Serum Levels of Glutamate, Aspartate, Glycine and Nitric Oxide as Early Biochemical Predictors for Poor Outcome in Ischemic Stroke Patients

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ABSTRACT

Objective: This prospective study was done to detect whether serum levels of glutamate, aspartate, glycine or nitric oxide could be used as early biochemical predictors for poor outcome in patients with acute ischemic stroke. Subjects and Methods: Sixty-two patients with first supra-tentorial ischemic stroke presented within 24 hours from onset of symptoms were included in the study. They were 38 (61.3%) males and 24 (38.7%) females. Their age ranged from 39 to 79 years (mean 55.8±9.5). On admission, all patients were subjected to assessment of: stroke severity by Scandinavian Stroke Scale, infarction volume by brain computed tomography and serum levels of glutamate, aspartate, glycine and nitric oxide. All subjects were followed up for three months. After three months stroke outcome was assessed by Glasgow Outcome Score. Results: After 3 months 9 patients (14.5%) had good recovery, 25 patients (40.4%) had moderate disability, 15 patients (24.2) had severe disability, no vegetative patient (0%) and 13 patients (20.9%) were dead. There was a significant relation between elevated admission serum levels of glutamate, glycine and nitric oxide and poor ischemic stroke outcome, stroke severity and infarction volume. There was no significant relation between admission serum aspartate level and ischemic stroke outcome stroke severity or infarction volume. Conclusion: Elevation of glutamate, glycine, and nitric oxide serum levels in the early onset of ischemic stroke can predict poor stroke outcome.

INTRODUCTION

For early prediction of ischemic stroke outcome, several studies were done and most of them considered clinical variables such as age¹, level of consciousness², severity of clinical picture³, hyperthermia⁴, hypertension⁵ and hyperglycemia⁶. Other studies considered radiological findings such as infarction site and size⁷ and midline shift⁸. The idea of the neurotoxicity of certain amino acids was introduced by Olney and Sharpe⁹. Subsequently, Meldrum¹⁰ and Rothman and Olney¹¹ postulated that this mechanism is involved in the pathogenesis of ischemic cellular lesion. Using microdialysis techniques, Bullock et al.¹² found elevated concentrations of glutamate and aspartate in a patient with a massive cerebral infarction. Kanthan et al.¹³, found marked elevation of glutamate concentrations immediately after ischemia in five patients subjected to surgical resections for untreatable epilepsy. Castillo et al.¹⁴, demonstrated elevation of CSF and plasma levels of glutamate and glycine during acute phase of cerebral infarction and found good correlation between CSF and plasma levels. They suggested that there is a good diffusion of neurotoxic amino acids through the altered blood brain barrier during cerebral ischemia. Thus, measurement of serum excitatory amino acids glutamate and aspartate and inhibitory amino acid glycine appear to be useful predictors of ischemic stroke outcome.¹⁵

Nitric oxide (NO) is an important signaling molecule. It is a free radical, which makes it very reactive and unstable. It is synthesized from...
arginine and oxygen by various nitric oxide synthase (NOS) enzymes. Its role was first discovered by attempting to identify the agent responsible for promoting blood vessel relaxation. This agent was termed endothelium-derived relaxing factor (EDRF). The discovery that EDRF was in fact NO has led to explosion of interest in this field.\textsuperscript{16}

NO plays a role in a variety of biological processes. The endothelium of blood vessels use it to signal the surrounding smooth muscle to relax. It also serves as a neurotransmitter between nerve cells. Unlike other neurotransmitters the small NO molecules can diffuse all over and can act on several nearby neurons. Its biological effects are mediated through its reaction with a number of targets such as haem groups, sulfhydryl groups and iron and zinc clusters.\textsuperscript{17}

While NO mediates normal synaptic transmission, its excess level is neurotoxic. It has been assumed that NO is a key pathological agent in developing ischemic stroke.\textsuperscript{18} Thus, its serum level could be an early predictor of ischemic stroke outcome.

Therefore, this study was done to detect whether serum levels of glutamate, aspartate, glycine or nitric oxide could be used as early biochemical predictors for poor outcome in patients with acute ischemic stroke.

**SUBJECTS AND METHODS**

This prospective follow up study included 62 patients admitted to the department of Neurology, Suez Canal University Hospital, from April 2003 to April 2004 with clinical diagnosis and radiological evidence of first supratentorial acute ischemic stroke presented within 24 hours from onset of symptoms. They were 38 males (61.3\%) and 24 females (38.7\%). Their age ranged from 39-79 years (mean 55.8±9.5). Patients with another brain pathological lesion, patients with history of previous cerebral stroke or with radiological evidence of old cerebral infarctions, patients with evidence of recent angina or myocardial infarction, patients recovered from neurological deficit within 24 hours of onset (TIA) and patients transferred to another hospital were excluded from the study.

*On admission all patients were subjected to:*
- Thorough history taking and physical and neurological examination.
- Assessment of stroke severity by Scandinavian Stroke Scale (SSS)\textsuperscript{19} on admission, weekly during the hospital stay, at discharge, and at three months of the onset.
- Brain computed tomography (CT): at the time of admission to exclude cerebral hemorrhage or any pathology other than cerebral ischemia and one week later to determine site and volume of infarction. All scans were performed on Siemens Somaton Balance scanner. The size of the lesion was calculated according to the formula 0.5\(\times\)A\(\times\)B\(\times\)C (where A and B are the largest perpendicular diameters measured on CT and C is the slice thickness (10 mm)).\textsuperscript{20}
- Electrocardiogram and routine laboratory investigations (Serum level of glucose, complete blood picture (CBC), erythrocyte sedimentation rate (ESR), lipid profile and serum creatinine).
- Biochemical analysis: Blood samples were drawn within 24 hours of onset of symptoms and sera were extracted and kept in \(-70^\circ\)C for following laboratory tests:

1. Determination of serum levels of amino acids: Serum levels of glutamate, aspartate and glycine using high performance liquid chromatography technique (HLPC).\textsuperscript{21} Concentrations of amino acids expressed in micromoles per liter (µM/L) were calculated with the use of computerized system.
2. Determination of nitrite /nitrate level: Nitrate was reduced to nitrite using nitrate reductase from Aspergillus Species and then by a Colorimetric assay using the diazotization procedure according to Bartholomew\textsuperscript{22}. Concentrations of nitrite were determined by comparison to standard curve of sodium nitrite solution. The curve was linear from 0.2 µM to 100 µM.
After admission all the patients received standard care including general care for comatose patients, anticoagulants for embolic stroke and for stroke in evolution, antiplatelet agents, antihypertensive drugs, hypoglycemic medications for diabetic patients and fluids.

All subjects were followed up for three months. Follow up information were collected during hospitalization (mean period of hospitalization was 14.85±5.64 days) as SSS was performed weekly during hospitalization period and at discharge. Patients who survived were followed up through repeated hospital visits after discharge.

Glasgow Outcome Scale (GOS) was used to assess stroke outcome after three months of onset. The five categories of the original scale are: good recovery, moderately disabled, severely disabled, vegetative and dead. Patients with good recovery and with moderate disability were classified as good outcome group. While, patients with severe disability, vegetative and those who died were classified as poor outcome group.

Data collected were coded, entered and analyzed using Microsoft Excel software, then imported into (SPSS 10.0) software for analysis. P value was set at <0.05 for significant results and <0.01 for highly significant results.

RESULTS

By the end of 3-months follow-up period 9 patients had good recovery (14.5%), 25 patients had moderate disability (40.4%), 15 patients had severe disability (24.2), no vegetative patients (0%) and 13 patients were dead (20.9%) (Table 1).

Table (2) shows the relation between admission serum levels of glutamate, aspartate, glycine and nitric oxide and outcome according to Glasgow outcome scale. Serum level of glutamate on admission was significantly (P<0.01) higher in dead patients (279.5±19.7 µM/L) and in patients with severe disability (203.1±16.7 µM/L) than in patients with good recovery (117.8±2.9 µM/L) and with moderate disability (154.5±28.2 µM/L).

There was no significant difference (P>0.05) between serum level of aspartate on admission in dead patients (11.9±1.8 µM/L) and patients with severe disability (11.9±1.4 µM/L) and in patients with good recovery (13.2±0.7 µM/L) and with moderate disability (12.4±0.5 µM/L).

Serum level of glycine on admission was significantly (P<0.01) higher in dead patients (229.2±14.4 µM/L) and in patients with severe disability (199.5±15.6 µM/L) than in patients with good recovery (164.9±3.1 µM/L) and with moderate disability (177.3±3.8 µM/L).

Serum level of NO on admission was significantly (P<0.01) higher in dead patients (14.3±0.5 µM/L) and in patients with severe disability (10.8±1.0 µM/L) than in patients with good recovery (6.1±0.2 µM/L) and with moderate disability (6.9±1.5 µM/L).

Table (3) shows correlation between admission serum levels of glutamate, aspartate, glycine and nitric oxide and Scandinavian stroke scale (SSS) scores. There was highly significant (P<0.01) inverse correlation relation between serum glutamate levels on admission and SSS scores on admission (Fig. 1), after two weeks of onset, on discharge and after 3 months of onset of stroke.

There was also highly significant (P<0.01) inverse correlation relation between serum glycine levels on admission and SSS scores on admission (Fig. 1), after two weeks of onset, on discharge, and after 3 months of onset of stroke.

There was non significant (P>0.05) correlation relation between serum aspartate levels on admission and SSS scores on admission, after two weeks of onset, on discharge, and after 3 months of onset of stroke.

There was highly significant (P<0.01) inverse correlation relation between serum NO levels on admission and SSS scores on admission (Fig. 1), after two weeks of onset, on discharge and after 3 months of onset of stroke.

Table (4) shows correlation between admission serum levels of glutamate, aspartate, glycine and nitric oxide and infarction volume. There was highly significant (P<0.01) direct correlation relation between admission serum glutamate, glycine and NO levels and infarction volume (Fig. 2). There was non
significant (P>0.05) correlation relation between serum aspartate levels on admission and infarction volume.

Table 1. Three months outcome according to Glasgow outcome scale.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>N=62 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good Recovery</td>
<td>9 (14.5)</td>
</tr>
<tr>
<td>Moderate Disability</td>
<td>25 (40.4)</td>
</tr>
<tr>
<td>Severe Disability</td>
<td>15 (24.2)</td>
</tr>
<tr>
<td>Vegetative</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Dead</td>
<td>13 (20.9)</td>
</tr>
</tbody>
</table>

Table 2. Relation between admission serum levels of glutamate, aspartate, glycine and nitric oxide and outcome according to Glasgow outcome scale.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Glutamate Mean±SD</th>
<th>Moderate disability Mean±SD</th>
<th>Severe disability Mean±SD</th>
<th>Dead Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good Recovery</td>
<td>117.8±2.9</td>
<td>154.5±28.2</td>
<td>203.1±16.7**</td>
<td>279.5±19.7**</td>
</tr>
<tr>
<td>Moderate Dis.</td>
<td>13.2±0.7</td>
<td>12.4±0.5</td>
<td>11.9±1.4</td>
<td>11.9±1.8</td>
</tr>
<tr>
<td>Severe Dis.</td>
<td>164.9±3.1</td>
<td>177.3±3.8</td>
<td>199.5±15.6**</td>
<td>229.2±14.4**</td>
</tr>
<tr>
<td>Dead</td>
<td>6.1±0.2</td>
<td>6.9±1.5</td>
<td>10.8±1.0**</td>
<td>14.3±0.5**</td>
</tr>
</tbody>
</table>

Serum levels are expressed in micro moles/Liter (µM/L).
Mann-Whitney U test was used to determine the difference between study groups.
** Highly statistical significance (P<0.01).
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**Fig. (1):** Correlation between admission serum glutamate, glycine and nitric oxide and admission Scandinavian stroke score (SSS).

**Fig. (2):** Correlation between admission serum glutamate, glycine and nitric oxide and volume of infarction.

**Table 3.** Correlation between admission serum levels of glutamate, aspartate, glycine and nitric oxide and Scandinavian stroke scale (SSS) scores.

<table>
<thead>
<tr>
<th></th>
<th>Admission SSS (R value)</th>
<th>2 wk SSS (R value)</th>
<th>Discharge SSS (R value)</th>
<th>3 Months SSS (R value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate</td>
<td>-0.806**</td>
<td>-0.794**</td>
<td>-0.708**</td>
<td>-0.739**</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.349</td>
<td>0.376</td>
<td>0.310</td>
<td>0.254</td>
</tr>
<tr>
<td>Glycine</td>
<td>-0.781**</td>
<td>-0.774**</td>
<td>-0.644**</td>
<td>-0.712**</td>
</tr>
</tbody>
</table>
Nitric oxide  -0.767**  -0.731**  -0.581**  -0.589**

Pearson’s correlation coefficient was used to determine the correlation relation.

** Correlation is highly significant at P value <0.01.

Table 4. Correlation between admission serum levels of glutamate, aspartate, glycine and nitric oxide and infarction volume.

<table>
<thead>
<tr>
<th>Infarction volume (R value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate 0.913**</td>
</tr>
<tr>
<td>Aspartate  -0.376</td>
</tr>
<tr>
<td>Glycine 0.905**</td>
</tr>
<tr>
<td>Nitric oxide 0.891**</td>
</tr>
</tbody>
</table>

**Correlation is highly significant at P value <0.01 level.

DISCUSSION

In the current study, the neurotoxic amino acid glutamate was found significantly in higher concentrations in sera of died patients and in patients with severe disability. Similarly, glycine though considered as an inhibitory amino acid was found significantly in excess in sera of poor outcome groups.

These findings came in agreement with those of Skvortsova et al., who found a significant increase in CSF levels of glutamate, glycine, and aspartate in the first 6 hours of ischemic stroke. The level and duration of these increments were correlated with the severity of the insult. Castillo et al., reported that levels of glutamate and glycine in serum and CSF were significantly higher in ischemic patients with higher degree of neurological deficits and in patients with larger brain infarctions. They postulated the excitotoxic hypothesis of ischemic cerebral injury that the decline in cerebral perfusion to below critical values gives rise to accumulation of glutamate and other amino acids within and around the ischemic zone. Castillo et al., also found that early neurological progression of acute ischemic stroke is associated with high concentrations of glutamate and glycine in blood and CSF. They stated that measurement of these amino acids could be useful for the early detection of patients who will deteriorate during 48 hours after onset of ischemic stroke. Even with lacunar infarctions, Serena et al., reported that glutamate concentrations >200 µmol/L and GABA concentrations <240 µmol/L in blood within the first 24 hours after the onset of symptoms are powerful predictors of subsequent deterioration.

On the contrary to glutamate and glycine, though considered an excitatory amino acid, aspartate was found to have low concentrations in sera of both good and poor outcome groups with no significant statistical difference. However, in animal models of focal cerebral ischemia the concentration of aspartate has been shown to be high. This difference could be attributed to the fact that the existence of aspartate in serum after ischemic cerebral lesion is very transitory, while glutamate levels remain high for a longer time and glycine levels progressively increase during ischemia. The persistence in the elevated levels of glutamate and glycine but not of the other amino acids could be explained by conditions of chronic excitotoxicity, as described in experimental focal ischemia and in many patients with traumatic brain injury probably due to prolonged and fluctuating ischemia. Indirect evidence for the persistence of glutamate excitotoxicity is also supported by effect of some NMDA receptor inhibitors demonstrated in the treatment of experimental cerebral ischemia.

There was a significant inverse relationship between serum levels of glutamate and glycine in the first 24 hours of onset and stroke severity as assessed by the SSS on admission, at week two, at discharge and at three months of onset. There was
no significant relation found in respect to aspartate that could be owing to its transitory presence in serum. These findings confirm also the neurotoxic effect of glutamate and glycine in cerebral ischemia and impress the association between this neurotoxicity and neurological deterioration.

In a study by Aliprandi et al.\textsuperscript{32}, higher serum glutamate levels were found to be inversely correlated with neurological improvement between day 3 and 15 of onset of brain infarction and they assumed glutamate level increments, which might be linked to altered platelet functions, to excessive release of the amino acid or impaired uptake. Glutamate is released in high concentrations in the core of the cerebral infarction and in the penumbral cortex, leading to a massive influx of calcium that activates a variety of catabolic processes that subsequently produce cell death.\textsuperscript{33} Plasma glutamate concentrations $>200$ µmol/L within the first 24 hours of the onset of symptoms have been associated with subsequent progression of cortical and subcortical ischemic stroke.\textsuperscript{25} Similarly, in Egypt, Tag El-Din et al.\textsuperscript{34}, found that increased serum and CSF levels of glutamate and glycine were associated with increased risk for progression of ischemic stroke. There was a significant direct association between infarction size and serum levels of glutamate and glycine in the first 24 hours of onset of ischemic stroke suggesting increased release of these amino acids from larger ischemic lesions. While aspartate did not show similar association. Supporting our findings Castillo et al.\textsuperscript{14}, found a correlation between infarction volume and levels of glutamate and glycine in plasma. Experimental studies by Takagi et al.\textsuperscript{33} and Mitani and Kataoka\textsuperscript{35} have shown also a clear correlation. However, Serena et al.\textsuperscript{26} reported that glutamate accumulation within or outside the ischemic area does not depend exclusively on infarction volume.

In the current study serum nitric oxide (NO) on admission was found to be a significant predictor of poor outcome and higher serum levels of NO were significantly correlated with stroke severity assessed by the SSS (at admission, at week two, at discharge and at 3 months) and also significantly correlated with larger infarction volume. These findings suggest the important role of NO generation in acute ischemic stroke. However, the method we used in this study could not allow determination of which portion of NO concentration derived from (endothelial, neuronal or other cells).

Supporting our findings Castillo et al.\textsuperscript{20} found higher concentrations of NO in CSF of patients with acute ischemic stroke than in controls and in those with early neurological deterioration than in those with stable stroke. They also found a significant correlation between NO CSF levels and infarction volume and they stated that increase NO levels in CSF were associated with a greater brain injury and early neurological deterioration.

Interestingly, Tarkowski et al.\textsuperscript{36}, found that levels of nitrate measured at stroke onset were negatively correlated to the final size of infarction volume measured by MRI. In addition, patients with large infarcts displayed significantly lower levels of nitrate in CSF compared to patients with small infarcts. In contrast, the CSF levels of nitrate were significantly positively correlated to the neurological deficit in the late stage of the stroke (after 3 days). They postulated that early NO production is associated with a smaller infarction volume suggesting a protective effect, whereas late NO production is associated with severe neurological deficits, suggesting a neurotoxic effect.

Similarly, Beridze et al.\textsuperscript{37}, found a significant negative correlation between NO initial blood levels and ischemic lesion size. They stated that endothelial derived NO has a positive impact on restoration of cerebral blood flow in the initial stage of acute brain ischemia.

Foster et al.\textsuperscript{38}, reported that NO synthase (NOS) activity and NO release are greatly increased in the acute human ischemic brain. The effect of selective antagonists of NOS isoforms on acute cerebral ischemia\textsuperscript{39} and the effect of cerebral ischemia on mouse models in which a
single NOS isoform gene is not expressed (knockout mice) have clarified the protective role of endothelial NOS activity and the neurotoxic role of neuronal NOS and inducible NOS activity. The former is associated with smaller cerebral infarctions and the latter with increased infarction volume.

The present study shows that NO plays a part in early neurological deterioration, a fact that could be attributed to the expansion of the ischemic area. These findings suggest a direct neurotoxic effect of NO on the propagation of ischemic penumbra and neuronal death. It has been shown that immediately after ischemia, NOS is much more active in the core than in the surrounding area, which gives rise to an increasing concentration of NO that gradually extends from the core to the vulnerable neighboring neurons in the penumbra. This phenomenon may explain in part the progressive expansion of the brain injury in the absence of relevant changes in cerebral blood flow. Although the neurotoxic mechanisms mediated by NO have been only partially elucidated, free radical damage by formation of peroxynitrite may have an important role. Peroxynitrite decomposes to other reactive oxygen species, such as radical hydroxyl and nitrogen dioxide, which cause lipid peroxidation and thus destroy cell membranes. This mechanism may be particularly important in the peri-infarct region, because oxygen delivery during reperfusion facilitates a delayed generation of NO and a higher production of peroxynitrite due to the reduction of NO by the superoxide anion.

REFERENCES


الملخص العربي

قياس مستوي الجلوتامات والأسبرتات والجليدين والنيتريك أوكسيد في المصل كمترتبات كيميائية مبكرة للإصابة السريرية في مرض السكتة الدماغية الانسدادية

تم إجراء هذا البحث بهدف دراسة إمكانية استخدام قياس مستوي الجلوتامات-الأسبرتات-الجليدين والنيتريك أوكسيد في المصل كمترتبات كيميائية مبكرة للإصابة السريرية في مرضى السكتة الدماغية الانسدادية. وقد تم إجراء هذا البحث على 62 مريضاً. وقد خضع المرضى إلى الفحوصات التالية خلال 24 ساعة من حدوث الأعراض:
- قياس السكتة الدماغية.
- أنشطة مقطعة على المخ للتحديد حجم الجلطة.
- قياس مستوي الجلوتامات-الأسبرتات-الجليدين والنيتريك أوكسيد في المصل.

النماذج التي غذت نتائج البحث:
- 9 مرضى تحسنت حالتهم، 25 مريضاً كانت نسبة الإعاقة لديهم متوسطة، 15 مريضاً كانت نسبة الإعاقة لديهم شديدة، 13 مريضاً فارقوا الحياة.
- ارتفاع مستوي الجلوتامات والأسبرتات والنيتريك أوكسيد في المصل كان مرتبطًا بحالة السكتة الدماغية.
- لا توجد علاقة إحصائية بين مستوى الأصبات في المصل وحجم السكتة الدماغية.
- توجد علاقة طردية بين ارتفاع مستوي الجلوتامات والأسبرتات والنيتريك أوكسيد وشدة أعراض السكتة الدماغية وحجم الجلطة.