

Short Communication

Urinary citrulline in very low birth weight preterm infants receiving intravenous nutrition

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(Submitted 21 July 2011 – Final revision received 1 November 2011 – Accepted 3 November 2011 – First published online 12 December 2011)

Abstract

As gut immaturity precludes full enteral feeding, very low birth weight (VLBW) preterm infants receive parenteral nutrition (PN) during the first few weeks of life. Weaning VLBW infants off PN, however, is a top priority since PN is associated with a high risk of complications. The decision making is purely empirical, as there is currently no suitable index of gastrointestinal (GI) maturity. Plasma citrulline concentration is considered an index of GI function in conditions such as short-bowel syndrome and coeliac disease in adults. To identify the factors determining urinary citrulline excretion, and determine whether urinary citrulline excretion could be used as a non-invasive index of GI tolerance to enteral feeding, nutritional intake and urinary citrulline were monitored bi-weekly in forty-seven preterm infants < 1500 g (interquartiles 880–1320 g), during their stay in the Neonatology unit. Median urinary citrulline was 24.7 μmol/mmol creatinine (14.5–38.6 μmol/mmol creatinine). No relationship was observed with the percentage of energy tolerated enterally. In multivariate regression analysis, weak correlations were found with post-conceptual age ($P=0.001$), parenteral amino acid supply ($P=0.001$) and the daily volume of enteral mixture administered ($P=0.043$). A significant correlation was found with urinary nitrite + nitrate excretion ($r\ 0.47$; $P<0.001$). We conclude that in preterm infants: (1) one of the major determinants of urinary citrulline may be the biosynthesis of citrulline from arginine by NO-synthase; (2) urinary citrulline cannot be used to predict GI tolerance. This is consistent with the observations that, in neonatal gut, citrulline is converted to arginine *in situ* rather than exported towards the kidneys as observed in adults.

Key words: Premature infants: Gastrointestinal function: GC-MS: Stable isotopes

Ensuring adequate postnatal growth remains a challenge in the care of very low birth weight (VLBW) infants, defined by a birth weight below 1500 g. For instance, a preterm infant born with an average weight of 1000 g at a gestational age of 30 weeks must triple its weight in less than 3 months. Yet, gut immaturity precludes full enteral feeding: intravenous support (parenteral nutrition, PN) therefore is mandatory to cover the infants' tremendous protein requirements. Due to the high risk of sepsis, thrombosis and cholestasis associated with prolonged PN, weaning infants off PN is, however, a top priority for neonatologists^(1,2). The decision making is purely empirical, based on the occurrence (or lack) of abdominal distension, vomiting or the presence of large

gastric residues, as there is currently no suitable biological index of intestinal maturity to help predict the tolerance to enteral feeding. A non-invasive marker of gastrointestinal (GI) maturity that would predict the tolerance to enteral feeding would clearly be highly desirable.

Citrulline is a non-essential amino acid that is not incorporated into protein, and is not present in enteral or parenteral nutrient mixtures. Citrulline has long been thought to arise predominantly from the conversion of glutamine in the gut; once released into portal vein, citrulline escapes uptake by the liver, and is taken up in the kidney, where it is converted to arginine which is released into systemic circulation. Arginine, in turn, can be converted to citrulline by

Abbreviations: GI, gastrointestinal; PN, parenteral nutrition; VLBW, very low birth weight.

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NO-synthase, as arginine is the sole endogenous precursor of NO, a ubiquitous mediator⁽³⁾. In healthy adults, glutamine and glutamate supply 50 and 80% of the carbon⁽⁴⁾ and nitrogen⁽⁵⁾, respectively, required for citrulline synthesis, and <10% of endogenous citrulline nitrogen is produced from arginine by NO-synthase⁽⁵⁾. Accordingly, plasma citrulline has been shown to reflect the mass or function of the small intestine in several conditions such as short-bowel syndrome⁽⁶⁾, coeliac disease⁽⁷⁾, intestinal transplantation⁽⁸⁾ or paediatric short-bowel syndrome⁽⁹⁾.

Recent studies, however, challenge the dogma that citrulline production predominantly reflects small-intestinal metabolism. First, the contribution of proline as a precursor of citrulline exceeds that of glutamine in both adult humans⁽¹⁰⁾, and preterm infants receiving full enteral feeding⁽¹¹⁾. This is in keeping with earlier evidence that, in neonatal piglets, the process of arginine synthesis takes place entirely within the small intestine⁽¹²⁾, contrary to what happens in the adult pig. If the inter-organ cycle of citrulline metabolism involving gut and kidney is not operational in the first few weeks of life, then circulating citrulline would not be an index of intestinal function.

The aims of the present study therefore were: (1) to identify the main factors determining urinary citrulline excretion in very low birth weight infants receiving intravenous nutrition; (2) to determine whether urinary citrulline excretion could be used as a non-invasive index of GI tolerance to enteral feeding in this population.

Methods

Patient population

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the local Institutional Review Board (Comité de Protection des Personnes dans la Recherche Biomédicale des Pays-de-la-Loire, Nantes, France). Written informed consent was obtained from all parents before inclusion. Preterm infants enrolled in the present study were part of a larger controlled trial on probiotic supplementation⁽¹³⁾. The trial was registered at www.clinicaltrials.gov, under ID #NCT 00290576. Preterm infants admitted to the neonatal intensive care units at the Mère-Enfant Hospital (Nantes, France) and at the Institut de Puériculture (Paris, France) were eligible for enrolment if they met the following inclusion criteria: a gestational age <32 weeks, a birth weight <1500 g, a postnatal age no greater than 2 weeks, and the absence of any disease other than those linked to prematurity. Infants were fed with human milk and/or with a preterm formula. They were randomly assigned to receive from the start of enteral feeding until discharge from the Neonatal Intensive Care Unit four daily capsules of a supplement containing either maltodextrin alone or 10⁸ lyophilised cells per unit of *Lactobacillus rhamnosus* GG (Valio Limited) and *Bifidobacterium longum* BB536 (Morinaga Milk Industry Company Limited) and maltodextrin. Detailed results of the trial have been published elsewhere⁽¹³⁾.

Clinical parameters

Collected clinical parameters included gestational age, birth weight, sex, mode of delivery (vaginal or caesarean section), intra-uterine growth retardation, 5 min Apgar score, maternal treatment (corticoids and antibiotics). In addition, postnatal age, type of enteral feeding (own mother's milk, bank milk or preterm formula), postnatal treatment (antibiotics), volume of enteral feeding (ml/kg per d) and number of enteral feeding interruptions were recorded before each urine sample was collected. Poor tolerance to enteral feeding was defined as a suspension of enteral feeding for any day during the week following the day the urine sample had been obtained. After clinical assessment, enteral feeding was interrupted if there were significant residues in gastric aspirates, abdominal distension and/or blood in stool. As enteral feeding is routinely suspended when ibuprofen is administered (to promote the closure of *ductus arteriosus*), the planned, on purpose interruptions of enteral feeding motivated by the prescription of ibuprofen treatment, were not considered as an event of poor GI tolerance.

A total of 220 urine samples were obtained from forty-seven infants at weekly intervals from the 6th day of life. Plasma samples were not obtained, due to the ethical constraints in blood draws for research purposes in VLBW infants.

Analytical methods

Citrulline concentration was determined by electron impact GC-MS using ²H₇-citrulline as an internal standard as described^(14,15). Urinary nitrates and nitrites were quantified by a commercial kit (Roche-Diagnostic), following the Griess method.

Statistical analysis

Statistical analysis was performed using SPSS[®] version 14.0 (SPSS). Results are reported as median and interquartiles, and percentages were presented as percent (95% CI). χ^2 and Mann-Whitney *U* tests were used to compare proportion and quantitative values, respectively. Spearman's correlation test was used to evaluate the relationship between selected perinatal or neonatal characteristics and urinary citrulline values. All variables that showed a statistically significant correlation with urinary citrulline by univariate analysis were subsequently analysed together in forward and backward stepwise, multivariate regression analyses, after log-transformation of urinary citrulline values. Statistically significant differences were assumed when $P < 0.05$.

Results

Patients

A total of forty-seven preterm infants (twenty-nine boys and eighteen girls) were enrolled in the present study. They had a median gestational age of 29 weeks (interquartile, 27–29 weeks), a median birth weight of 1100 g (interquartile, 880–1320 g). Of these infants, sixteen were born by caesarean

section and thirty-one by vaginal delivery. Median Apgar score at 5 min was 10 (interquartile, 8–10). During follow-up, seventeen infants were fed solely with human milk, twenty-nine with human milk combined with a preterm infant formula, and one with a preterm infant formula. As this study was part of a larger clinical trial on probiotic supplementation, twenty-five of the forty-seven infants received milk supplemented with the probiotic strains from the start of enteral feeding until discharge from hospital. No severe intestinal disease, such as necrotising enterocolitis or perforation, occurred during their hospital stay. None of the patients experienced rectal bleeding or required surgery. A large fraction of the infants enrolled nevertheless required one or several courses of antibiotics (81%) or corticosteroids (28%).

The median time to full enteral feeding (100% of total energy supplied via the enteral route) was 20.0 d (interquartile, 14.5–30.0). Infants received 55 (SD 32) and 76 (SD 34)% of overall nutritional intake via the enteral route at 14 and 21 d of life, respectively.

Urinary citrulline excretion

A total of 220 urine samples were analysed for citrulline concentration (Table 1). The median urinary citrulline concentration was 24.7 $\mu\text{mol}/\text{mmol}$ of urinary creatinine (interquartile: 14.5–38.6 $\mu\text{mol}/\text{mmol}$ creatinine), with a wide inter-subject, and between-day variability within a given subject as well. The first urine sample was obtained at a postnatal age of 9 (SD 3) d. As shown in Fig. 1, urinary citrulline did not correlate with the fraction of overall energy intake received enterally ($P=0.8$).

Relationship with age, weight and growth rate

In univariate regression analysis, no correlation was observed between urinary citrulline and postnatal age ($P=0.6$). A weak positive correlation ($r\ 0.17$, $P=0.01$) was found with post-conceptual age, defined as the sum of

gestational + postnatal age. Urinary citrulline did not depend on postnatal growth rate, expressed as change in weight z -score between birth and the time of the last citrulline measurement ($P=0.36$).

Impact of nutrition

No correlation was found between the enteral intake of energy or protein, or the volume of milk received over the 48 h before the time of urinary sampling. Similarly, urinary citrulline did not correlate with parenteral energy or amino acid intake. Only total protein intake (enteral + intravenous) approached significance ($r\ 0.12$, $P=0.09$).

Impact of nitric oxide production

As shown in Fig. 1, a significant correlation was observed between urinary citrulline and nitrite + nitrate (NO_2+NO_3) excretion ($r\ 0.47$, $P<0.0001$), suggesting that the conversion of arginine to citrulline by NO-synthase affects citrulline excretion. Accordingly, when the population of patients was divided in four subgroups based on quartiles of urinary citrulline excretion, NO_2+NO_3 excretion was higher in the upper quartile than in the lower quartile (258 (SD 200) *v.* 120 (SD 109) $\mu\text{mol}\ \text{NO}_2+\text{NO}_3/\text{mmol}$ creatinine, respectively; $P=0.001$).

Multivariate analysis

Multivariate analysis was carried out after excluding explanatory factors that were tightly correlated with each other (with an $r > 0.7$). In the regression model selected (Table 2), the positive correlation with urinary NO_2+NO_3 persists ($P<0.001$). The model also suggests an effect of post-conceptual age ($P=0.02$) and intravenous amino acid intake ($P=0.01$). Taken together, the sum of all factors retained in the model explains 24% ($r^2\ 0.24$) of the variability in measured urinary citrulline.

Table 1. Selected parameters analysed per quartiles of urinary citrulline excretion (Mean values and standard deviations)

| Quartiles of urinary citrulline... | 1st | | 2nd | | 3rd | | 4th | | P |
|---|--------|------|-----------|------|-----------|------|--------|------|--------|
| | < 14.6 | | 14.7–24.7 | | 24.8–38.7 | | > 38.8 | | |
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | |
| Urinary citrulline ($\mu\text{mol}/\text{mol}$ creatinine) | 8.9 | 3.2 | 20.2 | 3.1 | 31.6 | 4.3 | 76.5 | 41.6 | |
| $\text{NO}_2 + \text{NO}_3$ ($\mu\text{mol}/\text{mmol}$ creatinine) | 120 | 109 | 154 | 155 | 217 | 156 | 258 | 200 | 0.0001 |
| Postnatal age (d) | 21.1 | 12.2 | 29.2 | 18.6 | 29.1 | 15.6 | 25.4 | 13.3 | 0.023 |
| Post-conceptual age (weeks) | 30.5 | 2.1 | 30.5 | 2.5 | 30.8 | 2.5 | 31.8 | 2.5 | 0.021 |
| Weight (kg) | 1.4 | 0.3 | 1.3 | 0.4 | 1.3 | 0.3 | 1.4 | 0.3 | 0.151 |
| Milk intake (ml/kg per d) | 78 | 42 | 90 | 47 | 85 | 44 | 75 | 46 | 0.285 |
| Enteral protein intake (g/kg per d) | 1.4 | 0.9 | 1.6 | 1.0 | 1.5 | 0.9 | 1.3 | 1.0 | 0.476 |
| Energy via enteral route (%) | 67 | 37 | 67 | 37 | 68 | 37 | 63 | 36 | 0.911 |
| Intravenous amino acids (g/kg per d) | 0.8 | 1.0 | 1.1 | 1.4 | 1.0 | 1.3 | 1.3 | 1.4 | 0.276 |
| Overall energy intake | | | | | | | | | 0.196 |
| kcal/kg per d | 110.1 | 20.4 | 117.6 | 18.6 | 115.6 | 15.0 | 116.1 | 19.6 | |
| kJ/kg per d | 460.7 | 83.7 | 492.0 | 77.8 | 483.7 | 62.8 | 485.8 | 82.0 | |
| Overall amino acid and protein intake (g/kg per d) | 3.0 | 0.7 | 3.3 | 0.7 | 3.4 | 0.7 | 3.5 | 0.7 | 0.002 |

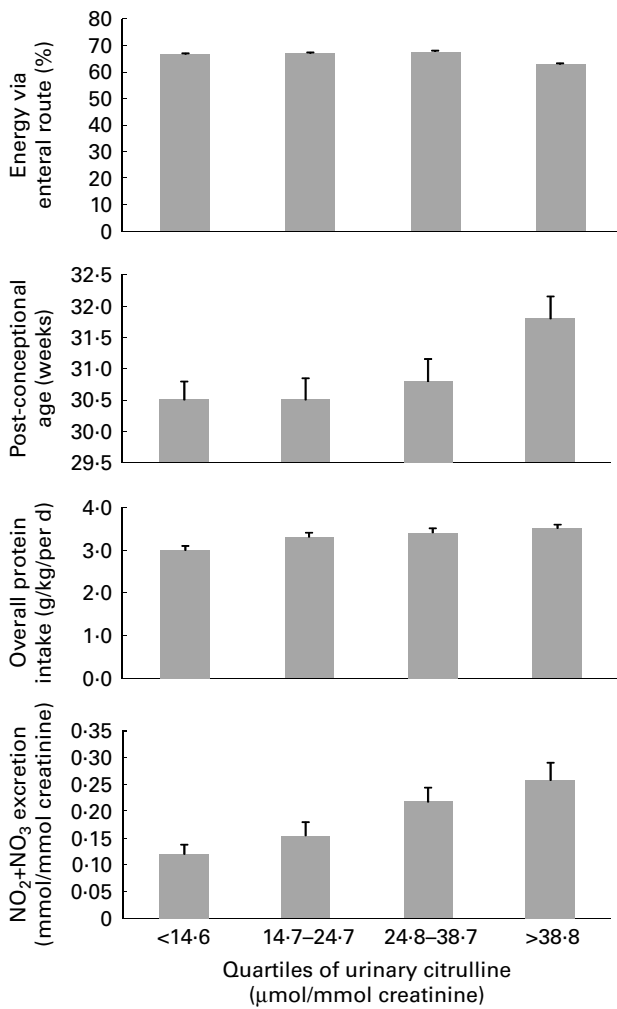


Fig. 1. Relationship between urinary citrulline excretion and selected parameters.

Discussion

The present pilot study explored the determinants of urinary citrulline excretion in premature infants. The findings suggest that: (1) expressed per mmol of creatinine, urinary citrulline is >10-fold higher in VLBW infants than in healthy adults; (2) urinary citrulline cannot be used as a predictor of digestive tolerance to enteral feeding in this population; (3) in VLBW preterm infants, conversion of arginine to citrulline by NO-synthase – rather than citrulline synthesis from glutamine metabolism – may be the predominant source of endogenous citrulline.

Despite a wide variability, urinary citrulline excretion was dramatically higher in the premature infants enrolled in the present study than in healthy adults (34 (SD 33) *v.* 0.9 (SD 0.1) μmol/mmol creatinine; *P*<0.0001), measured using the same assay^(14,15), and higher than the 0–11 μmol/mmol creatinine reported in term infants⁽¹⁶⁾. Such high levels of citrulline excretion may arise from either impaired citrulline tubular reabsorption, or impaired conversion of citrulline to arginine, as very low activity of argininosuccinate synthase

and argininosuccinate lyase, the two key enzymes involved in citrulline conversion to arginine, has been reported in the renal proximal tubule of premature piglets⁽¹⁷⁾.

We failed to observe any correlation between urinary citrulline and gut function, as assessed by the fraction of overall nutrient intake delivered enterally. This finding suggests that urinary citrulline cannot be used as a non-invasive marker to predict the tolerance to enteral feeding in intravenously fed preterm infants. This contrasts with the fact that plasma citrulline is a reliable marker of intestinal function in infants with short-bowel syndrome⁽⁹⁾. Very few studies, however, have assessed citrulline in preterm infants. In a study by Roig *et al.*⁽¹⁸⁾, plasma citrulline was higher among preterm infants receiving 100% of their feeding through the enteral route, compared with those receiving both enteral and intravenous nutrition (29 *v.* 9 μmol/l; *P*<0.05). This suggests that either citrulline indeed reflects intestinal function, or, alternatively, citrulline rises with age and maturation, since infants who had achieved full enteral feeding were obviously older. An obvious limitation of the present study is the fact that, due to ethical constraints in the sampling of blood for research purposes in preterm infants, we did not measure plasma citrulline. Whether plasma citrulline is a marker of GI tolerance in preterm infants, therefore, remains to be determined. This is, however, unlikely, for several reasons. First, contrary to what happens in the adult pig, in newborn piglets, the endogenous citrulline produced in the gut is converted locally to arginine in enterocytes rather than exported to be converted in the kidneys^(12,19). Secondly, recent studies suggest that proline, rather than glutamine, may be the predominant precursor of citrulline in preterm infants⁽¹¹⁾. If the inter-organ cycle of citrulline metabolism involving gut and kidney does not operate in the first few weeks of life, circulating citrulline cannot possibly be an index of intestinal function in that specific population.

In our sample, urinary citrulline correlated with overall protein intake, even though PN admixtures were devoid of citrulline, and enteral feeding supplies were very little (0.5 μmol/l in the case of human milk) or there was no citrulline at all in the case of formulas⁽²⁰⁾. This suggests that citrulline can be produced from the conversion of other exogenous amino acids. Oral glutamine administration indeed can raise citrulline concentration in adults⁽²¹⁾, and proline may be a better precursor than glutamine or glutamate for citrulline synthesis in human infants⁽¹¹⁾.

In multivariate analysis, urinary NO₂ + NO₃ excretion was the factor that contributed most to explaining the variability in urinary citrulline. NO₂ + NO₃ excretion is commonly accepted as an index of endogenous NO synthesis⁽²²⁾.

Table 2. Multivariate analysis model

| Variables | Standardised coefficients | <i>P</i> |
|--|---------------------------|----------|
| <i>r</i> ² 0.24, <i>n</i> 146 | | |
| Nitrites/nitrates | 0.403 | 0.0000 |
| Parenteral amino acid intake | 0.237 | 0.0112 |
| Post-conceptual age | 0.24 | 0.0161 |

During NO synthesis by NOS, NO and citrulline are produced in an equimolar fashion from arginine. The present finding is consistent with the view that the conversion of arginine to NO may be a major contributor to citrulline synthesis in the population of premature infants.

Acknowledgements

The present work was supported by grants from Agence Nationale de la Recherche (ANR PNRA-05-009), the Programme Hospitalier de Recherche Clinique (PHRC) and the Délégation à la Recherche Clinique (DRC), CHU de Nantes, France. Aurélie Bourdon and Carole Rougé were supported by doctoral fellowship grants from the Ministère de la Recherche et de l'Enseignement Supérieur (Paris, France) and from Nestec (Vevey, Switzerland), respectively. The authors' contributions to this study were as follows: A. B., C. R., D. D., H. P., A. L., Mi. V., Ma. V. and J.-C. R. contributed to the study design; A. B., C. R., H. P., A. L., C. D. R., A. L., Mi. V., Ma. V. and J.-C. R. to the acquisition of data; A. B., C. R., H. P., C. D. R., A. L., P. N., Mi. V., Ma. V., D. D. and J.-C. R. to the analysis and interpretation of the data. Statistical analysis was performed by J.-C. R. and A. L. The manuscript was drafted by A. B., J.-C. R. and D. D., and critically revised by all authors. H. P., Ma. V., J.-C. R. and D. D. were in charge of the overall supervision of the study. None of the authors had any conflict of interest.

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