

# Cytochrome C oxidase activity and nitric oxide synthase in the rat brain following aluminium intracerebral application

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## Abstract

*In the present study we investigated the cytochrome C oxidase (CO) activity and nitric oxide synthase (NOS) isoenzyme expression after intrahippocampal AlCl<sub>3</sub> application in selective vulnerable brain structures. A single dose of AlCl<sub>3</sub> was applied in the CA1 sector of rats hippocampus. For biochemical analysis, the animals were killed 10 min and three days after the treatment and forebrain cortex, basal forebrain and hippocampus were removed. Activity of CO was decreased bilaterally in the AlCl<sub>3</sub>-treated groups in all examined brain structures. We also applied immunohistochemical techniques to identify changes induced by AlCl<sub>3</sub> injection after survival periods of 10 min and three days. Both the nNOS and eNOS stains were detected in the hippocampus of controls and AlCl<sub>3</sub>-treated animals, but iNOS labelling was present in the hippocampus only three days after AlCl<sub>3</sub> application. An increased iNOS expression three days post AlCl<sub>3</sub> administration could be involved in the mechanism of CO activity depletion.*

**Key words:** aluminium, brain, cytochrome C oxidase, immunohistochemistry.

## Introduction

Aluminium (Al) compounds are neurotoxic and have been shown to induce experimental neurodegeneration although the mechanism of this effect is unclear [3,8]. Aluminium has the ability to promote formation and accumulation of insoluble beta amyloid (A beta) and hyperphosphorylated tau [27,28]. Also, Al has been implicated as a destabilizer of cell membranes. Due to its high reactivity, Al<sup>3+</sup> is able to interfere with several biological functions, including enzymatic activities in key metabolic pathways. Aluminium reacts with positive groups of protein components placed on heads of membrane phospholipids, inducing in that way irreg-

ular opening of the cell membrane, leading to a change of membrane fluidity [15].

Three routes have been identified triggering neuronal death under physiological and pathological conditions. Excess activation of ionotropic glutamate receptors cause influx and accumulation of Ca<sup>2+</sup> and Na<sup>+</sup> that result in rapid swelling and subsequent neuronal death within a few hours. The second route is caused by oxidative stress due to accumulation of reactive oxygen and nitrogen species [26]. Apoptosis under mitochondrial control has been implicated in a progressive and selective loss of neurons, which involve the release of cytochrome c into the cytoplasm and initiation of the apoptosis cascade [7].

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Mitochondria are both targets and sources of oxidative stress [2,6]. Under physiological conditions, the production of nitric oxide (NO) by mitochondria has an important implication for the maintenance of the cellular metabolism, i.e. modulates the oxygen consumption of the organelles through the competitive (with oxygen) and reversible inhibition of cytochrome C oxidase (CO) [25]. Nitric oxide is an enzymatic product of NO-synthesizing enzyme NO synthase (NOS), which is present in the brain in three different isoforms, two constitutive enzymes (i.e. neuronal, nNOS, and endothelial eNOS) and one inducible enzyme (iNOS) [10,19]. The expression and activity of iNOS play a pivotal role in sustained and elevated NO release. Many published data suggest that neurons can respond to proinflammatory stimuli and take part in brain inflammation [11]. In addition, CO is the terminal complex of the mitochondrial respiratory chain, and passes electrons from cytochrome c to O<sub>2</sub> [1,5]. Within cytochrome oxidase NO binds to the reduced form of cytochrome a<sub>3</sub> and probably also to Cu<sub>B</sub>. This is the same site and form of the enzyme to which O<sub>2</sub> binds. Nitric oxide inhibition of cytochrome oxidase potentially may occur in physiological and/or pathological conditions [21].

In this regard, we wanted to examine the CO activity on a biochemical level on the one hand, as well as early changes in the expression of all three forms of the NOS (nNOS, eNOS, iNOS) enzyme evoked by AlCl<sub>3</sub>. In presented time-course study post AlCl<sub>3</sub> intrahippocampal injection, the CO activity and NOS isoenzyme expression have been investigated in different brain regions.

## Material and methods

### Animals

The experimental animals were treated according to the Guidelines for Animal Study, No. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia).

Male adult Wistar rats, 4 months old, with body mass of 500 ± 50 g, were used for experiments. Groups of two or three rats per cage (Erath, FRG) were housed in an air-conditioned room at a temperature of 23 ± 2°C with 55 ± 10% humidity and with lights on 12 h/day (07.00-19.00 h). The animals were given a commercial rat diet and tap water ad libitum.

### Experimental procedure

Animals were anesthetized by intraperitoneal injections of sodium pentobarbital (0.0405 g/kg b.w.). They

were divided into sham-operated group (CG group) and aluminium chloride (AlCl<sub>3</sub>)-treated group (Al group). Both groups were subdivided into 10 minutes' treated (CG 10 min and Al 10 min) and three days' treated (CG 3 days and Al 3 days). A single dose of AlCl<sub>3</sub> (3.7 × 10<sup>-4</sup> g/kg b.w. in 0.01 ml of deionized water), was injected into CA1 sector of the hippocampus, using a Hamilton microsyringe, using stereotaxic instrument for small animals (coordinates: 2.5 A; 4.2 L; 2.4 V) [16]. The second group received the same volume but only 0.9% saline solution and served as a control (sham-operated). 10 min and three days of saline or AlCl<sub>3</sub> injection, animals from each group were sacrificed and tissues were collected for biochemistry and immunohistochemistry. The total number of animals in the study was 40. In all treated animals the injected intracerebral volume was 10 × 10<sup>-6</sup> ml and it was always injected into the same – left side.

### Brain tissue preparation

The brains were dissected from two animals from all experimental groups, rapidly removed from the skull, fixed in 4% paraformaldehyde for at least 24 h and cryoprotected in graded sucrose at 4°C. Brains were frozen in methylbutane and stored at -70°C until cryosectioning (CRIO CUT-E Reichert-Yung). From the other animals, the removed brain tissue was washed in PBS and 10% homogenates of the both ipsi- and contralateral forebrain cortex, basal forebrain and hippocampus, were homogenated in sucrose on ice with teflon pounder and stored at -70°C for later biochemical analysis.

### Biochemical analysis

The brain structures – forebrain cortex, basal forebrain and hippocampus were dissected on ice, and each tissue slice (approximately 0.1 g) was transferred into a tube of 1 ml of cold, buffered sucrose medium (0.25 M/L sucrose with 0.1 mM/L EDTA in 50 mM K-Na phosphate buffer, pH 7.2). Homogenization of the tissue in the sucrose medium was performed by a homogenizer (Tehtnica, Zelezniki, Slovenia) at 800 rotation/min, on ice. The homogenates were centrifuged at 1000 × g, for 15 min at 4°C. The precipitates were redispersed in the sucrose medium and centrifuged again. The supernatants were centrifuged at 2500 × g for 30 min at 4°C and the obtained precipitates were redispersed in 1.5 mL of deionized water. After that, the samples were centrifuged at 2000 × g for 15 min at 4°C and the supernatants (crude mitochondrial fractions) were stored at -70°C [9].

Cytochrome C oxidase activity, the last component of the mitochondrial respiratory electron-transport chain, was measured as the decrease of absorbance during oxidation of ferrous cytochrome C to ferric cytochrome C. Kinetics was followed in the potassium-phosphate buffer (0.05 M, pH = 7.1) for 3-5 min at 550 nm. Samples of investigated brain structures were pre-treated with 7.5% deoxycholate. Reduction of cytochrome C was mediated by 1 mM Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> – sodium dithionite. Reaction was started with addition of a prepared sample (0.05 ml) to the solution of 0.95 ml of reduced cytochrome C [13]. The results were expressed as mg cytochrome C per mg proteins.

The content of protein in the rat brain homogenates (forebrain cortex, basal forebrain and hippocampus, ipsi- and contralateral) was measured by the method of Lowry using bovine serum albumin as standard [18].

### Processing of brain tissue and immunohistochemistry

Frozen, 8 µm thick sections were deposited on Poly-L Lysine coated slides and allowed to air dry. Dako-Cytomation EnVision + System-HRP kit was used in a two-step IHC staining technique. Cryostat sections were fixed in acetone and endogenous peroxidase activity was blocked by peroxidase block (0.03% hydrogen peroxide containing sodium azide) (DakoCytomation) for 15 minutes. Slides were incubated with appropriate dilutions of monoclonal antibodies – mouse NOS1 antibody 1 : 50 and mouse NOS2 antibody 1 : 25, as well as polyclonal antibody – rabbit NOS3 antibody 1 : 50, for 60 min. After that, slides were incubated with the labelled polymer (DakoCytomation) conjugated to goat anti-mouse and goat anti-rabbit immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent with addition of 5% normal rat serum for 30 min. Staining is completed by a 5-10 minute incubation with 3,3'-diaminobenzidine (DAB) + substrate-chromogen (DakoCytomation) which results in a brown-coloured precipitate at the antigen site. In negative controls, slides were incubated with PBS, in the absence of the primary antibody. Finally, slides were counterstained with hematoxylin and mounted with Kaiser gel.

### Reagents

All used chemicals were of analytical grade. The following compounds were used in this study: saline solution (0.9% w/v) (Hospital Pharmacy Military Medical

Academy, Belgrade, Serbia); paraformaldehyde (TAAB Laboratory Equipment, Aldermaston, UK); deoxycholate (Kemika, Zagreb, Croatia); Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> – sodium dithionite (Riedel-de Haen Ag. Seelze-Hannover, Germany); AlCl<sub>3</sub> and bovine serum albumin were purchased from Sigma-Aldrich, Inc., USA; cytochrome C and Kaiser gel were purchased from Merck (Darmstadt, Germany); mouse NOS1 antibody, mouse NOS2 antibody and rabbit NOS3 antibody were purchased from Santa Cruz Biotechnology, Inc., USA. All drug solutions were prepared on the day of the experiment.

### Statistical analysis

After verifying a normal distribution in all groups, using Kolmogorov-Smirnov test, the data presented were mean ± SD or mean ± SEM. Biochemical and immunohistochemical data were analyzed statistically by one-way ANOVA using Dunnett's C test. Statistical significance was defined as  $p < 0.05$ .

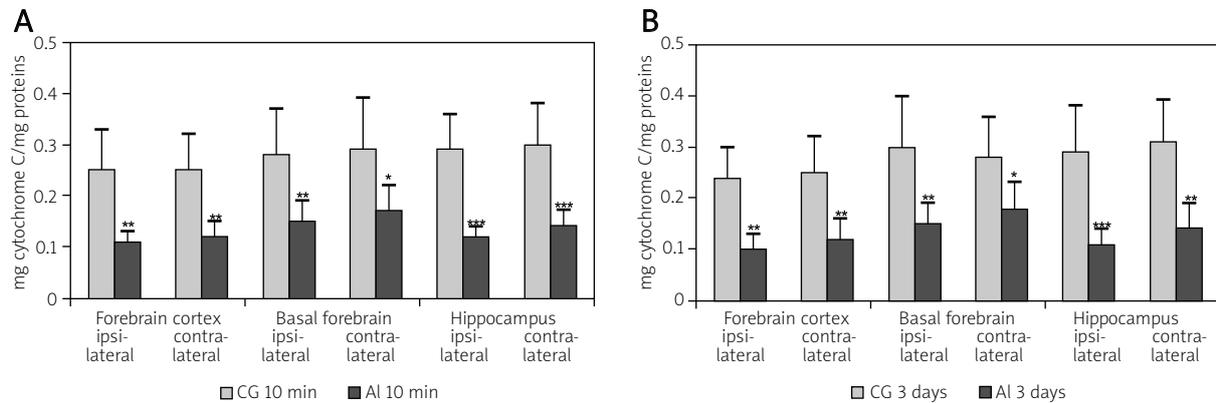
## Results

### Cytochrome C oxidase activity in the rat brain 10 min and three days after intrahippocampal AlCl<sub>3</sub> injection

The obtained results of biochemical analysis showed that CO activity was significantly decreased 10 min after AlCl<sub>3</sub> application in both ipsi- and contralateral forebrain cortex, basal forebrain and hippocampus, compared to control-injected rats (Fig. 1A). Results obtained for CO activity 3 days after AlCl<sub>3</sub> injection (Fig. 1B) show almost an identical trend of changes as 10 min (Fig. 1A) after neurotoxin application.

### NOS expression

Injection of AlCl<sub>3</sub> was performed unilaterally into CA1 subfield of the hippocampus of rats. Both nNOS (Figs. 2A, B) and eNOS (Figs. 2C, D) stains were detected in the hippocampus of control animals (Fig. 2A and Fig. 2C, respectively), as well as in AlCl<sub>3</sub>-treated rats 10 min (data not shown) and 3 days (Fig. 2B and Fig. 2D, respectively) after neurotoxin injection. iNOS labelling was present in the hippocampus of AlCl<sub>3</sub>-treated animals three days (Fig. 2F) after AlCl<sub>3</sub> application. The staining was faint in brain parenchyma. However, moderate staining was seen in macrophage-like cells localized predominantly in perivascular tissue.



**Fig. 1. A)** Activity of cytochrome C oxidase (mg cytochrome C/mg proteins) in the rat ipsi- and contralateral forebrain cortex, basal forebrain and hippocampus of the control group (CG) and  $\text{AlCl}_3$ -treated (Al) Wistar rats, sacrificed 10 min after the treatment. Significance to corresponding control values: \* $p < 0.05$  (Al vs. CG in contralateral basal forebrain), \*\* $p < 0.01$  (Al vs. CG in ipsi- and contralateral forebrain cortex and ipsilateral basal forebrain), \*\*\* $p < 0.001$  (Al vs. CG in ipsi- and contralateral hippocampus), One Way ANOVA, Dunnett's C test. **B)** Activity of cytochrome C oxidase (mg cytochrome C/mg proteins) in the rat ipsi- and contralateral forebrain cortex, basal forebrain and hippocampus of the control group (CG) and  $\text{AlCl}_3$ -treated (Al) Wistar rats, sacrificed 3 days after the treatment. Significance to corresponding control values: \* $p < 0.05$  (Al vs. CG in contralateral basal forebrain), \*\* $p < 0.01$  (Al vs. CG in ipsilateral forebrain cortex and basal forebrain and contralateral forebrain cortex and hippocampus), \*\*\* $p < 0.001$  (Al vs. CG in ipsilateral hippocampus), One Way ANOVA, Dunnett's C test.

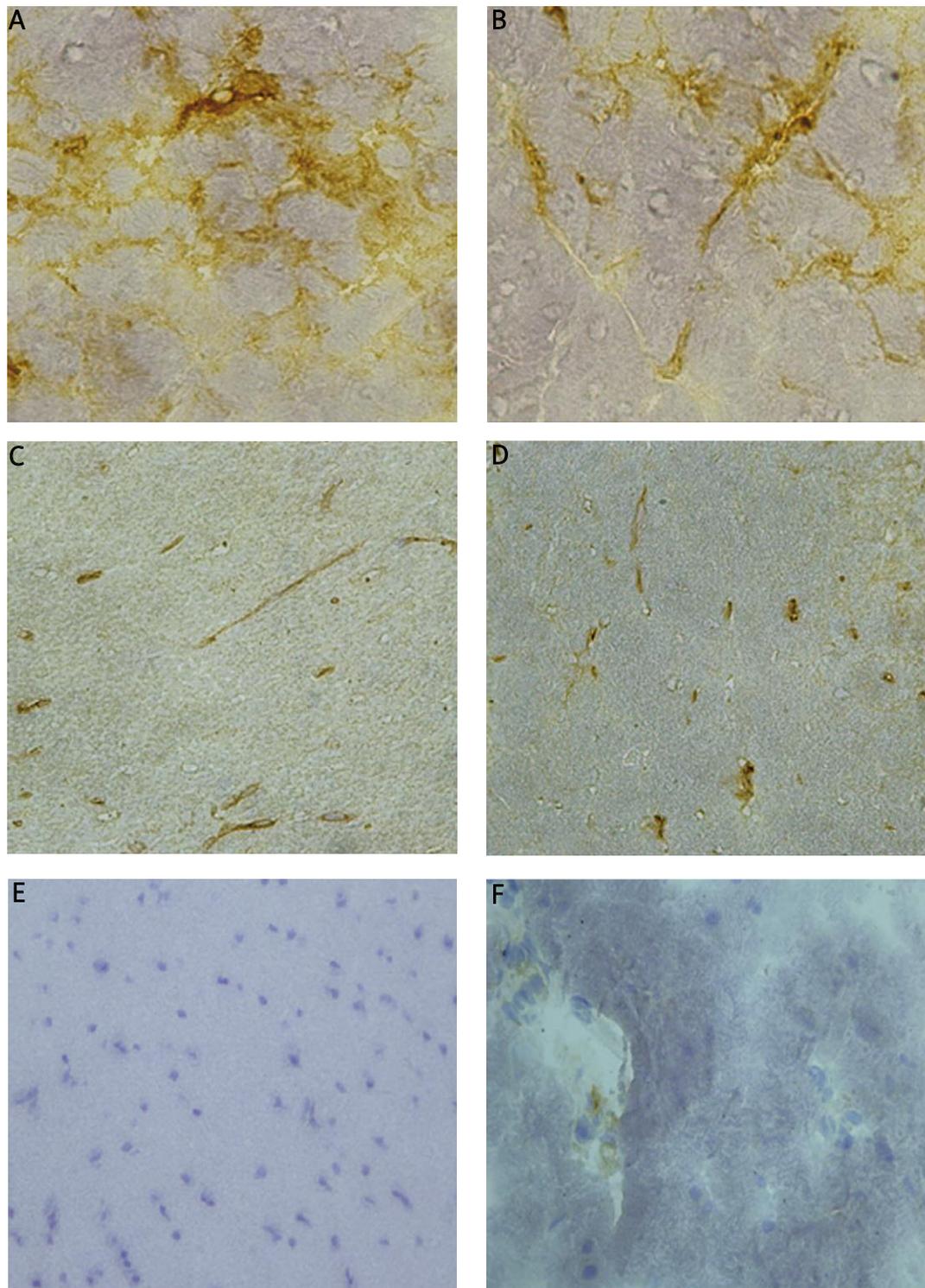
## Discussion

Bilateral decrease of CO activity in the forebrain cortex, basal forebrain and hippocampus both 10 min (Fig. 1A) and three days (Fig. 1B) after  $\text{AlCl}_3$  application suggest that Al neurotoxicity involves a specific impairment of CO activity. The decreased activity of CO indicates a deficiency in reducing equivalents with consequent diminishing of proton gradient i.e. re-entering of protons to the mitochondria, followed by insufficient ATP synthesis. This is in accordance with the study of Bosetti *et al.* which showed that CO subunit III (COX III) mRNA was significantly reduced by 70% after addition of  $1 \mu\text{M}$   $\text{AlCl}_3$  [4]. Our previous studies show that CO activity decreased bilaterally at both seven days and 12 days after  $\text{AlCl}_3$  application in the selective vulnerable brain structures [14,15]. Decreased activity of CO in  $\text{AlCl}_3$ -treated animals suggests the existence of oxidative stress. This process appears due to mutual effects of  $\text{AlCl}_3$ . One is mediated through direct diminishing synthesis of NADH, and the other one is indirect, via increased utilization of reducing equivalents because Al increased lipid peroxidation.

Krugel *et al.* show that the activity of the mitochondrial enzyme CO was diminished seven days after

the lesioned rat hippocampus [17]. Subcellular fractionation showed that the activity of this enzyme was affected in the synaptosomal as well as in the extrasynaptosomal mitochondria indicating a loss of neuronal input and also vulnerability of intrinsic hippocampal neurons and/or non-neuronal cells. The recovery of the mitochondrial enzyme activity in the animal model at later post lesion intervals may be a result of compensatory responses of surviving cells or of sprouting of other non-affected inputs.

Immunostaining for NOS (nNOS, eNOS, iNOS) showed no labelling of cells 10 min after saline- and  $\text{AlCl}_3$ -injection in rat hippocampus (data not shown), indicating that this period is too early for detection of any changes in NOS expression. Such a hypothesis is supported by the results of Heneka *et al.* which demonstrated an up-regulation of iNOS expression in brain tissue as early as four hours following intrastriatal microinjection of interferon- $\gamma$  and bacterial lipopolysaccharide in the rat [11]. Immunohistochemistry examination of constitutive NOS isoenzymes (nNOS – Fig. 2, section A, B and eNOS – Fig. 2, section C, D) at three days did not demonstrate any difference between saline control (Fig. 2, section A, C;  $p < 0.05$ ) and  $\text{AlCl}_3$ -treat-



**Fig. 2. A-F** Immunohistochemical detection of nNOS (Figs. 2A-B), eNOS (Figs. 2C-D) and iNOS (Figs. 2E-F) expression within the hippocampus three days after injection of the saline solution (A, C, E) and AlCl<sub>3</sub> (B, D, F). Both nNOS (A, B) and eNOS (C, D) stains were observed in control rats and AlCl<sub>3</sub>-treated animals. The iNOS was not detected in the hippocampus of control animals (E), but it was obvious three days after AlCl<sub>3</sub> application (F). (Original magnification x 400).

ed (Fig. 2, section B, D;  $p < 0.05$ ) animals. These results could be evidence for minor participation of these compounds in CO activity decline due to Al intoxication.

The influence of iNOS seems to have an essential position in the mechanism of mitochondrial damage, which was demonstrated by CO activity reduction. In our previous studies, we found iNOS positive cells in hippocampus 30 days after both saline solution and  $\text{AlCl}_3$  administration [22]. Also, we previously revealed a greater inflammatory response over immunoreactivity of astrocytes and phagocytic microglia in  $\text{AlCl}_3$ -injected animals [23]. In this study,  $\text{AlCl}_3$  application in hippocampus increased iNOS expression three days after the treatment (Fig. 2, section F;  $p < 0.05$ ). That could be a result of brain blood barrier disruption and induced inflammation in the rat brain. Inducible NOS-positive cells in the hippocampus detected in perivascular cells resembled macrophages, according to their morphological characteristics and localization.

It is known that  $\text{NO}^\bullet$  binds to cytochrome oxidase, inhibiting the mitochondrial respiratory chain and as a consequence increasing mitochondrial superoxide release, leading to peroxynitrite ( $\text{ONOO}^-$ ) formation [24]. The continuous production of  $\text{ONOO}^-$  by mitochondria may contribute significantly to the elevated oxidative stress sustained by mitochondria relative to the rest of the cell. The literature data implicate that chronic stress induced an increase in NO production via an expression of iNOS in the brain [12]. Inducible NOS inhibition produces an effect of mitochondrial respiratory chain protection as well as prevents NO accumulation, lipid peroxidation and glutathione depletion induced by stress [20]. Sustained overproduction of NO via iNOS is responsible, at least in part, for the inhibition of the mitochondrial respiratory chain caused by stress and that this pathway also accounts for the oxidative stress.

Decreased CO activity in the both ipsilateral and contralateral forebrain cortex, basal forebrain and hippocampus post  $\text{AlCl}_3$  injection suggest the early impairment of enzyme activity as a result of Al neurotoxicity. Unchanged NOS isoenzyme expression in the early time-point suggests no involvement of NO in CO activity, but increased iNOS expression on the 3<sup>rd</sup> day could have an influence on CO activity, following the significant influence of NO metabolism in energy metabolism upset.

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