Original articles

Haemoglobin concentration depends on protein intake in small preterm infants fed human milk

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SUMMARY Studies have shown that early anaemia of prematurity cannot be prevented by iron or vitamin supplementation. We studied 35 infants of birthweight less than 1520 g and mean gestational age 30.4 weeks who were fed either human milk alone or human milk supplemented with human milk protein. The vitamin and iron status were the same in both groups but the concentration of haemoglobin was significantly higher at the ages 4 to 10 weeks in the protein supplemented infants. Reticulocytosis occurred earlier in the protein supplemented infants. The findings on haemoglobin and reticulocytes were similar in 18 infants who received no blood transfusions. We conclude that human milk protein supplementation can increase the haemoglobin concentration of very low birthweight infants in the early weeks of life and that the protein content in human milk may be insufficient to satisfy their needs.

Blood haemoglobin concentration declines rapidly after birth and results in even lower concentrations at approximately 6 to 8 weeks in low birthweight infants.^{1,2} Some very low birthweight infants may be symptomatic and have difficulties in maintaining adequate tissue oxygenation.³ Although the erythropoietin values in serum seem to respond to hypoxia even in preterm infants, it seems that the response is of a different magnitude to that in adults.⁴ It has been reported that the degree of anaemia is dependent on birthweight, iron excess, and the composition of dietary fat, especially under conditions where vitamin E nutrition is suboptimal.²

We investigated whether protein intake limits the concentration of haemoglobin in human milk fed infants whose birthweights were less than 1520 g and who were given a recommended amount of iron and vitamin supplementation.

Subjects and methods

Subjects. Preterm infants treated with exchange transfusion after birth ($n=12$) and those who received blood transfusions after age 6 weeks ($n=4$) were excluded from the study. In this way infants in whom red cell transfusions were used to compensate for blood samples drawn for laboratory investigations were included, and those who developed

anaemia later, for other reasons, were excluded. The final study population consisted of 35 preterm infants whose birthweights ranged from 710 to 1520 g (mean (SEM) 1232 (33) g). The mean gestational age was 30.4 weeks and ranged between 27 and 35 weeks (Table 1). Twelve infants were small for gestational age; 18 infants required no red blood cell transfusions; and 17 received 8 to 44 ml (mean 19 ml) of red blood cells (10 during the first two weeks of life, 11 during the second and the fourth weeks, and four during the fourth and sixth weeks of life) (Table 2).

All 35 infants were followed to 6 weeks of age and thereafter until their discharge from hospital. Twenty one were followed to 8 weeks, 18 to 10 weeks, and 12 to 12 weeks of age. After hospital discharge the feeding regimens and practices diverged and not all infants were available for study.

Feeding regimens. Mothers were encouraged to express breast milk while their infants were in hospital. All infants were fed with human milk only and received either fresh, pooled mature human milk from the milk bank or the mother's own preterm milk, or both. The latter was pasteurised before use, because it was usually collected at home. The final volume of milk intake was 200 ml/kg/day, which was reached at between 1 and 4 weeks of age.

Table 1 Birthweight; gestational age; and vitamin, selenium, and iron status of protein supplemented and unsupplemented infants at age 6 weeks. Values mean (SEM)

	Protein unsupplemented (n=16)	Protein supplemented (n=19)	P
Boys: girls	9:7	4:15	NS*
Gestational age (weeks)	30.3 (0.6)	30.5 (0.4)	NS
Birthweight (g)	1248 (42)	1218 (51)	NS
Weight gain by 6 weeks (g)	490 (48)	633 (73)	0.018†
Fat intake at age 6 weeks (g/kg/d)	8.0 (0.6)	7.5 (0.5)	NS
(Range)	(6.0-10.4)	(4.2-10.1)	
<i>Plasma/serum concentrations</i>			
Folic acid (µg/l)	27.3 (4.2)	23.2 (2.5)	NS
Vitamin B ₁₂ (ng/l)	874 (110)	796 (129)	NS
Vitamin C (mg/l)	1.60 (0.14)	1.46 (0.11)	NS
Vitamin E (mg/l)	13.3 (0.9)	15.9 (1.0)	NS
Selenium (µg/l)	30.7 (2.0)	36.2 (1.9)	NS
Iron (µmol/l)	16.2 (1.9)	18.1 (3.8)	NS
Ferritin (µg/l)	142 (14)	142 (42)	NS
Transferrin (g/l)	1.7 (0.1)	2.7 (0.2)	<0.001

* χ^2 with Yates's correction; † analyses of variance for repeated measures.

Table 2 Volume of red cells transfused and estimated volume of blood taken from the infants by age 6 weeks. (Infants who received no transfusions are also indicated in the Table).

	Total material		Transfused		Untransfused	
	Protein unsupplemented (n=16)	Protein supplemented (n=19)	Protein unsupplemented (n=7)	Protein supplemented (n=10)	Protein unsupplemented (n=9)	Protein supplemented (n=9)
Transfused erythrocytes by age 6 weeks (ml)	8.5 (3.1)	9.9 (2.3)	19.4 (4.3)	18.8 (3.1)	—	—
Blood for laboratory samples by age 6 weeks (ml)	20.2 (2.8)	22.1 (2.7)	27.3 (4.7)	29.5 (3.6)	14.7 (2.0)	13.8 (1.7)

The mean values did not differ statistically in the protein unsupplemented and supplemented infants.

The infants were randomised to one of two feeding regimens—human milk alone or human milk supplemented with human milk protein. The amount of protein supplement was equal to 0.9 g/dl, which roughly doubled the protein content of the milk. Protein intake was estimated at three ages. At 2 weeks it was mean (SEM), 1.8 (0.08) and 3.1 (0.25) g/kg/day, at 6 weeks 1.9 (0.04) and 3.5 (0.12) g/kg/day, and at 12 weeks 1.8 (0.01) and 3.6 (0.01) g/kg/day in unsupplemented and supplemented infants, respectively.

Fat intake varied due to the great variation in the fat contents of the milks: half the infants received fat supplementation of 1 g/dl as medium chain triglyceride oil. The mean fat intake in protein supplemented and unsupplemented infants did not differ significantly (Table 1). We found no evidence that fat intake had any influence on the concentration of haemoglobin. Centile changes in haemoglobin concentration were similar in fat supplemented and unsupplemented infants at all ages.

Iron supplementation. Iron supplementation was started at 2 weeks of age and by 6 weeks had

reached its maximum. Supplements were given as follows: birthweight 1000 g, or less, 4 mg/kg/day; birthweight 1001 g, or more, 3 mg/kg/day of oral ferrous sulphate in three divided doses.⁵

Vitamin supplementation. Vitamin supplementation was started on the third day of life. By age 2 weeks the daily doses were as follows: Vitamin A 1500 IU, vitamin D 1000 IU, vitamin E 10 mg, B₁₂ 1 µg, and folic acid 50 µg. Vitamin C, 24 to 48 mg/day, was given from the third day. Half the infants were given additional vitamins: 20 mg/kg/day of vitamin E (im) during the first three days, 0.5 mg/kg/day of folic acid (im) five times during the first month, and 0.3 mg/day of vitamins B₁, B₂, and B₆ from the third day of life. The vitamin status did not differ statistically in protein supplemented and unsupplemented infants (Table 1).

Blood samples. Haemoglobin, reticulocyte count, and red blood cell indices were obtained from capillary samples drawn at 1 to 10 weeks of age. The rest of the blood samples were taken intravenously at 1, 2, 4, 6, 8, 10, and 12 weeks of age.

Methods. Haemoglobin concentration and red blood cell indices were measured by Coulter counter. The reticulocyte count was determined microscopically. Serum ferritin was determined by radioimmunoassay,⁶ serum transferrin by immunoturbidimetry,⁶ and serum iron by spectrophotometry.⁶ Serum selenium concentrations were measured by direct electrothermal atomic absorption spectrometry;⁷ total serum protein was quantitated by a Biuret method;⁸ and plasma concentration of ascorbic acid and α -tocopherol were estimated by chemical assays.^{9,10} Plasma folic acid and vitamin B₁₂ were measured by radioligand assays (Dualcount Diagnostic Products, Los Angeles). The preparation of protein concentrate from human milk was performed as described earlier.¹¹

Statistical analyses were performed using Biomedical computer programs by University of California at Los Angeles.⁶ Tests used were one way analysis of variance, simple linear regression and correlation, partial correlation, and analysis of variance for repeated measurements. The values are given as mean (SEM).

Parents gave informed consent to the feeding regimen and blood sampling, and the ethics committee of the hospital approved the study protocol.

Results

Protein supplementation of very low birthweight infants fed human milk resulted in an increase in the concentration of haemoglobin (Fig. 1). The mean concentrations ranged between 1.3 and 1.7 g/dl between the ages of 4 and 10 weeks. A statistically significant difference was found in haemoglobin concentrations from age 4 to 10 weeks ($P > 0.05$ – 0.01) (Fig. 1). The pattern was similar in both appropriate and small for gestational age infants. The observation was also essentially similar if we analysed only the results from infants who had not undergone transfusion. The mean concentrations of haemoglobin were statistically different at 4 ($P < 0.05$) and 6 weeks of age ($P < 0.01$). Individual values are shown in Fig. 2. Even the increased concentrations remained lower, however, than the respective haemoglobin values obtained in healthy term infants.⁶

Fig. 3 shows the correlation between individual concentrations of haemoglobin at age 8 weeks and total serum protein at age 4 weeks in all infants. The correlation was also similar with regard to haematocrit values and red blood cell counts.

Reticulocytosis started about two weeks earlier in the supplemented infants and the values also peaked and fell earlier (Fig. 4).

Fig. 1 Influence of human milk protein supplementation on the haemoglobin concentration.

P values represent the statistical significance between the protein supplemented (—) and unsupplemented infants (---), * $P < 0.05$, ** $P < 0.01$.

Fig. 2 Haemoglobin concentrations in untransfused infants.

Fig. 3 Correlation between the haemoglobin concentration (at age 8 weeks) and the preceding total serum protein value (at age 4 weeks) in 21 infants ($r=0.74$, $P<0.001$).

The correlation was similar in the untransfused infants ($r=0.87$, $P<0.01$).

Fig. 4 Influence of human milk protein supplementation on reticulocyte count. (Same infants as in Fig. 1).

The observation was similar in the untransfused infants, although the mean curves diverged more from 4 to 8 weeks of age.

We found no difference in the concentrations of serum ferritin or iron between the supplemented and unsupplemented infants. The serum concentration of transferrin was higher, however, in the

supplemented infants. The mean (SEM) values at age 6 weeks were 2.7 (0.2) g/l and 1.7 (0.1) g/l ($P<0.001$) and at 8 weeks 2.9 (0.2) g/l and 1.7 (0.2) g/l ($P<0.01$) in supplemented and unsupplemented infants respectively. Serum transferrin and total serum protein correlated highly significantly at ages 6 and 8 weeks ($r=0.77$ and 0.72 , $P<0.001$).

There were no differences in the mean plasma concentrations of vitamins C, E, B₁₂, or folic acid between the supplemented and unsupplemented infants at 2, 6, or 12 weeks of age. In contrast, the mean concentration of serum selenium was higher in the supplemented infants at 2 weeks ($P<0.05$), but there was no statistically significant difference at 6 or 12 weeks. Total serum protein and serum selenium correlated at the same ages ($r=0.42$, $P<0.05$; $r=0.61$, $P<0.01$; $r=0.40$, $P<0.05$, respectively).

Plasma vitamin concentrations did not correlate with haemoglobin or reticulocyte values at any age studied. An exception was vitamin E, which correlated with haemoglobin at age 6 weeks ($r=0.42$, $P<0.05$).

Mean corpuscular volume and mean corpuscular haemoglobin values did not differ in either group of infants, except that at age 10 weeks mean corpuscular haemoglobin was higher in protein supplemented infants (31 (0.7) pg v 28 (0.4) pg, $P<0.05$). Analysis of variance for repeated measurements (from 1 to 10 weeks), however, did not show any significant difference between the groups ($P=0.31$).

We were able to estimate the volume of blood drawn for laboratory tests and also the volume of red cells transfused into the infants during the first six weeks (Table 2). The blood drawn from the untransfused infants was about half of that from those infants who needed transfusions during the study.

Discussion

The present results indicate that protein intake is a rate limiting factor which determines the concentration of haemoglobin during the second and third months of life in very low birthweight infants fed human milk. Furthermore, we have shown that by supplementing milk with protein isolated from human milk, the haemoglobin concentration increased significantly, in both appropriate and small for gestational age preterm infants.

It is possible that very low birthweight infants who are fed human milk suffer from real protein malnutrition. Consequently, the impaired globin synthesis may be a rate limiting factor in haemoglobin synthesis. The general hypoproteinaemia¹¹ found in very low birthweight infants fed human milk also

suggests the possibility of nutritional protein deficiency.

Kumar *et al*¹² have studied the effect of progressive protein deprivation in the monkey to determine which laboratory tests are affected early and which reflect the severity of protein deprivation. They found reduced haematocrit values after four weeks of protein deprivation. Total serum protein and transferrin were affected later, from six to 16 weeks and seemed to reflect the severity of protein deprivation. In our study we found deprivation in total serum protein, transferrin, haematocrit, erythrocytes, and haemoglobin as early as age 4 weeks in protein unsupplemented infants. This phenomenon is in accordance with the even greater need to use protein for growth in these very low birthweight infants.

Protein supplemented infants gained weight faster than the unsupplemented infants (Table 1). This development probably changed the requirement for haemoglobin synthesis and increased the haemoglobin mass in supplemented infants, further emphasising our findings on haemoglobin concentrations.

That vitamin E deficiency in preterm infants is associated with haemolytic anaemia has been well documented.¹³ In this study, however, there was no evidence of deficiency, since every infant was supplemented with water soluble vitamin E, and the plasma concentrations of vitamin E were normal throughout the study period. Similarly, there was no evidence of vitamin B₁₂, folic acid, or vitamin C deficiency in the infants. Based on these data it is unlikely that the vitamin status would explain our findings.

Evidence of haemolysis has been shown in children with protein-energy malnutrition.¹⁴ The haemolysis may be caused by decreased activity of superoxide dismutase and glutathione peroxidase enzymes in erythrocytes. Decreased activities of these enzymes seem also to be related to a reduced availability of copper and selenium. In a study by Rudolf *et al*,¹⁵ however, an early decline in the concentration of haemoglobin did not correlate with red blood cell selenium values or glutathione peroxidase activity in very low birthweight infants. In our study design the intake of selenium and the subsequent concentration of red blood cell selenium were dependent on protein intake. Thus it is not surprising that both intakes correlated with haemoglobin concentrations, even if we feel that the weaker correlation with selenium is a secondary phenomenon. The patterns in reticulocyte counts also argue against haemolysis in the unsupplemented infants.

Plasma concentrations of albumin and cerulo-

plasmin are low in very low birthweight infants compared with those found in normal term infants.^{16 17} Both proteins are active in copper transport. The albumin is involved in the transport of ingested copper to the liver and ceruloplasmin in the copper donation for extrahepatic tissues.¹⁸ Hågå *et al*¹⁷ have shown that neither ceruloplasmin nor superoxide dismutase activity seem to play a role in the aetiology of the early anaemia of prematurity.

The blood drawn for vital laboratory studies and the consequent blood transfusions should not be ignored in studies of haemoglobin concentration. We estimated the cumulative volumes in all subjects, and these were similar in protein supplemented and unsupplemented infants. Most of the blood was drawn and transfused soon after birth, having less influence on later haemoglobin concentrations. Further, our observations were similar in the 18 preterm infants who required relatively uniform and modest numbers of laboratory tests and who did not receive any transfusions. These findings support our conclusion that the blood transfusions had little or no influence on results.

The question of potential benefits of the quality of human milk protein fed to the very low birthweight infant remains open. The present results may suggest indirectly that human milk protein is superior because of the consequent concentration of haemoglobin. Nevertheless, our data indicate strongly that the protein content in a mixture of mature or preterm human milk is insufficient to satisfy the needs of very low birthweight infants.

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