

Influence of Different Intravenous Lipid Emulsions on Hepatobiliary Dysfunction in a Rabbit Model

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ABSTRACT

Objectives: Long-term total parenteral nutrition (TPN) in children is often complicated by the development of cholestasis, liver fibrosis, and liver failure. High doses of intravenous lipids may be involved in the pathogenesis of hepatobiliary dysfunction. The purpose of this study was to determine whether the use of 2 newly developed lipid emulsions could reduce liver damage.

Materials and Methods: Three groups of prepubescent rabbits received TPN including a lipid emulsion either based on soybean oil, olive oil, or soybean oil with n-3 fatty acids added. Enterally fed animals served as controls. After 21 d the animals were killed. Serum samples were obtained at the beginning and end of the study period. Specimens were processed for histological evaluation using a specific score to assess the severity of liver damage.

Results: Biochemical parameters did not predict the extent of liver damage. Hydropic degeneration as an indicator of toxic

liver injury was the predominant histological alteration regardless of the type of lipids infused. The extent of fibrosis did not significantly differ among treatment groups except for animals infused with n-3 fatty acids exhibiting increased fibrotic transformation as compared with controls.

Conclusion: In our animal model, the use of a lipid emulsion with a reduced amount of polyunsaturated fatty acids was not superior to a lipid emulsion based on soybean oil. Long-term application of n-3 fatty acids was associated with more extensive fibrosis. Therefore, intravenous n-3 fatty acids containing lipid preparations (fish oil) should not be used in patients for long-term TPN. *JPGN* 44:237–244, 2007. **Key Words:** Total parenteral nutrition—Hepatobiliary dysfunction—Intravenous lipid emulsion—n-3 fatty acids. © 2007 by European Society for Pediatric Gastroenterology, Hepatology, and Nutrition and North American Society for Pediatric Gastroenterology, Hepatology, and Nutrition

INTRODUCTION

Since the introduction of catheters for long-term central venous access, long-term total parenteral nutrition (TPN) has become a treatment option for newborn infants with extreme short bowel syndrome and severe intestinal motility disorders. Children may now survive for many years despite intestinal insufficiency that prevents complete enteral nutrition.

Hepatobiliary dysfunction and subsequent liver cirrhosis are well-known complications of long-term TPN and represents the most common organ failure during TPN.

Typically, signs of cholestasis, portal inflammation, and fibrosis are observed in histological sections of liver biopsies (1). Due to the immaturity of liver functions, newborn and especially premature infants are more susceptible than older children and adults to this potentially life-threatening complication (2). The cause of hepatobiliary dysfunction is considered multifactorial. Elimination of enteral food intake, systemic infections, components of the TPN solution, toxic biliary acids, and toxic substances such as plasticizers of polyvinyl chloride-containing tubing or phytosterols have been implicated (3–5). Moreover, it has been shown that the type of lipid emulsion may also play an important role (6,7).

Most commercially available intravenous lipids are based on soybean oil with a high content (62%) of polyunsaturated fatty acids (PUFA) and γ -tocopherol. PUFA induce free radical formation and increase oxidative stress. A lipid emulsion partially based on olive oil is now available which contains 20% PUFA

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and 60% monounsaturated fatty acids (MUFA) as well as α -tocopherol, which is more biologically active than γ -tocopherol. Supplemental fish oil emulsions contain predominantly n-3 fatty acids. An increased ratio of n-3 to n-6 fatty acid intake inhibits the metabolism of arachidonic acid and results in reduced production of proinflammatory cytokines such as interleukin (IL)- α and - β , IL-6, IL-8, tumor necrosis factor (TNF)- α and other inflammatory mediators such as platelet activating factor and adhesion molecules (8). TNF has been directly implicated in the pathogenesis of TPN-associated cholestasis (TPN-AC) (9).

In this study, the effects of 3 different lipid emulsions on hepatotoxicity in an established rabbit model of TPN-induced cholestatic liver disease were compared by evaluating the extent of induced liver damage on histological sections and by measuring corresponding biochemical parameters. We hypothesized that an emulsion with a reduced amount of PUFA would be less toxic than a lipid emulsion based on soybean oil, and that the addition of fish oil would be protective because of its anti-inflammatory properties.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits (body weight 1000–1500 g, from Charles River, Germany) were housed under controlled conditions (12 h light-dark cycle, room temperature 18°C, relative humidity 55%) in individual cages. Following anesthesia with ketamine (40 mg/kg) and xylazine (4 mg/kg) administered intramuscularly, a 3F silicone catheter (Access Technologies, Zevenaar, The Netherlands) was inserted into the right internal jugular vein under sterile conditions and tunneled to leave the skin behind the ears. A blood sample was drawn. To protect it from being bitten by the rabbits, the catheter was threaded through a metal sheath and connected to a swivel in the roof of the cage, allowing the rabbits unrestricted movement.

Treatment Groups

Animals were divided into 4 groups. Six rabbits were enterally fed with unrestricted access to a standard rabbit chow (group 1). To exclude unspecified effects from parenterally applied fluids, control animals received 48 mL/d of a standard pediatric infusion containing glucose and electrolytes which delivered \approx 1 g/kg/d of glucose.

Starting from 24 h after implantation of the catheter, the remaining animals received a TPN solution delivered at 5 mL/kg/h that provided 2.5 g protein, 14 g glucose, 3 g fat, and 388 kJ/kg/d with 29% of energy from fat. Electrolytes and trace elements (Peditrace, Baxter Deutschland GmbH, Erlangen, Germany) were added. Vitamins (Vitalipid infant N, Soluvit, Fresenius Kabi AG, Bad Homburg, Germany) were added to the lipid emulsion and infused separately. Syringe and tubing used for the lipid solutions were protected against light and did not contain polyvinyl chloride. All of the animals had free access to

tap water but were not fed enterally. Intravenous lipids based on soybean oil (Intralipid, Baxter Deutschland GmbH, Erlangen, Germany) were given to 8 animals (group 2). Nine animals received either lipids based on olive oil with reduced amounts of PUFA (ClinOleic, Baxter Deutschland GmbH, Erlangen, Germany) (group 3) or Intralipid combined with 0.2 g/kg of a lipid emulsion that contained 50% n-3 fatty acids (Omegaven, Fresenius Kabi AG, Bad Homburg, Germany) (group 4). All of the solutions were delivered by metered pumps as continuous infusion.

Histological, Biochemical, and Hematological Parameters

After 3 weeks of TPN animals were killed. Blood was drawn from the inferior vena cava for the determination of liver-related biochemical (cholesterol, triglycerides, glutamic-oxaloacetic transaminase [GOT], γ -glutamyl transferase [GGT], bilirubin) and hematological parameters (hemoglobin, leukocytes, thrombocytes). Liver specimens were taken from the anterior left hepatic lobe and fixed in 4% buffered formalin solution for histological evaluation. Sections from paraffin-embedded material were processed for routine histology (hematoxylin-and-eosin staining). In addition, Azan and Masson-Goldner trichrome staining was performed to assess the extent of fibrosis. Sudan III staining was obtained from sections of frozen liver tissue to demonstrate intracellular lipid storage. The histological score according to Curran et al (10) was used to grade tissue alterations with respect to location and intensity of inflammation, fibrosis, bile duct proliferation, intracellular lipid inclusions, and cellular changes such as hydropic degeneration or necrosis. The score was adapted to take into account the specific features of normal rabbit liver histology (Table 1). A pathologist blinded to the assigned treatment group examined the tissue sections.

Statistical Analysis

Statistical analysis was performed using SPSS 11.5 statistical analysis system (SPSS Inc, Chicago, IL). Differences between means were determined by using Student *t* test for paired and unpaired data. A probability level of <0.05 was considered significant. The study was approved by the animal research committee of the Universitaetsklinikum Schleswig-Holstein.

Conflicts of Interest

The lipid emulsions were sponsored by the manufacturers (Fresenius Kabi AG, Baxter Deutschland GmbH). The companies did not have any influence on study design, the experiments, nor analysis and interpretation of results.

RESULTS

Gross Morphology

Animals on TPN gained less weight than those with free access to food (Fig. 1). On gross morphological evaluation, all of the groups showed a normally configured

TABLE 1. Histological scoring system

Grading	Hydropic degeneration	Intracellular lipid inclusions	Cellular necrosis	Inflammation	Fibrosis	Bile duct proliferation
0	None	None	None	None	None	<2 bile ducts/portal area
1	<25% of cells	<25% of cells	Single cell <4	<20 leukocytes/portal area	Fine septa within lobule (normal rabbit liver)	>2 bile ducts/portal area
2	<50% of cells	<50% of cells	Single cell ≥4	>20 leukocytes/portal area,	Mild proliferation limited to portal area	>5 bile ducts/portal area
3	<75% of cells	<75% of cells	Cell cluster	Infiltrate beyond portal area	Moderate fibrosis with enlargement of portal area	Irregular bile duct epithelium
4	≥75% of cells	≥75% of cells			Severe fibrosis with bridging between portal areas	
5					Cirrhosis	

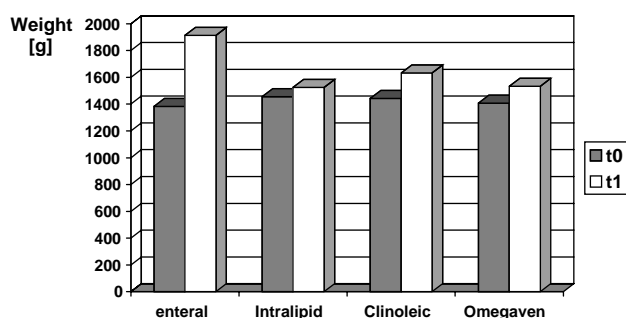


FIG. 1. Weight gain during parenteral nutrition.

liver anatomy. Although gall bladders were more distended in groups treated with TPN, sludge or bile stones were not observed.

Hematological and Biochemical Parameters

A significant decrease in hemoglobin occurred in all of the study groups, but not in control animals. A rise in the leukocyte count was seen in all of the groups, whereas thrombocytes remained unaffected (Table 2). As a consequence of intravenous lipid administration, a fall in serum cholesterol levels was not observed in parenterally fed animals, and enterally fed animals had significantly lower cholesterol levels at the end of the study period. Other biochemical parameters, such as GOT, bilirubin, triglyceride levels, or albumin, remained unchanged in

both enterally fed animals and groups treated with TPN, regardless of the type of lipid emulsion administered. A significant fall of GGT was noted in all of the groups except group 3. In this group 1 animal had a GGT of 87 U/L at the end of the study. Differences in cholesterol and triglyceride levels between treatment groups at the beginning of the study period were not statistically significant (Table 3).

Histological Findings

The most obvious and significant histological alteration consisted of extensive hydropic degeneration of hepatocytes in all parenterally fed animals, which was not observed in controls (Fig. 2A–D). Swollen hepatocytes were predominantly located adjacent to the central vein and exhibited vacuolated or “empty” cytoplasm. The degree of hydropic degeneration ranged between 2.3 (ClinOleic), 2.4 (Omegaven), and 2.7 (Intralipid) (Table 4).

Moreover, TPN-treated animals were characterized by signs of fibrosis (Fig. 3A–D) and showed higher mean values for the degree of fibrotic changes (Table 4). In animals that had received Omegaven, fibrosis was significantly more extensive compared with that in control animals, whereas the degree of fibrosis did not reach statistical significance in groups 2 (Intralipid) and 3 (ClinOleic). The development of fibrosis was independent of an inflammatory infiltrate or concomitant cell necrosis. Only 1 animal group (group 3, ClinOleic)

TABLE 2. Hematological parameters before and after TPN

	n	Hemoglobin [g/L]			Leukocytes [/ μ L]			Thrombocytes [/ nl]		
		T0	T1	P	T0	T1	P	T0	T1	P
Enteral	6	124 ± 4.8	112 ± 11.5	ns	283 ± 46	575 ± 198	0.03	468 ± 208	266 ± 210	ns
Intralipid	8	118 ± 2.5	102 ± 9.5	0.05	295 ± 71	582 ± 101	0.005	288 ± 81	406 ± 44	ns
ClinOleic	9	130 ± 9.5	104 ± 10.2	0.02	384 ± 132	895 ± 193	0.01	355 ± 114	424 ± 119	ns
Omegaven	9	130 ± 10.5	101 ± 17.7	0.01	362 ± 95	937 ± 672	ns	366 ± 125	233 ± 194	ns

T0, beginning of study period; T1, end of study period. Values expressed as mean ± standard deviation.

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TABLE 3. Biochemical parameters before and after TPN

n	Cholesterol [mmol/L]			Triglycerides [mmol/L]			GOT [U/L]			GGT [U/L]			Bilirubin [μ mol/L]			Albumin [g/L]		
	T0	T1	P	T0	T1	P	T0	T1	P	T0	T1	P	T0	T1	P	T0	T1	P
Enteral	182 ± 70	124 ± 62	0.009	110 ± 52	158 ± 107	ns	9 ± 3.5	17 ± 11	ns	6.8 ± 2.1	3.5 ± 0.5	0.006	<15	79 ± 34.5	ns	37.2 ± 2.1	39.6 ± 4.3	ns
Intralipid	133 ± 15	149 ± 84	ns	96 ± 76	112 ± 60	ns	14 ± 7.6	13 ± 10	ns	8.4 ± 4.8	4.1 ± 2.1	0.02	<15	<15	ns	35.3 ± 1.9	35.9 ± 3.2	ns
ClinOleic	206 ± 74	235 ± 145	ns	249 ± 201	162 ± 77	ns	10 ± 4.2	23 ± 52	ns	7.2 ± 5.0	19.8 ± 38	ns	<15	78.5 ± 137	ns	35.9 ± 3.7	34.1 ± 2.6	ns
Omegaven	222 ± 85	202 ± 90	ns	118 ± 39	111 ± 63	ns	9.8 ± 6.7	130 ± 232	ns	7.5 ± 3.4	5.0 ± 3.6	0.003	<15	71.4 ± 133	ns	37.8 ± 2.1	35.6 ± 6.9	ns

T0, beginning of study period; T1, end of study period. Values expressed as mean ± standard deviation.

showed severe histological changes characterized by extensive fibrosis as well as inflammatory infiltrates extending beyond the portal area and clusters of single cell necrosis. Intracellular lipid inclusions were not observed in controls and were only scarcely distributed in TPN-treated animals. Bile duct proliferation was neither found in the control group nor in the parenterally fed groups.

Statistical comparison between the TPN-treated groups concerning the 3 different lipid emulsions applied (Intralipid, ClinOleic, Omegaven) revealed that each of the observed histological alterations did not differ significantly among the groups.

Correlation between Biochemical and Histological Parameters

Five animals had abnormal liver function tests. Two of them were in the control group with normal blood films and liver histology, thus no sound explanation for the elevation of transaminases and bilirubin could be given. In liver sections of 1 animal with elevated liver enzymes (Clinoleic) marked steatosis, a granulocytic infiltrate and extensive fibrosis were observed. The other 2 animals had received Omegaven. In 1 case, histology only demonstrated hydropic degeneration. The other animal had marked leukocytosis, thrombocytopenia, little hydropic degeneration, but severe fibrosis. On overall analysis elevation of liver enzymes or bilirubin did not predict the extent of histological alterations or exclude liver damage.

DISCUSSION

In this animal model of long-term TPN, prepubertal rabbits received differently composed lipid emulsions for 21 d. The potential effects of Intralipid (high content of PUFA), ClinOleic (reduced content of PUFA), and Omegaven (increased n-3/n-6 fatty acid ratio) on hepatotoxicity were evaluated by means of a histological scoring system and both biochemical and hematological parameters.

This animal model was developed to study the hepatotoxicity of TPN. Loff et al demonstrated similar histological finding in infants affected by TPN-associated cholestasis and completely parenterally fed rabbits (11). In clinical practice complete intolerance of enteral nutrition with intact bowel reflects the situation of a subgroup of patients on parenteral nutrition with severe chronic intestinal pseudoobstruction, who also develop TPN-associated cholestasis. We therefore regarded the animal model in its present form valid to draw conclusions with respect to our patients, most of whom tolerate some enteral feeding, which protects against cholestasis.

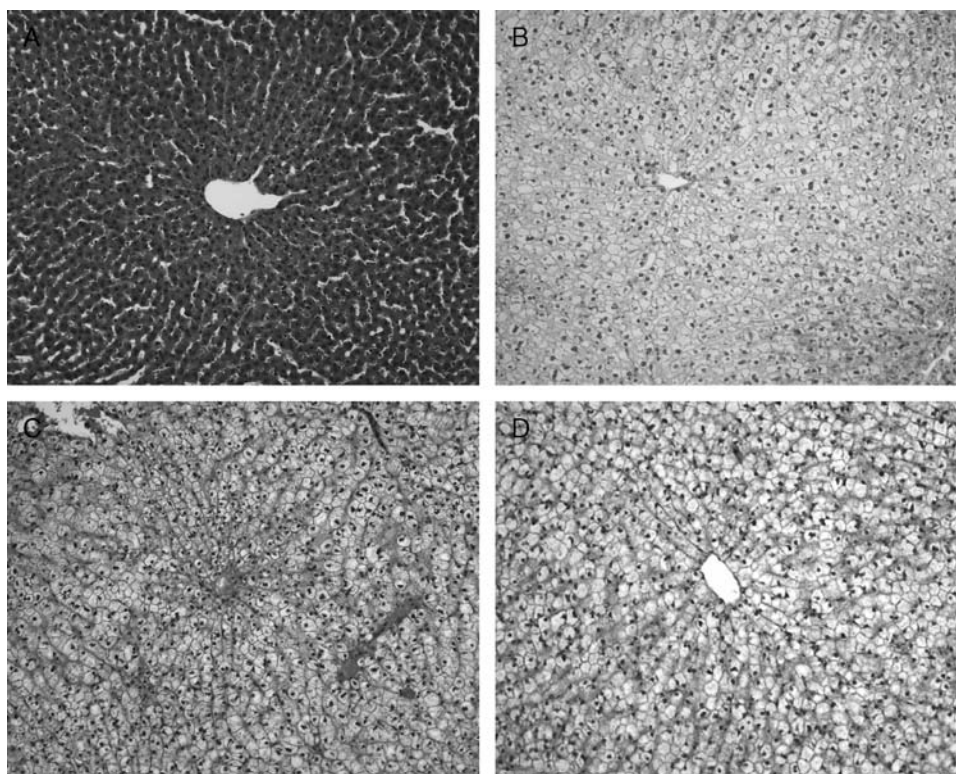


FIG. 2. (A) Normal liver lobule of a control animal (H&E staining). (B) Centrilobular accentuated hydropic degeneration of nearly all hepatocytes (Intralipid) (H&E staining). (C) Moderate hydropic degeneration predominantly located adjacent to the central vein. Hepatocytes are swollen and exhibit clear cytoplasm (ClinOleic) (H&E staining). (D) Extensive hydropic degeneration of the perivenular hepatocytes (Omegaven) (H&E staining).

Animals in the control group gained more weight than parenterally fed animals. Because energy consumption in control animals, which had unlimited access to rabbit chow, was not measured, we cannot decide whether these animals had lower energy requirements or the parenterally fed rabbits experienced a lack of nutrients. In the treatment groups, liver damage may have been aggravated because of malnutrition. These animals received the same amounts of all nutrients and thus, differences of liver pathology caused by different lipid emulsions were not influenced by hypocaloric nutrition.

The observed decrease in hemoglobin under TPN was most likely the result of low iron supplementation.

Cholesterol levels remained high under lipid-containing infusion regardless of the type of lipid administered. Liver-specific biochemical parameters remained unaffected after TPN, again independent of the type of lipid emulsion. In particular, neither bilirubin levels nor transaminases correlated with the severity of histological liver damage and thus may not be considered to be reliable parameters to monitor early stages of hepatocellular injury in rabbits. This observation is in accordance with previous data reported in rabbits (12) and also in infants receiving long-term TPN. In children, only direct hyperbilirubinemia but not elevation of transaminases is a predictor of worsening fibrosis (1). Despite intracellular

TABLE 4. Histological findings based on the scoring system*

	n	Hydropic degeneration	P	Intracellular lipid inclusions	P	Inflammation	P	Fibrosis	P	Bile duct proliferation	P
Enteral	6	0.4 ± 0.5		0		1.1 ± 0.3		0.8 ± 0.4		1	
Intralipid	8	2.7 ± 0.5	<0.001	0.3 ± 0.7	ns	1.1 ± 0.3	ns	1.6 ± 1.4	ns	1.1 ± 0.3	ns
ClinOleic	9	2.3 ± 1.2	0.001	1.3 ± 2.1	ns	1.2 ± 0.7	ns	1.3 ± 1.1	ns	1	ns
Omegaven	9	2.4 ± 0.9	0.001	0.1 ± 0.4	ns	1 ± 0	ns	2.1 ± 1.6	0.03	1	ns

* See Table 1. P values refer to the control group (enterally fed animals).

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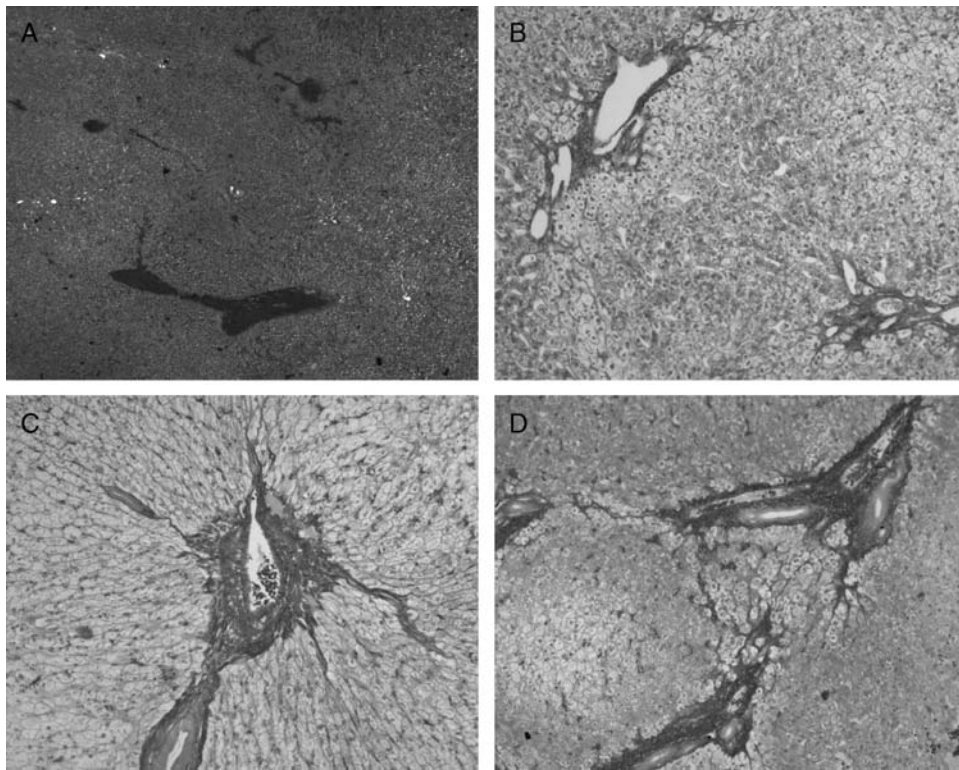


FIG. 3. (A) Liver section of a control animal without fibrosis or hydropic degeneration (Azan staining). (B) Moderately enlarged fibrotic portal tracts with adjacent thin bands of sinusoidal fibrosis (Intralipid) (Azan staining). (C) Enlarged fibrotic portal tract with extension of the fibrosis to the portal-portal septa and minimal pericellular fibrosis (ClinOleic) (Azan staining). (D) Enlarged fibrotic portal tracts extend into the interlobular space and surround the periphery of liver lobules (Omegaven) (Azan staining).

damage, hydropic degeneration of hepatocytes may not cause a significant release of liver enzymes into the bloodstream.

In contrast, at the histomorphological level several correlates of both parenchymal and stromal alterations could be observed after TPN. In comparison to controls, all parenterally fed animals developed hydropic swelling of hepatocytes. The extent of hydropic degeneration was not determined by the type of lipid emulsion. Although signs of fibrosis were more pronounced (higher mean values) in TPN-treated groups, the findings did not differ significantly at statistical analysis except for animals receiving n-3 fatty acids (Omegaven), which developed significantly higher degrees of hepatic fibrosis as compared with controls.

Intravenous lipid preparations are indispensable components for patients on long-term TPN. Only the combination of glucose, lipids, and amino acids provides enough substrate to meet daily requirements for energy supply. Lipids also provide essential fatty acids (13). Although high parenteral lipid intake is a risk factor for the development of TPN-associated cholestasis in children and adults (6,14), it is not evident from these clinical data which component of the lipid emulsion causes liver injury.

Lipid emulsions based on soybean oil with a high content of PUFA induce free oxygen radical formation and oxidative stress, which may render them more hepatotoxic (15,16). In adult patients, TPN with a lipid infusion rich in PUFA increased oxidative stress and lowered α -tocopherol concentration (17). In preterm infants, lipid peroxidation during TPN was associated with hepatocellular injury (18).

ClinOleic, which contains a larger proportion of MUFA as well as α -tocopherol, should reduce oxidative stress and preserve antioxidant capacity. Lipid peroxidation has been shown to be lowered in rats treated with ClinOleic as compared with a medium-chain/long-chain triglycerides mixture based on soybean oil (19). Clinical studies with ClinOleic in long-term TPN are scarce. In premature infants vitamin E status was improved with ClinOleic, but malondialdehyde as a marker for oxidative stress was equally elevated in infants receiving lipids based on olive oil or soybean oil (20). In older children the peroxidation index was lower with ClinOleic (21). Both studies, however, were not designed to compare the extent of liver damage during treatment with the 2 different lipid emulsions. Despite the potential advantages of MUFA, we did not observe a protective effect on liver cell damage in our animal model.

In the liver, irrespective of etiology, the release of proinflammatory cytokines inhibits hepatocellular bile secretion and induces cholestatic hepatitis. The clinical spectrum of inflammation-induced cholestasis includes viral- and drug-induced hepatitis, systemic bacterial infections, and alcohol (22). Systemic and intrahepatic synthesis of TNF- α and other proinflammatory cytokines initiate a cascade of events that eventually leads to extensive liver fibrosis (23). Inflammatory mediators are elevated in patients receiving long-term TPN (24). In this context, TPN-related liver damage may therefore also be regarded as an inflammatory reaction.

Fifty percent of lipids in the intravenous fish oil preparation Omegaven are n-3 fatty acids, mainly docosahexaenoic acid and eicosapentaenoic acid. In combination with a regular lipid emulsion, the ratio of n-3 to n-6 fatty acids is increased. This results in a shift from prostaglandin synthesis via the arachidonic acid pathway to the synthesis of metabolites of docosahexaenoic acid and eicosapentaenoic acid and subsequently in reduced secretion of proinflammatory mediators (25). In clinical trials with intravenous Omegaven, the inflammatory response was less severe and outcome was improved in patients with early sepsis (26) and after major abdominal surgery (27). We expected a beneficial effect of the anti-inflammatory properties of Omegaven on the development and progression of liver damage during TPN. However, in our animal model, the addition of Omegaven was not protective but led to increased fibrosis.

Cell culture experiments and some of the short-term clinical studies used pure fish oil preparations. High doses of n-3 fatty acids uniformly lowered cytokine production in cell culture. However, in patients serum levels of proinflammatory cytokines were highly variable and independent of the infused lipid preparation (28). In our model, only a fraction of the lipid emulsion was given as n-3 fatty acids, and the maximum dose for adult patients of 0.2 g/kg as recommended by the manufacturer was not exceeded. Because 2.8 g/kg were given as Intra-lipid, the ratio of n-3/n-6 fatty acids was 1:6, whereas the optimal ratio is thought to be 1:2 to 1:3 (29). The supply of n-6 fatty acids may have been too high to suppress cytokine synthesis from n-6 fatty acids precursors with the relatively small amounts of fish oil given in our model. Alternatively, long-term application of n-3 fatty acids may exert adverse effects by accelerating the development of fibrosis in the setting of toxic liver damage. Because we did not measure free fatty acids, membrane fatty acid composition, or cytokines, this explanation for the ineffectiveness of fish oil to prevent TPN-associated cholestasis remains speculative.

The initial change after inflammation-induced liver damage is intrahepatic cholestasis. In infants with TPN-associated cholestasis, biliary stasis, bile duct proliferation, portal inflammation mainly with eosinophilic granulocytes, and fibrosis have been described (1). This is in

contrast to our findings because we found only inflammation and fibrosis but no signs of cholestasis. In a newborn rabbit model TPN induced marked cholestasis within 7 d of initiation (30), corresponding to the findings in premature and newborn infants, whereas the liver of older rabbits reacted differently. Hydropic degeneration, a sign of toxic liver damage, was predominant. As observed in our animal model, intrahepatic cholestasis was not a prerequisite for the development of hydropic degeneration and fibrosis. The present results do not support the initial hypothesis of the hepatotoxic effects of a high content of polyunsaturated fatty acids in TPN. Prepubertal rabbits may be more resistant to oxidative stress, and therefore the lipid itself may not have been as toxic to the liver as other components of the parenteral solution, such as amino acids or plasticizers (4,31).

In conclusion, a lipid emulsion with a reduced content of PUFA and increased levels of α -tocopherol did not lower the extent of TPN-induced liver injury in prepubertal rabbits. The addition of small amounts of n-3 fatty acids to a soybean oil-based lipid emulsion did not reduce histological inflammation and increased the development of subsequent fibrosis. Although we cannot conclude from our experimental findings that ClinOleic is beneficial for newborn infants requiring long-term TPN, there are clinical data that demonstrated that this lipid emulsion is safe in pediatric patients and may be advantageous with respect to oxidative stress. Clinical data on long-term application of intravenous fish oil or data on fish oil in children are not available. On the basis of the present results, the use of Omegaven in long-term TPN cannot be recommended.

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