IJN
Iranian Journal of Neonatology
http://ijn.mums.ac.ir

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Original Article

Evaluation of Cerebrospinal Fluid and Plasma Amino Acids Levels in Neonates with Refractory Seizures: A Prospective Cohort Study

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ABSTRACT

Background: Altered concentrations of cerebrospinal fluid (CSF) and blood amino acids may be related to epilepsy or the severity of the seizure. In the present study, we assessed the concentrations of amino acids in CSF and plasma in neonates with refractory seizures.

Methods: In this prospective cohort study, 27 neonates aged 1 to 56 days with refractory seizures were included. Blood and CSF samples were collected from each neonate within 24 hours after receiving 2nd antiseizure medications. All plasma and CSF samples were sent to the laboratory to measure amino acid concentrations. The associations between CSF and plasma amino acid levels with different variables were evaluated.

Results: Except for leucine (P=0.15) and isoleucine (P=0.07), the levels of all amino acids were significantly higher in plasma than CSF. Significant associations were observed between types of seizure and plasma citrulline (P=0.008) and leucine concentrations (P=0.04). The mean of CSF glutamic acid was also statistically different among neonates with different EEG results (P=0.02).

Conclusion: Our findings indicate that several plasma and CSF amino acids could be candidate biomarkers for neonatal refractory seizures. Further studies with larger sample size are to confirm our findings.

Keywords: Amino acid, Cerebrospinal fluid, Plasma, Seizure

Introduction

Seizure is the most prevalent neurological complication among neonates who admitted in NICU (neonatal intensive care units) (1). It is well-documented that repetition of seizure can disrupt the development of the central nervous system (2). Infection, ischemic hypoxic encephalopathy, cerebral hemorrhages, and inherited metabolic diseases are the most important causes of the seizure in neonates (3).

It has been shown that several amino acids are directly or indirectly involved in synaptic transmission of the nervous system. Analyses of amino acids in cerebrospinal fluid (CSF) have revealed their associations with inherited metabolic disorders, especially unexplained seizures (4-6). Previous studies demonstrated

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Please cite this paper as:
Kadivar M, Sangsari R, Zarkesh MR, Ashrafi MR, Shamel Sh, Mirnia K, Saeedi M, Rosmati P. Evaluation of Cerebrospinal Fluid and Plasma Amino Acids Levels in Neonates with Refractory Seizures: A Prospective Cohort Study. Iranian Journal of Neonatology. 2024 Apr: 15(2). DOI: 10.22038/IJN.2024.71069.2384

the roles of glutamate, gamma-aminobutyric acid (GABA), and aspartate in seizure pathology (7, 8). During an epileptic episode, the alterations of these amino acids could result in stimulatory or inhibitory neurotransmitters imbalance (8). Moreover, in some studies alterations of blood amino acids were associated with human epilepsy. For instance, in the patients with generalized epilepsy, the increased concentration of glutamate has been reported (7, 9).

Neonatal seizure is one of the predictive causes of cerebral palsy and delayed neurodevelopment. Early diagnosis and timely treatment are crucial in improving neurodevelopmental outcomes. It is supposed that assessing the CSF and plasma amino acids can provide important information regarding pathophysiology, as well as the severity of neurological diseases. Hence, the aim of present study was to investigate the association/ correlation of CSF and plasma amino acid concentrations with neonatal refractory seizures.

Methods

This prospective cohort study was conducted at the NICU of the Children's Medical Center Hospital affiliated with Tehran University of Medical Sciences in 2020.Neonates aged 1 to 56 days who admitted in neonatal intensive care unit (NICU) due to refractory seizure were included in the study. Diagnosis of refractory seizure was made by a neonatologist considering epilepticus status controlled with at least two anticonvulsant drugs (10). The neonates with gestational age<35 weeks, those who received less than two anticonvulsant medications and inadequate drug dosage, and those who had contaminated CSF samples were excluded from the study. Moreover, no intravenous amino acids were administered for included neonates. Epileptic neonates with electrolyte imbalance, hypoglycemia, or uncorrelated electrographic results (to describe events) were not also included.

Two ml of venous blood and 200 µl of CSF samples were collected to assess the concentrations of amino acids profile. It is important to note that all samples were collected from each neonate within 24 hours after receiving 2nd antiseizure medication and stabilizing of neonate. The concentrations of amino acids, including Aspartic acid, Asparagine, Serin, Glutamine, Histidine, Glysine, Tyrosine, Aminobutyric acid, Tryptophan, Methionine, Valine, Isoleucine, Ornithine, Lysine, Alanine, Glutamic acid, Phenylalanine, Threonine, Arginine, Taurine, Citrulline, and eucine were assessed in CSF and plasma samples.

Participants' demographic and clinical data were recorded in the researchers-made questionnaire. The types of seizure, including tonic, clonic, apnea & cyanosis, tonic-clonic, or spastic, were also recorded. All findings related to electroencephalography (EEG) examination (categorized in Normal, Epileptic seizure, and Burst suppression groups) and laboratory tests (CSF and plasma amino acids concentrations) were collected and recorded, as well.

Our primary outcome was to assess plasma and CSF amino acids in neonates with refractory seizures. The relationships between amino acid levels with neonates' demographic and clinical characteristics were also evaluated as the secondary outcome.

Sample size

Using Cochran's sample size formula and based on studies available in this area, 27 neonates were considered for the present study. Considering p & q= 0.5, d= 0.01, and Z= 1.96, the study had a power of 95% with the proposed sample size.

$$n = \frac{\frac{Z^2 pq}{d^2}}{1 + \frac{1}{N}\left(\frac{z^2 pq}{d^2} - 1\right)}$$

Statistical analysis

Statistical analysis was performed by STATA software Version 14. The Kolmogorov–Smirnov and Shapiro–Wilk tests were used to show data normality. The student's t-test or Mann–Whitney U test was applied for parametric and non-parametric amino acids, respectively. The Mann-Whitney U test and Kruskal-Wallis test were used to compare two and more than two groups, respectively. We reported median and interquartile ranges (IQR) for variables with skewed distribution. The significant levels were considered P< 0.05.

Ethical approval

The present study was approved by the Ethics Committee of the Tehran University of Medical Sciences (Ethical Code: IRTUMS.CHMC.REC. 1399.020). Informed consent was obtained from each neonate's parents, no extra costs were imposed, and data confidentiality was considered.

Results

Based on the defined criteria, 27 neonates with the mean weight of 3085.4±483.3 grams were included. The majority of neonates (88.4%) were term (37-39 weeks: n=11; 39-40 weeks: n=9; >40 weeks: n=3) and only 3 neonates (11.6%) were preterm. Among them, 14.6% had Apgar score <7. Approximately one out of five neonates had a positive familial history of seizure. The history of jaundice was also reported in 33.3%. The most frequent type of seizure was clonic with 44.4%. Regarding EEG results, 11 (40%), 13 (59.2%), and 2 (0.8%) neonates (60%) showed normal, epileptic, and burst suppression patterns, respectively. Demographic and clinical characteristics of neonates with refractory seizure are shown in Table 1.

As Table 2 shows, except for leucine (P=0.15) and isoleucine (P=0.07), the concentrations of all amino acids in plasma were significantly higher than CSF amino acids (P<0.05).

Table 1. Participants' demographic and clinical characteristics

Variable	N (%)
Sex	
Male	13 (48.2)
Female	14 (51.8)
Gestational age (weeks	
35-36	3 (11.6)
37-38	1 (3.8)
38-39	10 (38.5)
39-40	9 (34.6)
>40	3 (11.5)
Family history of seizure	
Yes	6 (22.2)
No	21 (77.8)
Type of seizure	
Clonic	12 (44.4)
Subtle	4 (14.8)
Tonic	2 (7.4)
Tonic-clonic	3 (11.1)
Spastic	6 (22.2)
History of jaundice	
Yes	9 (33.3)
No	18 (66.7)
Consanguinity of parents	
Yes	14 (51.8)
No	13 (48.2)
Type of Delivery	
NVD	9 (33.3)
CS	18 (66.7)
Apgar	
≥7	23 (85.2)
<7	4 (14.8)

As all detailed data are presented in Tables 3 and 4, there were no significant relationships between plasma amino acids with participants' sex(P>0.05), gestational age (P>0.05), low Apgar scores (P>0.05), and EEG findings (P>0.05). The associations between these factors and CSF amino acids were not also significant (P>0.05), except for CSF taurine, which significantly increased by gestational age (P=0.02) and the mean of glutamic acid that was statistically different among cases with varying EEG findings (P=0.02). The mean CSF glutamic acid in the patients with epileptic pattern was significantly higher than this factor in cases with normal (7.9vs. 4.9 µmol/L; P=0.006) and burst suppression (7.9 vs. 4.9 µmol/L; P=0.08) patterns.

Data analyses showed significant relationships between plasma citrulline (P=0.008) and leucine concentrations (P=0.04) with types of seizure. The mean plasma citrulline level in the patients with spastic seizure was significantly higher than this factor in cases with clonic (P=0.03), apnea & cyanosis (P=0.06), and tonic-clonic seizures (P=0.03). Patients with spastic seizure also had significantly higher levels of plasma leucine than cases with clonic seizure (P=0.01). Conversely, the CSF amino acids concentrations were not correlated to seizure types (P>0.05).

The results showed that the consanguinity of parents was a significant factor in alterations of CSF-phenylalanine (P= 0.05) and plasma-serine & glutamine (P value= 0.05); infants with such a

Table 2. Comparison of blood and CSF-amino acids

Amino acids	Serum mean (SD)	SCF mean (SD)	P value
Aspartic acid	5.81 (2.3)	1.20 (1.5)	< 0.0001*
Glutamic acid	118.47 (50.6)	7.27 (3.2)	< 0.0001*
Asparagine	40.79 (14.6)	12.52 (6.2)	< 0.0001*
Serin	148.44 (75.0)	69.11 (20.4)	< 0.0001*
Glutamine	527.25 (254.0)	750.72 (213.5)	0.001*
Histidine	65.06 (25.1)	26.04 (11.6)	< 0.0001*
Glysine	416.36 (572.7)	62.89 (122.9)	0.003*
Threonine	146.71 (74.8)	42.43 (24.4)	< 0.0001*
Citrulline	15.67 (6.9)	5.77 (4.1)	< 0.0001*
Arginin	58.99 (30.7)	16.47 (8.1)	< 0.0001*
Taurine	56.59 (70.2)	14.28 (7.7)	0.003*
Alanine	244.59 (152.8)	39.76 (20.3)	< 0.0001*
Tyrosine	59.17 (73.9)	21.76 (12.6)	0.01*
Aminobutyric acid	20.65 (18.9)	5.11 (4.6)	0.0001*
Tryptophan	41.58 (20.0)	9.22 (4.9)	< 0.0001*
Methionine	29.75 (62.5)	5.60 (3.0)	0.05*
Valine	168.70 (178.7)	31.94 (27.9)	0.0003*
Phenylalanine	52.49 (15.0)	29.09 (13.1)	< 0.0001*
Isoleucine	70.89 (142.9)	19.27 (44.2)	0.07
Leucine	439.61 (1078.3)	123.82 (318.6)	0.15
Ornithine	76.80 (39.8)	24.47 (14.1)	< 0.0001*
Lysine	125.16 (76.4)	31.63 (19.5)	<0.0001*

Table 3. The relationships between CSF-amino acids and different variables

Amino acids	Sex (P value)	Gestational age (P value)	Low Apgar scores (P value)	EEG findings (P value)	Type of seizure (P value)	Consanguinity of parents (P value)	Fimilal history of seizure (P value)
Aspartic acid	0.22	0.84	0.78	0.39	0.43	0.25	0.36
Glutamic acid	0.23	0.24	0.41	0.007*	0.14	0.34	0.43
Asparagine	0.38	0.93	0.50	0.46	0.66	0.16	0.56
Serin	0.70	0.43	0.78	0.40	0.75	0.17	0.77
Glutamine	0.63	0.77	0.89	0.48	0.50	0.07	1.00
Histidine	0.16	0.16	1.00	0.07	0.14	0.77	0.91
Glysine	0.59	0.23	0.50	0.15	0.57	0.44	0.86
Threonine	1.00	0.69	0.22	0.52	0.94	0.73	0.91
Citrulline	0.30	0.16	0.27	0.64	0.09	0.90	0.38
Arginine	0.32	0.33	0.59	0.91	0.61	0.70	0.03*
Taurine	0.46	0.02*	0.41	0.44	0.81	0.37	0.60
Alanine	0.18	0.74	0.59	0.35	0.41	0.73	0.82
Tyrosine	0.39	0.53	0.68	0.21	0.24	0.47	0.73
Aminobutyric acid	0.88	0.62	0.71	0.87	0.34	0.38	0.66
Tryptophan	0.80	0.67	0.61	0.43	0.42	0.08	0.29
Methionine	0.55	0.80	0.27	0.94	0.57	0.47	0.19
Valine	0.26	0.38	0.84	0.50	0.07	0.21	0.91
Phenylalanine	0.10	0.80	0.59	0.22	0.76	0.05*	0.91
Isoleucine	0.27	0.70	0.66	0.71	0.58	0.58	0.21
Leucine	0.22	0.62	0.95	0.60	0.23	0.63	0.66
Ornithine	0.60	0.15	0.68	0.68	0.80	0.90	0.04*
Lysine	0.61	0.16	0.31	0.74	0.87	0.59	0.32

history had lower concentrations of these amino acids compared with their other counterparts.

Infants with a positive familial history of seizure had significantly lower levels of plasma histidine (0.01), CSF arginine (P=0.03), and ornithine (P= 0.04) compared to other cases without such a history.

The results showed no differences in plasma and CSF amino acid levels of infants with different APGAR score subgroups(P>0.05).

Table 4. The relationships between blood-amino acids and different variables

Amino acids	Sex (P value)	Gestational age (P value)	Low Apgar scores (P value)	EEG findings (P value)	Type of seizure (P value)	Consanguinity of parents (P value)	Fimilal history of seizure (P value)
Aspartic acid	0.34	0.89	0.41	0.93	0.25	0.10	0.06
Glutamic acid	0.26	0.14	0.59	0.89	0.99	0.16	0.86
Asparagine	0.64	0.75	0.59	0.60	0.98	0.21	0.64
Serin	0.58	0.76	0.95	0.82	0.99	0.05*	0.24
Glutamine	0.38	0.47	0.63	0.96	0.77	0.05*	0.64
Histidine	0.83	0.34	0.89	0.26	0.34	0.21	0.01*
Glysine	0.50	0.82	0.22	0.21	0.70	0.81	0.95
Threonine	0.63	0.58	0.25	0.11	0.46	0.11	0.48
Citrulline	0.75	0.49	0.84	0.17	0.008*	0.63	0.60
Arginine	0.46	0.69	0.79	0.95	0.37	0.70	0.82
Taurine	0.47	0.69	0.19	0.68	0.84	0.44	0.73
Alanine	0.77	0.80	0.27	0.99	0.94	0.07	0.60
Tyrosine	0.47	0.68	0.59	0.20	0.22	0.09	0.38
Aminobutyric acid	0.72	0.89	0.32	0.34	0.69	0.08	0.10
Tryptophan	0.44	0.25	0.45	0.39	0.78	0.96	1.00
Methionine	0.38	0.58	0.89	0.19	0.85	0.81	0.47
Valine	0.85	0.60	0.78	0.96	0.13	0.63	0.41
Phenylalanine	0.06	0.14	0.08	0.14	0.62	0.44	0.82
Isoleucine	0.26	0.54	0.78	0.77	0.13	0.56	0.77
Leucine	0.33	0.47	0.49	0.69	0.04*	0.66	0.48
Ornithine	1.00	0.16	0.89	0.69	0.57	0.66	0.70
Lysine	0.90	0.83	0.08	0.99	0.91	0.58	0.50

Discussion

Neonatal seizure is one of the most common nervous system disorders that often lead to severe neurological complications (11). Generally, neonatal seizure is a predictable and manageable condition and its misdiagnosis may lead to irreversible damages (12). The brain uses amino acids to synthesize neurotransmitters, and the alteration of amino acids has been proposed in the pathomechanism of seizure (13, 14). The

synthesis of neurotransmitters in the brain is influenced by the concentration of amino acids in plasma. This is a mechanism of action for some antiepileptic drugs which can control the seizures through the down-regulation of amino acid receptors.

Changes in concentrations of amino acids also affect therapeutic approaches and the severity of drug-resistant epilepsy (6). Therefore, assessing CSF and blood amino acids in epileptic patients would be critical for timely diagnosis and treatment (6, 15). It has been indicated that drug-treated patients with mixed types of epilepsies had lower levels of several amino acids than untreated patients (6). Furthermore, it has been demonstrated that the ketogenic diet, as a treatment for pediatric refractory epilepsy, significantly altered the levels of various CSF amino acids (16). Accordingly, the present study was designed to investigate the concentrations of CSF and plasma amino acids in neonates with refractory seizures.

Our results showed that the frequency of refractory seizure was more frequent among female than male neonates; however, the difference was not significant between the two genders. Moreover, no relationship was observed between the amino acid levels and the neonates' sex. Although this finding was confirmed by another study from Iran (17), two investigations have reported a high frequency of seizures among male neonates. The authors pointed to the male gender could be a possible risk factor for seizure (18, 19).

In the current study, about 60% of epileptic neonates had abnormal EEG results. Neonatal seizure is one of the predictive causes of cerebral palsy and delayed neurodevelopment, so early diagnosis is essential to improve the prognosis (20). Various brain-studying techniques are implemented for early diagnosis and subsequent management (21). EEG examination as a necessary diagnostic tool is suggested for every patient with seizures in emergency departments (22). This examination would be more beneficial when used within 48 hours after an attack due to the probability of interictalepileptiform discharges (IED) in the first hours/days following a seizure (23, 24) We also observed that 40% of neonates had a normal EEG. This notable rate demonstrates the importance of using more diagnostic tools in such high-risk subjects. Therefore, in order to find brain lesions, brain CT scan has been suggested for infants with postpartum hypoxia, localized seizures, and seizures occurring during the first /few days of life (25).

In the present study, clonic (44%) and spastic (22%) seizures were the most common types. Consistent with our findings, another study from Iran showed clonic seizure as the most common type of seizure (26). Conversely, other studies showed different results; for instance, two studies showed tonic and subtle as the most common type of seizure (27, 28).

Findings of the present study showed significant associations/correlations between the plasma citrulline and leucine and the types of seizure in neonates studied. Similarly, in a study conducted by Origuchi et al., a10-time increase of serum citrulline (citrullinemia) was reported in a 16-year-old case with uncontrolled epilepsy and high voltage slow activity of EEG finding (29). It has been shown that the concentration of citrulline may be inversely correlated with the efficacy of antiepileptic drugs (30). Another study on the role of plasma amino acid profiles in the pathogenesis of refractory epilepsy also showed a significant increase in plasma levels of leucine in patients with refractory epilepsy. However, in addition to leucine and citrulline, the higher levels of other amino acids, such as valine, ornithine, aspartate, arginine, and alanine were found in the plasma of epileptic patients compared to healthy subjects (31). In another study, the concentrations of plasma glutamate, glycine, GABA, phenylalanine, aspartate, ornithine, serine, and alanine were significantly higher in patients with refractory epilepsy compared to the healthy group (6).

We found that there is a significant relationship between the CSF level of glutamic acid and EEG results; glutamic acid was significantly higher in the epilepsy group compared to the normal and burst suppression groups. Glutamate is the most abundant neurotransmitter in the nervous system, and alteration in glutamate transport may contribute to the pathogenesis of genetic and acquired seizure types. The knowledge related to factors involved in neonatal seizures is crucial in determining of the treatment plan. Failure to detect such factors may lead to dysfunction of the nervous system, which can cause severe long-term neurodevelopmental impairments (17, 32). Consistently, in the study by Pu *et al*. the level of CSF glutamine/glutamate in neonates with hypoxic-ischemic encephalopathy and seizure was significantly higher compared to neonates with hypoxic-ischemic encephalopathy without seizure

(31). Also, a study conducted by Jensen et al., suggested that the increased level of glutamate in the brain can induce seizure (33).

Our findings showed that the consanguinity of parents and a positive familial history of seizure were significant factors for alterations of several CSF and plasma amino acids. These results may relate to inborn errors of metabolism that may pass down to the next generation and affect brain biochemical pathways causing neonatal epilepsy (34).

Few biomarkers have been known to diagnose refractory neonatal seizures. Considering alterations of plasma or CSF amino acids like glutamic acid, citrulline, and leucine may also propose new biomarker candidates. In addition, implementing therapeutic approaches to balance such changes may be beneficial in the control of seizures. Antiepileptic approaches could also be selected based on the drug's effect on blocking or stimulating specific amino acid receptors (15). It is supposed that these attempts will impact neonatal outcomes and the cost of treatment.

Limitation

The main limitation of this study was the small sample size. We were unable to include more neonates with refractory seizures from multicenter due to budget constraints. Due to small sample size, we did not categorize the neonates based on different underlying etiologies, which may influence the amino acid concentrations in CSF and blood samples. Because CSF sampling by lumbar puncture was an invasive procedure, we could not include a control group to compare the amino acids profile between neonates with refractory seizure and healthy ones. The last but not least limitation was that we didn't assess the concentration of amino acids in neonates who responded to 1st medication and those with different underlying seizure etiologies.

Conclusion

Our findings indicate that CSF glutamic acid and plasma citrulline/leucine concentrations were associated with patterns of refractory seizure in neonates. The above-mentioned amino acids may play a role in the pathogenesis of refractory seizures which can be suggested as new potential biomarker candidates.

These potentially active roles of amino acids in the pathogenesis of refractory seizures may suggest new biomarker candidates. Moreover, implementing therapeutic approaches to balance levels of amino acids may help the control seizures. However, further studies with larger sample sizes are warranted to confirm our findings.

Acknowledgments

We want to thank all parents of the neonates, the NICU staff of Children's Medical Center, and those who had collaboration in the present study process. The authors also thank to Dr. Zahra Farahani and Dr. Pantea Nazeri for their kind collaborations.

Conflicts of interest

The authors declare that there is no conflict of interest.

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