN with enteral lipid. Liver fat content as measured by magnetic resonance spectroscopy was significantly lower in the control and enteral lipid groups when compared with mice receiving PN alone or with IV lipid. Mice receiving enteral lipid had significantly lower levels of serum aspartate aminotransferase and alanine aminotransferase compared with animals receiving PN alone.

Conclusions: These data provide preliminary evidence that lipid administered through the enteral route protects against PN-associated hepatic injury in an animal model.

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Parenteral nutrition; Lipid; Liver; Steatosis; Magnetic resonance spectroscopy

Despite decades of study, the etiology of parenteral nutrition (PN)–associated hepatic injury remains unresolved. This process is characterized by fatty deposits within the liver; hepatocyte injury resulting in cholestasis and elevation of serum liver enzymes; and, ultimately,
Materials and methods

1.1. Animal model

Experiments were performed on C57BL/6 mice 5 to 6 weeks old (Taconic, Germantown, NY). Mice were housed 5 animals to a cage and were acclimated to their environment for 72 hours before the initiation of each experiment. Animal protocols were approved by the Children’s Hospital Boston Animal Care and Use Committee.

Animals in the experimental groups were fed ad libitum with a PN solution placed in one water bottle per cage as previously described [23,24]. Experimental animals did not receive any additional sources of nutrition or hydration. The bottles were replaced daily with fresh PN solution, and the volume of PN solution consumed was measured daily. The PN solution used throughout the study was a typical clinical formula used at Children’s Hospital Boston containing 20% dextrose and a commercial mixture of 2% essential and nonessential amino acids (TrophAmine, B. Braun Medical, Irvine, Calif.). Each liter of PN contained 30 mEq sodium, 20 mEq potassium, 15 mEq calcium (as gluconate), 10 mEq magnesium, 10 mmol/L phosphate, 5 mEq acetate, 30 mEq chloride, 0.2% pediatric trace elements (American Regent, Shirley, NY), and 0.5% pediatric multivitamins (MVI Pediatric, aaiPharma, Wilmington, NC). The experimental period lasted 19 days. Mice were individually weighed every fourth day.

Control mice received normal mouse chow and water ad libitum. All experimental groups were fed PN solution ad libitum. Parenteral nutrition–only (PN-O) mice received no additional treatment. In addition to oral PN solution, high per gavage (HPG) mice received 20% Intralipid (Baxter Healthcare/Fresenius Kabi Clayton LP, Clayton, NC) via orogastric gavage at a dose of 120 mg (6 mg/g, 0.6 mL) every other day; low per gavage (LPG) mice received 40 mg (2 mg/g, 0.2 mL) of 20% Intralipid via orogastric gavage every other day; and IV mice received 120 mg (6 mg/g, 0.6 mL) 20% Intralipid IV via sterile tail vein injection every other day.

1.2. Specimen collection

At the conclusion of the 19-day experiment, mice were anesthetized with 400 μL of 2.5% Avertin by intraperitoneal injection. Four hundred microliters of blood was collected from each mouse via retro-orbital puncture. Next, blood specimens were placed into serum separator tubes (Becton Dickinson, Franklin Lakes, NJ) at 4°C and centrifuged at 8000 rpm for 10 minutes to collect serum. Serum was frozen at −80°C and delivered to the Clinical Laboratory at Children’s Hospital for measurement of alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). After blood collection, midline laparotomy was performed and livers were excised and weighed. Random biopsies were then taken from the liver. One set of liver tissue was fixed in 10% formalin overnight, washed with phosphate buffered saline, and then embedded in paraffin. After cutting 5-μm sections, slides were stained with H&E and periodic acid–Schiff (PAS). A second set of biopsies was collected for frozen section. Random biopsies were taken from different areas of the liver and placed in embedding medium (Optimal Cutting Temperature OCT, Sakura Fenetek, Torrance, Calif) and immersed in cold 2-methylbutane (Aldrich, Milwaukee, Wis) on dry ice and stored at −80°C. Sections were stained with Oil Red O at the Harvard Rodent Pathology Facility and the Department of Pathology, Children’s Hospital Boston. All histologic sections were reviewed by a pathologist blinded to treatment assignment.

1.3. Magnetic resonance imaging

The remaining liver was evaluated by magnetic resonance spectroscopy to determine percentage liver fat content. Livers were frozen at −80°C after harvest and delivered to the Magnetic Resonance Laboratory at the...
Liver samples were stored at –80°C and allowed to thaw to room temperature immediately before magnetic resonance testing. Magnetic resonance imaging and spectroscopy were performed on a Bruker 8.5 T magnet. Spin-lattice relaxation time $T_1$ measurements were made with the saturation recovery

Fig. 1  Histologic analyses of liver specimens from experimental groups using both H&E and PAS staining are illustrated. Control tissues showed normal hepatic architecture. Livers from PN-O and IV mice revealed diffuse macrovesicular and microvesicular steatosis with minimal PAS staining. High per gavage animals, treated with the highest concentration of enteral lipid, demonstrated a minimal degree of histologic liver injury with rare fat microvacuoles and no histologic evidence of steatosis.
approach using spin echo images with an echo time of 6.4 milliseconds and 8 repetition times ranging from 0.05 to 4000 milliseconds. Three 2-mm-thick slices were imaged for each sample and the saturation recovery curves were generated from signal intensities measured in identical 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 and a 10-second repetition time. Spectra were obtained by Fourier transformation and were manually phased. It is established in magnetic resonance imaging that T1 recovery times for fat are short relative to water. Thus, increases in fat within a given sample should result in shortening of T1 times. The percentage fat content was determined relative to water by numerical integration of the areas under the lipid and water peaks by a blinded reviewer.

1.4. Statistical analyses

Comparisons of means between 2 experimental groups were made using 2-tailed independent t tests. Comparisons between all experimental groups were performed using a 1-tailed analysis of variance test with the Bonferroni post hoc analysis test. P < .05 was used for statistical significance. All statistical tests were performed using SPSS software (SPSS, Inc, Chicago, Ill). Values are listed as mean ± SEM.

2. Results

2.1. Gross animal and liver findings

All animals survived the experimental protocol without gross signs of morbidity. Average daily intake of PN solution per mouse was 15 mL. There were no differences in PN consumption between experimental groups. All animals gained weight on the PN solution with a mean weight gain of 10% ± 1% during the 19-day study period. There were no significant differences in rate of weight gain between groups during the study protocol.

Upon harvest, livers from PN-O and IV mice revealed a striking pale yellow color, suggesting fatty liver changes. Livers from HPG mice, in contrast, had a darker red appearance that was similar to the gross appearance of control livers that did not receive PN. Livers from LPG mice demonstrated a mild pale color that was less dramatic than that observed in PN-O livers. There were no significant differences in liver weights between groups.

2.2. Histology

H&E staining was used to examine histopathologic findings, and PAS staining was used to identify the presence of glycogen. For selected specimens, Oil Red O staining was performed to confirm the H&E findings and to qualitatively estimate hepatic fat content. There was no evidence of acute inflammatory change, bile duct proliferation, or fibrosis in any group on H&E sections.

Fig. 1 presents histologic results from all groups. Control mice showed normal hepatic architecture and no evidence of hepatic steatosis. In contrast, livers from PN-O mice had diffuse macrovesicular and microvesicular steatosis seen predominantly in the periportal and midzone hepatocytes. Central vein hepatocytes were spared of steatosis for a 2- to 3-cell thickness. Parenteral nutrition-only livers revealed minimal PAS positivity in a 2- to 3-cell layer around the central vein. High per gavage livers had well-preserved hepatic architecture with rare fat microvacuoles. No definitive steatosis was identified on H&E sections. PAS staining for glycogen was strongly positive and diffuse but most prominent within hepatocytes around the portal and central vein regions. Low per gavage livers also had well-preserved hepatic architecture with mild to moderate microvesicular and macrovesicular steatosis in the periportal and midzone hepatocytes. There was no steatosis seen in the central vein hepatocytes. PAS stain was diffusely positive, most prominently around the central vein hepatocytes. Intravenous livers had severe, diffuse involvement of hepatocytes by macrovesicular steatosis. Most of the cell cytoplasm was occupied by large clear

![Fig. 2](image)

Oil Red O staining revealed lipid droplet accumulation in PN-O and IV livers that was not present in HPG mice that received enteral lipid supplementation.
vacuoles. There was no zonal distribution of the steatosis. The PAS stain was weakly positive throughout all regions of the liver parenchyma.

Oil Red O staining for lipid accumulation revealed minimal staining in control livers (Fig. 2). Parenteral nutrition–only mice showed severe, diffuse accumulation of fat particles throughout the liver parenchyma. In contrast, livers from HPG mice treated with enteral lipid revealed minimal fat content by Oil Red O stain.

2.3. Radiological fat measurements

The T1 measurements made from saturation recovery imaging experiments in all of the liver samples were generally found to correlate with the percentage fat calculated from the magnetic resonance spectroscopy data ($R^2 = 0.66$). Fig. 3 presents the liver fat content values from spectroscopic measurements of all experimental groups. Administration of PN without lipid supplementation (PN-O mice) resulted in a significant increase in liver fat content compared with control mice (15.78% ± 3.74% vs 5.39% ± 1.10%, $P < .001$). After concomitant administration of enteral lipid, the liver fat content of HPG animals was significantly lower when compared with PN-O animals (8.08% ± 4.28% vs 15.78% ± 3.74%, $P < .001$) and HPG animals (18.13% ± 4.84% vs 8.08% ± 4.28%, $P < .05$).

2.4. Serum liver function tests

In addition to evaluation by histology and magnetic resonance spectroscopy, serum liver function tests were measured as a marker of intrinsic liver damage. Results of serum liver function tests are listed in Tables 1 and 2. Total bilirubin levels were within normal range in all animals. Serum levels of ALT and AST revealed marked differences between experimental and control groups with the lowest levels found in control and HPG mice. When compared with the control group, PN-O mice had the most dramatic elevations noted in ALT (108 ± 29 vs 34 ± 2 U/L, $P < .05$) and AST (187 ± 21 vs 61 ± 9 U/L, $P < .01$). Intravenous mice showed a trend toward elevations in both serum ALT and AST. High per gavage mice had significantly lower serum transaminase levels when compared with PN-O animals for AST (99 ± 15 vs 187 ± 21 U/L, $P < .01$) and ALT (38 ± 6 vs 108 ± 29 U/L, $P = .01$). In addition, the PN-O animals had significantly elevated serum levels of alkaline phosphatase when compared with all other experimental groups ($P < .01$, data not shown).

![Fig. 3](image-url) Mean percentage fat content with standard error is shown for each of the treatment groups. Both control and HPG livers had significantly lower liver fat content levels than livers from both PN-O and IV mice (*$P < .05$), demonstrating a protective effect of enteral lipid, but not IV lipid, against PN-induced hepatic injury in this model.

3. Discussion

The etiology of PN-associated hepatic injury remains unclear despite years of focused investigation. The lack of conclusive data has led to the assumption that the origin of PN-associated liver injury is multifactorial, and at present, there is no direct treatment or prevention for this condition [10,11].

Recent evidence has implicated a role for the IV lipid emulsion in the development of PN-associated hepatic dysfunction, and the aim of this study was to assess whether

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<td>Group</td>
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<td>Control</td>
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<td>PN-O</td>
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Similar to the findings for ALT, PN-O mice had significant elevations in serum levels of AST as compared with control animals and HPG animals treated with enteral lipid. $P$ values were calculated for comparison between experimental groups and control groups.
the route of lipid administration affects PN-induced liver injury. To evaluate this question, a modified, previously established rodent model of PN dependence was used, which incorporates the ad libitum feeding of oral PN solution [23,24]. This rodent model is known to cause marked hepatic steatosis, which likely represents the first stage in PN-associated liver injury, within 19 days. In this way, the model avoids the use of invasive central venous catheters that raise the confounding risk of systemic infection and also allows for longer exposure of the animal to PN solution. The same lipid emulsion used in clinical PN regimens (Intralipid) was administered by the enteral and IV routes in doses sufficient to avoid essential fatty acid deficiency. Two dosing regimens of enteral lipid provision were used to preliminarily explore the possibility of a dose-dependent pattern.

The study found a consistent pattern of (1) protection against PN-associated hepatic steatosis with the use of enteral lipid supplementation and (2) persistent, severe steatosis with IV lipid. As expected from previously published data [23,24], PN-O mice fed only PN solution without lipid developed hepatic steatosis on histology and magnetic resonance spectroscopy along with significant elevations in serum transaminases. High per gavage mice that received the same volume of PN solution with the addition of enteral lipid had a marked decrease in the extent of overall liver injury. In all areas of this investigation, mice treated with enteral lipid most closely resembled control mice that did not receive PN as part of the experimental protocol. These results were in complete contrast to the extensive hepatic injury found in IV mice that were fed PN solution with IV lipid. The IV group developed severe hepatic steatosis with diffuse macrovesicular fatty infiltration as well as elevations in spectroscopic liver fat content and serum transaminase levels. Intravenous mice did not exhibit any improvement in liver injury and appeared to have a higher degree of steatosis on histology when compared with mice receiving PN without lipid. In addition, there was a dose-dependent effect of enteral lipid supplementation. Low per gavage mice, which received one third the dose of enteral lipid, had improved liver histology but demonstrated liver injury by spectroscopy and liver function tests, whereas HPG mice were significantly protected from liver injury with the full dose of enteral lipid.

The nutritional model used in this study fed all mice with PN solution ad libitum as their only source of nutrition and fluid. Importantly, there were no differences in weight gain parameters and volume of PN consumed between experimental groups. Each milliliter of PN solution provided 0.2 g (0.68 kcal) of dextrose and 0.02 g (0.08 kcal) of amino acid. From this formula, mice were ingesting approximately 456 kcal/kg per day and 15 g/kg per day of protein. This caloric and protein provision is similar to the established dietary needs of the mouse [25,26]. For lipid treatment, all animals except the LPG group received 1.2 kcal (48 kcal/kg) of lipid every other day. Low per gavage mice, which received the lowest dose of enteral lipid, were treated with 0.4 kcal (16 kcal/kg) of lipid every other day. The lipid emulsion was provided as approximately 5% of total caloric intake to prevent essential fatty acid deficiency in the full lipid dose.

The scope of the present study did not allow for identification of the mechanism behind the protection of the liver from enterally administered lipid. However, one explanation for the severe steatosis found in the PN-O animals is essential fatty acid deficiency because this group received only PN without dietary lipid supplementation during the 19-day study protocol. Previous studies have shown that essential fatty acid deficiency alone can cause steatosis in the liver [27] and that reversing essential fatty acid deficiency can prevent steatosis [28]. To measure essential fatty acid status, we performed fatty acid analyses on serum from all study groups. Using the common definition for essential fatty acid deficiency as a Mead acid (20:3 n-9) to arachidonic acid (20:4 n-6) ratio greater than 0.2, these fatty acid profiles demonstrate that PN-O animals were indeed essential fatty acid deficient, but lipid supplementation in both HPG and IV mice prevented essential fatty acid deficiency (data not shown). These findings are expected because the concentration of lipid used in these mice was sufficient to meet the murine requirement for essential fatty acid provision [25].

There were no differences, then, in the dose of lipid administered to or the essential fatty acid status of HPG and IV mice. Therefore, the dramatic disparity in liver injury is likely because of the route of lipid administration. Penco et al [15] obtained similar findings when they administered either enteral or IV lipid supplementation to chow-fed rabbits; whereas the IV lipid group developed histologic fat accumulation throughout the liver parenchyma, the enteral lipid–treated livers were spared from injury. These differences in hepatic steatosis from the route of lipid administration may be explained, in part, by reduced or altered liver metabolism of the IV lipid emulsion. There is accumulating evidence that the metabolism of the artificial chylomicron found in IV lipid emulsions differs from that of the native chylomicron manufactured in the enterocyte after ingestion of fat [21,29]. A significant portion of the artificial chylomicron may be metabolized by extrahepatic tissue instead of the liver [30], and this may be related to different ratios of apolipoproteins on the surface of the artificial chylomicron [21,22,31]. Alternatively, IV lipid particles may compete with endogenous lipoproteins, resulting in the accumulation of plasma triglycerides and subsequent deposition into hepatocytes [32]. Regardless of the mechanism, the data from this study support the notion that IV lipid emulsions may not be metabolized in a physiological manner by the liver.

Further indirect evidence supports the findings presented here. Previous studies have shown that IV lipid emulsions composed of different fatty acid ratios may reduce the risk of PN-induced liver injury. In particular, lipid emulsions
rich in ω-3 fatty acids have been associated with less extensive liver injury in PN-dependent animal models when compared with standard lipid emulsions [12,33,34]. Second, a growing body of literature is investigating the role of plant phytosterols, a common ingredient in lipid emulsions used with PN, and their association with liver injury during PN administration [19,20]. It is possible that these compounds are toxic to the hepatocyte when administered IV but not when absorbed through the gastrointestinal tract. In addition, it has been established clinically that the risk of PN-associated hepatic dysfunction is reduced when partial enteral provision of nutrition is tolerated [23,33,35]. Although the nutritional basis for this finding has not been studied extensively, it can be hypothesized that lipid is the macronutrient in enteral nutrition that reduces the risk of PN-associated hepatic injury.

Alternative explanations for the findings presented here exist and deserve mention. For example, enteral lipid may impart a state of satiety on the mouse, so that animals receiving enteral lipid may have ingested a smaller quantity of the PN solution. Our intake measurements, however, did not show an appreciable difference in PN consumption between experimental groups, and the amount of fat administered was minimal relative to total caloric intake. Alternatively, it is possible that certain animals took in more PN solution than others. In this way, the data observed here may include the confounding effects of carbohydrate overfeeding. In addition, the lipid dosing schedule used in this study departs from the continuous lipid infusion commonly administered in the clinical setting; it is likely, however, that continuous administration of lipid would only worsen the liver changes demonstrated in the IV mice.

It is also possible that the protective effects of enteral lipid are unique to the mouse model used in this study. Although this model does not deliver the dextrose and amino acid components of PN in standard IV fashion, the model produces consistent liver injury with parenchymal changes demonstrated in the IV mice. The accumulation of fat within the liver parenchyma is thought to represent an early stage in the evolution of PN-associated hepatic injury and nonalcoholic fatty liver disease in general [3]. In this way, persistent liver injury after the “first hit” of steatosis may subsequently lead to hepatic fibrosis and cholestasis. Indeed, the steatosis observed in this model is similar to early histologic changes found in patients with PN-associated liver injury. Therefore, although the model used in this study has its limitations, it represents a novel technique to study the early toxic effects of PN on the liver.

Pending further study, the finding that enteral lipid protects the liver against hepatic steatosis during PN administration may have implications to the clinical setting. Although lipid provision is required in a PN regimen to provide a calorically dense source of calories and to avoid essential fatty acid deficiency, the use of limited amounts of enteral lipid provision to PN-dependent patients may be feasible. In the present study, the highest dose of enteral lipid was 120 mg (0.6 mL of a 20% emulsion) every other day. For comparison in the human patient, a 5-kg baby would require 12 g (60 mL) of enteral lipid supplementation daily, or approximately 2.5 mL/h of a 20% emulsion, to achieve a comparable dosage. In this way, it may be possible to aggressively wean patients on PN from parenteral to enteral lipid. Moreover, there may be additional benefit from the use of enteral lipid in the PN-dependent population. A recent study has shown that enteral formulas with higher concentrations of polyunsaturated fatty acids may improve small bowel adaptation in the setting of short bowel syndrome [36].

This study provides preliminary evidence in an animal model that enteral lipid protects the liver from the development of PN-induced steatosis and that IV lipid supplementation does not improve PN-associated hepatic injury. Further study is needed to clarify these findings in more advanced animal models and to identify the mechanistic pathways.

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