Postnatal Variations in Blood Free and Acylcarnitines

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Background: Alteration in concentrations of blood carnitine and its esters are diagnostic of a number of inherited metabolic disorders. Acylcarnitine (AC) profiles of newborns obtained from dried blood spots by tandem mass spectrometric analysis are being used for the diagnosis of these disorders. There are no data of the postnatal variations of free carnitine (FC) and AC in Indian neonates. **Objectives:** Evaluation of postnatal variations in free and AC levels in newborns. **Methods:** Blood FC and AC levels were evaluated in 2,727 healthy neonates of postnatal day 2–30 by electrospray ionization tandem mass spectrometry. **Results:** Blood C2, C5DC, C16, C16:1, C18, C18:1, C18:2, and C18:OH carnitines

were increased in groups A (aged 8–14 days) and B (aged 15–30 days), compared with the control group (aged 2–7 days), whereas C3, C4, C4OH, C6, C6DC, and C12 carnitines were increased only in group B. No sex-related differences were found except for C3DC, C4, and C5 carnitine concentrations, which were higher in female neonates. **Conclusions:** Our data can be used as a reference for the assessment of carnitine status in Indian newborns, hence reducing the risk of misdiagnosis of fatty acid oxidation disorders and organic acidemias during interpretation of the results of tandem mass spectrometry-based newborn screening. *J. Clin. Lab. Anal.* 25:126–129, 2011. © 2011 Wiley-Liss, Inc.

Key words: free carnitine; acylcarnitines; tandem mass spectrometry; newborns

INTRODUCTION

L-Carnitine (L-3-hydroxy-4-*N,N,N*-trimethylaminobutyrate), a quaternary ammonium compound, is an essential metabolic mediator which has a number of vital roles in intermediary metabolism (1). It is responsible for the transport of acyl groups across the mitochondrial membrane so that they can be oxidized to produce energy, ketone bodies, and possibly regulation of certain aspects of nitrogen metabolism. L-carnitine also plays a key role in removing potentially toxic acyl-CoA esters out of this cellular organelle by formation of acylcarnitine (AC) esters and thereby releasing coenzyme A and restoring mitochondrial homeostasis. Carnitine is present in free as well as in esterified forms in the tissues and body fluids. Reduction in free carnitine (FC) and increase of AC in blood can occur in inherited metabolic disorders such as organic acidemias and fatty acid oxidation disorders. These disorders may manifest in the neonatal period and early infancy, and estimation of FC and ACs in plasma or blood spots provides a powerful selective newborn screening tool for their identification (2). Electrospray ionization tandem mass spectrometry (ESI-MS-MS) allows quantitative

determination of FC and various ACs in blood (2). In recent years, ESI-MS-MS has been applied in newborn screening programs around the world in order to detect aminoacidopathies, organic acidemias, and fatty acid oxidation disorders. This method is highly sensitive and specific, and whole blood FC and ACs levels can be obtained using very small, dried blood samples. However, knowledge of postnatal variations in carnitine concentration is essential for the accurate diagnosis and optimal management of these disorders.

Several studies have been carried out in the Western population to determine variations in the carnitine levels in the postnatal period (3). No such data are available on age-related carnitine levels in neonates in India, a country in which the prevalence of genetic disorders is expected to be high owing to a high birth rate and

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avored consanguineous marriages (4). In this study, we aimed at using ESI-MS-MS to investigate the changes in neonatal carnitine concentrations in blood during the first month of life in Indian newborns.

MATERIALS AND METHODS

The study was approved by the Institute Ethics Committee of the National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, India. The study group composed of 2,727 healthy appropriate-for-gestational-age neonates selected from 3,100 infants screened by Newborn Screening Program conducted in Bangalore, between March 2007 and August 2009. Premature and low birth weight babies, newborns with acute or chronic illnesses, congenital malformation syndromes, and as well as those on any medication were excluded. Written informed consents were obtained from parents of the neonates. Specimens of dried blood spots were collected by heel prick from infants from postnatal day 2–4 weeks, and were spotted on Schleicher & Schuell (S&S) 903 filter paper. Demographic information, such as age in days, gender, gestational age, birth weight, and time of sampling, were collected at the time of sample collection.

The study group was divided into three sub-groups based on the age at the time of sampling. The neonatal screening group of 2–7 days and gestational age of 39.6 ± 1.2 weeks represented the Control group. Group A was composed of infants of 8–14 postnatal days and gestational age 39.1 ± 0.9 days. Infants of 15–30 postnatal days were grouped into Group B with gestational age of 38.9 ± 1.7 weeks.

All dried blood spots were analyzed by electrospray-tandem mass spectrometry method using nonderivatized MS/MS kits (Neogram AA/AC kits, Perkin Elmer, Turku, Finland) as per the manufacturer's instruction. The internal standards included deuterated FC (d_9 -C0) and 12 ACs (d_3 -C2, d_3 -C3, d_3 -C4, d_9 -C5, d_3 -C6, d_3 -C8, d_3 -C10, d_6 -C5DC, d_3 -C12, d_3 -C14, d_3 -C16, and d_3 -C18). A Waters Micromass Quattro MicroTM API

mass spectrometer was used for the analysis. The FC and AC concentrations were calculated by comparing the ion mass spectra of the different analytes with the spectra of their corresponding internal standards using the Neolynx software (Waters, Massachusetts).

The statistical analyses were carried out using SPSS (version 11.0 for Windows). The mean of the three groups were computed by an ANOVA model and Levene's statistics was applied to test the homogeneity of variances of different groups. Tukey test procedure was used to test the differences between the control group mean and means of other groups. Statistical significance was defined as a P value < 0.05 .

RESULTS

The mean values for different carnitines computed from 2,204 samples of infants aged 2–7 days (control group) were comparable to standard reference values measured by tandem mass spectrometry cited in the literature (3). The concentrations of different carnitines for groups A and B were compared with the control group and presented in Tables 1 and 2.

FC concentration in group A did not differ significantly from the mean FC concentration of the control group; however, it was significantly higher in group B. Total carnitine (TC) and total acylcarnitine (tAC) concentrations were significantly higher in groups A and B in comparison with the control group. Short and medium-chain ACs were found to be higher only in group B, whereas long-chain ACs were increased in both groups A and B compared with the control group.

Short-chain ACs, C2 and C5DC were increased in both groups A and B, whereas C3, C4, and C4OH were increased only in group B compared with the control group. Of the medium-chain ACs, C6 and C6DC were increased in group B. No significant difference was found between group A and the control group. C16, C16:1, C18, C18:1, C18:2, and C18:OH long-chain ACs were significantly higher in groups A and B in comparison with the control group. No sex-related

TABLE 1. Concentrations of Free Carnitines, Total Carnitines, and Total Acylcarnitines

Carnitines	Control group, Age: 2–7 days, $n = (2,204)$ $\mu\text{mol/l}$	Group A, Age: 8–14 days, $n = (295)$ $\mu\text{mol/l}$	Group B, Age: 15–30 days, $n = (228)$ $\mu\text{mol/l}$
	Mean $\pm$ SD	Mean $\pm$ SD (P)	Mean $\pm$ SD (P)
Free carnitine	26.08 ± 7.73	27.58 ± 9.36 (0.08)	29.37 ± 9.9 ($< 0.01^*$)
Total acylcarnitine	25.48 ± 6.9	30.37 ± 7.2 ($< 0.001^*$)	31.85 ± 8.4 ($< 0.001^*$)
Total carnitine (free carnitine + total acylcarnitine)	51.51 ± 16.5	57.95 ± 18.6 ($< 0.05^*$)	61.16 ± 20.8 ($< 0.001^*$)
Short-chain acylcarnitines	18.09 ± 8.19	19.23 ± 14.22 (0.13)	22.29 ± 14.37 ($< 0.001^*$)
Medium-chain acylcarnitines	1.24 ± 0.44	1.26 ± 1.59 (0.16)	1.34 ± 0.40 ($< 0.01^*$)
Long-chain acylcarnitines	6.76 ± 2.41	7.84 ± 3.50 ($< 0.01^*$)	8.26 ± 3.31 ($< 0.001^*$)

*Significant.

TABLE 2. Concentrations of Short-, Medium-, and Long-Chain Acylcarnitines

Acylcarnitines	Control group (2–7 days), <i>n</i> = (2,204) $\mu\text{mol/l}$ Mean $\pm$ SD	Group A (8–14 days) <i>n</i> = (295) $\mu\text{mol/l}$ Mean $\pm$ SD (<i>P</i>)	Group B (15–30 days) <i>n</i> = (228) $\mu\text{mol/l}$ Mean $\pm$ SD (<i>P</i>)
C2	14.85 $\pm$ 7.32	15.97 $\pm$ 12.86 (<0.05*)	18.75 $\pm$ 12.86 (<0.001*)
C3	1.44 $\pm$ 0.93	1.36 $\pm$ 1.21 (0.39)	1.63 $\pm$ 1.48 (<0.01*)
C4	0.52 $\pm$ 0.40	0.51 $\pm$ 0.33 (0.92)	0.56 $\pm$ 0.35 (<0.05*)
C5	0.23 $\pm$ 0.15	0.25 $\pm$ 0.22 (0.12)	0.24 $\pm$ 0.14 (0.88)
C3:DC	0.31 $\pm$ 0.23	0.32 $\pm$ 0.70 (0.54)	0.30 $\pm$ 0.29 (0.9)
C5:DC	0.11 $\pm$ 0.12	0.14 $\pm$ 0.39 (<0.05*)	0.15 $\pm$ 0.16 (<0.01*)
C4:OH	0.36 $\pm$ 0.34	0.34 $\pm$ 0.25 (0.57)	0.40 $\pm$ 0.28 (<0.05*)
C5:OH	0.27 $\pm$ 0.17	0.29 $\pm$ 0.34 (0.31)	0.27 $\pm$ 0.15 (0.9)
C6	0.02 $\pm$ 0.03	0.02 $\pm$ 0.03 (0.99)	0.04 $\pm$ 0.04 (<0.01*)
C8	0.14 $\pm$ 0.09	0.15 $\pm$ 0.18 (0.41)	0.16 $\pm$ 0.10 (0.14)
C10	0.15 $\pm$ 0.09	0.16 $\pm$ 0.30 (0.12)	0.15 $\pm$ 0.08 (0.69)
C12	0.17 $\pm$ 0.14	0.17 $\pm$ 0.29 (0.94)	0.20 $\pm$ 0.09 (<0.05*)
C6:DC	0.12 $\pm$ 0.10	0.13 $\pm$ 0.10 (0.18)	0.14 $\pm$ 0.12 (<0.01*)
C8:1	0.22 $\pm$ 0.13	0.23 $\pm$ 0.26 (0.26)	0.24 $\pm$ 0.18 (0.19)
C10:1	0.17 $\pm$ 0.10	0.18 $\pm$ 0.25 (0.37)	0.18 $\pm$ 0.09 (0.9)
C10:2	0.08 $\pm$ 0.09	0.09 $\pm$ 0.19 (0.15)	0.08 $\pm$ 0.08 (0.95)
C12:1	0.17 $\pm$ 0.10	0.16 $\pm$ 0.22 (0.6)	0.18 $\pm$ 0.10 (0.37)
C14	0.21 $\pm$ 0.10	0.22 $\pm$ 0.13 (0.2)	0.21 $\pm$ 0.11 (0.57)
C16	3.50 $\pm$ 1.44	3.59 $\pm$ 1.84 (<0.05*)	3.60 $\pm$ 2.01 (<0.05*)
C18	0.92 $\pm$ 0.41	0.96 $\pm$ 0.50 (<0.05*)	0.97 $\pm$ 0.50 (<0.05*)
C14:OH	0.04 $\pm$ 0.07	0.04 $\pm$ 0.19 (0.32)	0.03 $\pm$ 0.02 (0.9)
C14:1	0.11 $\pm$ 0.09	0.10 $\pm$ 0.19 (0.53)	0.11 $\pm$ 0.07 (0.51)
C16:OH	0.06 $\pm$ 0.06	0.07 $\pm$ 0.17 (0.39)	0.07 $\pm$ 0.04 (0.55)
C16:1	0.29 $\pm$ 0.14	0.32 $\pm$ 0.29 (<0.01*)	0.34 $\pm$ 0.17 (<0.05*)
C18:OH	0.05 $\pm$ 0.16	0.07 $\pm$ 0.23 (<0.05*)	0.09 $\pm$ 0.04 (<0.05*)
C18:1	1.30 $\pm$ 0.52	1.38 $\pm$ 0.71 (<0.01*)	1.39 $\pm$ 0.67 (<0.01*)
C18:1-OH	0.06 $\pm$ 0.11	0.07 $\pm$ 0.20 (0.26)	0.07 $\pm$ 0.05 (0.88)
C18:2	0.21 $\pm$ 0.18	0.27 $\pm$ 0.25 (<0.05*)	0.28 $\pm$ 0.13 (<0.05*)

*Significant. C2, acetylcarnitine; C3, propionylcarnitine; C3DC, malonylcarnitine; C4, butyrylcarnitine; C5, isovalerylcarnitine; C5DC, glutaryl carnitine; C4OH, hydroxybutyrylcarnitine; C5OH, hydroxyisovalerylcarnitine; C6, hexanoylcarnitine; C6DC, methylglutaryl carnitine; C8, octanoylcarnitine; C8:1, octenoylcarnitine; C10, decanoylcarnitine; C10:1, decenoylcarnitine; C10:2, decadienoylcarnitine; C12, Dodecanoylcarnitine; C12:1, Dodecenoylcarnitine; C14, Tetradecanoylcarnitine; C14:1, Tetradecenoylcarnitine; C16, Hexadecanoylcarnitine; C16:1, Hexadecenoylcarnitine; C18, stearoylcarnitine; C18:1, oleylcarnitine; C14OH, 3-OH-tetradecenoylcarnitine; C16OH, 3-OH-hexadecenoylcarnitine; C18OH, 3-OH-octadecenoylcarnitine; C18:1OH, 3-OH-oleylcarnitine; C18:2, Linoleylcarnitine.

differences were found except for C3DC ($P < 0.01$), C4 ($P < 0.05$), and C5 ($P < 0.05$) concentrations, which were higher in female neonates.

DISCUSSION

Fatty acids are an important source of energy for the newborns. Numerous studies have shown that the carnitine pool in the neonate is limited (5). The activity of gamma-butyrobetaine hydroxylase, the enzyme that catalyzes the final step of the carnitine synthetic pathway is very low in the early days of life. Thus, the fetus obtains carnitine by transplacental transfer (6). Without an exogenous supply of carnitine, preterm infants cannot achieve carnitine homeostasis. With the initiation of milk feeding, neonatal metabolism switches to a high-fat diet, thus increasing the carnitine pool in the postnatal period. Carnitine deficiency can be part of a number of inherited and acquired metabolic diseases.

Maternal nutrition could contribute to the carnitine levels in newborns. Alteration in blood carnitine levels are also a diagnostic feature in fatty acid oxidation disorders and organic acidemias (2). Normal levels of carnitine in blood have been determined for all ages in the population of developed countries (3), but no published data are available on the carnitine levels in healthy newborns in India, a rapidly developing country. Thus, this study was intended at determining age-related variation in carnitine levels in Indian newborns for accurate diagnosis and optimal management of metabolic disorders.

In this study, we used tandem mass spectrometry for quantitative determination of FC and various carnitine esters like ACs to evaluate the changes in neonatal carnitine levels and fatty acid metabolism during the first month after birth in Indian neonates. The whole-blood concentration of FC was unchanged in the first week after birth, whereas TCs and tACs were significantly

higher in older neonates. Studies of carnitine levels within the neonatal period and early infancy are few. Some studies have shown increase in plasma levels of TC, FC, and tAC 1–4 weeks after birth (7) and significantly higher levels in older children when compared with neonates (8). Meyburg et al. (9), found no differences between FC concentrations in cord blood and 5-day-old infants, whereas TC and tAC were significantly higher in postnatal day 5. In another study, Cavedon et al. reported higher FC, TC, and tAC values in older children compared with newborns (10). Our results are in agreement with these previous observations. Within the first 2 weeks after birth, levels of most of the ACs increased, whereas the concentration of FC remained unchanged in the first week. No significant sex-related differences were found in the concentrations of carnitines and its esters in our study, except C3DC, C4, and C5 concentrations, which were higher in female neonates. This is also consistent with the findings of Covedon et al. who observed only minor sex-related differences (10).

In conclusion, our study provides normative values for blood FC and AC in Indian newborns between the ages of 2 and 30 days. These values can be used as a reference for the assessment of carnitine status in Indian neonates, thus reducing the risk of misdiagnosis during interpretation of the results of expanded, tandem mass spectrometry-based newborn screening for disorders of fatty acid oxidation and organic acidemias. The data may be of particular relevance while interpreting screening results when blood samples are collected after the first postnatal week. Our data are consistent with

previously reported findings in European, Chinese, Japanese, and Korean newborns.

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