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ORIGINAL ARTICLE

Plasma amino acids in preterm infants fed different human milk diets from a human milk bank

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Summary
Background & aims: The enzymatic immaturity of the amino acid metabolic pathways in preterm infants makes these children vulnerable to deficiencies or amino acid excess. The purposes of this study was to evaluate the plasma amino acid profile of preterm infants fed one of three diets, and then compare them to the reference standards for preterm infants.
Methods: Thirty low-birthweight preterm infants were randomly assigned to diets: unmodified banked human milk ($n = 10$), banked human milk evaporated to 70% ($n = 10$), and banked human milk fortified with a bovine whey protein hydrolysate ($n = 10$). Amino acid concentrations were analyzed by high efficiency liquid chromatography.
Results: No significant statistical differences in the amino acid profiles were found across groups. With few exceptions (arginine and glutamic acid), plasma amino acid concentrations in the three groups were lower than or reached the minimum values of references found in the literature for preterm infants.
Conclusions: The diets utilized led to deficiencies in amino acids, relative to the reference standards. It can be concluded that the supplies of these nutrients were below the needs of the infants in all groups.

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Introduction

Scientific and technological advances in medicine, particularly in perinatal intensive care, have reduced the mortality rate among preterm infants, especially in those with birth-

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weights of less than 1500 g. This achievement has led to the investigation of a number of related issues, particularly in the area of nutritional support.[1] In 1985, the American Academy of Pediatrics[2] stated that the main objective of preterm infant nutrition was to achieve postnatal growth rates similar to those found *in utero*. In 1992, Steer et al.[3] pointed out that nutrition should also target the long run to normal neurological development. The concept of optimal protein nutrition emerges when the adjusted protein intake has to be not only quantitative, but also qualitative, taking into account the enzymatic immaturity of the amino acid metabolic pathways, which are peculiar in preterm infants, making these children vulnerable to deficiencies or toxicity resulting from any amino acid excess.[4] Research has provided evidence of the inadequacy of human milk as the sole nutritional source for preterm infants.[5] An alternative to this inadequacy is to fortify human milk, which significantly increases growth rates, as expressed by weight gain, linear length, and head circumference.[3] Another option is to increase human milk concentration by controlled evaporation, resulting in a diet that provides much higher protein and energy intake. The purpose of this study was to evaluate the plasma amino acid profile of preterm infants fed one of three diets: banked human milk (BHM), banked human milk evaporated to 70% (EBHM), and banked human milk fortified with FM85 (FBHM) and to compare them with conventional reference standards for preterm infants, in order to identify toxicity risks and/or nutritional deficiencies.

Patients and methods

Thirty preterm infants of both genders at 34 weeks or less of gestational age (GA), as evaluated by the Ballard method,[6] with birthweights of up to 1750 g were included in the study. Birthweight was appropriate for GA according to the curves of intrauterine growth designed by Bataglia and Lubchenco.[7] The infants were admitted to the Neonatal Intensive Care Unit of the Teaching Hospital of Universidade Federal de Mato Grosso do Sul (UFMS), Campo Grande, MS, Brazil, in the period from March 2000 to June 2002. Written informed consent was obtained from parents. The study protocol was approved by the local Ethics Committee. Carriers of congenital pathologies or intercurrent diseases (infectious and/or metabolic disorders) were excluded, in accordance with election criteria. The patients were randomly assigned to three groups of 10 infants, each group receiving one of three diets, all processed by the local milk bank:

(a) Diet BHM was obtained from milk donors in different periods of lactation, pasteurized, and frozen.
(b) Diet EBHM was prepared by concentrating banked human milk under controlled evaporation to 70% of its initial volume using a Buchi Rotavapor RE vacuum rotating evaporator. Each milk sample to be evaporated (400 mL at a time) was maintained at 56 °C under a vacuum pressure ranging from 62 to 63 cmH_2O. The 70% reduction in sample volume (from 400 to 280 mL) was achieved in about 30–40 min, and the resulting milk was frozen and stored. The procedure was always carried out by the same investigator.
(c) Diet FBHM was prepared from banked human milk with the addition of protein supplied as a bovine whey protein hydrolysate associated with a fortifier containing maltose–dextrine and minerals (FM85, Nestlé, Switzerland).

Before the period of study, the infants were fed banked human milk associated with small volumes of their own mothers' milk, whose production was not yet sufficient to meet daily requirements. The children were included in the study after 11 ± 1 days of life, when they had already been on full enteral feeding for at least 72 h. Each group received the assigned diet for a period of 10–12 days. According to their individual ability, the children were fed orally or by gavage. Daily intakes were of 170–180 mL/kg, in 3-h feeding intervals, for all groups. Intake volume was defined according to the protocol of the service, without researchers' interference. The macronutrient and energy content of the banked breast milk fed to the infants was not analyzed. After the period of 10–12 days, preprandial venous blood samples of 1 mL were taken 150–180 min after the last feeding. Plasma was separated by centrifugation, identified, and frozen at −20 °C. Plasma amino acids were determined by High-Efficiency Liquid Chromatography in a high-pressure liquid chromatograph (Shimadzu, model LC-10, Japan).

The amino acids analyzed were arginine, alanine, aspartic acid, glutamic acid, glutamine, serine, histidine, glycine, threonine, tyrosine, methionine, valine, phenylalanine, leucine, isoleucine, and lysine. The reagent used in the pre-column reaction was orthophthaldialdehyde (OPA), a compound that reacts with the amino group of amino acids yielding a colored molecular complex (isoindole) that can be detected by a fluorescence reader. The compound used in the pre-column reaction does not react with the amino acids proline, cystine, or tryptophan, none of which were quantified by reason of this technical limitation.

Body weights were measured with an electronic balance of 5-g sensitivity (Filizola, Brazil) immediately before and after the period of study—i.e., at 10–12 days and at 22 ± 2 days of age.

Descriptive statistics was expressed as medians, minimums, and maximums for the variables that characterize the population studied. No sample size calculation was performed; the power achieved with the number of infants included in the study was not estimated. Medians and first and third quartile values were taken into account for analysis of weight variation and plasma amino acid levels. The differences were tested with nonparametric procedures, Mood's median test[8] for the quantitative variable and with Fisher's exact test[9] for the qualitative variable. No correction for multiple testing was performed. A p-value of less than 0.05 was considered significant.

Results

Table 1 reports the variables that characterize the population studied: GA, gender, birthweight, length, head circumference, and type of delivery. Statistical analysis showed that the groups were similar ($p > 0.05$).

Table 2 shows that no significant statistical differences in plasma amino acid concentrations were found across groups,

Table 1 Profile (gestational age, gender, birth-weight, length, head circumference, type of delivery, and initial weight) of preterm infants fed one of three milk diets: BHM, EBHM and FBHM.

Variable	Groups			*p*
	BHM ($n = 10$)	EBHM ($n = 10$)	FBHM ($n = 10$)	
Gestational age (weeks)	31.0 (28–34)	31.0 (30–33)	32.0 (29–32)	0.67
Gender*				
Females	5 (50%)	4 (40%)	4 (40%)	0.87
Males	5	6	6	
Birthweight (g)†	1.640 (1250–1750)	1.410 (1265–1620)	1.505 (850–1715)	0.17
Length (cm)	40.0 (37–45)	39.5 (37–42)	39.0 (35–41.5)	0.06
Head circumference (cm)	28.8 (27–30)	28.0 (27–31)	29.0 (24–30)	1.00
Caesarean section*	7 (70%)	5 (50%)	6 (60%)	0.66
Initial weight (g)†	1492 (1060–1785)	1375 (1175–1680)	1382 (770–1680)	0.30

*Fisher's exact test was applied to verify similarities according to gender and type of delivery ($p < 0.05$).
†Values are expressed as medians. Minimum and maximum values are shown in parentheses.

Table 2 Concentrations of plasma amino acids in preterm infants fed one of three milk diets: BHM, EBHM and FBHM.

Amino acid	Groups			*p*
	BHM (μmol/L)	EBHM (μmol/L)	FBHM (μmol/L)	
Aspartic acid (Asp)	6.1 (4.2–8.9)	10.5 (6.4–11.7)	11.6 (5.3–18.1)	0.06
Glutamic acid (Glu)	87.4 (41.8–159.5)	93.6 (70.5–157.5)	67.0 (59.9–108.3)	0.67
Serine (Ser)	43.7 (33.1–51.8)	63.2 (37.8–75.9)	46.7 (36.9–82.6)	0.20
Histidine (His)	19.6 (14.6–29.7)	29.0 (11.6–52.7)	20.5 (9.1–69.6)	0.67
Glycine (Gly)	33.6 (30.6–50.6)	44.4 (26.2–57.3)	45.2 (22.0–63.9)	0.30
Threonine (Thr)	49.8 (30.0–69.6)	58.9 (41.6–108.5)	73.6 (49.7–87.8)	0.20
Arginine (Arg)	67.3 (50.2–105.6)	102.3 (76.3–146.9)	70.9 (47.1–131.5)	0.30
Alanine (Ala)	21.4 (15.1–30.6)	35.0 (30.4–42.1)	16.4 (14.5–23.9)	<0.01
Tyrosine (Tyr)	30.5 (23.2–45.4)	36.9 (22.0–52.8)	30.8 (15.6–43.6)	0.20
Methionine (Met)	8.8 (6.8–14.8)	12.4 (9.0–18.6)	9.5 (5.3–17.1)	0.20
Valine (Val)	45.8 (41.1–65.0)	63.8 (41.2–74.4)	39.3 (31.3–83.1)	0.30
Phenylalanine (Phe)	22.0 (19.3–24.6)	25.5 (19.4–33.6)	17.2 (12.4–30.1)	0.67
Isoleucine (Ile)	20.5 (17.5–43.3)	25.4 (22.1–56.7)	30.8 (21.4–49.3)	0.67
Leucine (It read)	29.1 (24.2–40.0)	39.8 (27.9–51.2)	22.7 (20.5–46.1)	0.30
Lysine (Lys)	21.0 (16.7–34.2)	15.6 (12.4–24.1)	21.5 (14.3–32.3)	0.30
Glutamine (Gln)	16.5 (15.1–25.3)	20.8 (16.8–31.4)	17.8 (11.9–21.6)	0.67

Values are expressed as medians. Values of 1st and 3rd quartiles are shown in parentheses ($p < 0.05$).

Table 3 Total and daily weight gains of preterm infants fed one of three milk diets: BHM, EBHM and FBHM.

Weight gain	Weight (g)			*p*
	BHM	EBHM	FBHM	
Total	90.0 (32.5–117.5)	188.0 (128.8–257.5)	230.0 (178.8–288.8)	<0.01
Daily	8.2 (3.3–10.8)	18.8 (12.3–25.0)	19.8 (17.4–21.9)	<0.01

Values are expressed as medians. Values of 1st and 3rd quartiles are shown in parentheses ($p < 0.05$).

with the exception of alanine, which had a significantly higher concentration in the EBHM group ($p < 0.01$).

Table 3 shows the values of total and daily weight gains in the three groups. Statistically significant differences were found in median total weight gains and in median daily weight gain across groups ($p < 0.01$). Group FBHM had the highest total and daily weight gains (230 and 19.8 g, respectively), whereas group BHM had the lowest (90 and

8.2 g, respectively). Group EBHM had total (188 g) and daily (18.8 g) weight gains well above those of group BHM and very close to those of FBHM.

Figure 1 compares the plasma amino acid profiles of each group with Pohlandt's reference values (blood amino acids from umbilical cord[10] and plasma amino acids from preterm infants receiving maternal milk exclusively).[11] Overall, plasma amino acids in the three groups were below or very near to the minimum value (10th percentile) of both reference ranges. Glutamic acid was the only amino acid whose values were within both reference ranges in all groups. Arginine values were within the reference ranges in groups BHM and FBHM, but exceeded the 90th percentile of the two references in group EBHM.

Figure 2 compares the plasma amino acids of each group with McIntosh's reference values (fetal plasma amino acids at 18–29 weeks of GA).[12] Plasma amino acids had highly similar profiles in all three groups: glutamine, lysine, alanine, valine, threonine, glycine, serine, leucine, histidine, and phenylalanine values were below the fetal reference range. The concentrations of isoleucine, tyrosine, aspartic acid, and methionine coincided with the lower limit

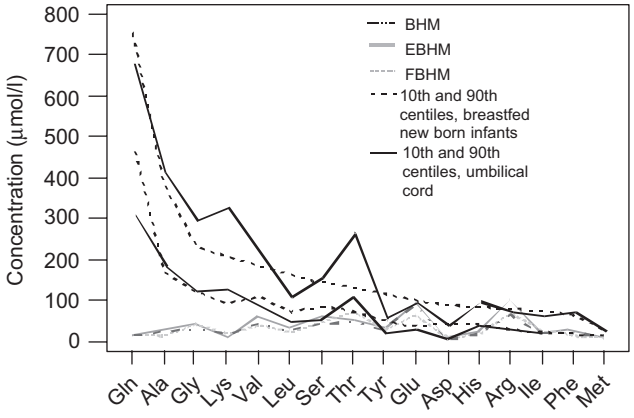

Figure 1 Median concentrations of plasma amino acids in preterm infants fed one of three milk diets and reference values for plasma amino acids.[11]

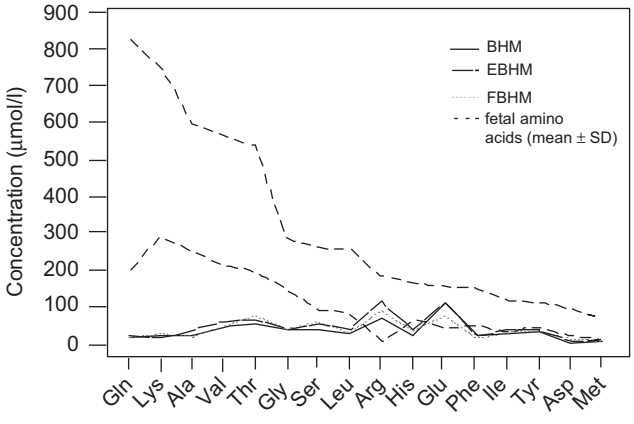

Figure 2 Median concentrations of plasma amino acids in preterm infants fed one of three milk diets and reference values for fetal plasma amino acids.[12]

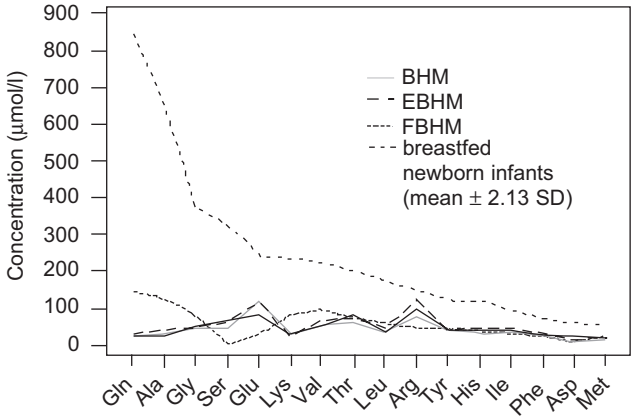

Figure 3 Median concentrations of plasma amino acids in preterm infants fed one of three milk diets and reference values for plasma amino acids in exclusively breastfed newborn infants.[13]

(mean−1SD) of the reference range. Only arginine and glutamic acid were within the fetal reference range.

Figure 3 compares the plasma amino acids of each group with Wu's reference values (amino acids in exclusively breastfed term newborns).[13] Glutamine, alanine, glycine, lysine, valine, and leucine levels were below the lower limit (mean−2.13SD) of the reference range. Serine, glutamic acid, and arginine levels were within the reference range. Tyrosine, histidine, isoleucine, phenylalanine, aspartic acid, and methionine were at the lower limit (mean−2.13SD), but still within the reference range. Threonine was below the lower limit of the reference range in groups BHM and EBHM, but coincided with the lower limit in group FBHM.

Discussion

Only one of the 30 infants weighed less than 1000 g at birth. The absence of infants with extremely very-low birthweight in the population studied is due to specific characteristics of these children. They usually demand longer periods of intensive care and they normally do not reach a sufficiently steady clinical condition to satisfy the inclusion criteria described. In 13 infants, birthweights were in the range of 1001–1499 g; in 16, in the range of 1500–1750 g. The study therefore was characterized by a population of low birthweight preterm infants with 43.3% of very low birthweight infants,[14] though distributed into the three groups in a homogeneous manner. The volumes of milk per day per infant were equivalent in all groups (170–180 mL/kg/day), but not the protein intakes, since the three diets had their own peculiarities. Banked human milk was either evaporated or fortified in order to increase protein and energy content, either by increasing by 30% the concentration of macronutrients (EBHM) or by adding FM85 bovine whey protein, maltose–dextrin, and minerals (FBHM). Protein quality was the same in groups EBHM and BHM; in group EBHM, however, protein intake was higher. The diet of group FBHM differed qualitatively in protein content (human milk protein fortified with a bovine whey protein hydrolysate); therefore protein intake was higher in this group than in group BHM. These differences in quantity (higher supplies in

groups EBHM and FBHM) and in quality (group FBHM) in protein intake among the three regimens were not reflected in the plasma amino acid concentrations of the groups. Although amino acid profile is influenced both by quantitative and qualitative protein supplies,[15] no statistically significant differences were found in these results (Table 2).

Polberger and Räihä[16] found for banked mature human milk a mean protein concentration of 1.3 g/dL, within a range of 0.9–1.7 g/dL, which agrees with the mean concentration of 1.25 g/dL published by the ESPGAN in 1987.[17] In 1990, Michaelsen et al.[18] studying the variation of macronutrients in banked human milk in 2554 samples from 224 mothers, found that less than 15% of the samples had protein contents higher than 1.05 g/dL. Such concentrations do not meet the needs of preterm infants, even when intake volume (in kg/day) is the maximum tolerated in terms of gastric capacity and volume tolerance.[2,17] In the present study, the unexpected similarity of plasma amino acid concentrations in all three groups can be related to the wide, unforeseeable variation in the protein content of banked human milk[16] and to a predominance of mature milk with low protein concentration.[18] The attempt to improve supply by concentrating milk or by adding fortifier was not sufficient to reach the needs of preterm infants. The fortifier used in group FBHM did not determine a better adjusted amino acid profile, and the data available in the literature are in agreement with this finding.

Fortifier FM85 is a bovine whey protein hydrolysate composed of peptides and free amino acids. The generally lower concentrations found for infants who were given fortified milk may be attributed to the differing absorption kinetics of the amino acids contained in proteins and peptides.[19] Protein hydrolysate usage reduces the availability of nitrogen and induces irregularities in plasma amino acid concentrations.[20]

The statistically significant difference found was related to alanine predominating in group EBHM, though quite below the minimum recommended in the literature. This difference did not confer a better quality to diet EBHM, as it was determined by an unexpected variability in amino acid content in banked human milk, as previously described.

The similarities found in the amino acid profile of the three groups were not accompanied by similarities in weight gains (Table 3). Median total weight gains in groups FBHM and EBHM were 2.5 and 2 times as great, respectively, as in group BHM. Despite greater weight gain in groups FBHM and EBHM than in group BHM, daily weight gains (19.8, 18.8, and 8.2 g, respectively) were still below the minimum value advocated for preterm infants, according to intrauterine grown rates.[2] Not even the two diets (EBHM and FBHM) modified to enhance protein and calorie content were enough to ensure satisfactory daily weight gain.

Weight gain *per se* does not reflect actual nutritional status, as revealed also in the present study. Despite greater gains in groups EBHM and FBHM than in group BHM, amino acid profiles were below reference levels. Therefore, weight gains achieved with higher energy supply but lower protein supply are probably explained by fat deposition.

The adoption of a metabolic standard marker such as plasma amino acid concentration in preterm infants has been a focus of discussion. The reference values more frequently used are plasma values of fetuses of the same postconceptional age, levels found in the umbilical cord at the moment of birth, or those found in exclusively breastfed term infants.[21] The comparison of the present results with Pohland's references[10,11] (Fig. 1) reveals that the great majority of amino acids were below or close to the minimum reference range (10th percentile), in all three groups. Glutamic acid (Glu) and arginine (Arg) were within the reference ranges, in all groups, whereas arginine was the only amino acid that slightly exceeded the 90th percentile of the reference in group EBHM. Such behavior led us to extend the comparative analysis of arginine by including other sources of reference—McIntosh's (Fig. 2) and Wu's (Fig. 3), based respectively on fetal plasma amino acids[12] and plasma amino acids in term infants fed maternal milk exclusively[13]—in an attempt to identify whether its concentration might pose any toxicity risks. Arginine concentrations, however, remained within the reference ranges.

In Polberger's study of 1990,[21] essential plasma amino acids were evaluated in two groups of preterm infants given either unenriched human milk or human milk enriched with human milk protein. The milk used as the basis for both diets was not pasteurized. In the group receiving fortified milk, mean protein intake was 3.6 g/kg/day, with a volume of 170 mL/kg/day, leading to plasma amino acid levels below those established by the same references adopted in the present study. Protein supplies in the three diets in the present study were lower or similar to those in Polberger's investigation, resulting in low concentrations of amino acids—in agreement with the well established view, found in the literature, that banked unmodified human milk is unsuitable for low-weight preterm infants, because of its low protein content.

The peculiar results of the present study show that the protein levels of banked human milk can be so low that not even the attempt to concentrate it (by evaporation) or to enrich it with nutrients (by fortification) are sufficient to improve protein intake so as to meet the nutritional requirements of preterm infants.

In the present study, no statistically significant differences were found in the plasma amino acids concentrations across groups. When the plasma amino acid profiles were compared with reference values from healthy term infants fed exclusively maternal milk *ad libitum*,[11] and compared with values found in umbilical cord blood from normal-delivery preterm infants,[10] the concentrations, except in a few cases, were found to be lower than or nearly equal to the minimum reference range (10th percentile). Also, no potentially toxic concentrations occurred, considering the plasma amino acid reference values for preterm infants.[10–13] The diets utilized led to deficiencies in certain amino acids, relative to the reference standards adopted.[22] It can be concluded that the supplies of these nutrients were below the needs of the infants in all three groups.

The results suggest that human milk pooling should be performed in a more standardized manner—an approach known to reduce macronutrient variation[18]—associated with controlling the nutritional quality of banked human milk. Implementation of this procedure would make banked milk more promptly available for modification when increased protein and mineral concentrations are required, as milk pools would be more easily selected based on their calorie and protein contents. The present results warrant

further research in this area, in order to find alternatives that may enable banked human milk to meet the nutritional requirements advocated by the American Academy of Pediatrics.[2] Banked human milk is an important alternative for preterm infants when feeding them their own mothers' milk cannot be easily sustained,[1] as it ensures the availability of species-specific milk and its biological advantages.

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The contributions of authors were as follows: Sandra Christo dos Santos: design study, manuscript preparation, data analysis, practical performance; Carmen Martimbianco de Figueiredo: data analysis; Sônia Maria Oliveira de Andrade: manuscript preparation, practical performance; Durval Batista Palhares, design study, data analysis.

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