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BRAIN STEM AND THALAMUS ANTIOXIDATIVE DEFENSE IN EXPERIMENTAL SEPSIS

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Although brain complications in sepsis are not rare, early pathophysiologic events had not been made clear yet. We have considered antioxidative components-glutathione peroxidase (GSHPx) activity and reduced glutathione (GSH) concentration in two brain integrative centers, i.e the brain stem (BS) and thalamus. Sepsis was induced in adult male Wistar rats (200-250 g) by cecal ligation and perforation (CLP) with inoculation of Escherichia coli suspension (ATCC 25922) (n=40). The control group was sham operated (n=40). For each time point (0, 12, 24 and 72 hours) after treatment, ten animals within each group were decapitated. In BS, GSHPx activity increased at 12 and 24 hours after CLP, while in the thalamus, GSHPx activity increased at 72 hours, compared to controls. In BS, GSH concentration decreased at the 12th and 24th hour, and in the thalamus it decreased at the 72nd hour. Changed oxidative status in BS, recorded as soon as the 12th hour, reflects a prompt reaction of the central nervous system. This could be of great consequence for disturbed vasomotor response during sepsis.

Key words: brain stem, glutathione, glutathione peroxydase, rat, sepsis, thalamus

INTRODUCTION

Numerous cellular and metabolic mechanisms that initiate sepsis, leading it toward septic shock, are not completely understood. Following microbes blood invasion, release of different toxic products emerges (Vallance P and Chan N, 2001). Humoral reactions (complement and coagulation systems) and cellular components (endothelium, monocytes/macrophages, neutrophils) activate, participating with the release of numerous mediators such as: cytokines, arachidonic acid metabolites, nitric oxide (NO), reactive oxygen species (ROS) etc. Such events additionally amplify the inflammatory response and reflect on all tissues (Cuzzocrea *et al*., 2001). The typical haemodynamic pattern in sepsis is reflected in high cardiac output, low systemic vascular resistance and

hypotension refractory to vasopressing agents (Brady, 1993). The state of oxidative stress in inflammation is provoked by blood redistribution, oxygen consumption misbalance and respiratory "burst", which occurs as a result of circulatory changes and increased immune activity. Beside oxygen consumption discrepancy between organs, inside the same organ local ischemia zones can happen as well (Gutierrez, 1993).

The central nervous system (CNS) is protected by the blood-brain barrier (BBB), but it still can be affected by mediators from the site of infection, from peripheral tissue nerve afferences, as well as from the reflected signals inside the brain (Anning *et al*., 1999). Although brain complications in sepsis are not so rare, early pathophysiologic events in some regions of the brain have not been made clear yet (Papadopoulous *et al*., 2000).

Respiratory and circulatory centers are located in the brain stem. Taking into consideration the phenomenon of vasoparesis during sepsis, the state of the circulatory center is of great importance for sepsis development (Abu-Zidan *et al*., 2002). The thalamus is also an integrative brain structure. It gathers cortical and subcortical information closely connected with motor and sensor functions (Marinković *et al*., 1989). The thalamus shows dense projective and intrathalamic connections. Intrathalamic connections merge some of the nuclei inside the thalamus, while projective fibers include massive thalamocortical and corticothalamic connections. Some thalamic nuclei join output projections of basal ganglia, while some of them are part of the limbic system (Graybiel, 1990).

Considering unlightening early biochemical events in CNS during sepsis, as well as common brain comlications which appear during or after survived sepsis, we were urged to investigate the oxidative status in these two brain structures (Barichello *et al*., 2005).

MATERIALS AND METHODS

Experimental procedure

Adult Wistar rats, males, 11 weeks old, weighting between 200 and 250 g, were used in the experiments. The animals were housed inside a climate-controlled facility. Food and water were available *ad libitum*. All animals were cared for in strict agreement with good laboratory practice and in accordance to guidelines for the humane care of animals, ethical principles of Military Medical Academy, and valid federal statute (Sl. list SRJ, 1998).

The animals were randomized into sepsis and control groups. Within both groups, there were four subgroups, with 10 animals in each subgroup. Before the treatment, all animals were anaesthetized with ether.

Operative procedure

Sepsis had been induced by cecal ligation and perforation (CLP model) with intracecal inoculation of 1mL of *Escherichia coli* suspension (ATCC 25922) (Stojanović *et al*., 2004). The operation procedure was as following: the middle incision was made at the venter, the cecum was taken out, ligated, and a suspension of *E. coli* (14-gauge puncture) was applied. The cecum was then

removed and the venter had been sutured up at two layers. Control animals were sham operated (middle laparotomy and cecal exposure without any manipulation). All treated animals were housed in single cages with free access to food and water.

The animals from the appropriate subgroups were decapitated at time points of 0 (immediately after the operation), 12, 24 or 72 hours from the operation. The heads were at once frozen in liquid nitrogen and stored at -70°C until subsequent analysis.

Sample preparation

Brain structures-brain stem and thalamus were dissected on ice. For GSHPx activity samples were homogenized in cold buffered sucrose- [0.25 M sucrose; (Serva, Feinbiochemica, Heidelberg, New York)], 10 mM phosphate buffer pH 7.0 and 1 mM EDTA (Sigma chem. co. St. Louis, USA). The homogenates were centrifuged at 2000x g for 15 min at 4°C. Crude sediments were redissolved in sucrose medium and centrifuged again. The supernatants were centrifuged at 3200x g for 30 minutes at 4°C, and the obtained sediments were resolved in deionized water. After one hour of incubation, the samples were centrifuged at 3000x g for 15 minutes at 4°C, and supernatants (crude mitochondria fractions) were stored at –70°C (Gurd *et al*., 1974). For reduced glutathione (GSH) determination, samples were homogenized in 10% sulfosalicylic acid (Sigma chem. co. St. Louis, USA). Proteins were determined by the Lowry method using bovine serum albumin as standard (Lowry and Passonneau, 1974).

Glutathione peroxidase activity

Glutathione peroxidase activity (EC 1.11.1.9; GSH-Px) was measured spectrophotometrically at 340 nm. The reaction was based on the oxidation of GSH to its oxidized form (GSSH), in the presence of glutathione reductase (GSHR, E.C. 1.6.4.2, Sigma, St. Louis, USA). The reaction was followed in the potassium-phosphate buffer (50 mM pH- 7.0; Serva, Feinbiochemica, Heidelberg, New York), containing 1 mM EDTA (Sigma, St. Louis, USA) (Maral *et al*., 1987).

Reduced glutathione determination

Reduced glutathione was determined using 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 36.9 mg in 10 mL of methanol), which previousli reacted with aliphatic tiol compounds in Tris-HCl buffer (0.4 M, pH 8.9), thus making a yellow colored p-nitrophenol anion. Spectrophotometric measurements of GSH concentration were carried out at 412 nm (Anderson, 1986).

Statistical analysis

Descriptive data were expressed as the mean±standard deviation (SD). Statistical analysis was performed using a statistical software program (Statistic 5.0 for Windows). Groups were compared by Student's t-test and two-way analysis of variance. Differences were considered significant at $p<0.05$

RESULTS

GSHPx activity in the brain stem, was found to be increased at 12th and 24th hour (developed stage of sepsis), compared to controls at appropriate time points, as well as compared to values at the beginning. At early terms, as well as at the late term (72nd hour), there were no changes in GSHPx activity (Figure 1).

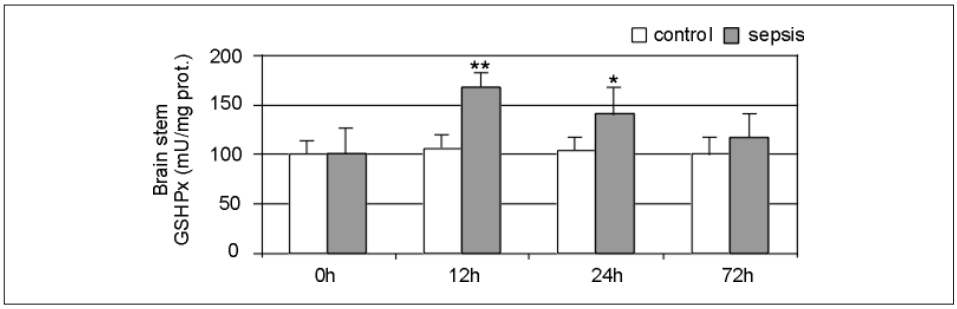

Figure 1. Glutatione peroxidase activity (GSHPx) in the brain stem of Wistar rats in sepsis. Values are given in mU/mg proteins, mean±SD.
$p<0.05$; $^{**}p<0.01$ Significance to corresponding controls

Contrary to the increases in GSHPx activity during the developed stage of sepsis in the brain stem, GSHPx did not increase in the thalamus until 72 hours from the operation, compared to controls (Figure 2).

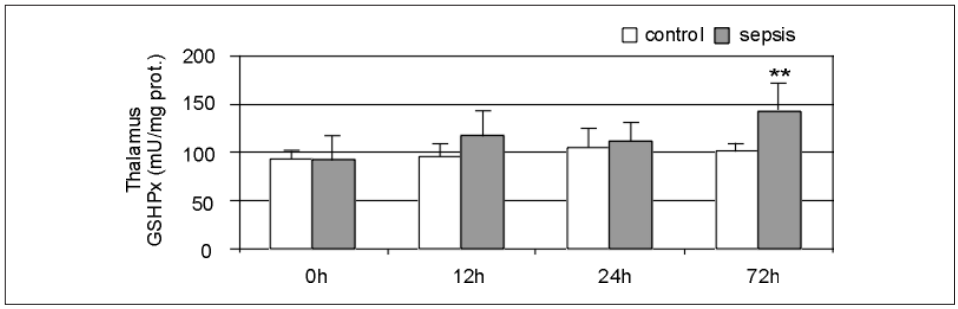

Figure 2. Glutathione peroxidase activity (GSHPx) in the thalamus of Wistar rats in sepsis. Values are given as mU/mg protein, mean±SD.
$p<0.05$; $^{**}p<0.01$ Significance to corresponding controls

Reduced glutathione concentration in the brain stem decreased at 12th and 24th hour compared to controls, but also compared to 0-hour concentrations. At early and late stages of sepsis, no changes in GSH concentration were found (Figure 3).

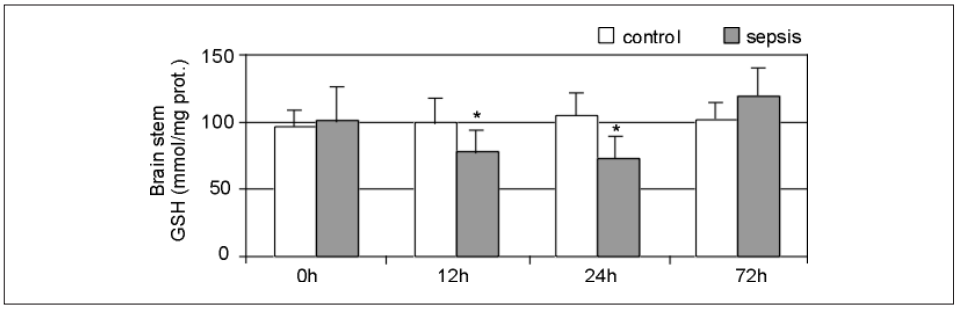

Figure 3. Reduced glutathione (GSH) concentration in brain stem of Wistar rats in sepsis. Values are given in nmol/mg protein, mean±SD.
p<0.05; **p<0.01 Significance to corresponding controls

Reduced glutathione concentration showed no changes in the thalamus at the early and developed stages, until 72nd hour. After this time point it decreased, compared to controls (Figure 4).

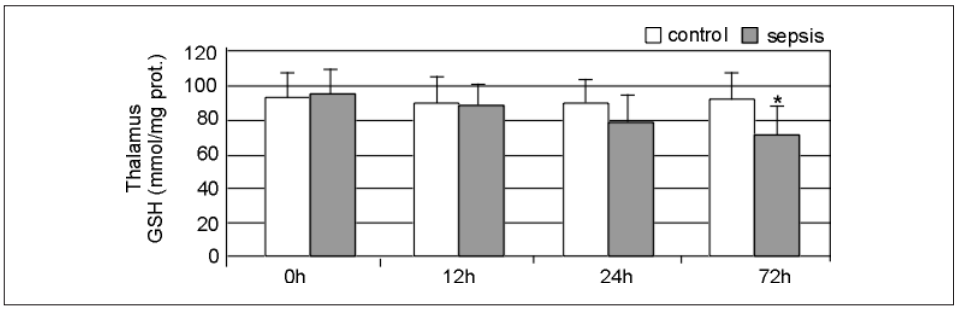

Figure 4. Reduced glutathione (GSH) concentration in thalamus of Wistar rats in sepsis. Values are given in nmol/mg protein, mean±SE.
p< 0.05; **p<0.01 Significance to corresponding controls

DISCUSSION

The products arising in the microcirculation due to neutrophil activation, play an important role in spreading tissue damage in sepsis (Cohen, 1995). In conditions of anaerobic metabolism, as it happens during tissue hypoxia, glycolisis restriction and energy disruption arise (Darley-Usmar and Halliwell, 1996). Information about peripheral tissue destruction through nerve afferences challenge CNS reactions, but also overtake endothelial cells in cerebral capillaries inside BBB (Curzen *et al*., 1994). The main site of tissue reaction in sepsis is the endothel (Benjamim *et al*., 2000).

Antioxidative defense maintains ROS metabolism rate in the brain. Glutathione peroxidase is a critical intracellular enzyme, involved in detoxification

of hydrogenperoxide (H_2O_2) to water. It has been found that knock-out of GSHPx may be adequately compensated under nonstressed conditions, but administration of mitochondrial toxins increases the need of oxygen radicals detoxification by GSHPx (Smith *et al.*, 1996). This enzyme, coupled to reduce nicotine adenine diphosphate (NADP) regenerating systems via GSHR, is virtually able to guarantee an adequate protection of biological structures against oxidative attack (Wolin *et al*., 2005). Increased GSHPx activity in the brain stem at a developed stage of sepsis (12^{th} and 24^{th} hour) confirms a fast reaction of structures with vital centers, that is in accordance to the exchange of information between periphery and CNS. Energy crisis obviously causes oxidative stress in which H_2O_2 production is increased. Glutathione peroxidase eliminates H_2O_2, reducing it, in reaction with GSH, to GSSH and water. Glutathione peroxidase also reduces other hydroperoxides, including hydroperoxide of oiled acids. On such a way, it prevents damage caused by H_2O_2 and H_2O_2-dependant producing ROS (Shan *et al*., 1990). Increased GSHPx activity at different times - in the brain stem at 12^{th} and 24^{th} and in the thalamus at 72^{nd} hour, could be the result of increased needs (increased superoxide anione production, with consequent increased H_2O_2 production) and adequate antioxidative defense in the investigated structures. In addition, it can be the result of modified signalisation inside cells i.e. activated nuclear factor - κB, which is reflected in GSHPx upregulation on the genetic level (Connellu *et al*., 2001).

Decreased GSH concentration in the same period (12^{th} and 24^{th} hour in the brain stem and at 72^{nd} hour in the thalamus) could be the measure of early disrupted tissue environment, but also increased consumption of GSH for increased maintenance of GSHPx activity. Unchanged GSHPx activity and GSH concentration in the thalamus until 72^{nd} hour (late stage sepsis) confirm good antioxidative potentials of this integrative structure in rats brain during early and developed stages of sepsis.

Reactivity of ROS overlaps NO metabolism, whose overproduction occours in sepsis (Vallet, 2000). The state of oxidative stress in the cell can activate the transcription factor NF-κB, which than can induce transcription of genes, that code proteins included in inflammation (such is the enzyme inducible nitric oxide synthase) (Mac Donald *et al*., 2003). Decreased GSH could be the result of its reaction with peroxynitrite ($ONOO^-$). Peroxynitrite originates from the reaction of superoxide anion ($O_2^{\bullet-}$) and NO (Bolanos *et al*., 1995). It is known that $ONOO^-$ is the most powerful cause of energy disruption, because it can irreversibly inhibit the mitochondrial electron chain by binding to Fe-S groups. Reduced glutathione possesses high affinity for reactive nitric species (among them $ONOO^-$) and ROS. It is known that GSH depletion increases NO-dependent cytotoxicity. In addition, nitrosoglutathione, which originates from GSH reaction with nitrogen agents, may inhibit numerous enzyme pathways, even enzymes included in GSH maintenance. Moreover, nitrosoglutathione can be regarded as a kind of nitric reservoir, emerging feature of NO (Boveris *et al*., 2002).

It is known that oxidative or nitrifying modifications of sensitive target proteins in the cell may have a dual role. Under conditions of moderate oxidative stress, cysteine (Cys) oxidation can lead to the reversible formation of mixed

disulfides between protein thiol groups and low-molecular-mass thiols, such as GSH. Reversible modifications (at Cys and methionine residues) can modify protein function, but also protect them from irreversible modifications. Therefore, GSH is not only a cofactor of GSHPx, but also is a tripeptide with great defensive potentials against irreversible molecular damages (Smith *et al*., 1996). The process of S-glutathionylation is a regulatory reaction, which can serve as a mean of storing GSH, as well as redox-sensitive post-translation modification, that play a key regulatory role in signal transduction (Giustarini *et al*., 2004). Decreased GSH concentration in the brain stem and thalamus, besides consumption by GSHPx could be the result of regulatory defense mechanisms against irreversible functional disturbances.

Dynamic of antioxidative defense misbalance during sepsis reflects on the brain stem at developed stage of sepsis. According to the here presented viewpoint, integrative functions of the thalamus are protected up to 24 hours. However, antioxidative changes demonstrated in this work could participate in the development of brain damage during sepsis.

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ANTIOKSIDATIVNA ZAŠTITA U MOŽDANOM STABLU I TALAMUSU U EKSPERIMENTALNOJ SEPSI

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SADRŽAJ

Moždane komplikacije u toku sepse nisu retke, ali patofiziološki događaji u nekim delovima mozga još uvek nisu razjašnjeni. U ovom radu su razmatrani rezultati dobijeni određivanjem antioksidativne komponente – aktivnost glutation peroksidaze (GSHPx) i koncentracije redukovanog glutationa (GSH) u integrativnim moždanim centrima, moždanom stablu i talamusu. Odraslim Wistar pacovima, mužjacima (200-250 g) sepsa je izazivana cekalnom ligacijom i perforacijom (CLP) uz inokulaciju suspenzije *Escherichia coli* (ATCC 25922) (n=40). Kontrolne životinje su bile lažno operisane (n=40). U vremenski određenim terminima (0, 12, 24 i 72 časa) nakon tretmana, po deset životinja iz svake grupe je bilo dekapitovano. U moždanom stablu, aktivnost GSHPx povećala se u 12. i 24. času nakon CLP, dok se u talamusu, aktivnost GSHPx povećala u 72. času u odnosu na kontrolu. U moždanom stablu, koncentracija GSH smanjila se u 12. i 24. satu, a u talamusu se smanjila u 72. satu, u odnosu na kontrolu. Promenjen oksidativni status u moždanom stablu, već nakon 12 h od CLP pokazuje brzu reaktivnost centralnog nervnog sistema, što bi mogao da bude značajan faktor poremećaja vazomotornog odgovora u sepsi.