Carnitine Concentrations in Term and Preterm Newborns at Birth and During the First Days of Life

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Abstract: Carnitine plays an important role in energetic metabolism. The aim of the study was to characterize the carnitine status in term and preterm newborns with respect to gestational age, birth weight, haematocrit and red blood cell count (RBC). The effect of nutrition on carnitine levels in the first week of life was also studied. Total blood pool of free carnitine (FC), acylcarnitines (AC) and total carnitine (TC) were analysed in whole cord blood and postnatally in capillary blood obtained at the day 4–6 in 33 term newborns and at the day 7–10 in 27 preterm newborns using tandem mass spectrometry. Plasma level of carnitine in the cord blood was measured using radioenzymatic method. Cord plasma levels of FC, AC and TC were higher in preterm newborns in comparison with term newborns (p<0.01), but the total blood pool of FC and TC in whole cord blood was lower in preterm newborns than in term newborns (p<0.01) and positive correlation was found between FC and gestational age or birth weight (p<0.05). In addition, positive correlation was found between AC and red blood cell count or haematocrit (p<0.05). During the first week of life, blood pool of FC and TC in term newborns and AC and TC in preterm newborns decreased regardless of the type of enteral or parenteral nutrition. Our results indicate that preterm newborns are born with limited carnitine store. Interpretation of carnitine analyses in whole blood relies in addition to gestational age and birth weight on the haematocrit, especially in newborns with anaemia or blood hyperviscosity.

Introduction

Glucose is the main source of energy for the foetus before birth. In neonates, the successful adaptation to extrauterine life depends critically on the rapid switch in metabolic pathways relevant to energy provision from foetal anaerobic glycolysis to the neonatal oxidative phosphorylation [1, 2]. After birth the role of glucose as energy substrate decreases and the shift from glucose to carbohydrate-fat mixture as the energy source is observed. Almost half of the energy expenditure in neonates after birth is provided by fat oxidation.

Carnitine is ubiquitous in mammalian tissues and plays an important role in mitochondrial metabolic pathways especially in beta-oxidation of fatty acids. Carnitine transports the long-chain fatty acids to the mitochondrial matrix. Carnitine is also a cofactor for the transport of short-chain and medium-chain fatty acids in the heart and skeletal muscle but not in the liver. On the contrary, carnitine removes acyl-groups from mitochondria and thus regulates the acyl-CoA/CoA ratio [3, 4, 5]. It was shown in animal studies, that carnitine also increases the activities of respiratory chain complexes I+III, II+III and IV [6] and stimulates the metabolism of the branched-chain amino acids. Carnitine is water-soluble and is readily filtered by the renal glomerulus. Tubular carnitine reabsorption is the main regulatory mechanism of the carnitine plasmatic level. [7].

The total level of carnitine (TC) is composed from free carnitine (FC) and acylcarnitines (AC). Whereas radioenzymatic method (RE) measures plasmatic

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carnitine concentrations, the electrospray ionisation-tandem mass spectrometry method (ESI-MS/MS) measures whole carnitine blood pool including the indispensable intraerythrocytic carnitine, which constitutes up to 75 – 85 % of the whole blood carnitine content. In healthy newborns the blood level of AC represents about 25 % of TC [8, 9, 10]. However, the ratio between AC and FC varies and reflects the actual metabolic turnover of fatty acids. Therefore the AC/FC ratio may be used for the detection of fatty acid oxidation disturbances and in many countries is ESI-MS/MS method used for routine neonatal screening of inherited metabolic disorders [11, 12, 13].

   Lower availability of free carnitine, lower carnitine tissue stores, immature transport system for carnitine and potential restriction of fatty acid oxidation capacity may have negative influence on early postnatal adaptation, especially in preterm newborns. The aim of the study was to characterize the carnitine status in term and preterm newborns at birth with respect to gestational age, birth weight, haematocrit and red blood cell count (RBC). Using two methods, we analysed the levels of free carnitine (FC), acylcarnitines (AC) and total carnitine (TC) at birth in the whole cord blood and cord plasma in 33 term newborns (38–42 weeks of gestation) and 27 preterm newborns (24–37 weeks of gestation). In addition, the effect of nutrition within the first week of life on carnitine levels was studied in term newborns at the age of 4–6 days and in preterm newborns at the age of 7–10 days using capillary blood from Guthrie cards intended for routine neonatal screening of inherited metabolic disorders including phenylketonuria (PKU).

Material

Patients

Altogether, 60 newborns were investigated: 33 healthy term newborns (16 boys, 17 girls, birth weight 3485 ± 309 g) and 27 preterm newborns (12 boys, 15 girls, gestational age 24–37 week, birth weight 1855 ± 765 g with range 590–2620 g). All term newborns and 19 preterm newborns were delivered vaginally, 8 preterm newborns were delivered by Caesarean section. In all term newborns early postnatal adaptation was uneventful with the Apgar score at least 7 and 9 in the first and the fifth minute, respectively. Nine mothers of term newborns and 11 mothers of preterm newborns received antibiotics before the delivery due to the premature rupture of membranes (>18 hour). Five mothers were smokers during pregnancy, no one was vegetarian. All children, except three preterm newborns who developed multi-organ failure, had good clinical outcome. All term newborns were breast fed and the mean milk intake of 100–120 ml/kg of body weight was achieved at the end of the first week of life. Parenteral nutrition without carnitine supplementation in combination with gradual increase in the amount of mother milk or adapted milk formula for preterm neonates was initiated in preterm newborns and the mean milk intake at the end of the first week of life was 50 ml/kg/day (range 20–100 ml/kg/day) according to individual tolerance.

Small-for-gestational-age newborns and newborns with congenital malformations, perinatal asphyxia or metabolic diseases, and children of mothers with gestational diabetes mellitus or symptoms of acute infection were excluded from the study.

Samples
After delivery, two drops of cord blood from the placental side of the umbilical vein were spotted on the Guthrie card. Substantial part of cord blood was collected into the tubes containing sodium citrate, transferred to the laboratory and centrifuged. Fresh plasma was frozen at –80° C. Cord plasma samples were obtained from all 33 term newborns and only from 14 preterm newborns due to limited amount of cord blood in preterm newborns. The blood count was evaluated in cord blood of 27 infants. In all children the second sample was obtained from the Guthrie cards intended for neonatal screening of inherited metabolic disorders including PKU at the age of 4–6 days in term newborns and at the age of 7–10 days in preterm newborns.

Method
Free carnitine (FC), acylcarnitines (AC) and total carnitine (TC) were analysed by two different methods – electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) and radioenzymatic technique (RE). All samples from dried blood spots were investigated on API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX) with TurboIonSpray (TIS) interface in combination with a PE 200 Autosampler and a PE series 200 microgradient system was used [14]. One 3-mm diameter dot was punched from each 10-mm diameter dried blood spot specimen into a single well of a 96-well microtiter filter plate to which was added 200 μl of a methanol stock solution of internal deuterated standards (containing 0.76 μmol/l [2H3]carnitine, 0.19 μmol/l [2H3]acetylcarnitine, 0.038 μmol/l each of [2H3]propionylcarnitine, [2H3]butyrylcarnitine, [2H3]isovalerylcarnitine, [2H3]octanoylcarnitine, [2H3]myristoylcarnitine, 0.076 μmol/L [2H3]palmitoylcarnitine, 2.5 μmol/l each of [2H3]alanine, [2H8]valine, [2H3]leucine, [2H3]methionine, [2H8]phenylalanine, [2H4]tyrosine, [2H3]glutamate, [2H6]ornithine and 12.5 μmol/L [2H3]glycine). After 25 minutes, the eluate was evaporated to dryness and 100 μl 3N HCl in butanol were added. The microtitration plates were sealed and incubated at 60° C for 30 minutes. After removal of the seal, excess HCl-butanol was evaporated to dryness. The derived samples were reconstituted with 200 μl acetonitril/water (1:1) containing 0,02 % formic acid. The sample, 40 μl, was injected directly to tandem mass spectrometer at a solvent flow rate of 60 μl/min, resulting in a run-time of 2,5 min for each sample. The detection of acylcarnitines was carried out using the precursor-ion scan of m/z 85 and scanning from m/z 200 to 550. Each acylcarnitine was quantified using the signal intensity ratio of the compound to its internal standard

and related to concentrations using the slope derived from standard curves. The levels of short-chain acylcarnitines (C2-5), medium-chain acylcarnitines (C6-12) and long-chain acylcarnitines (>C12) were summarised to yield total acylcarnitines concentrations.

Carnitine in plasma was assayed by a radioenzymatic technique on Tri-Carb Liquid Scintillation Analyzer Models 2500TR (Cambera Packard) [15]. For total carnitine, to 50 μl of plasma is added 50 μl of 0.2 mol/l KOH and the samples are placed in water bath at 50° C for 30 min. At the end of the hydrolysis period 50 μl of 0.5 mol/l (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) buffer is added. The pH of samples is checked to confirm a value of about 7.4. For free carnitine, 50 μl of plasma is diluted with 100 μl distilled water. For carnitine measurement, samples are mixed with 100 μl of reagent mixture (containing 0.01 mol/l N-ethylmaleimide; 0.5 mol/l (N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), pH 7.6; 0.1 mol/l EDTA; 0.5 mol/l acetyl-CoA; 5000 dpm/μl [1-^{14}C] acetyl-CoA; 1 μl carnitine acetyltransferase) and incubated at room temperature for 30 min. Samples are placed on top of column of Dowex 1-X8 (Cl form 200–400 mesh) contained in a Pasteur pipette. The column is washed twice with 0.5 ml distilled water and with 1 ml 96% ethanol. The eluate is collected in scintillation vial, and 5 ml of a scintillation fluid is added to the vial, mixed, and counted. Carnitine was quantified using the slope derived from standard curves.

Ethics
The study was approved by the Medical Ethics Committee in the Faculty Hospital. Samples were obtained after informed consent from the parents.

Statistical Analysis
Student's test for unpaired samples was used to test the differences in carnitine level in cord plasma and whole cord blood. Simple linear regression was used to test for the correlation between carnitine level and gestational age, birth weight or blood count. Differences were regarded as significant at a $p < 0.05$. T-test for paired samples was used to compare the changes in carnitine level in cord blood and Guthrie cards.

Results
Cord plasma level of FC, AC and TC estimated by radioenzymatic method was significantly higher in preterm newborns in comparison with term newborns (Table 1) and negative correlation between cord plasma level of FC, AC or TC and birth weight or gestational age was found ($p < 0.05$ for all parameters) (Figure 1 C, D). On the contrary, the total blood pool of FC and TC in cord blood estimated by ESI-MS/MS method were significantly lower in preterm newborns in comparison with term newborns (Table 1) and the positive correlation between cord blood level of FC and gestational age or birth weight was observed ($p < 0.05$

Table 1 – Free carnitine (FC), acylcarnitines (AC) and total carnitine (TC) levels and their ratios in plasma from cord blood estimated by radioenzymatic method (RE) and in the whole cord blood estimated by electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) in term newborns (B, D) and preterm newborns (A, C)

Group*	A	B	C	D		
n	14	33	27	33		
Material	Cord plasma		Cord blood			
Method	RE		ESI-MS/MS			
					p-value	
Carnitine	(μmol/l)				A:B	C:D
FC	20.8 ± 16.5	6.61 ± 2.4	32.9 ± 6.7	46.87 ± 13.9	<0.01	<0.001
AC	15.6 ± 13.6	3.63 ± 1.46	17.4 ± 3.9	14.49 ± 8.7	<0.01	NS
TC	36.4 ± 19	10.2 ± 3.5	50.3 ± 10	61.36 ± 20.3	<0.01	<0.01
AC/FC	0.78 ± 0.37	0.59 ± 0.31	0.53 ± 0.07	0.31 ± 0.13	NS	<0.001
FC/TC	0.58 ± 0.08	0.65 ± 0.09	0.65 ± 0.03	0.77 ± 0.07	NS	<0.001

* Mean±SD-birth weight (g), gestational. age (weeks): A-1652 ± 728, 32 ± 2.8; B-3485 ± 309, 40 ± 1.4; C-1855 ± 765, 32.6 ± 3.2; D-3485 ± 309, 40 ± 1.4
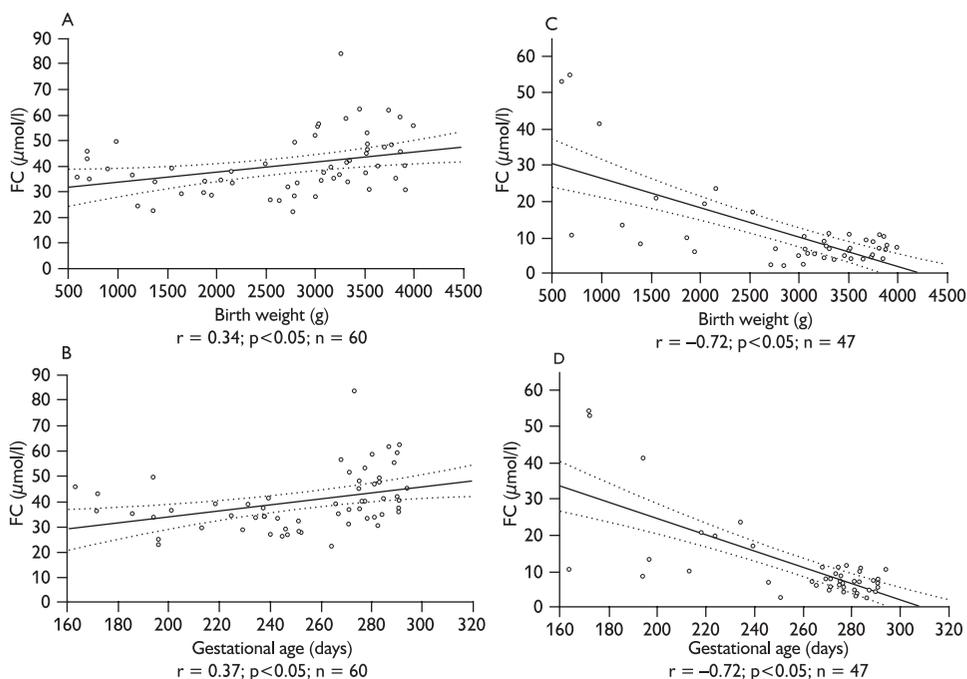

Figure 1 – The correlation between free carnitine (FC) level in the whole cord blood and birth weight (A) or gestational age (B) estimated by electrospray ionisation-tandem mass spectrometry (ESI-MS/MS) and the correlation between free carnitine (FC) level in plasma and birth weight (C) or gestational age (D) estimated by radioenzymatic method (RE).

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for both parameters) (Figure 1 A, B). No correlation was observed between red blood cell count, haematocrit or white blood cell count and the level of FC or TC in the whole cord blood (data not shown), but positive correlation was found between AC level and RBC or haematocrit (p<0.05 for both parameters) (Figure 2).

The TC level decreased significantly from birth to the postnatal day 4–6 in term newborns and from birth to the postnatal day 7–10 in preterm newborns (Figure 3). During the same period FC level decreased in term newborns and AC level decreased in preterm newborns. No relation was found between carnitine level and the amount of maternal milk or adapted milk formula intake with or without parenteral nutrition within the first week of life in term newborns at the age of 4–6 days and in preterm newborns at the age of 7–10 days (data not shown).

No differences in carnitine level were found between the boys and girls, between the newborns born to non-smoking or smoking mothers, and between the newborns of mothers receiving and not receiving antibiotics for premature rupture of membranes. No differences in carnitine level were found in three
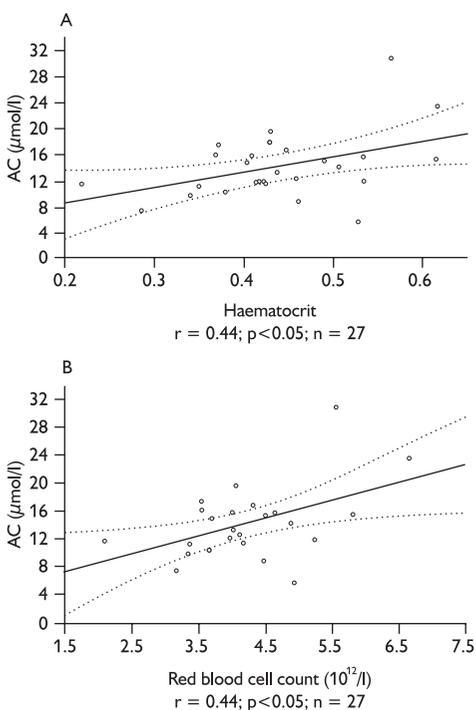

Figure 2 – The relation between acylcarnitines (AC) level in the whole cord blood and haematocrit (A) or red blood cell count (B).
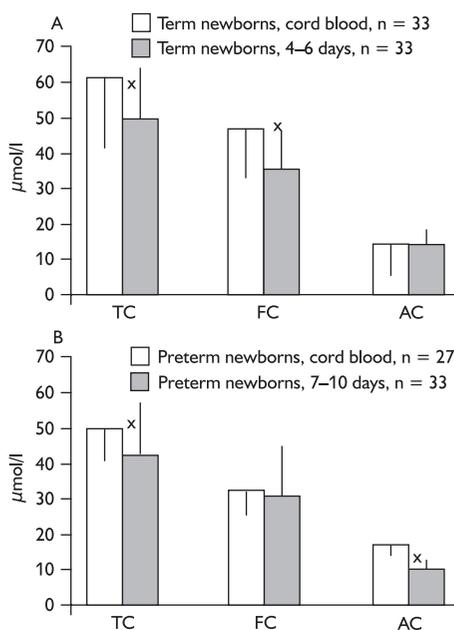

Figure 3 – The comparison between total carnitine (TC), free carnitine (FC) and acylcarnitines (AC) levels in pairs of samples from the cord blood and the blood obtained in term newborns at the postnatal day 4–6 (A) and in preterm newborns at the postnatal day 7–10 (B). (^{x}p< 0.05).

preterm newborns that died during the first week of life due to multi-organ failure (data not shown).

Discussion

In our study we analysed carnitine status in term and preterm newborns at birth in the whole cord blood and cord plasma using two methods. In addition, carnitine was also investigated in the Guthrie cards obtained at the age of 4–6 days in term newborns and at the age of 7–10 days in preterm newborns.

Placental transfer is the main source of carnitine for the foetus and for the newborn during the early postnatal period. The plasma carnitine concentration in pregnant women decreases during gestation and reaches the lowest levels near the end of the gestation [16, 17]. Similar to other studies using RE method, we observed higher level of carnitine in cord plasma in our group of preterm newborns in comparison with term newborns resulting from a higher placental carnitine transfer from the mother in earlier stages of gestation [18]. In contrast with plasma carnitine level, total carnitine pool in muscle tissue is positively correlated with gestational age [19] and it further increases with age to adulthood [20]. It was already presented using ESI-MS/MS method that the total blood pool of carnitine in preterm newborns is lower in comparison with term newborns [21] and it further increases during childhood [22]. We also observed higher FC and TC levels in cord blood in term newborns in comparison with preterm newborns and significant correlation between FC and gestational age or birth weight. It supports the hypothesis, that increasing store of carnitine at the end of gestation is supposed for the postnatal participation of carnitine at the mitochondrial fatty acid oxidation. Lower cord blood carnitine pool in preterm newborns in comparison with term newborns might indicate that preterm newborns are born with limited carnitine store [21]. Intrauterine growth retardation and perinatal asphyxia are additional risk factor for diminishing carnitine stores [9, 23].

During foetal development red blood cell count, haemoglobin and haematocrit increase and red blood cell volume decreases [24, 25]. Acylcarnitines in red blood cells represent the major pool of carnitine (65–75 %) in blood, whereas only 1–3 % of acylcarnitines are present in leucocytes and platelets [8, 26]. Several theories tried to explain the role of carnitine in erythrocytes [8], but AC in erythrocytes may represent freely exchangeable reservoir of promptly available activated acyl-groups. We found positive correlation between AC level and red blood cell count and haematocrit. Therefore not only gestational age and foetal growth, but also changes in red blood cell count and haematocrit may influence the results of carnitine measurement if ESI-MS/MS method is used.

Different trends of blood carnitine levels in the neonatal period in comparison with carnitine levels in the cord blood were described [21, 22, 27]. Moreover, prolonged parenteral nutrition without carnitine supplementation in premature newborns may further diminish carnitine reserves and leads to carnitine deficiency

[28, 29, 30]. About 75% of the daily carnitine requirement (20–100 mg/day) is provided by enteral nutrition and one quarter is from endogenous carnitine synthesis in the liver and the kidneys in a healthy child under normal circumstances. Carnitine content in colostrum (1–1.4 mg/100 ml) and mature human milk (0,6–1,0 mg/ /100 ml) is lower in comparison with the cow's milk (2 mg/100 ml) [31].

We compared carnitine levels in cord blood with carnitine levels in Guthrie card supposed for neonatal screening of PKU. During the first week of life, the average blood levels of TC and FC decreased in term newborns and TC and AC decreased in preterm newborns resulting in similar AC/FC and FC/TC ratios in both group of newborns. We did not found any correlation between carnitine levels in blood and the amount of milk intake during the first days of life.

Conclusion

Preterm newborns in comparison with term newborns are born with limited carnitine store. It may be of importance to notice, that the results of acylcarnitine analyses in the whole blood in newborns may partially rely not only on the gestational age and birth weight but also on the actual haematocrit, especially in newborns with anaemia or blood hyperviscosity.

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