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Plasma Levels of Conjugated Bile Acids in Newborns After a Short Period of Parenteral Nutrition

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Background: Patients receiving parenteral nutrition (PN) frequently exhibit liver dysfunction. The authors previously reported that plant sterols of lipid emulsions added to the nutritional solution of newborns receiving PN accumulate in plasma and cell membranes and may contribute to the development of cholestasis. Conjugated bile acids (BA) have been shown to be useful markers of cholestasis. Plasma levels of several BA in newborns were quantified after administration of PN for less than 2 weeks. *Methods:* Plasma samples from 15 healthy control infants (CN), 22 patients who had received PN for 3–15 days (T1), and 9 patients scheduled to receive PN (T0) were analyzed. After a simple extraction procedure, plasma BA were analyzed by liquid chromatography–tandem mass spectrometry using a quantitative isotope dilution method. *Results:* The concentrations of BA did not differ significantly between controls and patients before PN (CN vs T0), with the exception of glycocholic acid (GCA; 2.30 ± 2.60 µM vs 7.29 ± 5.39 µM,

respectively). There was a significant difference in several BA between controls and patients after PN (2.30 ± 2.60 µM vs 7.61 ± 6.46 µM for GCA, respectively; 4.02 ± 3.49 µM vs 11.88 ± 11.05 µM for taurocholic acid [TCA], respectively; and 4.81 ± 3.49 µM vs 13.58 ± 12.22 µM for taurochenodeoxycholic + taurodeoxycholic + tauroursodeoxycholic acids [TCDCA+TDCA+TUDCA], respectively). *Conclusions:* In newborns receiving PN, a short period of PN is associated with an early increase of some conjugated BA. These results suggest that GCA, TCA, and TCDCA+TDCA+TUDCA levels could be used as early markers of PN-related cholestasis. (*JPEN J Parenter Enteral Nutr.* 2010;34:538–541)

Keywords: total parenteral nutrition; glycine-conjugated bile acids; taurine-conjugated bile acids; conjugated bile acids; high-performance liquid chromatography–tandem mass spectrometry.

Parenteral nutrition (PN) is a life-saving therapeutic approach that provides adequate nutrition to children and newborns with severe intestinal failure or who require an intestinal rest period due to necrotizing enterocolitis, intestinal atresia, pseudo-obstruction, or other motility disorders.[1,2] PN is also used as nutrition support for preterm neonates.[3] Unfortunately, in newborns, PN induces sometimes irreversible and even fatal liver injury.[4] Many studies have reported an association between

liver dysfunction and PN, with an incidence ranging from 15% to 85%. In pediatric aged patients, fibrosis, portal inflammation, cholestasis, proliferation of bile ducts, and hydropic degeneration are the main histological findings.[5-10] In preterm babies, canalicular cholestasis is the first histological sign of PN-induced hepatobiliary dysfunction and can be detected as early as 5 days after initiation of PN. After 2 weeks of PN, intracellular cholestasis is detected,[9] and proliferation of bile ducts and fibrosis develops mainly after 8 weeks of PN, with incidences as high as 100% after 28 weeks. The risk of developing liver complications is higher in underweight newborns treated with PN for more than 2 weeks as compared with normal-weight infants.[10,11-13] However, despite numerous studies that have suggested several potential etiological factors, including infections, toxins, excess of sugars and calories, or bile acid (BA) toxicity, the pathogenesis of PN-induced liver complications has yet to be precisely defined.[4] There is evidence that reduced enterohepatic circulation of BA and increased levels of phytosterols, which are highly concentrated in

Table 1. Clinical and Biochemical Characteristics in Healthy Control (CN) Infants and in Patients Before (T0) and After (T1) Parenteral Nutrition (PN) Reported as Mean (SD)

	CN (n = 15)	Before PN, T0 (n = 9)	After PN, T1 (n = 22)
Gestational age, wk	35 (4)	31 (5)	34 (4)
Birth weight, g	2531 (982)	1554 (586)[a]	2223 (965)
Total bilirubin, mg/dL	1.6 (0.7)	3.66 (1.99)[a]	3.73 (3.15)[a]
γ-glutamyltransferase, IU/L	72 (19)	155 (104)	122 (99)
Alanine transaminase, IU/L	10 (4)	16 (8)	21 (25)

[a]$P < .05$ vs CN (analysis of variance with Bonferroni correction).

many lipid emulsions used for PN, might increase the risk of cholestasis.[14,15] However, PN-associated liver dysfunction due to lipids has been attributed mainly to impaired immune defenses and altered inflammatory responses.[16] Another possibility is that an immature hepatic secretory apparatus, particularly in premature newborns, predisposes the infant to cholestasis.[17] Thus, insults such as PN might have a substantial impact on hepatic secretory function, resulting in conjugation but not excretion of BA into bile and subsequent increases in serum concentrations. Elevated concentrations of serum BA represent the earliest and most sensitive indicator of cholestasis, occurring 7–29 days before cholestatic histological changes are seen.[18]

Here we quantified plasma tauro-conjugated and glyco-conjugated BA in preterm and term newborns receiving PN for prematurity, respiratory diseases, or intolerance to enteral feeding. The aim of the study was to determine whether conjugated BA could be used early as sensitive markers of cholestasis and, therefore, potentially for monitoring the effects of therapy. Levels of BA in plasma were analyzed by liquid chromatography–tandem mass spectrometry (LC-ESI/MS/MS) using the isotope dilution technique.

Experimental Procedure

Subjects

Study participants consisted of 22 term and preterm newborns who had received 3–15 days of PN (T1) and 15 healthy newborns as a control (CN). Blood samples were also collected from patients requiring PN before the initiation of artificial nutrition (T0; n = 11). The patients of the T0 group were not randomized, and inclusion was based on the possibility, related to clinical condition of patient, of taking a blood sample before starting PN. Two infants in the T0 group were excluded because of insufficient plasma samples. Clinical characteristics and biochemical plasma markers of liver disease for each group are listed in Table 1. A full description of the patients and protocol has been published previously.[19] Informed written consent was obtained from the parents of enrolled newborns. The study protocol was approved by the ethics committee of Fatebenefratelli Hospital.

Analysis of Conjugated BA

Plasma levels of glycine- and taurine-conjugated BA were analyzed by LC-ESI/MS/MS using the isotope dilution technique. Samples from the T1 group were taken 12 hours after the end of the last lipid infusion.

The procedure for extraction of BA from plasma was as follows. Sample (0.05 mL) was mixed with 0.1 mL of methanol containing fixed amounts of the following 4 internal standards labeled with stable isotopes: trihydroxylated taurocholic and glycocholic acids ([*d*-4]TCA, 5 µM and [*d*-4]GCA, 6 µM, respectively), and dihydroxylated taurochenodeoxycholic and glycochenodeoxycholic acids ([*d*-4]TCDCA, 3 µM and [*d*-4]GCDCA, 5 µM, respectively). Samples were vortexed and then sonicated for 10 minutes, followed by the addition of 0.85 mL of pure methanol. Samples were sonicated again for 20 minutes and then subjected to centrifugation at $6,000 \times g$ for 10 minutes at 8°C. Supernatants were collected and dried at 37°C under a gentle stream of nitrogen. The resultant residue was resuspended in 0.2 mL of mobile phase, and an aliquot (0.03 mL) was removed for analysis.

A high-performance liquid chromatography (HPLC) system with an autosampler (Alliance 2695; Waters, Milford, MA) was coupled with a triple quadrupole mass spectrometer equipped with an electrospray ion source operating in negative mode (Micromass Quattromicro; Waters). The mobile phase consisted of 2-propanol/water (60/40), and the flow rate was maintained at 0.07 mL/min. Mass spectrometry was carried out as follows. Briefly, glycine- and taurine-conjugated di- and trihydroxylated BA and internal standards were individually dissolved in methanol at a final concentration of 1 mg/mL. These standards were diluted to a concentration of 50 µg/mL and used to determine the working conditions for HPLC and mass spectrometer for optimal resolution and abundance of ion signals. The conditions were as follows: capillary voltage, 3.20 kV; cone voltage, 30 V; source temperature, 120°C; desolvation temperature, 250°C;

Table 2. Plasma Levels (µM) of Conjugated Bile Acids in Healthy Control (CN) Infants and in Patients Before (T0) and After (T1) Parenteral Nutrition (PN), Reported as Mean (SD)

Conjugated Bile Acids	CN (n = 15)	Before PN, T0 (n = 9)	After PN, T1 (n = 22)
GLCA	0.15 (0.06)	0.15 (0.11)	0.14 (0.11)
GCDCA+GDCA+GUDCA	1.83 (1.30)	1.34 (0.75)	5.18 (7.35)
GCA	2.30 (2.60)	7.29 (5.39)[a]	7.61 (6.46)[a]
TLCA	0.11 (0.07)	0.23 (0.20)	0.21 (0.20)
TCDCA+TDCA+TUDCA	4.81 (3.49)	6.46 (5.14)	13.58 (12.22)[a]
TCA	4.02 (3.49)	5.81 (4.13)	11.88 (11.05)[a]

GCA, glycocholic acid; GCDCA + GDCA + GUDCA, glycochenodeoxycholic + glycodeoxycholic + glycoursodeoxycholic acids; GLCA, glycolithocholic acid; TCA, taurocholic acid; TCDCA+TDCA + TUDCA, taurochenodeoxycholic + taurodeoxycholic + tauroursodeoxycholic acids; TLCA, taurine-conjugated lithocholic acid.

[a]$P < .05$ vs CN (analysis of variance with Bonferroni correction).

desolvation gas flow, 300 L/h; gas cell pirani, 0.0028 mbar. Collision cell energy was 40 eV and 65 eV for glycine- and taurine-conjugated BA, respectively. For quantitative analysis, precursor ions of the 74 m/z fragment for glycine conjugates and of the 80 m/z fragment for taurine conjugates were monitored using the specific scan mode (PI) of the MS/MS system. Acquisition mass ranges were from 420 to 500 and from 470 to 550 for glycine and taurine conjugates, respectively, and the run time was 3 minutes. For each analyte, a calibration curve was used to calculate the concentration of BA in samples. Calibration points were obtained using a plasma pool enriched with the following standards: GCDCA (40 µM), GCA (50 µM), TCDCA (12 µM), and TCA (30 µM). The enriched plasma was diluted (1:2 to 1:64) with unenriched plasma to obtain a range of concentrations. Correlation coefficients (r) of the calibration curves ranged from 0.998 to 0.999. Method imprecision was calculated for 3 different concentrations of enriched plasma as follows: GCDCA, 40 µM, 10 µM, and 0.625 µM; GCA, 50 µM, 12.5 µM, and 0.78 µM; TCDCA, 12 µM, 3 µM, and 0.016 µM; and TCA, 30 µM, 7.5 µM, and 0.47 µM. Values ranged 4.8%–8.7% for GCDCA, 3.3%–12.6% for GCA, 6.3%–17.5% for TCDCA, and 4.1%–14.5% for TCA.

Statistical Analysis

Differences in plasma levels of conjugated BA among CN, T0 (before PN), and T1 (after PN) infants were analyzed by analysis of variance with Bonferroni correction and Student's *t* test. Data represent means and standard deviation (means ± SD). A *P* value of ≤.05 was considered statistically significant.

Results and Discussion

Table 1 shows the clinical and biochemical characteristics of CN, T0, and T1 infants. Treatment of the patient groups

ranged from 3–15 days (median, 6 days) and consisted of at least 75% of the required energy delivered via PN. Lipids were provided through a soybean oil–based emulsion (Intralipid 20%; FreseniusKabi, Italy). The average amounts of lipid, glucose, and nitrogen administered were1.38 ± 0.74, 6.8 ± 1.79, and 1.7 ± 0.44 g/kg/d, respectively.[19] There were no differences in clinical characteristics among the 3 groups, with the exception of birth weight of the T0 group and total bilirubin of the T0 and T1 groups as compared with CN infants. Table 2 shows the plasma concentrations of glycine- and taurine-conjugated BA for each group. Plasma concentrations of glycine- and taurine-conjugated BA in term and preterm newborns before PN and at least 12 hours after the end of the last infusion were measured to determine whether there were differences between these 2 groups of patients and healthy newborns.

BA concentrations in the CN and T0 groups were not statistically significant with the exception of GCA, which was significantly elevated (approximately 3.5-fold) in the T0 group, perhaps due to individual variability in that particular group, since it included premature newborns (7 of 9 subjects were premature). Moreover, the comparison of BA concentrations in the T0 and T1 groups was not statistically significant even though some BA in the T0 group, on average, were lower than in the T1 group (Table 2; GCDCA+GDCA+GUDCA, TCDCA+TDCA+TUDCA, and TCA). By comparison, after PN, patients had significantly increased levels of GCA, TCDCA+TDCA+TUDCA, and TCA as compared with CN infants. There were no significant differences among the groups in the concentrations of glycolithocholic acid (GLCA), GCDCA+GDCA+GUDCA, and taurine-conjugated lithocholic acid (TLCA). Additionally, in the T1 group, the concentrations of alanine transaminase, γ-glutamyltransferase, and total bilirubin in plasma did not correlate with levels of BA; however, the concentration of TCA correlated positively with duration of PN ($r = 0.547$; $P < .01$). These results indicate that plasma BA profiles could potentially serve as indicators of metabolic disorders and liver disease, as well sensitive indicators of cholestasis.

PN is associated with changes in plasma BA composition; the excess amounts of some BA cause hepatotoxic effects related to PN, and the administration of taurine can decrease the toxicity of BA. Conjugation of BA to taurine and glycine is mediated by the same competitive enzyme,[20] and in preterm infants, where the taurine is a provisionally essential amino acid, due to either an inability of endogenous synthesis or a nutrition deficit, conjugation of BA with glycine would be expected to increase. In fact, in the current study, GCA concentrations were significantly elevated in the T0 group (Table 2) as compared with healthy controls, which we attribute to the number of preterm infants in the T0 group (7/9). It is well documented that patients with liver disease have high levels of taurine conjugation, while high levels of glycine conjugation are typical in patients with malabsorption and intestinal bile loss.[21] Our data demonstrate that there is an early increase in glycine- and taurine-conjugated BA in the plasma of newborns receiving PN for 3–15 days. Even if only 2 of the patients who received PN developed cholestasis during or after the administration of parenteral solution, our results indicate that changes in the levels of plasma BA could serve as an early marker of liver injury. These results are consistent with literature reports that increased levels of conjugated BA are indicative of hepatic diseases and are in line with the proposal by Demircan et al[22] that serum BA may be more useful for the early diagnosis of PN-induced cholestasis than liver markers such as γ-glutamyltransferase and alkaline phosphatase. In addition, even though we did not measure the sulfated BA, our results are in agreement with those reported by Farrell et al,[23] in which serum sulfated lithocholate levels were elevated in 18 of 55 patients during PN in infants and children. Importantly, even if the number of patients in each group is small, the data obtained from this pilot study suggest for the first time that in newborns, a short period of PN is associated with an early and significant increase in glycine- and taurine-conjugated BA. Finally, the results may encourage researchers to perform larger randomized trials, which could lead to identifying the role of BA as marker for cholestasis and PN-associated liver disease.

Acknowledgments

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