RESEARCH ARTICLE

Correlation between plasma levels of arginine and citrulline in preterm and full-term neonates: Therapeutical implications

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Background: Preterm neonates exhibit several deficiencies that endanger their lives. Understanding those disturbances will provide tools for the management of preterm neonates. The present work focuses on arginine and citrulline which has been flagged among the biochemical landmarks of prematurity.

Methods: We examined blood samples of preterm newborns as compared with mature neonates to determine the levels of arginine and citrulline by capillary zone electrophoresis with laser induced fluorescence detection (CZE-LIFD).

Results: Significantly lower levels of arginine and citrulline were found in preterm neonates than in mature neonates ($P < .01$). Interestingly there was a highly significant correlation between the two amino acids in mature neonates ($P < .0001$). Such correlation was present in preterm neonates too ($P < .01$). Pearson coefficient showed that 60% of the citrulline concentration depends on arginine concentration in mature neonates. Only 20% of the citrulline concentration depends on arginine concentration in preterm neonates. Although the ratio arginine/citrulline was lower in preterm neonates than in mature neonates the difference was not statistically significant.

Conclusions: These results suggest that less arginine is converted to citrulline to form nitric oxide in preterm than in full-term neonates. The result is discussed in terms of the immature enzymatic systems in the preterm neonate.

KEYWORDS

amino acids, capillary, electrophoresis, fluorescence, infant, premature, nitric oxide

1 | INTRODUCTION

Premature neonates are born with immature anabolic and catabolic biochemical systems.¹⁻⁵ They cannot synthesize several amino acids and for this reason non-essential amino acids in mature neonates are considered essential in premature neonates thus making parenteral amino acids administration common practice.^{6,7} Arginine and citrulline are two of these amino acids. Their essential nature is suggested by the

fact that supplements of arginine help to avert necrotizing enterocolitis (NEC) a potentially lethal condition in preterm neonates⁸⁻¹⁰ even though some authors do not find a consistent protective effect.^{11,12} Citrulline also is an amino acid intimately related to arginine metabolism.¹³ The enzyme nitric oxide synthase (NOS) converts arginine into citrulline and produces nitric oxide (NO).^{14,15} Citrulline reaction with aspartate to form argininosuccinate is catalyzed by the enzyme argininosuccinate synthetase (ASS).¹⁶ Then the enzyme argininosuccinate lyase (ASL) breaks the molecule of argininosuccinate into fumarate and arginine.¹⁷ Citrulline and arginine supplements protect the tight junctions of intestinal epithelium during ischemic episodes.¹⁸ Therefore supplements of citrulline have been recommended to prevent NEC in preterm neonates. Although low levels of arginine¹⁹⁻²¹ and citrulline²²

Abbreviations: ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CPS-1, carbamoyl phosphate synthase; CZE, capillary zone electrophoresis; LIFD, laser induced fluorescence detection; NEC, necrotizing enterocolitis; NO, nitric oxide; NOS, nitric oxide synthase; OCT, ornithine carbamoyl phosphate transferase; P5CR, pyrroline-5-carboxylate reductase.

have been reported and deficiencies of ASS and ASL have been proposed,²¹ little attention has been paid to the relationship between arginine and citrulline in preterm neonates. In this study, it was investigated the plasma level of arginine and citrulline in preterm neonates as compared with full-term neonates. Therefore, a correlation between arginine and citrulline was analyzed.

2 | METHODS

The subjects were 11 female and 18 male (29 total) preterm neonates and 11 female and 19 male (30 total) normal neonates. None of the preterm neonates suffered Necrotizing Enterocolitis. Fourteen of them (48%) had Respiratory Distress Syndrome due to Hyaline Membrane Disease; ten of them (34.4%) had sepsis diagnosed by lymphocytosis the first day and three days later confirmed by hemoculture. Their body weight was 1583 ± 82 g (mean $\pm$ standard error of the mean) with a range 870–2350 g. None of them had received parenteral nutrition nor were breast fed since the samples were collected when they were admitted. All of them were free of congenital defects. Their estimated gestational age according to the Capurro test was 32.3 ± 0.47 weeks (mean $\pm$ standard error of the mean) and the range was 27–34 weeks. The full-term neonates had a body weight of 3053 ± 61 g (mean $\pm$ standard error of the mean) with a range 2560–3800 g. Their estimated gestational age according to the Capurro test was 38.6 ± 0.21 weeks (mean $\pm$ standard error of the mean) and the range was 37–41 weeks. The number of patients used in this study corresponds to the number of neonates born on a lapse of 15 days. However, retrospectively analyzing our data we can determine that the standardized difference (effect size) for the results in arginine and citrulline was 1.28 and 1.09, respectively, and assuming a power over 90% then the number of subjects needed in the study would be approximately 16 per group (for arginine difference) and 22 per group (for citrulline difference), well under the number of patients sampled in this study (29 preterm and 30 term babies).

Blood samples were collected from the central via in the premature neonates and from the umbilical cord in the mature neonates. Preterm newborn blood samples were collected, centrifuged at 735 g and the supernatant collected (plasma) and placed on ice as soon as they were admitted into the Premature Service at 8:00 AM. Two and a half hours later the plasma samples (still on ice) were taken to our laboratory for deproteinization and derivatization. Full-term neonate blood samples were collected at 7:00 AM; 12:00 m and 5:00 PM in the labor room at most one hour after birth. They were immediately centrifuged and 2 hours later the plasma samples were transported under refrigeration to our laboratory. Then they were deproteinized and derivatized. Deproteinization consisted in pipetting an aliquot of 100 μ L of plasma into an Eppendorf tube and adding 100 μ L of acetonitrile to precipitate plasma proteins. After vortex the mixture was centrifuged at 4°C and 100 μ L of the supernatants free of proteins were extracted.

A derivatizing solution was prepared by mixing 1 mg of FITC isomer I dissolved in 1 mL of acetone with 1 mL of 20 mmol/L Carbonate

buffer. One hundred microliters of this solution was added to the 100 μ L of de-proteinized plasma. After 18 hours in the dark at room temperature the mixture was ready to be analyzed. The capillary zone electrophoresis with laser induced fluorescence detection (CZE-LIFD) technique is extremely sensitive and for this reason we had to dilute each sample between 80 and 160 times.

The analytical procedure used was CZE-LIFD. The running buffer was a 20 mmol/L Carbonate buffer at pH 10. 27 KV were applied between the two ends of the buffer filled capillary. The fluorescent bands were detected with a Cobolt solid state, 488 nm, continuous wave laser focused on the window of a fused silica capillary, which was 350 mm external diameter and 25 mm inside diameter by means of a Newport 60X, 0.85 NA objective. The fluorescence was collected by the same objective (collinear arrangement) filtered through a high pass filter centered in 510 nm and transformed in an electric signal by a Photomultiplier.

The mixture was loaded by hydrodynamic injection by applying a -10 psi negative pressure for one second at the cathodic end of the capillary, whereas the anodic end was immersed in the mixture.

Standard solutions of arginine and citrulline were prepared by dissolving 1 mg of the amino acid in 1 mL of 20 mmol/L carbonate buffer. Five microliters of the derivatizing solution was added to each amino acid solution to obtain a 1.5 mmol/L solution of the thiocarbamate derivative of the amino acid. This solution was diluted in steps of two to obtain 40.1, 80.3, 160.7, 321.4, and 642.8 nmol/L solutions of fluorescein thiocarbamate arginine and 20.0, 40.1, 80.3, 160.7, 321.4, and 642.8 nmol/L solutions of fluorescein thiocarbamate citrulline. Alternatively, 80, 170, 350, 710, 1420, and 2850 nmol/L solutions of arginine and 70, 140, 290, 590, 1180, and 2370 nmol/L solutions of citrulline were prepared and derivatized.

The arginine and citrulline peaks were identified by spiking the samples. To spike a sample this was injected according to the procedure described above followed by an injection of the 90 nmol/L solution of the fluorescein thiocarbamate amino acid. The concentration of arginine and citrulline in the samples were calculated by comparing with the concentration vs fluorescence curve, ie, solving the linear equation for a given fluorescence and finding the concentration. Peaks that partially overlap were measure by deconvoluting the Gaussian curves with previously reported software.²³

The statistical analysis was carried out with the software package IBM SPSS Statistic Visor. Student's *t*-tests were used to compare the concentrations of the amino acids in the samples collected from the preterm neonates with the concentrations in the samples of the mature neonates. Regression analysis was used to test the correlation between the arginine and the citrulline concentrations both in preterm and full-term neonates. The goodness of fitting was tested with one way ANOVA. The variability in citrulline respect arginine was calculated by the Pearson coefficient. The percent variation in citrulline attributable to arginine concentration was tested by *t*-test and the interval of confidence were estimated both for preterm and full-term neonates. Finally the quotient arginine concentration/citrulline concentration (Arg/Cit) was calculated for each baby and compared by *t*-test.

FIGURE 1 Preterm neonates have significantly less citrulline and less arginine than full-term neonates. Dot plot shows each individual response. The horizontal line across the dots represents the mean of the results

3 | RESULTS

The concentrations of arginine and citrulline were significantly lower in the plasma of preterm neonates plasma, as compared to that of the full-term neonates: arginine full-term vs preterm: 71.1 ± 8.5 vs 28.3 ± 2.9 mmol/L, mean $\pm$ SEM; citrulline full-term vs preterm: 19.6 ± 2.4 vs 8.9 ± 1.1 μ mol/L, mean $\pm$ SEM.

The difference of these concentrations were statistically significant when compared by a *t*-test: arginine full-term vs preterm: $P < .0001$; and citrulline full-term vs preterm: $P < .0001$ (Figure 1).

There was a significant correlation between arginine and citrulline in full-term neonates, $F(1, 28) = 41.7$; $P < .0001$, and there was a significant correlation between the two amino acids in the preterm neonates too, $F(1, 27) = 11.4$; $P < .003$ (Figure 2). The Pearson correlation coefficient for full-term neonates was larger for than the Pearson correlation coefficient for preterm neonates (0.768 vs 0.529, respectively). The adjusted *r* squared (r^2) value was also higher for full-term than for preterm neonates (0.576 vs 0.265, respectively). The non-standardized coefficient for full-term neonates was 0.22 and it was

FIGURE 2 Arginine concentration vs citrulline concentration correlated significantly both in full-term neonates (open circles) and in preterm neonates (solid circles)

FIGURE 3 The Arg/Cit quotient of preterm neonates was smaller than the Arg/Cit quotient in full-term neonates but the difference was not statistically significant. The horizontal line across the dots represents the mean of the results

statistically significant ($t=6.465$; $P<.0001$; limits of confidence 0.151 and 0.290). The non-standardized coefficient for preterm neonates was 0.206 and it was statistically significant too ($t= 3.387$; $P<.003$; .082 and .331). The quotient Arg/Cit was smaller in preterm neonates (3.73 ± 0.29) than in full-term neonates (4.36 ± 0.47). The difference was statistically not significant (Figure 3).

4 | DISCUSSION

The present report confirms previous findings showing lower levels of arginine and citrulline in preterm neonates than in full-term neonates.^{8-10,24-26} The low levels of arginine and citrulline in the premature neonates suggest that they are not capable of synthesizing the required amount of arginine. In fact, there is a low expression of the genes coding carbamoyl phosphate synthase (CPS-1) ornithine carbamoyl phosphate transferase (OCT), pyrroline-5-carboxylate reductase (P5CR),^{27,28} argininosuccinate synthase (ASS)²⁹, and argininosuccinate lyase (ASL)²¹ in preterm neonates. Moreover, enterocytes of preterm neonates do not adequately synthesize arginine from its main precursors glutamate and proline. Previous studies have shown that the glutamate arginine synthesis pathway is inoperative in preterm neonates and that only the proline arginine pathway produces arginine.³⁰ Due to the low expression of the genes coding for Pyrroline-5-carboxylate synthase, ASS and ASL there is not enough enzyme to catalyze the synthesis of arginine which is the substrate for the synthesis of citrulline.

The correlation between arginine and citrulline both in full-term and preterm neonates suggest that these neonates: 1) convert arginine in citrulline to produce NO, and 2) transform arginine into ornithine to react with carbamoyl phosphate to synthesize citrulline. The Pearson correlation coefficient suggests a stronger correlation of arginine and citrulline plasmatic concentrations in full-term neonates than in preterm neonates. This suggests that the mechanisms of conversion of arginine in citrulline are more efficient in full-term than in preterm neonates. The corrected r^2 value was higher in full-term neonates than

in preterm neonates (0.576 vs 0.265, respectively). This suggests that while 57.6% of conversion of arginine in citrulline explains the correlation in full-term neonates only 26.5% conversion explains the correlation of arginine with citrulline. However, the rates of conversion were very similar in both groups. The non-standardized coefficients were 0.22 and 0.206 for full-term and preterm neonates. It means that for each change in plasmatic arginine concentration of 1 $\mu\text{mol/L}$ it would be expected a change in plasmatic citrulline concentration of 0.22 $\mu\text{mol/L}$ in full-term neonates and 0.20 $\mu\text{mol/L}$ in preterm neonates. This similarity explains why the correlation lines were so similar in both groups as can be appreciated in Figure 2. Interestingly, the limits of confidence for the non-standardized coefficients were closer in full-term neonates (the difference between confidence limits was 0.139) than in preterm neonates (the difference was 0.249). This suggests that the mechanisms of conversion of arginine into citrulline are better regulated in full-term neonates than in preterm neonates. The immature mechanisms of conversion of arginine into citrulline might be due to the lower levels of NOS and will cause the lower levels of NO reported in preterm neonates when compared with full-term neonates^{31,32} as well as the direct correlation that exists between gestational age and plasma NO level.³²

Although there was a trend for the arginine/citrulline quotients to be smaller in preterm neonates than in full-term neonates, the difference was not statistically significant.

If the speculations here presented are correct, there are several clinical and therapeutic implications. The most important is that if the enzymatic deficit is not corrected, the administration of arginine and citrulline to preterm neonates might have limited value to treat the consequences of the low arginine and citrulline levels. The consequences include serious conditions contributing to high morbidity and mortality of preterm neonates. Such conditions include impairment of the synthesis of proteins in which arginine is an essential building blocks (such as histones) hyperammonemia^{21,33,34} necrotizing enterocolitis,⁸⁻¹⁰ respiratory distress syndrome,^{35,36} and intraventricular hemorrhage.³⁷ In some cases, such as hyperammonemia, these conditions might be corrected with arginine and citrulline administration but in other conditions may not be corrected. However, the fact that most of the enzymes are expressed in preterm neonates²⁹ and arginine and citrulline supplements have not been proven to be toxic, it is advisable to provide such supplements to preterm neonates.

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