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The Effect of Neuronal Nitric Oxide Synthase Inhibitor 7-Nitroindazole on the Cell Death Induced by Zinc Administration in the Brain of Rats*

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Aim: To evaluate the effect of the neuronal nitric oxide synthase (nNOS) inhibitor 7-nitroindazole (7-NI) on hippocampal cell loss induced by zinc hemisulfate salt ($ZnSO_4 \cdot 7H_2O$) treatment.

Materials and Methods: Rats were divided into 3 groups (n = 21; 7 rats in each group): Control, Zinc, and Zinc+7-NI. In the Zinc and Zinc+7-NI (pretreatment) groups, $ZnSO_4 \cdot 7H_2O$ was applied intracortically to the left sensory motor cortex. The 7-NI (50 mg/kg) was injected into the third group (Zinc+7-NI) intraperitoneally 20 min prior to zinc application and daily for the next 7 days. Cell loss in the left and right hippocampal hemispheres was quantified by optical fractionator, which is a relatively unbiased and reliable stereological counting method.

Results: Zinc produced decreases of 43.5% and 46.1% in the number of viable neurons in the left and right hippocampal hemispheres, respectively, in comparison with control values. In the Zinc+7-NI group, cell losses were 16.2% and 18.5% in the left and right hemispheres, respectively, in the same conditions. Thus, 7-NI exerted a significant neuroprotective effect against zinc-induced cell loss in the rat hippocampus ($P < 0.05$).

Conclusions: These results suggest that nitric oxide contributes to the hippocampal cell loss induced by zinc sulfate and that the prevention of nitric oxide formation by nNOS blockade can reduce this cell loss.

Key Words: Zinc, cell death, hippocampus, 7-nitroindazole, stereology, rat

Sıçan Beyninde Çinkonun Sebep Olduğu Hücre Ölümüne Nöronal Nitrik Oksit Sentaz İnhibitörü 7-Nitroindazolün Etkisi

Amaç: Bu çalışmanın amacı; çinko sülfat ($ZnSO_4 \cdot 7H_2O$) uygulanması ile oluşturulan hipokampusa ait hücre ölümüne nöronal nitrik oksit sentaz (nNOS) inhibitörü 7-Nitroindazolün (7-NI) etkisinin araştırılmasıdır.

Yöntem ve Gereç: Sıçanlar (n = 21; her grupta yedi rat olmak üzere) Kontrol, Çinko ve Çinko+7-NI olmak üzere üç gruba ayrıldı. Çinko ve Çinko+7-NI gruplarında $ZnSO_4 \cdot 7H_2O$ sol somatomotor korteks içine intrakortikal yolla uygulandı. Üçüncü gruba (Çinko+7-NI) çinko uygulanmasından 20 dakika önce ve bunu takip eden 7 gün süresince 7-NI (50 mg/kg dozunda) her gün intraperitoneal yolla enjekte edildi. Sağ ve sol beyin yarımkürelerine ait hipokampal hücre kayıpları tarafsız ve daha güvenilir stereolojik bir sayım metodu olan optik parçalama kullanılarak hesaplandı.

Bulgular: Çinko uygulanması sonucu, kontrol değerleri ile karşılaştırıldığında sol ve sağ hipokampustaki sağlam nöron sayısındaki azalma sırasıyla % 43,5 ve % 46,1 oranlarında bulundu. Çinko+7-NI grubunda ise aynı koşullarda sol ve sağ beyin yarımküreleri için bu değerler sırasıyla % 16,2 ve % 18,5 olarak saptandı. Böylece, 7-NI'nın sıçan hipokampusunda çinko ile oluşturulan hücre ölümüne karşı nöron koruyucu etkisinin anlamlı olduğu bulundu ($P < 0,05$).

Sonuç: Bu sonuçlar bize nitrik oksidin çinko sülfat ile oluşturulan hipokampal hücre ölümüne katkıda bulunduğunu ve nNOS enziminin inhibisyonu sonucu nitrik oksit üretiminin önlenmesi ile bu etkinin azaltılabileceğini göstermektedir.

Anahtar Sözcükler: Çinko, hücre ölümü, hipokampus, 7-Nitroindazol, stereoloji, sıçan

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Introduction

The release of zinc from presynaptic terminals is a key step in neurotoxic injury produced by status epilepticus (1) or ischemic brain states (2). Zinc is located within synaptic vesicles of some glutamatergic neurons, with the highest brain zinc concentrations in the CA3 region and dentate gyrus of the hippocampus (3). In the brain, zinc can be released as a direct transmitter and can modulate postsynaptic signaling pathways through -aminobutyric acid (GABA) and glutamate-sensitive N-methyl-D-aspartate (NMDA) receptors (3,4).

Zinc is known to contribute to excitotoxic neuronal death; chelating extracellular Zn^{2+} during and after ischemic episodes or epileptic brain insults (5) can reduce or prevent cell death. Intracellular zinc neurotoxicity may inhibit energy metabolism, create oxidative stress, or activate apoptotic cascades. For example, zinc can trigger plaque maturation in Alzheimer's disease (6).

Nitric oxide (NO) is a retrograde second messenger transmitter molecule in the central nervous system (CNS) and regulates several brain processes (7). Neuronal nitric oxide synthase (nNOS) converts L-arginine to NO and citrulline in an NADPH-dependent reaction (8). The activation of ionotropic glutamate receptors was shown to stimulate NO production via a calcium-dependent mechanism, leading to the hypothesis that NO is involved in certain neurotoxic disorders (9). NO mediates NMDA-related glutamatergic cortical and hippocampal neurotoxicity under several pathological conditions such as stroke, trauma, and epilepsy (10,11). However, both neurotoxic and neuroprotective effects of nNOS inhibitors have been reported; this discrepancy may be attributable to a number of factors, including dose and animal model (12,13).

The primary goal of this study was to determine whether neuronal nitric oxide (nNO) molecule is involved in the hippocampal neurotoxicity produced by zinc hemisulfate salt ($ZnSO_4 \cdot 7H_2O$).

Materials and Methods

We used 16- to 20-week-old male Wistar rats weighing 200-250 g each (n = 21). The rats were housed in plastic transport cages in a regulated environment with a 12-h light/dark cycle. Free access to standard laboratory chow pellets and tap water was allowed at all times. The Animal Utilization Committee of the university approved the experimental procedure according to "Principles of Laboratory Animal Care." Ketamine hydrochloride (Ketalar 50 g/ml) was purchased from Eczacıbaşı, Turkey.

The nNOS inhibitor 7-nitroindazole (7-NI) and peanut oil were obtained from Sigma (N-7778 and P-2144, respectively). The 7-NI was dissolved in peanut oil (12.25 mg/ml). Both drugs were freshly prepared and used immediately.

The rats were randomly assigned to 3 groups: Control, Zinc, and Zinc+7-NI. The rats were food-deprived for 12 h before surgery, anesthetized with ketamine hydrochloride (100 mg/kg) injected intraperitoneally (i.p.) and immobilized in a stereotaxic apparatus (David Kopf Instruments). Body temperature was maintained at 36.5-37.5 °C with a homeothermic blanket (Harvard Apparatus), and mean arterial blood pressure (MABP) was continuously monitored by a mercury manometer introduced into the right femoral artery. The skull was exposed, and a hole was drilled 2.0 mm lateral to midline, 1.5 mm rostral to bregma, and 1.2 mm below the cranial surface, using a special dental burr. Areas of excessive bleeding were cauterized with an electronic coagulator (Electro-Mag 75). Zinc sulfate (200 mg/kg in 2 µl) was applied intracortically (i.c.) into the left sensory motor cortex using a Hamilton microinjector (Z-12035; Aldrich Instruments). Control animals received saline i.c. The needle was left in place for 3 min post-injection to prevent the spreading of the drug along the needle track.

The rats in the Zinc+7-NI group received a single injection of 7-NI (50 mg/kg in 1.0 ml, i.p.) 20 min before surgery. Following surgery, these rats were administered the same dosage i.p. of 7-NI once a day for 7 days. The rats in the Control and Zinc groups received peanut oil at a similar volume with a similar dosing regimen.

One week after zinc administration, all rats were perfused intracardially with neutral formalin. After dehydration, the brains were embedded in paraffin blocks and cut into 40-µm sections, which were then stained with Cresyl violet for histological analysis (14).

The total number of pyramidal cells in the hippocampus was estimated using an optical fractionator, which combines an efficient and unbiased stereological counting method (optical dissector) with an unbiased

sampling scheme (stereological work station units; Histology and Embryology Department, Ondokuz Mayıs University, Samsun, Turkey). The first section in the series to be analyzed was chosen at random from among the first 8 sections. Each subsequent 8th section was analyzed, giving a section sampling fraction (ssf) of 1/8. Approximately 15-20 sections per brain are sufficient for estimating the total neuron number using the optical fractionator method (15). This method ensures that each neuron is counted only once. Neurons were counted using a specialized stereological analysis system (16) consisting of a microscope (Carl Zeiss Jena/Jenaval, Germany), a video camera (Loewe, Japan), 2 dial indicators, a microcator (Heidenhain, Traunreut, Germany), and a video monitor. All counts were performed at a final magnification of 3000 .

Although the pyramidal cell layer of the hippocampus also includes the cell bodies of glia and basket cells, these cells can be easily distinguished from the pyramidal cells. Pilot studies indicated that the optimal ssf, area sampling fraction (asf), and dissector depths (h) were 1/8, 400 μm^2/22,500 μm^2, and 15 µm, respectively. The total number of pyramidal neurons (N) in each hippocampus was estimated using the following equation:

$$N = \Sigma Q^- \times 1/ssf \times 1/asf \times 1/tsf,$$

where ΣQ^- is the total number of pyramidal neurons counted in the selected pyramidal neuron layer and tsf is the thickness sampling fraction (h/t). The pyramidal cell layer volume of the hippocampus was also evaluated using Cavalieri's principle (17). The parameters used for the stereological estimation of the total neuron number are given in Table 1.

The dry ashing method was used to determine the zinc content of the brain. Tissue zinc levels were assayed. The brain was removed, and the total wet weight of the intact brain and the individual hemispheres were recorded. The brain tissue was heated in an oven at 100 °C for 2 days, and the remaining ash was dissolved in 3 ml of 3 N HCl. The solution was filtered and diluted with 0.36 N HCl. Care was taken to avoid zinc contamination from the atmosphere. The ash solution was analyzed at 213.9 nm using a flame atomic absorption spectrophotometer (Model 2280; Perkin-Elmer).

Statistical Evaluation

Data are presented as means ± SD. Student's t-test was used to compare means and to test for significant differences in the neuron count and zinc level between the hemispheres. Kruskal-Wallis and post-hoc Dunn tests were used to analyze differences between groups.

Results

Stereological examination of the hippocampus and neuronal cell counting in the CA1, CA2, and CA3 fields of the pyramidal cell layer in the left and the right hippocampus were performed 7 days after perfusion. The mean total numbers of neurons in the left and right hemispheres are shown in Table 1. The injection of zinc i.c. caused neuronal cell death in both the left and right hippocampal hemispheres. There was no significant difference in the number of pyramidal neurons between the left and right hippocampus of the rats within each group.

In the left hippocampal hemisphere, the total numbers of viable cells in the Zinc and Zinc+7-NI groups were 56.5% and 83.8% of the number of viable cells in the Control group, respectively; in the right hemisphere, the total numbers of viable cells in the Zinc and Zinc+7-NI groups were 53.9% and 81.5% of the control values, respectively.

Thus, the injection of zinc produced a 43.5% decrease in the total number of pyramidal cells in the left hemisphere and a 46.1% decrease in the right hemisphere, in comparison with the control values. The differences between the experimental groups and the Control group were significant ($P < 0.01$). Pretreatment

Table 1. Estimated total pyramidal cell number in the left and right hemispheres of the hippocampus (CA1-CA3).

	GROUPS (n = 21)	Total Pyramidal Cell Number (mean ± SD)
Left Hippocampus	Control (n = 7)	672,756 ± 14,328
	Zinc (n = 7)	380,590 ± 12,524*
	Zinc + 7-NI (n = 7)	564,278 ± 16,986*
Right Hippocampus	Control (n = 7)	653,486 ± 18,988
	Zinc (n = 7)	352,624 ± 11,425*
	Zinc + 7-NI (n = 7)	532,985 ± 13,738*

*$P < 0.05$ compared with corresponding control values

with 7-NI 50 mg/kg significantly reduced the neuronal cell death in both the left and right hemispheres in comparison with the Zinc group values ($P < 0.05$). Peanut oil vehicle alone produced no cell death (data not shown).

Data on the volume and the mean total neuron number of pyramidal cell layers of the left and right hippocampus in each group are shown in Tables 2 and 3. There was a significant difference observed between volumes, neuron densities, and the mean total neuron number of Zinc and Zinc+7NI groups in the left and the right hemispheres ($P < 0.05$).

The zinc levels in the brain of the Control, Zinc, and Zinc+7-NI groups were measured after the 7-day zinc application. There was no significant difference between the same side hemispheres of the Zinc and Zinc+7-NI groups or between hemispheres of the Control group (Table 4). However, the zinc level 7 days after the application was significantly different between left and right hemispheres of the Zinc and Zinc+7-NI groups ($P < 0.05$).

Discussion

The present study examined the effects of the specific nNOS inhibitor 7-NI on zinc-induced cell death in rat hippocampus as assessed using a stereological counting method. Stereological cell counting methods were developed more than 20 years ago and represent a well-established means of assessing total cell number in a given brain area (18,19). In this study, the number of neurons in the hippocampus was estimated using an optical fractionator. This technique combines the counting ability of an optical dissector with the systematic sampling ability of a fractionator (15,18) and allows unbiased and efficient estimation of the total number of neurons in any brain region (19). With this method, all types of neurons have the same chance of being sampled, independent of cell size, shape, and orientation, and independent of tissue heterogeneity. The neuron counts obtained are therefore free of the methodological bias that is a major confounding factor with conventional counting methods.

Table 2. Comparison of total volume, neuron density, and total neuron number of the left hippocampal hemisphere in the Zinc and Zinc+7-NI groups.

Parameter	Zinc-L (n = 7)	Zinc+7-NI-L (n = 7)	P
Total volume (V, mm^3)	0.82 ± 0.01	0.89 ± 0.01	< 0.05
Total neuron number (N, × 10^6)	0.326 ± 0.003	0.459 ± 0.003	< 0.05
Numerical neuron density (N_V, cell/mm^3)	380,590 ± 12,524	564,278 ± 16,986	< 0.05

Data are given as mean ± SD

Table 3. Comparison of total volume, neuron density, and total neuron number of the right hippocampal hemisphere in the Zinc and Zinc+7-NI groups.

Parameter	Zinc-L (n = 7)	Zinc+7-NI-L (n = 7)	P
Total volume (V, mm^3)	0.84 ± 0.01	0.90 ± 0.01	< 0.05
Total neuron number (N, × 10^6)	0.299 ± 0.003	0.428 ± 0.003	< 0.05
Numerical neuron density (N_V, cell/mm^3)	352,624 ± 11,425	532,985 ± 13,738	< 0.05

Data are given as mean ± SD

Table 4. Zinc levels in the left and right hippocampal hemispheres of the Control, Zinc, and Zinc+7-NI groups.

Groups	Left Hemisphere (μg/g)	Right Hemisphere (μg/g)	P
Control (n = 7)	14.82 ± 0.02	14.17 ± 0.03	> 0.05
Zinc (n = 7)	30.32 ± 1.33	16.45 ± 0.38	< 0.05
Zinc+7-NI (n = 7)	30.06 ± 1.31	16.03 ± 0.37	< 0.05

Data are given as mean ± SD

Demir et al. produced chemical neurotoxicity in the rat hippocampus via intracortical zinc sulfate (500 μg/kg), causing pyramidal cell loss characterized by variability across sections (20). The concentration of zinc required for cell death in the brain is not known, and different mechanisms for this process have been proposed (21,22).

Electron microscopy studies have demonstrated that tubulin is damaged in neurons and glia treated with toxic levels of zinc (23).

Zinc applied to the cortex might produce neurotoxicity by enhancing the effects of excitatory neurotransmitters while attenuating the effects of inhibitory ones. Another possibility is that zinc in the extracellular fluid may cause cell death by increasing excitatory transmitter release through its action at presynaptic terminals (24). The intrahippocampal administration of zinc chloride in rats produced pyknosis and hippocampal pyramidal cell loss. An increase in excitatory amino acids such as glutamate and enhanced release of retrograde messenger molecules may be a possible mechanism for zinc-induced neurotoxicity (25).

Several studies have examined the relationship between NO and neurotoxicity (26-28); however, whether NO is neuroprotective or neurotoxic is not yet conclusive. Conflicting results are likely to be attributable to differences in the neurotoxic and epileptic models used, brain regions of interest, routes of drug administration, dosages, and experimental methods. The most crucial factor may be the transformation of NO to its reduced or oxidized form. Determining the amount of NO present in a tissue under baseline or stimulated conditions will be important for understanding its physiological and pathological effects. NO is produced in small amounts under normal physiological conditions and is known to stimulate guanylyl cyclase, increase intracellular cGMP levels, relax blood vessels, prevent thrombocyte adhesion, and affect ion channels. In contrast, the production of pathologically large amounts of NO activates iron-based enzymes owing to iron related to cellular respiration and reproduction (29).

In this study, we used a NOS inhibitor different from those used previously in the above studies and yet obtained a similar result: nNOS inhibition reduced zinc-induced cell death. Our results also highlight the role of NO in zinc-induced neuronal cell death. Further research with new techniques using other specific and nonspecific NOS inhibitors should shed light on the pathophysiological mechanisms of neuronal loss and may contribute to the development of new clinical treatments.

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