Reduced Plasma Citrulline Levels in Low Birth Weight Infants With Necrotizing Enterocolitis

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Background: Citrulline, a nonprotein amino acid, is an intermediate of the urea cycle and synthesized in small intestine. Lower plasma citrulline levels were associated with reduced function of enterocytes. Necrotizing enterocolitis (NEC) causes high morbidity and mortality, and leads impaired intestinal functions. **Methods:** Plasma citrulline levels of neonates with a gestational age <32 weeks and ≤1,500 gm who developed NEC stage II/III were measured by high-performance liquid chromatography. **Results:** We enrolled 36 preterm infants including 20 with NEC and 16 controls. Median citrulline levels of NEC and control groups were 8.6 and 20.18 $\mu\text{mol/l}$ ($P < 0.05$), and cut off level of citrulline was

13.15 $\mu\text{mol/l}$ with a sensitivity of 80% and a specificity of 82%. Median arginine levels of NEC and control groups were 22.02 and 39.89 $\mu\text{mol/l}$ ($P < 0.05$), and cut off level of arginine was 28.52 $\mu\text{mol/l}$ with a sensitivity of 70% and a specificity of 75%. Blood sampling day, gender, parenteral, and enteral nutrition did not affect the amino acid levels. **Conclusion:** We found lower plasma citrulline and arginine levels in preterm infants with NEC. Further studies are needed to determine most appropriate levels to predict recovery and prognosis of NEC, and treatment options with these amino acids in preterm infants. J. Clin. Lab. Anal. 27:328–332, 2013.

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INTRODUCTION

Necrotizing enterocolitis (NEC) causes high morbidity and mortality especially in premature infants (1, 2). Pathophysiology of NEC is still not understood and multifactorial. Prematurity is the most important risk factor because premature infants have limited motility, digestion, circulation of intestines, humoral and cell-mediated immunity, and local host defense (1). Clinical symptoms may be nonspecific as well as abdominal distension, signs of intestinal perforation, or multiorgan failure (2). Impaired intestinal functions are seen as acute and chronic complication of NEC.

Citrulline, a nonprotein amino acid, is an intermediate of the urea cycle (3). Citrulline is synthesized in the liver and small intestine. Main source of circulating citrulline is small intestine because citrulline produced in liver is ca-

tabolized in situ and enterocytes produce citrulline (4, 5). Many studies showed that lower plasma citrulline levels were associated with reduced function of enterocytes in diseases, such as short bowel syndrome (6, 7), villous atrophy associated small bowel disease (8), active Crohn's disease (9), intestinal graft versus host disease (10–12), and radiation enteritis (13). A recent study showed low plasma citrulline levels in preterm infants with NEC (14).

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Arginine is synthesized from citrulline and has unique properties and key roles in protein synthesis, ammonia detoxification, creatine and polyamine synthesis, and nitric oxide production (15). It is proposed that premature intestines had lower arginine synthesis capacity because of limited expression of key synthetic enzymes (16, 17). Arginine deficiency was described in infants with NEC (18, 19).

Reference values of plasma and dried blood spot (DBS) citrulline concentrations in healthy newborns and children were described (20). We previously observed that healthy premature infants had lower citrulline levels in DBS samples than pediatric age group (21). This finding may be related to immaturity of gastrointestinal system and enterocyte function.

As many studies pointed to citrulline as a marker of functional enterocyte mass, lower plasma citrulline levels are expected in preterm neonates with NEC. The aim of this study was to evaluate plasma citrulline levels in preterm infants with NEC and investigate the usefulness of plasma citrulline as a marker of intestinal function.

MATERIALS AND METHODS

This prospective study was conducted at the neonatal intensive care unit (NICU) of Zekai Tahir burak Maternity Teaching Hospital, the biggest tertiary unit in Turkey, between March 2011 and February 2012, and approved by local ethical committee of Zekai Tahir Burak Maternity Teaching Hospital. Neonates with a gestational age <32 weeks and $\leq 1,500$ gm who developed NEC were enrolled in the study. Diagnosis and classification of NEC were made by Bell's criteria (22). Stage I NEC: nonspecific systemic and abdominal signs, including increased gastric residuals, abdominal distension, or bloody stools. Stage II NEC: fulfilled criteria of suspected NEC and abdominal X-ray demonstrating pneumatosis intestinalis. Stage III NEC: multiorgan failure, occurrence of ascites, peritonitis, and intestinal perforation. Neonates with NEC stage II or III were included in the study.

The control group consisted of stable preterm infants of similar gestational age and birth weight. Blood sampling times were similar with study group. Exclusion criteria included congenital anomalies and dysfunction of intestines, liver and kidney, inborn errors of metabolism, and exchange transfusion.

Human milk and preterm formula (PreAptamil® Milupa) were used for enteral feeding. The starting amount was 20 ml/kg in the first day of life and gradually increased as 10–20 ml/kg. Parenteral amino acids were administered as Primene 10% (Baxter-Clintec, France) with an arginine content of 84 mg/100 ml (citrulline and glutamine free amino acid solution). The starting dose

was 0.5 gm/kg/day and gradually increased over the first week of life.

Blood sampling was performed on diagnosis day of NEC. In enterally fed neonates, blood samples were taken 1 hr before feeding because of fluctuations of amino acids after feeding. Quantitative measurements of plasma amino acids were performed by high-performance liquid chromatography (HPLC) using Aracus amino acid analyzer (membraPure GmbH, Bodenheim, Germany).

Statistics

Statistical analyses were performed using the SPSS statistical package (v. 20.0). Categorical variables between groups were analyzed using the χ^2 test. Comparison of means between two groups was examined by Mann–Whitney *U* test, where the data were nonnormal distributions. For comparison of more than two groups, ANOVA was used for normal distributions and the Kruskal–Wallis test for nonnormal distributions. Receiver operating characteristic curve analysis was used to determine the power of variables to differentiate groups, and the area under the curve was calculated; significant cutoff levels were calculated using a Youden index. A *P*-value of <0.05 was deemed to indicate statistical significance.

RESULTS

We enrolled 36 preterm infants including 20 with NEC and 16 controls in the study. Clinical characteristics of patients are listed in Table 1. Male:female ratio, gestational age, birth weight, blood sampling days were similar between groups (*P* > 0.05).

Plasma citrulline and arginine levels were significantly lower in NEC group than control group (*P* < 0.05), whereas glutamine levels did not differ (*P* > 0.05). Table 2 shows plasma amino acid levels. Median citrulline levels

TABLE 1. Clinical Characteristics of Patients

	Necrotizing enterocolitis (NEC, <i>n</i> = 20)	Controls (<i>n</i> = 16)
Male:female	10:10	8:8
Birth weight (gm)	1176 $\pm$ 468	1185 $\pm$ 570
Gestational age (week)	30.3 $\pm$ 3.1	29.6 $\pm$ 3
Blood sampling day (median)	10.5 (3–48)	13.5 (5–34)
SGA	4 (20%)	4 (25%)
APGAR score at 5 min $\leq$ 6	5 (25%)	4 (20%)
PDA	10 (50%)	7 (43%)
Sepsis	8 (40%)	6 (37.5%)
Mortality	6 (30%)	1 (6.3%)

SGA, small for gestational age; PDA, patent ductus arteriosus; APGAR, Appearance, Pulse, Grimace, Activity, Respiration.

TABLE 2. Plasma Citrulline, Arginine, and Glutamine Levels ($\mu\text{mol/L}$, Median, Minimum–Maximum)

	Necrotizing enterocolitis (NEC, $n = 20$)	Controls ($n = 16$)	<i>P</i>
Citrulline	8.6 (4.07–21.68)	20.18 (10.32–42)	<0.05
Arginine	22.02 (8.9–48.11)	39.89 (17.14–119.5)	<0.05
Glutamine	119.2 (25.92–665.2)	92.06 (49.31–390.4)	>0.05

of NEC and control groups were 8.6 and 20.18 $\mu\text{mol/L}$, respectively (Fig. 1). The area under the receiver operating characteristic (ROC) curve for citrulline to differentiate NEC from controls was 0.88 (95% CI, 0.77–0.99). Cut off level of citrulline was 13.15 $\mu\text{mol/L}$ with a sensitivity of 80% and a specificity of 82%. Median arginine levels of NEC and control groups were 22.02 and 39.89 $\mu\text{mol/L}$, respectively (Fig. 1). The area under the ROC curve for arginine to differentiate NEC from controls was 0.79 (95% CI, 0.64–0.94). Cut off level of arginine was 28.52 $\mu\text{mol/L}$ with a sensitivity of 70% and a specificity of 75%. Median glutamine levels did not differ between NEC and control groups (119.2 vs. 92.06 $\mu\text{mol/L}$).

There was no correlation between birth weight and citrulline/arginine levels in each group ($P > 0.05$). We performed a subgroup analysis that consisted of sampling time, before and after 10 days to evaluate the development of intestines, and median amino acid levels of NEC group did not differ, but there was a slight increase of citrulline levels (7.58 vs. 10.5 $\mu\text{mol/L}$), and also there was no difference between levels of control group before and after 10 days ($P > 0.05$). In each time point, citrulline levels were higher in control group ($P < 0.05$). Plasma citrulline and arginine levels were not associated with increased mortality ($P > 0.05$). In the condition of enteral feedings $>50\%$ of total caloric intake, median plasma citrulline levels of NEC group were higher than $<50\%$ of total caloric intake, but not statistically different (10.53 vs. 8.15 $\mu\text{mol/L}$). Plasma citrulline and arginine levels were not associated with the day of reaching total enteral feeding after NEC ($P > 0.05$). Gender did not affect the amino acid levels ($P > 0.05$). We found no correlation between amino acid levels and duration of parenteral nutrition ($P > 0.05$). There was no correlation between amino acid levels and the number of days to reach full-enteral nutrition after NEC recovery ($P > 0.05$).

Fig. 1. Plasma citrulline and arginine levels of control and necrotizing enterocolitis (NEC) groups.

DISCUSSION

In this study, we evaluated plasma citrulline, arginine, and glutamine levels in premature infants with NEC. Plasma citrulline and arginine levels were found to be lower in NEC group than healthy preterm infants. Several studies reported low plasma citrulline levels in diseases, which lead to enterocyte dysfunction. Plasma citrulline levels correlate with functional intestinal mass in short bowel syndrome, intestinal graft versus host disease, radiation enteritis, celiac disease, Crohn's disease, and villous atrophy-associated intestinal disease (20, 23). Intestinal functions of premature infants are immature and have lower capacity to synthesize citrulline and arginine (21, 24, 25). Immaturity of enterocytes and key enzymes, limited enteral feeding are responsible for low levels of amino acids, and marked structural and functional maturations happen in postnatal period (5, 17, 26).

Ioannou et al. (14) reported that plasma citrulline levels of NEC and healthy preterm infants on postnatal day 7, 14, and 21 were 16.9 ± 4.3 versus 20.5 ± 4.5 , 18 ± 4.2 versus 23.5 ± 4.3 , and 17 ± 2.5 versus 30 ± 5.7 $\mu\text{mol/l}$, respectively. We found median plasma citrulline levels of NEC and control groups as 8.6 (4.07–21.68) and 20.18 (10.32–42) $\mu\text{mol/l}$, respectively. In our study, citrulline levels of NEC group were lower than their study whereas similar in control groups. This may be associated with lower birth weight of our study group than their study. We performed a subgroup analysis and found that there was a slight increase of plasma citrulline levels after day 10 (7.58 vs. 10.5 $\mu\text{mol/l}$) like previous studies (14, 19). They also found lower plasma arginine levels in NEC group (59 ± 16 vs. 85 ± 26 $\mu\text{mol/l}$) like in our study. In our study, median arginine levels of NEC and control groups were 22.02 (8.9–48.11) and 39.89 (17.14–119.5) $\mu\text{mol/l}$, respectively, and seemed to be lower than their study. Similar to Ioannou et al., we also found no difference of plasma glutamine levels.

Becker et al. (19) reported that citrulline, arginine, and glutamine levels were lower in preterm infants with NEC similar with several other amino acids. Our citrulline and arginine levels of NEC group were lower than their study (on day 7, 14, and 21, citrulline levels: 10 ± 1 , 13 ± 3 , and 13 ± 2 $\mu\text{mol/l}$, and arginine levels: 45 ± 5 , 54 ± 9 , and 84 ± 13 $\mu\text{mol/l}$). But they did not find a statistical difference in citrulline levels between groups whereas there was statistically difference in arginine levels on day 7 and 14. Blood sampling day did not affect the results in our study. In contrast to our study, they found statistically reduced glutamine levels on day 7, 14, and 21 as 445 ± 512 versus 234 ± 88 , 451 ± 38 versus 177 ± 19 , 435 ± 49 versus 231 ± 34 $\mu\text{mol/l}$, respectively.

We found a slight increased citrulline levels in patients whose enteral feedings were $>50\%$ of total caloric intake,

but there were no statistical differences on parenteral and enteral nutrition amounts and durations in contrast to Ioannou et al. This may be explained by our aggressive enteral nutrition policy. We started enteral nutrition as soon as possible and this may play a role in maturity of intestinal functions, such as endocrine, motility, absorption, and digestion. Citrulline levels were not associated with the number of days to reach full-enteral nutrition after NEC recovery. This result may lead us to use plasma citrulline to diagnose intestinal dysfunction and not to use in prediction of recovery intestinal function. But, we did not perform repeated analysis and this was a limitation of our study. Ioannou et al. (14) reported that plasma citrulline levels of NEC group increased after enteral feeding was started, but never reached the levels of control groups. We found a lower cut off level of citrulline to diagnose NEC than Ioannou et al. as 13.5 versus 17.75 $\mu\text{mol/l}$ with similar sensitivity and specificity. Some studies showed that plasma citrulline levels were a good predictor of remnant intestinal length in short bowel syndrome, intestinal dysfunction in Celiac and Crohn's diseases, catheter-related bloodstream infections in children with intestinal failure (7, 23, 27).

Our study showed that plasma citrulline and arginine levels reflect reduced enterocyte function and intestinal dysfunction of patients with NEC. In premature infants, measurements of these amino acids should be evaluated carefully because immaturity of intestinal and enzymatic functions affects the results and results should be evaluated according to patient's clinical status.

In conclusion, we found lower plasma citrulline and arginine levels in preterm infants with NEC. Further studies are needed to determine most appropriate levels to predict recovery and prognosis of NEC, and treatment options with these amino acids in preterm infants.

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