

Nitric oxide synthase inhibitors partially inhibit oxidative stress development in the rat brain during sepsis provoked by cecal ligation and puncture

Milica B. Ninković¹, Živorad M. Maličević¹, Ankica Jelenković², Mirjana M. Đukić³, Marina D. Jovanović¹ and Ivana D. Stevanović¹

¹ *Laboratory of Clinical Physiology, Institute of Medical Research, Military Medical Academy, Belgrade, Serbia*

² *Institute of Biology Research “Siniša Stanković”, Belgrade, Serbia*

³ *Department of Toxicological Chemistry, Faculty of Pharmacy, Belgrade, Serbia*

Abstract. Oxidative stress development in different brain structures and the influence of nitric oxide (NO) overproduction during sepsis was investigated using male Wistar rats. Rats were subjected to cecal ligation and puncture (CLP) or were sham-operated. To evaluate the source of NO production, 30 min before the operation septic and control animals were treated with single intraperitoneal doses of NO synthase (NOS) inhibitors: L-NAME and aminoguanidine (AG) (10, 30 or 90 mg/kg) and 7-nitroindazole (7-NI) (30 mg/kg). The concentration of GSH in the cortex, brain stem, cerebellum, striatum and hippocampus significantly decreased post CLP at both early and late stage sepsis. Lipid peroxidation also occurred in the cortex and cerebellum in late stage sepsis. Pre-treatment with a non-selective NOS inhibitor (L-NAME) and with selective inducible and neuronal NOS inhibitors (AG and 7-NI) revealed benefit effects on the GSH concentrations. Unexpectedly, NOS inhibition resulted in diverse effects on TBARS concentrations, suggesting the importance of specific quantities of NO in specific brain structures during sepsis.

Key words: Sepsis — Brain — Nitric oxide synthase inhibitors — Rat

Introduction

Sepsis and multiple organ dysfunctions are the leading causes of death among hospitalized patients in critical care units. Despite the availability of specialized therapy, mortality rates remain unacceptably high (Levy et al. 2003). Unfortunately, pathophysiological mechanisms underlying sepsis development (systemic inflammation, coagulopathy and systemic vascular dysfunction) are complex and are often undetectable until symptoms become visible at which time therapy is initiated (Rivers et al. 2005). One of the characteristic hallmarks of sepsis is tissue damage which is a consequence of gross systemic disturbance. A hyperactive immune system coupled with misbalanced overproduction of a number of endogenous

mediators, are two examples of the host response (Hotchkiss and Karl 2003).

Nitric oxide (NO) overproduction is a known occurrence in sepsis (Feihl et al. 2001). Due to the fact that NO is a key regulator of homeostasis in almost all known tissues, understanding both its direct and indirect actions are of particular interest during sepsis conditions (Poon et al. 2003). In addition to NO's well-documented roles in vascular homeostasis (vasodilatation, hemostasis, coagulation and immunomodulation) that influence septic processes, NO regulates a number of functions in the central nervous system (CNS) (Esplugues 2002).

NO serves as a neurotransmitter and a neuromodulatory mediator for inter-neuronal communication (Wiklund et al. 1997). Furthermore, NO participates in neuroendocrine regulation as well as in long-term potentiation that form the foundations for complex processes including learning, memory, sleep regulation, pain and appetite (Garthwaite and Boulton 1995). It is also crucial for correct cerebellar activity. The influence of NO on synaptic plasticity, long-term

Correspondence to: Milica B. Ninković, Laboratory of Clinical Physiology, Institute of Medical Research, Military Medical Academy, Crnotravska 17, 11002 Belgrade, Serbia
E-mail: ninkomj@ptt.yu

potentiation, receptor neuromodulation as well as in the above-mentioned processes within blood vessels all together influence whole body homeostasis (Liaudet et al. 2000).

Brain complications during or after sepsis are not rare (Angus et al. 2001). Barichello and colleagues demonstrated that rats that survived sepsis showed decreased cognitive functions (Barichello et al. 2005). Reciprocal interactions between the immune and CNS seems to be the major components of the host response to sepsis. However, the dynamic of changes remain unresolved (Sharshar et al. 2005). They are related to neurochemical interaction complexity in the CNS, anatomical and functional brain defense that has developed throughout evolution as well as control of physiological functions that are crucial for maintaining homeostasis and orchestrating the host response at autonomic, neuroendocrine and behavioral levels (Sharshar et al. 2004). The oxidative milieu inside brain tissue reflects brain function during sepsis progression. Although brain tissue is approximately 2% of the total body weight, blood flow through the brain contributes to almost 15% of the total body blood flow. Cells within the blood brain barrier possess a high antioxidative capacity (consisting of enzymes: superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase and non-enzyme components such as glutathione) (Gherzi-Egea et al. 1994). During sepsis, immune cells in the circulation exhibit features characteristic of an oxidative burst (as a defense mechanism) and contribute to conditions of enhanced oxidative stress at the blood brain barrier. At the same time, increased NO production can lead to nitrosative stress. Tissue homeostasis within the brain stem during system inflammation is of huge importance (Ninkovic et al. 2008). In addition, some brain structures possess selective vulnerability to oxidative as well as nitrosative stress development. These include the cerebral cortex (especially the third layer), the striatum (the caudate nucleus and the putamen), the hippocampus (CA1 and CA3 sector) and the cerebellum (Purkinje cells). Therefore, we sought to answer the following questions: In which brain regions do conditions of oxidative stress arise during sepsis progression? Does the blood brain barrier protect the oxidative milieu inside brain tissue during sepsis progression and what are NO's roles in such events?

Materials and Methods

The study was performed in accordance with the Guidelines for Animal Study No. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia). Eleven-week-old male Wistar rats weighing between 250 and 300 g were purchased from the laboratory animal care centre located in the Military Medical Academy. *Escherichia coli* suspension – ATCC 25922 (33% agar in 0.7% saline solution) was

manufactured in the Institute of Immunology and Virology, Torlak, Serbia.

The rats were housed five per cage with free access to food and water. For adaptation purposes they were maintained under standard laboratory conditions (room temperature and a circadian regime of light/dark ratio of 13 : 11 h) for at least seven days before the study.

Sepsis was induced by a modified method of cecal ligation and puncture (CLP) (Stojanovic et al. 2002). The procedure was performed in a sterile manner under brief diethylether anaesthesia. After midline laparotomy, the content of the cecum was squeezed towards the ileocecal valve. The cecum was tied with a 3-0 silk suture one centimetre from the distal end leaving the ileocecal valve free. *Escherichia coli* suspension (1 ml) was injected distal to the point of ligation using a 14-gauge needle. The above-mentioned procedure was performed inside the opened abdominal cavity ensuring that a small amount of *E. coli* suspension leaked from the puncture. The abdominal wall was then closed with silk sutures consisting of two layers. Sham operations were performed without CLP. After surgical procedures all animals had free access to food and water.

Animals were randomly assigned to the sepsis (CLP) group or to the sham-operated (control) group. Animals were also treated with non-selective/selective NO synthase (NOS) inhibitors before surgery. The non-selective NOS inhibitor, L ω -nitro-L-arginine methyl ester (L-NAME) (Sigma) and the selective inducible NOS (iNOS) inhibitor aminoguanidine (AG) (Sigma) were dissolved in 0.9% saline solution. The selective neuronal NOS (nNOS) inhibitor, 7-nitroindazole (7-NI) (Sigma) was dissolved in olive oil (Robert et al. 1997). All three inhibitors were administered intraperitoneally (i.p.) 30 min before surgery. The doses, the routes of administration and the pre-incubation times of the NOS inhibitors were chosen on the basis of published data and according to experience in our laboratory (Benjamim et al. 2002).

Experimental subgroups were defined according to the dose of the applied NOS inhibitor. Rats in the L-NAME 10 + CLP, the L-NAME 30 + CLP and the L-NAME 90 + CLP as well as those in the L-NAME 10 + control, the L-NAME 30 + control and the L-NAME 90 + control subgroups received L-NAME 30 min before surgery at a dose of 10, 30 or 90 mg/kg i.p.

Rats in the AG 10 + CLP, the AG 30 + CLP and the AG 90 + CLP as well as those in the AG 10 + control, the AG 30 + control and the AG 90 + control subgroups received AG 30 min before surgery at a dose of 10, 30 or 90 mg/kg i.p.

Rats in the 7-NI 30 + CLP and the 7-NI 30 + control subgroups received 7-NI 30 min before surgery at a dose of 30 mg/kg i.p.

To study the time-course of inflammation during sepsis, each subgroup was randomly subdivided into early and late stage sepsis, each consisting of 10 animals killed after 3 h or

48 h, respectively, after surgery. Rats were killed by decapitation. Their heads were immediately frozen in liquid nitrogen and stored at -70°C until use. Brain structures (cortex, brain stem, cerebellum, striatum and hippocampus) were removed on ice and then homogenised in cold buffered sucrose medium (0.25 mol/l sucrose (Serva Feinbiochemica), 10 mmol/l phosphate buffer (pH 7.0) and 1 mmol/l EDTA (Sigma)) (Gurd et al. 1974). Proteins were determined by the Lowry method using bovine serum albumin as a standard (Sigma) (Lowry et al. 1951).

Thiobarbituric acid reactive species (TBARS) were measured spectrophotometrically at 533 nm (Girotti 1991).

GSH in brain samples was measured spectrophotometrically at 412 nm (Ellman 1959).

Statistical analysis of the data was performed using Statistic 5.0 for Windows. Descriptive data are expressed

as the mean \pm standard deviation (SD). Data among the groups were analysed by two-way analysis of variance and the Students *t*-test. Differences were considered statistically significant when $p < 0.05$ and highly statistically significant when $p < 0.01$.

Results

A highly significant decrease in GSH in brain tissue was found in early stage sepsis when compared with control rats ($p < 0.01$) (Table 1). Pre-treatment with L-NAME followed by induction of sepsis led to a significant decrease in GSH in almost all the brain structures, (the exception being in the cortex in rats pre-treated with 90 mg/kg of L-NAME). L-NAME-mediated dose-dependent increase in brain stem GSH in septic rats

Table 1. Glutathione concentration in rat brain structures in early stage sepsis: effect of i.p. pre-treatment with NOS inhibitors

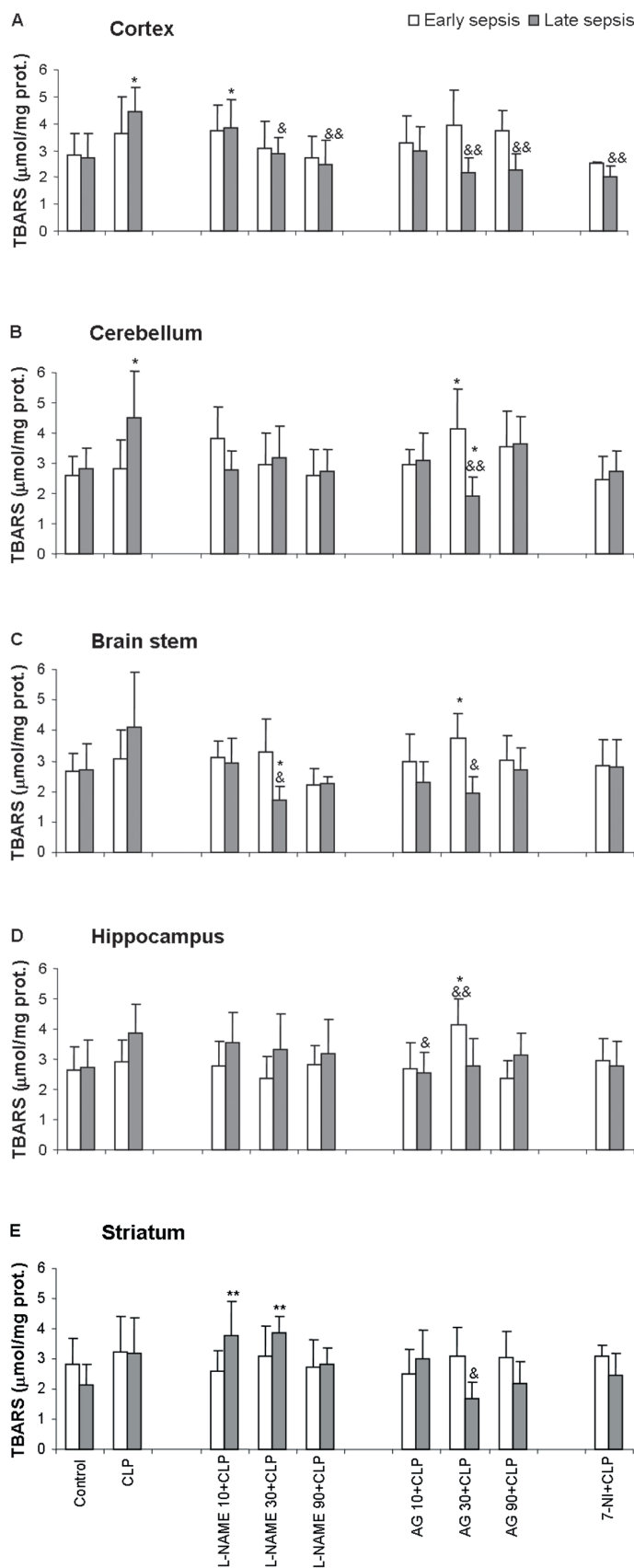
Group	Dose	Cortex	Brain stem	Cerebellum	Striatum	Hippocampus
Control	–	11.20 \pm 4.70	15.17 \pm 4.88	22.56 \pm 7.58	16.36 \pm 5.70	17.58 \pm 4.84
CLP	–	3.45 \pm 1.03**	2.75 \pm 0.80**	4.35 \pm 1.27**	4.21 \pm 1.40**	4.73 \pm 0.79**
L-NAME + CLP	10	5.74 \pm 1.51**	5.83 \pm 1.67**‡	7.07 \pm 2.06**	5.62 \pm 1.64**	6.13 \pm 0.45**
	30	5.94 \pm 1.89*	6.80 \pm 2.12**‡	5.68 \pm 1.43**	4.79 \pm 1.23**	5.22 \pm 1.36**
	90	8.13 \pm 2.37 ⁺	10.27 \pm 0.83**‡	7.01 \pm 1.53**	8.52 \pm 1.67* ⁺	9.41 \pm 2.58*‡
AG + CLP	10	8.50 \pm 1.38‡	8.97 \pm 2.62‡	8.74 \pm 2.64*‡	4.94 \pm 1.47**	7.36 \pm 0.73**‡
	30	14.31 \pm 4.73‡	17.70 \pm 4.73‡	19.20 \pm 5.44‡	10.76 \pm 4.84* ⁺	16.97 \pm 0.33‡
	90	10.40 \pm 2.04* ⁺	8.20 \pm 2.46‡	12.10 \pm 2.92‡	12.37 \pm 3.10‡	8.04 \pm 2.17**‡
7-NI + CLP	30	13.75 \pm 3.17‡	14.23 \pm 3.21‡	17.72 \pm 2.91‡	16.89 \pm 2.50‡	15.01 \pm 2.59‡

Data are expressed as mean \pm SD nmol/mg protein ($n = 10$ for each group). Control, rats that underwent sham laparotomy; CLP, rats that underwent cecal ligation and puncture; L-NAME + CLP, rats that received L-NAME (10, 30 or 90 mg/kg b.w.) and underwent cecal ligation and puncture; AG + CLP, rats that received AG (10, 30 or 90 mg/kg b.w.) and underwent cecal ligation and puncture; 7-NI + CLP, rats that received 7-NI (30 mg/kg b.w.) and underwent cecal ligation and puncture. * $p < 0.05$, ** $p < 0.01$ compared with control; ⁺ $p < 0.05$, ‡ $p < 0.01$ compared with CLP.

Table 2. Glutathione concentration in rat brain structures in late stage sepsis: effect of i.p. pre-treatment with NOS inhibitors

Group	Dose	Cortex	Brain stem	Cerebellum	Striatum	Hippocampus
Control	–	20.04 \pm 4.25	21.85 \pm 5.35	21.38 \pm 4.86	16.12 \pm 4.14	20.18 \pm 4.77
CLP	–	7.39 \pm 2.25**	4.16 \pm 1.22**	5.55 \pm 0.64**	6.03 \pm 1.13**	5.62 \pm 1.61**
L-NAME+CLP	10	8.59 \pm 2.47*	14.13 \pm 1.40*‡	13.19 \pm 1.52**‡	14.83 \pm 3.22‡	12.80 \pm 3.72**‡
	30	7.46 \pm 2.07**	10.18 \pm 3.37**‡	9.92 \pm 2.82* ⁺	10.15 \pm 2.55**‡	8.90 \pm 2.01** ⁺
	90	11.63 \pm 3.18*	17.89 \pm 5.07** ⁺	9.47 \pm 3.43*	5.69 \pm 2.79**	10.35 \pm 4.14*‡
AG+CLP	10	13.45 \pm 1.38‡	7.37 \pm 1.85**‡	10.65 \pm 1.95**‡	9.70 \pm 2.72* ⁺	15.44 \pm 3.15**‡
	30	14.31 \pm 4.73‡	16.40 \pm 4.18‡	22.41 \pm 7.00‡	15.35 \pm 4.09‡	14.46 \pm 2.25‡
	90	10.40 \pm 2.04* ⁺	7.72 \pm 1.65** ⁺	12.39 \pm 1.99*‡	12.21 \pm 3.03‡	15.14 \pm 3.16‡
7-NI+CLP	30	13.75 \pm 3.17‡	16.58 \pm 2.38‡	17.32 \pm 1.43‡	16.62 \pm 1.71‡	15.14 \pm 3.16‡

Data are expressed as mean \pm SD nmol/mg proteins ($n = 10$ for each group). Control, rats that underwent sham laparotomy; CLP, rats that underwent cecal ligation and puncture; L-NAME+CLP, rats that received L-NAME (10, 30 or 90 mg/kg b.w.) and underwent cecal ligation and puncture; AG+CLP, rats that received AG (10, 30 or 90 mg/kg b.w.) and underwent cecal ligation and puncture; 7-NI+CLP, rats that received 7-NI (30 mg/kg b.w.) and underwent cecal ligation and puncture. * $p < 0.05$; ** $p < 0.01$ compared with control; ⁺ $p < 0.05$; ‡ $p < 0.01$ compared with CLP.



was apparent when compared with the CLP subgroup ($p < 0.01$). The increase in GSH was also evident in the cortex ($p < 0.05$), striatum ($p < 0.05$) and hippocampus ($p < 0.01$) in subgroups treated with 90 mg/kg of L-NAME. Increases in GSH concentrations were observed in other brain structures in septic rats pre-treated with L-NAME. However, these increases were not statistically significant when compared with the CLP subgroup. Pre-treatment with AG followed by induction of sepsis caused elevations in the GSH concentrations in almost all the investigated brain structures in early stage sepsis compared with the CLP subgroup. The exception was in the striatum from rats treated with 10 mg/kg AG, where the GSH concentration stayed low. Pre-treatment with 7-NI followed by induction of sepsis caused elevations in the GSH concentrations in all the investigated brain structures in early stage sepsis compared with the CLP subgroup ($p < 0.01$).

At a late (48 h) stage of sepsis, the concentration of GSH in brain structures stayed very low compared with control rats (Table 2). L-NAME pre-treatment prior to CLP increased the concentration of GSH compared with the CLP group. The exceptions were the cortex in all three septic groups pre-treated with L-NAME and both the cerebellum and striatum in septic groups pre-treated with 90 mg/kg L-NAME. Pre-treatment with AG led to an increase in the GSH concentration in all the investigated brain structures regardless of the applied AG dose. Moreover, pre-treatment with 30 mg/kg AG prior to CLP maintained the GSH concentration at a concentration found in control-treated rats. Similar effects were found in septic rats pre-treated with 7-NI. GSH concentrations in brain structures isolated from 7-NI-treated late stage septic rats were significantly elevated compared to the CLP group ($p < 0.01$) and were not significantly different to the concentrations found in control-treated rats.

To determine whether NOS inhibitors had any effect on oxidative stress development during sepsis, the levels of TBARS within brain tissue were examined during

◀ **Figure 1.** (A-E) TBARS concentrations in rat brain structures in early and late stage sepsis and the effect of i.p. pre-treatment with NOS inhibitors. Control, rats that underwent sham laparotomy; CLP, rats that underwent cecal ligation and puncture; L-NAME + CLP, rats that received L-NAME (10, 30 or 90 mg/kg b.w.) and underwent cecal ligation and puncture; AG + CLP, rats that received AG (10, 30 or 90 mg/kg b.w.) and underwent cecal ligation and puncture; 7-NI + CLP, rats that received 7-NI (30 mg/kg b.w.) and underwent cecal ligation and puncture. Each experimental group consisted of 10 rats ($n = 10$). * $p < 0.05$; ** $p < 0.01$ compared with control; & $p < 0.05$; && $p < 0.01$ compared with CLP.

both early and late stage sepsis in all experimental groups of rats. TBARS increased in the cortex (Figure 1A) and the cerebellum (Figure 1B) during late stage sepsis ($p < 0.05$).

In the two above-mentioned brain structures, pre-treatment with all three doses of L-NAME decreased TBARS, returning the levels back to control values. Only the lowest dose of L-NAME had no effect on the TBARS level in the cortex during the late sepsis period, causing it to remain significantly higher compared with control-treated rats ($p < 0.05$). All three doses of L-NAME maintained the control TBARS levels found after CLP in the hippocampus (Figure 1D). Non-elevated levels of TBARS were also found in the brain stem of septic rats. Surprisingly, pre-treatment with 30 mg/kg L-NAME led to a decrease in the level of TBARS in the brain stem (Figure 1C). Pre-treatment with 10 and 30 mg/kg L-NAME elevated the concentration of TBARS during the late stage of sepsis in the striatum. CLP itself failed to increase the concentration of TBARS in the striatum (Figure 1E).

The concentrations of TBARS in brain structures from AG pre-treated septic rats were different from L-NAME pre-treated septic rats. The most prominent effects observed were those in rats pre-treated with 30 mg/kg AG. This AG dose increased the concentration of TBARS in early sepsis in the cerebellum, brain stem and hippocampus compared to control-treated rats ($p < 0.05$). During late sepsis, the concentrations of TBARS in the cortex, cerebellum, brain stem and striatum of rats treated with 30 mg/kg AG decreased compared with the CLP group ($p < 0.05$). Septic rats pre-treated with AG (both 10 mg/kg as well as 90 mg/kg) presented no differences in the concentrations of TBARS in brain structures compared with control rats. The exceptions were the cortex isolated from rats pre-treated with 90 mg/kg and the hippocampus isolated from rats pre-treated with 10 mg/kg. Surprisingly, the concentration of TBARS decreased compared with CLP.

7-NI application before induction of sepsis did not affect the concentration of TBARS in almost all of the examined brain structures at both early and late stages of sepsis when compared with control rats. Only during late stage sepsis the concentration of TBARS was markedly reduced in the cortex in rats pre-treated with 7-NI when compared with the CLP group ($p < 0.01$).

The concentrations of TBARS in brain structures isolated from control-treated mice pre-treated with NOS inhibitors were not significantly different from each other (results not shown).

Discussion

The current study focused on oxidative stress development in brain structures as a consequence of CLP-induced sepsis.

The major finding was a reduction in the concentration of GSH in both early and late stage sepsis. Lipid peroxidation, as revealed by the concentration of TBARS, is a common process in the cortex and cerebellum during late stage sepsis following CLP. Non-selective L-NAME-mediated NOS inhibition demonstrated the participation of the NO system in destructive events in the cortex during late stage sepsis. The lowest dose of L-NAME was not sufficient to abolish the increased TBARS in the cortex. Both 30 and 90 mg/kg b.w. L-NAME decreased TBARS accumulation back to control concentrations in late stage sepsis. Selective iNOS inhibition by AG and nNOS inhibition by 7-NI both inhibited CLP-induced TBARS elevation, suggesting the importance of eNOS in cortex homeostasis during late stage sepsis. In addition, non-selective L-NAME-induced NOS inhibition, as well as selective nNOS inhibition by 7-NI, stabilised the concentration of TBARS in the cerebellum during late stage sepsis.

The same effects caused selective iNOS inhibition induced by the lowest and the highest doses of AG. Unexpectedly 30 mg/kg b.w. AG pre-treatment increased TBARS in early stage sepsis and then decreased TBARS in late stage sepsis compared with controls. However, in the brain stem and the hippocampus similar effects of increased TBARS in early stage sepsis were noted with 30 mg/kg b.w. AG pre-treatment. We cannot fully explain such an inverse effect. However, pharmacological effects of AG can be independent from iNOS inhibitory effects (Nilson 1999). Compared with other rat brain structures the cortex possesses the most effective system for cyclic guanosine monophosphate (cGMP) degradation. In addition, the granular layer of the cerebellum is sufficient for NOS activity as a high concentration of soluble guanylate cyclase exists (Pepicelli et al. 2004). The actions of the second messenger cGMP are largely under the influence of NO activity, underlining the importance of the correct functioning of the cortex and cerebellum. In addition to the apparent influence of the NO system on glutamatergic transmission, the unchanged concentration of TBARS in the hippocampus indicated its stability in both early and late stage sepsis. This could be a consequence of less cGMP-mediated signalling/activity, which has been suggested (Burgunder and Cheung 1994).

The most unpredictable results were obtained in the striatum. Besides decreased GSH, TBARS remained unchanged during sepsis. Non-selective NOS inhibition by low-dose L-NAME resulted in increased TBARS in late stage sepsis. While such changes were not as a consequence of selective iNOS and nNOS inhibition, they could be discussed of importance of endothelial NOS (eNOS) in striatum constancy. The striatum is a selectively excitable structure with a specific biochemical organisation containing robust antioxidant defence mechanisms as a consequence of its high iron content which can be prooxidative in nature (Mizuno

and Ohta 1986). According to our results, non-selective NOS inhibition during sepsis could be detrimental to cell membranes, suggesting the importance of eNOS-mediated NO participation in the antioxidant defence system in the striatum.

Research aimed at understanding the pathophysiological mechanisms underlying sepsis are complicated due to hypotension during early stage sepsis (Matsuda and Hattori 2007). Among other endogenous mediators, NO participates in vasodilatation during sepsis. Most of the CLP models therefore use early saline resuscitation to abolish the hypotensive effect. According to our predictions, such action is too simple and does not sufficiently eliminate hypotension. At the same time, early saline resuscitation changes the natural defense mechanism reactions, which does not permit us to examine real time recovery and entry into early stage sepsis. There are sporadic and variable data concerning alterations in brain activity in early stage sepsis (Konsman et al. 1999), most of which are only related to the different models used and types of early saline resuscitation employed. Barichello and colleagues noted the oxidative stress development in the rat brain 6 h after CLP-induced sepsis with early saline resuscitation, prior to that found in other organs (Barichello et al. 2006). This time point fits in the stage of the developing sepsis, but not within early stage sepsis. We found a decrease in GSH 3 h post CLP, without any increase in TBARS in any of the examined brain structures. S-glutathionylation could have been a defence response to protect critical protein sulphhydryl groups against irreversible destruction (Giustarini et al. 2004). Reactive oxygen species (ROS) interact, modify and inactivate numerous critical cells components including proteins, lipids and nucleic acids. In addition to lipid peroxidation, ROS can inactivate sulphhydryl group-rich enzymes that participate in the Krebs cycle.

In all the investigated brain regions GSH concentrations remained very low in late stage sepsis. In the cortex and cerebellum TBARS increased. Our results are not totally consistent with those previously published concerning early changes in the cerebral capillaries of septic rats. However, they demonstrate that the detrimental process can pass the blood-brain barrier (Ninkovic et al. 2006). One could conclude that our results provide the basis for the effects of hypotension and consecutive brain ischaemia. However, published data has already demonstrated early oxidative stress development in several brain regions regardless of resuscitation and abolished hypotension (Barichello et al. 2006).

Non-selective NOS inhibition by L-NAME demonstrated a beneficial effect on the oxidative stress development, particularly at a dose of 90 mg/kg before CLP. This effect could have been due to suppression of hypotension. The same beneficial effect was also found in septic rats pre-treated with AG as well as 7-NI. Non-selective NOS inhibition

by L-NAME indicated that the NO system participated in destructive events in the cortex. A low L-NAME dose was not sufficient enough to abolish NO's effects. Doses of 30 and 90 mg/kg decreased late stage sepsis-induced TBARS elevation. Selective iNOS inhibition by all three doses of AG, and nNOS inhibition by 7-NI suppressed sepsis-induced TBARS elevation. This suggested the importance of constitutively active NOS activity in the homeostasis of the cortex during sepsis.

Due to its high affinity for both ROS and reactive nitrogen species, GSH participates in host defense mechanisms to combat oxidative/nitrosative stress during sepsis (Das and Maulik 2003). It is known that GSH depletion significantly increases NO cytotoxicity (Nelson et al. 2003). Nitrosothiols, including nitrosoglutathione, also serve as inhibitors of enzymes included in GSH storage (Wink and Mitchell 1998).

Moreover, AG's variable ability to decrease CLP-mediated oxidative stress was also dose-dependent which we mainly observed in late stage sepsis. Early time point discovered impaired oxidative status. Our results confirm the crucial role of both iNOS and eNOS. High concentrations of AG can be not only selective, but also a non-selective NOS inhibitor (Pheng et al 1995). In addition, it has been demonstrated that AG expresses pharmacological effects that are not related to NOS inhibition. These include inhibition of glycated end product production (typical in sepsis) and catalase inhibition, the latter leading to increased hydrogen peroxide production that could assist host defense mechanism during sepsis (Nilsson 1999).

Adjacent to neurons and nerve fibres which innervate larger brain arteries and pia blood vessels nNOS also exists in glial cells. It has been shown that dendrites and axons are the source of nearly 85% of nNOS activity in brain (Acki et al. 1993). nNOS activation is regulated by a number of factors, particularly glutamate (Huang et al. 1999). Besides eNOS participation in blood flow control, it is partly influenced by nNOS in physiological as well pathophysiological conditions (Estrada and Defelipe 1998).

One must consider that NO inhibition in all the investigated brain regions may suppress NO's hypotensive effect in early stage sepsis. However, in late stage sepsis the effect of NOS inhibition may be weaker due to the long distance time between NOS inhibitor application and animal sacrifice.

Despite the significant decrease in the hippocampal GSH concentration, the concentration of TBARS discreetly changed when NOS inhibitors were present. As the hippocampus is an extremely sensitive structure to oxidative stress the results were unexpected. However, mechanisms by which endotoxin-induced neuronal destruction are different from well-known mechanism of excitotoxicity (De Bock et al. 1998). This could be the explanation for the mild changes that occurred in the hippocampus during sepsis and in the presence of NOS inhibitors.

We conclude that the measured oxidative stress status parameters in the cortex, cerebellum, brain stem, hippocampus and striatum indicated oxidative stress development post CLP which resulted in a significant reduction in GSH concentration in both early and late stage sepsis. Lipid peroxidation was evident in late stage sepsis in both the cortex and the cerebellum. Pre-treatment with a non-selective NOS inhibitor (L-NAME) and with selective iNOS and nNOS inhibitors (AG and 7-NI) revealed benefit effects on the GSH concentrations. Unexpectedly, NOS inhibition resulted in diverse effects on TBARS concentrations, suggesting the importance of specific quantities of NO in specific brain structures during sepsis.

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References

- Acki C., Fenstermaker S., Lubin M., Go C. G. (1993): Nitric oxide synthase in the visual cortex of monocular monkeys as revealed by light and electron immunocytochemistry. *Brain Res.* **620**, 97–113
- Angus D. C., Musthafa A. A., Clermont G. (2001): Quality-adjusted survival in the first year after the acute respiratory distress syndrome. *Am. J. Respir. Crit. Care Med.* **163**, 1389–1394
- Barichello T., Martins M., Reinke A., Feier G., Ritter C., Quevedo J., Dal-Pizzol F. (2005): Cognitive impairment in sepsis survivors from cecal ligation and perforation. *Crit. Care Med.* **33**, 221–223
- Barichello T., Fortunato J. J., Vitali A. M., Feier G., Reinke A., Moreira J. C. F., Quevedo J., Dal-Pizzol F. (2006): Oxidative variables in the rat brain after sepsis induced by cecal ligation and perforation. *Crit. Care Med.* **34**, 886–889
- Benjamim C. F., Silva J. S., Fortes Z. B., Oliveira M. A., Ferreira S. H., Cunha F. Q. (2002): Inhibition of leukocyte rolling by nitric oxide during sepsis leads to reduced migration of active microbicidal neutrophils. *Infect. Immun.* **70**, 3602–3610
- Boveris A., Alvarez S., Navarro A. (2002): The role of mitochondrial nitric oxide synthase in inflammation and septic shock. *Free Radic. Biol. Med.* **33**, 1186–1193
- Burgunder J. M., Cheung P. T. (1994): Expression of soluble guanylyl cyclase gene in adult rat brain. *Eur. J. Pharmacol.* **6**, 211–217
- Das K. D., Maulik N. (2004): Conversion of death signal into survival signal by redox signaling. *Biochemistry (Moscow)* **69**, 10–17
- De Bock F., Derijard B., Dornand J., Bockaert J., Rondouin G. (1998): The neuronal death induced by endotoxic shock but that induced by excitatory amino acids requires TNF- α . *Eur. J. Neurosci.* **10**, 3107–3114
- Ellman G. L. (1959): Tissue sulphhydryl groups. *Arch. Biochem. Biophys.* **74**, 443–450
- Esplugues J. V. (2002): NO as a signalling molecule in the nervous system. *Br. J. Pharmacol.* **135**, 1079–1095
- Estrada C., Defelipe J. (1998): Nitric oxide-producing neurons in the neocortex: morphological and functional relationship with intraparenchymal microvasculature. *Cereb. Cortex* **8**, 193–203
- Feihl F., Waeber B., Liaudet L. (2001): Is nitric oxide overproduction the target of choice for the management of septic shock? *Pharmacol. Ther.* **91**, 179–213
- Garthwaite J., Boulton C. L. (1995): Nitric oxide signalling in the central nervous system. *Annu. Rev. Physiol.* **57**, 683–706
- Gherzi-Egea J. F., Leininger-Muller B., Suleman G., Siest G., Minn A. (1994): Localization of drug-metabolising enzyme activities to blood-brain interfaces and circumventricular organs. *J. Neurochem.* **62**, 1089–1096
- Ghafourifar B., Bringold U., Klein S. D., Richter C. (2001): Mitochondrial nitric oxide synthase, oxidative stress and apoptosis. *Biol. Signals Recept.* **10**, 57–65
- Giustarini D., Rossi R., Milzani A., Colombo R., Dalle-Donne I. (2004): S-glutathionylation-from redox regulation of protein functions to human diseases. *J. Cell. Mol. Med.* **8**, 201–212
- Gurd J. W., Jones L. R., Mahler H. R., Moore W. J. (1974): Isolation and partial characterization of rat brain synaptic membrane. *J. Neurochem.* **22**, 281–290
- Hotchkiss R. S., Karl I. E. (2003): The pathophysiology and treatment of sepsis. *N. Engl. J. Med.* **348**, 138–150
- Huang P. L., Gyurko R., Zhang L. (1999): Cardiovascular effects of nitric oxide: lessons learned from endothelial nitric oxide synthase knockout mice. In: *Endothelium, Nitric Oxide and Atherosclerosis*. (Eds. J. A. Panza and R. O. Cannon), pp. 37–48, Futura Publishing Co., Armonk, New York
- Konsman J. P., Kelley K., Dantzer R. (1999): Temporal and spatial relationships between LPS-induced expression of FOS, interleukin-1 β inducible and inducible nitric oxide synthase in rat brain. *Neuroscience* **89**, 535–548
- Levy M. M., Fink M., Marshall J. C. (2003): SCCM/ESICM/ACCP/ATS/SIS international sepsis definitions conference. *Crit. Care Med.* **34**, 1250–1256
- Liaudet L., Soriano F. G., Szabo C. (2000): Biology of nitric oxide signaling. *Crit. Care Med.* **28**, N37–52
- Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J. (1951): Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
- Matsuda N., Hattori Y. (2007): Vascular biology in sepsis: pathophysiological and therapeutic significance of vascular dysfunction. *J. Smooth Muscle Res.* **43**, 117–137
- Girotti M. J. (1991): Early measurement of systemic lipid peroxidation products in the plasma of major blunt trauma patients. *J. Trauma* **31**, 32–34
- Mizuno Y., Ohta K. (1986): Regional distributions of thiobarbituric acid-reactive products, activities of enzymes regulating the metabolism of oxygen free radicals, and some of the related enzymes in adult and aged rat brains. *J. Neurochem.* **46**, 1344–1352

- Nelson J. E., Connolly J, McArthur P. (2003): Nitric oxide and S-nitrosylation: excitotoxic and cell signaling mechanism. *Biol. Cell* **95**, 3–8
- Nilsson B. O. (1999): Biological effects of aminoguanidine: an update. *Inflamm. Res.* **48**, 509–515
- Ninković M., Maličević Ž., Jelenković A., Jovanović D. M., Đukić M., Vasiljević I. (2006): Oxidative stress in the rats brain capillaries – the influence of 7-nitroindazole. *Acta Physiol. Hung.* **93**, 315–323
- Ninković M., Maličević Ž., Stojanović D., Vasiljević I., Jovanović D. M., Đukić M. (2008): Brain stem and thalamus antioxidative defense in experimental sepsis. *Acta Vet. (Belgrade)* **58**, 129–137
- Pepicelli O., Raiteri M., Fedele E. (2004): The NOS/sGC pathway in the rat central nervous system: a microdialysis overview. *Neurochem. Int.* **45**, 787–797
- Pheng L. H., Francoeur C., Denis M. (1995): The involvement of nitric oxide in a mouse model of adult respiratory distress syndrome. *Inflammation (N. Y.)* **19**, 599–610
- Poon B. Y., Raharjo, E., Patel, K. D., Tavener, S., Kubes, P. (2003): Complexity of inducible nitric oxide synthase. Cellular source determines benefit versus toxicity. *Circulation* **2**, 1107–1112
- Rivers E. P., McIntyre L., Morro D. C., Rivers K. K. (2005): Early and innovative interventions for severe sepsis and septic shock: taking advantage of a window of opportunity. *CMAJ* **173**, 1054–1065
- Robert P., Koedel U., Pfister H. (1997): 7-Nitroindazole inhibits pial arteriolar vasodilatation in a rat model of Pneumococcal meningitis. *Metabolism* **17**, 985–991
- Sharshar T., Annane D., de la Grandmaison G. L., Brouland J. P., Hopkinson N. S., Gray F. (2004): The neuropathology of septic shock. *Brain Pathol.* **14**, 21–33
- Sharshar T., Hopkinson N. S., Orlikowski D., Annane D. (2005): Science review: the brain in sepsis-culprit and victim. *Crit. Care* **9**, 37–44
- Stojanović D., Maličević Ž., Ašanin M. (2002): The use of a new model for the investigation of sepsis. *Acta Vet. (Belgrade)* **52**, 125–132
- Wiklund N., Cellek S., Leone A., Iversen H., Gustadsson L., Brundin L., Furst V., Flock A., Moncada S. (1997): Visualisation of nitric oxide released by nerve stimulation. *J. Neurosci. Res.* **47**, 224–232
- Wink D. A., Mitchell J. B. (1998): Chemical biology of nitric oxide: insights into regulatory, cytotoxic and cytoprotective mechanisms of nitric oxide. *Free Radic. Biol. Med.* **25**, 434–456