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Plasma Antioxidant Activity as a Marker for a Favourable Outcome in Acute Ischemic Stroke

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1. Introduction

Ischemic stroke (IS) is a leading cause of mortality and disability in industrial countries, only overwhelmed by cardiac disease and cancer (Donnan et al., 2008; Doyle et al., 2008; Flynn et al., 2008). In Western countries stroke causes 10-12% of all deaths (Bonita, 1992). Stroke is also the leading cause of adult disability, because 76% of people survive their stroke. Of these survivors, 50% have hemiparesis, 26% are dependent in activities of daily living, and 26% are forced into a nursing home. Thus stroke is a lethal disease, but it disables more than it kills (Carmichael, 2005). This fact has led a recent effort to develop strategies for neural repair after stroke and to search for neuroprotective therapies to reduce cell death and infarct volume after stroke.

Many studies have been directed to understanding the molecular events involved in cerebral ischemia and developing agents for neuroprotective therapies. These studies result in the concept that early injury due to the loss of energy substrates is followed by secondary inflammation, which produces tissue damage (del Zoppo et al., 2000). As the inflammatory process develops during a span of hours to days, there is a potential window for therapeutic treatment. There is very little treatment for stroke. At present it was shown, that treatment with tissue plasminogen activator (rt-PA) can improve outcome in patients with acute ischemic stroke (Clark et al., 1995). However due to a narrow time window and fear of hemorrhagic complications, this treatment is effective in the first hours of stroke and is only appropriate for a very limited number of patients (Clark et al., 1995; Grophen et al., 2006).

Focal ischemia can be caused by systemic hypoperfusion or by occlusion of an artery in the brain by thrombosis or embolism from the heart. Other causes are abrupt occlusion of small penetrating arteries (at lacunar stroke), arterial dissection, and various genetical and haematological disorders (Hossman, 1994). Sudden decrease or loss of blood circulation to

an area of the brain results in insufficient oxygen and glucose delivery to support cellular homeostasis. This produces complex series of events that lead to cell death: excitotoxicity, acidotoxicity and ionic imbalance, peri-infarct depolarization, oxidative and nutritive stress, inflammation (Doyle et al., 2008; Gonzalez et al., 2006; Sims & Muyderman, 2010). Each of these processes usually goes on for minutes, hours or days. Within the core of the ischemic area blood flow is more severely restricted, less than 20% of normal, necrotic death occurs within minutes. In the periphery of the ischemic area, where perfusion takes place, a lesser ischemia develops. The blood flow is reduced 20-40% of normal flow (Back et al., 2004; Belayeev et al., 1997; Hossman, 1994). In this area, which is potentially salvageable, called penumbra, the degree of ischemia and timing of reperfusion determine the outcome for individual cells. In ischemic penumbra cell death occurs less rapidly via apoptosis and inflammation (Gonzalez et al., 2006).

Restoration of the blood circulation has a decisive importance for the reverse of an arterial occlusion. However, the restoration results in secondary damage, called reperfusion injury, which is a recognized complication of restoring blood flow to ischemic tissue (Hallenbeck & Dutka, 1990). One of the mechanisms of the secondary damage consists in the increased generation of reactive oxygen species (ROS) initiated during the reoxygenation from parenchymal and endothelial cells and from infiltrating leucocytes. There is considerable evidence that reactive oxygen and nitrogen species are important mediators of tissue injury in acute ischemic stroke (Cuzzocrea et al., 2001; Warner et al., 2004).

Oxidative stress is defined as an imbalance between the production and removal of reactive oxygen species (Halliwell & Gutteridge, 1999). Oxygen is inevitable component of aerobic life. Incomplete reduction of oxygen to water during normal aerobic metabolism generates reactive oxygen species, which have one or more unpaired electrons. The main ROS such as superoxide anion, singlet oxygen, hydrogen peroxide and nitric oxide, which reacts with superoxide anion producing different types of reactive nitrogen species (RNS), are very transient species and play an important role in many physiological and pathological processes. Reactive oxygen and nitrogen species differ to each other, e.g. superoxide is a single electron oxidant of only moderate strength and crosses cell membrane via the anion channel (Kontos, 2001), and hydrogen peroxide is lipid soluble and easily crosses cell membrane via diffusion, as it is a neutral particle. Hydroxyl radical has only one unpaired electron and represents the most reactive oxygen radical, it cannot diffuse and causes its damaging effect in the vicinity of the biomacromolecules. Each of the reactive oxygen and nitrogen species has specific reactivity and properties and accordingly can activate different specific signalling pathways and biological responses.

One of the most popular theories to explain oxygen toxicity has been the superoxide theory, which proposes that oxygen toxicity is due to overproduction of superoxide anions (Halliwell & Gutteridge, 1999). Mitochondria are the organelles in eukaryotic cells responsible for aerobic respiration, and they are the most common source of ROS. In normal cells, 1-2% of electrons carried by the mitochondrial transport chain leak from this pathway and pass directly to oxygen generating superoxide anion, which can be a source of the ROS by developing different type of chain reactions (Curtin et al., 2002). Abnormal electron

leakage is connected with perturbation of mitochondrial metabolism and inflammatory responses to injury (Halliwell & Gutteridge, 1999). Although mitochondria is a main source of superoxide, superoxide anions can be also produced by auto-oxidation of tissue components such as small molecules, haemoglobin and myoglobin or generated by intracellular oxidative enzymes such as oxidases, peroxidases, oxygenases, metal catalyzed reactions, inflammatory cell activation (neutrophils and macrophages) (Dalton et al., 1999). Superoxide rapidly dismutates to hydrogen peroxide or reduces Fe(III) to Fe(II) releasing the iron from storage sites. Although dismutation of superoxide is the main source of hydrogen peroxide in tissue, the later can be produced directly by several oxidases such as glycolate oxidase, urate oxidase, flavoprotein dehydrogenase, localized in peroxisomes (Halliwell & Gutteridge, 1999). Hydroxyl radical is generated from hydrogen peroxide in the presence of transition metals (e.g. Fe(II) or Cu(I) ions) via Fenton and Haber-Weiss reactions (Halliwell & Gutteridge, 1999). In this case superoxide is essential because it serves to reduce transition metal, which is then oxidized in the reaction that produces hydroxyl radical. As a result, the cycle can be repeated.

Thus, superoxide and hydrogen peroxide are unavoidable by-products of aerobic metabolism. The most biomolecules resist univalent redox reactions and are nonreactive with superoxide. One way in which superoxide is believed to cause toxicity is through its participation in hydroxyl radical production representing an extremely powerful oxidant. Of particular importance at ischemic stroke is the interaction of superoxide with nitric oxide, a water and lipid soluble free radical, which is produced by nitric oxide synthases (NOS). Nitric oxide combines with superoxide anion generating very strong oxidant peroxynitrite anions (Beckman & Koppenol, 1996; Dugan & Choi, 1994).

Excessive ROS are harmful because they react with and modify all classes of cellular macromolecules causing wide-ranging cellular effects such as lipid peroxidation, protein denaturation, inactivation of enzymes, nucleic acid and DNA damage, release of calcium ions from intracellular stores, damage of cytoskeleton, chemotaxis. Oxygen radicals have significant vascular effects. Superoxide, hydrogen peroxide and peroxynitrite are strong cerebral vasodilators. Cerebral vascular effects of these radicals include vasodilation, increased platelet aggregability, increased endothelial permeability, and focal destructive lesions of endothelial cell membranes. Vascular effects are very important for cerebral blood flow. The registration of these effects offers the convenient monitoring of ROS presence and action (Kontos, 2001).

The effect of ROS is balanced by antioxidant systems, which provide either direct or indirect protection of cells against adverse effects on different biological sites. The cellular protective antiradical mechanisms consist of multiple interacting enzymatic such as superoxide dismutases (SODs), catalase, and glutathione peroxidases (GPx) and non-enzymatic antioxidants such as glutathione (GSH), vitamin A, vitamin C, vitamin E, uric acid etc.

The brain contains 2% of total body, but utilizes 20% of the oxygen consumed by the body, indicating that the brain can be the source of many more free radicals than the other tissues (Dringen, 2000; Margail et al., 2005). However, the antioxidant level of the brain is low (Chan, 2001; Kelly et al., 2008; Polidori et al., 1998). As a result the brain can be very vulnerable to oxidative stress especially at ischemic stroke.

The techniques, which are usually used for detection of free radical generation, as spin trapping, electron paramagnetic resonance are not applicable for human brain. Because of the transient nature of oxygen radicals and technical difficulties in measuring their brain levels, experimental strategies have been focused on the use of pharmacological agents and antioxidants, seeking a correlation between an exogenous supply of specific free radical scavengers (e.g. SOD, catalase) and the subsequent protection of cerebral tissue from ischemic injury.

Human studies evaluate the presence of either oxidized molecules or antioxidants in blood, urine or cerebrospinal fluids (CSF). Antioxidant activity is known to reflect the altered redox balance of affected fluids, tissues or organs in several pathological processes including brain ischemia (Cherubini et al., 2005). A biomarker of oxidative stress is classically defined as a biological molecule whose chemical structure has been modified by ROS. Additionally, any biological process influenced by ROS could be used as an oxidative stress biomarker. Therefore, antioxidant concentration or degree of antioxidant activity can be useful to estimate the extent of oxidative stress.

The prediction of outcome in ischemic stroke is important for clinicians, patients, and researchers. The pathogenesis of ischemic stroke (IS) is highly complex. Oxidative stress is proposed as a fundamental mechanism of brain damage at ischemic stroke. Measurements of antioxidants in plasma can allow revealing a new pathological feature of formation the ischemic stroke seat and can be considered as noninvasive tools in the monitoring of the disease, as cellular changes may be reflected in body fluids. We studied a wide spectrum of components of antioxidant system in plasma of healthy volunteers (controls) and patients within the first 72 h of acute ischemic stroke onset, including enzymatic and non-enzymatic antioxidants, and discriminate of their activity and quantity for the establishment of possible correlation. The obtained correlations can be considered as biomarkers during the acute phase of ischemic stroke (IS) and corroborate the existing clinical prognostic models to predict the outcome in individual patients with stroke, which are not enough accurate (Counsell et al., 2004).

2. Experimental

2.1. Clinical study

Case subjects are selected from the all acute stroke patients admitted to the Sarajishvili Institute of Neurology and Neurosurgery (SINN). 42 eligible subjects (22 males and 20 females; 69 ± 15 years of age) were selected from 70 patients with suspected acute stroke admitted to either Clinical or Critical Care departments of the SINN. Reasons for exclusion were: final diagnoses other than stroke (7 cases), admission after 72 hours of stroke onset (4 cases), hemorrhagic stroke (10 patients) and patients' refusal to participate in the study (7 cases). All study subjects underwent the following investigations: detailed neurological examination (special stroke scales for evaluating the stroke severity and functional state were used according to the study protocol), CT, Extracranial Dopplerography, EKG and

detailed laboratory work-up including routine blood and urine analysis, coagulation tests, venous hematocrit, routine blood biochemistry (glucose and total cholesterol). Patients were clinically evaluated using GOS (Glasgow Outcome Scale), GCS (Glasgow Coma Scale), Barthlet-Rankin (Scale), Allen (Scale). Additionally, patients were stratified according to the NIHSS (National Institute of Health Stroke Scale) score and the Oxfordshire Community Stroke Project (OCSP) classification. The Oxfordshire Community Stroke Project classification is widely used for stroke pathophysiology classification. This classification divides cerebral infarction into four categories: total anterior circulation infarction (TACI), partial anterior circulation infarction (PACI), lacunar infarction (LACI), and posterior circulation infarction (POCI). From all patients 14 patients were in the category TACI, 14 patients in the category PACI, and 14 patients in the category LACI. It was not sufficient cases in the category POCI for the statistical analysis.

Healthy individuals without stroke are selected randomly from outpatients paying visits to the Polyclinic of the Sarajishvili Institute of Neurology and Neurosurgery. This study includes plasma samples from 15 healthy individuals (11 males and 4 females; 43 ± 30 years of age). Controls were persons without acute stroke/history of stroke and without current acute or chronic inflammatory illness. Blood samples were drawn in sterile tubes and then centrifuged for the further analyses of the plasma. Besides, the plasma of 17 healthy donors from the Blood Bank of Jo Ann's Medical Center was used as the control subjects. The study protocol was approved by the local ethics committee, and written informed consent was obtained from each participant or their relatives before inclusion in the study.

2.2. ELISA method for the quantification of Cu, Zn-SOD in plasma

Cu,Zn-SOD assay ELISA kit (IBL International, Germany) based on the monoclonal antibodies to human Cu,Zn-SOD has been used to quantify Cu,Zn-SOD in plasma. We have followed the manufacturer's instructions.

2.3. Quantification of SOD activity in plasma by the spectrophotometric method

Superoxide Dismutase Assay (IBL International, Germany) based on colorimetric superoxide radicals detection has been used to quantify total SOD activity in plasma. Superoxide radicals are generated by the xantine oxidase and hypoxanthine pair. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radicals. The SOD assay measures total SODs (Cu,Zn-SOD, Mn-SOD, and extracellular SOD) activity in plasma.

2.4. Quantification of catalase activity in plasma

Hydrogen peroxide created after superoxide radical disproportionation by SOD can be neutralized in blood by catalase, which along with peroxidases may regulate hydrogen peroxide either generated in blood or coming from other tissues. Catalase spectrometric measurement at 240 nm based on the ability of catalase to oxidize hydrogen peroxide

proposed by the method of (Beers & Sizer, 1952). The conditions for catalase measurement in plasma were set. Briefly the method is as follows: 2.25 ml of potassium phosphate buffer (50 mM, pH 7.0 or 65 mM, pH 7.8) was added to 0.05 ml of plasma (diluted (1:10) by potassium phosphate buffer (50 mM, pH 7.0)) and incubated at 25°C for 30 min. 650 µl of hydrogen peroxide (to get 7.5 mM final) were added to initiate the reaction. The change in absorbency was measured at 240 nm for 3 min. The catalase activity was expressed in IU. One international unit (IU) of catalase is the enzyme activity, which decomposes one µmol of hydrogen peroxide per minute at 25°C.

Western blots were prepared from total blood plasma protein samples diluted 1:25 times in Laemmli loading buffer and separated on 12% SDS-polyacrylamide gels (normalized to 75 µg per lane) and blotted on Hybond-C Extra membrane (Amersham, USA). Membranes were blocked in 3% (w/v) Ovalbumin (Sigma) in 1xPBS for 1 h, washed in 1xPBS and 0.02% Tween 20 and incubated with diluted antibodies (IgG) against human erythrocytes catalase (500 ng/ml) (Oxis, USA) overnight at 4°C and then secondary goat anti-rabbit IgG (1:2500) (Sigma, USA) for 2 h. After further washing with 1xPBS and 0.02% Tween 20, chromogenic detection was performed using the chromogen 4-chloro-1-naphthol.

2.5. Total glutathione quantity in plasma

The BIOXYTECH GSH/GSSG kit (OXIS, USA) was used to estimate the content of GSH and GSSG in plasma of healthy volunteers and patients with ischemic stroke. The kit procedure is based on the use of Elman's reagent – DTNB (5,5'-Dithiobis-(2-nitrobenzoic acid)). The color developed was read at 412 nm.

2.6. Total thiols concentration in plasma

A 96-well plate method of thiols' quantification using DTNB optimized for plasma was used to estimate the content of total thiols in plasma of healthy volunteers and patients with ischemic stroke (Hawkins et al., 2009). The procedure is based on the use of Elman's reagent (DTNB), which interacts with SH groups producing chromogenic substrate. The range of GSH concentration 0 – 0.5 mM serves as the standards. The color developed was read at 412 nm.

2.7. Methods of analysis

All values are expressed as means and medians by using Origin for Windows, version OriginPro8, and were analyzed using the Mann-Whitney *U* test (two-tailed). Correlation between variables implies a statistical test carrying out under the null hypothesis. A null hypothesis is a precise statement relating to the research question to be tested, expressed in terms which assume no relationship (association) or difference between variables. Correlations between variables were determined by Spearman's rank test and Pearson's rank test. Spearman's rank correlation coefficient (r_s) provides a measure of how closely two sets of rankings agree with each other. Pearson's correlation coefficient (r_p) is a measure of

the strength of the association between the two variables. A P value <0.05 was taken to be of statistical significance; P value <0.01 was taken to be of significant difference; P value ≥ 0.05 was not taken to be of statistical significance.

3. Results and discussion

3.1. Enzymatic antioxidants at ischemic stroke in plasma

3.1.1. SOD activity

The wide distribution of superoxide dismutase among aerobic organisms points to the special role of this enzyme (Fridovich, 1995). SOD plays a central role in protecting cells against harmful effect of superoxide radicals. Their sole function is to remove the superoxide and thus protect cells against oxygen toxicity. SOD catalyzes the superoxide dismutation to hydrogen peroxide and oxygen by alternate reduction and reoxidation of the transition metal at the active site (Hsieh et al., 1998; Mates, 2000). Based on the metal ion requirement and the atomic distribution two main types of endogenous SOD exist. Cu,Zn-SOD is a homodimeric enzyme (32 kDa), containing one copper Cu(II) per subunit joined to buried Zn(II) by a bridging histidyl imidasolate group (Fridovich, 1989). It is found in the cytosolic and lysosomal fractions, but it exists also in the mitochondrial intermembrane space (Mates et al., 1999; Okato-Matsumoto & Fridovich, 2001). Mn-SOD homotetramer (96 kDa), containing one manganese per subunit, is found in the mitochondrial matrix (Mates et al., 1999). Extracellular SOD (EC-SOD) is the secretory, tetrameric (130 kDa) Cu,Zn-containing glycoprotein explaining SOD activity in extracellular fluids (Enghild et al., 1999). EC-SOD also expressed in brain tissue, but its concentration is substantially lower than Cu,Zn-SOD and Mn-SOD (Marklund, 1984). EC-SOD is secreted into extracellular fluids, such as plasma and lymph, by cells such as fibroblasts, endothelial cells and smooth muscles, and binds with sulphated polysaccharides such as heparin and heparin sulphate (Marklund, 1984) as well as other matrix components (Fattman et al., 2003). The arterial wall contains exceptionally large amount of EC-SOD as a result of EC-SOD binding to the surface of endothelial cells and the extracellular matrix. The EC-SOD content is about 100 times higher compared with other tissues such as muscle or fatty tissues, suggesting a special function of EC-SOD within the vascular walls, which can be seriously damaged at the ischemic stroke.

Cu,Zn-SOD has been extensively used to reduce brain injury caused by ischemia and reperfusion by its exogenous supply and the subsequent protection of cerebral tissue from ischemic injury. However, the use of free unmodified SOD was not successful. The extremely short half-life of exogenous Cu,Zn-SOD (6 min) in circulating blood and its failure to pass the blood-brain barrier (BBB) makes it difficult to use enzyme therapy in cerebral ischemia. The modified enzyme with an increased half-life, such as polyethylene glycol-conjugated SOD has been successfully used to reduce infarct volume in rats that have been subjected to focal cerebral ischemia (He et al., 1993). Liposome-entrapped SOD has an increased half-life (4.5 hours), BBB permeability, and cellular uptake, and it has also proved

to be an effective treatment in reducing severity of traumatic and focal ischemic brain injuries (Chan et al., 1987; Imaizumi et al., 1990). But in some instances, modified SOD has been used with conflicting results caused by hemodynamic, pharmacokinetic, and possible toxic effects of drugs, as well as their blood-brain barrier permeability properties.

The alternate and more direct method for the study of oxidative stress in ischemia and reperfusion injury is to use transgenic/knockout technology to alter the levels of prooxidants, antioxidants, and oxidant related enzymes or proteins and to study the role of a specific oxidant or antioxidant in ischemic brain injury. Knockout and overexpressing mutants for both Cu,Zn-SOD and Mn-SOD isozymes have been created. Experiments with transgenic mice overexpressing Cu,Zn-SOD reveal reduction of ischemic damage resulting from ischemia/reperfusion at middle cerebral artery occlusion (Yang et al., 1994). Neither Cu,Zn-SOD overexpression, nor Cu,Zn-SOD targeted deletion alter the outcome from permanent focal ischemia (Chan et al., 1993), indicating the requirement of reperfusion for this enzyme to play role. However, Mn-SOD targeted deletion worsens outcome from both temporary and permanent middle cerebral artery occlusion (Kim et al., 2002; Murakami et al., 1998). EC-SOD overexpressing mice have increased tolerance to both local and global cerebral ischemia (Sheng et al., 1999a; Sheng et al., 2000), while EC-SOD knockout exhibits enhanced damage (Sheng et al., 1999b).

As to endogenous SOD level or activity in cerebrovascular ischemia the data are contradictory: SOD activity in brain tissue after ischemia/reperfusion has been found both to be decreased (Tokuda et al., 1993) and increased (Sutherland et al., 1991).

In the human study the data are also contradictory: SOD concentration after stroke was unchanged in serum (Adachi et al., 1994). Some studies observed an augmentation of SOD concentration in plasma (Gruener et al., 1994) or CSF (Strand, & Marklund, 1992). Strand and Marklund (1992) reported good correlation between the increased Cu,Zn-SOD activity in CSF with the size of the infarct and functional impairment. Besides, the SOD activity has been monitored in human erythrocytes at IS, and the decrease (Demirkaya et al., 2001) or no modification (Alexandrova et al., 2004) has been observed.

We evaluated the changes of plasma SODs activity after stroke to determine their utility in predicting outcome in terms of survival and functional status. In our study we used colorimetric method to detect the total SOD (including Cu,Zn-SOD, Mn-SOD and EC-SOD) activity in plasma of healthy controls and patients at the early stage of ischemic stroke. It was established that total SOD activities were significantly lower in patients compared to healthy controls ($P=0.0018$) (Fig. 1).

The decrease of SOD activity in stroke patients has been detected in plasma (Cherubini et al., 2000), in serum (Spranger et al., 1997) and in red blood cells (Demirkaya et al., 2001). In these studies SOD activity was inversely correlated with the size of infarction and the severity of neurological deficit, the lower SOD activity was associated with the worst outcome.

The reason of the low SOD activity in plasma can be related to the exhaustion of enzymes owing to ROS scavenging or the inhibition of enzymes caused by ROS (Escobar et al., 1996).

To quantify Cu,Zn-SOD level in plasma we used assay based on the monoclonal antibodies to human Cu,Zn-SOD. The reliable increase of Cu,Zn-SOD content in blood plasma of patients within the first 72 h of ischemic stroke onset in comparison with control samples has been detected. The difference between the ischemic stroke and healthy groups was statistically significant ($P=0.0079$) (Fig. 2).

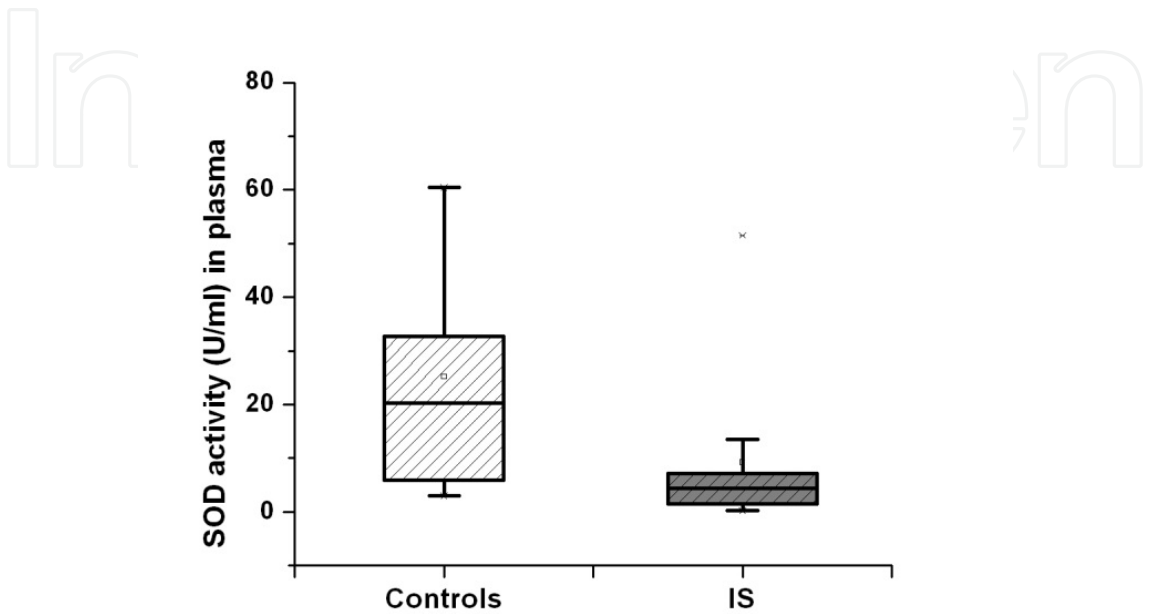


Figure 1. SOD activity in plasma of healthy volunteers (Controls) and patients with ischemic stroke onset (IS), $P=0.0018$.

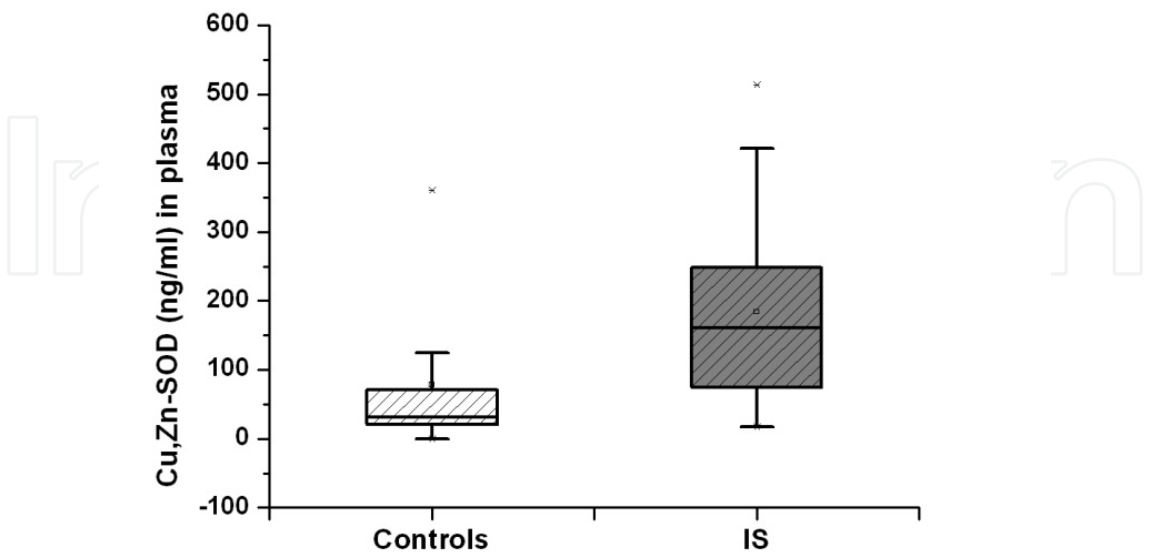


Figure 2. Cu,Zn-SOD activity in plasma of healthy volunteers (Controls) and patients with ischemic stroke onset (IS), $P=0.0079$.

The increased Cu,Zn-SOD activity was detected in CSF (Strand, & Marklund, 1992) in stroke patients. Following arterial occlusion, infarcts initially develop in the core tissue but continuous to penumbral regions (Back et al., 1995; Heiss et al., 1994). The differences in the severity of the ischemia in the core and penumbra result in the switching on the different mechanisms of the cell death – necrosis and apoptosis. Necrosis is the predominant mechanism follows acute occlusion, whereas milder injury, particularly within the ischemic penumbra often results in apoptosis. Severe oxidative stress causes cell death through necrosis while moderate oxidation can trigger apoptosis (Evans & Cooke, 2004; Liu et al., 1996). The activity of Mn-SOD and Cu,Zn-SOD in apoptotic cells does not significantly differ from normal ones, if apoptosis is caused by oxidative conditions (Asatiani et al., 2004). Enzymes of low molecular weight may passively leak from the intracellular space within hours from ischemically disturbed membranes, which may explain why the increase of SOD activity/concentration was frequently found within 8-36 hours after symptom onset in extracellular fluids (Marklund, 1984; Strand, & Marklund, 1992). Besides, the damages in endothelial cells may also accompany the IS, and the damaged endothelial cells cannot be excluded as the source of intracellular SOD in plasma of IS patients.

3.1.2. Catalase activity

Pathological conditions, which increase the rate of hydrogen peroxide production, will lead to the accumulation of hydrogen peroxide in cytosol and mitochondria. Safe disposal of hydrogen peroxide is carried out by catalase and glutathione peroxidase. The former is located only in peroxisoms, the latter functions in the cytosol and mitochondria. Elevation of hydrogen peroxide results in harmful consequences, such as depletion of ATP, GSH, NADPH pools and induction of mitochondrial permeability, disrupting mitochondrial membrane potential that trigger the apoptotic pathway (Kroemer & Reed, 2000). Catalase is a tetrameric protein (240 kDa), which contains a ferric (Fe(III)) haem group per subunit bound to its active site (Mates, 2000). Both catalase and GPx are present in the brain, although GPx activity is greater than that of catalase.

In developing brain catalase and GPx are poorly expressed. When the Cu,Zn-SOD overexpression was studied in the neonatal mice, it was shown that excess of hydrogen peroxide, produced by Cu,Zn-SOD cannot be scavenged neither by catalase, nor by GPx, and the outcome from ischemia/reperfusion was worsened (Fullerton et al., 1998). Cu,Zn-SOD overexpression in adult mice improves the outcome (Yang et al., 1994). Thus, the improvement of the outcome points to sufficient concentration of catalase and GPx in the adult mice brain to defence brain against hydrogen peroxide produced owing to superoxide dismutation. Although it is difficult to say which one (catalase or GPx) plays a central role for the brain defence? In animal models estimation of endogenous antioxidant system in brain tissue showed a significant decrease of catalase activity at the reperfusion stage, as well as a 48 h delayed decline in GPx activity.

In human total blood catalase and GPx activities did not reflect the severity of neurological deficit (Alexandrova et al., 2004). However, the ischemic patients' blood showed significantly higher catalase and GPx activity in comparison to the control group.

In our study the increase of catalase activity was observed in the patients' plasma that points to the oxidative stress developed under ischemic conditions, and the difference between the ischemic stroke and healthy groups was statistically significant ($P=0.0089$). It was also estimated GPx activity in patients' plasma. The increased activity was detected (data not shown). The catalase activity in plasma was expressed in IU/ml. The data are presented in Fig. 3.

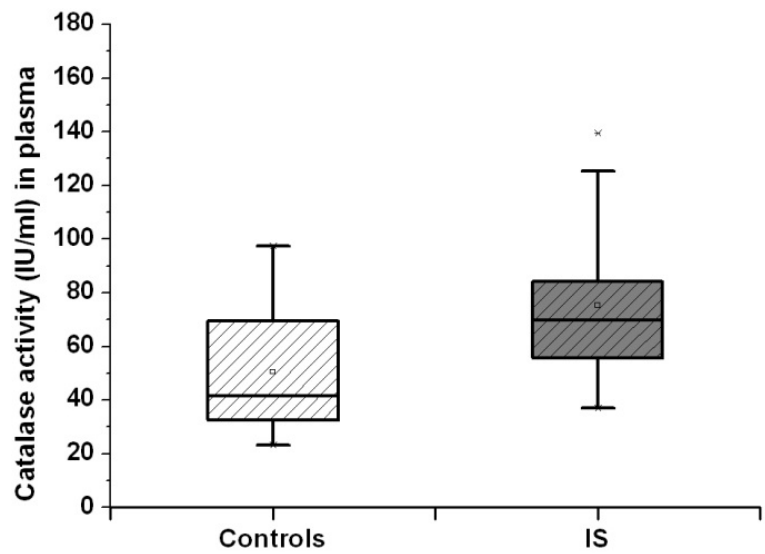


Figure 3. Catalase activity in plasma of healthy volunteers (Controls) and patients with ischemic stroke onset (IS), $P=0.0089$.

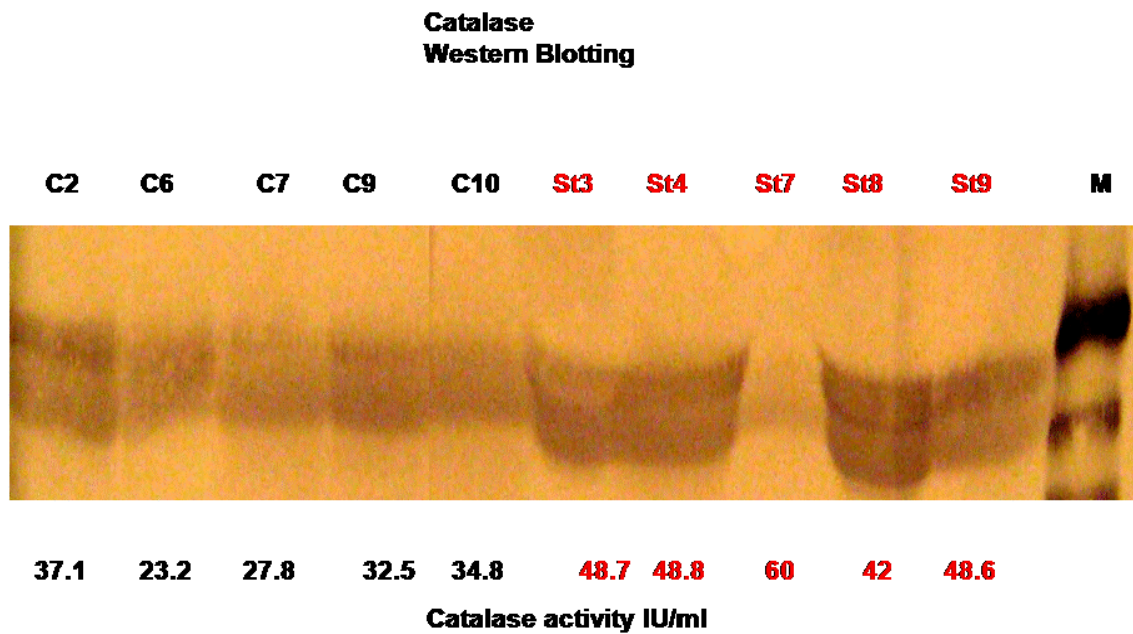


Figure 4. Western blot, stained with anti-catalase. C-control; St – IS patients; M – marker, catalase from bovine liver (Oxis, USA).

We have used Western blotting to estimate, if in case of ischemic strokes, characterizing by the increased catalase activity, the observed activation of catalase is connected with quantitative increase of catalase in plasma, or the increased catalase activity is the result of catalase activation under the oxidative stress conditions at IS. The results are presented in Fig. 4.

The results demonstrate activation of catalase in case of IS 7 as the response to oxidative stress (low quantity but high activity) and activation of catalase in case of IS 3,4,8,9 as the result of the quantitative increase of catalase (high quantity and high activity) in blood plasma of IS patients in comparison with controls. Thus the both possibilities of the increase of the catalase activity take place at the ischemic stroke conditions: by the increase of quantity and by activation under oxidative stress conditions.

Three investigated enzymatic antioxidants (Cu,Zn-SOD, catalase and GPx) are activated in the first 72 h in plasma of patients diagnosed with acute ischemic stroke in our study.

3.1.3. Plasma enzymatic antioxidant profile as the predictor of functional outcome

A disturbance in the oxidant/antioxidant balance in favor of antioxidants may be implicated as a prognostic factor in human stroke. For this reason we have stratified the patients in accord with Glasgow Outcome Scale of ischemic stroke development. According to GOS ischemic patients are subdivided into five groups (GOS is a 5-level score): (1)- Dead; (2)- Vegetative state (meaning the patient is unresponsive, but alive); (3)- Severely disabled (conscious, but the patients requires others for daily support due to disability); (4)- Moderately disabled (the patient is independent but disabled); (5)- Good recovery (the patient has resumed most normal activities but may have minor residual problems. Our patients were subdivided into three groups: dead and severely disabled (1+3), moderately disabled (4) and recovered (5).

In all three cases SOD activity decreased (with different degree) compared to control and was statistically different ($P < 0.05$). Cu,Zn-SOD level reliably increased in all cases, but is significantly different ($P < 0.01$) at moderate disability and recovery (Table 1).

It should be noted that whereas Cu,Zn-SOD and Mn-SOD are found in very small amounts in human extracellular fluids, EC-SOD is a major one (Marklund et al., 1982). EC-SOD is considered to serve for defence against superoxide, which can be produced by membrane-bound NADPH or secreted by inflammatory cells into the extracellular space (Oury, 1992). In spite of the relatively low EC-SOD concentration in whole brain, it may be important for ischemic event. The extracellular compartment is small and thus EC-SOD concentration in the extracellular compartment may be sufficient to provide defence (Cherubini et al., 2005). Perhaps in a case like that, augmentation of Cu,Zn-SOD is overlapped by decrease of EC-SOD level which predominates in extracellular plasma.

In all three cases catalase activity of patients increased compared to controls and correlates with recovery ($P < 0.01$) and moderate state ($P < 0.05$).

The activated antioxidants (Cu,Zn-SOD and catalase) can be considered as adequate markers for the positive outcome in the range of Glasgow Outcome Scale within the early phase of ischemic stroke development. As total SOD activity in plasma of IS patients reliably decreases in all cases, it cannot be considered as an adequate marker of IS outcome.

	Control	Min, IStroke Max (1+3)	Min, IStroke Max (4)	Min, IStroke Max (5)	Min, IStroke Max (5)	Min, IStroke Max (5)	Min, IStroke Max (5)	P (Control & IS (1+3))	P (Control & IS (4))	P (Control & IS (5))	
SOD activity (U/ml)	25,21 (23,3) ±19,7	3; 60.4	11.61 (3.4) ±17.66	0.71; 51.5	7.82 (4.65) ± 11.31	0.873; 39.8	8.98 (6.35) ± 12.72	0.24; 51.5	0.011 (are marginally significant P < 0.05)	0.0031 (are significantly different P<0.01)	0.0137 (are marginally significant P < 0.05)
Cu,ZnSOD (ng/ml)	78,5 (36,9) ±101,8	0; 360	130.84 (92.5) ±81.85	41.7; 249	236.1 (230.5) ±129.01	20,8; 422	219.31 (173.5) ±151.5	42.1; 513	0.07 (are not significantly different P≥0.05)	0.00187 (are significantly different P<0.01)	0.0038 (are significantly different P<0.01)
Catalase activity (IU/ml)	50,6 (41,7) ±25,9	23,2; 97,5	55.25 (48.8) ±24.13	28; 98.2	66.746 (69.5) ±14.15	42; 97.5	85.63 (83.6) ±32.88	41.7; 139.3	0.27 (are not significantly different P≥0.05)	0.029 (are marginally significant P < 0.05)	0.0038 (are significantly different P<0.01)

Table 1. Plasma levels of enzymatic antioxidants in control and ischemic stroke patients stratified according to GOS. Values (means) are analyzed using the Mann-Whitney *U* test (numbers in the parentheses are medians) ±SD (standard deviation).

3.1.4. The correlation between the enzymatic antioxidants

We have considered the relation between catalase and Cu,Zn-SOD at the different functional outcomes in accord to GOS. The results are presented in Table 2.

Correlation				
$y=a+bx$	r_s	P	r_p	P
Cu,ZnSOD vs. Catalase (1+3) GOS $y = 56.81+0.92x$	0.41	0.27	0.26	0.51
Cu,ZnSOD vs. Catalase (4) GOS $y = 105.6 + 1.63x$	0.23	0.38	0.21	0.44
Cu,ZnSOD vs. Catalase (5) GOS $y = -111.55+3.5x$	0.62	0.01	0.71	0.002

Table 2. Correlations of plasma antioxidants in the IS patients with different functional outcome. Correlations were determined by combining data from 42 patients and 32 controls using Spearman rank correlation analysis (r_s – coefficient of correlation, P – significance levels) and Pearson rank correlation analysis (r_p – coefficient of correlation, P – significance levels).

In case of moderate recovery and a poor functional outcome a correlation between catalase and Cu,Zn-SOD in plasma has not been observed ($P>0.05$). In patients with ischemic stroke the increased catalase activity and Cu,Zn-SOD levels are associated with a positive functional outcome and are significantly correlated with each other only in case of recovery

($P < 0.05$). The tandem action of Cu,Zn-SOD and catalase is clearly elicited in plasma of IS patients and their activation is necessary for a recovery after IS.

3.1.5. Plasma enzymatic antioxidant profile in ischemic stroke patients stratified according to OCSF classification

IS pathophysiology classification of the patients in accord with Oxfordshire Community Stroke Project has revealed, that Cu,Zn-SOD only increased in plasma of patients with partial anterior circulation infarction. For catalase activity, it increases only in patients with PACI and LACI (Table 3).

	Control	Min, Max	I Stroke (TACI)	Min, Max	I Stroke (PACI)	Min, Max	I Stroke (LACI)	Min, Max	P (Control & IS (TACI))	P (Control & IS (PACI))	P (Control & IS (LACI))
SOD Activity (U/ml)	25,21 (23,3) ±19,7	3; 60.4	11,93 (3,85) ±15,64	2,1; 44,6	13,08 (5,6) ± 16,7	1,5; 60,7	4,30 (5,03) ±3,44	0.24; 10	0.059 (are not significantly different P≥0.05)	0.036 (are marginally significant P < 0.05)	0.0056 (are significantly different P<0.01)
Cu,ZnSOD (ng/ml)	78,5 (36,9) ±101,8	0,5; 360	141,9 (136,1) ±111,8	0; 311,8	226,3 (210,2) ±137,6	20,8; 513	113,4 (89) ±98,43	17,6; 280	0.19 (are not significantly different P≥0.05)	0.00057 (the difference is highly significant P<0.001)	0.29 (are not significantly different P≥0.05)
Catalase (IU/ml)	50,6 (41,7) ±25,9	23,2; 97,5	50,48 (48,8) ±17,20	28; 83,6	73,94 (69,65) ±19,1	48,6; 125,3	74,66 (69,7) ±19,3	48,7; 97,5	0.402 (are not significantly different P≥0.05)	0.0052 (are significantly different P<0.01)	0.025 (are marginally significant P < 0.05)

Table 3. Plasma levels of enzymatic antioxidants in control and ischemic stroke patients stratified according to OCSF classification. Values (means) are analyzed using the Mann-Whitney U test (numbers in the parentheses are medians) ±SD.

The exclusive role of Cu, Zn-SOD is elicited in case of such brain damage location as PACI.

3.2. Non-enzymatic antioxidants at ischemic stroke in plasma

3.2.1. Glutathione

The major water soluble non-enzymatic antioxidant glutathione is localized in both the cytosol and the mitochondria of cells. Glutathione exists in two major forms: reduced (GSH) and oxidized (GSSG). GSH has a potent electron-donating capacity as indicated by the high negative redox potential of the GSH/GSSG redox couple. The reducing power of GSH is a measure of its free radical scavenging, electron-donating, and sulfhydryl-donating capacity. The ratio of GSH/GSSG plays an important role in regulating the cellular redox status, since it is the most abundant thiol-disulfide redox buffer in a cell.

Glutathione is a tripeptide (γ -L-glutamyl-L-cysteinylglycine). Oxidation of the cysteine sulfhydryl groups joins two glutathione GSH (reduced form of glutathione) molecules with a disulfide bridge to form glutathione disulfide GSSG (oxidized form of glutathione). Glutathione reacts directly with radicals (Wefers & Sies, 1983) in non-enzymatic reactions and is the electron donor in the reduction of peroxides by GPx. The product of the oxidation is GSSG, which is reduced by glutathione reductase (GR). Thus GSH is recycled during this process (Dringen et al., 2000).

Glutathione system plays a very special role in the brain defense. Its content and localization varies in different regions of the brain, e.g. astrocytes appear to contain higher GSH level than neurons both *in vivo* and *in vitro* (Cooper, 1997). Astrocytes in culture can decompose hydrogen peroxide with a rapid oxidation of GSH, forming GSSG (Dringen et al., 2000; Kussmaul et al., 1999).

Plasma contains both forms of glutathione: reduced and oxidized. The plasma soluble components and formed elements of blood do not destroy plasma circulating glutathione. Extracellular glutathione defends SH-groups of proteins, constituting the blood formed elements' plasma membrane, from oxidation, preventing the formation of S-S bonds. Thus, it is very important to estimate not only changes in GSH and GSSG content in plasma at acute ischemic stroke conditions, but also to estimate the correlation between these two forms. The correlation between GSH and GSSG in plasma can testify to the extent of oxidative conditions, developing in tissues and organs, and to the reversibility of redox balance distortion in tissues and organs.

It was shown that concentration of GSH decreases early after ischemia (Cooper et al., 1980; Rehncrona et al., 1980) in rats, plasma concentration of protein thiols is associated with the degree of neurological impairment (Leinonen et al., 2000), ischemic outcome is worsened by pharmacological depletion of GSH (Vanella et al., 1993). Depletion of the total GSH and decrease of GSH/GSSG ratio are markers for oxidative stress in ischemic brain and as long as 72 h may be required to restore concentrations to normal values following an ischemic insult according to (Namba et al., 2001; Park et al., 2000).

In our study GSH and GSSG levels were estimated in plasma of healthy volunteers and patients within the first 72 h of ischemic stroke. The data are presented in Fig. 5. As it follows from the picture, GSH concentration does not differ ($P=0.275$) at both studied conditions. But these conditions are characterized by the different GSSG concentration. GSSG content increases highly significant at acute ischemic stroke ($P=0.00098$). It is known, that in a cell GSSG may form mixed disulfides with thiol-containing enzymes, disrupting their normal activity (Mieyal et al., 2008). However, a cell effectively opposes the development of oxidative stress by getting rid of GSSG either by glutathione reductase reduction or by active export of the disulfide from a cell (Nur et al., 2011). We can suppose that the increased GSSG concentration in plasma is the result of GSSG export from neural tissue and blood formed elements into plasma for the maintenance of redox potential of a cell.

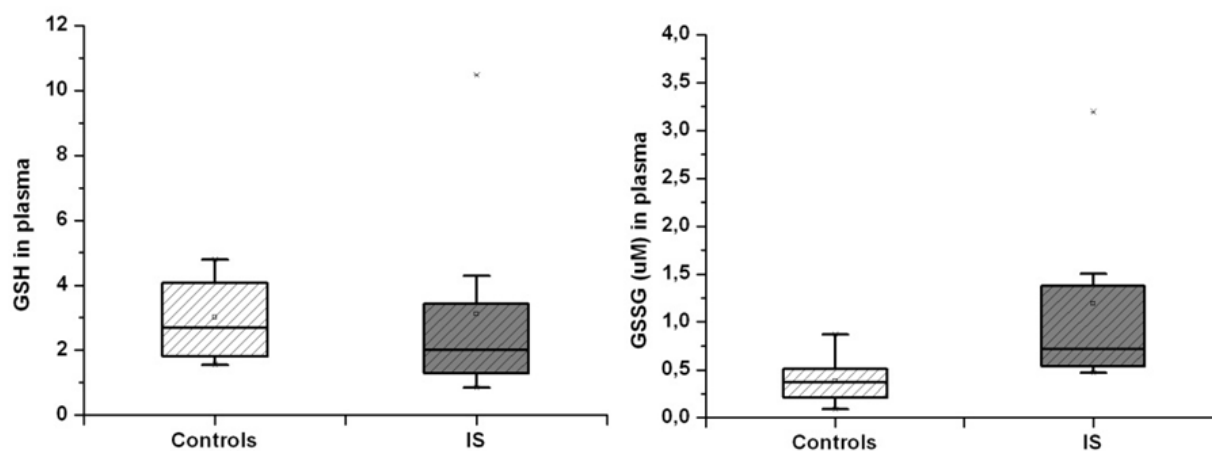


Figure 5. GSH and GSSG levels in plasma of healthy volunteers (Controls) and patients with ischemic stroke onset (IS)

3.2.2. Total thiols

Thiol (SH-group)-containing compounds are the important components maintaining redox homeostasis in cells, tissues and biological fluids in an organism. SH-group modification of membrane proteins changes membrane permeability; oxidative modification of SH-groups in enzymes or in their coenzymes influences on enzymatic activity. Reversible modification of SH-groups is considered as nonspecific defense mechanism of organism in response to the extreme conditions. Thus, SH-groups of blood plasma proteins can quench up to 50% of peroxy radicals and as the result inhibit the process of lipid peroxidation taking place under the oxidative conditions (Wayner et al., 1987). SH-group containing compounds are the subject of oxidative stress in the first place and provide first line of defense by direct scavenging of hydroxyl radicals. Thiol's autooxidation, leading to cellular and tissue hypoxia, is considered as the protective effect of thiol-containing compounds against irradiation and its accompanying oxidative stress. The oxidized thiols make a valuable contribution to the neurodestruction mechanisms, namely displaying thiol-disulfide system to the augmentation of the oxidized thiols concentration, that in turn decrease reducing potential of a cell. At the irreversible displacement of the thiol-disulfide ratio the expression of the pro-apoptotic proteins is also possible. Thus, study of the concentration changes of glutathione and total thiols can provide an understanding of processes at the ischemic stroke

The total thiols concentration in blood plasma at ischemic stroke can serve as an indirect indicator of the oxidative conditions developing under the stroke circumstances. It was shown in our study, that the total thiols level at ischemic stroke within the first 72 h of ischemic stroke onset was decreased. The data are presented in Fig. 6. As it follows from the analysis and the picture, the total thiols concentration decreases at acute ischemic stroke, and the values between the total thiols concentration in healthy subjects and IS patients are significantly different ($P=0.0083$).

Figure 6. The total thiols (RSH) concentration in plasma of healthy volunteers (Controls) and patients with ischemic stroke onset (IS), $P=0.0083$.

The decrease of the total thiols and the increase of GSSG points to the necessity of the redox regulation of the intracellular and extracellular processes for the good outcome at IS (Table 4).

Table 4. Plasma levels of non-enzymatic antioxidants in control and ischemic stroke patients stratified according to GOS. Values (means) are analyzed using the Mann-Whitney *U* test (numbers in the parentheses are medians) \pm SD.

As it follows from the Table 4, in the case of a favorable outcome the concentration of the oxidized glutathione increased in plasma. This could be an evidence of active export of GSSG from the cells in case of ischemic stroke and the possible restoring of the redox balance in the cells of damaged tissue areas. A reliable decrease of the thiols concentration in plasma of the IS patients with a favorable outcome (score 5) points to their oxidative modification. This may indicate to the defense reactions at the early stages of ischemic stroke, which is reflected in the participation to run multiple signaling pathways leading to neutralization impact, and in case of irreparable damage to the elimination of cells (apoptosis) (Circu & Aw, 2010).

3.2.4. Plasma non-enzymatic antioxidant profile in ischemic stroke patients stratified according to OCSF classification

It was not revealed the specific connection of the non-enzymatic antioxidants activation and the division of cerebral infarction into four categories in accord with the Oxfordshire Community Stroke project classification, widely used for stroke pathophysiology classification.

Control		Min, IStroke Max (TACI)	Min, IStroke Max (PACI)	Min, IStroke Max (LACI)	Min, IStroke Max (& IS (TACI))	P (Control & IS (TACI))	P (Control & IS (PACI))	P (Control & IS (LACI))			
Total Thiols (mM)	0.51 (0.476) ±0.12	0.315; 0.797	0.58 (0.51) ±0.17	0.45; 0.885	0.476 (0.445) ±0.25	0.132; 1.111	0.356 (0.343) ±0.096	0.22 (are not significantly different P≥0.05)	0.178 (are not significantly different P≥0.05)	0.084 (are not significantly different P≥0.05)	
GSH (μM)	3.02 (2.7) ±1.25	1.56; 4.8	2.1 (2.1) ±1.9	0.759; 3.44	3.044 (1.869) ±3.24	0.852; 9.528	2.262 (1.29) ±1.765	1.194; 4.3	0.43 (are not significantly different P≥0.05)	0.27 (are not significantly different P≥0.05)	0.28 (are not significantly different P≥0.05)
GSSG (μM)	0.38 (0.367) ±0.23	0.09; 0.871	0.987 (0.72) ±0.63	0.536; 1.704	1.64 (1.66) ±1.26	0.47; 2.768	1.88 (1.375) ±1.15	1.08; 3.196	0.038 (are marginally significant P<0.05)	0.039 (are marginally significant P<0.05)	0.006 (are significantly different P<0.01)

Table 5. Plasma levels of non-enzymatic antioxidants in control and ischemic stroke patients stratified according to OCSF classification. Values (means) are analyzed using the Mann-Whitney *U* test (numbers in the parentheses are medians) ±SD.

3.3. Plasma CRP levels in ischemic stroke patients as a valuable diagnostic marker at acute IS

C - reactive protein (CRP) is an acute-phase protein. A CRP concentration in plasma is widely used by clinicians as a marker for acute inflammation and tissue necrosis. CRP is produced exclusively in the liver, and in case of acute inflammation it starts to rise within the 6 hours in plasma. Biological half-life of this protein is 24 hours. Plasma concentration of CRP increases significantly in cases of both infectious and non-infectious inflammation, of

tissue damage and necrosis. CRP is present in the acute stages of inflammatory disorders like rheumatoid arthritis, systemic lupus erythematosus, polyarteritis nodosa, inflammatory bowel disease. Thus, CRP is considered as a very specific inflammatory marker, but it is non-specific for the kind and place of inflammation.

As among the pathological processes in acute ischemic stroke are inflammation, neuronal and glial injury, CRP concentration in plasma was estimated throughout to correlate with and predict infarct growth in acute ischemic stroke and stroke progression. The elevated plasma level is currently accepted as an outcome-predicting factor at IS (Di Napoli et al., 2001; Kuhlmann et al., 2009). We prospectively measured the CRP concentration in plasma of IS patients in our study (≤ 24 hours from symptom onset) and compared it with the CRP plasma level of healthy persons. The plasma CRP concentration, ranging up to 10 $\mu\text{g/ml}$, is considered as normal concentration. The data are presented in the Tables 6&7. As it follows from the Tables 6 and 7, our data are in accordance with the generally accepted view on the CRP as the predictor of the poor outcome in IS. However, the elevated level of CRP was observed in patients, in whom the score 4 in GOS (moderate disability) was appropriated as well. When we stratified the IS patients in accord with the Oxfordshire Community Stroke project classification, the patients with total anterior circulation infarction and partial anterior circulation infarction are characterized by the significantly elevated levels of CRP, and only in plasma of patients with lacunar infarction CRP level is in the range of normal concentration.

	Control	Min, I Stroke Max (1+3)	Min, I Stroke Max (4)	Min, I Stroke Max (5)	Min, I Stroke Max (6)	P (Control & IS (1+3))	P (Control & IS (4))	P (Control & IS (5))
CRP ($\mu\text{g/ml}$)	3,84	0;	25,51	14,84	6,53	0.000002	0.0002	0.117
≤ 10	(1,51)	19	(20,65)	(8,32)	(5,71)	(the difference is highly significant $P < 0.001$)	(are significantly different $P < 0.001$)	(are not significantly different $P \geq 0.05$)
$\mu\text{g/ml}$	$\pm 4,99$		$\pm 23,07$	$\pm 17,19$	$\pm 5,59$			
norm								

Table 6. Plasma level of CRP in control and ischemic stroke patients stratified according to GOS. Values (means) are analyzed using the Mann-Whitney U test (numbers in the parentheses are medians) \pm SD.

	Control	Min, I Stroke Max (TACI)	Min, I Stroke Max (PACI)	Min, I Stroke Max (LACI)	Min, I Stroke Max (LACI)	P (Control & IS (TACI))	P (Control & IS (PACI))	P (Control & IS (LACI))
CRP ($\mu\text{g/ml}$)	3,84	0;	28,74	13,81	6,84	0.000002	0.00034	0.091
≤ 10	(1,51)	19	(22,8)	(9,6)	(4,46)	(the difference is highly significant $P < 0.001$)	(are significantly different $P < 0.001$)	(are not significantly different $P \geq 0.05$)
$\mu\text{g/ml}$	$\pm 4,99$		$\pm 23,41$	$\pm 15,90$	$\pm 8,63$			
norm								

Table 7. Plasma level of CRP in control and ischemic stroke patients stratified according to OCSP classification. Values (means) are analyzed using the Mann-Whitney U test (numbers in the parentheses are medians) \pm SD.

However, the question whether the elevated CRP levels are induced by stroke or reflect pre-existing inflammatory conditions is still open. Recently, participation of CRP in blood-brain barrier disruption and its mechanisms are specified by Kuhlman (Kuhlmann et al., 2009). It was shown that the clinically relevant concentrations 10 and 20 $\mu\text{g/ml}$ cause a disruption of BBB in a cell coculture BBB model and in the guinea pig isolated whole brain preparation. CRP induces activation of surface Fc γ receptors CD16/32 followed by p38-mitogen-activated protein kinase-dependent ROS formation by NAD(P)H-oxidase. The oxidative conditions activate the contractile machinery involving phosphorylation of myosin light chain and as the result the disruption of tight junctions takes place.

4. Conclusion

According to the current conception, the neurodestruction at ischemic stroke is accompanied by the complicated metabolic cascades in neurons, which switch on the neuronal death program. The switching of the death program can be accomplished by ROS, oxidized thiols and products of oxidized modification of proteins and nucleic acids. We observed the decrease of the total thiols concentration and the significant increase of the oxidized glutathione concentration at acute ischemic stroke, what could point to the displacement of the reduced/oxidized balance to the increased oxidized thiols concentration. The activation of catalase at acute IS of the disease onset points to the development of the oxidative conditions. Vasodilatation from hydrogen peroxide could be under control of the catalase activity, and the study of the antioxidant system is a key moment in the understanding of the correct therapeutic strategy at the ischemic stroke. It was revealed, that among the studied spectra of antioxidants the tandem activation of Cu,Zn-SOD and catalase is necessary for a recovery after IS. The behavior of the non-enzymatic antioxidants (GSSG and total thiols) correlates with the behavior of the main enzymatic antioxidants (Cu,Zn-SOD and catalase) in case of the IS positive outcome directed to the protection against the oxidative stress. The exclusive role of Cu,Zn-SOD is elicited in case of such brain damage location as PACI. According to our results, antioxidants in plasma can be not only markers of oxidative stress at IS, but also the markers of brain tissue damages. All these observations corroborate strategies targeting antioxidants for the therapeutic intervention in clinical settings. Possibly, the unprotected by antioxidant defense system oxidative conditions, developed in case of IS poor outcome, participate in the CRP activation registered in these conditions.

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