Supplementation of Fat Emulsion Improves Fatty Liver due to Total-Parenteral Nutrition in Young Adult Rats

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Conscious young adult male rats were given total-parenteral nutrition (TPN) with or without a soybean fat emulsion for 4 days and those that did not receive the emulsion (fat-free TPN group) developed severe fatty liver with hyperglycemia, hyperinsulinemia, and hypotriglyceridemia. These disorders were improved by administering TPN supplemented with soybean fat, equivalent to 20% of total calories, with glucose correspondingly reduced. In the fat-free TPN group, the expression of sterol regulatory element binding protein 1c (SREBP1c), acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) mRNAs in the liver were coordinately up-regulated, resulting in an increase in non-essential fatty acids and a decrease in essential fatty acids in the liver, due to the fat supply drying up. In contrast, expression of carnitine-palmitoyltransferase (CPT) 1a mRNA decreased. However, in the group given the fat emulsion in their TPN, the expressions of all of the above types of mRNA remained similar to those in an oral diet control group.

It appeared that the hyperinsulinemia following infusion of excess glucose had induced lipogenesis and a decrease in fatty acid β -oxidation in the liver and that these changes were prevented by supplementation of soybean fat equivalent to 20% of total calories (with a corresponding reduction in glucose). Therefore, it may be appropriate to routinely supplement fat-free TPN with soybean fat in the clinical situation, substituting it for part of the glucose. In addition, serum glucose and insulin levels should be monitored during TPN infusions.

Key words total-parenteral nutrition, soybean fat emulsion, fatty liver, lipogenesis, fatty acids

Introduction

Total-parenteral nutrition (TPN) is widely used to provide complete nutritional support to patients who are unable to eat. It is well known that liver dysfunction with steatosis is a common complication after long-term TPN, in both infant and adult patients¹⁻⁴. In a randomized clinical trial, Buchman et al⁵, found that the use of a balanced energy source TPN solution prevents the liver abnormalities, and a TPN solution in which 40% of total calories were derived from lipid was more effective than one in which 7.5% of total calories were from lipid. Oshita et al⁶, suggested that optimal fat intake may be between 20% and 40% of calories in infant rats. Nishimura et al⁷, reported that metabolic complications in the liver of infant rats owing to excessive administration of fat-free TPN could be prevented by sup-

plying 20% of the total calories from soybean oil. However, in Japan, the clinical situation is still not satisfactory⁸; in our hospital, only about 30% of all TPN prescriptions include lipid emulsion supplementation. In the present study we prepared model rats, which continually received drip infusion through the right jugular vein and were able to freely move in metabolic cages. We used these conscious young-adult rats to examine the mechanisms of the hepatic changes arising from fat-free TPN administration, comparing fat-free TPN with TPN in which 20% of non-protein calories was derived from soybean fat.

Materials and Methods

1. Animals and diets

Male Sprague-Dawley rats at 6 weeks of age were purchased from Nippon SLC Inc. (Hamamatsu, Japan). The rats

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were housed in a light-controlled room (light on from 8 : 45 to 20 : 45) at a temperature of 25 ± 2 and humidity of $60 \pm 10\%$, and allowed free access to water and a standard diet containing 54.7 starch, 18.8 protein, 3.9 fat, 6.6 fiber, and 6.9 mineral and vitamin mixture (LABO MR STOCK®, Nosan Co., Yokohama, Japan) for one week. All animal procedures were in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at the Takara-machi campus of Kanazawa University, Japan.

2. Surgery and TPN solution preparation

Rats were divided into three groups (five in each group). Each rat in the sham-operated control group received a standard oral diet (total energy 259.2 kcal/100 g) and tap water freely in a stainless steel wired metabolic cage. In the TPN groups, the rats were fasted overnight, then a polyethylene catheter (I.D.0.58 mm, O.D.0.96 mm) for the infusion of parenteral nutrition was placed, under anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally), in the right jugular vein into the superior vena cava and routed to the back of the neck to exit through a coil spring attached to a swivel. When the rats recovered consciousness, they were housed in stainless steel wire metabolic cages, in which tap water was freely available. The TPN groups continuously received an increasing amount in a stepwise manner using an infusion pump (BRIFSYS-R 1, Bioresearch Center Co., Tokyo, Japan) : 5 mL/kg body weight/h (120 kcal/kg/day) from just after cannulation for the first 24 h, then 10 mL/kg/h (240 kcal/kg/day) for the next 24 h (2nd day), and 15 mL/kg/h (360 kcal/kg/day) for the 3rd to 4th day. The compositions of the TPN solutions are shown in **Table 1**. The fat-free TPN group received only the basal TPN solution (Amiparen®, Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan) and the TPN with fat group received basal solution supplemented with soybean fat equivalent to 20% of total calories (Intralipos®, Otsuka Pharmaceutical), with a corresponding decrease of glucose concentration. Vitamedin IV® (Daiichi-Sankyo Co. Ltd., Tokyo, Japan) was used to supply vitamins.

Table 1 . Formulation of TPN solutions.

	Fat-free TPN	TPN with fat
Glucose (g)	182	140
Fat (g)	0	18
Amino acids (g)	36	36
Na (mEq)	35	35
K (mEq)	27	27
Mg (mEq)	5	5
Ca (mEq)	7.8	7.8
Cl (mEq)	54	54
P (mmol)	6.5	7.8
Zn (μmol)	20	20

TPN calories per unit volume : 880 kcal/880 mL.

Caloric ratio of carbohydrate calories to nitrogen : 130.

Added fat emulsion is consist of 55% linoleic acid(18 : 2), 25% oleic acid(18 : 1), 8% palmitic acid(16 : 0), 8% γ -linolenic acid(18 : 3), and 3% stearic acid(18 : 0).

3. Biochemical and pathological examinations

Blood samples were collected from the left jugular vein, and centrifuged to provide serum samples, which were stored at -80 until assay. The animals were killed immediately after blood sampling under deep diethyl ether anesthesia. After weighing liver, a part of liver specimen was fixed in 10% buffered formalin and stained with Oil red O and hematoxylin-eosin according to the standard methods, and other parts were used for biochemical examinations.

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (γ -GTP), phospholipids (PL) and fatty acids were measured by SRL Co., Ltd. (Tokyo, Japan). Triglyceride (TG), total cholesterol (TC), free fatty acid (FFA), glucose, and insulin were measured in our laboratory using commercially available assay kits (Wako Pure Chemical, Co., Osaka, Japan).

Hepatic lipids were extracted with chloroform : methanol (2 : 1), according to a published method⁹⁾. The extract was dissolved in water and subsequently analyzed for TG, TC, and FFA using respective kits (Wako).

4. Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from the liver by using an Isogen Kit (Nippon Gene, Tokyo, Japan). Synthesis of cDNA from the isolated total RNA was carried out using RNase H- reverse transcriptase (GIBCO BRL, Rockville, MD). Reverse transcription (RT) reactions were carried out in 40 mM KCl, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP, and dTTP, 10 units of RNase inhibitor (Promega, Madison, WI), 100 pmol of random hexamer, total RNA and 200 units of the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Berlin, Germany) in a final volume of 50 μ L at 37 for 60 min. Polymerase chain reaction (PCR) was carried out in a final volume of 20 μ L, containing 1 μ L of RT reaction mixture, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 10 μ M each of the mixed oligonucleotide primers, and 1 unit of Taq DNA polymerase (Gibco-BRL). Primers used for rat *SREBP-1 c*¹⁰⁾ were 5'-GGA GCC ATG GAT TGC ACA TT-3' and 5'-AGG AAG GCT TCC AGA GAG GA-3' (191 bp), those for rat *ACC I*¹¹⁾ were 5'-CAG ACA GGT TCA AGC TGA AGT CCC T-3' and 5'-GCT GTG CTG CAG GAA GAT TGA CAT C-3' (383 bp), those for rat *FAS*¹²⁾ were 5'-TGC AAC TGT GCG TTA GCC ACC-3' and 5'-TGT TTC AGG GGA GAA GAG ACC-3' (710 bp), those for rat *CPT I a*¹³⁾ were 5'-AGT GAG GAC CAT AAG CAG AG-3' and 5'-ACG TCT GGA CCT AAA GCA GAG-3' (590 bp), and those for rat β -actin were 5'-TTC TAC AAT GAG CTG CGT GTG GC-3' and 5'-CTC (A/G)TA GCT CTT CTC CAG GGA GGA-3' (456 bp), as previously reported by Waki et al¹⁴⁾. Each cycle consisted of 60 sec at 95 , 60 sec at 62 , and 60 sec at 72 for *SREBP-1 c*, 60 sec at 95 , 60 sec at 65 , and 60 sec at 72 for *ACC I*,

60 sec at 95 °C, 60 sec at 62 °C, and 60 sec at 72 °C for *FAS*, 60 sec at 95 °C, 60 sec at 55 °C, and 60 sec at 72 °C for *CPT 1a*, and 60 sec at 95 °C, 60 sec at 62 °C, and 60 sec at 72 °C for β -actin. PCR reaction was run for 26 cycles for *SREBP-1c*, *ACC 1*, and *FAS*, and for 22 cycles for β -actin, respectively.

5. Data analysis

Student's *t*-test was used to compare the unpaired mean values of two sets of data. Regression analysis was performed using Pearson's correlation coefficient test. *P* values less than 0.05 were considered statistically significant.

Results

1. Pathologic observations of the liver

When young-adult rats were received TPN with or without fat for 4 days, the body weight was not different among three groups, but the liver weight of the group given fat-free TPN was enlarged to double as compared with that of the oral diet control group (Table 2). The liver weight of the TPN with fat group was intermediate between those of the control group and the fat-free TPN group. In pathological finding, severe steatosis was observed in the liver of rats received fat-free TPN, whereas fat supplementation improved the hepatic lipid accumulation (Fig. 1).

2. Biochemical findings

Fig. 2 shows the lipid levels in the liver. The contents of TG and TC were significantly increased in rats given fat-free

TPN and were moderately increased in the TPN with fat group. In contrast, the content of PL was decreased in the fat-free TPN group.

Among hepatic biomarkers, serum AST and ALT were significantly increased after fat-free TPN and were improved by fat supplementation (Table 2). Blood glucose and insulin levels were also significantly increased by fat-free TPN. Fat-free TPN significantly decreased the serum concentrations of TG, TC, and FFA, and fat supplementation in the TPN almost completely blocked the decrease in the lipid concentrations, except for TG (Table 2). Other laboratory data were hardly influenced by TPN with or without fat.

3. mRNA expression

The changes of expression of lipid metabolism-related mRNAs in the liver after TPN infusion were examined using semi-quantitative RT-PCR (Fig. 3). The expression of mRNAs of a transcriptional regulator of fatty acid synthesis, sterol regulatory element binding protein 1c (SREBP1c)¹⁵⁾, and lipogenic enzymes, acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) was significantly up-regulated after fat-free TPN infusion. In contrast, the mRNA expression of carnitine palmitoyltransferase-1a (CPT-1a), which is the rate-limiting enzyme of β -oxidation in liver mitochondria¹⁶⁾, was down-regulated by fat-free TPN. However, fat supplementation completely suppressed these changes.

4. Fatty acid composition in the liver

As shown in Table 3, in rats that had received fat-free TPN for 4 days, the hepatic contents of non-essential fatty

Table 2 . Body and liver weight and biochemical data

	Control	Fat-free TPN	TPN with fat
Body weight (g)	200 ± 7	194 ± 6	195 ± 5
Liver weight (g)	8.0 ± 0.9	16.7 ± 1.8**	12.2 ± 1.0**, ##
Liver weight ratio (g/100 g body weight)	3.8 ± 0.4	8.6 ± 0.8**	6.3 ± 0.5 **, ##
Serum			
AST (IU/L)	89 ± 6	204 ± 67*	98 ± 7 #
ALT (IU/L)	53 ± 5	96 ± 21*	39 ± 14 ##
ALP (IU/L)	1122 ± 223	1274 ± 241	1175 ± 220
γ -GTP (IU/L)	< 2	4 ± 2	3 ± 1
Glucose (mg/dL)	117 ± 4	304 ± 83**	147 ± 57 ##
Insulin (μ IU/mL)	73 ± 21	248 ± 16 **	142 ± 14 *, ##
TG (mg/dL)	60 ± 9	7.7 ± 2.0**	21 ± 5 **, ##
TC (mg/dL)	87 ± 15	36 ± 13 **	74 ± 10 ##
FFA (μ Eq/L)	294 ± 110	130 ± 42**	246 ± 56 ##
PL (mg/dL)	111 ± 7	107 ± 24	113 ± 20

Data are represented the means $\pm$ S.D. of 5 rats.
* , **Significantly different from the oral diet control group at *P* < 0.05 and 0.01, respectively.
, ##Significantly different from the fat-free TPN group at *P* < 0.05 and 0.01, respectively.

a) Control

b) Fat free

c) With fat

Fig. 1 . Light micrographs of the liver after 4 days administration (Hematoxylin and Oil red O double stain. The original magnification was X 200).

Fig 2 . Hepatic lipid contents after oral diet (■), fat-free TPN (□) and TPN with fat (▨) for 4 days.

Data are presented the means $\pm$ S.D. of 5 rats.

*, **Significantly different from the oral diet control group at $P < 0.05$ and 0.01, respectively.

##Significantly different from the fat-free TPN group at $P < 0.01$.

Fig 3 . Expression lipid metabolism-related mRNAs after oral diet (■), fat-free TPN (□) and TPN with fat (▨) for 4 days.

Data are presented the means $\pm$ S.D. of 5 rats.

**Significantly different from the oral diet control group at $P < 0.01$.

#, ##Significantly different from the fat-free TPN group at $P < 0.05$ and 0.01 , respectively.

acids, palmitic acid, palmitoleic acid, oleic acid, and eicosatrienoic acid were significantly increased, while the concentrations of essential fatty acids, including linoleic acid and arachidonic acid, were decreased compared with those of the oral diet control group, as reported in deficiency of essential fatty acids¹⁷⁾. Although the supplemented fat contains high levels of linoleic acid and oleic acid, the composition of these fatty acids in the liver was increased only slightly, but with statistical significance, and the supplemented fat appeared mainly to inhibit the changes of fatty acid composition resulting from fat-free TPN administration. The supplementation of TPN with fat completely blocked the decrease in essential fatty acids caused by fat-free TPN.

Recent studies have shown that long-chain polyunsaturated fatty acid (LCPUFA), which is produced through the pathways for desaturation and elongation of the essential fatty acids linoleic acid and α -linolenic acid, (i) inhibits the transcription of lipogenic genes, such as SREBP-1c¹⁸⁾, and that (ii) activates peroxisome proliferator-activated receptor (PPAR γ), a key up-regulator of peroxisomal and mitochondrial fatty acid β -oxidation, leading to improvement of

fat accumulation^{19, 20)}. Both n-6 and n-3 LCPUFAs were decreased in the liver of rats received fat-free TPN, whereas fat supplementation fully recovered hepatic contents of LCPUFAs (Table 3). These results are consistent with the observation that fat-free TPN up-regulated genes for lipogenic enzymes and down-regulated CPT-1a mRNA.

Discussion

Our results confirmed that fat-free TPN caused severe fatty liver with hyperglycemia, hyperinsulinemia, and hypolipidemia in conscious young-adult rats after infusion for only 4 days, whereas these disorders were much less severe in rats given TPN supplemented with soybean fat emulsion equivalent to 20% of total calories. Administration of TPN with fat also ameliorated the increase in hepatic biomarkers (Fig. 2, Table 2). We suggest that the biochemical mechanisms leading to lipid accumulation in the liver after fat-free TPN are as follows: the infusion of excess glucose causes hyperinsulinemia, and the insulin increases lipogenesis and decreases β -oxidation, resulting in high synthesis and accu-

Table 3 . Fatty acids composition of the liver

		Control	Without fat	With fat
Palmitic acid	16:0	4.65 ± 0.21	27.4 ± 6.1 **	14.4 ± 2.0 **, #
Palmitoleic acid	16:1,n-7	0.23 ± 0.10	11.4 ± 3.5 *	2.53 ± 0.94*, #
Stearic acid	18:0	5.19 ± 0.35	4.87 ± 0.12	5.19 ± 0.06 #
Oleic acid	18:1,n-9	1.18 ± 0.33	15.7 ± 2.1**	7.01 ± 1.38**, ##
Linoleic acid	18:2,n-6	4.06 ± 0.35	0.50 ± 0.03**	5.22 ± 0.42 *, ##
α -Linolenic acid	18:3,n-3	0.03 ± 0.01	0.00 ± 0.00 **	0.23 ± 0.08 *, ##
γ -Linolenic acid	18:3,n-6	0.04 ± 0.00	0.04 ± 0.00	0.09 ± 0.01 *, #
5-8-11 Eicosatrienoic acid	20:3,n-9	0.03 ± 0.01	0.25 ± 0.04 **	0.04 ± 0.01 ##
Arachidonic acid	20:4,n-6	3.13 ± 0.36	1.45 ± 0.07*	3.08 ± 0.20 ##
Eicosapentaenoic acid	20:5,n-3	0.13 ± 0.01	0.08 ± 0.00 **	0.11 ± 0.03
Docosahexaenoic acid	22:6,n-3	1.27 ± 0.14	0.71 ± 0.04 **	1.08 ± 0.09 ##
Total LCPUFA		4.58 ± 0.49	3.35 ± 0.07 **	4.32 ± 0.29 ##
Total n-6 LCPUFA		3.13 ± 0.36	1.50 ± 0.07 **	3.08 ± 0.20 ##
Total n-3 LCPUFA		1.39 ± 0.13	0.80 ± 0.04 **	1.20 ± 0.10 ##

Data are represented the means ± S.D. of 5 rats.

n-6 LCPUFA is 20 : 4, n-6 ; n-3 LCPUFA are 20 : 5, n-3 and 22 : 6, n-3.

*, ** Significantly different from the oral diet control group at $P < 0.05$ and 0.01 , respectively.

#, ## Significantly different from the fat-free TPN group at $P < 0.05$ and 0.01 , respectively.

mulation of TG in the liver. It has been known that insulin promotes the gene expression of lipid synthesis-related enzymes, such as ACC and FAS, through activation of transcription factors SREBPs²¹⁾. SRBP1c is essential for induction of ACC and FAS, but not other lipogenic enzymes, glucose-6-phosphatase dehydrogenase, glycerol-3-phosphate acyltransferase, etc. This study indicated significant increase in the expression of SREBP1c, ACC, and FAS (Fig. 3), resulting increase in the contents of non-essential fatty acids, such as palmitic acid, palmitoleic acid, stearic acid and oleic acid, in the liver of rats given fat-free TPN (Table 3). In addition, the decreased essential fatty acids in the liver caused by depletion of fat component in TPN seems to impair LCPUFA production, resulting in liver steatosis. These changes were improved by TPN with soybean fat equivalent to 20% of total calories, with a corresponding reduction of glucose (the formulation is shown in Table 1).

Among lipids in the liver, only PL was decreased by administration of fat-free TPN (Fig. 2). Large amounts of PL and lecithin are synthesized using plant choline, and fat-free TPN has been reported to induce choline deficiency and to decrease PL^{22, 23)}. Because the soybean fat emulsion (Intralipos®) used in this study contains egg-lecithin as an emulsifier, it seems that the PL content and choline deficiency were improved by administration of TPN supplemented with fat, compared with fat-free TPN.

CPT1 is located at the outer membrane of mitochondria and is the rate-limiting enzyme in the β -oxidation of long-chain fatty acids. CPT1a is the liver-type isoform of CPT¹⁶⁾. In this study, the expression of mRNA of CPT1a was found to be significantly decreased after fat-free TPN, but

not TPN with fat (Fig. 3), although it is not yet clear whether the expression of CPT is regulated by insulin and glucose. Consequently, administration of fat-free TPN not only increased lipogenesis as a result of hyperglycemia and hyperinsulinemia, but also decreased β -oxidation, resulting fatty liver. Moreover, ACC stimulated by insulin has been reported to suppress the CPT-1 activity through increased synthesis of malonyl-CoA²⁴⁾. These results and evidence indicated that hyperinsulinemia lowered the ability of CPT to transport fatty acid into mitochondria and decreased β -oxidation. On the other hand, while lipid was accumulated in the liver after fat-free TPN, serum lipids were significantly lowered (Table 2). The low serum lipid concentration was not seen when TPN supplemented with soybean fat was used. Lipids synthesized in the liver assemble with apo B through the action of microsomal triglyceride transfer protein (MTP) and are secreted as very low density lipoprotein (VLDL) into the bloodstream²⁵⁾. It has been reported that acute hyperinsulinemia suppresses the hepatic secretion of VLDL^{12, 13)}. Thus, the low serum lipid concentration might have been caused by hyperinsulinemia after fat-free TPN, and supplementation of TPN with fat in place of a part of the glucose may improve insulin secretion and lipid metabolism. However, it remains to examine the correlation among activities of MTP, VLDL-degradation protein, and lipoprotein lipase, and the infusion of TPN with or without fat.

In conclusion, we found that fat-free TPN caused severe fatty liver with hyperglycemia, hyperinsulinemia, and hypolipidemia after administration for only 4 days, and these disorders were clearly less severe when TPN supplemented with soybean lipid, equivalent to 20% of total calories, was

used. Therefore, we should avoid the use of fat-free TPN in the clinical context. Instead, TPN should be routinely supplemented with soybean fat at an appropriate level in place of a part of the glucose. It may also be necessary to monitor serum glucose and insulin concentrations during TPN infusion.

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