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Original paper

CYTOCHROME C OXIDASE IN PATIENTS WITH ACUTE ISCHAEMIC BRAIN DISEASE

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Summary: The aim of this study was to examine temporal dynamics of cerebrospinal fluid and erythrocyte haemolysate activity of cytochrome c oxidase in acute period of ischaemic brain disease. The study included 85 patients of both sexes, mean age 65 8 years. Control group consisted of 15 patients with radicular lesions of discal origin, subjected to diagnostic radiculography, without signs of interruption in the passage of cerebrospinal fluid. Results showed that during the first seven days of ischaemic brain disease significant decrease in cytochrome c oxidase activity in cerebrospinal fluid occurred. The decrease was highest during the first two days compared to control group (1.22 0.358 cyt c/mL). Significant increase in cytochrome c oxidase activity was established in erythrocyte haemolysate. The increase was the highest in brain infarction, somewhat lower in reversible ischaemic attack, and the lowest in transient ischaemic attack compared to controls. We concluded that cytochrome c oxidase activity in the cerebrospinal fluid and erythrocyte haemolysate of patients with acute ischaemic brain disease could be an indicator of metabolic dysfunction and neuronal cell damage.

Key words: ischaemic brain disease, cytochrome c oxidase, oxidative stress, cerebrospinal fluid

Introduction

Ischaemic brain disease (IBD) is a complex aetiopathogenetic process with many diverse clinical phenomena, and thus the recognition, confirmation and treatment of the clinical syndrome are extremely difficult. Molecular occurrings in ischaemia are mutually conditioned dynamic processes that, modulating each other, initiate a cascade of metabolic reactions responsible for cell adaptation and its survival, or in the case of exhaustion of cell defense capacities, they are responsible for its damage and cell death. The mechanisms of cell damage and death in ischaemia are complex and comprise all up till now known processes and mediators that cause nerve cell damage: disturbance of transport system of hydrogen ions and electrons in mitochondria, reduction of concentration of adenosine thriphosphate (ATP), acidosis, increase in intracellular concentration of free calcium (Ca^{2+}), uncontrolled formation of free radicals, lipid mediators derivatives of arachidonic acid, etc. (1, 2). One of the mediators of the brain parenchyma damage in ischaemic episodes are excitatory aminoacids that are released (glutamate, aspartate) and act as endogenic excitotoxins (3, 4). Process of excitotoxicity leads to cell death and damage of surrounding cells. Ca^{2+} triggers many toxic changes, including the activation of oxidoreductive stress, what makes pathophysiologic actions disperse in time and space like cascade reactions, while primary effects are enhanced (4 7).

It is absolutely proven that free radicals are included in ischaemic neuron damage, and that their damaging reactions may be induced with a cascade of glutamate-calcium (6, 7). Free radicals cause damage of cell membrane, while the process has the character of chain reaction, with rapid multiplication of free radicals, what disintegrates more the cell. Further complication, although a paradox, is additional formation of large quantities of free radicals and intensification of the process of lipid peroxidation in postischaemic reperfusion, when oxygen supply is increased what can aggravate the neurologic damage. If ischaemia is not complete and if there is a certain residual oxygen supply of ischaemic regions, and especially in the period

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of reperfusion when the oxygen supply is reestablished, oxidative neuron damage occurs (8). A cascade of oxidative stress reactions means further potential of free radicals production in the respiratory chain, in reactions of xanthine oxidase, and in reactions of autooxidation of catecholamine (8). Neutralization of free radicals is disabled because of a decreased content of antioxidants (tocopherol, vitamin C, glutathion, superoxide dismutase) (9 11).

In the cases of oxidoreductive stress, transport chain of electrons in mitochondria is the main source of superoxide radicals (.O_2); the place of their release is the complex NADH-coenzyme of Q reductasis and reduced forms of Q coenzyme. At the same time, the inhibition at the level of complex I of the respiratory chain reduces the energy production in the cell. During electrons transport through the respiratory chain, the proton translocation makes a proton starting force, at the same time which depends on the difference in proton concentration, and difference in charge between the two sides of interior mitochondria membrane. A new entrance of protons into the mitochondria matrix is necessary for the synthesis of ATP and is realized with the complex V (ATP-synthesis). Numerous experimental studies have establistied that mitochondria respiratory chain is most sensitive to the ischaemic damage (12 20).

In the cases of oxidoreductive stress, it is important to monitor the function of the ultimate component in the respiratory chain of mitochondria- complex IV, cytochrome c oxidase. All cytochromes, except cytochrome c, are integral proteins of mitochondria membrane. Cytochrome c is a mobile transmitter of electrons connecting the complexes III and IV. According to its structure cytochrome c oxidase is a haemo--lipo-cupro-protein, where copper (Cu) has a crucial role in the change of iron (Fe) valence in hem during the process of oxygen activation. It is established that the functional unit of this enzyme is composed of two Cu atoms and two cytochromes, while one Cu atom is firmly linked to the cytochrome a, and the other is loosely connected to the cytochrome a_3 and always follows the change of Fe valence (when Fe^{2+} + out of a_3 becomes Fe^{3+}, loosely connected Cu out of cupro becomes a cupri form). This enzyme catalyzes the four-electron reduction of the molecular oxygen into water, followed with the translocation of protons into intermembrane space of mitochondria: O_2 + 4e + $4H^+ \rightarrow 2H_2O$ (12, 21 25).

The aim of the study was to monitor the time dynamics of the change of cytochrome c oxidase activity in cerebrospinal fluid (CSF) and haemolysate of erythrocytes in patients with different kinds of acute IBD.

Methods

The study included 85 patients with acute IBD, they were of both sexes, mean age 65 8 years. Twenty four patients had transient ischaemic attack (TIA), 26 reversible ischaemic attack (RIA) and 35 brain infarction (BI). Diagnosis was made on anamnestic data, clinical results and computerized head tomography. All patients gave informed consent to be included into the study. Adequate clinical and biochemical parameters were monitored during the first seven days since the occurrence of ischaemic episode.

Control group included 15 patients, of adequate age and sex, with radicular lesions of discal origin (lumbosacral area), subjected to diagnostic radiculography, without signs of obstacles in the passage of CSF. The study included only patients with abrupt development of motor deficiency, without pains, so that they received no anti-pain therapy. Patients with intensive radicular pains that used nonsteroid analgetics, and patients with anamnestic and clinical data on the present inflammatory, malignant, renal, hepatic, pulmonary, neurodegenerative and psychiatric diseases, were excluded from the study.

In all patients samples of blood and CSF were taken at 9 a.m. Samples of the peripheral vein blood were collected into test tubes at cold, previously prepared with five U/L heparin and five mmol/L Na_2EDTA. Plasma was selected with centrifugation of full blood, and in further processing haemolysate of erythrocytes was prepared (26). Samples of haemolysate were kept at 70 ^{2}C and melted at room temperature immediately before analyzing (26). Samples of CSF were put during lumbar puncture into test tubes that were kept on ice (+4 ^{2}C) and after centrifugation were kept at 70 ^{2}C until adequate analysis.

Cytochrome c oxidase (ferro cytochrome c: oxygen oxidoreductase; EC 1.9.3.1) activity was measured in CSF samples and haemolysate of erythrocytes. The principle of the method is based on ferrous cytochrome c oxidation into ferric cytochrome c with the activity of cytochrome c oxidase out of sample, what is followed with reduction of absorption during 3 5 minutes at the wave length of 550 nm (27). Cytochrome c oxidase activity was expressed as mg cyt c/mL CSF, or as mg cyt c/mg Hb (27). Results are shown as mean value Ò standard deviation and analyzed with the implementation of Students's t-test.

Results

During the first seven days after the occurrence of ischaemic attack in patients with acute ischaemic brain disease the activity of cytochrome c oxidase in CSF was remarkabely reduced compared to the value found in the control group of patients (1.22 0.358 mg cyt c/mL CSF) (*Figure 1*). Minimal value of acute ischaemic brain disease was noted within the first two

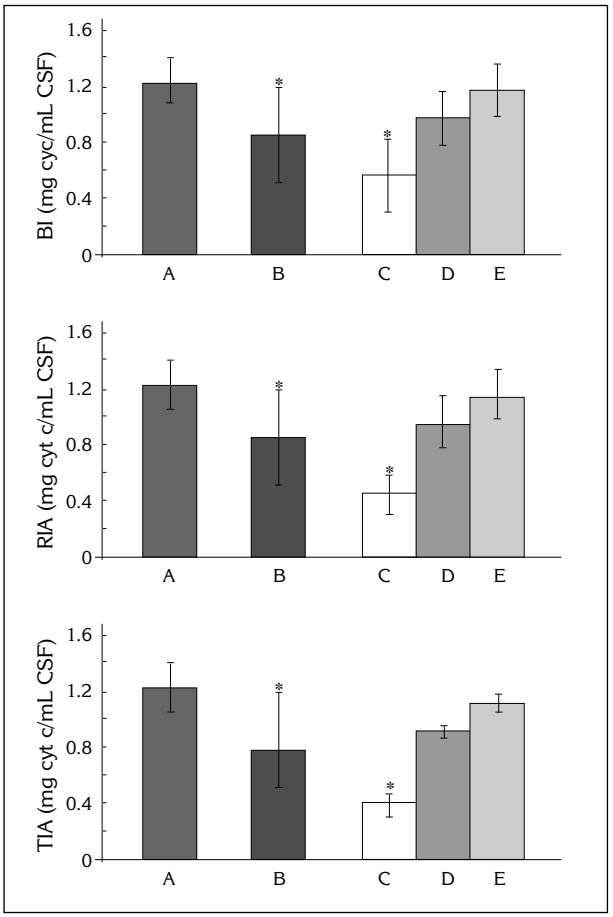

Figure 1. Activity of cytochrome c oxidase (mg cyt c/mL) in cerebrospinal fluid (CSF) in patients with acute brain infarction (BI), reversible ischaemic attack (RIA) and transient ischaemic attack (TIA). * $p<0.01$ in relation to control value (A). B activity in period within seven days, C on the first and second day, D on the third and forth day and E on the fifth up to seventh day after the onset of acute ischaemic insult.

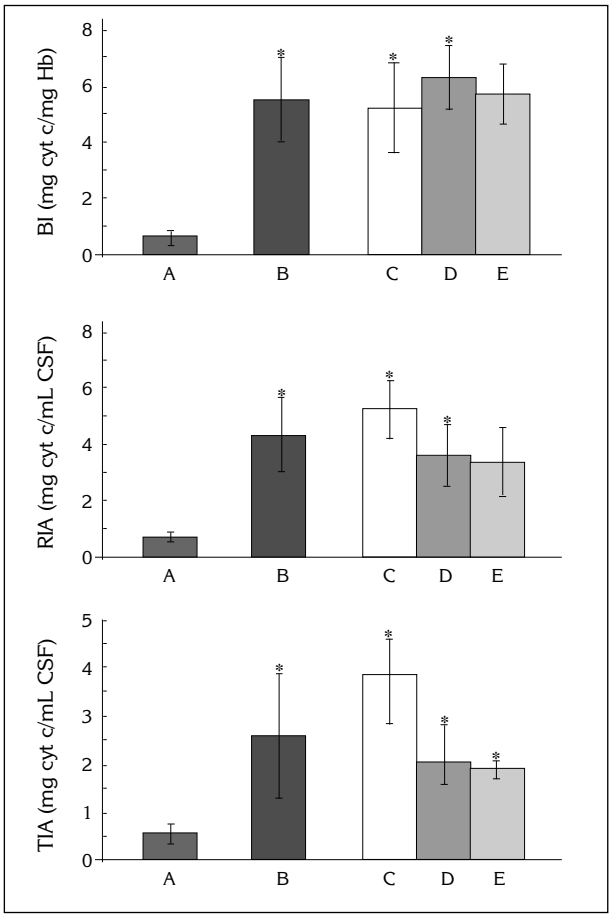

Figure 2. Activity of cytochrome c oxidase in haemolysate of erythrocytes (mg cyt c/mg Hb) in patients with acute brain infarction (BI), reversible ischaemic attack (RIA) and transient ischaemic attack (TIA). * $p<0.01$ in relation to control value (A). B activity within the first seven days, C on the first and second day, D on the third and fourth day and E on the fifth up to seventh day after the occurrence of acute ischaemic insult.

days after the occurrence of ischaemic attack (*Figure 1*). Minimal activity of cytochrome c oxidase in CSF patients with acute BI was 0.564 0.254 mg cyt c/mL CSF, in patients with RIA 0.456 0.128 mg cyt c/mL CSF, and in patients with TIA it was 0.401 0.063 mg cyt c/mL CSF, ($p<0.01$) (*Figure 1*).

Activity of cytochrome c oxidase in haemolysate of erythrocytes in patients with acute ischaemic brain disease measured within the first seven days after ischaemic stroke was significantly increased compared to the value reconded in the control group of patients (0.664 0.172 mg cyt c/mg Hb), ($p<0.01$) (*Figure 2*). In patients with BI the activity of cytochrome c oxidase in haemolysate of erythrocytes during the first seven days after the ischaemic attack was 5.506 1.49 mg cyt c/mg Hb ($p<0.01$) (*Figure 2*). Maximal value was noticed in the period of the third and fourth day after infarction, when it was 6.289 1.133 mg cyt c/mg Hb ($p<0.01$) (*Figure 2*).

Activity of cytochrome c oxidase in haemolysate of erythrocytes of patients with RIA within the first seven days after ischaemic stroke was 3.975 1.243 mg cyt c/mgHb, and in patients with TIA it was 2.595 1.225 mg cyt c/mgHb ($p<0.01$) (*Figure 2*). Maximal value in patients with RIA and TIA was established within the first two days after ischaemic stroke (*Figure 2*).

Discussion

Ischaemia is a metabolic disorder in brain functioning, caused by the reduction of circulation that ends in morphologic damage of nerve elements. Primary stimulus that triggers a cascade of metabolic changes that end in nerve cell death is energetic crisis and/or excessive (uncontrolled) depolarization (10). Numerous studies have established that transition of reversible into irreversible neuron damage depends, above all, on the gravity and length of ischaemia dura-

tion (2, 3, 6, 7, 28). Efforts of contemporary acute brain ischaemia therapy are directed to the protection of brain tissue before the development of irreversible damage takes place (2, 6, 7).

As it has already been mentioned, one of the main pathophysiologic mechanisms included into the development of central nervous system (CNS) damage in IBD is the formation of active metabolites of oxygen. In reactions with biological molecules, they provoke damage in a series of cell systems and functions. They cause damage of nucleine acids, and in processes of lipid peroxidation they damage biomembranes, protein enzyme components and transport systems, as well as polysaccharides and detoxic cell systems. Metabolic cascade of free radicals reactions causes in certain moments of its propagation the transition of disorders into irreversible and cells death (1, 6 8).

In ischaemia, the balance between the level of oxygen free radicals and endogen antioxidants is disturbed. The level of oxidative stress or balance between oxidants and antioxidants is determined by redox state in a cell. Balance between oxidative stress on one hand and metabolic potential on the other hand present a homeostatic mechanism inside the cell that protects or damages oxidants or antioxidants depending on which of these two events prevail (1, 2, 7). The results of our study have shown that the activity of cytochrome c oxidase in CSF is decreased during the acute IBD period. Activity is the lowest during the first two days after ischaemic stroke, then it gradually rises, and in the period from the fifth to the seventh day after ischaemia it returns to the control value. This may be the neuron attempt at the level of respiratory chain to compensate the increased formation of free radicals induced with ischaemia.

During the acute brain ischaemia, besides development of oxidoreductive stress and disorder of mitochondrial energetic metabolism there is also activation of microglia (29). Some new results point to the fact that toxic materia formed during microglia response cause inactivation of key enzymes of Crebs cycle and energetic metabolism, and this effect causes the inhibition of complex IV (22, 23, 29). Inhibition of complex IV causes increase in concentration of reactive oxidative species, what, further on, induces and complicates damage of nerve elements (29).

The latest studies of gerbils showed that the level of mRNK of the sub-unit I cytochrome c oxidase is reduced in neurons of CA1 sector of hypocampus after brain ischaemia (12, 16). Also, activity of cytochrome c oxidase shows early reduction in neurons of CA1 sector. This is achieved with reduction of the level of adequate DNK after ischaemia and detects damage of mitochondrial DNK expression at the level of transcription. This disturbance in expression of mitochondrial DNK has, as its consequence, damaged formation of energy, what can lead to neuron death in cases of ischaemia (12, 16).

Taking into account that redox state of cytochrome c oxidase changes when oxygen and glucose lack and in ATP depletion, then the degree of the activity of cytochrome c oxidase may be an indicator of cell dysfunction in acute period of brain ischaemia. Besides, it has been established that reperfusion after brain ischaemia leads to transient hyperoxigenation and tissue hyperemia, and to hyperoxigenation of mitochondria electronic bearers (12, 21 25). It is possible that the formed mitochondrial hyperoxigenation induces residual intracellular changes that take part in the recovery of metabolism and electric activity in neurons in recirculation period. On the other hand, mitochondrial hyperoxigenation may induce release of a great amount of free radicals that caused the already existing damage of nerve elements. Balance between these two events in acute period of brain ischaemia determines the direction and outcome of damage caused by ischaemia (30).

Results of our study have shown that the activity of cytochrome c oxidase in haemolysate of erythrocytes has increased remarkabely within the first seven days after the occurence of ischaemia. The increase is the greatest in patients with BI, somewhat lower in RIA and the lowest in TIA. These changes may be understood as a systematic response of organism to acute brain ischaemia and an attempt of organism to compensate local disorders. Organism tries by supply into periphery tissues to recompense the lack of substrates necessary for keeping energetic and antioxidative brain status in the cases of ischaemia. Brain infarction, as the most serious aspect of brain ischaemia with the highest damage of brain parenchyma causes, as its consequence, the most expressed general reaction, i.e. organism attempt to compensate disturbed homeostasis.

The results of our study have shown that monitoring of the activity of cytochrome c oxidase in CSF and haemolysate of erythrocytes in patients with IBD in acute period may be relevant in early diagnosis of this desease. Biochemical changes in CNS caused by acute brain ischaemia (demonstrated with changes in CSF), cause relevant changes in elements of system circulation (erythrocytes). First, this indicates that there is a connection of circulatory and neuron compartments. Second, these changes may indicate the degree of neurologic damage, and changes at the systemic level during brain ischaemia. In addition, our results point to the role of cytochrome c oxidase in IBD pathogenesis, and the importance of polyvalent therapy which includes the administation of antioxidants in acute IBD period.

CITOHROM C OKSIDAZA U BOLESNIKA SA AKUTNOM ISHEMIJSKOM BOLEŠĆU MOZGA

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Kratak sadržaj: U istraživanju je praćena vremenska dinamika aktivnosti citohrom c oksidaze u cerebrospinalnoj tečnosti i hemolizatu eritrocita bolesnika sa akutnom ishemijskom bolešću mozga. Ispitivano je 85 bolesnika, oba pola, prosečne starosti 65 8 godina. Kontrolna grupa imala je 15 pacijenata sa radikularnim lezijama diskalnog porekla, podvrgnutih dijagnostičkoj radikulografiji, bez znakova smetnji u pasaži cerebrospinalne tečnosti. Rezultati su pokazali da se tokom prvih sedam dana akutne ishemijske bolesti mozga značajno snižava aktivnost citohrom c oksidaze u cerebrospinalnoj tečnosti a sniženje je najveće tokom prva dva dana u odnosu na kontrolu (1.22 0.358 cit c/mL). U hemolizatu eritrocita zabeležen je značajan porast aktivnosti citohrom c oksidaze. Maksimalan porast je zabeležen kod bolesnika s infarktom mozga, nešto niži kod reverzibilnog ishemijskog ataka i najniži kod tranzitornog ishemijskog ataka. Zaključeno je da aktivnost citohrom c oksidaze u cerebrospinalnoj tečnosti i hemolizatu eritrocita bolesnika s akutnom ishemijskom bolešću mozga može biti pokazatelj metaboličke disfunkcije i oštećenja nervnih ćelija.

Ključne reči: ishemijska bolest mozga, citohrom c oksidaza, stres oksidativni, cerebrospinalna tečnost

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