TÜBİTAK

ORIGINAL ARTICLE

Turk J Med Sci
2007; 37 (2): 75-81
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E-mail: medsci@tubitak.gov.tr

Increased Lipid Peroxidation and Decreased Antioxidant Response in Serum and Cerebrospinal Fluid in Acute Ischemic Stroke

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Background and Aim: The pathogenesis of brain damage in ischemic stroke is highly complex. The aim of the present study was to investigate the presence of oxidative stress in ischemic stroke and to determine whether the measured oxidative stress markers in serum and cerebrospinal fluid (CSF) were associated with infarction volume, and the Glasgow Coma Scale (GCS) and Glasgow Outcome Scale (GOS).

Materials and Methods: The study included 32 patients with acute ischemic stroke within 48 h of onset and 18 suitable controls. Superoxide dismutase (SOD) and glutathione peroxidase (GPX) activity, glutathione (GSH) and malondialdehyde (MDA) level, and total antioxidant capacity (TAC) were measured in both serum and CSF samples of the participants.

Results: GPX activity, GSH level, and TAC in the serum and CSF samples of stroke patients were significantly lower than those in the controls. In the patient group, SOD activity in serum was lower and SOD activity in CSF was higher compared to the control group. Both serum and CSF MDA concentrations were significantly higher among stroke patients as compared to the controls.

Conclusions: The lower activity levels of antioxidant molecules measured in this study could have resulted from increased free radical generation, which may confirm the presence of oxidative stress in acute ischemic stroke; however, the levels of oxidative stress markers in serum and CSF may not always be indicative of neurological deficit.

Key Words: Ischemic stroke, oxidative stress, cerebrospinal fluid, malondialdehyde, total antioxidant capacity

Akut İskemik Strokta Serum ve Beyin Omurilik Sıvısında Artmış Lipid Peroksidasyonu, Azalmış Antioksidan Aktivite

Giriş ve Amaç: İskemik strokta beyin hasarının patogenezi oldukça karmaşıktır. Bu çalışmanın amacı iskemik strokta oksidatif stres varlığını araştırmak ve beyin omurilik sıvısı (CSF) ve serumda ölçülen oksidatif stres belirteçleriyle infarkt volumu, Glasgow Koma Skalası ve Glasgow Sonuç Skalası ile ilişkisinin olup olmadığını araştırmaktı.

Yöntem ve Gereç: Bu çalışma strok başlangıcından 48 saat içinde hastanemize başvuran 32 akut iskemik stroklu hasta ve uygun kontrol grubu (n = 18) üzerinde yürütüldü. Serum ve CSF örneklerinde superoksit dismutaz (SOD) ve glutatyon peroksidaz (GPX) aktiviteleri ve total antioksidan kapasite (TAC), glutatyon (GSH) ve malondialdehit (MDA) düzeyleri ölçüldü.

Bulgular: Hastaların serum ve CSF örneklerinde, GPX aktiviteleri ve GSH düzeyleri ve TAC kontrol grubuna göre anlamlı şekilde daha düşüktü. Kontrol grubu ile karşılaştırıldığında hasta grubu serum örneklerinde azalmış SOD aktivitesi ve CSF örneklerinde ise artmış SOD aktivitesi bulundu. Kontrol grubu ile karşılaştırıldığında stroklu hastalarda hem serum hem de CSF MDA konsantrasyonu daha yüksekti.

Sonuç: Bu çalışmada ölçülen antioksidan moleküllerin azalmış konsantrasyonları veya aktiviteleri artmış serbest radikal oluşumundan kaynaklanmış olabilir, ki bu durum da akut iskemik strokta oksidatif stresin varlığını doğrulayabilir. Bununla birlikte, serum ve CSF'de ölçülen oksidatif stres belirteçlerinin düzeyleri her zaman nörolojik hasarın bir göstergesi olmayabilir.

Anahtar Sözcükler: İskemik strok, oksidatif stres, beyin omurilik sıvısı, malondialdehit, total antioksidan kapasite

Received: November 06, 2006
Accepted: March 22, 2007

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Introduction

Stroke is the third most common cause of death in industrialized countries, following coronary heart disease and cancer (1). Ischemic stroke accounts for about 75% of all strokes. Cerebral infarction in ischemic strokes may be due to vessel occlusion by an embolus or to primary thrombosis in an artery (2). The pathogenesis of brain damage in ischemic stroke is highly complex, and involves impaired blood-brain barrier permeability, energy failure, loss of cell ion homeostasis, acidosis, increased intracellular calcium, excitotoxicity, and free radical-mediated toxicity. Ischemia rapidly causes a cascade of events that eventually lead to neuronal damage and death (3,4).

Oxidative stress states occur when cellular antioxidant defenses are insufficient to keep the levels of reactive oxygen species (ROS) below a toxic threshold. This may be due to excessive production of ROS or the failure of antioxidant defenses, or both (5). Oxidative stress in cerebral ischemia/reperfusion is a subject of intensive investigation and is considered to be one of the mechanisms involved in neuronal damage due to ischemia/reperfusion in stroke patients (6). Yet, the mechanisms that participate in the development of oxidative stress in ischemic stroke are not completely known. In other words, it is not fully evident if oxidative stress contributes to the pathogenesis of stroke or if it is a consequence of pathophysiological processes following ischemic stroke. Controversial results, which will be further discussed, are even found in the literature concerning antioxidant status and lipid peroxidation in stroke patients (7-10).

Cerebrospinal fluid (CSF) contains both powerful enzymatic and non-enzymatic antioxidants, and CSF analysis provides some important clues about the physiological or pathological changes in the central nervous system (11). The assessment of lipid peroxidation products and antioxidant status in CSF may be important in predicting free radical-induced cerebral injury in stroke patients. To the best of our knowledge, there is little information in the literature on the simultaneous evaluation of oxidative stress markers in serum and CSF. Therefore, we measured superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities, glutathione (GSH) and malondialdehyde (MDA) levels, and total antioxidant capacity (TAC) in both serum and CSF samples in patients with acute ischemic stroke within 48 h of onset, and compared them to a suitable control group. This study was performed with 2 aims: [1] to investigate the presence of oxidative stress in ischemic stroke by measuring the level and activity of several antioxidants, and MDA concentrations in serum and CSF samples, and [2] to determine whether the measured oxidative stress markers in serum and CSF were associated with infarction volume, and the Glasgow Coma Scale (GCS) and Glasgow Outcome Scale (GOS).

Materials and Methods

The study included 32 patients (17 male, 15 female) with acute ischemic stroke, who were admitted to the Department of Neurology at Atatürk University Hospital within 48 h of the onset of symptoms. Control subjects were 18 age- and sex-matched volunteers without organic disorders of the nervous system, who underwent lumbar punctures for intrathecal anesthetic drug administration during orthopedic surgery. 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.

On admission, all strokes were initially diagnosed on the basis of full physical and neurological examinations. The diagnoses were then confirmed by an MRI or CT scan of the brain. Vascular risk factors, including hypertension, diabetes mellitus, and smoking and alcohol habits, were recorded. Patients with hemorrhagic stroke, other neurological diseases, liver disease, renal failure, or severe medical disease were excluded.

Patients were clinically evaluated using the GCS, and functional outcome at discharge was assessed using the GOS. The GOS evaluates clinical outcome with a functional status scale comprised of 5 items; one point represents the best score (recovery), whereas 5 points constitutes the worst result (death). On the basis of clinical and neuroradiological criteria, it was possible to distinguish patients with lacunar or non-lacunar syndromes, including total anterior, partial anterior, and posterior syndromes.

Blood samples were collected in vacutainer tubes without any additive on the second day following stroke onset and also from the control subjects. We collected 4 ml of CSF from the patients and controls in a clean tube for biochemical analyses. All blood samples were centrifuged at 3500 x *g* for 5 min at 4 °C. We mixed 500

µl of serum with an equal volume of 10% metaphosphoric acid for measurement of GSH before freezing at –80 °C. For the other biochemical measurements, serum aliquots were stored at –80 °C until analysis. CSF samples were immediately stored at –80 °C until the biochemical analyses were performed.

MDA level in CSF and serum was used as the indicator of lipid peroxidation, and were determined by the thiobarbituric acid (TBA) method, as previously described (12). Briefly, 0.2 ml of sample was combined with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid, 1.5 ml of 0.8% TBA solution, and 0.6 ml of distilled water, and incubated at 95 °C for 1 h. After *n*-butanol/pyridine (15:1, v/v) extraction, absorbance of the pink chromophore was read at 532 nm. Tetramethoxypropane (5 nM) was used as the standard solution, and MDA levels were expressed as µmol/l.

SOD activity was measured in serum and CSF using the SOD Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. The assay uses a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine, which yields a chromophore with a maximal absorbance at 525 nm. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. SOD activity was expressed as U/ml. GPX activity was quantified in serum and CSF samples using a kinetic colorimetric assay from Cayman Chemical. The assay kit measures GPX activity indirectly by a coupled reaction with glutathione reductase; oxidized glutathione is converted to the reduced form in the presence of glutathione reductase and NADPH, while NADPH is oxidized to $NADP^+$. The rate of decrease in the absorbance of NADPH at 340 nm is directly proportional to GPX activity in a sample. GPX activity was expressed as nmol/min per ml. The GSH level was measured spectrophotometrically by a glutathione reductase recycling method, using a commercial assay kit, according to the manufacturer's instructions (Cayman Chemical). Briefly, the samples were deproteinated by an equal volume of 10% metaphosphoric acid reagent before assaying. Into appropriate wells we pipetted 50 µl of sample or standard, and 150 µl of freshly prepared Assay Cocktail, according to the manufacturer's instructions [MES buffer, cofactor mixture ($NADP^+$ and glucose-6-phosphate), enzyme mixture (glutathione reductase and glucose-6-phosphate dehydrogenase) and 5-5'-dithiobis-2-nitrobenzoic acid]. After incubation in the dark on a shaker, the absorbance in the wells was measured at 405 nm using a plate reader, and the concentrations of samples were calculated by the end point method. TAC was measured by a commercially available kit (Cayman Chemical). The assay method relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{®\bullet+}$ by metmyoglobin. The amount of $ABTS^{®\bullet+}$ produced can be monitored by reading the absorbance at 750 nm. The capacity of the antioxidants in the sample to prevent ABTS® oxidation is compared to that of Trolox and is quantified as millimolar Trolox equivalents.

All absorbance values were read in an ELISA plate reader and the concentration of samples were automatically calculated by software (Power Wave XS; BIO-TEK Instrument, Inc., KC Junior software). All samples were run in duplicate, and the 2 measurements were averaged for statistical analysis.

Data are stated as means ± standard deviation (SD). When appropriate, differences between group means were tested by Student's t-test, chi-square test or Mann-Whitney U-test (in the data of non-normal distribution). Correlations between variables were determined by Spearman's rank test. All statistical analyses were performed using SPSS for Windows (version 11.5, Chicago, IL, USA). A value of $P < 0.05$ was considered statistically significant.

Results

There were no significant differences in terms of age between the control group and stroke patients. The clinical and laboratory characteristics of the patients and controls are summarized in Table 1. The mean volume of cerebral infarction was 9.5 ± 9.1 cm^3 (range: 1-39 cm^3; median: 6.5 cm^3).

The antioxidant parameters and MDA results (in serum and CSF) are presented in Table 2. In the serum and CSF samples of the stroke patients, GPX activity, GSH level, and TAC were significantly lower compared to those in the controls. In the patient group, SOD activity in serum was lower and SOD activity in CSF was higher compared to those in the control group. Both serum and CSF MDA concentrations were significantly higher in the stroke patients than in the controls.

Table 1. The characteristics of patients with acute ischemic stroke and control subjects.

Characteristics	Controls (n = 18)	Patients (n = 32)	P [a]
Age (years, mean ± SD)	55.7 ± 9.3	59.6 ± 10.6	> 0.05
Sex (male/female)	10/8	17/15	> 0.05
Infarct volume, cm^3	-	9.5 ± 9.1	-
GCS on admission	-	10.3 ± 2.1	-
GOS at month			
Dead		6	-
Vegetative state		4	-
Severely disabled		7	-
Moderately disabled		8	-
Good recovery		7	-
Infarct type			
Lacunar / Non-lacunar		13/19	-
Risk factors			
Smoking	7 (38.8%)	12 (37.5%)	> 0.05
Alcohol habit	-	-	-
Hypertension	-	18 (56.2%)	-
Diabetes mellitus	-	5 (15.6%)	-
ECG evidence of atrial fibrillation	-	7 (21.9%)	-

[a] Calculated using Mann-Whitney U-test or chi-square test as appropriate.
ECG: electrocardiography.

Table 2. Level or activity of some parameters of antioxidants and MDA concentration in serum and CSF in patients with acute ischemic stroke and controls.

Parameters	Serum			CSF		
	Controls (n = 18)	Patients (n = 32)	P	Controls (n = 18)	Patients (n = 32)	P
SOD (U/ml)	0.221 ± 0.021	0.188 ± 0.031	< 0.001	0.063 ± 0.018	0.078 ± 0.018	< 0.05
GPX (nmol/min per ml)	212.5 ± 39.9	179.9 ± 38.8	< 0.01	36.7 ± 11.2	30.5 ± 12.6	< 0.05
GSH (µmol/l)	11.8 ± 3.1	9.1 ± 2.0	< 0.005	1.6 ± 1.1	0.8 ± 0.4	< 0.05
TAC (mM Trolox)	0.268 ± 0.043	0.215 ± 0.042	< 0.001	0.081 ± 0.020	0.066 ± 0.016	< 0.05
MDA (µmol/l)	7.1 ± 2.1	8.6 ± 1.8	< 0.01	4.2 ± 1.6	5.9 ± 2.2	< 0.01

Data are expressed as mean ± SD. CSF: cerebrospinal fluid; SOD: superoxide dismutase; GPX: glutathione peroxidase; GSH: glutathione; TAC: total antioxidant capacity; MDA: malondialdehyde.

Serum TAC had a nearly significant correlation to CSF TAC in stroke patients ($r = 0.348$, $P = 0.051$) (Figure 1). A significant negative correlation was observed between TAC and MDA concentration in CSF samples of the patients ($r = –0.478$, $P = 0.006$) (Figure 2). In patients with ischemic stroke, CSF MDA level, and CSF antioxidant activity and level were not associated with infarction volume. In serum samples, a significant correlation between infarction volume and the measured antioxidant parameters was also not observed. GCS or GOS did not correlate significantly with any of the measured individual serum and CSF oxidative stress markers (SOD, GPX, GSH, TAC, and MDA) (data not shown).

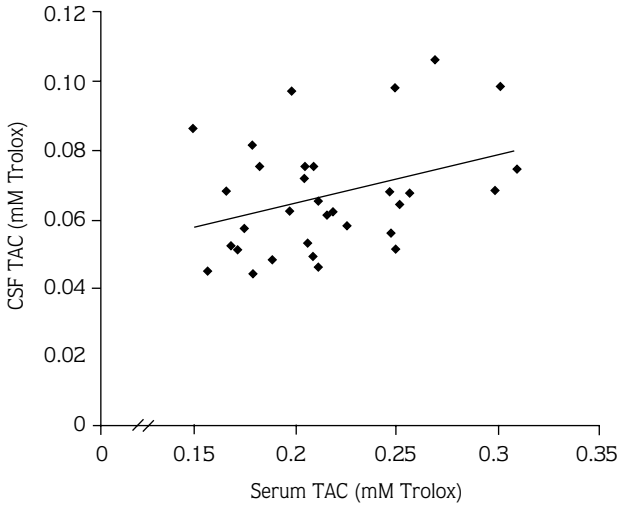

Figure 1. The relationship between CSF TAC and serum TAC in patients with acute ischemic stroke (r = 0.348, P = 0.051). TAC: total antioxidant capacity.

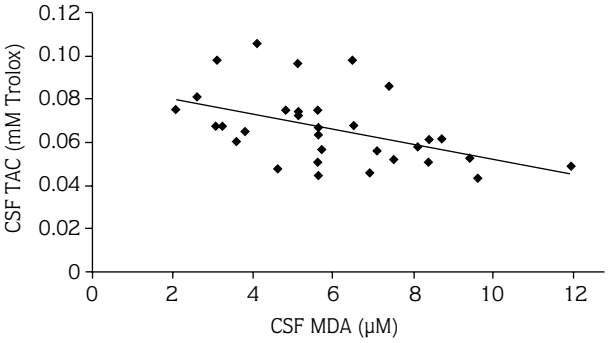

Figure 2. The correlation between CSF TAC and CSF MDA levels in patients with acute ischemic stroke (r = -0.478, P = 0.006).

Discussion

The brain consumes a large quantity of oxygen because of the high metabolic rate of neurons, making it particularly susceptible to oxidative stress. Under normal physiological conditions, there is equilibrium between the antioxidants and oxidants produced by aerobic cellular systems. A number of studies documented that ischemic stroke was associated with increased production of free radicals in animal and human models (6,13). The direct measurement of free radicals in biological samples is difficult because they are extremely reactive and have a short half-life. Therefore, particularly in human studies, indirect approaches have been used to demonstrate free radical production during cerebral ischemia, measuring the products of free radical reaction with other molecules, such as lipids, proteins, and DNA, and the level or activity of antioxidant molecules (14,15).

Lipid peroxidation is a free radical chain reaction, which arises from the oxidative conversion of polyunsaturated fatty acids by $HO^{\cdot}$ to lipid peroxides, which in turn can damage biological membranes (16). MDA level is widely utilized as a marker of lipid peroxidation in states of elevated oxidative stress. In the current study, MDA level in the serum and CSF of the stroke patient group were higher than those in the control group, which confirmed previously published data (17,18). Several possible mechanisms involved in the elevated lipid peroxidation in ischemic stroke have been proposed. First, cellular membranes in the brain are very rich in polyunsaturated fatty acids, which are especially sensitive to free radical attack (19). Second, the brain has a low content of antioxidant enzymes (i.e. GPX and catalase), while it contains a significant amount of iron ions, which stimulate free radical generation (20). In addition, it has been claimed that lipid peroxidation products are key mediators of neuronal apoptosis induced by oxidative stress (21). Alexandrova et al. (22) reported that the blood concentration of TBA reactive material is an indicator of the severity of neurological deficit, and is associated with infarct size and the severity of stroke. Moreover, Polidori et al. (23) reported a significant negative correlation between lipid hydroperoxides and GCS. These reports appear to contradict our results; however, the absence of a correlation between MDA (TBA reactive substance) and infarct volume, and GCS or GOS of the stroke patients in our study may have been due to different assay methods.

SOD plays a protective role toward cerebral damage induced by ischemia (24); however, data associated with SOD activity after acute ischemic stroke are controversial. SOD activity in the serum of patients with acute stroke was reported to be decreased (7) and unchanged (8),

while SOD activity after stroke was reported to be increased in CSF (9), and increased in both CSF and serum (10). In the present study, we noted reduced serum SOD activity and increased CSF SOD activity in stroke patients, which is in accordance with published data (7,9); however, we did not observe a significant correlation between infarction volume and CSF SOD activity. This appeared to contradict the findings reported by Strand et al. (9). This discrepancy could be explained by the different SOD isoforms (Cu/Zn-, Mn-, and Fe-SOD) and differences in the methodology used, as we measured total SOD (all 3 types of SOD).

GSH is a free radical scavenger and a proton donor for GPX, which is known to have a neuroprotective role. It is reported that depletion of total GSH and a decreasing GSH to GSSG ratio are markers for oxidative stress in an ischemic brain (5,25). In the present study, the level of serum and CSF total GSH in acute stroke patients were significantly lower compared to those in the controls. In an experimental study, Anderson et al. (26) reported that intracerebroventricular infusion of glutathione monoethyl ester reduced infarct volume by more than 60% in a rat model of stroke. Nevertheless, no correlation between infarct volume and GSH level in stroke patients was observed in our study. Weisbrot-Lefkowitz et al. (27) stated that GPX has protective effects on ischemic brain injury and reduced GPX activity is associated with an increased stroke risk. We found a decline in the activity of serum and CSF GPX in the stroke patients as compared to the healthy controls; however, our results provide no information about the association between GPX activity and GSH level, or the risk of stroke, but these data may point to an important role for GPX and GSH in acute stage stroke.

The measurement of TAC has been used to indirectly assess free-radical activity in biological samples, and may offer more relevant biological information compared to that obtained by the measurement of individual antioxidant components in plasma and body fluids (28,29). Nonetheless, experimental and clinical studies of stroke have focused on individual antioxidant molecules rather than overall antioxidant defense, and to the best of our knowledge there is no information in the literature on the simultaneous evaluation of TAC in serum and CSF in acute ischemic stroke. In this study we found that TAC was reduced in stroke patients compared to controls, which indicates that the observed decrease in TAC was partly due to the decreased activity or level of SOD, GPX, and GSH, and the accumulation of lipid peroxidation products in serum and CSF. Gariballa et al. (1) reported that TAC was reduced in the serum of patients 48-72 h following stroke onset compared to controls, which is in accordance with our results. Leinonen et al. (6) examined total peroxyl radical-trapping potential in the plasma and CSF of patients with acute ischemic stroke by a chemiluminescence-enhanced method. Their data showed an association between plasma total antioxidant activity and the volume of ischemic cerebral infarction. Nevertheless, in this study we did not find any correlation between infarction volume with serum or CSF TAC in stroke patients.

In summary, once generated, free radicals can react with all cellular macromolecules, including proteins, and protein oxidation, particularly of enzymes, can lead to impairment of their function (30). In the present study, the decreased activity or level of antioxidant molecules measured could have resulted from increased free radical generation, which may confirm the presence of oxidative stress in acute ischemic stroke. Despite the data in the scientific literature, we did not find any significant correlation between infarct volume, GCS or GOS, and the measured individual serum and CSF oxidative stress markers. Therefore, SOD and GPX activity, and TAC, GSH, and MDA levels in serum and CSF in patients with acute ischemic stroke may not always be an indicator of the severity of neurological defect.

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