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Effects of various nitric oxide synthase inhibitors on $AlCl_3$-induced neuronal injury in rats

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Abstract: The present study was aimed at determining the effectiveness of nitric oxide synthase (NOS) inhibitors: *N*-nitro-L-arginine methyl ester, 7-nitroindazole and aminoguanidine in modulating the toxicity of $AlCl_3$ on superoxide production and the malondialdehyde concentration of Wistar rats. The animals were sacrificed 10 min and 3 days after the treatment and the forebrain cortex was removed. The results show that $AlCl_3$ exposure promotes oxidative stress in different neural areas. The biochemical changes observed in the neuronal tissues show that aluminum acts as pro-oxidant, while NOS inhibitors exert an anti-oxidant action in $AlCl_3$-treated animals.

Keywords: aluminum; forebrain cortex; NOS inhibitors; superoxide production; lipid peroxidation.

INTRODUCTION

Aluminum has the ability to produce neurotoxicity by many mechanisms. In addition to the promotion of insoluble beta-amyloid (A beta) and hyperphosphorylated tau protein formation and accumulation, Al can alter neuronal signal transduction pathways associated with glutamate receptors.[1,2] In cerebella neurons in culture, long term-exposure to Al added *in vitro* impaired the glutamate (Glu)–nitric oxide (NO)–cyclic GMP (cGMP) pathway, reducing the glutamate-induced activation of NO synthase (NOS) and NO-induced activation of the cGMP generating enzyme, guanylate cyclase. These findings suggest that the impairment of the Glu–NO–cGMP pathway in the brain may be responsible for some of the neurological alteration induced by Al.[3–5]

Free radicals (oxidative toxins) have been implicated in the destruction of cells through the process of lipid peroxidative damage of the cell membranes. Aluminum has been shown to alter the Ca^{2+} flux and homeostasis, and facilitate

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peroxidation of membrane lipids.[6] After exposure to Al, a statistically significant increase in malondialdehyde (MDA), an index of lipid peroxidation, was observed.[7,8]

Molecular oxygen is the primary biological electron acceptor that plays vital roles in fundamental cellular functions. However, the beneficial properties of O_2 are accompanied by the inadvertent formation of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•).[9,10] Evidence is being amassed that iron (Fe) accumulates in the brain and catalyzes O_2^- formation, which reacts with NO to form the very harmful peroxynitrite ion ($ONOO^-$), that nitrates tyrosine residues to form nitrotyrosine.[11]

The NO-synthesizing enzyme NOS is present in the mammalian brain in three different isoforms, two constitutive enzymes (*i.e.*, neuronal nNOS and endothelial eNOS) and one inducible enzyme (iNOS). All three isoforms are aberrantly expressed after Al intoxication giving rise to elevated levels of NO, which is apparently involved in neurodegeneration by various different mechanisms, including oxidative stress and activation of intracellular signaling mechanisms.[12]

Cell death and changes in neurite morphology were partly reduced when NO production was inhibited by NOS inhibitors.[13–17] In view of the above, the present study was undertaken to examine whether O_2^- production and MDA concentration after receiving intracerebral injections of $AlCl_3$ can be modulated by the co-administration with various NOS inhibitors: non-specific inhibitor NOS (*N*-nitro-L-arginine methyl ester – L-NAME) and specific inhibitors: neuronal NOS inhibitor (7-nitroindazol – 7-NI) and inducible NOS inhibitor (aminoguanidine – AG).[18–20]

EXPERIMENTAL

Materials and animals

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

Male adult Wistar rats with a body mass 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 the procedure were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

Experimental procedure

For biochemical analysis, the rats were divided into eight basic groups (according to drug treatment). Each basic group consisted of two different subgroups (according to survival times – 10 min and 3 days) and each subgroup consisted of 10 animals. Animals were anesthetized by intraperitoneal injections of sodium pentobarbital (0.04 g/kg b.w.). Using a stereotaxic instrument for small animals, the chemicals were administered by a Hamilton microsyringe and injected into the CA1 sector of the hippocampus (coordinates: 2.5 A; 4.2 L; 2.4 V).[21] The NOS inhibitors (L-NAME, 7-NI or AG) were immediately applied before a neuro-

toxin/saline solution. In all treated animals, the injected intracerebral volume was 10 µl (Table I) and it was always injected into the same left side. All animals were decapitated and the heads were immediately frozen in liquid nitrogen and stored at –70 °C until use. Then the ipsilateral and contralateral forebrain cortex (FC) was quickly isolated and homogenized in ice-cold buffer containing 0.25 M sucrose, 0.10 mM EDTA, 50 mM K-Na phosphate buffer, pH 7.2. The homogenates were centrifuged twice at 1580 rpm for 15 min at 4 °C. The supernatant (crude mitochondrial fraction) obtained by this procedure was then frozen and stored at –70 °C.[22]

Table I. All the experimental animal groups with the applied dosage for the appropriate treatment

Number	Group	Dosage
1	Control	0.9% saline solution
2	$AlCl_3$	3.7×10^{-4} g/kg b.w. dissolved in 0.010 ml of deionizied water
3	L-NAME +$AlCl_3$	1×10^{-4} g dissolved in saline solution + $AlCl_3$
4	7-NI+$AlCl_3$	1×10^{-4} g dissolved in olive oil + $AlCl_3$
5	AG+$AlCl_3$	1×10^{-4} g dissolved in saline solution + $AlCl_3$
6	L-NAME	1×10^{-4} g dissolved in saline solution + saline solution
7	7-NI	1×10^{-4} g dissolved in saline solution + saline solution
8	AG	1×10^{-4} g dissolved in saline solution + saline solution

Biochemical analysis

The superoxide anion content was determined through the reduction of nitro blue tetrazolium (Merck, Darmstadt, Germany) in a nitrogen saturated alkaline medium. Kinetic analysis was performed at 550 nm.[23]

The lipid peroxidation index was measured as the quantity of produced malondialdehyde (MDA). Thiobarbituric acid reagent (TBAR–15 % trichloroacetic acid (Merck, Darmstadt) + + 0.375 % TBA + 0.25 % mol HCl) reacted with MDA, which had been produced from polysaturated fatty acids in the process of peroxidation. The product of reaction – MDA, was measured spectrophotometrically at 533 nm.[24]

The protein content in the rat brain homogenates (forebrain cortex-FC, ipsilateral and contralateral) was measured by the Lowry method using bovine serum albumin (Sigma) as the standard.[25]

Data presentation and analysis

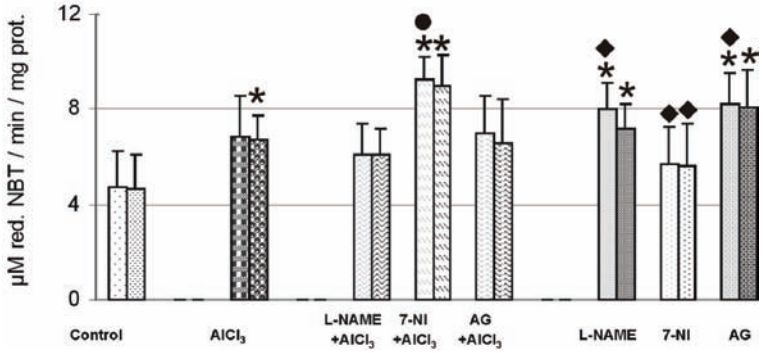
Statistical analysis was performed using the statistical software program, Statistic 5.0 for Windows. Descriptive data are expressed as the mean±standard deviation (*SD*). The statistical significance was determined as $p < 0.05$ using either the Student's *t*-test or ANOVA followed by the Tukey's *t*-test.

RESULTS

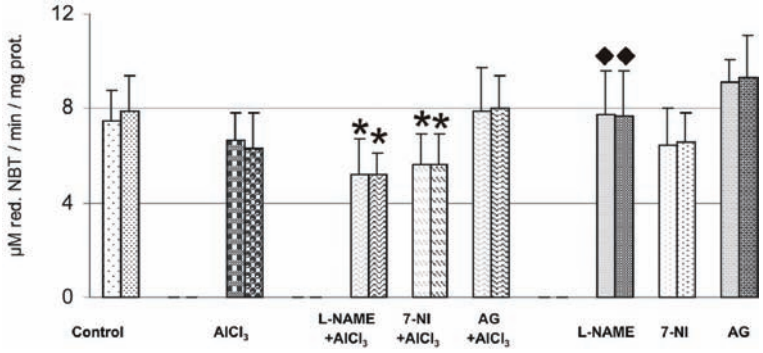
Superoxide production in the rat forebrain cortex (FC)

The O_2^- levels (µM red. NBT min^{-1} mg^{-1} proteins) bilaterally in the rat FC homogenates at 10 min (Fig. 1A) and 3 days (Fig. 1B) after the treatment are presented in Fig. 1. At the early tested time (10 min), $AlCl_3$ injection resulted in higher levels of O_2^- production in the contralateral FC, compared to the control animals ($p < 0.05$). Also, 7-NI + $AlCl_3$ application, as well as L-NAME injection and AG injection, resulted in an increase in the production of O_2^- bilaterally in

the FC after 10 min, compared to the control groups. However, after 3 days, L-NAME + $AlCl_3$ and 7-NI + $AlCl_3$ injection resulted in lower levels of O_2^- production bilaterally in the same brain structure, compared to the control groups. At 10 min after 7-NI + $AlCl_3$ application, the O_2^- levels showed increase in the ipsilateral FC, compared with the $AlCl_3$-treated animals. L-NAME application resulted in an increase of the O_2^- levels ipsilaterally in the FC after 10 min and bilaterally in this brain structure after 3 days, compared to the L-NAME + $AlCl_3$--treated groups. Also, AG injection resulted in an increase of O_2^- production in the ipsilateral FC after 10 min, compared to the AG + $AlCl_3$-treated group. However, 7-NI injection resulted in a decrease of O_2^- production bilaterally in the FC after 10 min, compared to the 7-NI + $AlCl_3$-treated groups (Fig. 1).



(A)

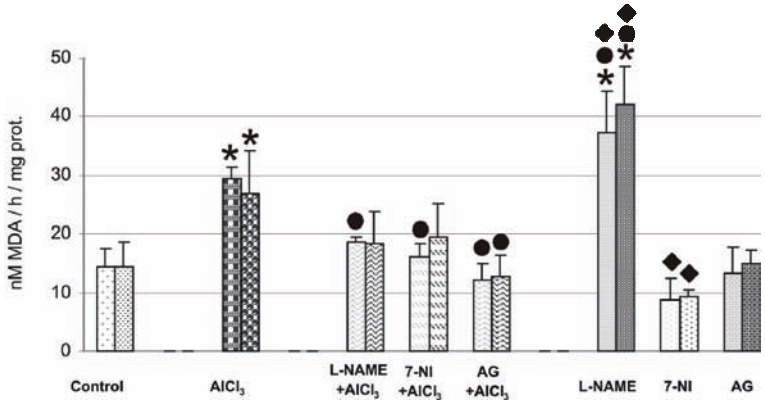


(B)

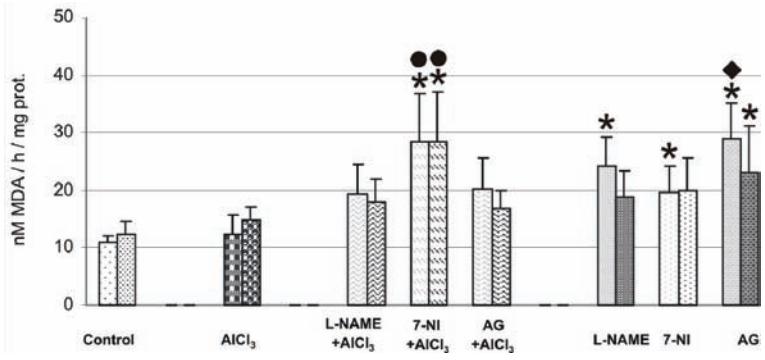
Fig. 1. The effect of intrahippocampal drug injection on O_2^- production (µM red. NBT/min/mg protein) in the rat ipsilateral and contralateral FC at different survival times – 10 min (A) and 3 days (B) after treatment. Data are means ±*SD* of 10 animals. *A statistically significant difference between the treated ($AlCl_3$-, NOS inhibitors + $AlCl_3$- and NOS inhibitors-treated) and the control (sham-operated) animals ($p < 0.05$). •A statistically significant difference between the NOS inhibitors + $AlCl_3$-treated and the $AlCl_3$-treated animals ($p < 0.05$). ♦A statistically significant difference between the NOS inhibitors-treated and the NOS inhibitors + $AlCl_3$-treated animals ($p < 0.05$).

Malondialdehyde concentration in the rat forebrain cortex (FC)

The MDA concentration (nM MDA h^{-1} mg^{-1} proteins) in the ipsilateral and contralateral FC homogenates at 10 min (Fig. 2A) and 3 days (Fig. 2B) after the treatment are shown in Fig. 2. Ten minutes after the injection of $AlCl_3$ injection and of L-NAME, the MDA concentration was bilaterally increased in the FC compared to controls, with the difference being statistically significant (Student's *t*-test; $p < 0.05$). Also, in the same brain structure, after 3 days, the MDA concentration was increased bilaterally after 7-NI+$AlCl_3$ and AG injection, as well as ipsilaterally after L-NAME and 7-NI application, compared to the control groups.



(A)



(B)

Fig. 2. The effect of intrahippocampal drug injection on the MDA concentration (nM MDA/h/mg protein) in the rat ipsilateral and contralateral FC at different survival times – 10 min (A) and 3 days (B) after treatment. Data are means ±*SD* of 10 animals. *A statistically significant difference between treated ($AlCl_3$-, NOS inhibitors + $AlCl_3$- and NOS inhibitors-treated) and the control (sham-operated) animals ($p < 0.05$). •A statistically significant difference between the treated (NOS inhibitors + $AlCl_3$- and NOS inhibitors-treated) and the $AlCl_3$-treated animals ($p < 0.05$). ♦A statistically significant difference between the NOS inhibitors-treated and the NOS inhibitors + $AlCl_3$-treated animals ($p < 0.05$).

Ten minutes after L-NAME + $AlCl_3$ and 7-NI + $AlCl_3$ injection, lower MDA concentrations were measured in the ipsilateral FC, and after AG + $AlCl_3$ injection in the ipsilateral and contralateral FC, compared to the $AlCl_3$-treated group. However, after 3 days, 7-NI + $AlCl_3$ injection resulted in a higher MDA concentration bilaterally in the same brain structure, compared to the $AlCl_3$-treated group. At the early tested time (10 min), L-NAME application resulted in an increase in the MDA concentration bilaterally in the FC, compared with the $AlCl_3$-treated animals, as well as compared to the L-NAME + $AlCl_3$-treated groups ($p < 0.05$). Also, AG injection resulted in an increase in the MDA concentration in the ipsilateral FC after 3 days, compared to the AG + $AlCl_3$-treated group. However, 7-NI injection resulted in a decrease in the MDA concentration bilaterally in the FC after 10 min, compared to the 7-NI + $AlCl_3$-treated group (Fig. 2).

DISCUSSION

The application of $AlCl_3$ to the CA1 sector of the hippocampus resulted in a significant increase in O_2^- production and the MDA concentration in the FC. This suggests that inhibition of NOS by L-NAME, 7-NI or AG can modulate $AlCl_3$ poisoning and, therefore, may limit the retrograde and anterograde spread of toxicity.

In this study, $AlCl_3$ application produced a rapid (within 10 min) increase in O_2^- production contralaterally in the FC, compared to the control (Fig. 1A). Literature data suggest that Al is suspected to be associated with oxidative stress, possibly due to the pro-oxidant properties of A beta in the senile plaques.[26,27] The underlying mechanism by which this occurs is not well understood although interactions between amyloid and Fe have been proposed. The presence of low molecular weight Fe compounds can stimulate free radical production in the brain. Both Al and A beta can potentiate free radical formation by stabilizing Fe in its more damaging ferrous (Fe^{2+}) form, which can promote the Fenton reaction. The rate at which Fe^{2+} is spontaneously oxidized to Fe^{3+} was significantly slower in the presence of Al salts.[28]

There are several lines of evidence that show a key role of ROS in both intracellular signaling and intracellular communication, processes involved in maintaining homeostasis. Some experimental data indicate that ROS-mediated lipid peroxidation, protein oxidation and oxidative alterations to nucleic acids are crucial events of the unfavorable actions of ROS.[29,30] Lipid peroxidation is a measure of tissue destruction. Literature data suggest that Al may facilitate increases in intracellular Ca^{2+} and ROS, and potentially contribute to neurotoxicity induced by other neurotoxicants.[6] In this study, it was shown that 10 min after intrahippocampal $AlCl_3$ injection, the application of a neurotoxicant produced an increase in the MDA concentration bilaterally in the FC, compared to control animals (Fig. 2A).

Neuropharmacological data indicate that A beta toxicity is mediated by an excitotoxic cascade involving blockage of astroglial glutamate uptake, sustained activation of NMDA receptors and an overt intracellular Ca^{2+} influx.[31] These changes are associated with increased NOS activity in cortical target areas that may directly lead to the generation of free radicals. A sustained overproduction of NO *via* NOS expression may be responsible, at least in part, for some of the neurodegenerative changes caused by stress and support a possible role for NOS inhibitors in this situation.[32]

Decreased O_2^- production bilaterally in the FC at 3 days in the L-NAME + + $AlCl_3$ group and the 7-NI + $AlCl_3$ group compared to the controls (Fig. 1B), along with decreased SOD activity (results not shown) confirm the achieved anti--oxidative defense.

In addition, the decreased MDA concentration ipsilaterally in the same brain structure at 10 min in the NOS inhibitors + $AlCl_3$ groups, compared to the $AlCl_3$--treated animals, (Fig. 2A), suggests activation of the anti-oxidative system, resulting in an aggressive blockage of the oxidative mechanisms initiated by neurotoxicant application.

The significant increase in O_2^- production after L-NAME and after AG injection (at 10 min bilaterally in the FC, compared to the controls), which correlates with unchanged O_2^- levels after 7-NI application in the same brain structure (Fig. 1A), indicates the permanent and long-lasting effect of nNOS blocking at the early tested time. These findings suggest that treatment with 7-NI leads to the protection of brain neurons against neuronal injuries by impairment of cellular energy metabolism and oxidative stress.[33–35]

The present data, which indicates an increased MDA concentration 10 min after L-NAME application bilaterally in the FC compared to the controls (Fig. 2A), suggests that NOS inhibition exhibits protective effects for cellular membrane. This only occurs during the presence of oxidative stress caused by $AlCl_3$, meaning that peroxidation after L-NAME application is not developed through NO, but through the creation of $^{\bullet}OH$ from O_2^- and H_2O_2.[36]

In addition, it was shown in this study that L-NAME injection after 10 min produced an increase in the MDA concentration, compared to the L-NAME + + $AlCl_3$-treated group (Fig. 2A). It is known[37] that glutamate excitotoxicity, oxidative stress, and mitochondrial dysfunctions are common features leading to neuronal death after Al intoxication. Nitric oxide, alone or in cooperation with O_2^- and $ONOO^-$, is emerging as a predominant effector of neurodegeneration.

CONCLUSIONS

In conclusion, the present data revealed that NO is included in the toxicity induced by $AlCl_3$ application in the CA1 hippocampal sector, resulting in both

temporal and spatial spreading of damage to the FC, so that NOS inhibitors (L-NAME, 7-NI, AG) could have potentially beneficial effects.

И З В О Д

ЕФЕКТИ РАЗЛИЧИТИХ ИНХИБИТОРА АЗОТ-ОКСИД-СИНТАЗЕ НА ОШТЕЋЕЊЕ НЕУРОНА ИЗАЗВАНО $AlCl_3$

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У експерименту је одређивана ефикасност инхибитора азот-оксид-синтазе (NOS): метил-естра *N*-нитро-L-аргинина, 7-нитроиндазола и аминогванидина у модулацији токсичности $AlCl_3$ на стварање супероксидног анјона и концентрацију малондиалдехида код Wistar пацова. Животиње су жртвоване 10 min и 3 дана након третмана и изолована је кора великог мозга. Резултати показују да излагање $AlCl_3$ покреће оксидативни стрес у различитим можданим регионима. Биохемијске промене описане у неуронском ткиву показују да алуминијум делује као про-оксидант, док инхибитори NOS имају антиоксидативно дејство код животиња третираних $AlCl_3$.

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