

Neurotoxic Effects of Elevated CSF Aspartic and Glutamic Acids in Cerebral Malarial Patients

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Background and Objective: Cerebral malaria (CM) is the most serious and life-threatening complication of malaria, caused by *Plasmodium falciparum*. To know the significance of excitatory amino acids, the Aspartic acid (Asp) and the Glutamic acid (Glu) in causing neurotoxic effects, we analyzed their levels in the CSF of cerebral malarial patients.

Material and Methods: We developed a High-performance liquid chromatographic method, based on a pre-column derivatization with ophthal aldehyde to quantitate levels of those amino acids.

Results: In comparison to the control subjects, the levels of both amino acids (Asp and Glu) were found to be highly elevated in cerebral malarial patients. The patients showed aggravating neurogenic signs and symptoms.

Conclusion: The significant elevation reflects the neurotoxic effects of these amino acids in cerebral malarial patients.

Keywords: Cerebral malaria; Aspartic acid; Glutamic acid; CSF; HPLC; OPA

Introduction

CM is a fatal complication of malarial infection, caused by *Plasmodium falciparum* parasite. Around 400 million people suffer from malaria each year throughout the world and 2.3 million people die due to this fatal complication. Clinical signs and symptoms of cerebral malaria are related to disorders of central nervous system (CNS) which include motor dysfunctions, seizures and coma [1,2]. The pathogenesis of CM is not fully understood yet. Amino Acids are known to play an important role in neurotransmission [3]. Among those, Glu and Asp are well-known excitatory neurotransmitters, which belong to non-essential amino acids group. These are the most abundant of the free amino acids in the mammalian brain and exert their excitatory effects [4-6]. The receptors for Glu are distributed throughout the CNS. Glu binds to its receptors and activate intracellular signaling pathways to exert its excitatory effects [7]. Asp acts as an excitatory neurotransmitter and causes the release of Glu to cause excitation effects [8]. As in CM, role of Glu and Asp has not been investigated in causing the signs and symptoms in humans yet, we investigated the levels of the both amino acids in CSF of CM patients and their co-relation with the signs and symptoms in CM patients in the presented study.

Materials and Methods

Chemicals and reagents

Individual crystalline salts of Asp, Glu, Hydroxylysine (Schlaru), 2-Mercaptoethanol (Merck) and Orthophthaldehyde (OPA) were obtained from Pierce Eurochemie (Oud- Beijerland, Netherlands). Potassium di-hydrogen phosphate, Boric Acid, Sulfosalicylic acid, Methanol, absolute Ethanol and all other chemicals and reagents of analytical grade were obtained from Merck, Germany.

1mM standard stock solution of each Amino acid was prepared in Absolute Ethanol by the addition of few drops of 0.1M hydrochloric acid (HCl) to dissolve. The standard Mixture was diluted to 2,4,6,8 and 10 μ M to construct the Calibration Curve (p.height vs. concentration). The entire standard kept on freeze when not in use. Boric acid (3 ml) was added to purified water (90 ml) and the pH was adjusted to 10.5 with potassium hydroxide. In a separate container OPA 0.05 g was dissolved in 1.0ml ethanol with 0.05 ml 2-mercaptoethanol. The solution were mixed and diluted to 100 ml with purified water. Mixture of standard was mixed with OPA in equal volume (25 μ l). Ophthaldehyde (OPA) forms fluorescent derivatives in aqueous solution when reacted with amino acids in the presence of the reducing agent 2-mercaptoethanol.

Apparatus

Experiments were performed on HPLC Agilent 1100 Series (Waldbron Germany). Detection was made on fluorescence detector at Excitation wavelength= 340nm and Emission wavelength=455 nm. Separation of Amino acids was performed on a Column (Merck Germany).

Chromatographic conditions

Analysis of Amino acids was performed with multigradient programme by using 100% Mobile phase A with potassium dihydrogen phosphate buffer (0.05M) pH=4.5 and methanol with 3:2 ratio for the separation and mobile phase B was Deionized water and methanol with 192:18 ratio for washing of HPLC. Both solvents were filtered through filter membrane and sonicated for 10 minute before use. The flow-rate was maintained at 0.8 ml/min.

Sample preparation and run

Lumbar CSF samples were collected from 20 control subjects (7 women and 13 men, aged 36-67 years) and 20 patients with clinically

defined cerebral malaria, diagnosed microscopically to have *P. falciparum* in peripheral blood smear (7 women and 13 men aged 25-65 years). None of the subjects was on any medication.

CSF samples were treated with an equal volume of cold 10% sulphosalicylic acid (SSA). 50 μ L hydroxy lysine (20 nmol/L) was added as an internal standard. The mixture was centrifuged at 1500 rpm for 20 min. Supernatant was collected and stored at -70°C , if not analyzed immediately. The derivatization of OPA with CSF sample or standard (25 μ L each) was injected onto column within 3 to 4 minutes to avoid decomposition of the reagents.

Separation of standard OPA-derivatized Aspartic and Glutamic acid

Triplicates of each amino acid standard were run. The results showed 20 minutes retention time and the derivatives prepared were confirmed using spectrophotometric method to know the precise wave length for the detection of the complexes of standards for Asp and Glu in different concentrations with OPA.

Results

Asp and Glu were separated from CSF of cerebral malaria patients and control subjects by standard OPA-derivatized HPLC. Total 40 subjects (20 CM patients and 20 control subjects) were evaluated. For both amino acids, high levels were found in the CSF of CM patients in comparison to normal subject. Figure 1 A and B show the chromatograms of control and CM patients, respectively. Figure 2 shows increased levels of Asp in CM patients (35.5 to 223 $\mu\text{mol/l}$) in comparison to control subjects (2.3 to 8.8 $\mu\text{mol/l}$). Figure 3 shows increased levels of Glu in CM patients (15.7 to 116.6 $\mu\text{mol/l}$) in comparison to control subjects (4.3 to 10.2 $\mu\text{mol/l}$).

Discussion

In the present study, we found higher concentrations of Asp and Glu in the CSF of CM patients. We observed worse neurological symptoms, such as, increased muscle tone and seizures in the CM patients, who had more increased levels of these amino acids.

Previously, it was shown that increased Glu levels cause excitatory behavioral changes in experimental mice, suffering from cerebral malaria [5]. Sanni LA et al. showed that Glu and other amino acids, such as, aspartic and quinolinic acids that act via glutamate receptors exert excitotoxic effects on CNS to cause neurological and cognitive symptoms in CM experimental mice. They also showed that these neurological symptoms are produced due to blockade of microcirculation by parasitized erythrocytes and increased cerebrospinal fluid lactate in the CNS of these mice [9,10]. One group also showed that Glu was significantly high in the plasma of children, who died of malaria due to cerebral complications [11]. High amounts of glutamate release in intersynaptic spaces were also shown to cause neurodegeneration and neuronal cell death [12]. Therefore, in the presented study, as increased levels of Glu in CM patients exerted more aggravating effects on CM patients, it might be possible that these patients had toxic effects of Glu to cause worse neurological symptoms. There is also evidence of immune activation in the CM patients with altered neurological changes [13]. In the current study, we could not measure IgM concentration to know the role of immune activation as the patients were diagnosed by performing microscopy of peripheral blood. Therefore, in addition to increased levels of Asp and

Glu acids, activation of immune system might also have excitatory effects on CNS. Excitatory role of Asp and Glu in CNS was indicated by Haibin Yuan, et al, who showed that there were increased levels of these amino acids in the patients, suffering from spastic and athetotic cerebral palsies [14].

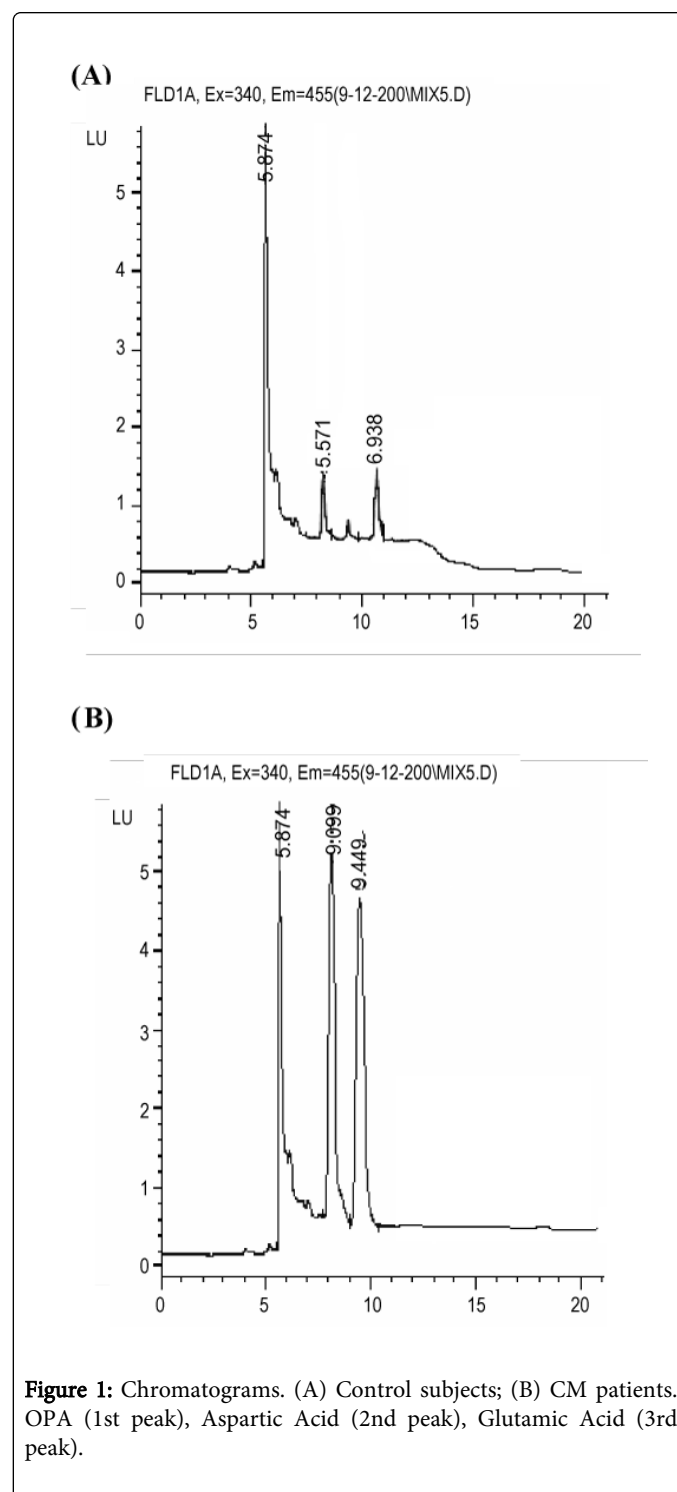


Figure 1: Chromatograms. (A) Control subjects; (B) CM patients. OPA (1st peak), Aspartic Acid (2nd peak), Glutamic Acid (3rd peak).

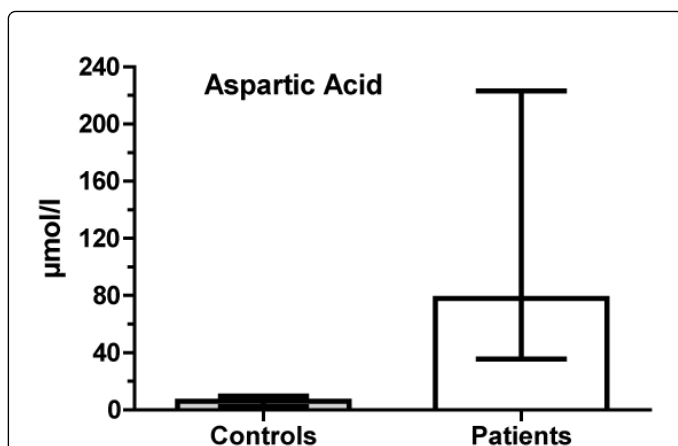


Figure 2: Aspartic Acid concentrations in CSF. In control subjects, 2.3 to 8.8 µmol/l and in cerebral malaria patients 35.5 to 223 µmol/l were found.

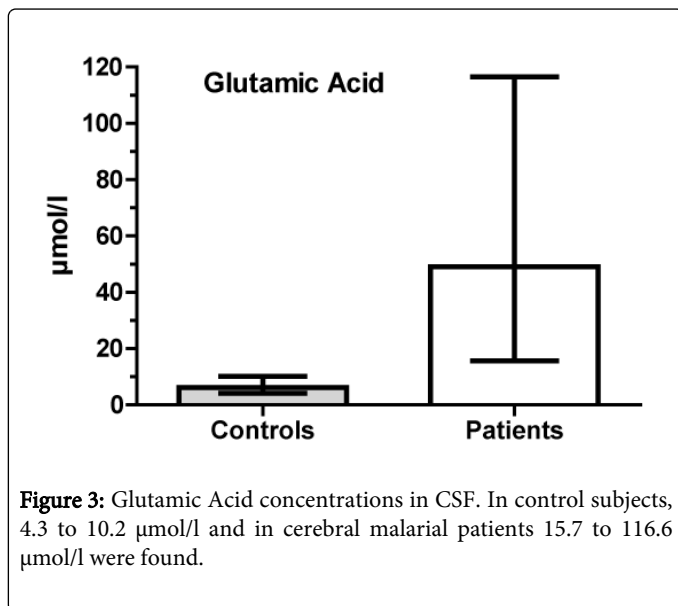


Figure 3: Glutamic Acid concentrations in CSF. In control subjects, 4.3 to 10.2 µmol/l and in cerebral malarial patients 15.7 to 116.6 µmol/l were found.

To our knowledge, the correlation between increased Glu and Asp levels in CSF and the neurological symptoms has not been clearly defined in CM patients. Here, we investigated and observed that the higher concentrations of these amino acids correlated with the exaggerating neurological symptoms in CM patients. No significant differences in both amino acid concentrations between male and female patients were observed. Thus, our findings suggest that CM patients should be investigated for CSF concentrations of Glu and Asp to know the disease progress and plan further treatment to prevent the neurological complications.

Conclusion

The results of our study show that Asp and Glu levels are raised in the CSF of CM patients. Quick and low cost method of HPLC for the detection of these amino acids can be used as a test for the disease progress of cerebral malaria to employ the proper therapeutic regimen for the patients.

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