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THE EFFECT OF 7-NITROINDAZOLE ON ALUMINIUM TOXICITY IN THE RAT BRAIN

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Summary

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We investigated the influence of 7-nitroindazole (7-NI), a selective neuronal nitric oxide synthase (nNOS) inhibitor *in vivo*, on nitrite concentration, superoxide production and superoxide dismutase activity in $AlCl_3$-treated Wistar rats. The observed biochemical changes in neuronal tissues showed that aluminium served as pro-oxidant, while 7-NI had antioxidant effects in $AlCl_3$-treated animals. The protective effects of 7-NI against the cellular damage caused by $AlCl_3$-induced oxidative stress, together with its low toxicity, make 7-NI worthy of investigation as a potential supplement in the treatment of neurological disorders in which the oxidative stress effects have to be minimized.

Key words: 7-nitroindazole, aluminium, nitric oxide, superoxide, superoxide dismutase

INTRODUCTION

Aluminium (Al) is a trivalent cation that does not undergo redox changes. The exact mechanism of Al toxicity is not known. However, accumulating evidence suggests that it can potentiate prooxidative and inflammatory events, leading to tissue damage (Campbell, 2002).

The supposed mechanism of Al entrance in brain involves transferrin-receptor mediated endocytosis and a more rapid process transporting small molecular weight Al species. Aluminium has the ability to produce neurotoxicity by many mechanisms. Beside 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. In cerebellar neurons in culture, long-term exposure to Al added *in vitro* 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. These findings suggest that the impairment of the Glu-NO-cGMP pathway in the brain may be responsible for some of the neurological alterations induced by Al (Cucarella *et al.*, 1998; Hermenegildo *et al.*, 1999; Liansola *et al.*, 1999; Canales *et al.*, 2001; Rodella *et al.*, 2001).

However, both Al and A beta potentiate free radical production, maintaining the more damaging ferrous (Fe^{2+})

form (Yang *et al.*, 1999). Also, Al increases iron-induced oxidative injury (Yokel, 2000). Iron catalyzes the formation of superoxide anion ($O_2^{\cdot-}$), which reacts with NO to form the very harmful peroxynitrite anion ($ONOO^-$) (Johnson, 2001). Peroxynitrite serves as a strong oxidizing and nitrating agent that could react with all classes of biomolecules. In the CNS it can be generated by microglial cells (Torreilles *et al.*, 1999).

Aluminium has been shown to alter calcium (Ca^{2+}) flux and homeostasis, and to facilitate peroxidation of membrane lipids. Aluminium may facilitate increases in intracellular Ca^{2+} and reactive oxygen species (ROS), and potentially contributes to neurotoxicity induced by other neurotoxicants (Mundy *et al.*, 1997). Reactive oxygen species are an integral part of signal transduction essential for intracellular communication. The balance between pro- and antioxidative processes determines normal cellular metabolism manifested by genes activation and/or protein expression in response to exo- and endogenous stimuli. The disturbed balance leads to the state known as oxidative stress inducing damage of DNA, proteins, and lipids (Stanczyk *et al.*, 2005).

In view of the above, the present study was undertaken to examine whether the production of NO and $O_2^{\cdot-}$, as well as SOD activity, due to intracerebral injections of $AlCl_3$, can be modulated with 7-nitroindazole (7-NI) pretreatment, the selective neuronal nitric oxide synthase (nNOS) inhibitor (Vasiljević *et al.*, 2002; Jovanović *et al.*, 2006).

MATERIALS AND METHODS

Animals

Male adult Wistar rats, weighting 500 ± 50 g, were used for experiments. Animals were housed two/three per cage (Erath, FRG), in an air-conditioned room at a temperature of 23 ± 2 ºC 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*.

Animals used for procedure were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

Experimental procedures

Animals were anesthetized by intraperitoneal injections of sodium pentobarbital (0.04 g/kg b.w.). A single dose of $AlCl_3$ (Sigma, USA) (3.7×10^{-4} g/kg b.w. in 0.01 mL deionized water), was injected into the CA1 sector of the hippocampus (coordinates: 2.5 A; 4.2 L; 2.4 V) (König & Klippel, 1963). Using stereotaxic instrument for small animals, chemicals were applied by means of Hamilton microsyringe. The second group was treated with 7-NI (Sigma Chemical Co. USA; 1×10^{-4} g dissolved in purified olive oil) + $AlCl_3$. The third group received 7-NI + saline solution. 7-NI was applied immediately before the neurotoxin/saline solution. The fourth group (n=10) received the same volume of 0.9 % saline solution and served as control (sham-operated). In all treated animals, the injected intracerebral volume was 10×10^{-6} mL and it was always injected into the same left side.

Four basic groups (according to drug treatment), were divided into four subgroups each (n=10). At several time intervals – 10 min, 3 h, 3 days and 30 days from the treatment, the animals from subgroups were decapitated. Heads were immediately frozen in liquid nitrogen and stored at −70 ºC until use. Then ipsi- and contralateral hippocampuses and ipsi- and contralateral basal forebrains (BF) were

quickly isolated and homogenized in ice-cold buffer (0.25 M sucrose, 0.1 mM EDTA, 50 mM K-Na phosphate buffer, pH 7.2). Homogenates were centrifuged twice at 1580×g for 15 min at 4 ºC. The supernatant obtained by this procedure (crude mitochondrial fraction) was then frozen and stored at −70 ºC (Gurd *et al.*, 1974).

Biochemical analysis

Superoxide anion ($O_2^{\cdot-}$) content was determined through the reduction of nitroblue-tetrazolium (Merck, Darmstadt, Germany) in the alkaline nitrogen saturated medium. Kinetic analysis was performed at 550 nm (Auclair & Voisin, 1985).

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured spectrophotometrically, as inhibition of epinephrine spontaneous autooxidation at 480 nm. The kinetics of sample enzyme activity was followed in a carbonate buffer (50 mM, pH 10.2; Serva, Feinbiochemica, Heidelberg, New York), containing 0.1 mM EDTA (Sigma, St. Louis, USA), after the addition of 10 mM epinephrine (Sigma, St. Louis, USA) (Sun & Zigman, 1978).

Production of NO was analyzed after sample deproteinizing procedure (using 150 mM $ZnSO_4$ and 110 mM NaOH). Nitrite and nitrate levels were measured together, nitrate being previously transformed to nitrite by cadmium reduction. Nitrite was 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)ethylendiamine dihydrochloride in distilled water) (Navarro-Gonzalvez *et al.*, 1998).

The protein content of samples was measured by the method of Lowry using bovine serum albumin (Sigma) as standard (Lowry & Passonneau, 1974).

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

Data presentation and analysis

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

RESULTS

Superoxide production in the rat hippocampus

The results presented on Fig. 1 show the $O_2^{\cdot-}$ levels (μM red. NBT/min/mg proteins) in ipsilateral (A) and contralateral (B) hippocampal homogenates, respectively. $AlCl_3$ injection resulted in higher levels of $O_2^{\cdot-}$ production after 3 h and 30 days in the ipsilateral and after 3 h and 3 days in the contralateral hippocampus, compared to control group ($P<0.05$). In 7-NI+$AlCl_3$ group at day 30, $O_2^{\cdot-}$ production decreased in both ipsi- and contralateral hippocampus, compared both to the control ($P<0.05$), and to neurotoxin-treated group ($P<0.05$) (Fig. 1A, B). Thirty days after 7-NI application, $O_2^{\cdot-}$ production decreased in both the ipsi- and contralateral hippocampus, compared to control ($P<0.05$), as well as to $AlCl_3$-treated animals ($P<0.05$) (Fig. 1A, B).

Superoxide production in the rat basal forebrain

The effect of intrahippocampal drug injection upon $O_2^{\cdot-}$ production in BF is shown

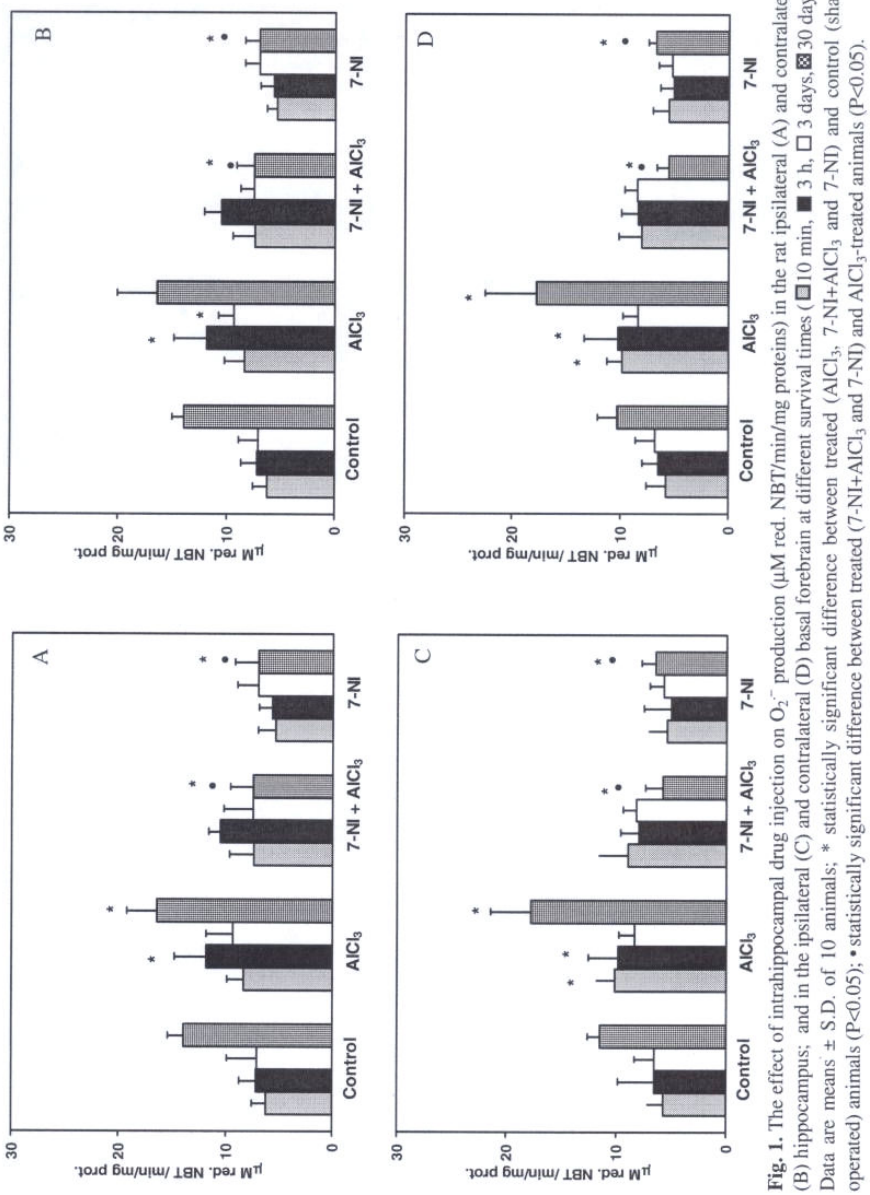

Fig. 1. The effect of intrahippocampal drug injection on $O_2^{\cdot-}$ production (μM red. NBT/min/mg proteins) in the rat ipsilateral (A) and contralateral (B) hippocampus; and in the ipsilateral (C) and contralateral (D) basal forebrain at different survival times (□10 min, ■ 3 h, □ 3 days, ⊠ 30 days). Data are means ± S.D. of 10 animals; * statistically significant difference between treated ($AlCl_3$, 7-NI+$AlCl_3$ and 7-NI) and control (sham-operated) animals ($P<0.05$); • statistically significant difference between treated (7-NI+$AlCl_3$ and 7-NI) and $AlCl_3$-treated animals ($P<0.05$).

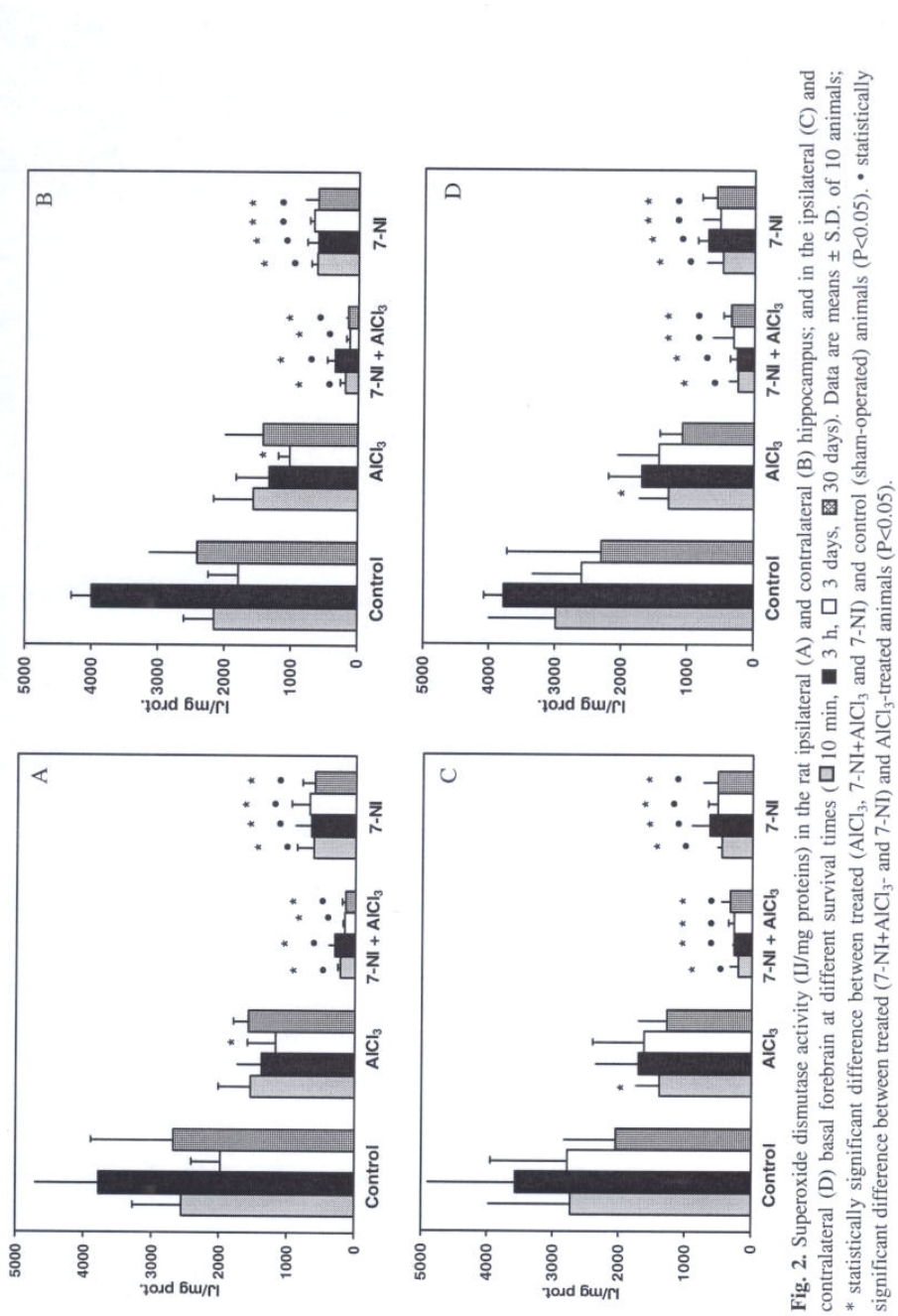

Fig. 2. Superoxide dismutase activity (IJ/mg proteins) in the rat ipsilateral (A) and contralateral (B) hippocampus; and in the ipsilateral (C) and contralateral (D) basal forebrain at different survival times (□ 10 min, ■ 3 h, □ 3 days, ▩ 30 days). Data are means ± S.D. of 10 animals; * statistically significant difference between treated ($AlCl_3$, 7-NI+$AlCl_3$ and 7-NI) and control (sham-operated) animals ($P<0.05$). • statistically significant difference between treated (7-NI+$AlCl_3$- and 7-NI) and $AlCl_3$-treated animals ($P<0.05$).

on Fig. 1C, D. There was no statistically significant difference between mean $O_2^{\cdot-}$ levels obtained from each hemisphere in this brain structure. After 10 min, 3 h and 30 days, the $AlCl_3$ injection resulted in generally higher levels ($P<0.05$) of $O_2^{\cdot-}$ production, compared to control group (Fig. 1C, D). Thirty days after of 7-NI+$AlCl_3$ injection, $O_2^{\cdot-}$ levels were lower, than in controls ($P<0.05$), as well as vs the $AlCl_3$-treated group ($P<0.05$). By the 30th day, 7-NI application resulted in lower $O_2^{\cdot-}$ production bilaterally in BF, compared to both control ($P<0.05$), and $AlCl_3$-treated rats ($P<0.05$) (Fig. 1C, D).

Superoxide dismutase activity in the rat hippocampus

The results presented on Fig. 2A, B show the SOD activity (U/mg proteins) in the ipsi- and contralateral hippocampal homogenates, respectivelly. There was no significant difference between mean SOD activity between hemispheres. SOD activity decreased bilaterally in the hippocampus 3 days after $AlCl_3$ injection, compared to control ($P<0.05$) (Fig. 2A, B). $AlCl_3$ injection followed by 7-NI, clearly reduced SOD activity in this brain structure, compared to both control ($P<0.05$), and $AlCl_3$-treated groups, with statistically significant differences ($P<0.05$) at all times tested (Fig. 2A, B). 7-NI injection resulted in lower SOD activity , compared to control and neurotoxin-treated rats ($P<0.05$) (Fig. 2A, B).

Superoxide dismutase activity in the rat basal forebrain

The results obtained for the ipsi- and contralateral BF were similar. SOD activity decreased in the contralateral BF, 10 min after $AlCl_3$ application, compared to controls ($p<0.05$) (Fig. 2D). Intrahippocampal 7-NI+$AlCl_3$ injection resulted in markedly decreased SOD activity at all times tested, compared to control ($P<0.05$), as well as to $AlCl_3$-treated animals ($P<0.05$) (Fig. 2C, D). Single 7-NI injection resulted in lower SOD activity, compared to control and neurotoxin-treated animals ($P<0.05$) (Fig. 2C, D).

Nitrite levels in the rat hippocampus

Nitrite levels (nM/mg proteins) in ipsilateral (A) and contralateral (B) hippocampal homogenates respectively, are presented on Fig. 3A, B. $AlCl_3$ injection resulted in higher nitrite production at 3 h ipsilaterally, as well as at min 10, hour 3 and day 3 contralaterally, compared to control group ($P<0.05$). At the same time intervals, 7-NI+$AlCl_3$ treatment resulted in higher nitrite levels bilaterally in the hippocampus, compared to controls ($P<0.05$) (Fig. 3A, B). In the same experimental group, nitrite levels were increased at 10 min and 3 days ipsilaterally, and at 3 days contralaterally, compared to $AlCl_3$-treated group. Single 7-NI injection resulted in decreased nitrite production at 10 min and 30 days in the ipsilateral and at 30 days in the contralateral hippocampus, compared to control group ($P<0.05$) (Fig. 3A, B). In the same exprimental group, nitrite production was lower at 10 min, 3 days and 30 days in both ipsi- and contralateral hippocampus, compared to $AlCl_3$-treated animals ($p<0.05$) (Fig. 3A, B).

Nitrite levels in the rat basal forebrain

The effect of intrahippocampal drug injection on nitrite production in BF is shown in Fig. 3C, D. $AlCl_3$ injection resulted in higher nitrite production after 3 h and 3 day ipsilaterally as well as after 3 h contralaterally, than in controls ($P<0.05$) (Fig. 3C, D). At 10 min and 3 h ipsilaterally, and at 10 min, 3 h and 3 day contralaterally, 7-NI+$AlCl_3$ injection in

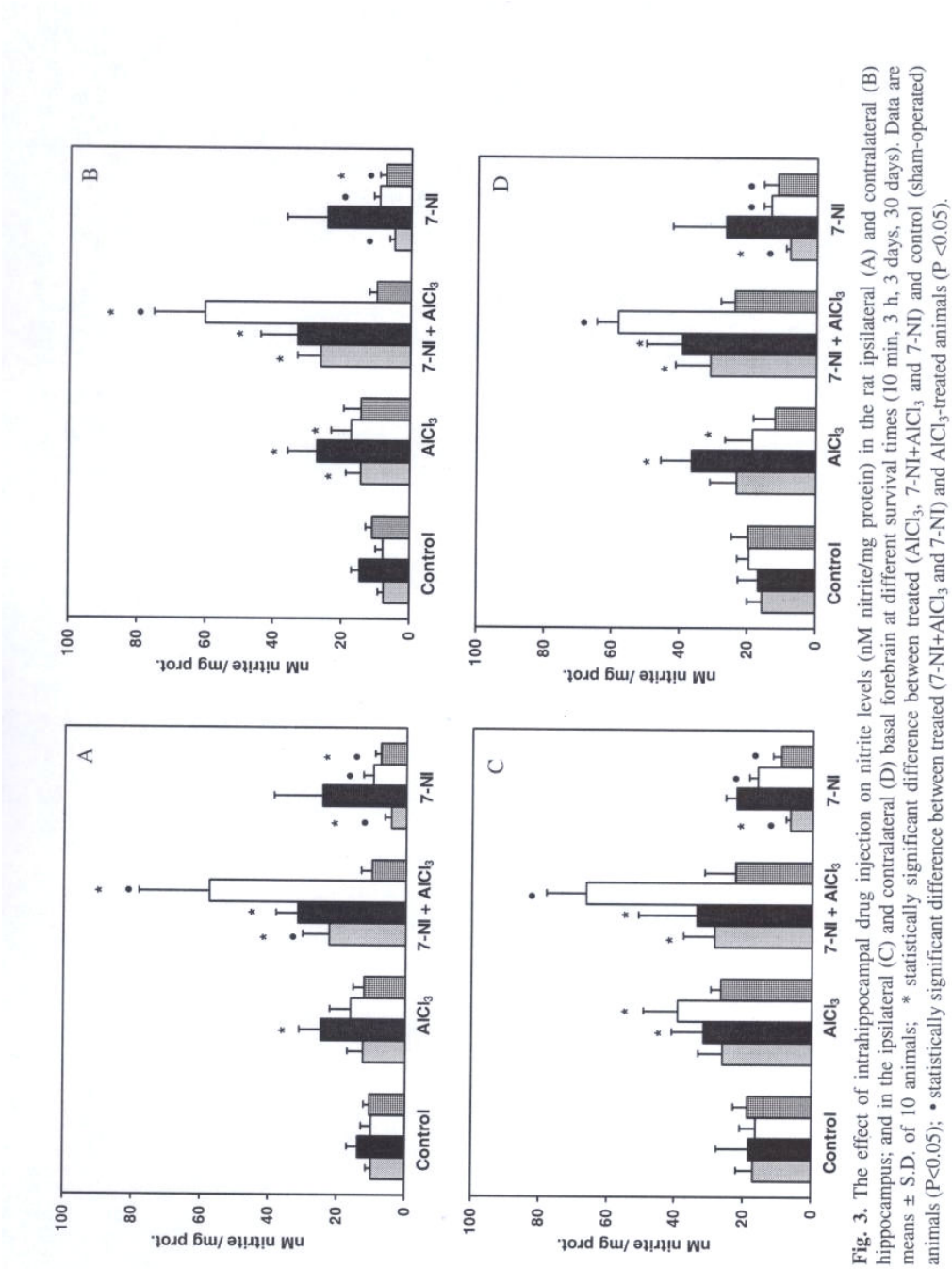

Fig. 3. The effect of intrahippocampal drug injection on nitrite levels (nM nitrite/mg protein) in the rat ipsilateral (A) and contralateral (B) hippocampus; and in the ipsilateral (C) and contralateral (D) basal forebrain at different survival times (10 min, 3 h, 3 days, 30 days). Data are means ± S.D. of 10 animals; * statistically significant difference between treated ($AlCl_3$, 7-NI+$AlCl_3$ and 7-NI) and control (sham-operated) animals (P<0.05); • statistically significant difference between treated (7-NI+$AlCl_3$ and 7-NI) and $AlCl_3$-treated animals (P <0.05).

creased nitrite concentrations vs controls ($P<0.05$) (Fig. 3C, D). $AlCl_3$ injection followed by 7-NI, increased considerably nitrite production after 3 days bilaterally in the BF, compared to $AlCl_3$-treated animals ($P<0.05$). Nitrite levels were significantly reduced after 7-NI treatment, especially by the 10^{th} min ipsilaterally, and after 10 min and 30 days contralaterally, compared to controls ($P<0.05$) (Fig. 3C, D). At 10 min, 3 days and 30 days, 7-NI treatment decreased nitrite levels in the ipsilateral and at 10 min in the contralateral BF, compared to the $AlCl_3$-treated group ($P<0.05$) (Fig. 3C, D).

DISCUSSION

Application of $AlCl_3$ to the CA1 sector of the hippocampus resulted in significant bilateral decrease in SOD activity, as well as increased $O_2^{\cdot -}$ production and nitrite concentration in the hippocampus and basal forebrain, while 7-NI could have a protective effect on the propagation of oxidative stress caused by $AlCl_3$.

Under the conditions of this experiment, $AlCl_3$ application produced a rapid (within 3 h) increase in $O_2^{\cdot -}$ production bilaterally in both the hippocampus and BF, compared to controls (Fig. 1). The literature data suggest that Al is probably associated with oxidative stress, possibly due to the pro-oxidant properties of A beta in the senile plaques. 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 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 shown to be significantly slowed in the presence of Al salts (Yang *et al.*, 1999).

It was previously demonstrated (Tohgi *et al.*, 1998) that production and oxidation of NO in the brain increased in early stage of disease, while decreasing with elevating loss of neurons. Increased $O_2^{\cdot -}$ production followed by increased nitrite concentration in the same brain structures 3 h after $AlCl_3$ application (Fig. 3), suggested that redox balance was not obtained, and therefore, an inadequate antioxidative defence.

The significant increase in $O_2^{\cdot -}$ production after $AlCl_3$ injection (at the 3^{rd} day contralaterally in the hippocampus and by the 10^{th} min contralaterally in the BF) correlated with decrease of SOD activity in the same brain structures (Fig. 2B, D), and indicated oxidative stress development. This is not necessarily a consequence of inappropriate function, but of achieved enzyme saturation, so that the relation $O_2^{\cdot -}$/SOD presents oxidative stress intensity in tissues in the best way.

Unchanged nitrite concentration and $O_2^{\cdot -}$ production along with decreased SOD activity in the ipsilateral hippocampus 3 days after $AlCl_3$ application indicated harmful peroxynitrite ($ONOO^-$) formation. Peroxinitrite causes considerable damage that exacerbates the damage caused by the hyperactivated microglia, accelerating the progress of neurodegeneration (Johnson *et al.*, 2001). Nitric oxide shows a greater affinity toward $O_2^{\cdot -}$ compared to SOD, which could explain decreased enzyme activity caused by lack of substrate.

The literature data implicate neuronal NO generation in the pathogenesis of both direct and secondary excitotoxic neuronal injuries *in vivo*. Thus, it is hypothesized that neuronal NOS inhibitors may be

useful in the treatment of neurological diseases in which excitotoxic mechanisms participate. In the brain, nNOS has been detected in cerebellum, hypothalamus, striatum, hippocampus and medulla oblongata (Torreilles *et al.*, 1999). Cortical areas are known to contain the highest packing densities of nNOS-positive interneurones such as the piriform and entorhinal cortices (Bidmon *et al.*, 1999). It indicates that, physiological neurotransmission and probably cognitive information processing, would be affected by the pharmacological modulation of NO production. 7-NI+$AlCl_3$ treatment which caused a significant increase in nitrite, especially by the 3rd day, compared to $AlCl_3$-treated animals (Fig. 3), suggested that excessive production of NO was involved in the mechanisms triggering seizures and neurodegeneration produced by Al (Bagetta *et al.*, 1995; Bush & Pollack, 2001).

Decreased SOD activity in both hippocampus and BF at the 30th day in 7-NI+$AlCl_3$ group was probably a consequence of reduced enzyme substrate ($O_2^{\cdot -}$) production. Obtained results confirmed NOS inhibitory antioxidative effects after intracerebral Al intoxication, as well as the adequate redox balance, meaning that an appropriate level of antioxidative defence was achieved. Nitric oxide synthase inhibitors break the cascade of increased $O_2^{\cdot -}$ production induced by Al.

The decreased $O_2^{\cdot -}$ production bilaterally in the hippocampus and BF by 7-NI suggested permanent and long-lasting effects of nNOS blocking, maintained until the 30th day. The result of decreased $O_2^{\cdot -}$ production as substrate is evidenced by the reduced activity of antioxidant enzyme SOD in the same brain structures after 7-NI application (Fig. 2).

The present data, indicating that the inhibition of nNOS by 7-NI can effectively decrease the NO production bilaterally in the hippocampus and contralaterally in the BF 30 days after the treatment, compared to controls, are relevant to explain a similarly efficient effect of 7-NI in all tested brain structures in supressing nitrite accumulation (Fig. 3). Also, the results suggest that excitotoxicity could be modulated by an extremely fine regulation of levels in the different neural cell types. The pathophysiological significance of decreased NO activity in selected areas of the brain is that the treatment with 7-NI protects brain neurons against neuronal injuries consequently to impairment of cellular energy metabolism and oxidative stress (Thorns *et al.*, 1998; Strorch *et al.*, 2000; Hartlage-Rubsamen *et al.*, 2001).

Taken together, higher nitrite levels after 7-NI plus $AlCl_3$ application, compared to 7-NI-injected animals, suggested that the applied dose of the nNOS inhibitor (7-NI) did not eliminate $AlCl_3$ toxic effects, mediated by NO.

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