

# The role of blood nitrogen and oxygen radical species in acute ischemic stroke

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## ABSTRACT

Research purposed to investigate the pathogenic role and prognostic value of several acute phase factors reflecting the severity of oxidative stress in blood of acute ischemic stroke patients. 95 patients with acute ischemic stroke investigated. The basic neurological impairment assessed applying the international scales NIHSS and GCS. Patients divided into 3 groups: with severe stroke (GCS<9, NIHSS>15), stroke with moderate severity (GCS=14,15; NIHSS=10-15), mild stroke (GCS=15, NIHSS<10). Visualization of ischemic injury performed by conventional MRI at 48 hours. NO levels measured by Electron Paramagnet Resonance method (EPR). NO<sub>2</sub> and Superoxidismutase (SOD) levels defined by Spectrophotometer. The high EPR signals of free NO were noticed in all groups of patients compared to control, though the significant differences between groups were not found. Increased blood levels of SOD and NO<sub>2</sub> were detected in severe stroke patients compared to control, but - significantly decreased in comparison with moderate severity and mild stroke groups. Significant negative correlation was found between the initial bloods levels of NO<sub>2</sub> and the ischemic lesion size at 48 hours as well as with functional outcome at 1 month from stroke onset ( $r=-0,71$   $p<0,01$  and  $r=-0,52$   $p<0,05$  respectively). Apparently the blood levels of NO<sub>2</sub> in acute period of ischemic stroke might be considered as the important predictor of initial infarct size and the functional outcome at 1 month from stroke onset.

**KEYWORDS:** stroke, ischemia, nitric oxide, nitrates, antioxidants, necrosis, apoptosis

In the past decade called as „Brain Decade” the basic mechanisms of neuronal injury in acute brain ischemia has been emphasized. At present the basic and clinical stroke research is directed toward neurovascular unit in the context of integrative tissue response by dynamic interactions between endothelial cells, vascular smooth muscles, matrix elements, astroglia, microglia and neurons. These interactions create many sources of initiation of free toxic radicals and reactive oxygen species.

In physiological conditions endogenous antioxidative protective mechanisms stabilize the levels of free oxygen radicals and reduce the oxidative stress reaction. Failure of antioxidative protective system in acute brain ischemia assists to the accumulation of arachidonic acid, prostaglandins, superoxid anion, nitric oxide and other aggressive substrates, which lead to the destabilization of cellular membranes, damage of blood-brain barrier, disintegration of DNA and ultimately, to the neuronal death [3-5].

In acute brain ischemia cytotoxicity and inflammatory stimuli upregulate the genes of 3 isoforms of NO-synthase: Neuronal (NOS-1), inducible (NOS-2), and endothelial (NOS-3).

In normal conditions NO is the transmitter of vasodilatation signal from endothelial cells to vascular smooth muscles, retrograde mediator of glutamatergic neurons and the key element of nonspecific immune reactions. In acute brain ischemia expression of proinflammatory cytokines (Tumor necrosis factor- $\alpha$ ) stimulate the transcriptional activation NOS-2. Simultaneously, cytotoxicity and inflammatory stimuli upregulate the genes of NOS-1 and NOS-3 that leads to the long-lasting generation of NO and therefore- to the activation of enzymatic pathways of necrosis and apoptosis [6,7]. Induction of necrotic or apoptotic neuronal death depends on many factors and among them on levels of NO, superoxid anion (O<sup>2-</sup>), growth factors and antioxidative enzymes in the microenvironment of neuronal and glial cells. Reaction between NO and O<sup>2-</sup> with formation of extremely neurotoxic radical peroxynitrite (ONOO<sup>-</sup>) results in suppression of first and second mitochondrial respiration

chain enzymatic complexes and depletion of adenine nucleotides that lead to the perishing of neurons via necrosis. NO-induced enzymatic cascade leading to apoptosis comprises the activation of pro apoptotic protein P-53, deregulation of P-21 and fragmentation of DNA by ONOO<sup>-</sup> [2,8]. Apparently, inhibition of NOS and action on NO enzymatic systems might lead to the effective therapeutic strategies of stroke treatment.

The present research purposed to investigate the pathogenic role and prognostic value of several acute phase factors reflecting the severity of nitrosative/oxidative stress reaction in blood of acute ischemic stroke patients.

## MATERIALS AND METHODS

95 ischemic stroke patients aged 45 to 75 years, 54 female and 41 male have been investigated in neurological clinic of Georgian State Medical Academy during 2000-2004. Exclusion criteria comprised acute inflammatory and autoimmune disorders, cancer, severe somatic pathology, and coma. Control consisted with 25 age-matched healthy persons, who did not reveal any significant signs of cerebrovascular pathology. Etiology of stroke classified according to TOAST criteria [1]. Several non-modifiable and modifiable risk factors of stroke were studied retrospectively (age, sex, inheritance, history of TIA or previous stroke, hypertension, atherosclerosis, atrial fibrillation, diabetes mellitus, smoking, alcohol abuse, acute infections 1-2 months before stroke, psychological stress). Blood flow in extra- and intracranial arteries were evaluated by duplex- scanning (HDI Ultramark 9-linear multi-frequent transducer 7-11MHz) and by transcranial dopplerography (DWL Multi-Dop T with pulse-wave transducer 2 MHz). Visualization of ischemic region was performed at 48 hour of stroke onset by conventional MRI (magnet operating 0,2 Tesla, Vision, Siemens) providing axial T1, T2 images with slice thickness of 5 mm. The whole lesion volume was evaluated by neuroradiologist multiplying the area of focal hyperintensity by interslice gap. Therapy was directed towards correction of central and cerebral hemodynamics, hemoreological indexes and against cerebral edema.

Initial severity of disease was evaluated by international scales: Glasgow Coma Scale (GCS) and National Institute Health Stroke Scale (NIHSS). Patients were divided into three groups: 1<sup>st</sup> group- 27 patients with severe stroke (GCS>9, NIHSS>15), 2<sup>nd</sup> group -39 patients with stroke of moderate severity (GCS=14-15; NIHSS=10-15) and 3<sup>rd</sup> group- 29 patients with mild stroke (GCS=15, NIHSS <10). Functional outcome of stroke at 1 month was evaluated by Glasgow Outcome Scale (GOS) and Barthel Index (BI). High compensation was considered when GOS=1; BI=19,20, moderate disability – when - GOS=1,2; BI=12,20, severe disability - when GOS>2; BI<12.

For special laboratory investigations 7 ml. blood was taken within first 48 hours from patients and from controls. Blood samples (1 ml) were processed by Diethyldithiocarbamic acid as NO trap (Dosage 0,35 mg/200 mkl) and 1ml samples without processing were frozen and preserved in liquid nitrogen until assay. For detection of free NO, hydroxyl anion (OH<sup>-</sup>), metal ions of (Fe<sup>2+</sup>, Mn<sup>2+</sup>, and Mo<sup>5+</sup>) and iron transporting enzymes (Fe<sup>3+</sup> Transferine, Oxidized Ceruloplasmine) Electron Paramagnet Resonance (EPR) method (spectrophotometer RA-1307, with modulation frequency of 50 KHz and TM-110 cavity) was used. Signal intensity was measured in millimeters and accounted on milligram blood matter. For NO<sub>2</sub> detection 3 ml blood samples were centrifuged on 3000 g for 5 min. and processes by 20% GRIES reagent. Spectrophotometer CF-46 LOMO was applied for colorimetry. Optical density detected on 540 wavelengths. NaNO<sub>2</sub> (5 mkl) was used for drawing the calibrating curve. Additional 2 ml blood samples were used for detection of SOD levels by spectrophotometric assay [12].

The data obtained were analyzed by computer software SPSS 10.0. All data were expressed as means ±SD. Student's t-paired test was used for analysing of differences between means. Normally distributed continuous variables were compared with one-way analysis of variances (ANOVA) and Krushkall-Wallis test was used to compare abnormally distributed variables. Pearson correlation and multiple logistic regression analysis (forward stepwise conditional model) were applied, when all researched factors entered into the model. Hosmer and Lemeshow test assessed the goodness of fit of each model.

## RESULTS

All 3 groups of patients revealed the increased blood EPR signals of free spin-labeled NO in 48 hours of stroke onset compared to control (p<0,05). Significant statistical differences were not found between groups (p<0,5), though the first group showed the decreased levels of NO compared to 2<sup>nd</sup> and 3<sup>rd</sup> groups. High intensity blood EPR signals of metals- inductors of oxidative processes (Fe<sup>2+</sup> and Mn<sup>2+</sup>) were found in 1<sup>st</sup> group against 2<sup>nd</sup> and 3<sup>rd</sup> groups (p<0,05), while the same signals were not detected in control. Increased blood EPR signals of Mo<sup>5+</sup> containing complexes were detected in the same group against 2<sup>nd</sup> and 3<sup>rd</sup> groups (p<0,05). Significantly less intensive blood signals of Fe<sup>2+</sup>, Mn<sup>2+</sup>, Mo<sup>5+</sup> revealed the patients of 2<sup>nd</sup> and 3<sup>rd</sup> group. Statistical differences were not found between these two groups (p<0,5).

Increased blood EPR signals of OH<sup>-</sup> were found in 1<sup>st</sup> group compared to 2<sup>nd</sup> group (p<0,05), while the same

signals were not detected in 3<sup>rd</sup> group and control. The blood EPR signals of iron transporting proteins were increased in all 3 groups against control (p<0,01). Statistical differences were found between all groups (p<0,05). Intensive blood EPR signals of FeNO and HbNO were found in 1<sup>st</sup> group compared to 2<sup>nd</sup> group (p<0,05). The same signals were not detected in control and 3<sup>rd</sup> group of patients (Tab.1).

The 1<sup>st</sup> group of patients showed decreased blood levels of SOD against control, 2<sup>nd</sup> and 3<sup>rd</sup> groups (p<0,05). Statistical differences were found between all 3 groups and control (p<0,05).

Blood levels of NO<sub>2</sub> were increased in all groups of patients against control (p<0,05). Though, the levels of NO<sub>2</sub> found to be significantly decreased in 1<sup>st</sup> group compared to 2<sup>nd</sup> group and 3<sup>rd</sup> groups (p<0,01). Statistical differences between 2<sup>nd</sup> and 3<sup>rd</sup> groups were not revealed (p<0,5).

Significant differences were found between all 3 groups in relation with initial ischemic lesion sizes (cm<sup>3</sup>) on MRI scans (42,8±19,1 against 21,7±9,6 and 16,2±2,2 respectively p<0,05) and functional outcome of stroke at 1 month: (Barthel Index 11±4 against 15±3 and 19±1 respectively p<0,05).

Multivariate logistic regression showed the negative correlation of blood initial NO<sub>2</sub> levels with ischemic lesion size at 48 hours of stroke onset: r=-0,71 p<0,01 (Fig.1) and with functional outcome of stroke at 1 month: r=-0,52, p<0,05 (Fig.2).

Apparently, blood NO<sub>2</sub> levels in acute stage of ischemic stroke have the significant prognostic value in predicting the clinical course and functional outcome of disease at 1 month from symptoms onset. The blood levels of free NO in given clinical groups did not correlate significantly (p<0,5) with initial ischemic lesion size and with functional outcome of stroke.

## DISCUSSION

Proinflammatory stimuli, Glutamate toxicity and induction of genes of different forms of NO – synthase create the strong generators of free radicals and reactive oxygen species in penumbra region. Changes of paramagnetic centers of CSF and blood reflect the intensity of free radical oxygenation in mentioned region. Intensive blood EPR signals of FeS centers of NADH-dehydrogenase indicate to the inhibition of electron transportation in the region of NADH- ubiquinonyreductase. As a result accumulates ubisemiquinon, which is the active inductor of free radical species. Disruption of electron transport respiratory chain diminishes the mitochondrial energy producing function, activates hypoxanthin-xanthin-oxidase system, assists to the accumulation of intracellular calcium and to the transformation of xanthindehydrogenase in xanthinoxidase that is revealed by intensive signals of blood Mo<sup>5+</sup> containing complexes [13,15].

In physiological conditions NO consumption by red blood cells (RBCs) is reduced due to the effect of intravascular flow with probable formation of RBC free zone near the vessels wall, but in oxidative stress conditions the violation of CBF and accumulation of deoxy- forms of hemoglobin can lead to the increased consumption of NO by RBCs. In the present study appearance of blood HbNO complexes in severe stroke

patients indicates to the increased binding of free NO by hemoglobin. On one hand it reduces NO toxicity, on the other hand it inhibits vasodilatation and antiaggregation effects of NO and aggravates hypoxia. Formation of firm complexes of HbNO inhibits its disintegration to NO<sub>2</sub> that is revealed by low concentrations of NO<sub>2</sub> and NO in severe stroke cases [10,14].

Suppression of antioxidative protection system and damage of cellular membrane structures in severe stroke group is evident by reduced activity of free radical scavenger SOD, iron transporting proteins (Fe<sup>3+</sup> transferine, oxidized ceruoplasmine) and increased EPR signals of variable valence metals (Fe<sup>2+</sup>, Mn<sup>2+</sup>). Indicated metal ions are the strong inductors of oxidative stress reaction, when nitrosohemoglobin transforms in methemoglobin and NO<sub>2</sub> releases in blood [2].

In acute brain ischemia development of glutamate toxicity in glutamatergic neurons and stimulation of NMDA (N-methyl D-aspartat) -receptors open the voltage dependent calcium channels and induce the accumulation of excessive cytosolic calcium. The last one links with the receptor calmodulin and activates neuronal NO-synthase (NOS-1) in postsynaptic membrane [3]. If consider that proinflammatory stimuli activate simultaneously the inducible (NOS-2) and endothelial (NOS-3) NO-synthase the long-lasting NO generation should exceed the levels, which have the protective effect in initial stages of acute brain ischemia [9,13].

As mentioned above the toxic action of NO highly depends on ratio of NO and O<sub>2</sub><sup>-</sup> in microenvironment of

cells and on levels of growth factors, glucose, antioxidant enzymes as well.

In cases when levels of NO significantly prevail the levels of O<sub>2</sub><sup>-</sup>, reaction between them is directed toward restoration of NO<sub>2</sub> from ONOO<sup>-</sup> and toxic action of NO transforms in neuroprotection [11]. In such conditions NO acts as antioxidant. Such interpretation corresponds to the results of our research as the expression of high quantities of aggressive oxygen radicals in severe stroke patients could invert the ratio NO/O<sub>2</sub><sup>-</sup> toward O<sub>2</sub><sup>-</sup> that leads to the production of ONOO<sup>-</sup> and to the product of its disruption OH<sup>-</sup>. Probably the high signals of OH<sup>-</sup> in patients of 1<sup>st</sup> group reflect the mentioned processes. Indicated metabolites suppress the mitochondrial respiration through inhibition of cytosolic Aconitase resulting in depletion of adenindinucleotides and disruption of DNA. These changes could be related to the larger sizes of ischemic injury on MRI scans and thus to the severe disability at 1 month from symptoms onset.

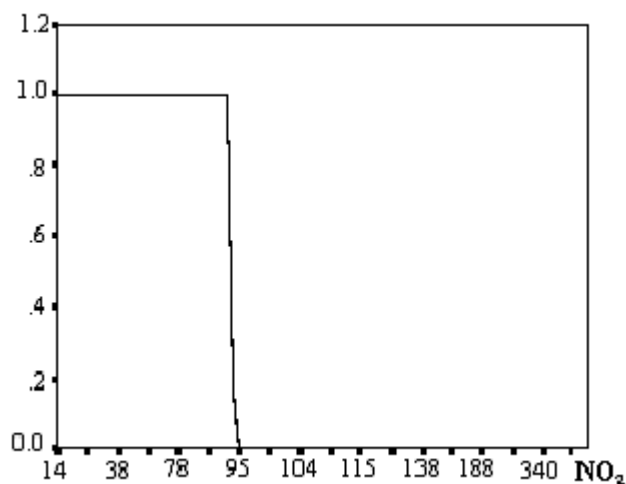
In relatively mild stroke cases defence mechanisms (antioxidant enzymes) can suppress the high expression of reactive oxygen spices and shift the ratio NO/O<sub>2</sub><sup>-</sup> toward NO prevalence and thus, toward protective effect of nitric oxide. The ultimate effect of NO in penumbra region depends on many factors. For example, nitrosonium (NO<sup>+</sup>) nitrosilates thiol groups of NMDA receptors and accordingly diminishes the Glutamate toxicity. In cases of prevalence of reactive oxygen spices nitrosonium transforms in nitrogenmonooxidaze that activates apoptosis [2,8].

Data	Control	1 <sup>st</sup> group	2 <sup>nd</sup> group	3 <sup>rd</sup> group
NO mm/mg	11±2,7	*17±2,3	19± 3,1	20± 2,2
NO <sub>2</sub> µmol/l	102±15,9	*121±4,56	**158±3,13	161±2,3
SOD µmol/l	0,02±0,006	*0,038±0,04	*0,051±0,012	*0,058±0,003
Mn <sup>2+</sup> mm/mg	(-)	4,8±1,4	*2,2±0,8	2,7±0,5
Mo <sup>5+</sup> mm/mg	(-)	5,5±1,3	**1,7±0,4	1,5±0,7
Fe <sup>2+</sup> mm/mg	(-)	60±19	**42±4,8	37±4,6
OH <sup>-</sup> mm/mg	(-)	3,9±0,7	*2,1±0,4	*0,9±0,04
Fe <sup>3+</sup> transferine mm/mg	20±2,6	*26±3,8	**52±4,7	*56±3,4
Oxidized ceruoplasmine mm/mg	31±4,1	*37±4,4	**46±5,2	*53±3,8

Note: Numbers represent means (SD)

\* - p < 0,05; \*\* - p < 0,01; (-) – EPR signal is not found.

**Tab.1** Comparison of several acute phase factors of CSF in patients and control in acute period of ischemic stroke.

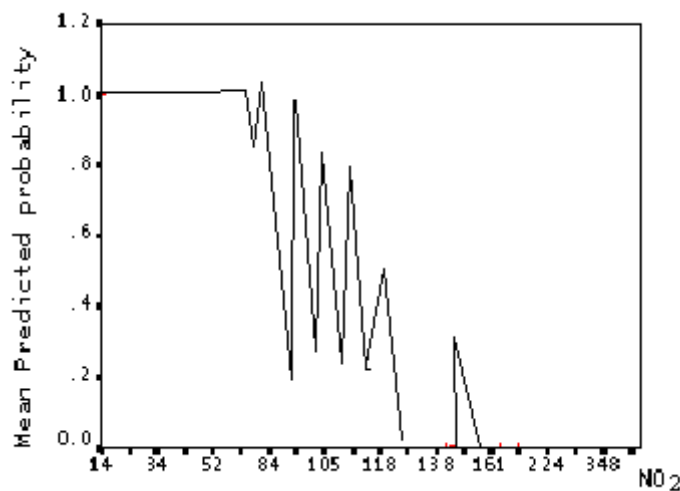


Multivariate logistic regression (entered stepwise model) when all researched risk factors and acute phase factors entered into the regression analysis.

Chi-squar-94.117; Sig. - 0.000

Correlation coefficient by Pearson  $r = -0,71$ ;  $p < 0,01$ .

**Fig.1** The negative correlation of blood initial  $NO_2$  levels with ischemic lesion size at 48 hours in acute period of ischemic stroke.



Multivariate logistic regression (entered stepwise model). All researched risk factors and acute phase factors entered into the regression analysis.

Chi-squar 101.223; Sig. - 0.000

Correlation coefficient by Pearson  $r = -0,52$ ;  $p < 0,05$ .

**Fig.2** The negative correlation of blood initial  $NO_2$  levels with stroke outcome at 1 month.

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## **Роль свободных реактивных радикалов азота и кислорода в острой стадии ишемического инсульта**

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### **Р Е З Ю М Е**

Целью исследования являлось изучение патогенетической роли и прогностического значения некоторых факторов крови в острой фазе ишемического инсульта, отражающих силу азотзависимого окислительного стресса. Обследовано 95 больных с острым ишемическим инсультом. Для определения тяжести заболевания использовались международные шкалы NIHSS и GCS. Больные были подразделены на 3 группы: 1) с тяжелым инсультом (балл по GCS > 9, по NIHSS > 15); 2) со средней тяжестью (балл по GCS = 14, 15; по NIHSS = 10-15) и 3) со сравнительно легким течением (балл по GCS = 15; по NIHSS < 15). Визуализацию мозгового поражения проводили на 2-й день после развития инсульта методом МРТ. Содержание NO в крови определяли методом электронно-парамагнитного резонанса (ЭПР). Содержание NO<sub>2</sub> и супероксиддисмутазы (СОД) в крови - спектрофотометрически. В обеих группах в течение 48 ч после развития заболевания в крови появлялись ЭПР-сигналы свободного NO, значительно превышавшие по интенсивности контрольные данные, тогда как достоверных различий между отдельными группами больных не обнаружено. Содержание СОД и NO<sub>2</sub> в крови у больных с тяжелым инсультом было повышено в сравнении с контрольной группой, но было значительно ниже чем во II и III группах. Значительная отрицательная корреляция выявлена между содержанием NO<sub>2</sub> в крови и исходным размером ишемического очага, а также функциональным исходом заболевания через месяц. Следовательно, содержание NO<sub>2</sub> в крови в остром периоде ишемического инсульта можно рассматривать как важный прогностический фактор, определяющий начальный размер инфаркта и функциональный исход заболевания уже через один месяц после развития инсульта.

**Ключевые слова:** инсульт, ишемия, оксид азота, нитраты, антиоксиданты, некроз, апоптоз