The Role of Parenteral Lipids in the Development of Hepatic Dysfunction and Hepatic Steatosis in a Mouse Model of Total Parenteral Nutrition

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Summary Parenteral nutrition-associated liver disease, a common and life-threatening complication among people who require long-term parenteral nutrition, has been associated with abnormal liver function, cholestasis, steatosis and fibrosis. Intravenous soybean lipids may be associated with the development of liver disease. We therefore examined whether different doses of parenteral lipids would affect the development of liver disease, and further investigated the possible pathogenesis of it. C57BL/6J mice with a central catheter placed in the right jugular vein were divided into three groups. The control group received normal mouse chow with intravenous normal saline; The lipids group received parenteral nutrition solution (0.14 g lipids per day); the H-lipids group received parenteral nutrition solution with twice the amount of lipids (0.3 g lipids per day). Changes in body weight, serum biochemical parameters, liver histology and farnesoid X receptor gene expression in the liver were assessed. The values of serum direct bilirubin, total bilirubin and cholesterol were markedly increased in the H-lipids group at day 7. The predominant histologic finding in the H-lipids group was steatosis, and the steatosis score in the H-lipids group was much higher than in the other two groups at either day 5 or day 7. Hepatic expression of farnesoid X receptor mRNA decreased after 7 d of parenteral nutrition. High doses of parenteral lipids are more likely to develop liver disease in a mouse model of parenteral nutrition. Farnesoid X receptor may play a key role in the development of parenteral nutrition-associated liver disease.

Key Words total parenteral nutrition, parenteral nutrition-associated liver disease, lipids, steatosis, mice

Since its introduction into clinical practice, parenteral nutrition (PN) has profoundly impacted the prognosis and quality of lives for patients with intestinal failure (IF). However, its long-term use is associated with potentially fatal complications including septic infections, metabolic imbalances and hepatobiliary dysfunction. The hepatobiliary complications of PN are now recognized as parenteral nutrition-associated liver disease (PNALD), or intestinal failure-associated liver disease (IFALD). PNALD is defined as a decrease in bile flow that occurs independent of mechanical obstruction in patients receiving prolonged PN, and with no other specific cause of liver injury (1, 2). It is diagnosed by a prolonged duration of PN administration (>2 wk), and elevations in direct bilirubin (>2 or 3 mg/dL) in the absence of any other underlying liver disease (3, 4). Cholestasis occurs more frequently in infants and children, whereas hepatic steatosis is more common in adults. The etiology is not well understood and is likely multifactorial. A number of factors, including low birth weight, the duration of PN, sepsis and cumulative

amount of lipids infusion may contribute to the development of PNALD in infants and children (5–7).

Intravenous lipids are a vital component of PN and provide a source of non-protein calories and prevent essential fatty acid deficiency (EFAD). However, there is growing evidence that PNALD may be, in part, due to the composition of the soybean oil-based lipid emulsions. The evidence from retrospective, cohort studies shows that limiting soybean oil-based lipid intake to <1 g/kg/d as opposed to the conventional 2.5–3 g/kg/d reduces the bilirubin level in infants with established cholestasis (4, 8, 9). More recently, they showed that a replacement of soybean by fish oil was associated with reversal of cholestasis in infants (10, 11). In our previous clinical study, we also observed diffuse involvement of hepatocytes by severe macrovesicular and microvesicular steatosis in patients on long-term PN and the beneficial effects of partial replacement of soybean by fish oil on PNALD (12, 13). However, the molecular mechanisms underlying the soybean oil development of cholestasis and clinical benefits observed with fish oil are unclear.

Farnesoid X receptor (FXR) is a ligand-activated nuclear receptor, which plays a key role in bile salt

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homeostasis. Gene studies have demonstrated that FXR knockout mice lack these hepatoprotective mechanisms and are ultrasensitive to BA-induced injury (14).

Based on our clinical findings and previous studies, we believe intravenous soy bean lipids may be associated with the development of liver disease by decreasing FXR gene expression, and decreased FXR gene expression may play a key role in the development of PNALD. We therefore examined whether different doses of parenteral lipids would affect the development of liver disease, and further investigated the possible pathogenesis of it.

MATERIALS AND METHODS

Animal model, experimental design and TPN compositions. The study was approved by the Ethics Committee of the medical faculty of Jinling Hospital (approval number 2015DWLS-03-020). All animals received humane care in compliance with The Principles of Laboratory Animal Care formulated by the National Society of Medical Research and The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication no. 85-23, revised 1996).

Female C57BL/6 mice, 6 to 8 wk old, weighing 17 to 21 g were purchased from Model Animal Research Center of Nanjing University. The mice were housed 5 animals to a cage and were acclimated to their environment for 72 h before the initiation of each experiment. Mice were housed in a pathogen-free animal facility under a standard 12 h light/dark cycle and fed standard rodent chow and water ad libitum.

Catheterization was performed using the method described in our previous study (15). All surgical procedures were performed under general anesthesia using ketamine 100–120 mg/kg intraperitoneally. The hair on the neck was shaved. A vertical incision was made through the skin on the right side of the neck to expose the jugular vein. A silicon rubber catheter (0.3 mm inner diameter and 0.5 mm outer diameter; Anilab Software & Instruments Co., Ltd., Ningbo, China) was inserted into the vena cava through the right jugular vein by an operation microscope (Model JSZ6, Nanjing Jiangnan Novel Optics Co., Ltd., Nanjing, China). The proximal end of the catheter was tunneled subcutaneously from the neck to the skull of the head and attached to a swivel mechanism. The silicone catheter was protected by a specially designed stainless-steel spring. A polyethylene tube connected the swivel mechanism to a micro-infusion pump (WZ-50C2, Zhejiang University Medical Instrument Co., Ltd., China). This system allowed the mouse to move freely and access water. Sterile instruments and strict aseptic conditions were used during surgery.

After the operation, mice were randomly divided into three groups (20 mice per group): a control group receiving normal saline via the intravenous catheter and permitted free access to normal mouse chow (from the Animal Centre); a lipids group receiving TPN solution intravenously; and a H-lipids group receiving TPN solution with twice the amount of lipids.

Table 1. Composition of TPN (per 7.3 mL).

	Lipids	H-lipids
NPC, kcal/mL	0.3	0.7
8.5% Novamin (mL)	0.9	1.9
20% Intralipid (mL)	0.7	1.5
50% Glucose (mL)	0.6	1.2
Normal saline (mL)	5.0	2.6
Addamel N (mL)	0.03	0.03
Soluvit N (mL)	0.015	0.015
Vitalipid N Adult (mL)	0.015	0.015
Heparin (U)	7.5	7.5
Total (mL)	7.3	7.3

The control group received normal mouse chow, with water ad libitum. The lipids group were infused with a TPN admixture with 0.14 g/d of lipids and 110 kcal/kg/d (about 2.2 kcal per day) of non-protein calories (NPCs) (NPC : N ratio of 200 : 1). The H-lipids group were infused with twice the amount of lipids (0.3 g/d) and a TPN solution providing 255 kcal/kg/d (about 5 kcal per day) of NPCs with a similar NPC : N ratio. The source of nitrogen was from 8.5% Novamin (Sino-swed Pharmaceutical Corp. Ltd., Beijing, China) and the source of fat was from 20% Intralipid (Sino-swed Pharmaceutical Corp. Ltd.). In addition to this, all mice daily received fat- (Vitalipid; Sino-swed Pharmaceutical Corp. Ltd.) and water- (Soluvit, Sino-swed Pharmaceutical Corp. Ltd.) soluble vitamins as well as trace elements (Addamel N, Sino-swed Pharmaceutical Corp. Ltd.). The PN solution was prepared under sterile conditions and the details of PN composition are shown in Table 1.

Measurements and laboratory tests. Body weight was measured immediately before the start of catheterization and at the time of sacrifice and liver weight was measured. Blood was collected for liver biochemistry tests, and the mice were sacrificed using CO₂ at the end of the experiment, either on day 5 or day 7. The serum was prepared by centrifugation (10 min, 3,000 rpm) and was stored at –80°C until liver function tests were analyzed. Serum bilirubin, γ -glutamyl transferase (GGT), alanine aminotransferase (ALT), total plasma cholesterol and triglyceride were determined with an automated chemistry analyzer (Olympus AU400; Olympus, Tokyo, Japan).

After blood collection, the entire liver was rapidly excised and weighed. A random biopsy was then taken from the liver, and was fixed in 10% formalin overnight, washed with phosphate buffered saline, then embedded in paraffin, and stained with hematoxylin-eosin (H&E) and periodic acid–Schiff (PAS) for light microscopy. An experienced liver pathologist blinded to the study evaluated the liver histology. Liver steatosis was based on the classification reported by Zaman et al. (16). The degree of steatosis was rated on a scale of 1 to 4 (scale: 1=no steatosis; 2=mild steatosis in <25% of the liver; 3=moderate steatosis in 25–75% of the liver; 4=severe steatosis in >75% of the liver). About 50 mg of frozen

Table 2. Weight changes and liver/body weight.

Group	Control	Lipids	H-lipids
Pre-experimental body weights	19.03±1.97	19.53±1.30	19.79±0.90
Day 5			
Weight changes	0.09±0.53	-2.39±0.31*	-2.07±0.31*
Liver/body weight	0.05±0.01	0.06±0.01	0.06±0.02
Day 7			
Weight changes	0.30±0.71	-1.07±0.31	-0.87±0.51
Liver/body weight	0.05±0.01	0.06±0.01	0.06±0.01

Data are expressed as mean±SD. * $p<0.05$ versus Control.

Table 3. Serum and liver biochemical parameters from mice in three experimental groups.

Group	Control		Lipids		H-lipids	
	Day 5	Day 7	Day 5	Day 7	Day 5	Day 7
DB, $\mu\text{mol/L}$	0.34±0.28	0.42±0.39	1.01±1.12	1.56±1.69*	2.06±0.81*	2.89±0.97*
TB, $\mu\text{mol/L}$	1.58±1.95	1.82±3.92	2.46±2.64	3.05±3.62	5.92±2.73	6.72±2.48*
ALT, U/L	24.78±3.05	35.13±17.45	32.47±7.31	44.87±10.73	48.67±26.88	51.51±11.20
GGT, U/L	0.55±0.24	0.67±0.80	0.83±0.69	1.03±0.85	1.10±0.75	1.15±1.01
Serum TG, mmol/L	0.82±0.13	1.10±0.82	2.13±0.21*	2.89±0.40*	2.91±1.01*	3.11±0.55*
Hepatic TG, $\mu\text{mol/g}$	2.01±0.21	2.10±0.58	5.05±1.61	9.59±2.08*	21.32±8.13*	31.21±7.11**

DB, direct bilirubin; TB, total bilirubin; ALT, alanine aminotransferase; GGT, γ -glutamyl transferase; TG, total triglyceride. Data are expressed as mean±SD. * $p<0.05$ versus Control, ** $p<0.01$ versus Control.

liver tissue was homogenized in a closed tube with volumes of 0.9% (w/v) NaCl solution by two 10-s bursts of a tissue disintegrator at 13,500 rpm, and the homogenates were then centrifuged at $2,000 \times g$ for 15 min to obtain the supernatants. Forty microliters of the hepatic supernatants was used for measurements of triglyceride and total cholesterol level. Levels of hepatic triglyceride and total cholesterol were measured by using assay kits (Pierce, Rockford, IL).

Total RNA was isolated from liver tissue using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was transferred from RNA by performing reverse transcription using a PrimeScript RT reagent kit (Takara, Dalian, China). The qRT-PCR experiment used a volume of 20 μL and was performed using a SYBR Green PCR kit (Roche Diagnostics, Indianapolis, IN). The final reaction was carried out on a stepone Plus Real-time PCR System (Applied Biosystems, Foster City, CA). Hepatic FXR and downstream genes related to bile salt transport were then measured with a RT-PCR system. The following sequences were used: FXR forward: 5'-TGGGCTCCGAATCCTCTTAGA-3' and reverse: 5'-TGCTCCTCAAATAAGATCCTTGG-3'; Bsep forward: 5'-GCTGCCAAGGATGCTAATGC-3' and reverse: 5'-TTTGCTTCTGCCACCACTC-3'; Mrp2 forward: 5'-TGTAACATCAAGAGCACCGAGA-3' and reverse: 5'-GCAGTCCAATGGAGGCAATA-3'; *ost α* forward: 5'-AACAGAACATGGGATCCAAGTTT-3' and reverse: 5'-CAGGGCGGTCAGGATGA-3'. GAPDH was used as an endogenous control. Levels were determined

with iQ5 software (Bio-Rad, Hercules, CA).

Statistical analyses. For statistical analyses, SPSS 16.0 was used. A 2-tailed probability value of <0.05 was used as a criterion for statistical significance. Differences in the end-points between groups were determined by a Kruskal-Wallis ANOVA test and a Dunn-Bonferroni test for post hoc comparison. Data are expressed as mean±SD for parametric data, or median (interquartile range) for nonparametric data. A probability value of $p<0.05$ is considered significant. Groups of data that failed tests for normality and equal variance were analyzed by the nonparametric Kruskal-Wallis ANOVA.

RESULTS

There was no significant difference in body-weight before the experiment among groups but body weight decreased significantly in the Lipids and H-lipids groups at day 5. There were no significant differences between the three groups at day 7. There were no significant differences in liver/body weight among groups (Table 2).

Serum and liver biochemical parameters

Table 3 illustrates that serum bilirubin and liver functions gradually increased with time. The values of direct bilirubin were markedly increased in the H-lipids group at day 5 and 7, compared with the control group. In both the lipids and H-lipids groups, the results showed clear though insignificant increases in ALT and GGT with time. In addition, serum and liver cholesterol in the experimental groups significantly increased compared with the control at day 5 and 7, while serum and liver

Fig. 1. Gross and H&E stained sections of livers from experimental groups (liver sections at 200 magnification).

Fig. 2. PAS staining of liver specimens from experimental groups at day 7 (liver sections at 400 magnification). (a) Control group; (b) lipids group; (c) H-lipids group.

triglyceride in the H-lipids group significantly increased compared with the control at day 5 and 7.

Liver histology

Livers from control mice appeared grossly and microscopically normal with no steatosis at day 5 or day 7 (steatosis score 1(1–2)). After days of TPN, mice in the lipids group had no to mild steatosis which was most prominent within hepatocytes around the portal and central vein regions at day 5 or day 7 (steatosis score 1(1–2) and 2(1–3), respectively) while mice in the H-lipids group had mild to moderate steatosis (steatosis score 3(2–4) and 3(3–4), respectively). The steatosis scores in the H-lipids group were much higher than in the other two groups at either day 5 or day 7 ($p < 0.05$).

Control tissues showed normal hepatic architecture; in contrast, liver from the H-lipids group appeared grossly pale and had diffuse involvement of hepatocytes by macrovesicular and microvesicular steatosis on H&E sections (Fig. 1). Most of the cell cytoplasm in the TPN groups was occupied by clear vacuoles with minimal PAS staining (Fig. 2). There was no obvious acute inflammatory change, hepatocellular cholestasis or canalicular cholestasis, or fibrosis in any group on H&E sections.

FXR mRNA expression

FXR plays a key role in bile salt homeostasis. Seven days of continuous PN resulted in a decrease of mRNA expression of hepatic FXR, especially in the H-lipids group. Expression of the canalicular transporter of biliary bile salt, and downstream *Mrp2* and *Bsep* genes,

Fig. 3. Effect of PN on hepatic FXR and downstream mRNA expression in the liver. Data are expressed as mean $\pm$ SD. * $p < 0.05$ versus Control.

were also decreased after 5 and 7 d of PN, which may underlie conjugated hyperbilirubinemia after 5 and 7 d of PN. The expression of $\text{ost}\alpha$, a protein that stimulates sodium-independent bile acid uptake, was similar in all groups (Fig. 3).

DISCUSSION

In the present study, we found that a higher dose of soybean lipids is more likely to cause elevations in serum bilirubin and liver steatosis in a mouse model of TPN. After days of a high level of lipid infusion, the liver appeared grossly pale and had severe diffuse involvement of hepatocytes by macrovesicular and microvesicular steatosis. Steatosis may progress from hepatocytes around the portal and central vein regions to all regions of the liver parenchyma. It is consistent with our clinical findings and previous studies that soybean oil is injurious and dose dependent, and this is useful to further investigate the pathogenesis and treatment of PNALD.

Although PN is lifesaving, PNALD is a challenging problem that concerns all of us. PNALD is a common complication in patients receiving long-term PN and develops in 15–40% of adults compared with 40–60% of infants on long-term PN (17). It has become more general in clinical practice but the etiology is still not clear. Most of the reviews have considered it as multifactorial. A number of factors including low birth weight, the duration of PN, sepsis, excess energy, and the cumulative amount of soybean lipid infusion have been associated with liver dysfunction (1, 4, 5, 13). Deficiencies in essential fatty acids, choline, and taurine may also contribute to the development of PNALD (4, 18). The predominant histological finding in infants and children is intrahepatic cholestasis along with bile duct proliferation and fibrosis. Unlike in infants, the most common histologic finding in adults is liver steatosis (19, 20). This may reflect the immaturity of the liver in biliary excretion in infants. More recently, focus has been placed on the composition of the lipid emulsion administered with PN as a potentially modifiable risk factor. We concur that excess intravenous soybean lipid infusion may be a key factor in the development of liver disease. We therefore examined whether different doses of parenteral lipids would affect the development of liver disease, and further investigated the possible pathogenesis of it.

Conventionally we use a dose of 2.5 g/kg/d to prevent essential fatty acid deficiency in clinical practice. Fell et al. (21) showed mice had normal livers, spleens, and lungs after 19 d of soybean oil infusion at a dose of 2.4 g/kg/d. Judith et al. (22) intended to evaluate liver function with different caloric loads and different amounts of lipids in a rat model. They showed that a maximal infusion of 1,611 J/kg/d total energy and 15 g/kg/d lipids was not associated with abnormal liver function tests or with histological evidence of cholestasis. Li et al. (23) showed when given a dose of 0.3 g lipids intravenously per day, mice experienced significant increases in serum transaminase, direct bilirubin, and bile acids. In this study, we used two dose levels of lipids (about 0.14 g/d and 0.3 g/d, respectively), to study

whether different doses of parenteral lipids would affect the development of liver disease. The predominant histological change in this study was steatosis. No evidence of acute inflammation or hepatocellular cholestasis was observed. This study provides evidence that steatosis may be the first stage of liver damage in mice receiving high-dose or long-term intravenous lipids. The results of the study were in accord with our clinical findings. In our recent study, liver biopsy from patients on long-term PN showed diffuse steatosis with hepatocellular cholestasis while most patients infused with a TPN regimen containing fish oil lipid emulsion for 1 mo had their serum bilirubin normalized and showed pronounced histologic improvement (12). It indicates that soybean-based lipid emulsion may play a key role in the development of PNALD. Javid et al. (24) found that soybean lipids given intravenously could cause liver steatosis but little or no injury was induced when they were given orally in mice. Intravenous lipid emulsion may not be metabolized in a physiological manner and can cause liver steatosis. The role of intravenous lipid in the development of PNALD is not clear, but at least some factors may contribute to the problem. First of all, lipids contain more energy per unit and excess energy is associated with development of cholestasis. We have proved that a reduced non-protein energy supply was beneficial in decreasing cholestasis in both rats and severely ill patients in our previous study (25). Secondly, soybean oil-based lipids, which predominantly contain omega-6 polyunsaturated fatty acids (ω -6 PUFAs), have been shown to impair biliary secretion and generate a pro-inflammatory response (26). Furthermore, recent evidence suggests that one major contributing factor that predisposes patients to PNALD may be hepatotoxic phytosterols. Conventional soybean-based lipids contain significant quantities of phytosterols (327–383 mg/L from manufacturers' data), which act as antagonists to the nuclear receptors that are critically involved in hepatoprotection from cholestasis (27). Farnesoid X receptor (FXR) is a member of ligand-activated nuclear receptors and plays a key role in bile salt homeostasis. FXR serves as a sensor for bile acids and promotes enterohepatic clearance of bile acids by controlling the expression of genes involved in their transport and metabolism (28). Under cholestatic conditions, FXR induces transporters in the liver to promote hepatic efflux of bile acids into the bile and blood. Gene studies have demonstrated that FXR knockout mice lack these hepatoprotective mechanisms and are ultrasensitive to BA-induced injury (29). Activation of FXR in vivo was associated with increased hepatobiliary circulation of bile acids, inhibition of hepatic bile acid biosynthesis and reduction in plasma triglycerides. Similar to a previous study (30), 7 d of continuous PN resulted in a decrease of mRNA expression of hepatic FXR, especially in the H-lipids group. Expression of the canalicular transporter of biliary bile salt, and downstream Mrp2 and Bsep genes, were also decreased after 5 and 7 d of PN, which may underlie conjugated hyperbilirubinemia after 5 and 7 d of PN. The suppression of FXR target genes in the liver might play a role in the pathogenesis

of parenteral nutrition-associated liver disease induced by a high dose of lipids.

Nevertheless, this study also has certain limitations. Although it is indicated that soybean-based lipid emulsion may play a key role in the development of PNALD, the underlying mechanisms involved in it should be elucidated in follow-up studies. FXR functions as the chief sensor of intracellular bile acid levels. It is very necessary to study the changes of FXR and downstream genes in mouse lipid metabolism. We will still focus on uncovering the mechanism in our further research. In addition to the lipids themselves, other contaminating factors such as phytosterols that are present in most commercial lipids could also play an essential role. Furthermore, whether an increase in mortality related to central line infections after 7 or more days is unknown.

In summary, high doses of parenteral lipids are more likely to develop PNALD and liver steatosis is the predominant histological finding in mice with high doses of lipids. Farnesoid X receptor may play a key role in the development of parenteral nutrition-associated liver disease. This model is useful to further investigate the pathogenesis and treatment of PNALD.

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