

ANTIOXIDANT ENZYMES - POTENTIAL TARGETS IN INTRACEREBRAL HAEMORRHAGE

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Abstract

Intracerebral haemorrhage (ICH) is accompanied by a cascade of pathological events which involves the generation of oxygen and nitrogen reactive species. These very short lifetime molecules change the level of antioxidant mechanisms, including antioxidant enzymes. The aim of our study was to assess the superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (Gred), glutathione transferase (GTS) activities. The level of oxidative damage was underlined by malondialdehyde (MDA) as index of lipid peroxidation. We also assessed the total nitrite level.

The measurements were performed on cerebrospinal fluid (CSF) samples from 21 patients and 15 healthy volunteers.

We found significantly increased SOD and GPx and reduced Gred and GST activities in CSF of ICH patients. MDA and total nitrate levels were both increased.

Rezumat

Hemoragia intracerebrală e acompaniată de o cascadă de evenimente patologice care implică generarea speciilor reactive de oxigen și de azot. Aceste molecule cu viață foarte scurtă modifică nivelul mecanismelor de apărare inclusiv activitatea enzimelor antioxidante. În acest studiu au fost evaluate activitățile unor enzime: superoxid dismutazei (SOD), glutatation peroxidazei (GPx), glutatation reductazei (Gred), glutatation transferazei (GTS). Nivelul agresiunii oxidative a fost subliniat de creșterile semnificative ale nivelului malondialdehidei (MDA) ca indicator al peroxidării lipidice și de concentrația nitritului total.

Determinările au fost efectuate pe probe de lichid cefalorahidian provenind de la 21 de pacienți cu hemoragie intracerebrală și 15 voluntari sănătoși.

Au fost identificate creșteri semnificative ale activităților SOD și GPx concomitent cu reduceri semnificative ale activităților Gred și GST la pacienții cu hemoragie intracerebrală. La aceiași pacienți, nivelurile MDA și nitritului au fost semnificativ crescute.

Keywords: antioxidant enzymes, intracerebral haemorrhage, oxidative stress

Introduction

Intracerebral haemorrhage (ICH) has a double incidence comparative to subarachnoid haemorrhage (SAH) and leads more often to death or major disability than cerebral infarction or SAH, according to American Heart Association American Stroke Association (AHA/ASA) Guide lines for the Management of Spontaneous Intracerebral Haemorrhage [1].

In patients with intracerebral haemorrhage (ICH) brain is highly sensitive to reactive oxygen and nitrogen species (ROS/RNS). Soon after the initial cause of the blood extravasation, a second wave of injuries is related to oxidative stress which produces damages of proteins, lipids, carbohydrates, DNA, RNA, resulting in neurotoxicity [2].

Among the most effective antioxidant interventions, enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are responsible for the neutralization of ROS by several mechanisms [3].

These enzymes are present in cerebrospinal fluid (CSF) and their assessment and modulation may allow a better protection in ICH patients.

Materials and Methods

Patients with ICH were admitted to the Department of Neurosurgery of the Cerebrovascular Diseases Institute Bucharest - University of Medicine and Pharmacy, "Carol Davila", Bucharest, Romania, 48 h after the onset of symptoms. Before inclusion in the study, the written inform consent was obtained from each person or their relatives. The study protocol was approved by the local ethics committee.

All patients were the subject of complete physical and neurological examinations. ICH was confirmed by magnetic resonance imaging (MRI) or computerized tomography (CT) scan.

The study group included 21 patients both gender (10 male, 11 female) aged between 35-65 years old, with acute ICH. The control group consisted of 15 healthy subjects without any nervous diseases, to whom CSF was collected during lumbar punctures for spinal anaesthesia in other surgical interventions.

Exclusion criteria were: the presence of subarachnoid haemorrhage and/or intraventricular blood effusion confirmed by CT or MRI; post-traumatic brain injuries; haematological disorders; chronic diseases (lung, kidney); use of medications able to interfere in the oxidative equilibrium: (cortisol and derivatives) non-steroidal anti-inflammatory drugs, anticoagulants,

iron and derivatives, chemotherapy, vitamin E, ascorbic acid, other antioxidant substances.

All CSF samples – 5mL each-were collected in sterile, chemically inert (Eppendorf) recipients and frozen at -80°C . Each sample was examined in duplicate for the determination of GPx, SOD, glutathione transferase (GTS), glutathione reductase (Gred) activities, nitrit-nitrate and MDA level.

GPx stimulates glutathione formation and was spectrophotometrically assayed, based on the reduction of glutathione in the presence of Glutathione reductase in excess. The concomitant nicotinamide (NADPH) reduction was monitored for 3 minutes (340 nm) [4].

For the SOD assay it was used a spectrophotometric method based on the ability of the enzyme to inhibit pyrogallol autoxidation in the presence of EDTA. Readings were performed at 420 nm every 30 seconds [5].

SOD activity was expressed as U/mL where one unit of SOD is the amount of enzyme which inhibits pyrogallol autoxidation to 50%.

Glutathione reductase (Gred) catalyzes the reduction of oxidized glutathione (GSSG) in the presence of reduced NADPH.

Gred activity was determined *in vitro* in the presence of flavin adenine dinucleotide (FAD) and GSSG, by measuring the absorbance at 340 nm for 3 minutes.

Glutathione transferase activity was determined spectrophotometrically at 340 nm by measuring the transfer of CDNB (4-chloro 1,2 dinitro benzene) on GSH for 3 minutes. One unit of the enzymatic activity of GST is the quantity of enzyme that catalysis the formation of a one mol S- 2, 4 - dinitrophenyl glutathione/minute. The enzymatic activity was expressed as IU/ mg protein.

Malondialdehyde was measured using thiobarbituric acid method to evaluate lipid peroxidation [6]. MDA levels were expressed as $\mu\text{mol/mL}$. Total proteins were determined using Markwell method [7].

Nitrit-nitrate level was determined using Griess reactive [8].

Resulted data were expressed as means $\pm$ standard deviation (SD) and the differences between group means were tested by Student's t-test, where $p < 0.05$ was considered statistically significant.

Results and Discussion

Glutathione peroxidase was significantly reduced ($p < 0.05$) in ICH group 132.6 ± 58.7 U/mL compared to control 284.14 ± 44.8 U /mL (Figure 1). SOD was significantly reduced ($p < 0.001$) in the ICH group 2.76 ± 0.41 U/mL compared to the control group 1.73 ± 1.05 U/mL (Figure 2).

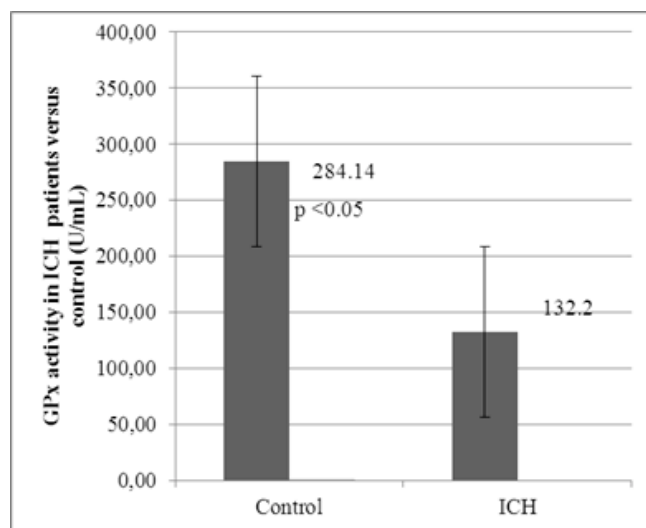


Figure 1.
GPx level in ICH patients *versus* control group

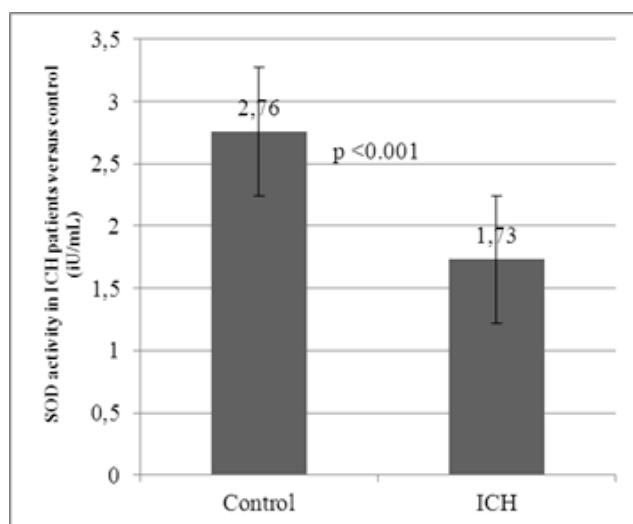
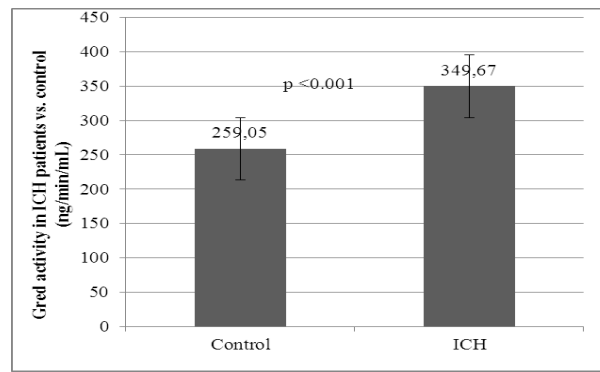
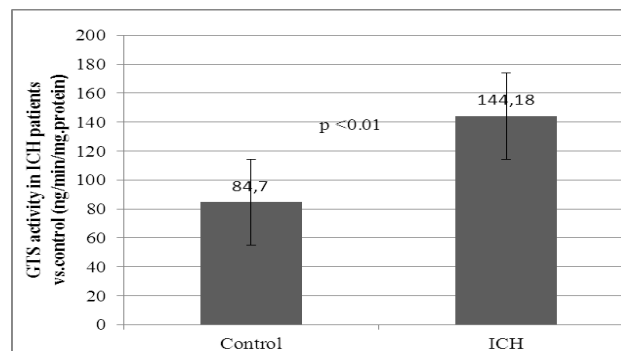
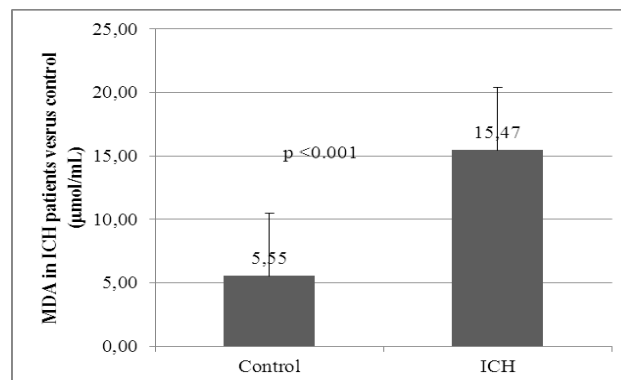


Figure 2.
SOD level in ICH patients *versus* control group

Gred activity was significantly increased ($p < 0.001$) in the ICH group 349.67 ± 67.6 ng/min/mL compared to control 259.05 ± 58.15 ng/min/mL (Figure 3). GTS was significantly increased ($p < 0.01$) in ICH group 144.18 ± 19.05 ng/min/mg protein compared to control 84.7 ± 22.86 ng/min/ mg protein (Figure 4).

**Figure 3.**Gred level in ICH patients *versus* control group**Figure 4.**GTS level in ICH patients *versus* control group

MDA level was significantly increased ($p < 0.001$) in ICH patients $5.55 \pm 3.15 \mu\text{mol/mL}$ *versus* control $15.47 \pm 2.56 \mu\text{mol/mL}$ (Figure 5).

**Figure 5.**MDA level in ICH patients *versus* control group

Nitrite nitrate levels were significantly increased ($p < 0.001$) in ICH patients 15.9 ± 2.54 ng/mL compared to control 2.06 ± 0.4 ng/mL (Figure 6).

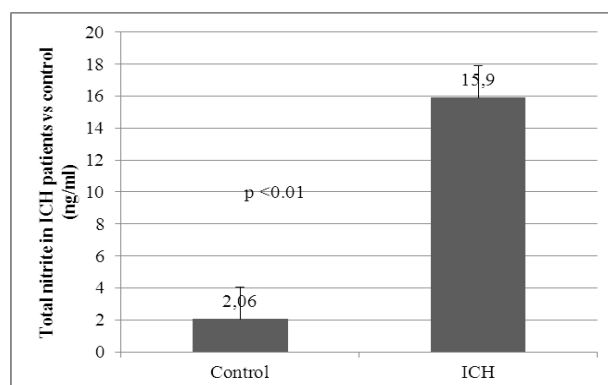


Figure 6.

Total nitrite level in ICH patients *versus* control group

Determination of the antioxidant enzymes activities in cerebrospinal fluid is an indirect method for the evaluation of the brain oxidative stress [9, 10].

SOD rapidly neutralizes free radicals resulted from the installation of an intracerebral hematoma and is overexpressed in the first 6 hours [11].

However, after that, SOD level is reflected by conflicting results in different studies. Some authors found maintenance of this enzyme at high values even after 24 hours, while others in contrast showed a reduction [12, 13].

Our data showed a reduced SOD activity, caused by superoxide anion excess [14].

Enzyme inactivation through distortion of protein structure or inactivation of the active site could be exerted by the increased concentration of lipid peroxidation products such as MDA [15].

GPx was also reduced in patients compared to healthy subjects; existing data showed that after brain injury, astrocytes release high amounts of ascorbate which is converted in dehydroascorbate (DHHA) in the presence of ROS. DHHA showed an inhibitory effect on GPx [16].

It was also showed that GPx is inactivated in contact with lipid peroxidation products (MDA and hydroxynonenal- HNE) [17].

Significant decreases in the activity of the main antioxidant enzymes SOD and GPx in the ICH group are primarily due to their active site inactivation. This mechanism has been documented experimentally for both SOD and GPx and was highlighted both on cultures of neurons and astrocytes. This significant decrease may be a marker of intense cerebral oxidative stress [18].

Gred significantly increased in ICH group compared to control. Due to oxidative stress, the reduction of intracellular GSH takes place and GSH reduction has a stimulatory effect on the activity Gred. Gred is also exposed to inactivation through oxidation of the two susceptible sulfhydryl groups. However, GST activity significantly increased in ICH group; in the presence of oxidative aggression GST activity doubled, this increase may a long-term adaptive response of the cerebral tissue [19].

The nitrite level was significantly increased in all ICH patients (100 %) *versus* control. This parameter reflects the generation of NO, secondary to N methyl D aspartate (NMDA) receptors activation [20]. Once synthesised, NO generates ONOO⁻, a much aggressive compound for both neurons and astrocytes [21, 22].

Conclusions

In ICH patients, the first line antioxidant enzymes activity decrease (SOD and GPx) is accompanied by a significant increase of Gred and GST. This process takes place on high oxidative aggression evoked by MDA and nitrite levels increase.

As long as oxidative stress worsens the clinical outcome, we propose the use of certain antioxidant enzymes as markers of the oxidative stress intensity in ICH patients.

References

1. Morgenstern L.B., Hemphill III J.C., Anderson C., Becker K., Broderick J.P., Connolly E.S., Greenberg S.M., Huang J.N. Macdonald L., Messe S.R., Mitchell P.H., Selim M., Tamargo R.J., on behalf of the American Heart Association Stroke Council and Council on Cardiovascular Nursing, AHA/ASA Guideline: Guidelines for the Management of Spontaneous Intracerebral Hemorrhage: A Guideline for Healthcare Professionals From the American Heart Association/American Stroke Association. *Stroke*, 2010; 41: 2108-2129.
2. Kelsey N., Wilkins H., Linseman D.A., Nutraceutical Antioxidants as Novel Neuroprotective Agents. *Molecules*, 2010; 15(11): 7792-7814.
3. Gilgun-Sherki Y., Melamed E., Offen D., Oxidative stress induced neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology*, 2001; 40(8): 959-975.
4. Mannervik B., Glutathione peroxidase. *Methods in Enzymology*, 1985; 113: 490-495.
5. Marklund S., Marklund G., Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *European J. of Biochemistry*, 1974; 47: 469-474.
6. Jentzsch A.M, Bachmann H., Fiirst P., Biesalski K.H., Improved analysis of malondialdehyde in human body fluids. *Free Radical Biology and Medicine*, 1996; 20(2): 251-256.
7. Markwell M., Haas S.M., Bieber L.L., A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.*, 1978; 87: 206-210.
8. Green L.C., Wagner D.A., Glogowski J., Analysis of nitrate nitrite in biological fluids. *Ann. Biochem.*, 1982; 126: 131-138.

9. Mihai L., Mitrea N., Papacoea R., Badarau A.I., Dynamic activity of some antioxidant enzymes in primary cultures of neurons under hypoxia and ischemia. *Farmacia*, 2012; 60(1): 49-57.
10. Mihai L.G., Mitrea N., Papacoea R., Ciornei C., Badarau A.I., Redox status in Wistar rat blood after hypoxia. *Farmacia*, 2012, 60 (3): 358-365.
11. Wang G., Yang Q., Li G., Time course of heme oxygenase-1 and oxidative stress after experimental intracerebral hemorrhage. *Acta Neurochir.*, Wien 2011; 153(2): 319-325.
12. Hussein A.S., Omayma A.T., Elwakil A., Biochemical Abnormalities in Brain Tissues during Acute Brain Stroke Induced in Wistar Rats. *Journal of Medical Sciences*, 2012; 12: 121-130.
13. Titova E., Ostrowski R.P., Rowe J., Effects of superoxide dismutase and catalase derivatives on intracerebral hemorrhage-induced brain injury in rats. *Acta Neurochir. Suppl.*, 2008; 105: 33-35.
14. Liu J., Teng L., Liu C., Augmented inhibitory effect of superoxide dismutase on superoxide anion release from macrophages by chemical modification with polysaccharide and attenuation effects on radiation-induced inflammatory cytokine expression *in vitro*. *J. Drug Target*, 2009; 3: 216-224.
15. Zheng W., Huang L.Z., Zhao L., Superoxide dismutase activity and malondialdehyde level in plasma and morphological evaluation of acute severe hemorrhagic shock in rats. *Am. J. Emerg. Med.*, 2008; 26(1): 54-58.
16. Nicolae A.C., Mitrea N., Drăgoi C.M., Constantinescu M.Z., Ciofrâncu C., Bărboi G., Arsene A.L., Murine studies regarding the variation of oxidative status in serum, hepatic and brain samples, after administration of some CNS active drugs. *Farmacia*, 2013; 61(4): 658-669.
17. Zhou H.G., Liu L., Zhang Y., Huang Y.Y., Tao Y.H., Zhang S., Su J.J., Tang Y.P., Guo Z.L., Hu R.M., Dong Q., Glutathione prevents free fatty acids-induced oxidative stress and apoptosis in human brain vascular endothelial cells through Akt pathway. *CNS Neurosci. Ther.*, 2013; 19: 252-261.
18. Papacoea T., Papacoea R., Bădăraș A., Oxidative stress and antioxidant therapy in intracerebral hemorrhage. *Therapeutics, Pharmacology and Clinical Toxicology*, 2011; XV(4): 270-273.
19. Aronowski J., Zhao X., Molecular pathophysiology of cerebral hemorrhage: Secondary brain injury. *Stroke*, 2011; 42(6): 1781-1786.
20. Sehba F.A., Bederson J.B., Nitric oxide in early brain injury after subarachnoid hemorrhage. *Acta Neurochir. Suppl.*, 2011; 110(1): 99-103.
21. Siuta M., Zuckerman S.L., Mocco J., Nitric oxide in cerebral vasospasm: theories, measurement, and treatment. *Neurology Research International*, 2013; ID Article: 972417.
22. Wang Z.P., Chen H.S., Wang F.X., Influence of plasma and cerebrospinal fluid levels of endothelin-1 and in reducing cerebral vasospasm after subarachnoid hemorrhage during treatment with mild hypothermia, in a dog model. *Cell Biochemistry and Biophysics*, 2011; 61(1): 137-143.

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