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THE EFFECT OF AMINOGUANIDINE, AN INDUCIBLE NITRIC OXIDE SYNTHASE INHIBITOR, ON $ALCL_3$ TOXICITY IN THE RAT HIPPOCAMPUS

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Abstract - The presented experiment was carried out to determine the effectiveness of the inducible nitric oxide synthase inhibitor - aminoguanidine in modulating the toxicity of aluminum chloride on the nitrite levels, malondialdehyde concentration, reduced glutathione content, as well as cytochrome c oxidase activity of Wistar rats. The animals were killed 3 h and 30 days after treatment and the hippocampus was removed. The biochemical results show that aluminum acts as a pro-oxidant, while aminoguanidine exerts an antioxidant action in aluminum chloride-treated animals. We have also applied immunohistochemical techniques to identify iNOS expression after the treatment. Our data suggest that aminoguanidine can be effective in the protection of toxicity induced by aluminum chloride.

Key words: Aluminum, hippocampus, aminoguanidine, nitric oxide

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INTRODUCTION

Aluminum (Al) is a neurotoxic metal that may be involved in the progression of neurodegenerative changes (Dave et al., 2002). Although the mechanism of action is not known, Al has been shown to alter calcium (Ca^{2+}) flux and homeostasis, and facilitate the peroxidation of membrane lipids (Bojarski et al., 2008). Lipid peroxidation is one of the consequences of free radical reactions which causes the dysfunction and damage of cell membranes. Also, Al has been implicated as an etiological factor in several neurodegenerative diseases and as a destabilizer of cell membranes (Zatta et al., 2002).

Brain Al entry from the blood may involve transferrin-receptor mediated endocytosis and a more rapid process of transporting small molecular weight Al species. There appears to be an Al efflux from the brain, probably as Al citrate. The interaction between transferrin and its receptor may function as a general metal ion regulatory system in the central nervous system (CNS) (Van Gelder et al., 1995).

Aluminum has the ability to produce neurotoxicity by many mechanisms: it promotes the formation and accumulation of insoluble amyloid beta (Abeta) and hyperphosphorylated tau (Kawahara et al., 2001). Aluminum exposure may not be sufficient to cause an abnormal production of the principal component of senile plaques directly, but it does exacerbate the underlying events associated with brain aging and thus could contribute to the progression of neurodegeneration (Ferreira et al., 2008; Mousavi and Hellström-Lindahl, 2009). Both Al and Abeta can potentiate free radical formation by stabilizing iron in its more damaging ferrous (Fe^{2+}) form which can promote the Fenton reaction. The velocity at which Fe^{2+} is spontaneously oxidized to Fe^{3+} was significantly slowed in the presence of Al salts (Yang et al., 1999).

Also, long-term exposure to Al impaired the glutamate-nitric oxide (NO)-cyclic GMP (cGMP)

pathway, reducing glutamate-induced activation of NO synthase (NOS) and NO-induced activation of the cGMP generating enzyme, guanylate cyclase (Sheldon and Robinson, 2007). Enzymatically derived NO has been implicated in numerous physiological and pathological processes in the brain (Law et al., 2001). Nitric oxide is an enzymatic product of the NO-synthesizing enzyme 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) (Stepanichev et al., 2008). The expression and activity of iNOS play a pivotal role in sustained and elevated NO release (Pannu and Singh, 2007). Most of the published data suggest that neurons can respond to proinflammatory stimuli and take part in brain inflammation (Heneka and Feinstein, 2000). Neuronal iNOS expression has been described in different experimental settings, including the cytokine stimulation of neuronal cell lines and primary neurons *in vitro*, as well as in animal models of stroke and neurodegeneration (Heneka and Feinstein, 2000).

Neurodegenerative processes are characterized by a progressive and selective loss of neurons. Apoptosis under mitochondrial control has been implicated in this neuronal death process, involving the release of cytochrome c into the cytoplasm and the initiation of the apoptosis cascade (Ghribi et al., 2001). Mitochondria are both targets and sources of oxidative stress (Fiskum et al., 2004).

The neurons are extremely sensitive to attacks by destructive free radicals. Intracerebral Al application has been linked to mitochondrial anomalies affecting cytochrome c oxidase (COX), and these anomalies may contribute to the abnormal production of free radicals (Christen, 2000). In normal cells, oxygen derivatives are neutralized or eliminated owing to the presence of a natural defense mechanism that involves enzymatic antioxidants (glutathione peroxidase, superoxide dismutase, catalase) and water or fat-soluble non-enzymatic antioxidants (vitamins C and E, glutathione, selenium) (Stanczyk et al., 2005). L-gamma-Glutamyl-L-cysteinyl-glycine, or glutathione (GSH), an antioxidant thiol, has a specific signaling role in redox regulation (Fratelli et al., 2005).

Our previous results demonstrated the positive effects of NOS inhibitors on the the development of neurotoxicity (Jovanović et al., 2006; Stevanović et al., 2008, 2009, 2010). In view of the above, the present investigation was undertaken to examine whether the production of NO, lipid peroxidation, GSH content and COX activity after receiving intracerebral injections of aluminum chloride ($AlCl_3$) can be modulated by pretreatment with aminoguanidine (AG), a specific iNOS inhibitor.

MATERIALS AND METHODS

Animals

Male adult Wistar rats, weighing 500 ± 50 g, were used for the 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*. The animals used for procedure were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

Experimental procedure

The animals were anesthetized by intraperitoneal injections of sodium pentobarbital (0.04 g/kg b.w.). The rats were divided into four basic groups (according to drug treatment) and each group consisted of 10 animals. Using a stereotaxic instrument for small animals, chemicals were applied by a Hamilton Microsyringe. The first group of animals received 10 μl of 0.9 % saline solution and served as a control. A single dose of $AlCl_3$ (Sigma, USA) (3.7×10^{-4} g/kg b.w. in 0.01 ml of deionized water), was injected into the CA1 sector of the hippocampus (coordinates: 2.5 A; 4.2 L; 2.4 V) (Konig and Klippel, 1963). The third and fourth group were treated with aminoguanidine (AG) (Sigma Chemical Co. USA; 1×10^{-4} g dissolved in saline solution) +$AlCl_3$ and AG+saline solution. Aminoguani-

dine was applied immediately before the neurotoxin/saline solution. In all treated animals the injected intracerebral volume was 10 μl and it was always injected into the same left side.

For biochemical analysis, the rats were decapitated 3 h and 30 days after treatment and the brains were immediately removed. The ipsi- and contralateral hippocampus from individual animals were quickly isolated and homogenized in an ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, 50 mM K-Na phosphate buffer, pH 7.2. Homogenates were centrifuged twice at 1580 x g for 15 min at 4 °C. The supernatant obtained by this procedure was then frozen and stored at -70 °C (Gurd et al., 1974).

For immunohistochemical analysis, the animals were decapitated 3 h after the treatment. The brains were removed from the skull, fixed in 4% paraformaldehyde (TAAB Laboratory Equipment, Aldermaston, UK) for at least 24 h and cryoprotected in graded sucrose at 4°C. The brains were frozen in methylbutane and stored at -70°C until cryosectioning (CRIOCUT-E Reichert-Yung).

Biochemical analyses

The hippocampus from each frozen brain was dissected bilaterally and a crude mitochondrial fraction was prepared from each region as previously described.

After deproteinization, the production of NO was evaluated by measuring the nitrite and nitrate concentrations. Nitrites were assayed directly spectrophotometrically at 492 nm, using the colorimetric method of Griess (Griess reagent: 1.5% sulfanilamide in 1 M HCl plus 0.15% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water). However, the nitrates were previously transformed into nitrites by cadmium reduction (Navaro-Gonzalvez et al., 1998).

The lipid peroxidation index was measured as a quantity of the produced malondialdehyde (MDA). The thiobarbituric acid reagent (TBAR-15% trichloroacetic acid (Merck, Darmstadt)+0.375% TBA+0.25% mol HCl) reacted with the MDA formed from polysaturated fatty acids in the process of peroxidation. The product of the reaction (MDA) was measured spectrophotometrically at 533 nm (Villacara et al., 1989).

The content of reduced glutathione (GSH) was determined using 5,5-dithiobis-2-nitrobenzoic acid (DTNB, 36.9 mg in 10 ml of methanol), which reacted with the aliphatic thiol compounds in a Tris-HCl buffer (0.4 mol, pH-8.9), thus forming a yellow-colored p-nitrophenol anion. The intensity of color was used for the spectrophotometric measurement of GSH concentration at 412 nm. Brain tissue was prepared in 10% sulfosalicylic acid for GSH-determination (Anderson, 1986).

Cytochrome c oxidase activity (COX) was measured as the decrease of absorbance during the oxidation of ferrous cytochrome c to ferric cytochrome c. The kinetics were followed in potassium-phosphate buffer (0.05M, pH-7.1) for 3-5 min at 550 nm. Samples were pretreated with deoxycholate (7.5%). Sodium dithionite (1mM $Na_2S_2O_4$) was used for the reduction of cytochrome c. Reaction started with the addition of the prepared sample (0.05ml) to the reduced solution of cytochrome c (0.95ml) (Hess and Pope, 1960).

The protein content in the rat brain homogenates (hippocampus, ipsi- and contralateral) was measured by the method of Lowry using bovine serum albumin (Sigma) as standard (Lowry et al., 1951).

Chemicals were purchased from Sigma (St. Louis, MO, USA). All used chemicals were of analytical grade. All drug solutions were prepared on the day of experiment.

Processing of brain tissue and immunohistochemistry

Frozen, 8 μm-thick sections were deposited on poly-L lysine coated slides and allowed to air dry. DakoCytomation 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 min. Slides were incubated with appropriate dilutions of mAb (mouse monoclonal iNOS antibody 1:25 (Santa Cruz Biotechnology, Inc.) for 60 min. After this, the slides were incubated with the labeled polymer (DakoCytomation) conjugated to goat anti-mouse immunoglobulins in Tris-HCl buffer containing stabilizing protein and an anti-microbial agent with addition of 5% normal rat serum for 30 min. Staining was completed by a 5-10 min incubation with 3,3`-diaminobenzidine (DAB) + substrate-chromogen (DakoCytomation) which resulted in a brown-colored precipitate at the antigen site. Finally, the slides were counterstained with hematoxylin and mounted with Kaiser gel (Merck). Control slides were incubated in the same way, using mouse isotype-matched irrelevant Ab (produced in the MMA, Belgrade).

Data presentation and analysis

Data are expressed as means ± S.D. Statistical significance was determined as $p<0.05$ using either the Student's t-test or ANOVA followed by Tukey's t-test.

RESULTS

Nitrite levels in the rat hippocampus

The results presented in Table 1 show the nitrite levels (nM/mg proteins) in ipsilateral and contralateral hippocampal homogenates. At the early tested time – 3 h, the $AlCl_3$ injection resulted in an increase of nitrite production in both the ipsi- and contralateral hippocampus compared to control group. Also, AG+$AlCl_3$ application resulted in an increase of nitrite production bilaterally in the hippocampus after 3 h, compared to the control group. However, after 3 h, the AG injection resulted in lower nitrite levels compared to the $AlCl_3$- and AG+$AlCl_3$-treated groups. At 30 days after AG application the levels of nitrite production showed decreases in the contralateral hippocampus compared with the control. Also, after 30 days the AG injection resulted in lower nitrite levels in both the ipsi- and contralateral hippocampus, compared to the $AlCl_3$- and AG+$AlCl_3$-treated animals (Table 1).

Table 1. Nitrite levels (nM nitrite/mg protein) in the rat ipsilateral and contralateral hippocampus, 3 h and 30 days after the treatment. Data are means ± S.D. of 10 animals.

Group	Time point	Hippocampus ipsilateral	Hippocampus contralateral
Control	3 hours	13.98 ± 3.1	14.64 ± 2.5
	30 days	10.57 ± 1.7	11.02 ± 1.9
$AlCl_3$	3 hours	24.94 ± 6.4*	27.31 ± 8.7*
	30 days	12.18 ± 3.1	14.54 ± 5.0
AG+$AlCl_3$	3 hours	22.62 ± 8.5*	23.55 ± 5.7*
	30 days	12.19 ± 4.2	12.37 ± 1.6
AG	3 hours	8.18 ± 1.6$^{\bullet,\blacklozenge}$	9.1 ± 1.7$^{\bullet,\blacklozenge}$
	30 days	7.91 ± 1.3$^{\bullet,\blacklozenge}$	6.85 ± 1.1$^{*,\bullet,\blacklozenge}$

*Indicates a statistically significant difference between treated ($AlCl_3$-, AG+$AlCl_3$- and AG-treated) and control (sham-operated) animals ($P<0.05$).

♦Indicates a statistically significant difference between AG+$AlCl_3$-treated and AG-treated animals ($P<0.05$).

Malondialdehyde concentration in rat hippocampus

The effect of an intrahippocampal drug injection of MDA concentration (nM MDA/h/mg protein) in the hippocampus is shown in Table 2. At the early tested time – 3 h, the $AlCl_3$ injection resulted in an increase of MDA concentration bilaterally in the hippocampus, compared to the control group. AG+$AlCl_3$ application resulted in a decrease of MDA concentration bilaterally in the same brain structure after 3 h, compared to the $AlCl_3$-treated group. After 3 h, AG injection resulted in a lower MDA concentration, compared to the control, $AlCl_3$- and AG+$AlCl_3$-treated animals. At 30 days after $AlCl_3$ application the MDA concentration showed increases in the contralateral hippocampus, compared with the control. Also, after 30 days the

Table 2. Lipid peroxidation (nM MDA/h/mg protein) in the rat ipsilateral and contralateral hippocampus, 3 h and 30 days after the treatment. Data are means ± S.D. of 10 animals.

Group	Time point	Hippocampus ipsilateral	Hippocampus contralateral
Control	3 hours	23.28 ± 6.0	24.53 ± 2.6
	30 days	19.09 ± 4.8	18.82 ± 3.8
$AlCl_3$	3 hours	45.21 ± 11.3*	52.76 ± 15.8*
	30 days	23.17 ± 3.7	25.94 ± 7.0*
AG+$AlCl_3$	3 hours	19.8 ± 4.1$^{\bullet}$	18.91 ± 3.3$^{\bullet}$
	30 days	22.47 ± 3.0	21.21 ± 3.9
AG	3 hours	14.1 ± 3.2$^{*,\bullet,\blacklozenge}$	13.98 ± 3.0$^{*,\bullet,\blacklozenge}$
	30 days	16.24 ± 2.6$^{\bullet,\blacklozenge}$	14.34 ± 1.5$^{\bullet,\blacklozenge}$

*Indicates a statistically significant difference between treated ($AlCl_3$-, AG+$AlCl_3$- and AG-treated) and control (sham-operated) animals (P<0.05).
$^{\blacklozenge}$Indicates a statistically significant difference between AG+$AlCl_3$-treated and AG-treated animals (P<0.05).

AG injection resulted in a lower MDA concentration in both the ipsi- and contralateral hippocampus, compared to the $AlCl_3$- and AG+$AlCl_3$-treated animals (Table 2).

Reduced glutathione content in rat hippocampus

The results presented in Table 3 show the GSH content (nM GSH/mg protein) in the ipsilateral and contralateral hippocampal homogenates. $AlCl_3$ injection resulted in higher levels of GSH concentration after 3 h bilaterally in the hippocampus, compared to the control group. After 3 h, AG+$AlCl_3$ application resulted in lower GSH content in both the ipsi- and contralateral hippocampus, compared to the $AlCl_3$-treated group. At the same tested time, AG application resulted in a lower GSH content in the ipsilateral hippocampus, compared to the control, as well as in both the ipsi- and contralateral hippocampus, lower values were measured compared to the $AlCl_3$- and AG+$AlCl_3$-treated animals. At 30 days after $AlCl_3$ application

Table 3. Reduced glutathione concentration (nM GSH/mg protein) in the rat ipsilateral and contralateral hippocampus, 3 h and 30 days after the treatment. Data are means ± S.D. of 10 animals.

Group	Time point	Hippocampus ipsilateral	Hippocampus contralateral
Control	3 hours	31.91 ± 10.7	34.27 ± 11.6
	30 days	25.15 ± 5.2	24.64 ± 9.2
$AlCl_3$	3 hours	155.57 ± 42.8*	164.16 ± 52.3*
	30 days	30.57 ± 9.9*	32.97 ± 10.5
AG+$AlCl_3$	3 hours	34.45 ± 7.5$^{\bullet}$	37.07 ± 10.3$^{\bullet}$
	30 days	3.19 ± 1.4$^{*,\bullet}$	3.33 ± 0.7$^{*,\bullet}$
AG	3 hours	22.05 ± 6.2$^{*,\bullet,\blacklozenge}$	20.18 ± 4.4$^{\bullet,\blacklozenge}$
	30 days	3.29 ± 1.1$^{*,\bullet}$	3.04 ± 1.1$^{*,\bullet}$

*Indicates a statistically significant difference between treated ($AlCl_3$-, AG+$AlCl_3$- and AG-treated) and control (sham-operated) animals (P<0.05).
$^{\bullet}$Indicates a statistically significant difference between treated (AG+$AlCl_3$- and AG-treated) and $AlCl_3$-treated animals (P<0.05).
$^{\blacklozenge}$Indicates a statistically significant difference between AG+$AlCl_3$-treated and AG-treated animals (P<0.05).

the GSH content was increased in the ipsilateral hippocampus, compared to the control. Intrahippocampal AG+$AlCl_3$ injections resulted in generally lower contents of GSH compared to the control and $AlCl_3$-treated animals. Also, after 30 days the AG injection resulted in lower GSH concentration, in both ipsi- and contralateral hippocampus, compared to the control and the $AlCl_3$-treated animals (Table 3).

Activity of cytochrome c oxidase in rat hippocampus

The activity of COX (mg cyt. c/mg protein) obtained for the ipsi- and contralateral hippocampus were similar. The COX activity was significantly lower bilaterally in the hippocampus after $AlCl_3$ application compared to the saline-injected animals at all tested times (3 h, 30 days) (Table 4). Intrahippocampal AG+$AlCl_3$ injection resulted in generally lower levels of COX activity compared to

Table 4. Cytochrome c oxidase activity (mg cyt.C/mg protein) in the rat ipsilateral and contralateral hippocampus, 3 h and 30 days after the treatment. Data are means ± S.D. of 10 animals.

Group	Time point	Hippocampus ipsilateral	Hippocampus contralateral
Control	3 hours	0.24 ± 0.07	0.22 ± 0.07
	30 days	0.35 ± 0.1	0.31 ± 0.08
$AlCl_3$	3 hours	0.17 ± 0.05*	0.15 ± 0.04*
	30 days	0.10 ± 0.03*	0.11 ± 0.03*
AG+$AlCl_3$	3 hours	0.19 ± 0.06*,•	0.18 ± 0.05*,•
	30 days	0.22 ± 0.07*,•	0.20 ± 0.06*,•
AG	3 hours	0.22 ± 0.07*,•	0.19 ± 0.06*,•
	30 days	0.30 ± 0.08*,•	0.25 ± 0.08*,•

*Indicates a statistically significant difference between treated ($AlCl_3$-, AG+$AlCl_3$- and AG-treated) and control (sham-operated) animals ($P<0.05$).
•Indicates a statistically significant difference between treated (AG+$AlCl_3$- and AG-treated) and $AlCl_3$-treated animals ($P<0.05$).

the control, while higher COX activity was measured at both tested times, compared to $AlCl_3$-treated animals. At both times a single AG injection resulted in lower COX activity bilaterally in hippocampus, compared to the controls, while higher COX activity were measured at the all tested times compared to the $AlCl_3$-treated animals (Table 4). There was no statistically significant difference between the COX activity obtained from each hemisphere after the treatment, although the injection site was in the ipsilateral hippocampus.

iNOS

Inducible NOS labeling was present in the control animals 3 h (Fig. 1A) after saline solution application and enhanced in the $AlCl_3$-treated animals 3 h (Fig. 1B) after injection. Photomicrographs of a hippocampus immunostained for iNOS after a delay of 3 h following AG+$AlCl_3$ is presented in Fig. 1C, and after injection of AG in Fig. 1D.

DISCUSSION

The application of $AlCl_3$ to the CA1 sector of the hippocampus resulted in the reduced activity of COX with a significant bilateral increase in the NO production, MDA concentration and GSH content in the hippocampus. This suggests that the inhibition of NOS by AG protects the cells in this region from $AlCl_3$-induced damage and may therefore limit the retrograde and anterograde spread of neurotoxicity.

Nitric oxide can react with superoxide to form the very harmful peroxynitrite ($ONOO^-$), a process referred to as oxidative stress, which is likely to play a role in neurodegenerative changes. Peroxynitrite causes considerable damage that exacerbates the damage caused by the hyperactive microglia. Under conditions of neurodegeneration, excessive activation of microglia can contribute to the neurodegenerative process by releasing potentially cytotoxic substances, including the cytotoxic free radical NO (Schubert et al., 2001).

In our study, $AlCl_3$ injection resulted in an increased nitrite production after 3 h, and in unchanged nitrite concentration after 30 days, bilaterally in hippocampus, compared to the controls (Table 1). It has been previously known (Tohgi et al., 1998) that the production and oxidation of NO in the brain increased in early stages of the disease while it was decreasing with elevating loss of neurons.

Under the conditions of this experiment, AG+$AlCl_3$ application produced a rapid (within 3 h) increase in the nitrite levels in the hippocampus compared to the control animals (Table 1). Reactive glial cell properties could contribute to the pathomechanisms underlying Al intoxication by favoring oxidative neuronal damage and Abeta toxicity. A critical step is apparently reached when pathological glial activation is no longer restricted to microglia and includes astrocytes, which may lose their physiological negative feed-back control on microglial NO production and even contribute to neurotoxic $ONOO^-$ formation.

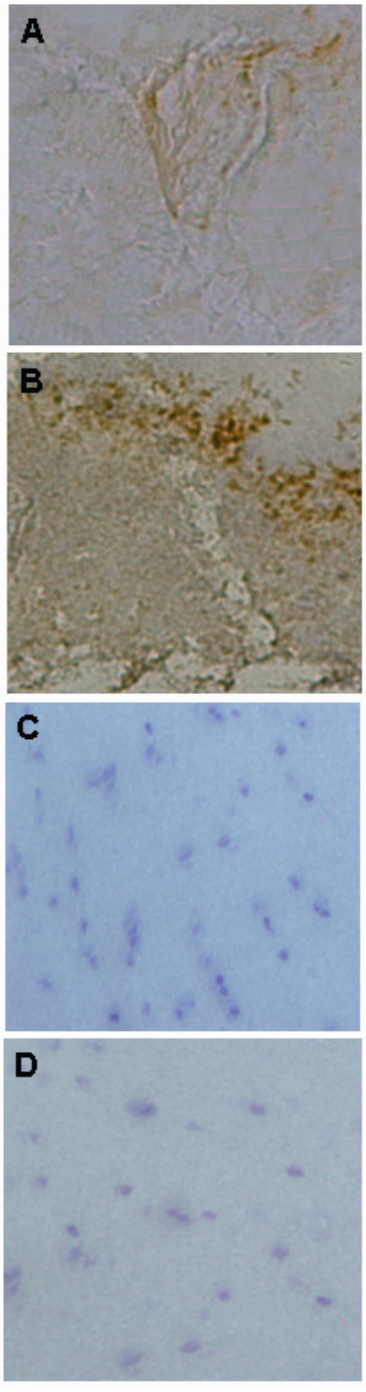

Fig. 1A-D. Immunohistochemical detection of iNOS expression within the hippocampus at 3 h after injection of saline solution (A), $AlCl_3$ (B), AG+$AlCl_3$ (C), as well as AG (D). The iNOS stain was observed in both saline solution-(A) and $AlCl_3$-injected animals (B). iNOS staining was not detected in the hippocampus of AG+$AlCl_3$-(C) and AG-injected rats (D). Magnification x 400.

Literature results imply that the inhibition of iNOS protects cells from the toxicity caused by the secretory products of microglia and monocytes (Combs et al., 2001). In our experiment, at various times following the injection of AG the nitrite production in the hippocampus decreased immediately (3 h), compared to the AG+$AlCl_3$-treated animals, and continued to decrease gradually throughout the experiment (30 days) (Table 1).

We have shown that 30 days after injection, the application of AG produced a decrease in nitrite production in the contralateral hippocampus compared to the control (Table 1). The results of Gorlach et al. suggest that the tissue nitrate/nitrite concentration was significantly attenuated by treatment with AG not only in the ipsilateral but also in the contralateral hemisphere (Gorlach et al., 2000). Aminoguanidine inhibits not only iNOS but also other enzymes, such as nNOS and advanced glycation end-product synthase. Administration of the selective iNOS inhibitor AG prevents NO-accumulation in the cortex and the impairment of glutamate uptake in synaptosomes. A sustained overproduction of NO via iNOS expression may be responsible, at least in part, for some of the neurodegenerative changes caused by stress and support a possible neuroprotective role for specific iNOS inhibitors in this situation (Olivenza et al., 2000).

There are several lines of evidence that show a key role of reactive oxygen species (ROS) in both intracellular signaling and intracellular communication, processes involved in maintaining homeostasis. On the other hand, when excessively produced *in vivo*, ROS are deleterious to the integral components of the cell and cause their dysfunctions. Some experimental data indicate that ROS-mediated lipid peroxidation, protein oxidation and oxidative alterations to nucleic acids are crucial events in the unfavorable actions of ROS (Juranek and Bezek, 2005). Lipid peroxidation is a measure of tissue destruction.

In our study, $AlCl_3$ injection resulted in an increased MDA concentration after 3 h bilaterally, as well as after 30 days contralaterally in the hippo-

campus compared to the controls (Table 2). Aluminum may facilitate increases in intracellular Ca^{2+} and ROS, and potentially contribute to the neurotoxicity induced by other neurotoxicants (Mundy et al., 1997).

The AG+$AlCl_3$ treatment which caused a significant decrease in MDA concentration, especially at 3 h, compared to $AlCl_3$-treated animals (Table 2), suggests activation of the antioxidative system, resulting in an aggressive oxidative mechanism blockade initiated by the neurotoxin application.

The present data indicate that the inhibition of iNOS by AG can effectively decrease the MDA concentration bilaterally in the hippocampus in all tested times (3 h, 30 days) after the treatment compared to $AlCl_3$-treated animals (Table 2), pointing to the balance of oxidative mechanisms.

It is conspicuous that there is a strong association between free radical accumulation and the evolution of inflammatory-related responses (Haddad and Harb, 2005). The reduced form of glutathione represents the main endogenous antioxidative compound responsible for control of the redox state in the cell, as well as extracellular space.

In our study, $AlCl_3$ injection resulted in an increased GSH content after 3 h bilaterally in the hippocampus, compared to the controls (Table 3). It has been previously shown (Li et al., 1998) that Al^{3+} alters the phosphorylation of tau and causes its aggregations. Phosphorylation sensitizes tau to Al^{3+} and phosphorylated tau transforms irreversibly into a phosphatase and protease resistant aggregate in the presence of this metal ion.

Decreased GSH content along with decreased MDA concentration in the ipsilateral hippocampus 3 hours after AG+$AlCl_3$ application compared to $AlCl_3$-treated group, suggest an intensive depletion of this tripeptide in antioxidative defense and the elevating of its oxidative form, generated in the reactions of oxidative stress. The obtained results confirm the NOS inhibitor antioxidative effects based on oxidative stress reduction, as well.

We have shown that 30 days after AG application the iNOS inhibitor produced a decrease in GSH content, compared to the control and $AlCl_3$-treated group (Table 3), showing inadequate antioxidative cell defense.

Mitochondria are essential to the cell for maintaining the normal voltage gradient across the cell membrane as well as a number of processes that control intracellular Ca^{2+}. Complex IV, COX, is the last component of the mitochondrial electron-transport chain. It catalyzes the four-electron reduction of molecular oxygen to water, which is accompanied by proton translocation into the intramembrane space of mitochondria. Nitric oxide or $ONOO^-$ could potentially be responsible for the mitochondrial damage occurring in the ischemic brain and at inflammatory sites. It has been known that NO binds to COX and it may act as an inhibitor of this enzyme at physiological concentrations. This reaction is reversible and competitive with oxygen. In contrast, $ONOO^-$ has little or no effect on COX but inhibits respiratory complex I-III in an apparently irreversible manner (Toiber and Soreq, 2005).

Our previous studies show that COX activity decreased bilaterally 7 and 12 days after $AlCl_3$ application to the hippocampus, forebrain cortex and basal forebrain (Jovanović et al., 2000, 2006).

Under the conditions of this experiment, the decrease in COX activity after 3 h and 30 days bilaterally in the hippocampus of the $AlCl_3$-treated animals compared to the controls (Table 4), indicates a deficiency in reducing equivalents with a consequent diminishing of the proton gradient, i.e. reentering of protons to the mitochondria, followed by insufficient ATP synthesis. Aluminum impedes glycolysis, followed by a decreased production of reducing equivalents, which are necessary for the regeneration/synthesis of the GSH form, as well as for the maintenance of the proton gradient and functionality of the mitochondrial electron-transport chain.

The decreased activity of COX in AG+$AlCl_3$-treated rats, compared to the control (Table 4),

suggests the existence of oxidative stress. This process appears to be due to the effects of $AlCl_3$. One of its effects could be mediated through a decrease in synthesis of NADH. However, we have shown that the application of AG+$AlCl_3$ produced an increase in COX activity, compared to the $AlCl_3$-treated animals (Table 4). Literature results (Madrigal et al., 2001), implicate that administration of the preferred iNOS inhibitor protects against the inhibition of the activity of complexes of the mitochondrial respiratory chain as well as preventing NO accumulation, lipid peroxidation and GSH depletion induced by stress.

We used immunohistochemistry to examine the expression of iNOS in the CNS of old adult rats (3 month-old). Inducible NOS-positive cells occurred in perivascular cells 3 h after saline solution administration (Fig. 1A). However, the maximal expression of iNOS occurred 3 h after $AlCl_3$ application in the hippocampus (Fig. 1B). This could be the result of brain-blood barrier disruption and induced inflammation in the rat brain (Heneka et al., 2000). Our data suggests that no labeling of cells for iNOS was seen 3 h after AG+$AlCl_3$-injection (Fig. 1C), or AG-injection in rat brain (Fig. 1D). Aminoguanidine, as a selective inhibitor of the inducible form of NOS, inhibits enzyme activity applied with neurotoxin or saline solution, respectively.

CONCLUSION

The decreased COX activity as well as increased nitrite levels, MDA concentration and GSH content after 3 h and 30 days of $AlCl_3$ treatment bilaterally in the hippocampus indicate the propagation of oxidative stress. Intrahippocampal administration of $AlCl_3$ could induce an aggregation of the cytoskeletal elements, forming very stable connections between phosphate groups of the hyperphosphorilated tau elements – low molecular weight microtubule associated proteins. These changes could cause retrograde trans-neuronal damage of the cholinergic neurons in the basal forebrain, with consequent damage of the cholinergic inputs in the hippocampus. In this case, NOS inhibitors such as AG could have a potentially neuroprotective effect.

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ЕФЕКАТ АМИНОГВАНИДИНА, ИНХИБИТОРА АЗОТ ОКСИД СИНТАЗЕ, НА ТОКСИЧНОСТ $ALCL_3$ У ХИПОКАМПУСУ ПАЦОВА

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У експерименту је одређивана ефикасност инхибитора индуцибилне форме азот оксид синтазе – аминогванидина, у модулацији токсичности алуминијум хлорида на ниво нитрита, концентрацију малондиалдехида, садржај редукованог глутатиона, као и активност цитохром ц оксидазе код Wистар пацова. Животиње су декапитоване 3 часа и 30 дана након одговарајућег третмана и изолован је хипокампус. Резултати добијени на биохемијском нивоу показују да алуминијум делује као про-оксидант, док аминогванидин показује антиоксидативно дејство код животиња третираних алуминијум хлоридом. Поред тога, коришћене су и имунохистохемијске технике за идентификацију iNOS експресије, 3 часа након примене одговарајућег третмана. Наши резултати показују да аминогванидин може спречити токсичност индуковану алуминијум хлоридом.