Experimental Studies

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Neurodegenrative changes in retina of rats with chronic epileptic syndrome under conditions of treatment with niacin-oxy-ethylidene-phosphonate germanate (MIGU-4)

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Background: *Epilepsy is accompanied by neurodegenerative changes, particularly those in the retina. Elucidation of the mechanisms of retinal alterations in a model of epileptic syndrome may allow for the development of new approaches to pharmacological vision correction.*

Purpose: *To assess the morphological characteristics of the retina, particularly treated with niacin oxy-ethylidene phosphonate germinate (MIGU-4), in the pentylenetetrazole (PTZ)-induced kindling model of chronic epileptic syndrome.*

Material and Methods: *PTZ was administered in Wistar male rats intraperitoneally (i.p.) at a daily dose of 35.0 mg/kg for 21 days. Rats with fully-developed generalized seizures were treated with MIGU-4 ip at a daily dose of 5.0 mg/kg or 25.0 mg/kg for 28 days. Thereafter, the animals were euthanized, their globes were enucleated, and retinal sections were prepared and stained with hematoxylin and eosin for morphological examination.*

Results: *Cell density in the retinal ganglion cell layer was 2.14 times lower, and in the inner nuclear layer and outer nuclear layer, 41.0% and 19.0%, respectively, lower for the rats with fully developed kindled seizures than for controls ($p < 0.05$). In the presence of treatment with MIGU-4 ip at a daily dose of 25.0 mg/kg, cell density in the retinal ganglion cell layer and in the inner nuclear layer was 38% and 30.5%, respectively, higher than for controls ($p < 0.05$). There was no significant difference in the cell density in the outer nuclear layer between rats treated with MIGU-4 ip at a daily dose of 5 mg/kg or 25.0 mg/kg and controls ($p > 0.05$).*

Conclusion: *The PTZ-induced kindling model of chronic epileptic syndrome is accompanied by degenerative changes in the eye. A course of treatment with MIGU-4 causes neuroptotective effects in a model of PTZ-induced retinopathy.*

Keywords:

pentylenetetrazol kindling, chronic epileptic syndrome, retinopathy, niacin-oxy-ethylidene-phosphonate germinate (MIGU-4), neuroprotection

Introduction

Epilepsy is a chronic neurological disorder that is characterized by repeated seizures, with the latter causing severe abnormalities in the cerebral neuromediating systems, bioelectrogenesis, synthesis and metabolism of macromolecular compounds in the brain. Neurodegenerative manifestations (particularly those arising in the retina) are typical in the disease.[1, 2]

Since these changes in the neural tissue originate from neuroinflammation [3], anti-inflammatory agents can be considered as promising candidates for the prevention of neurodegenerative changes in epilepsy.

In recent years, the anti-inflammatory activity of germanium containing organic compounds have been demonstrated.[4] In addition, germanium-containing agents have demonstrated anti-oxidative capacity and thus prevent degenerative cellular changes.[5, 6] Compounds of germanium with oxy-ethylidene phosphonic acid have a low toxicity and high biological activity. In addition, derivatives of these compounds have demonstrated a high clinical efficacy for the regulation of mineral metabolism, in anti-tumor and anti-inflammatory therapy and for detoxification purposes. Niacin oxy-ethylidene phosphonic germinate (MIGU-4) promptly enters the systemic circulation, is readily available to body tissues, have antioxidative and hepathoprotective effects, and decreases the excitability of brain structures.[7-9]

Apparent neurodegenerative abnormalities in brain structures have been demonstrated in the pentylenetetrazole (PTZ) kindling model of chronic epileptic syndrome.[10, 11] Until recently, there have been no studies on the state of the retina in the PTZ kindling rat model of chronic epilepsy.

Therefore, t**he purpose** of the study was to assess the morphological characteristics of the retina, particularly treated with MIGU-4, in the PTZ-induced kindling rat model of chronic epileptic syndrome.

Material and Methods

Experimental animals

Thirty-six Wistar male rats (age, 2-3 months; mass, 180–220 g) were used in this experimental study. They were maintained in a environmentally controlled room (23 ± 2 °C, 60% humidity) on a 12:12-h light:dark cycle, and fed and watered ad libitum. All animal experiments adhered to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and the Helsinki Declaration. The study was approved by the Bioethics committee of Odesa National Medical University (Meeting Minutes No.3 dated March 14, 2018).

PTZ-induced kindling and MIGU-4 introduction model

The PTZ kindling rat model of epilepsy was induced as previously reported.[12] PTZ (P6500, Sigma Aldrich, St. Louis, MO, USA) was dissolved in 0.9% NaCl (normal saline) and administered intraperitoneally (ip) at a daily dose of 35.0 mg/kg for 21 days (n=27). All rats included in the study exhibited seizures scored as class 4 to 5 as a response to each of the last three PTZ administrations. The rats of the control group (n=9) received ip normal saline.

After each PTZ injection, rats were gently placed in isolated transparent plexiglass cages for 30 min for behavioral seizure scoring using the 6-point scoring scale [12] as follows: stage 0, no response; stage 1, immobility, tremor and myoclonic jerks of isolated muscle groups; stage 2, whole body clonic seizures; stage 3, clonic seizures of the forelimbs associated with rearing; stage 4, generalized tonic-clonic seizures associated with loss of balance and falling; and stage 5, repeated stage 4 seizures or seizure-induced death.

Because of durable neural tissue changes caused by neuroimmune inflammation [13], MIGU-4 was introduced in the form of a four-week course beginning on the day after the last PTZ injection. MIGU-4 dissolved in normal saline was administered ip at a daily dose of 5.0 mg/kg (n = 9) and 25.0 mg/kg (n = 9). Nine intact control animals and nine kindling animals received similar ip doses of normal saline. Therefore, two groups of animals (n=18) received MIGU-4, and another two groups (intact and PTZ-kindled rats) were used as respective control groups.

Histological studies

On the day after the last MIGU-4 injection, the animals were euthanized by an overdose (100 mg/kg) of sodium pentobarbital, and their globes were enucleated. An enucleated globe was fixed in the solution containing picric acid, 15 parts; formalin, 5 parts, and acetic acid, 1 part. Thereafter, the cornea, intraocular fluid, lens and vitreous were removed to leave the posterior eyecup. The latter was dehydrated in a graded series of ethyl alcohol (70%–100%) and embedded in paraffin. In order to prepare 5-μm-thick retinal sections, the posterior eyecup was sectioned superiorly in the sagittal plane passing through the point located 1 mm above the optic disc margin.[14, 15] Sections prepared were stained with hematoxylin and eosin. Light microscopic specimens were examined under a Nikon Eclipse E-800 light microscope (Nikon Corporation, Japan) equipped with a Nikon DXM 1200 camera. An objective lens with ×40 and ×10 magnifications was used to take 1280x960 pixel sized images for the calculation of cells. Cells were counted manually or using image analysis software (ImageJ, free access). Mean number (± standard error of mean) of positive cells was expressed as counts/mm2.

The statistical software package Primer Biostatistics was used for statistical analysis. Values were compared using one-way ANOVA and Newman-Keuls test. P-values < 0.05 were considered significant. Data are reported as mean plus or minus standard error of mean.

Results

Behavioral characteristics of the seizures in kindled rats

The second to fourth PTZ injections resulted in seizures that progressed to the level of muscle clonus with the subsequent 2-3 PTZ administrations. The subsequent 4-9 PTZ administrations caused clonic forelimb seizures associated with rearing. The generalized clonic-tonic seizures emerged in experimental animals after 8 to 17 PTZ injections. During seizures, rats lost their balance fell on their side, and demonstrated post seizure depression on the EEG. Over the three weeks of daily PTZ administration in experimental rats, there was an increase in seizure severity to 4.3 ± 0.2 scored points (Fig. 1). The rats included in subsequent experiments demonstrated generalized seizures following each of the last three PTZ administrations.

Histological characteristics

Histological abnormality measures included the decrease in the number of cells, degree of cytoplasm vacuolization, and presence of edema, nuclear structure abnormalities and/or pyknotic nuclei. Such manifestations were typical in histological specimens of rats with fully developed kindled seizures (Fig. 2), but not seen in histological specimens of controls (Fig. 2). Typical abnormalities included cytoplasm vacuolization, nuclear structure abnormalities and/or pyknotic nuclei in retinal ganglion and inner nuclear layer cells. These abnormalities were less apparent in the rats that were administered MIGU-4 ip at a daily dose of 25.0 mg/kg (Fig. 2D).

Cell density in the retinal ganglion cell layer was 2.14 times lower for rats with fully developed kindled seizures than for controls ($p < 0.05$) (Fig. 3). In addition, when compared to controls, the cell density in the retinal ganglion cell layer for the rats that were administered MIGU-4 ip at a low daily dose of 5.0 mg/kg was 38.5% lower ($p < 0.05$), and for the rats that were administered

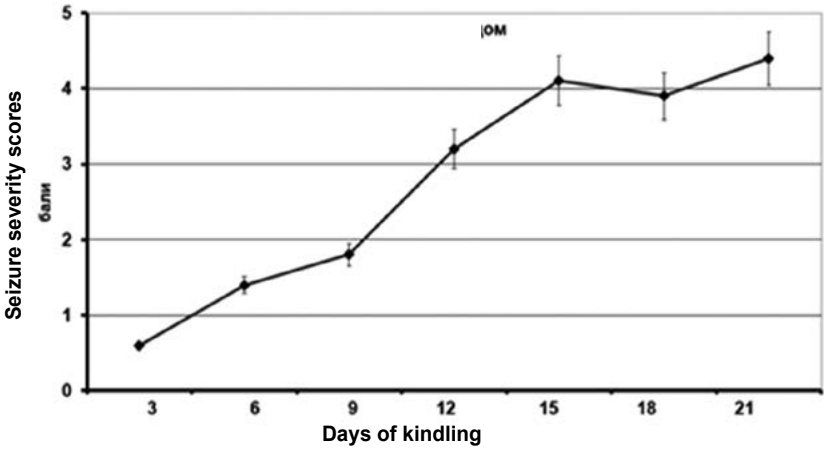

Fig. 1. Relationship between days of pentylenetetrazole (PTZ)-induced kindling and seizure severity scores.

Note: The abscissa indicates days of kindling, and the ordinate indicates seizure severity scores. Data are reported as mean plus or minus standard error of mean.

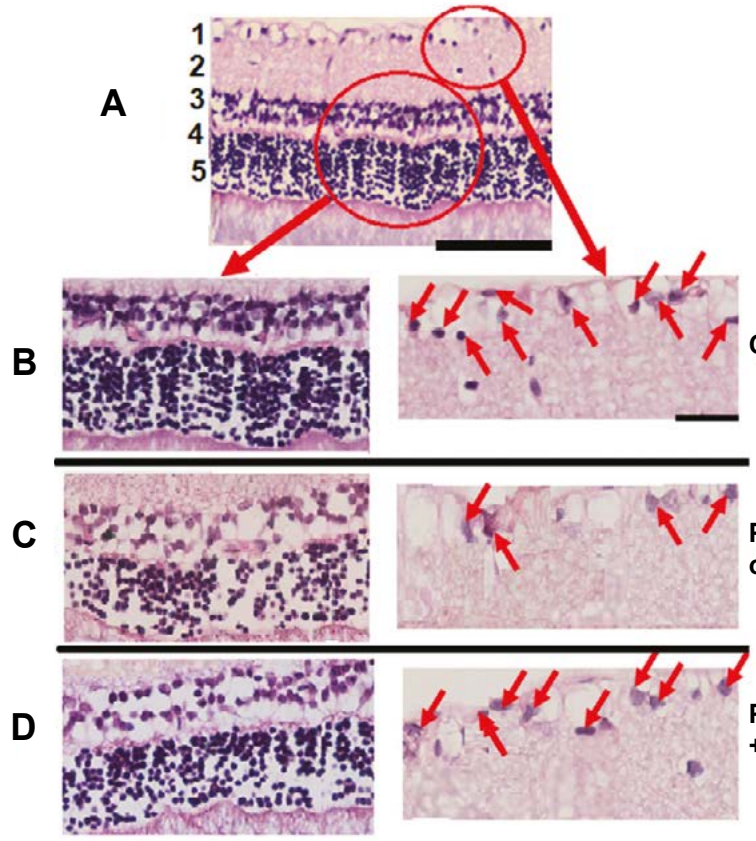

Fig. 2. Retinae of control rats and PTZ-kindled rats non-treated and treated with MIGU-4. Hematoxylin and eosin staining.

Notes: A, control: 1, ganglion cell layer; 2, inner plexiform layer; 3, outer inner layer; 4, outer plexiform layer; 5, outer nuclear layer.

B, control (rats that received intraperitoneally normal saline); C, kindled rats (rats that received intraperitoneally pentylenetetrazole); D, kindled rats treated with MIGU-4 at a daily dose of 25.0 mg/kg. Arrows denote the ganglion cells taken into account for determination of examined parameters.

The scale bar at the bottom right-hand corner in A and B represents 50 μm.

MIGU-4 ip at a high daily dose of 25.0 mg/kg, 24.8% lower ($p < 0.05$). Moreover, the cell density in the retinal ganglion cell layer was 38.0% higher ($p < 0.05$) compared to the group of kindling rats (Fig. 3).

Cell density in the inner nuclear layer was 41.0% lower ($p < 0.05$) for the rats with fully developed kindled seizures than for controls ($p < 0.05$) (Fig. 4). In addition, when compared to controls, the cell density in the retinal inner nuclear layer for the rats that were administered MIGU-4 ip at a daily dose of 5.0 mg/kg was 36.0% lower ($p < 0.05$), and for the rats that were administered MIGU-4 ip at a daily dose of 25.0 mg/kg, 15.3% lower ($p > 0.05$) (Fig. 4). Moreover, compared to the rats with fully developed kindled seizures, the parameter was 30.5% higher ($p < 0.05$) (Fig. 4).

Cell density in the retinal outer nuclear layer was 19.0% lower for rats with fully developed kindled seizures than for controls ($p < 0.05$) (Fig. 5). In addition, there was no significant difference in the cell density in the retinal outer nuclear layer between the kindling rats that were administered MIGU-4 ip at a daily dose of 5.0 mg/kg or 25.0 mg/kg and controls ($p > 0.05$) (Fig. 5).

Discussion

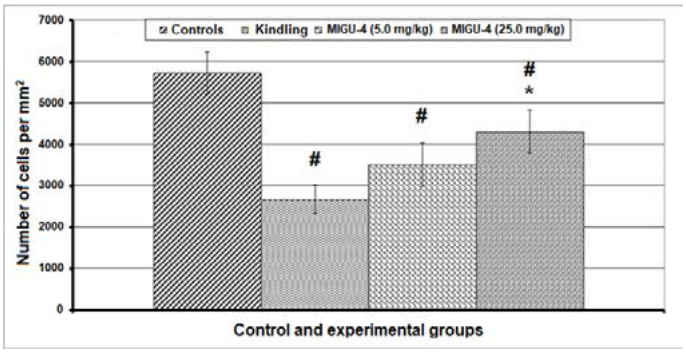

Fig. 3. Numbers of cells per mm^2 in the retinal ganglion layer for control rats, rats that received only PTZ, rats that received PTZ plus MIGU-4 at a daily dose of 5.0 mg/kg and rats that received PTZ plus MIGU-4 at a daily dose of 25.0 mg/kg
Note: #, significant difference compared to controls (P < 0.05); *, significant difference compared to rats with fully-developed kindling (P < 0.05; ANOVA+ Newman-Keuls test)

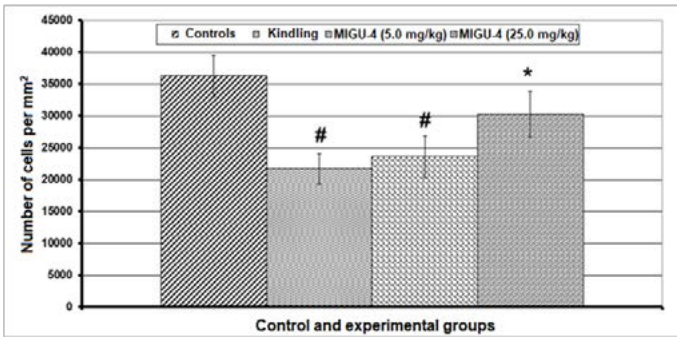

Fig. 4. Numbers of cells per mm2 in the retinal inner nuclear layer for control rats, rats that received only PTZ, rats that received PTZ plus MIGU-4 at a daily dose of 5.0 mg/kg and rats that received PTZ plus MIGU-4 at a daily dose of 25.0 mg/kg
MIGU-4 (5.0 mg/kg) Number of cells per mm^2
Note: #, significant difference compared to controls (P < 0.05); *, significant difference compared to rats with fully-developed kindling (P < 0.05; ANOVA+ Newman-Keuls test)

Therefore, the results obtained indicate the development of an increased susceptibility of the rats to the convulsive effect of PTZ in the course of administration of PTZ at a dose that initially did not cause seizures. This type of model is used in experimental studies as the most adequate model in terms of the reproduction of the clinical characteristics of epilepsy.[12] Unlike the acute epileptic syndrome induced by a single administration of an epileptogenic drug, the kindling model allows for studying the pathogenesis of chronic epilepsy that may originate from persistent neurodegenerative changes in brain tissue.[10] Until recently, it was the studies of brain structures that have confirmed this hypothesis, whereas the morphological and functional status of the retina has not been given attention.

The results obtained demonstrated degenerative changes in the retinal neurons in a PTZ-induced model of chronic epileptic activity. A mechanism of this type of abnormality is thought to be systemic neuroimmune changes including activation of microglia and an increase in the levels of pro-inflammatory cytokines and free radicals in the retina. [15] Accumulation of excitatory aminoacids at the site of inflammation has been implicated in these abnormalities. [3] These findings are in agreement with a study by Ahl and colleagues [13] who investigated the retinal morphology in a rat model of temporal status epilepticus. They noted that apparent neurodegenerative changes in the retina can be observed late after epileptic seizures. The morphological changes in the retina were preceded by the microglial activation typical of the onset of neuroinflammation which was most apparent in the inner retina.

In addition, in the current study, retinal degenerative changes were most prominent in the ganglion and inner nuclear layers, whereas in streptozotocin-induced diabetic retinopathy,[14] they were most prominent in the outer nuclear layer. Therefore, epileptogenic retinal neuronal degeneration is different from experimental streptozotocin-induced diabetic retinopathy in terms of morphological features.

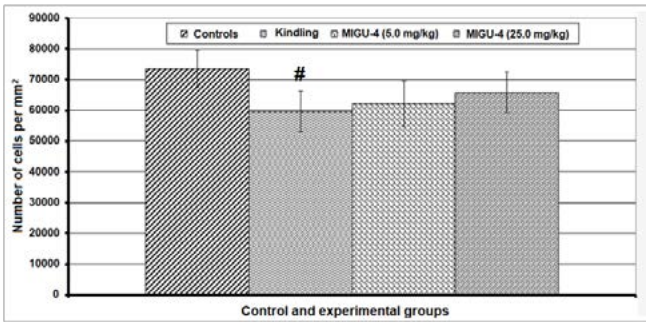

Fig. 5. Numbers of cells per mm2 in the retinal outer nuclear layer for control rats, rats that received only PTZ, rats that received PTZ plus MIGU-4 at a daily dose of 5.0 mg/kg and rats that received PTZ plus MIGU-4 at a daily dose of 25.0 mg/kg
Note: #, significant difference compared to controls (P < 0.05; ANOVA+ Newman-Keuls test)

Given that MIGU-4 contains nicotinic acid (a vasodilator), suppression of retinal ischemia during seizures constitutes a possible component of the neuroprotective effect of this preparation, in addition to the aforementioned antioxidative and anti-inflammatory effects. It is noteworthy that the neuroprotective efficacy of MIGU-4 that this study has revealed suggests the efficacy in the protection against neurodegenetative changes, both in the retina and in the brain.

Conclusion

First, the induction of the PTZ- kindling model of chronic epileptic syndrome caused retinal neurodegenerative changes which were most prominent in the ganglion and inner nuclear layers.

Second, the dose-dependent application of niacin oxy-ethylidene phosphonic germinate (MIGU-4) prevented retinal neurodegenerative disorders in rats with chronic epileptic syndrome.

Further studies have been planned on (a) neurophysiological characteristics (visual evoke potentials) as a marker for retinopathy, and (b) neuroprotective effects of MIGU-4 on brain structures in PDZ-induced kindling. This study was financially supported by the Ministry of Health of Ukraine (Research program: Improving the Efficacy of Control for Epileptic Activity through the Use of Pharmacological Medications and Non-Invasive Stimulation of Brain Structures; State Register Number: 0121U114510).

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Disclosures

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Conflict of interest. *The authors declare that they have no conflicts of interest that could influence their opinions on the subject matter or materials described and discussed in this manuscript.*

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