

7-NITROINDAZOLE, A SELECTIVE NEURONAL NITRIC OXIDE SYNTHASE INHIBITOR *IN VIVO*, PREVENTS KAINATE-INDUCED INTRAHIPPOCAMPAL NEUROTOXICITY

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Abstract - We investigated the effects of 7-nitroindazole (7-NI), a selective neuronal nitric oxide synthase inhibitor *in vivo*, on nitrite concentration after kainic acid injection unilaterally into the CA1 region of the rat hippocampus. The accumulation of nitrite, the stable metabolite of NO, was measured by the Griess reaction at different times in hippocampus, forebrain cortex, striatum, and cerebellum homogenates. 7-nitroindazole can effectively inhibit NO synthesis in rat brain after kainate-induced neurotoxicity and suppressed nitrite accumulation. The present results suggest that neuronal NO synthase inhibitors may be useful in the treatment of neurological diseases in which excitotoxic mechanisms play a role.

Key words: 7-nitroindazole, brain, glutamate receptors, hippocampus, kainate, neurological disorders, neuroprotection, neurotoxicity, nitric oxide, nitrite

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INTRODUCTION

Kainate (KA), a pyrrolidine excitotoxin isolated from the seaweed *Digenea simplex*, is a potent neuroexcitatory drug acting on glutamate receptors that after intracerebral or systematic injection leads to generalized limbic seizures in rats (Aubin *et al.* 1992; Lipton *et al.* 1994). Glutamate receptors are the primary excitatory neurotransmitter receptors in the vertebrate brain and are of critical importance in a wide variety of neurological processes (Woo *et al.* 1994). Kainate-induced seizures are accompanied by severe neuronal damage predominantly in the hippocampus and amygdala/pyriform cortex (Hollmann *et al.* 1994). Current models of KA toxicity support the hypothesis that the main cause of neurotoxicity is the activation of presynaptic KA receptors and the release of endogenous glutamate. The overstimulation of glutamate receptors has been implicated in the mediation of injury caused by neurotoxins and ischemia-related insults. Further, KA is also thought to mediate damage partly through an indirect mechanism, which may involve the overproduction of reactive forms of oxygen. The generation of free radicals appears to be pivotal

in KA neurotoxicity (Gratacos *et al.* 2001).

Stimulation of glutamate KA receptors induces neuronal nitric oxide (NO) release, which in turn modulates glutamate transmission (Alabadi *et al.* 1999; Nakaki *et al.* 2000). Nitric oxide is a highly reactive signal molecule in the CNS. The agent is a gaseous chemical messenger that acts on interneuronal communications, synaptic plasticity, memory formation, receptor function, intracellular signal transmission, and mediator release (Brown, 1999; Heales *et al.* 1999; Lei *et al.* 1999). However, pathological conditions may occur when higher fluxes of these mediators are generated, such as during the process referred to as excitotoxicity, i.e., the excessive activation of glutamate KA receptors. This is a condition common to both acute and chronic neurological diseases (Sengpiel *et al.* 1998; Brorson *et al.* 1999; Ciriolo *et al.* 2001).

In view of the above, the present study was undertaken to examine whether the production of NO after intracerebral KA injections can be modulated by pretreatment by 7-nitroindazole (7-NI), a selective neuronal nitric oxide

de synthase (nNOS) inhibitor.

MATERIALS AND METHODS

Animals

Adult rats of the Wistar strain (*Rattus norvegicus*) of both sexes, with body weight 200 ± 30 g, were used for experiments. Groups of two or three rats per cage (Erath, FRG), were housed in an air-conditioned room at temperature of $23 \pm 2^\circ\text{C}$ with $55 \pm 10\%$ humidity and with lights on 12 h/day (07.00-19.00). The animals were given a commercial rat diet and tap water *ad libitum*. These animals were anesthetized by giving intraperitoneal injections of pentobarbital sodium (0.0405 g/kg b.w.) and were placed in a stereotaxic frame.

Experimental procedure and intracerebral injection of drugs

The rats were divided into three basic groups (according to drug treatment), each basic group consisting of five different subgroups (according to survival times) and each subgroup consisting of eight animals. The first group received a unilateral KA injection (Sigma Chemical Co., U.S.A., 0.5 mg/ml, dissolved in 0.1 M saline, pH 7.2; 1 μL total volume) into the CA3 region of the hippocampus (coordinates from bregma: anteroposterior: - 3.3 mm, dorsoventral: 3.2 mm, and lateral: 3.0 mm) using a Hamilton microsyringe with a beveled tip. The second group received KA and 7-NI (Sigma Chemical Co., U.S.A., 0.5 mg/ml, dissolved in purified olive oil, pH 7.2; 1 μL). Finally, the third group received the same volume (1 μL) but only saline solution, and served as a control (sham-operated). The animals were allowed to survive from 5 min to seven days (5 min, 15 min, 2 h, 48 h, and 7 days). All animals were anesthetized, decapitated, and the brains immediately removed. The ipsi- and contralateral hippocampus, forebrain cortex, striatum, and cerebellum from individual animals were quickly isolated and homogenized in ice-cold buffer containing 0.25 M sucrose, 0.1 mM EDTA, and 50 mM K-Na phosphate buffer, pH 7.2. Homogenates were centrifuged twice at 1580g for 15 min at 4°C . The supernatant obtained by this procedure was then frozen and stored at -70°C .

Nitrite measurement

Nitrite and nitrate in biological material are increas-

ingly being used as markers of nitric oxide production. We detected nitrite in the rat brain homogenates by the Griess method (Guevara *et al.* 1998). Nitric oxide production was quantified by measuring nitrite, a stable oxidation end product of NO (Green *et al.* 1982). Briefly, nitrite production was determined by mixing 50 μL of the assay buffer with 50 μL of Griess reagent (1.5 % sulfanilamide in 1M HCl plus 0.15 % N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water, v:v). After 10 min of incubation at room temperature, the absorbance at 540 nm was determined and nitrite concentrations were calculated from the sodium nitrite (Sigma) standard curves. All measurements were performed in triplicate.

Protein measurement

The content of protein in the rat brain homogenates (hippocampus, striatum, forebrain cortex, and cerebellum, ipsilateral and contralateral) was measured by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as standard. All measurements were performed in triplicate.

Data presentation and analysis

All experiments were done with $n = 8$. Each assay was performed at least twice under identical conditions. Data are expressed as means \pm SD. The statistical significance of differences between groups was assessed by Student's *t*-test (paired and unpaired) for individual comparisons and regression analysis for overall significance (with $p < 0.05$ as significant and $p < 0.01$ as very significant).

Materials

Chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade. All drug solutions were prepared on the day of experiment. Animals used for procedures were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

RESULTS

Nitrite levels in the rat hippocampus

The results presented in Fig. 1 show the nitrite levels (mM/mg proteins) in ipsilateral and contralateral hippo-

campal homogenates, respectively. Injection of KA resulted in generally higher levels of nitrite production at all tested times with statistically significant difference (according to the Student *t*-test; $p < 0.05$). There was no statistically significant difference between mean nitrite levels obtained from each hemisphere, although the injection site was in the ipsilateral hippocampus. Treatment with 7-NI followed by KA, very clearly inhibited

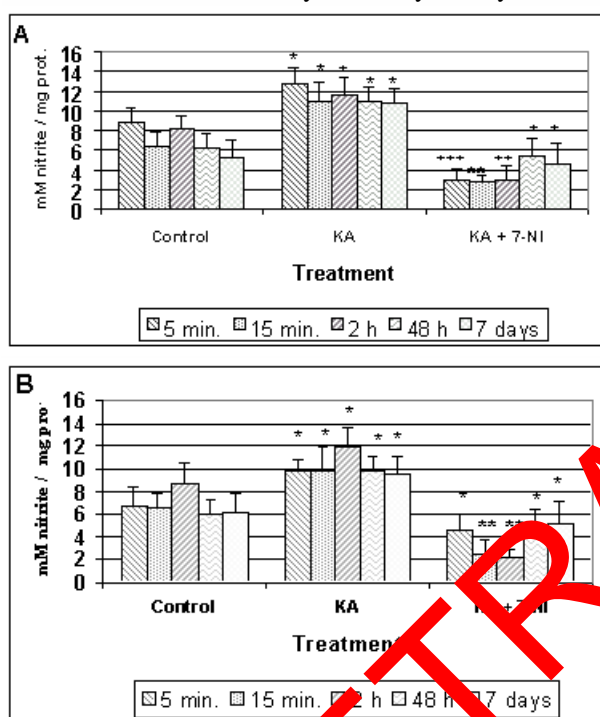


Fig. 1. The effect of intrahippocampal drug injection on nitrite levels (mM NO₂/mg prot.) in the rat ipsilateral (A) and contralateral (B) hippocampus at different survival times (5 min, 15 min, 2 h, 48 h, and 7 days). Data are means \pm S.D. of eight animals.

*Indicates a statistically significant difference between treated (KA- and KA+7-NI-treated) and control (sham-operated) animals ($p < 0.05$). **Indicates a statistically very significant difference between treated (KA- and KA+7-NI-treated) and control (sham-operated) animals ($p < 0.01$).

nitrite production in this brain structure. The early tested times (at 5 min, 15 min, and 2 h) showed statistically significant differences (according to the Student *t*-test; $p < 0.01$) compared with the equivalent control groups (Fig. 1A). The results obtained for the contralateral hippocampus were similar. Measurements at 15 min and 2 h showed statistically significant differences ($p < 0.01$) compared with the equivalent control groups (Fig. 1B).

Nitrite levels in the rat forebrain cortex

Intrahippocampal KA injection resulted in generally higher levels of nitrite production with statistically

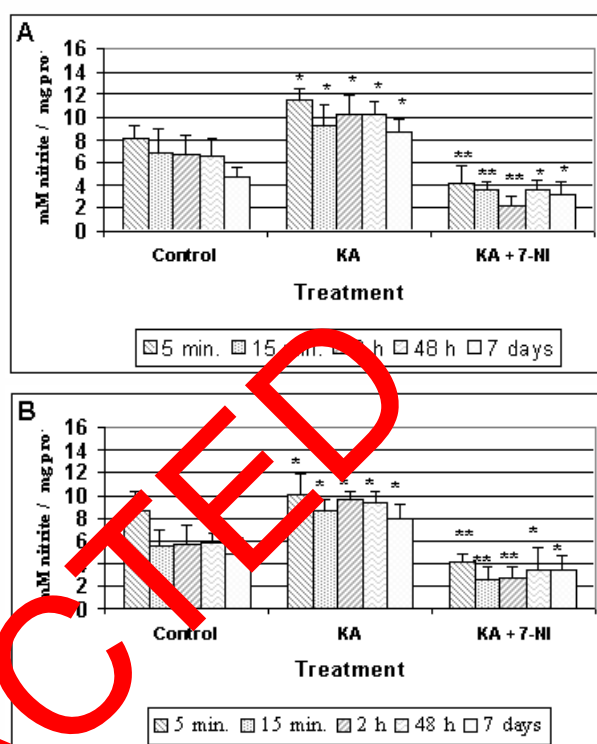


Fig. 2. The effect of intrahippocampal drug injection on nitrite levels (mM NO₂/mg prot.) in the rat ipsilateral (A) and contralateral (B) forebrain cortex at different survival times (5 min, 15 min, 2 h, 48 h, and 7 days). Data are means \pm S.D. of eight animals.

*Indicates a statistically significant difference between treated (KA- and KA+7-NI-treated) and sham-operated animals ($p < 0.05$).

significant difference, $p < 0.05$ at all tested times (Fig. 2). There was a significant reduction in nitrite levels after KA + 7-NI treatment at all tested times, especially at 5 min, 15 min, and 2 h in the ipsilateral and in the contralateral side of the brain. There was no statistically significant difference between mean nitrite levels obtained from each hemisphere.

Nitrite levels in the rat striatum

The striatum, the main component of the basal ganglia, receives glutamatergic inputs from the cortex and thalamus and considerable attention has therefore been given to the role of excitotoxicity in striatal disorders. The effect of intrahippocampal drug injection on nitrite production in the striatum is shown in Fig. 3. The effect of KA injection on nitrite levels measured at 5 min, 15 min, and 48 h and after 7 days for the ipsilateral and for the contralateral side was a significant increase, $p < 0.05$. Treatment with KA + 7-NI at all tested times showed a significant reduction in nitrite levels, especially at 5 min and 2 h for the ipsi- and contralateral sides, respectively

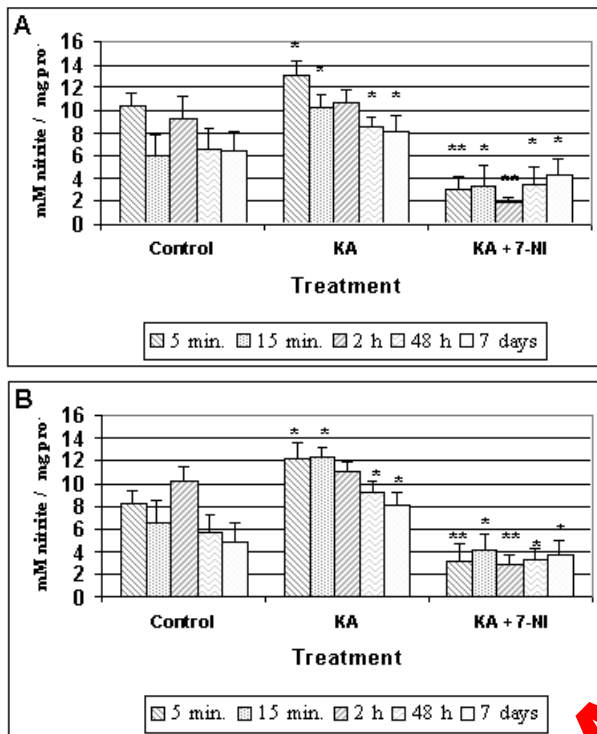


Fig. 3. The effect of intrahippocampal drug injection on nitrite levels ($\text{mM NO}_2^-/\text{mg prot.}$) in the rat ipsilateral (A) and contralateral (B) striatum at different survival times (5 min, 15 min, 2 h, 48 h, and 7 days). Data are mean \pm S.D. of eight animals.

*Indicates a statistically significant difference between treated (KA- and KA+7-NI-treated) and sham-operated animals ($p < 0.05$). **Indicates a statistically very significant difference between treated (KA- and KA+7-NI-treated) and control (sham-operated) animals ($p < 0.01$).

(Fig. 3). There was no statistically significant difference between mean nitrite levels obtained from each hemisphere.

Nitrite levels in the rat cerebellum

The results obtained for this brain structure were very similar to those for the striatum (Fig. 4). The effect of KA injection on nitrite levels measured for the ipsilateral and the contralateral side at almost all tested times showed a significant increase, $p < 0.05$. There was a significant reduction in nitrite levels after KA + 7-NI treatment at all tested times, especially at 5 min and 2 h ipsilaterally, and at 5 min, 15 min, 2 h, and 48 h contralaterally (Fig. 4B). There was no statistically significant difference between mean nitrite levels obtained from each hemisphere.

The behavioral changes after kainate injection

The purpose of this study was to investigate fine

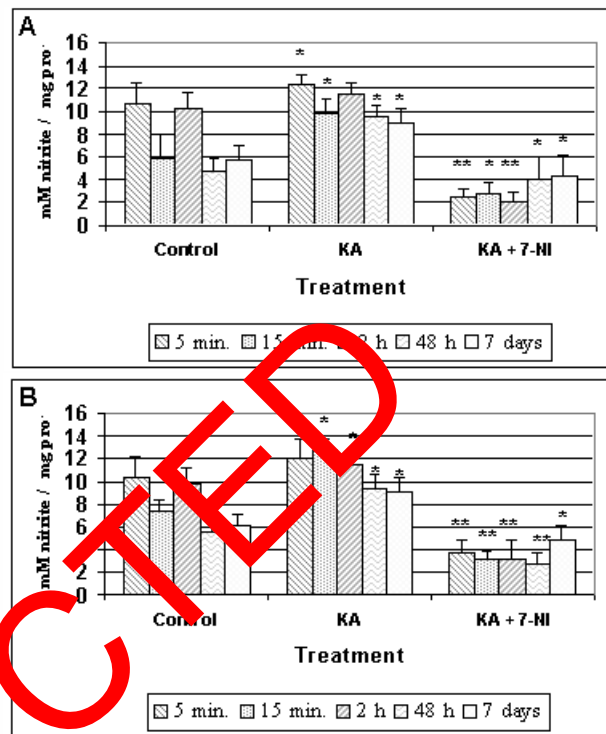


Fig. 4. The effect of intrahippocampal drug injection on nitrite levels ($\text{mM NO}_2^-/\text{mg prot.}$) in the rat ipsilateral (A) and contralateral (B) cerebellum at different survival times (5 min, 15 min, 2 h, 48 h, and 7 days). Data are mean \pm S.D. of eight animals.

*Indicates a statistically significant difference between treated (KA- and KA+7-NI-treated) and sham-operated animals ($p < 0.05$). **Indicates a statistically very significant difference between treated (KA- and KA+7-NI-treated) and control (sham-operated) animals ($p < 0.01$).

changes in NO levels during the process of excitotoxicity in various brain parts. Our aim was to inject KA (appropriate dose) but to avoid any behavioral changes ("wet dog shake", focal seizure of the limbs and neck, hypersalivation, or generalized convulsion) and typical limbic seizures evolving into status epilepticus, since during status epilepticus hippocampal blood flow, oxygen supply, and body temperatures are modified. These effects are accompanied by severe damage to all subfields of the hippocampal formation. It is a condition of intense metabolic activation and could interfere with our results and measurements. We did not measure epileptic activity by electroencephalogram. Only normal behavioral animals took place in experiments.

DISCUSSION

The role of NO in cerebral insult remains controversial. While numerous studies have used models of ischemia, hypoxia, and status epilepticus, few have examined NO in the KA model of excitotoxicity. Anim-

als exposed to KA-induced status epilepticus display a striking pattern of selective neuronal vulnerability in the hippocampus. Neurons in the hilus/CA3 and CA1 subfields appear particularly sensitive, whereas dentate gyrus granule cells are resistant (Becer *et al.* 1999; Lere *et al.* 2002), which is likely due to the high concentration of KA receptors on their membranes. Regional distribution of KA receptors of the rat brain was found to be highest in deep layers (layer 5) of the forebrain cortex, cerebellar granule cell layer, and caudate putamen (Carroll *et al.* 1998; Bailey *et al.* 2001), which is why we tested these particular brain regions: hippocampus, forebrain cortex, striatum, and cerebellum.

It was previously known (Montecot *et al.* 1998) that during status epilepticus 7-NI significantly reduced the increase in hippocampal blood flow and prevented an increase in the tissue partial pressure of oxygen. Also, seven days later, hippocampal damage in the CA1 and CA3 layers was significantly less in 7-NI-treated rats than in vehicle-treated rats. The authors concluded that the inhibition of nNOS by 7-NI protects neurons from seizure-induced toxicity despite reducing blood flow and oxygen supply to the hippocampus.

Kainate enhances hippocampal NO generation (Kashihara *et al.* 1998) and KA injection results in differential regulation of nNOS mRNA and NO formation in the rat hippocampus (Kashihara *et al.* 2000). The literature results implicate neuronal NO generation in the pathogenesis of both direct and secondary excitotoxic neuronal injuries *in vivo*. As such they suggest that nNOS inhibitors may be useful in the treatment of neurological diseases in which excitotoxic mechanisms play a role. Type nNOS has been detected in the cerebellum, the hypothalamus, the striatum, the hippocampus, and the medulla oblongata (Torreilles *et al.* 1999).

In the present study, an appropriate dose of KA (0.5 mg/ml) was used to cause small brain damage in the ipsilateral, but not contralateral, hippocampus, with no behavioral or epileptic effects. It has been previously shown that NO formation was determined in different regions of the rat brain during KA-induced seizures (Mulsch *et al.* 1994; Yasuda *et al.* 2001). In our experiments, at various times following intrahippocampal KA injection, nitrite levels were measured in the four rat brain structures. Cortical areas are known to contain the highest packing densities of nNOS-positive interneurons such as the pyriform and entorhinal cortices (Bid-

mon *et al.* 1999), indicating that, in normal animals, neurotransmission and probably cognitive information processing would be affected by the pharmacological modulation of NO production.

We have shown that NO end-product levels in the rat brain increased immediately after KA injection and continued to increase gradually throughout the experiments. Under conditions of normal behavior in the rat, the damage was localized mainly in the CA3 region of hippocampus, where neuronal loss occurred. 7-nitroindazole, a selective nNOS inhibitor *in vivo*, at any dose used did not affect basal nitrite levels before intracerebral KA injections.

In addition, pre-treatment with 7-NI was effective in modulating the production of NO following intrahippocampal KA injection. Under the conditions of this experiment, 7-NI produced a rapid (within 2 h) decrease in nitrite levels in all four brain regions. Previously, it was demonstrated that 7-NI can produce designated changes in brain NO content, facilitating the use of 7-NI to probe the pharmacological implications of NO in the CNS (Bush *et al.* 2001). It was suggested that excessive production of NO is involved in the mechanisms of KA-triggering seizures and neurodegeneration (Bagnetta *et al.* 1995). The results of Takei *et al.* (1999; 2001) suggest that NO is of major importance in the neurodestructive process in spite of its roles in maintaining both the cerebral blood flow and cerebral oxygenation during KA-induced seizures in the neonatal rabbit brain. They suggested that both 7-NI and L-NAME inhibited NO production and EEG abnormalities during the seizures that led to less neuronal damage to the hippocampus.

The study presented here shows that after intrahippocampal injection 7-NI produced an approximately 50% decrease in nitrite levels in all four brain regions, which was sustained. Correlation of the inhibitory effect was demonstrated in all tested brain structures, of which the striatum was shown to be most sensitive and the forebrain cortex most resistant to 7-NI activity. Decreased NO activity in selected areas of the brain suggests that treatment with 7-NI leads to protection of brain neurons against neuronal injuries by impairment of cellular energy metabolism and oxidative stress (Storch *et al.* 2000).

The present data indicate that inhibition of nNOS by 7-NI aseptically decreased NO production, at the early

tested times (from 5 min up to 2 h) in the rat brain after intracerebral KA application. These findings help to explain the equally efficient effect of 7-NI in all tested brain structures in suppressing nitrite accumulation. Also, the results suggest that extremely fine regulation of NO levels in the different neural cell types can modulate excitotoxicity. The NOS inhibitory effect of 7-NI following intracerebral injection should be taken into account when using this drug to evaluate NO central effects.

Finally, increased NO production in distinct brain regions, functionally connected via afferents and efferents suggests that these regions are affected by the injury. It suggests that 7-NI inhibition of nNOS protects the cells in these regions from KA-induced damage and therefore may limit the retrograde and anterograde spread of neurotoxicity.

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НЕУРОПРОТЕКТИВНО ДЕЈСТВО 7-НИТРОИНДАЗОЛА, ИНХИБИТОРА АЗОТ ОКСИД СИНТЕТАЗЕ У НЕУРОНИМА МОЗГА ПАЦОВА, ПОСЛЕ ИНТРАХИПОКАМПАЛНЕ АПЛИКАЦИЈЕ КАИНИЧНЕ КИСЕЛИНЕ

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Интрацеребрална апликација каиничне киселине, агонисте глутаматског рецептора, у селективно осетљив СА3 регион хипокампуса пацова доводи до ексцитотоксичног оштећења неурона у овој структури, посредовано стварањем азот оксида (NO) као медијатора оштећења. С обзиром да неуротоксичност доводи до оксидационог оштећења и повећаног стварања NO, праћење динамике стварања NO на нивоу различитих можданих структура представља сензитивни показатељ дисфункције неурона у различитим временским интервалима у односу на тренутак изазивања ексцитотоксичних ефеката. Стварање NO је праћено

преко акумулације нитрита, стабилних метаболита азот оксида, Griess-овом методом. На степен изазване неуротоксичности у свим праћеним можданим структурама неуропротективно је деловала примена 7-нитроиндазола, инхибитора NO-синтезе у неуронима. Најочигледнији ефекат 7-NI се постиже у раним праћеним терминима, што значи да веома брзо и ефикасно реагује на повећану акумулацију нитрита у смислу смањења и акумулације, па самим тим и превенције. На тај начин је потврђена наша претпоставка о избору ове супстанце у превенцији интоксикације каинатом.