

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

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Introduction

Necrotizing entero-colitis [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, digestive ability, 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 carbamoyl-phosphate synthetase 1(CPS1) enzyme.⁶ It has been noticed that the unavailability of L-arginine was associated with limitation in NO production and increased predisposition to NEC. In addition, numerous studies disclosed that plasma arginine concentration was declined in preemies with NEC.⁷⁻⁹ Moreover, it has been disclosed that arginine supplementation decreases the liability for NEC development.^[7] CPS₁ enzyme; which is encoded by CPS₁ gene on chromosome (2q34); is the rate-limiting enzyme catalyzing

the first step in urea-cycle.^{10,11} Among various formerly analyzed functional CPS₁ gene single nucleotide polymorphisms (SNPs), CPS₁ 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.^{11,12} 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.^{13,14} Up to our knowledge, this study 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 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 greater¹⁵ 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"¹⁶ 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, Aminoven infant 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, electrolytes 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 manufacturer 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, Penzberg, 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'-TAAATGCAGCTGTTGCCAC-3' and reverse primer 5'-GACTTGCATCAAGTAAGGTGAAA-3' as designed by Moonen et al.¹⁷ Polymerase chain reaction (PCR) amplification were carried out in a total volume of 25µL, containing 80ng genomic DNA, 12.5µL of 2 x Dream TaqTM Green Master Mix [Life Technologies, Eggenstein, 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]. Then 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 transillumination 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 wallis 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†		Birth Weight; Kg†	
Median(IQR)	1.32(8.91-1.78)	1.32(1.05-1.78)	0.668
Min-Max	850-1900	1000-2000	
Sex; n (%)‡		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	

Statistical analysis: †Mann-Whitney test, ‡chi square test

*P is significant.

C.S, caesarian section; IQR, inter-quartile range; NVD, normal vaginal delivery

Table 2 L-arginine level between preterm cases and controls

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

	Preterm with NEC n=30	Preterm without NEC n=30	P-value
Min-Max	3.76-16.56	6.56-20.87	

Statistical analysis: Man Whitney test.

*Significant at $P<0.05$.

IQR, inter-quartile range; NEC, necrotizing enterocolitis

Table 3 Genotype distribution of CPS 1 gene (4217 C>A) polymorphism among cases and controls

Preterm with NEC n=30	Preterm without NEC n=30	P-value	OR	95%CI
CPS1 (4217 C>A) genotype; n (%)				
C/C	20(66.6)	22(73.3)	Reference	Reference
C/A†	8(26.7)	6(20)	0.537	1.46
A/A‡	2(6.7)	2(6.7)	0.999	1.09
CPS1 (4217 C>A) alleles; n (%)†				
C	48(80)	50 (83.3)	0.637	1.25
A	12(20)	10(16.7)		

Statistical analysis: †chi square test, ‡ fisher's exact

*Significant at $P<0.05$.

CPS1, carbamoyl phosphate synthetase 1; 95% CI, 95% confidence interval; NEC, necrotizing enterocolitis; OR, odds ratio

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

CPS1 (4217 C>A) genotypes	L-arginine level ; (ng/ml)	P-value	
	Min-Max	Median(IQR)	
Preterm cases			
C/C	n =20	8.81(4.57-15.28)	3.67-16.56
C/A	n = 7	6.90(3.98-12.45)	3.98-12.45
A/A	n = 3	10.56(9.96-14.67)	9.96-14.67

Statistical analysis: Kruskal wall is test.

CPS1, carbamoyl phosphate synthetase 1; IQR, inter quartile range

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

	NEC survivors n=12	NEC non-survivors n=18	P-value
Median (IQR)	11(6.56-15.21)	7.16(3.95-13.13)	0.004*
Min-Max	5.67-15.45	3.76-16.56	
CPS1 (T1405N) genotype; n(%)‡			
C/C	7(58.3)	13(72.2)	0.082
C/A	3(25)	4(22.2)	
A/A	2(16.7)	1(5.6)	

NEC survivors n=12	NEC non-survivors n=18	P-value
CPS1 (T1405N) allele; n (%)§		
C 17(70.8)	30(83.3)	0.25
A 7(29.2)	6(16.7)	

Statistical analysis: †Mann-Whitney test, ‡fisher'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.¹⁸ 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.¹⁹ Arginine deficiency, particularly in preterm neonates, results in several metabolic and clinical disorders including hyperammonemia, cardiovascular, pulmonary, immunological, intestinal and neurological dysfunction.²⁰ It has been previously disclosed that arginine was recognized to be noticeably deficient in milk of humans and many animal species.²¹ 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.²² Thus, the endogenous synthesis of arginine through the expressed key enzymes is expected to play an essential role in maintain arginine homeostasis in 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.^{8,9,23} Richir et al.,²⁴ reported that preterm infants with NEC showed significantly low L-arginine level than those without NEC. Further, Amin et al.,²⁵ who investigate the role 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.,¹⁷ 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.,¹³ 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.¹⁷ 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.,¹⁴ 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 not in agreement with Pearson et al.,¹⁰ 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-arginine level with no significant difference in CPS1 genotype distribution when compared to survivors. Up to our knowledge, there were no researches conducted to investigate the relation between L-arginine level and severity and outcome of NEC patients. In a study conducted by Aydemir et al.,²⁶ on neonates with sepsis, he reported significant decrease in L-arginine level among septic neonates with septic shock when compared to those without septic shock; however, they found no significant difference in L-arginine level between survivors and non survivors. The limitations of this study were the small sample size of our patients. We are in great need for larger prospective cohort studies to detect the distribution of CPS1 gene distribution among Egyptian preterm neonates and its relation to L-arginine level.

Conclusion

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.

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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

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