Total Parenteral Nutrition Induces Liver Steatosis and Apoptosis in Neonatal Piglets

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Abstract

Total parenteral nutrition (TPN) induces a high rate of liver disease in infants, yet the pathogenesis remains elusive. We used neonatal piglets as an animal model to assess early events leading to TPN-mediated liver injury. Newborn piglets (n = 7) were nourished for 7 d on TPN or enteral nutrition (EN) and the liver tissue and isolated hepatocytes were subjected to morphologic and molecular analysis. Histological analysis revealed prominent steatosis (grade > 2) in 6 of 7 TPN pigs, whereas minimal steatosis (grade ≤ 1) was observed in only 2 EN pigs. Abundant cytosolic cytochrome C and DNA fragmentation were observed in hepatocytes from TPN compared with EN piglets. Markers of mitochondrial and Fas-mediated apoptosis were altered in TPN liver tissue, as indicated by a lower ATP concentration (P < 0.05), accumulation of ubiquitin, 9.9-fold activation of caspase-3 activity (P < 0.01), and increased cleavage of poly-(ADP-ribose) polymerase, caspase-8, -9, and -7 when compared with EN livers. Bcl-2 and proliferating cell nuclear antigen expression was downregulated, whereas Fas and Bax were upregulated in TPN livers. However, levels of caspase-12 and Bip/GRP78, both markers of endoplasmic reticulum-mediated apoptosis, did not differ between the groups. Short-term TPN induces steatosis and oxidative stress, which results in apoptosis mediated by the mitochondrial and Fas pathways. Thus, TPN-induced steatosis in newborn piglets may serve as a novel animal model to assess the pathogenesis of fatty liver and apoptosis-mediated liver injury in infants. J. Nutr. 136: 2547–2552, 2006.

Introduction

Since the introduction of total parenteral nutrition (TPN) in the 1960s, its use has become a vital clinical practice to prevent and reverse malnutrition in individuals with various diseases and conditions (1). TPN is used frequently for nutritional support of premature infants and other neonates with disorders of the gastrointestinal tract who cannot tolerate full enteral intake (2–4). However, recent studies have shown that TPN is linked to apoptosis in several tissues. A recent study demonstrated that continuous administration of TPN resulted in decreased oxidative phosphorylation in the hepatic mitochondria of immature rats, leading to speculation that this deterioration in mitochondrial function may contribute to hepatic dysfunction in infants (15). Furthermore, continuous administration of TPN resulted in decreased oxidative phosphorylation in the hepatic mitochondria of immature rats, leading to speculation that this deterioration in mitochondrial function may contribute to hepatic dysfunction in infants (15).

Several animal models of TPN including mice, rats, rabbits, and neonatal piglets have been used to study molecular mechanisms of TPN-induced intestinal injury as well as to screen novel ingredients for parenteral nutrition formulas in clinical use (2,3,16,17). In the current study, we used TPN-fed piglets as a model to assess early morphologic and molecular events in the development of TPN-mediated liver injury in infants. This model provides an opportunity to assess the relative importance of putative growth-regulatory and survival factors in a clinically...
relevant context, because the mechanisms observed in neonatal piglets that spontaneously develop TPN-induced liver disease are likely more relevant to human infants than outcomes demonstrated by experiments in adult rodents, including those with different genetic strains or transgenic manipulation.

Materials and Methods

Animals and diets. The study protocol was approved by the Animal Care and Use Committee of Baylor College of Medicine and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (DHHS publication no. NIH 85–23, revised 1985, Office of Science and Health Reports, DRR/NICHT, Bethesda, MD 2020). The pigs were 2- to 4-d-old, female crossbred piglets obtained from the Texas Department of Criminal Justice, Huntsville, TX. Piglets were surgically implanted with catheters in the jugular vein and adapted to their respective nutritional treatments within 36 h postsurgery, as described previously (18). Piglets were divided into 2 equal groups and were fed enterally or exclusively parenteral nutrition for 7 d. The enteral nutrition (EN) group (n = 7 piglets) was fed a liquid sow milk replacement formula (Advanced LiquiWean; Milk Specialties) at a rate of 50 g/kg body wt−1·d−1 for 7 d. The composition of the sow’s milk formula (g/kg dry matter) was 250 protein, 130 fat, 500 lactose; the ingredients included dried whey protein concentrate, dried whey product, dried whey, plasma protein, and animal and vegetable fat. The amino acid concentration (g/kg) was Ala, 10.72; Arg, 8.76; Asp, 23.22; Cys, 5.31; Glu, 37.36; Gln, 4.48; His, 14.02; Leu, 26.00; Lys, 21.95; Met, 5.69; Phe, 8.13; Pro, 12.83; Ser, 11.34; Thr, 16.53; Trp, 3.80; Tyr, 6.06; and Val, 13.35. The fatty acid concentration (g/kg dry matter) was C10:0-C15:0, 2.61; C16:0, 32.20; C16:1, 37.27; C18:0, 15.88; C18:1, 52.91; C18:2, 12.09; C18:3, 0.43; C20:0, 0.21; and C20:4, 0.42. The ratio of (n-6)/(n-3) fatty acids was 29.19. The second group was given TPN (n = 7 piglets) intravenously administered as an elemental diet containing free amino acids, dextrose, lipid, electrolytes, minerals, and vitamins as described previously (3). The lipid was provided as Intralipid (Fresenius Kabi), which is composed of (g/L) soybean oil, 200; egg yolk phospholipid, 12; and glyc erin, 22.5. The major component fatty acids (g/L) are linoleic, 106; oleic, 50; palmitic, 22; linolenic, 16; and stearic, 6. The daily fluid and micronutrient intakes/kg body wt of both the EN and TPN groups were ~240 mL, 13 g protein, and 900 kJ·kg−1·d−1. Piglets were weighed daily to maintain equal nutrient intake in both groups.

After 7 d of isocaloric and isonitrogenous feeding in both groups, piglets were anesthetized with an intravenous injection of phenobarbital (Fresenius Kabi), which is composed of (g/L) soybean oil, 200; egg yolk phospholipid, 12; and glyc erin, 22.5. The major component fatty acids (g/L) are linoleic, 106; oleic, 50; palmitic, 22; linolenic, 16; and stearic, 6. The daily fluid and micronutrient intakes/kg body wt of both the EN and TPN groups were ~240 mL, 13 g protein, and 900 kJ·kg−1·d−1. Piglets were weighed daily to maintain equal nutrient intake in both groups.

Harvesting primary pig hepatocytes. Primary pig hepatocytes (PPH) were harvested as previously described (19) with sequential perfusion of Liver Perfusion medium (Invitro) followed by HBSS, supplemented with 28 mg/L Liberase (Roche Diagnostics). After perfusion, the capsule was removed and hepatocytes were gently detached from the vascular tree by agitation in the Liberase solution at 37°C. Cells were plated into Hepatocyte Wash medium (Invitro), filtered through sterile gauze, and washed twice by low speed centrifugation (50 × g; 5 min). Cell viability was assessed by trypan blue exclusion.

Morphological and immunohistochemical evaluation. Liver tissues were fixed in 10% formalin and embedded in paraffin. Serial sections were stained with hematoxylin-eosin. Steatosis of liver tissues was graded and staged based on previous published criteria (20). Tissues were scored using a scale from 1 = mild to 4 = heavy steatosis. Sections were also immunostained with primary antibodies against ubiquitin (UB), Bcl-2, proliferating cell nuclear antigen (PCNA), and Bax (Santa Cruz Biotechnology). Glycojen in liver tissues was detected by the periodic acid-Schiff reaction according to the standard procedure (with and without diastase treatment) (21). To detect fat deposits in livers, cryo-sections were stained with Oil Red O (ORO). Transmission electron microscopy was performed as described previously (22).

Cell death detection. Cell apoptosis was measured using the Cell Death Detection ELISA (Roche Diagnostics). This assay is based on the quantitative sandwich–enzyme-immunnoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively.

Detection of DNA fragmentation. A DNA ladderizing method was used to evaluate internucleosomal DNA fragmentation for the confirmation of apoptosis. DNA was extracted using the DNAse Tissue kit-50 (Qiagen Sciences). DNA fragmentation was assessed by electrophoresis on 1.8% agarose gels as described (22).

Western-blot analysis. Western blot was performed as previously described (22). Blots were incubated with primary antibodies: anti-caspase-9, anti-Fas, anti-caspase-7, anti-caspase-3, anti-caspase-8, anti-PCNA, anti-Bcl-2, anti-Bip/GFP78, anti-Bax, anti-caspase-12, anti-poly-(ADP-ribose) polymerase (PARP) (Santa Cruz), and anti-cytochrome C (Pharmigen). After incubation with secondary antibodies, the protein bands were detected with the ECL (Amersham). The relative intensity of bands was measured using NIH image analysis software (ImageJ1.22d, NIH). To determine the cytochrome C levels in PPH by Western blot, the mitochondrial and cytosolic fractions were isolated using the mitochondrial fractionation kit (Active Motif).

Evaluation of caspase-3 activity and hepatic ATP concentration. Caspase-3 activity was measured using a 96-well format of Caspase-3 cellular activity assay kit (Calbiochem). Hepatic ATP concentration was evaluated with luciferase assay using ATP determination kit (Molecular Probes).

Statistical analysis. The significance of the difference between EN and TPN groups was evaluated by the two-tailed Student’s t test. Differences with P < 0.05 were considered significant.

Results

Morphological and immunohistochemical analysis. Body weights of pigs fed via EN and TPN were similar initially (1.62 ± 0.3 kg) and did not differ after 7 d of treatment (2.53 ± 0.3 kg in EN vs. 2.48 ± 0.4 kg in TPN). Livers from EN pigs were normal color, whereas TPN livers were yellowish in appearance, typical for fatty liver (Fig. 1A). The viability of PPH isolated from livers of TPN pigs was 49 ± 7.2%, whereas in EN pigs it was 88 ± 6.3% (P < 0.05). Histological examination revealed macro- or micro-vesicular steatosis in all TPN-liver tissues (Fig. 1B). Furthermore, ballooned, fatty degeneration of hepatocytes was noted in TPN tissues. The affected cells were enlarged and contain pale foamy cytoplasm. In 7 TPN liver specimens, 1 (14%) was graded as 1+, 4 (57%) as grade 2+, and 2 (29%) of 7 TPN livers were graded as 3+. Only 2 of 7 specimens obtained from EN pigs exhibited mild grade (≤+1) steatosis. Based on hematoxylin-eosin staining of liver sections, TPN pigs exhibited classical features of fatty liver disease that resembled nonalcoholic steatohepatitis (NASH) and alcoholic fatty liver disease (AFLD). Liver tissues from EN pigs were ORO stain negative; however, TPN liver tissues were ORO stain positive with massive accumulation of lipid droplets in hepatocyte cytoplasm (Fig. 1C). The amount of glycogen in all TPN livers was also markedly increased compared with EN (Fig. 1D).

Transmission electron microscopy revealed that in EN liver tissues, the hepatocyte architecture was normal (Fig. 2A). The subcellular organelles also had normal morphology. In contrast, the subcellular morphology was abnormal in 4 of 7 TPN liver specimens (Fig. 2B). These livers had morphological characteristics of fatty liver. The mitochondria were swollen, rounded,
and had a markedly abnormal morphology. The cytoplasm of hepatocytes contained accumulation of lipid droplets and granules of glycogen.

**Plasma AST, ALT, and bilirubin.** Plasma levels of AST (42.7 ± 17.1 IU/L in TPN and 31.4 ± 11.7 IU/L in EN; \( P = 0.08 \)) and ALT (20.7 ± 3.6 IU/L in TPN and 22.4 ± 3.2 IU/L in EN; \( P = 0.18 \)) did not differ between the groups. Reference ranges are: AST 10–42 IU/L, ALT 10–63 IU/L, and total bilirubin 2–12 mg/L. However, the plasma bilirubin concentration in TPN piglets was significantly higher (1.36 ± 0.50 mg/L) than in EN piglets (0.22 ± 0.05 mg/L; \( P < 0.001 \)). The data suggest that short-term TPN induces cholestasis without serious cellular injury.

**Assessment of apoptosis via mitochondrial pathway.** Whereas electrophoresis of DNA from 3 of 7 livers from TPN pigs revealed DNA fragmentation, DNA from all 7 EN livers was intact (Fig. 3A). These results were confirmed with quantitative Cell Death Detection ELISA that allows determination of the cytoplasmic histone-associated DNA fragments in cell lysates. This analysis revealed that apoptosis in freshly isolated PPH from all TPN piglets was 2.6-fold greater than in hepatocytes from all EN piglets (\( P < 0.05 \)).

Caspase-9 mediates apoptotic signals in response to mitochondrial damage and activation of caspase-9 requires cytochrome C (27,28). In comparison to EN, TPN livers contained 30% less procaspase-9 (47 kDa) as a result of cleavage and the formation of a 20-kDa cleaved protein (Fig. 3B). The amount of cytochrome C in the cytosolic fraction was markedly increased and that in the mitochondrial fraction comparably decreased in TPN hepatocytes compared with EN hepatocytes (Fig. 3B).

Caspase-3 and -7 are the key executioners of apoptosis. Western-blot analysis of tissues from TPN livers showed the processing of procaspase-7 accompanied by the formation of a new 17-kDa cleaved fragment (Fig. 3B).

**Figure 1** Macroscopic and histological illustration of liver tissues obtained from EN and TPN piglets after 7 days of feeding. Macroscopic appearance of livers: in contrast to normal appearance of EN livers, TPN livers had a distinctive pale yellow color that is typical for fatty liver (A). Hematoxylin and eosin staining of liver tissues (B). TPN liver tissues exhibited characteristics of hepatic steatosis with ballooned, fatty degeneration of hepatocytes. The affected cells were enlarged and contain pale foamy cytoplasm. Cryostat frozen liver sections stained with ORO (C). Arrows indicate fat deposits (Red stain) in TPN liver tissue. Periodic acid-Schiff staining (with and without diastase treatment) shows increased glycogen accumulation in the TPN livers (D). Original magnification of (B, C, D) ×400.

**Figure 2** Transmission electron micrographs of EN (panel A) and TPN (panel B) piglet livers. EN tissues showed normal appearance of mitochondria, nucleus (N), and cytoplasm in hepatocytes and presence of erythrocytes (E); arrows indicate mitochondria. TPN liver specimens showed morphological characteristics of fatty liver with swollen and rounded mitochondria and accumulation of the lipid droplets (L) and granules of glycogen (G) in cytoplasm. Original magnification ×2500. Black bar = 2 μm.

**Figure 3** Assessment of apoptosis in liver tissue and isolated PPH obtained from EN and TPN piglets. Analysis DNA fragmentation: lanes 1 and 2 are DNA from 2 EN livers, lanes 3 to 5 are DNA from 3 different TPN livers; results are representative of 3 independent experiments (A). Western-blot analysis of liver tissue extracts; lanes 1 and 2 are from 2 EN pigs; lanes 3, 4, and 5 are from 3 TPN pigs; β-actin was assessed for adjustment of protein loading (B). Cytochrome C in cytosolic fraction (Cyto-cyto C), Cytochrome C in mitochondrial fraction (Mito-cyto C), procaspases (Procasp), cleaved protein (cleaved), and PCNA. Immunoblots are representative of at least 3 independent experiments.
20-kDa cleaved protein. No activation of caspase-7 was detected in liver specimens from EN piglets (Fig. 3B). The detection of the cleavage of PARP, which is an intracellular substrate of caspase-3 protease, confirmed these results (Fig. 3B). In addition, there was a 9.9-fold increase in the activation of caspase-3 in TPN liver tissues as compared with EN \((P < 0.05)\). The hepatic ATP concentration of TPN liver tissues \((24.37 \pm 1.4 \, \text{nmol/g})\) was 24% lower than that ATP concentration of EN livers \((37.1 \pm 1.5 \, \text{nmol/g}; \, P < 0.05)\). The hepatic ATP concentration of TPN liver tissues \((24.37 \pm 1.4 \, \text{nmol/g})\) was 24% lower than that ATP concentration of EN livers \((37.1 \pm 1.5 \, \text{nmol/g}; \, P < 0.05)\).

Downregulation of Bcl-2 and upregulation of Bax were found in TPN compared with the EN liver samples (Fig. 3B). Furthermore, upregulation of Bax expression was confirmed by Bax immunostaining of liver tissues (Fig. 4A). All EN livers were negative for Bax. Whereas very little or no specific UB immunostaining was present in EN tissues, strong-positive UB immunostaining was identified in all 7 TPN tissues. In 1 of 7 TPN livers (14%), UB staining was graded as 1+, and in the other 6 tissue specimens (87%) it was 2+ (Fig. 4B). Thus, UB immunostaining of liver tissues from TPN pigs demonstrated the levels of cell injury. Cell proliferation in EN and TPN pig livers was assessed based on western-blot analysis of PCNA; we found that PCNA levels in TPN livers were reduced by 61% compared with EN livers (Fig. 3B).

Assessment of apoptosis via Fas- and endoplasmic reticulum-mediated pathways. Western-blot analysis revealed that TPN induced the cleavage of 55-kDa procaspase-8, which resulted in the formation of a 20-kDa cleaved protein (Fig. 3B). In contrast, no activation of caspases-8 was found in EN liver tissues. No cleaved caspase-12 was observed in liver specimens from either TPN or EN piglets. Furthermore, hepatic levels of the inducible chaperon Bip/GRP78, a marker of endoplasmic reticulum (ER) stress response, did not differ between the groups (data not shown).

**Discussion**

The pathogenesis of TPN-induced liver dysfunction is multifactorial and further compromised by necrotizing enterocolitis, sepsis, cardiac failure, shock, cytokines, hypotension, and genetic susceptibility. Lack of enteral feeding that leads to reduced gut hormone secretion, reduction of bile flow, and biliary stasis may be an important mechanism in the development of cholestasis, steatohepatitis, cirrhosis, and liver failure \((8,9,23)\). Most clinical and investigational studies have reported the late stages of TPN-induced liver injury during development of apparent signs of liver injury or decompensation. However, early events leading to clinically apparent TPN-induced liver dysfunction were rarely addressed. As a result, pathogenesis of TPN-induced liver injury remains poorly understood and few approaches have been developed to prevent TPN complications.

To gain insight into the impact of short-term TPN, we utilized TPN piglets as an animal model to assess early molecular events of liver injury. Piglets receiving TPN for 1 wk developed hepatic steatosis. Histology of liver tissues from TPN piglets exhibited cell swelling and an advanced degree of micro- and macro-vesicular steatosis (grade >2) in most animals. These data agree with previous reports suggesting that patients and animals on short-term TPN develop steatosis \((13,23)\). It has been suggested that fatty liver could result from a combination of various phenomena that affect lipid metabolism, namely, increased mobilization from depot fat, increased transport to liver, increased synthesis in liver, impaired transport from liver, and decreased oxidation of fatty acids \((7)\).

Several studies suggested that TPN-induced steatosis may also suppress the ability of hepatocytes to regenerate \((15,24)\). Hepatocytes from \(ob/ob\) mice were vulnerable to TNF-\(\alpha\)-induced cell death compared with normal mouse hepatocytes \((25)\). Furthermore, steatotic liver grafts are associated with a high incidence of primary nonfunction and initial poor function \((24)\). To assess the effect of steatosis and the mechanism(s) of low viability of hepatocytes from piglets on TPN, liver tissues were assessed for markers of apoptosis. DNA fragmentation, the PARP cleavage, and activation of caspase-3 and -7 were seen exclusively in livers of TPN piglets. Caspase-3 and -7 are the key executioners of apoptosis and both are partially or totally responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme PARP \((26,27)\). The fact that steatosis may result in apoptotic changes in diseased liver was also reported in several clinical conditions including NASH and AFLD \((28,29)\). Furthermore, association of steatosis with apoptosis was also confirmed in animal studies that demonstrated increased lipid peroxidation and apoptosis of hepatocytes following short-term TPN \((14)\).

To gain insight into the molecular mechanism leading to apoptosis, PPH and liver tissues were subjected to further analyses. There are three major pathways, including activation of death receptors (Fas ligand, TNF-\(\alpha\)), mitochondrial damage, and stress of the ER that culminate in activation of effector caspases, destruction of chromatin, and subsequent death by apoptosis \((27,30–32)\). Because mitochondria play a central role and interact with other pathways, initial studies were performed to assess the release of cytochrome C and cytochrome C-related caspases. Increased release of cytochrome C from mitochondria and cleavage of caspase-9 were observed in livers from TPN piglets when compared with EN pigs. The Bcl-2 family proteins mediate the major mitochondrial-associated apoptotic-signaling pathway. In this family, Bcl-2 is an antiapoptotic member and Bax is one of the proapoptotic members \((31)\). Proapoptotic proteins of the Bcl-2 family act on mitochondria and facilitate the release of cytochrome C \((33)\). Consistent with increased apoptosis, we found downregulation of Bcl-2 and overexpression of Bax were in all TPN livers as compared with EN. These data are consistent with recent studies that showed proapoptotic modulation of Bax and inhibition of Bcl-2 in mice models of fatty liver \((34)\). Additionally, in all livers from TPN piglets,
accumulation of UB was detected by immunochemistry. The UB-proteasome system, an indirect marker of mitochondrial injury, plays an important role in cellular defense by removing damaged proteins generated by oxidative stress. Apoptosis is an active process that requires ATP for its execution and ATP level is a determinant of cell death by apoptosis (35). We found lower ATP concentrations in livers of TPN piglets compared with EN piglets. These results are consistent with previous reports that demonstrated low ATP concentrations and increased expression of UB in a variety of clinical conditions associated with mitochondrial injury such as NASH and AFLD (20). Moreover, it has been shown that hepatocytes from ob/ob mice (a model for fatty liver) have a reduced ATP concentration and undergo increased cell death after in vitro exposure to TNF-α (25). Taken together, these findings suggest that mitochondrial injury plays a key role in the current neonatal model of TPN-induced liver injury.

Because recent studies suggested that TPN could induce apoptosis through the Fas pathway, liver tissues were subjected to further analyses for markers Fas-mediated apoptosis (28,29). Fas, a transmembrane receptor protein belonging to the TNF receptor family, contains a death domain that signals via apoptotic pathways. Caspase-8 is one of the initiator caspases associated with apoptosis involving death receptors. Increased levels of Fas expression and activation of caspase-8 were found in TPN liver tissues. Interestingly, increased levels of Fas in hepatocytes were recently reported in subjects with a variety of liver conditions with the features of steatosis (36). Furthermore, the levels of caspase-12 and Bip/GRP78, which are involved in the ER-mediated pathway, did not differ between EN and TPNlivers. Therefore, we suggest that it is unlikely that ER-stress response modulated by changes in intracellular calcium concentration and caspase-12 activation are involved in apoptotic events associated with neonatal TPN-induced steatosis and apoptosis.

In conclusion, this study revealed several cellular and molecular mechanisms associated with the pathogenesis of neonatal TPN-induced fatty liver disease. TPN may cause hepatic apoptosis mainly through both the mitochondrial and death receptor pathways rather than via the ER-stress pathway. The death program may be initiated at the cell surface with activation of Fas or TNFR1 (usually involving in receptor trimerization) by their respective ligands, Fas ligand and TNF-α, or may result from a primary disturbance of mitochondrial function. Clearly, defining specific pathways leading to TPN-induced cell injury and apoptosis may lead to development of targeted therapies with antiapoptotic properties. TPN-induced steatosis in newborn piglets may serve as a novel animal model to assess pathogenesis of this condition and provide insight for future intervention(s) to avoid TPN-induced liver injury and newborn morbidity and mortality.

**Literature Cited**