Carbamyol phosphate synthetase 1 gene (4217C>A) polymorphism and its relation to low plasma arginine level among preterm with necrotizing enterocolitis; a single center Egyptian study

Abstract

Background: Arginine deficiency contributes a significant metabolic problem in preterm babies especially those with necrotizing enterocolitis (NEC). A [C-to A-] nucleotide transverse in the gene encodes Carbamyol-Phosphate Synthetase 1 (CPS1) enzyme has been associated with low arginine level among term neonates but not in adult.

Aim of the study: We aimed at investigating the association between CPS1 gene (4217 C>A) polymorphism and plasma L-arginine level among Egyptian preterm infants with NEC.

Material & methods: A case-control study was conducted on 30 preterm infant (26-34 weeks of gestation) with NEC. Thirty sex and gestational age harmonized preterm babies without NEC were eligible as controls. Both cases and controls were subjected to plasma L-arginine level measurement using ELISA and genotyped for CPS1 gene (4217 C>A) using PCR-based RFLP-assay.

Results: There were significant decrease in plasma L-arginine concentration in preterm with NEC when compared to controls (P=0.002). The frequency distribution of CPS1 gene (4217 C>A) genotypes were in agreement with Hardy Weinberg (HW) equilibrium. The distribution of CPS1 gene (4217 C>A) C/C, C/A, A/A genotypes and A allele were 20(66.6%),8(26.7%), 2(6.7%) and 12(20%) among cases and 22(7.3%),6(20%),2(6.7%) and 10(16.7%) among controls with estimated odds ratio of 1.46 ,1.1 and 1.25 respectively. There was no significant relation between low L-arginine level and CPS1 gene (4217 C>A) genotypes. A significantly lower L-arginine levels were observed among NEC non-survivors when compared to survivors (P=0.004), however, there was no significant difference between 2 groups regarding CPS1 gene (4217 C>A) genotype distribution. (P=0.570).

Conclusion: A significantly lower L-arginine level was detected among preterm with NEC and significantly related to poor outcome. However, no significant relation was observed between low L- arginine level and CPS1 gene (4107 C>A) polymorphism.

Keywords: carbamyol phosphate synthetase 1 gene, CPS1 (4107 C>A) polymorphism, L-arginine; necrotizing enterocolitis

Introduction

Necrotizing enterocolitis [NEC] is the most prevailing and devastating acquired gastrointestinal emergency among premature neonates.1 Despite the observable worthy advancement in the care of small preemies over the foregoing decades, NEC; as yet; exists as a foremost leading predisposition for as certainable increase in mortality and poor outcome among tiny preemies in neonatal intensive-care-units [NICU].2 Although numerous predisposing factors have been well-settled, the explicit patho-etiological mechanisms of NEC is still unclear. It has been emphasized that the combination of immaturity & underdevelopment of intestinal motility, digestible activity, intestinal barriers, immune defense and intestinal microcirculatory regulation with an abnormal intestinal microbial colonization in the presence of genetic predisposition were the probable patho-etiological predispositions for development of NEC.1,3 Nitric oxide (NO); which is synthesized from amino acid (L-arginine) by NO synthetase enzyme (NOS), is the principle inhibitory neurotransmitter in the gastro-intestinal system. It has a crucial role in maintaining the vasodilator tone, regulate mucosal blood flow, and maintain intestinal mucosal integrity and barrier function.4,5 L-arginine, which is declared as a functionally essential amino-acid, is one of the urea-cycle intermediates that produced by the action of carbamyol-phosphate synthetase 1(CPS1) enzyme.6 It has been noticed that the unavailability of L-arginine was associated with NO production and increased predisposition to NEC. In addition, numerous studies disclosed that plasma arginine concentration was declined in preemies with NEC.1,3,7 Moreover, it has been disclosed that arginine supplementation decreases the liability of NEC development.[7] CPS1 enzyme; which is encoded by CPS1 gene on chromosome (2q34); is the rate-limiting enzyme catalyzing

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the first step in urea-cycle. Among various formerly analyzed functional CPS1 gene single nucleotide polymorphisms (SNPs), CPS gene (rs1047891) 4217C>A polymorphism with transposition of asparagines for threonine at (1405) position has been linked to numerous functional consequences affecting downstream availability of urea-cycle intermediates, including L-arginine. Few studies were antecedently conducted to ascertain the association between CPS1 gene (4217C>A) polymorphism and low L-arginine level and increased risk of NEC. Up to our knowledge, this was the first Egyptian study that aimed at investigating the relation between CPS1 gene (4217C>A) polymorphism and plasma L-arginine level among preemies with NEC.

Materials and methods

Materials

A prospective case-control study conveyed in 30 preterm cases (17 males &13 females) with gestational age ranged from 26 to 34 gestational weeks who developed established NEC in the first 2 weeks after birth. Thirty sex & age harmonized preterm babies of the same gestational age range who didn’t develop NEC were enrolled as control group. Both cases & controls were recruited from neonatal intensive care units (NICU) at Zagazig university polyvalent children hospital and Zagazig university maternity hospital, Zagazig University, Egypt during the period from January 2017 to Mai 2018. NEC were defined as Bell stage II disease or greater confirmed by clinical signs, laboratory data and radiological findings. Exclusion criteria were as follow: preterm babies with major congenital malformations, chromosomal abnormalities, perinatal asphyxia and recent blood product transfusion or exchange transfusion and those who were fed artificially as breast milk was not available. All preterm neonates enrolled in the study were subjected to detailed maternal, obstetric history taking and detailed clinical evaluation including assessment of gestational age using “Modified Ballard score” and detailed systemic examination. Regarding feeding protocols, both cases & controls were fed as follow: small amount of trophic breast feeding (1-2ml/kg every 3 hrs.) were started from 3 day of life or greater with rate of increment of 5-10ml/kg/day. Total caloric requirement were obtained from both breast feeding and total parenteral nutrition prepared by our clinical nutrition unit guided by clinical pharmacists. Daily arginine intake (mg/kg/day) at time of recruitment was calculated as follow: for preterm who was fed breast milk, each 100ml of breast milk contains 54mg of L-arginine and for preterm who received parenteral amino acid infusion, Aminovate atant 10% (Fresenius Kabi Oncology, Ltd., Austria), each 100ml contains 1.2gm L-arginine. Both cases and controls were subjected to routine laboratory investigations as recommended by our local protocols and guidelines specific for management and care of preemies including [Sepsis screen, electrolyte assessment, random blood sugar, serum total & direct bilirubin, bleeding profile, arterial blood gas, chest x ray, abdominal X ray, pelvi-abdominal U/S, routine Echo evaluation and routine trans-cranial U/S]. Further, they were subjected to plasma L-arginine level measurement and genotyped for CPS1 gene (4217C>A) polymorphism. They were followed during the period of NICU stay to record the prognosis and the outcome either survived or not. This work has been performed in accordance with the code of Ethics of the World Medical association (Declaration Helsinki) of 1964 as revised in 2008 and approved by the ethical committee of faculty of human medicine, Zagazig University. An informed written consent was obtained from parents of all participants in the study.

Methods

Blood sampling

Peripheral venous blood samples “2ml for each” were collected from all studied subjects then stored at-20°C until further analysis.

Plasma L-arginine assay

Plasma L-arginine level was analyzed by “Human Arginine (ARG) ELISA kit” supplied by [Bioassay Technology Laboratory] according to manufacture recommendations and instructions.

Analysis of CPS1 gene 4217C>A (rs 1047891) polymorphism

Genomic DNA extraction was done from EDTA whole blood using the total DNA Extraction Kit [Roche Applied Science DNA Isolation Kit, Penz berg, Germany] according to manufacturer’s instructions. The purified genomic DNA was stored at~80°C until use. The polymerase chain reaction were used to amplify a 214-bp fragment encompassing the C-to-A nucleotide trans version at position 4217 of exon 36 of the gene-encoding CPS1, using forward primer `5’-TAAATGCAAGCTGTTTGCCAC-3’` and reverse primer `5’-GACTTGGCAATCAAGTAAAGTGTTGAA–3’` as designed by Moonen et al.,

Polymerease chain reaction (PCR) amplification were carried out in a total volume of 25uL, containing 80ng genomic DNA, 12.5ul of 2 x Dream TaqTM Green Master Mix [Life Technologies, Eggenate in, Germany] and 25Pmol of each primer with cycling parameters as follows: initial denaturation at 95°C for 5min, 35 cycles at 94°C for 30s, annealing at 61°C for 30s and 72°C for 60s and one final cycle of extension at 72°C for 7min. Genotyping of the DNA sequence variants was performed using PCR-based RFLP assays through digestion of amplified PCR product of using specific restriction endonuclease enzymes according to manufacturer’s recommendation [New England Bio labs, Sch Wallach, Germany]. They were incubated at 37°C for 4h; then the digested fragments were separated in 2% agarose electrophoresis system then visualized with ethidium bromide staining under ultraviolet transilluminance to detect DNA fragments.

Statistical analysis

The assembled data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 18.0. Qualitative data were represented as frequencies and relative percentages. Quantitative data, which is not normally distributed, was expressed as median (inter quartile range). Chi square test and fisher’s exact test were used to calculate difference between qualitative variables in different groups and Odds ratio was calculated to estimate the risk of polymorphism. Mann Whitney test and Kruskal wall test were used to calculate differences between non parametric qualitative variables in 2 and 3 groups respectively. The significance level for all above mentioned statistical tests done and the threshold of significance is fixed at 5% level (P-value).

Results

The general demographic characteristics of both preterm cases with NEC (n=30) and preterm neonates without NEC (n=30) were demonstrated in Table 1. There was significant decrease in plasma L-arginine level in cases compared to controls (P=0.002). (Table 2) The Genotype frequency of CPS1 gene (4217C>A) polymorphism didn’t
deviate from Hardy Weinberg (HW) equilibrium among all studied subjects. The frequency distribution of CPS1 gene (4217 C>A) C/C, C/A, A/A genotypes and A allele were 59%, 33.3%, 7.7% and 24.4% among cases compared to 66.7%, 28.2%, 5.1% and 19.2% in controls respectively. Moreover, there were no significant difference between CPS1 gene (4217 C>A) mutant heterozygous C/A, homozygous A/A genotypes and an allele among cases when compared to controls. (Table 3) In the current study, Plasma L-arginine level didn’t differ significantly in relation to CPS1 gene (4217C>A) C/C, C/A and A/A genotypes among cases (P>=0.174). (Table 4) Further, there was significant decrease in plasma L-arginine level among NEC non-survivors compared to survivors (P<=0.004). On the other hand, there was no significant difference in CPS1 gene (4217 C>A) genotypes (P=0.570) and allele (P=0.250) distribution between survivors and non-survivors. (Table 5)

**Table 1** General characterization of studied groups

| Characteristics               | Preterm cases (n=30) | Preterm Controls(n=30) | P-value |
|-------------------------------|----------------------|------------------------|---------|
| **Gestational Age; Weeks‡**   |                      |                        |         |
| Median(IQR)                   | 29(26.1-33)          | 29(28-33.9)            | 0.391   |
| Min-Max                       | 26-34                | 28-34                  |         |
| **Birth Weight; Kg†**         |                      |                        |         |
| Median(IQR)                   | 1.32(0.91-1.78)      | 1.32(1.05-1.78)        | 0.668   |
| Min-Max                       | 850-1900             | 1000-2000              |         |
| **Sex; n (%)‡**               |                      |                        |         |
| Male                          | 17(56.7)             | 15(50)                 | 0.604   |
| Female                        | 13(43.3)             | 15(50)                 |         |
| **Mode of delivery; n (%)‡‡** |                      |                        |         |
| C.S                           | 9(30)                | 12(40)                 | 0.416   |
| NVD                           | 21(70)               | 18(60)                 |         |
| **Postnatal age ; days‡**     |                      |                        |         |
| Median(IQR)                   | 7(4.10-10.00)        | 7(6.00-11.90)          | 0.277   |
| Min-Max                       | 4-11                 | 6-14                   |         |
| **Glutamine intake; mg/kg‡‡** |                      |                        |         |
| Median(IQR)                   | 227.81(197.1-322.5)  | 229.72(190.2-300.5)    | 0.371   |
| Min-Max                       | 191.20-357.75        | 177.30-324.00          |         |
| **Onset of Feeding; days‡‡**  |                      |                        |         |
| Median(IQR)                   | 5(2-7)               | 5(3-7.9)               | 0.449   |
| Min-Max                       | 2-7                  | 3-8                    |         |

**Table 2** L-arginine level between preterm cases and controls

| L-arginine level, ng/ml | Preterm with NEC n=30 | Preterm without NEC n=30 | P-value |
|-------------------------|-----------------------|--------------------------|---------|
| Median(IQR)             | 8.71(4.57-14.7)       | 12.10(7.45-18.39)        | 0.002   |

**Table 3** Genotype distribution of CPS1 gene (4217 C>A) genotypes and allele among cases

| CPS1 (4217 C>A) genotypes | L-arginine level ; (ng/ml) | P-value | OR | 95%CI |
|----------------------------|----------------------------|---------|----|------|
| Preterm with NEC n=30      |                            |         |    |      |
| Preterm without NEC n=30   |                            |         |    |      |
| P-value                    |                            |         |    |      |
| C/C                       | 20(66.6)                   | 22(73.3) |    | Reference |
| C/A‡                      | 8(26.7)                    | 6(20)    | 0.537 | 1.46 | 0.43-4.96 |
| A/A‡                      | 2(6.7)                     | 2(6.7)   | 0.999 | 1.09 | 0.07-16.47 |
| CPS1 (4217 C>A) alleles; n (%)‡ |                      |         |    |      |
| C                      | 48(80)                     | 50 (83.13) | 0.637 | 1.25 | 0.49-3.16 |
| A                      | 12(20)                     | 10 (16.7) |    |      |         |

**Table 4** Relation between L-arginine level and CPS1 gene (4217 C>A) genotype distribution among cases

**Table 5** L-arginine level & CPS1 gene (4217 C>A) genotypes and allele among NEC survivors and non-survivors

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Carbamoyl phosphate synthetase 1 gene (4217C>A) polymorphism and its relation to low plasma arginine level among preterm with necrotizing enterocolitis; a single center Egyptian study

|                | NEC survivors n=12 | NEC non-survivors n=18 | P-value |
|----------------|--------------------|------------------------|---------|
| CPS1 (T14054N) allele n (%)§ |                    |                        |         |
| C              | 17(70.8)           | 30(83.3)               | 0.25    |
| A              | 7(29.2)            | 6(16.7)                |         |

Statistical analysis: †Mann-Whitney test, ‡fischer’s exact test, §chi square test
%Significant at P<0.05

IQR, inter quartile range; NEC, necrotizing enterocolitis.

**Discussion**

Plasma arginine homeostasis is a complex process affected by arginine intake, consumption and endogenous arginine production which occurs chiefly in the kidney through conversion of citrulline to arginine and in the gut through conversion of glutamine to arginine. Preterm infants have a distinct increased requirements of arginine that may be due to the abundance of arginine in various tissue proteins and the availability of multiple pathways of arginine utilization and consumption. Arginine deficiency, particularly in preterm neonates, results in several metabolic and clinical disorders including hyperammonemia, cardiovascular, pulmonary, immunological, intestinal and neurological dysfunction. It has been previously disclosed that arginine was recognized to be noticeably deficient in milk of humans and many animal species. In the first few weeks of life, preterm babies are mainly supported by TPN. Despite of the high amount of arginine supplementation in recently available TPN solutions, it remains inadequate and hypoargininemia remains to occur in preterm infant supported by current TPN solutions. Thus, the endogenous synthesis of arginine through the expressed key enzymes is expected to play an essential role in maintain arginine homeostasis in preterm neonates fed entirely and parentally. Therefore, it is crucial to identify the underlying mechanisms of hypoargininemia in preterm infants. In current study, we investigating the association between a single-nucleotide –polymorphism (C-to-A nucleotide transversion in exon 36) of the gene that encodes CPS1; the rate limiting enzyme in urea cycle; that control the production of the NO precursor “L-arginine” and plasma arginine level in preterm babies with NEC. We reported a significant decrease in plasma L-arginine level among preterm babies with NEC when compared to controls despite adequate amount of arginine intake in both groups. Several studies come in agreement with our results. Richir et al., reported that preterm infants with NEC showed significantly low L-arginine level than those without NEC. Further, Amin et al., who investigated the relation of enteral L-arginine supplementation in prevention of NEC, concluded that plasma L-arginine level was significantly low at time of diagnosis of NEC. Regarding CPS1 gene (4217C>A) polymorphism, we disclosed no significant difference in genotypes and allele distribution between preterm cases and preterm controls. In consonance with our results, a recent large prospective multicenter study conducted by Moonen et al., stated that allele and genotype frequencies of CPS1 gene (4217C>A) polymorphism didn’t differ significantly between preterm infants with or without NEC.

In contrary, a formerly conducted study by Moonen et al., on 17 preterm babies with NEC, reported that the C/C genotype and C allele of CPS1 gene (4217C>A) was associated with increased risk of NEC when compared to the grouped AA/CA genotypes and Allele. They used a small sample of preterm babies and when they used a large sample size from multicenter, they found no significant difference as previously mentioned. In the present study, we showed that there was no significant effect of CPS1 gene (4217C>A) on L-arginine concentration among preterm neonates with NEC. Our results were matched with Moonen et al., who investigated the relation between and L-arginine level among preterm infants; they reported that the level of L-arginine didn’t significantly differ between the three A/A, A/C and C/C genotypes. On the other hand, our results were in agreement with Pearson et al., who investigated L-arginine concentration to CPS1 genotypes among neonates with pulmonary hypertension, they reported that lower L-arginine level was found with homozygous C/C genotypes. In this study, we concluded that NEC non survivors expressed significantly lower L-arynine level with no significant difference in CPS1 genotype distribution when compared to survivors.

Despite there was no significant difference in arginine intake between cases and controls, low plasma L-arginine level was significantly present in preterm with NEC with no significant relation to CPS1 genotype distribution suggesting an increased metabolic demand for arginine or presence of underlying factor that limit endogenous intestinal synthesis and considered as a risk factor for NEC with poor outcome among Egyptian preterm neonates.

**Conclusion**

Authors thank all the participants of this study for their unstinted cooperation.

**Conflict of interest**

The author declares no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Declaration of funding interest**

The authors have no funding of interest to disclose.

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