Selected acute phase CSF factors in ischemic stroke: findings and prognostic value

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Abstract

Background: Study aimed at investigation of pathogenic role and prognostic value of several selected cerebrospinal fluid acute phase factors that can reflect the severity of ischemic brain damage.

Methods: Ninety five acute ischemic stroke patients were investigated. Ischemic region visualized at the twenty fourth hour by conventional Magnetic Resonance Imaging. Stroke severity evaluated by National Institute Health Stroke Scale. One month outcome of disease was assessed by Barthel Index. Cerebrospinal fluid was taken at the sixth hour of stroke onset. CSF pro- and anti-inflammatory cytokines were studied by Enzyme Linked Immunosorbent Assay. Nitric Oxide and Lipoperoxide radical were measured by Electron Paramagnetic Resonance. CSF Nitrate levels were detected using the Griess reagent. Statistics performed by SPSS-11.0.

Results: At the sixth hour of stroke onset, cerebrospinal fluid cytokine levels were elevated in patients against controls. Severe stroke patients had increased interleukin-6 content compared to less severe strokes (P < 0.05). Cerebrospinal fluid Electron Paramagnetic Resonance signal of nitric oxide was increased in patients against controls. Severe stroke group had an elevated Electron Paramagnetic Resonance signal of lipoperoxiradical compared to less severe stroke. Cerebrospinal fluid nitrate levels in less severe stroke patients were higher than those for severe stroke and control. Positive correlation was established between the initial interleukin-6 content and ischemic lesion size as well as with National Institute Health Stroke Scale score on the seventh day. Initial interleukin-6 and nitrate levels in cerebrospinal fluid found to be significant for functional outcome of stroke at one month.

Conclusion: According to present study the cerebrospinal fluid contents of interleukin-6 and nitrates seem to be the most reliable prognostic factors in acute phase of ischemic stroke.

Keywords: brain ischemia, inflammation, oxidative stress

Background

Modern concepts of acute cerebral ischemia highlight the role of neurovascular units and emphasize the importance of integrative tissue responses that result from dynamic interactions of endothelial cells, vascular sooth muscles, matrix elements, astroglia, microglia and neurons. By means of inflammatory stimuli and excitotoxicity, such interactions create many sources of free toxic radicals and reactive oxygen spices [1].

In physiological conditions, endogenous protective mechanisms stabilize the levels of free oxygen radicals and reduce the oxidative/nitrosative stress reaction. In conditions of severe ischemia, rapid failure of the antioxidation protective system assists in the accumulation of arachidonic acid, prostaglandins, superoxide anion, NO and other aggressive substrates, which lead to the destabilization of cellular membranes, further damage of the blood-brain barrier, disintegration of DNA and, ultimately, to neuronal death [2]. Current therapeutic options for acute brain ischemia are concentrated on thrombolytic treatment, but this therapy is restricted to a small proportion of patients [3]. There is a need to devise a more effective protective and repair strategy and cellular treatment. The precise neurochemical alterations that take place in human's stroke still remain to be clarified and the cerebrospinal fluid (CSF) is the...
closest environment reflecting the immediate immuno- 
biological changes in the ischemic brain tissue. The 
purpose of the present research was to investigate the 
importance and prognostic value of several selected CSF 
acute phase factors that are known to reflect the severity 
of ischemic brain damage.

Methods
A total of 95 acute ischemic stroke patients, 54 female 
and 41 male, aged 45-70 years, who had been admitted 
to the Neurological Clinic of Tbilisi State Medical 
University during 2005-2009 were studied. Exclusion 
criteria comprised acute inflammatory and autoimmune 
disorders, severe somatic pathology, cancer, coma, space 
occupying hemispheric and cerebellar ischemic strokes. 
Patients and controls that used the anti-inflammatory 
medications for the 1-year period prior to the current 
research were also excluded from the study. Upon 
admission, a conventional CT scan was performed to 
eclude a brain hemorrhage. The control group 
consisted of 25 age-matched patients with vertebral discop- 
pathies, who showed no signs of cerebrovascular 
pathology. The local ethics committee approved the pro- 
\cocol, and informed consent was obtained from all parti- 
cipants or their surrogates.
The etiology of stroke was classified according to TOAST criteria [4]. Medical records were retrospec- 
tively reviewed for selected non-modifiable and modifici- 
ble risk factors of stroke, including age, sex, 
inheritance, history of a transient ischemic attack (TIA) 
or a previous stroke, hypertension, atherosclerosis, atrial 
fibrillation, diabetes mellitus, smoking, alcohol abuse, 
acute infections 1-2 months before stroke, and psycholo- 
gical stress. The latter was ascertained by self-report in 
refuges from problematic regions of Georgia. Psycholo- 
gical stress was of interest to determine whether it can 
fluence the inflammatory markers or functional outcome 
of the disease [5,6].
Upon admission, body temperature and blood pressure 
were recorded. Next, chemistry, basic hematology, chest 
X-rays, and electrocardiography were performed. Selected 
patients did not display marked hyperthermia or infective 
complications. Patients were managed according to evi- 
\cidence-based stroke guidelines. Thrombolytics, hemodilu- 
ation, corticoids, and Nimodipine were not applied. 
Patients were strictly controlled and administered for 
\hypo- and hyperglycemia and hypertension. Antiplatelet 
drugs were used in atherothrombotic and lacunar infarcts 
and anticoagulants -in suspected cardioembolic infarcts 
when the initial CT or magnetic resonance imaging (MRI) 
scans did not show a large cerebral lesion.
The ischemic region was visualized at the twenty- 
fourth hour from stroke onset by conventional magnetic 
resonance imaging (MRI; magnet operating 1.5 Tesla, 
Vision, Siemens) that provided axial T1, T2 images with a slice thickness of 5 mm. Radiologists who were 
blinded to study protocol defined the whole lesion volume by multiplying the area of focal hyperintensity by interslice gap. Stroke severity at admission and on 
the seventh day was evaluated by the National Institute 
Health Stroke Scale (NIHSS). Patients were divided in 
two groups: Group 1 included the patients with severe 
stroke (NIHSS ≥15) and Group 2 included the patients 
with mild and moderate stroke (NIHSS <15) [7]. Func- 
tional outcome was evaluated by Barthel Index at 
1 month of stroke onset (BI) [8,9].

Immunological Assay
For special laboratory investigations, 10 ml of CSF was 
taken from patients and controls at the sixth hour from stroke onset. Eight-ml CSF samples were frozen at -20°C for further assays, and the other 2 ml of CSF 
were frozen in liquid nitrogen for electron paramagnet 
resonance (EPR) study. CSF (5 ml) levels of the pro- 
\inflammatory cytokines: interleukin-1β (IL-1β), interleu- 
kin-6(IL-6), tumor necrosis factor-α(TNF-α) and the 
anti-inflammatory cytokine interleukin-10 (IL-10) were 
detected by enzyme-linked immunosorbent assay 
(ELISA), by application of ELISA- RIDER. The rela- 
tionship between optical density and cytokine concentra- 
tions was defined using the standard curve according to kit instructions (Bender Med systems 
Diagnostics, Vienna, Austria).

Electron Paramagnetic Resonance (EPR) Study
Nitric oxide (NO) and liperoxide radical (LOO-) were 
measured by EPR spin labeling (radiospectrophotometer 
ESR-231 (X- band), with a modulation frequency of 
50 KHZ and a TM-110 cavity). Diethyldithiocarbamic 
acid (DETC) (Sigma) was used as an NO trap. CSF samples 
were incubated with Fe2+(DETC)2 stock solution. The 0.8 mM Fe2+ (DETC) colloid solution formed was 
yellow-brown in color and was used immediately after 
preparation. EPR specters of NO-Fe2+ (DETC)2 complex 
existed at the temperature of liquid nitrogen on a microwave power of 20 mVt. The amount of 
detected NO was determined from the calibration curve 
for integral intensity of the EPR signal of NOFe2+ 
(DETC)2, prepared at various concentrations (1-20 μM) 
of the NO-donor MAHMANONOate [10]. LOO- trap, 
α-phenil-tert-butilitron (PBN) (SIGMA) was used at a 
dosage of 50 ml/0.5 ml CSF. EPR specters of LOO- 
were defined at room temperature on a microwave 
power of 20 mVt. EPR signals of LOO- measured in 
arbitrary units (a.u.) (signal intensity in millimeters 
represented milliliters of CSF matter) [11].
Biochemical Assay
For nitrate (NO2) detection, 3 ml CSF samples were processed by 20% Griess reagent. We used a CF-46 LOMO spectrophotometer for colorimetric detection. Optical density was detected on a 540nm wavelength. NaNO2 (5 μmol/L) was used for drawing the calibrating curve [12].

Statistics
The obtained data were analyzed using SPSS 11.0 computer software. Normally distributed continuous variables were compared with repeated measure ANOVA, and the Kruskall-Wallis test compared abnormally distributed variables. The $\chi^2$-test was used to assess associations among categorical variables. The effect of acetyl salicylic acid (aspirin) and HMG-CoA reductase inhibitors (statins) was separated by partial correlation analysis. Spearman rank correlation and multiple logistic regression (forward stepwise conditional model) was used when all acute phase factors and stroke risk factors were entered into the model. Aspirin and Statins included in regression analysis as categorical covariate variables. The Hosmer and Lemeshow test was used to assess the goodness of fit of each model.

Results
The main characteristics of each clinical group are presented (Table 1). At the sixth hour from stroke onset, the CSF proinflammatory cytokine levels in both study groups were elevated compared to the control ($P < 0.05$). There was no significant difference in IL-1β and TNF-α contents between the two groups, while Group 1 had significantly increased IL-6 contents compared with Group 2 ($P < 0.05$). The anti-inflammatory cytokine IL-10 levels were not significantly elevated in the two study groups compared to the control ($P > 0.07$), although there was a trend towards an increase in Group 2 (Table 2).

The EPR signal intensity of NO was increased in Groups 1 and 2 compared to the Control ($P < 0.05$), but not between the study groups ($P > 0.50$) (Figure 1). The EPR signal intensity of LOO- in Groups 1 and 2 was significantly increased compared to the control ($P < 0.05$), and Group 1 had an elevated EPR signal intensity of LOO- compared to Group 2 ($P < 0.05$) (Figure 2). The NO2 levels for Group 2 were higher than those for Group 1 and the controls (Table 2). At the twenty-fourth hour from stroke onset, the mean ischemic lesion size ($\text{cm}^3$) for Group 1 was significantly increased as compared to Group 2 (Table 1). There was a significant positive correlation between the initial IL-6 contents and ischemic lesion size ($r = +0.34; P < 0.05$). The effect of Aspirin and Statins did not change the zero ordered correlations between study variables. Multivariate logistic regression analysis established a level of significance of IL-6 contents toward the mean predicted probability of ischemic lesion size at the twenty-fourth hour from stroke onset, after all the acute phase factors and risk factors that we examined were entered into the

### Table 1 Main Characteristics of ischemic stroke patients of Group 1 and 2

| Characteristics                        | Group 1 (n = 44) | Group 2 (n = 51) |
|----------------------------------------|------------------|------------------|
| Male (%)                               | 65               | 60               |
| Age (years)                            | 57.3 (12.2)      | 57.1 (12.9)      |
| Inheritance (%)                        | 37.8             | 14.7             |
| History of TIA or previous stroke (%)  | 18.9             | 6.4              |
| History of myocardial infarction (%)   | 4.2              | 2.1              |
| SBP, mm Hg                             | 160.6 (25.5)     | 165.8 (30.8)     |
| DBP, mm Hg                             | 90.2 (15.4)      | 92.4 (14.6)      |
| Body mass index, kg/m²                 | 27.2 (5.8)       | 28.4 (2.6)       |
| Total/HDL cholesterol, ratio           | 4.3 (1.2)        | 3.9 (1.4)        |
| Triglycerides, mg/dL                   | 136 (69)         | 131 (72)         |
| C reactive protein, mg/L               | 3.9 (1.2)        | 3.1 (0.6)        |
| Atrial fibrillation (%)                | 17.8             | 12.6             |
| Serum glucose, mg/dL                   | 157.1 (58.6)     | 156.2 (59.9)     |
| Smoking (%)                            | 32.6             | 35.7             |
| Alcohol abuse (%)                      | 4.2              | 5.3              |
| Acute infections 1-2 months before stroke (%) | 3.1       | 4.2            |
| Psychological stress (%)*              | 17.8             | 11.5             |
| Aspirin usage in current stroke %      | 32.6             | 34.8             |
| HMG-CoA reductase inhibitors in current stroke (%) | 10.5       | 8.4             |
| Fibrinogen, mg/dL                      | 422.1 (100.2)    | 410.4 (106.8)    |
| Leukocyte count, $\times 10^9$/L        | 8.8 (2.2)        | 7.9 (2.6)        |
| Temperature at admission (°C)          | 37.0 (1.4)       | 36.9 (1.7)       |
| Ischemic lesion volume ($\text{cm}^3$) | 888.1 (11.7)     | 417.9 (9.6)      |
| NIHSS score at admission **            | 20.2 (4.1)       | 8.6 (4.9)        |
| NIHSS score on 7th day **              | 18.5 (3.2)       | 7.3 (3.5)        |
| Infarct topography                     |                  |                  |
| Cortical (%)**                         | 30.5             | 17.8             |
| Subcortical (%)**                      | 16.8             | 35.7             |
| Stroke etiology                        |                  |                  |
| Large-artery atherosclerosis (%)       | 12.9             | 8.4              |
| Cardioembolism (%)**                   | 28.4             | 13.6             |
| Small vessel occlusion (lacunar) (%)** | 0                | 25.2             |
| Other determined etiology (%)          | 3.1              | 2.1              |
| Undetermined etiology (%)              | 3.1              | 4.2              |

Numbers represent mean (SD) or percentage as appropriate. NIHSS values represent median (interquartil range).

* $P < 0.05$ ** $P < 0.001$. 

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model (Figure 3). A positive correlation was established between the initial IL-6 CSF levels and the NIHSS scores on the seventh day of stroke \( (r = +0.52; P < 0.05) \). There was no significant correlation between the CSF inflammatory markers and cortical or sub-cortical ischemic lesion sites. Researched factors found to be dependent on each other once all of them were included in stepwise logistic regression analysis toward the stroke functional outcome. Only the initial IL-6 and NO2 levels retained significance for functional outcome of stroke at one month (Table 3), and cardiogenic strokes showed borderline significance \( (P = 0.057) \). Effect and interactions of Aspirin and Statins were not significant in the given model. There was a negative correlation between the initial IL-6 levels and functional outcome (BI) of stroke at 1 month \( (r = -0.45; P < 0.05) \).

**Discussion**

It is believed that the first local, glial immune response of the brain tissue to acute ischemia is the connection of CD4 T lymphocytes with astrocytes. Activated CD4 cells produce \( \gamma \)-interferon, which stimulates astrocytes to express HLA-II class antigens and to produce IL-1\( \beta \). The latter stimulates phagocyte activity in gial tissue and induces production of IL-6 and TNF-\( \alpha \), the cytokines of initial local inflammatory reactions that trigger the subsequent development of the pro- and anti-inflammatory cytokine cascade [13].

Experimental and clinical studies have demonstrated that the high CSF and blood concentrations of proinflammatory cytokines appear to reach a peak response by 24-48 hours from stroke onset [14]. The present study found elevated IL-1\( \beta \), IL-6 and TNF-\( \alpha \) level in CSF at the sixth hour from ischemic stroke onset. Previously published studies have demonstrated that elevated CSF and plasma levels of IL-1\( \beta \) correlate with larger brain infarcts and worse functional outcome [15,16]. The present study did not show any significant group differences in the IL-1\( \beta \) and TNF-\( \alpha \) CSF levels at six hours of ischemic stroke. However, the absolute number of these cytokines was elevated in the severe stroke group, suggesting that they are of the first proinflammatory response and may trigger the subsequent proinflammatory cascade.

The significant group differences in the initial IL-6 levels and the positive correlation with the size of the ischemic region support earlier experimental and clinical studies that revealed a correlation between increased initial CSF and plasma IL-6 levels, on one hand, and larger brain lesion volume and poor outcome on the other [17]. According to a number of experimental studies, most animals retain high blood IL-6 levels during the one-year period after ischemic brain injury. IL-6 has a mitogenic effect on astrocytes and induces reactive gliosis in later stages of brain ischemia [18]. Thus, according to the present research, IL-6 tends to reflect the severity of the stroke even at six hours post-injury and may play a key role in inflammatory damage caused by ischemia.

Previous experimental and clinical studies have shown that a high initial expression of TNF-\( \alpha \) is connected with larger brain infarcts, and TNF-\( \alpha \) knockout animals have larger infarcts and decreased neuronal survival [19]. Expression of TNF-\( \alpha \) during the critical period of a stroke may restrict aggressive immune responses because the TNF signaling pathway involving CD95-CD95L (ligand) interactions is considered to be the controlling mechanism of T cell expansion during the immune response [20].

### Table 2 Comparison of selected CSF acute phase factors in clinical groups and control at the sixth hour from stroke onset

|                | IL-1\( \beta \) (pg/ml) | IL-6 (pg/ml) | TNF-\( \alpha \) (pg/ml) | IL-10 (pg/ml) | NO2 (\( \mu \)mol/L) | NO (\( \mu \)mol/L) | LOO’ (a.u) |
|----------------|-------------------------|--------------|--------------------------|--------------|---------------------|---------------------|------------|
| Control        | *0.95 ± 0.02            | *1.9 ± 0.09  | *14 ± 2.3                | 3.6 ± 1.2    | *102 ± 15.9         | *2.78 ± 0.16       | 0          |
| Group I        | 34.14 ± 4.7             | *58 ± 4.6    | 44 ± 5.4                 | 5.9 ± 1.4    | *121 ± 4.56         | 33.8 ± 7.1         | *18 ± 4.1  |
| Group II       | 30.4 ± 7.3              | *21.8 ± 4.4  | 39.4 ± 9.4               | 7.1 ± 1.2    | *158 ± 3.13         | 30.18 ± 6.8        | *34 ± 7.1  |

Data are expressed as means (SD).

![Figure 1 Comparison of EPR signal intensity of CSF free NO between the study groups and control. Box plots represent mean values (SD). P < 0.05 between the Group1, Group 2 and control.](image)
As mentioned above, the CSF levels of IL-1β and TNF-α were found to be increased, but not significantly, in severe stroke patients at the time point examined in this study but might become significant at later stages of stroke. Because we could not find a significant correlation with these CSF markers and infarct size six hours after stroke onset, we hypothesize that CSF IL-6 could rapidly and specifically react in areas of ischemic damage with increased activity at later time points and retaining high meanings for a longer period. However, further studies are necessary to confirm this hypothesis.

The anti-inflammatory cytokine IL-10 reaches its peak expression between 2-7 days after stroke onset and limits the production of proinflammatory agents through negative feedback mechanisms [21]. We could not confirm previous studies where initial IL-10 levels in blood were linked with infarct topography [22], which may be due to the relatively short time for IL-10 expression (6 hours). In keeping with previous studies, this study also connected the borderline significance of cardioembolic stroke to poor outcomes in selected patients, which might be explained by the likely association of elevated cardiac inflammatory markers in this stroke subtype [23].

In conjunction with the release of pro-inflammatory agents and glutamate toxicity, local inflammation, as described above, results in free radical pathology that directly and indirectly damages neurons by destabilizing cell membranes, disintegrating DNA, and switching the pathways of delayed neuronal death [24]. In the present study, the high EPR signal intensity of free CSF NO may be caused primarily by deregulation of the neuronal form of NO-synthase. However, NO produced by inducible and endothelial forms of NO-synthase can also pass the damaged blood-brain barrier and accumulate in CSF. The toxic effects of NO in the ischemic brain depend on the cellular ratio of NO/O2 and the existence of growth factors in the surrounding tissues. The high EPR signal intensity resulting from the cell membrane lipids’ degradation product, lipoperoxide radicals, LOO-, and the increased level of IL-6 in Group 1 indicates a prevalence of oxidative stress in severe stroke patients. In conditions where NO is more prevalent than superoxide anion (O2·−), NO toxicity in neurons is decreased by restoration of peroxyinitrite (ONOO−) to NO2 [25,26]. The increased concentrations of NO2 in Group 2 patients and the relatively diminished EPR signals of LOO- indicate conditions in which NO can act as an antioxidant. The protective response of NO can also be obtained through nitrosonium (NO+), which nitrosylates the thiol groups of glutamate receptors and thus diminishes glutamate toxicity [27].

The limitation of this study is that we lack a comprehensive understanding of the complex action of NO in the blood and CSF in the acute stage of ischemic stroke. The initial endothelial NO expressed in the blood might
exhibit protective qualities, which is consistent with its ability to improve microhaemorheology [28]. Additionally, whether the CSF cytokine levels are dependent upon serum/blood concentrations and the blood-CSF barrier function and whether the CSF markers are synthesized purely intrathetically has yet to be evaluated.

Conclusions

The results of the present investigation demonstrate that nitrate (NO$_2$) content in the CSF appears to reflect the severity of the oxidative stress reaction that develops in the ischemic neurovascular unit in the first hours of stroke and can predict functional outcome. CSF IL-6 content seems to be the most reliable prognostic indicator in the acute phase of ischemic stroke, with regard to the probability of infarct size, the clinical course of disease and the functional outcome of stroke at one month.

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Authors’ contributions

MB designed the study, interpreted the data and drafted the manuscript. TS carried out the immunoassays and EPR analysis. RS participated in acquisition of data, interpretation and statistical analysis. NL conducted the additional statistics and interpreted the data. NB revised critically the intellectual content and gave the final approval for the given version of manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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