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PENTYLENETETRAZOL-INDUCED SEIZURES AND BRAIN NEUROINFLAMMATION MARKERS UNDER CONDITIONS OF PITOLISANT AND PIOGLITAZONE USE

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Abstract

The purpose of the study was to investigate the dynamics of seizure activity and markers of neuroinflammation in kindled rats under conditions of individual and combined use of pitolisant and pioglitazone. Under conditions of acute observation in rats kindled with pentylenetetrazol, repeated administration (35.0 mg/kg, i.p.) of the behavioral, electrographic seizure manifestations, the density of microvessels, and the level of HIF-1α in brain structures have been investigated. The data obtained showed that pitolisant use in previously ineffective dosage (5,0 mg/kg, i.p.) but against the background of the administration of pioglitazone (50,0 mg/kg, i.p.) was accompanied by the anticonvulsant effect, which was expressed as prevention of generalized tonic-clonic seizures as well as in reduction of seizure severity and triple shortage of the ictal discharges duration in brain frontal cortex. Also, combined administration of pitolisant (5,0 mg/kg, i.p.) and (50,0 mg/kg, i.p.) prevented kindling-induced angiogenesis in the frontal cortex by 55,2% and the level of HIF-1α in the hippocampal gyrus dentatus by 38,6%. Thus, complex antiepileptic effects significantly

exceeded those observed after separate drug administration have been described. These data support the synergistic antiepileptic action of the investigated compounds, favoring the rationality of exploring a combination of modulators of the histaminergic system with PPAR-γ agonists as antiseizure therapy.

Keywords: chronic epileptic syndrome; pathogenic mechanisms of seizures; inflammation; histamine; pitolisant; pioglitazone.

Epilepsy is one of the most severe diseases of the nervous system and is characterized by paroxysmal seizure manifestations, as well as the occurrence of comorbid conditions [4, 6]. The prevalence of epilepsy ranges from 4 to 10 per 1000 people [6]. As of the beginning of 2025, more than 169,000 people diagnosed with epilepsy were officially registered in Ukraine [17]. Given the severe manifestations, progressive nature of the disease, and social consequences, the problem of treating patients with epilepsy remains relevant. Even though more than thirty drugs are available to epileptologists, seizures remain uncontrolled in one-third of epilepsy patients and are defined as a form of epilepsy resistant to pharmacotherapy [4].

One approach to developing new methods of controlling epileptogenesis is the use of drugs, specifically neuromodulators that can inhibit neuroinflammation, which includes modulators of the brain's histaminergic system [15]. Immunohistochemical mapping of the brain indicates the presence of two main histamine compartments: neuronal, which is localized in the tubero-mammillary zone in the posterior third of the hypothalamus, and extra-neuronal, the source of which is mast cells - both resident brain tissue cells and mast cells are associated with the smooth muscle fibers of the brain vessels [14, 15, 20]. Moreover, under normal conditions, the majority (60-80%) of all histamine is found in the neuronal compartment [15]. The activity of histaminergic neurons ensures the fundamental brain functions of maintaining circadian rhythms of wakefulness and sleep, cognitive functions, and controlling the excitability of neuronal structures [6, 10, 15].

It should be noted that the modulation of the activity of the histaminergic system of the brain - both neuronal and mast cell compartments - is accompanied by a distinct effect on the brain's seizure readiness, and electrical stimulation of the tubero-mammillary zone of the hypothalamus, which is the main source of the neuronal histamine pool, is accompanied by suppression of seizure activity [20]. The efficacy of pitolizant, a blocker/inverse agonist of H3 histamine receptors, has been established to control the excitability of brain neurons [13, 16].

However, until recently, the effectiveness of its use on the manifestations of chronic epileptic syndrome, specifically pentylenetetrazole (PTZ)-induced kindling, which is based on the mechanisms of inflammatory tissue, has not been studied [18].

The aim of the work is to investigate the manifestations of PTZ-induced kindling under the condition of pitolisant administration, examining both behavioral, electrophysiological, and immunohistochemical aspects, including angiogenesis in brain structures as a marker of the inflammatory process. In addition, the study aimed to investigate the effectiveness of pitolisant in combination with pioglitazone, a PPAR-γ receptor agonist that is closely integrated with the histaminergic system of the brain in the development of neuroinflammation, and whose activation causes the suppression of epileptic activity [1, 5, 11, 12].

Materials and Methods

Animals.

38 male Wistar rats weighing 180-220 g were involved in the experiments. Before experiment, the animals were acclimated to laboratory conditions for at least seven days. They were permanently housed in standard conditions with tap water, a standard feed, a 12-hour light/dark cycle, and a constant temperature of 23 °C and 60% relative humidity. Planning recommendations from ARRIVE and the Basel declaration (http://www.basel-declaration.org), which includes the 3R idea, have been considered at this stage of experimental work. Before the start of the experiment, the Odessa National Medical University Bioethics Committee (UBC) approved it (protocol № 3, 05.05.2022).

Kindled convulsions were induced using the previously published method [5, 8]. For 21 days, PTZ ("Sigma Aldrich") was administered intraperitoneally (i.p.) once a day at a dose of 35.0 mg/kg. The following standards were used to assess the convulsions' severity: 0, no seizure symptoms; 1, a tremor in the face and distinct myoclonic jerks; 2, clonic convulsions affecting the entire body; 3, clonic convulsions involving the entire body with rearing; 4, generalized clonic-tonic convulsions involving the whole body with rearing and falling; and 5, repeated convulsions as at stage 4 or a fatal outcome following a seizure fit. Only rats with fully developed seizures (4-5 scored seizure severity) as a response to the two last (20^{th} and 21^{t}) PTZ administrations were included in the observation.

Electrode implantation and EEG registration.

Animals were anesthetized by injection of ketamine (100 mg/kg, s.c., Farmak, Ukraine). The rats were fixed in a stereotactic device, “SEZH-5” (Bogomolets Institute of Physiology, Kyiv, Ukraine), and the pressure points and tissue dissection area were

anesthetized by local infiltration of a 0.5% Novocaine solution (Darnitsa, Kyiv, Ukraine). A 2 cm long tissue dissection was performed along the median sagittal line, and soft tissue was removed in the area of recording electrode fixation. To insert the latter, holes with a diameter of 1.0-2.0 mm were drilled in the skull bones using a dental drill "Colt 1" (Kharkiv, Ukraine). The nichrome recording electrodes were implanted in the frontal cortex area (AP=1.7-2.2; L=2.5-3.0; H=0.5-1.0) of both cerebral hemispheres by stereotactic coordinates [9].

The electrical activity was recorded monopolarly using the Neuropack Four (Nihon Kohden, Japan) and DX-5000 (Kharkiv, Ukraine) computer systems, for which an indifferent electrode was attached to the nasal bones of the skull. The sampling rate of the channels was 256 impulses per second. The data were visualized on the screen and recorded on a hard disk for further offline processing. The frequency range of the signals was 0.5-40 Hz. Offline signal processing was performed using the program "Matlab 7.0".

Immunohistochemical studies

To perform immunohistochemical studies according to the previously described method [8], the following antibodies were used: sc-29010 (Santa Cruz Biotechnology) for Collagen IV and sc-144 (Santa Cruz Biotechnology) for HIF-1a. Brain tissues were fixed in 10% formaldehyde and subsequently paraffin-embedded. Sections of 5 μm thickness were made from the paraffin-embedded blocks using a rotary microtome and placed on poly-L-lysine-coated slides. To determine the differences in the expression of collagen IV, as a marker of vascular growth, in brain tissue, the avidin-biotin peroxidase technique was used [8].

Images were obtained using an Olympus BX53 light microscope and EVOS® FL Auto Imaging System (Life Technologies, Ltd.) and analyzed visually. The number of vessels was counted by the blind method. The number of microvessels (capillaries) of small diameter (7-10 μm in fields of 500 x 500 μm (0.25 mm2) in the frontal areas of 3 cortical slices obtained within 2.0-1.5 mm from the bregma of each rat was determined at a magnification of x400. The presence of angiogenesis was registered by the characteristic signs of "growth buds" and the presence of endothelial cell mitosis. The content of NIF-1α was determined by measuring the relative density of color of the preparations using the method described in [8].

Pitolizant (Selleck, USA) and pioglitazone (Actiza Pharmaceutical Pvt.Ltd., India) were dissolved in dimethyl sulfoxide (DMSO) and administered i.p. 30 minutes before the use of epileptogen. Control rats were treated with DMSO under the same conditions.

Statistical procedures

Statistical descriptive and analytical procedures were performed using the statistical program SPSS 21.00 (USA), employing the ANOVA method, which was supplemented by the post-hoc Tukey HSD test. The normality of the distribution was verified using the Shapiro-Wilk test. The “z” test was also used to compare two proportions. The severity of seizures was assessed using the Kruskal-Wallis test, supplemented by Dunn's post-hoc test. Differences between groups were considered significant at $P<0.05$.

Results

Convulsions manifestations

The use of pitolisant (5.0 mg/kg, i.p.) in rats with developed kindling was accompanied by an increase in the latent period of seizures by 8.8% compared to the control ($P<0.05$) (Table 1). Against the background of the use of pitolisant at a dose of 10.0 mg/kg , i.p., the prolongation of the latent period was 21.7% ($P<0.05$), which also exceeded the level in the group of rats with the use of pitolisant in a lower dose ($P<0.05$). The latency of the first seizures in rats treated with pioglitazone (50.0 mg/kg, i.p.) was 13.2% higher than in the control group ($P<0.05$). Against the background of the combined use of pitolisant (5.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.), the latent period exceeded its value in the control by 18.3%, and in the dose of pitolisant 10.0 mg/kg, i.p. by 23.9% ($P<0.05$). In both groups, the studied index was higher compared to the group with separate use of pitolisant (5.0 mg/kg, i.p.) by 10.3% and 16.5%, respectively ($P<0.05$). In addition, the latency of seizures in the group with pitolisant at a dose of 10.0 mg/kg, i.p. and pioglitazone (50.0 mg/kg, i.p.) exceeded that in the group with pioglitazone alone by 12.3% ($P<0.05$) (Table 1).

The severity of seizures with the combined use of pioglitazone and pitolisant in both study doses was significantly lower than in the control group ($P<0.02$) (Table 1). The use of pitolisant at a dose of 10.0 mg/kg, i.p. in combination with pioglitazone (50.0 mg/kg, i.p.) prevented the occurrence of generalized tonic-clonic seizures ($z=2.497$; $P=0.013$). In addition, the severity of seizures in the group with pitolisant at a dose of 10.0 mg/kg, i.p. and pioglitazone (50.0 mg/kg, i.p.) was less compared to the group of rats with pioglitazone (50.0 mg/kg) alone ($P<0.05$) (Table 1).

Table 1

Effects of pitolisant and pioglitazone on kindled PTZ-induced generalized seizures (M±SD)

	Groups of observation	Latency of seizures (sec)	Scored seizure severity					P (Kruskal Wallis+ Dunn)
			1	2	3	4	5	
1	Kindling-control (PTZ, 35.0 mg/kg, i.p.) (n=7)	67.3± 3.0	-	-	-	4	3	>0.05
2	Pitolisant (5.0 mg/kg, i.p.) (n=6)	73.8± 4.1*	-	-	1	4	1	>0.05
3	Pitolisant (10.0 mg/kg, i.p.) (n=6)	86.0± 5.3*#	-	-	2	3	1	>0.05
4	Pioglitazone (50.0 mg/kg, i.p.) (n=6)	77.5± 4.8*	-	-	2	4	-	$P_4 – P_6 = 0.047$
5	Pitolisant (5.0 mg/kg, i.p.)+ pioglitazone (50.0 mg/kg, i.p.) (n=7)	82.4± 5.7*#	-	1	2	3	-	$P_5 – P_1 = 0.015$
6	Pitolisant (10.0 mg/kg, i.p.)+ piogltazone (50.0 mg/kg, i.p.) (n=6)	88.4± 6.6 *#@	-	2	3	1	-	$P_6 – P_1 = 0.0005$; $P_6 – P_2 = 0.016$

Notes: *-P<0.05 – vs control; #- P<0.05 – vs pitolisant (5.0 mg/kg, i.p.); @-P<0.05 vs pioglitazone (50.0 mg/kg, i.p.) (for latency - ANOVA+Tukey HDS).

EEG seizure manifestations

In the control group, 3.7-9.0 min after the administration of PTZ (35.0 mg/kg, i.p.), synchronized spike potentials with a frequency of 18-11 per s were recorded in the frontal cortex, which lasted from 12.0 to 40.5 s (Fig. 1, 1). Against the background of the use of pitolisant in doses of 5.0 and 10.0 mg/kg, i.p., the latency of ictal discharge onset ranged from 3.0 to 12.4 and from 6.5 to 14.7 min, respectively, and their duration was from 4.5 to 14.0 (not shown) and from 7.9 to 20.2 sec (Fig. 1, 2).

Notes: 1 - 5.5 min after the application of PTZ at a dose of 35.0 mg/kg, i.p. in an fully kindled rat (control); 2 - 7.0 min after the application of PTZ against the background of pitolisant administration at a dose of 10.0 mg/kg, i.p.; 3- 7.5 minutes after the application of PTZ in a rat with pioglitazone (50.0 mg/kg, i.p.); 4- 6.5 minutes after the application of PTZ in a rat with combined administration of pitolizant (5.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.). The 10-second period of uninterrupted observation was removed from the illustration.

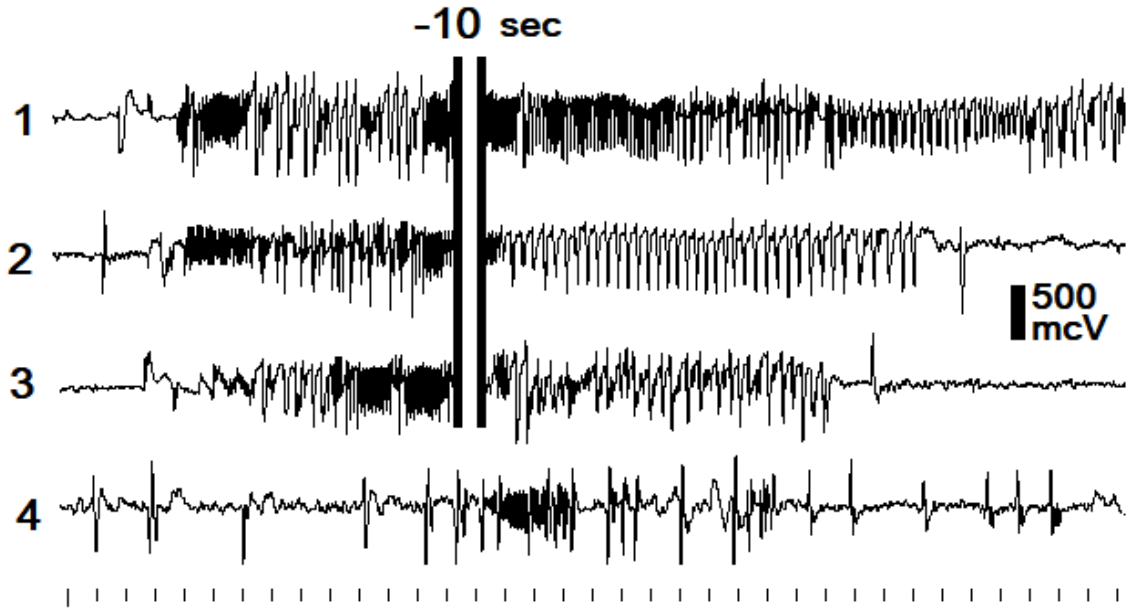

Fig. 1. Ictal potentials evoked in the frontal cortex of rats with developed PTZ-kindling under experimental treatment.

Time marks are 1 s, and the calibration signal is 500 μV.

Under the condition of administration of pioglitazone (50.0 mg/kg, i.p.), the administration of PTZ (35.0 mg/kg) was accompanied by the appearance of ictal potentials in 4.6-11.3 min, and the duration of the ictal potential was 5.5-11.5 sec (Fig. 1.3). Against the background of the combined administration of pitolizant (5.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.), the onset of ictal potentials was observed in 5.2-10.0 min after the injection of PTZ. The duration of ictal discharges ranged from 5.3 to 9.5 sec (Fig. 1, 4). Comparison of the mean values of the latent period of ictal potentials revealed no significant differences between the groups: $F_{(4, 22)}$=1.548; P=0.23 (Table 2).

At the same time, the study of ictal potential duration revealed significant differences between the groups: $F_{(4,45)}$=5.468; P=0.0012. With the use of pitolisant at a higher dose (10.0 mg/kg, i.p.), the duration of the ictal potential decreased by 40.2% compared to the control (P=0.029).

The decrease in the studied index was also significant in the setting of separate use of pioglitazone (50.0 mg/kg, i.p.) (P = 0.004) and its combined use with pitolisant at a dose of 5.0 mg/kg, i.p. (P = 0.006) (Table 2). Moreover, the combined use of drugs reduced the ictal potential by 3.1 times.

Table 2

Latency period and duration of ictal potential in rats with PTZ-induced kindling under the conditions of pitolisant and pioglitazone administration (M±SD)

	Groups of observation	Number of ictal discharges		Latency of ictal discharge (mins)	Duration of ictal discharges
		Per rats	Repeated		
1	Kindling-control (PTZ, 35.0 mg/kg, i.p.) (n=7)	7/7	3	6.70±3.31	22.55±9.76
2	Pitolisant (5.0 mg/kg, i.p.) (n=6)	5/6	1	6.88±3.50	11.85±5.73
3	Pitolisant (10.0 mg/kg, i.p.) (n=6)	4/6	1	11.25±3.74	13.48±5.6*
4	Pioglitazone (50.0 mg/kg, i.p.) (n=6)	4/6	-	7.40±2.89	8.38±2.97*
5	Pitolisant (5.0 mg/kg, i.p.)+ pioglitazone (50.0 mg/kg, i.p.) (n=7)	3/7	-	6.60±2.96	7.33±2.1*

Notes: * - P<0.05 vs control (ANOVA+Tukey HDS).

Thus, against the background of advanced PTZ-induced kindled seizures, both pitolisant (10.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.) were effective as separate treatments. However, the effect of reducing the duration of ictal discharges in the context of combined use of drugs was more pronounced.

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Angiogenesis and HIF-1α in brain structures

In rats with developed kindling, the density of microvessels, identified by their characteristic brown color, was significantly greater in the frontal cortex compared to the

control group. Specifically, the number of microvessels in kindling rats (52.7±4.8) was three times higher than in the control group (17.3±1.9), with a statistical significance of $P<0.05$ (Fig. 2, A, B).

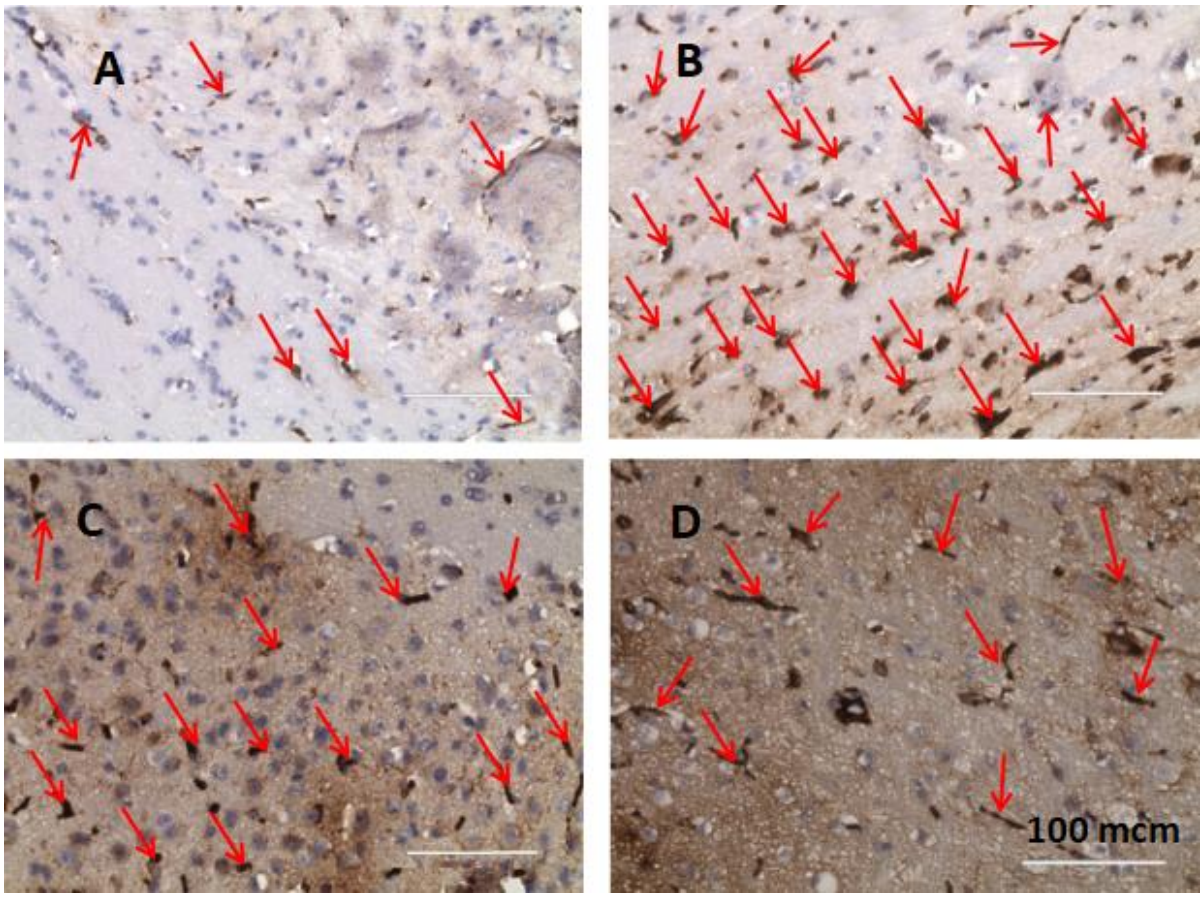

Fig. 2. Dynamics of immunohistochemical staining for collagen IV in the tissue of the frontal brain cortex in rats with PTZ-induced kindling under the conditions of pitolisant and pioglitazone administration.

Notes: A - intact animal with 0.9% saline NaCl injection; B - fully developed kindling; C - kindling under pitolizant (5.0 mg/kg, i.p.) administration; D - kindling under combined use of pitolizant (5.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.). Typical microvessels that were taken into account in the calculation are indicated by red arrows.

The calibration is 100 μm.

In the case of pitolisant (5.0 mg/kg, i.p.), such differences were 2.2 times (38.2±3.2 microvessels) ($P<0.05$). However, the studied index was significantly lower (by 27.5%) compared to that in the kindled rats ($P<0.05$; Fig. 3). At the same time, when pitolisant (5.0 mg/kg, i.p.) was administered against the background of preliminary administration of

pioglitazone (50.0 mg/kg, i.p.), the studied index exceeded that of the control group by 25.1% (P<0.05). It was less than in rats with kindling by 55.2% (P<0.05). In addition, the density of blood vessels was also lower compared to that in the group with one application of pitolisant, by 39.5% (P<0.05) (Fig. 3).

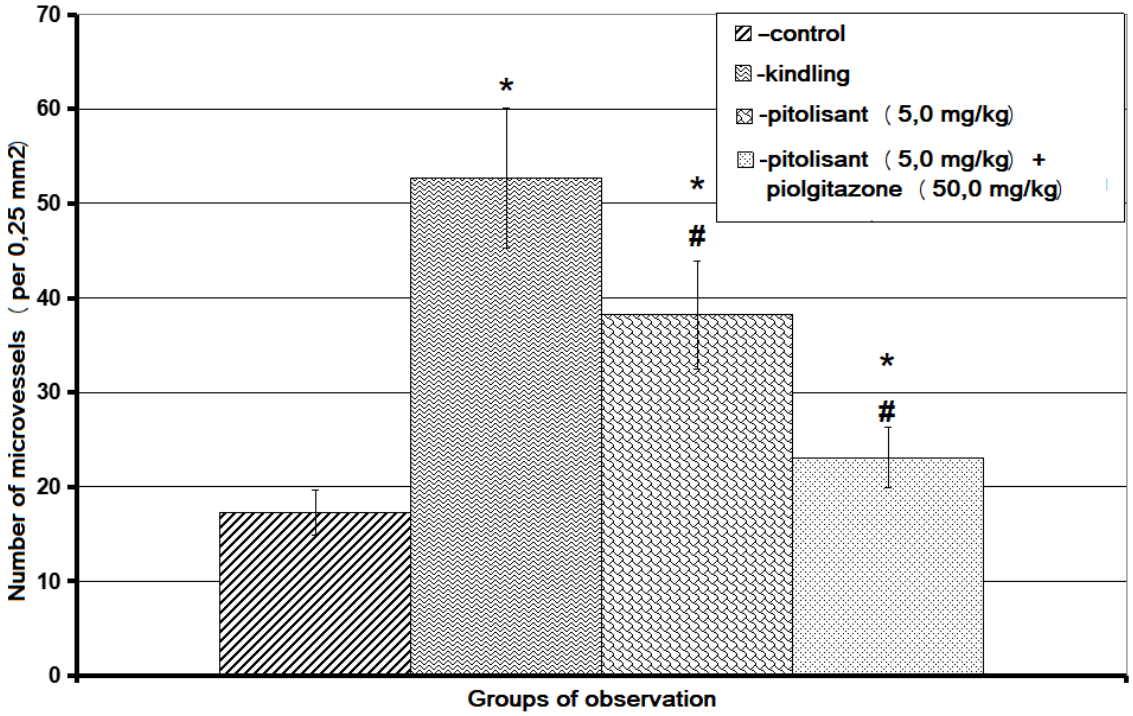

Fig. 3. Quantitative assessment of angiogenesis in the cerebral cortex of Kindling rats under the influence of pitolisant and pioglitazone.

Notes: Density of microvessels in % pertained to the group of intact rats, taken as 100% (ordinate) in the observation groups (abscissa). * - P<0.05 compared to the group of intact rats; # - P<0.05 compared to the group of kindled rats (ANOVA+Tukey HSD).

Determination of the HIF-1α content by the intensity of immunohistochemical staining in the hippocampal dentate gyrus showed its increase by 48.1% compared to the control (from 9.5±1.2 U to 18.3±1.5 U) (P<0.05) (Fig. 4). At the same time, against the background of the use of pitolisant (5.0 mg/kg, i.p.) and pitoglitazone (50.0 mg/kg, i.p.), the studied index was 11.2±1.4 U (P>0.05) (Fig.4, D).

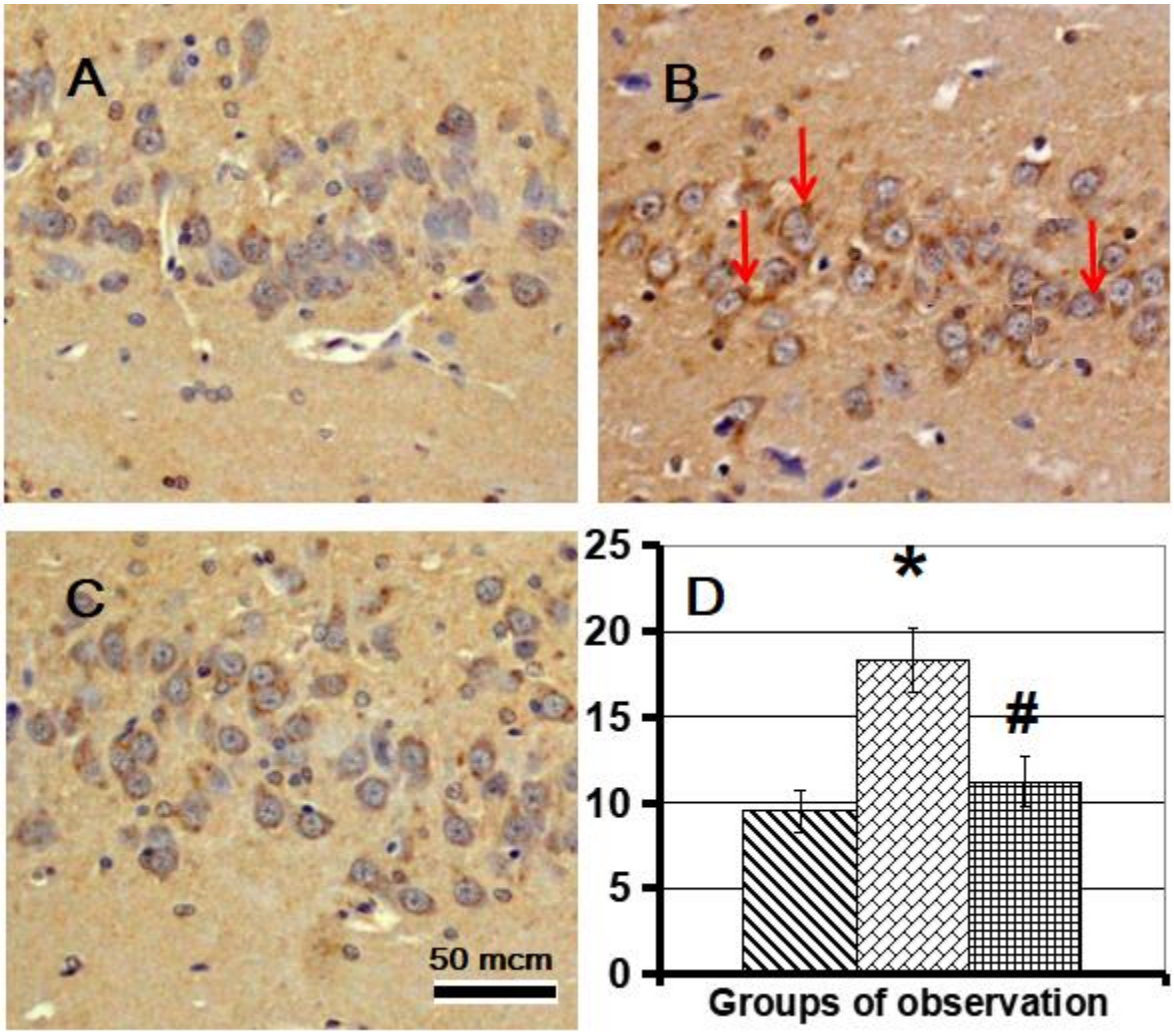

Fig. 4. HIF-1α content in the dentate gyrus of the hippocampus of rats with PTZ-induced kindling under experimental treatment.

Notes: A-control, B-kindling, C-kindling + administration of pitolisant (5.0 mg/kg, i.p.) and pioglitazone (50.0 mg/kg, i.p.). Red arrows indicate characteristic areas of coloration (B). G - % of staining intensity (ordinate, relative units (U)), abscissa - observation groups - in the order of histological image fragments; *-P<0.05 compared to control (A), #-P<0.05 compared to sham-operated (B) (ANOVA+Tukey HSD test).

Discussion

Thus, the results obtained determined the effectiveness of separate use of pitolisant and pioglitazone on the manifestations of kindled seizures, which were recorded in the form of prolongation of the latent period of the first seizures with more pronounced effectiveness for pioglitazone. Against the background of pioglitazone administration (50,0 mg/kg, i.p.) pitolisant (5,0 mg/kg, i.p.) caused pronounced antiseizure effects preventing generalized tonic-clonic fits and markedly - by three times reducing duration of ictal discharges registered

in frontal cortex. Greater dosage of pitolisant (10,0 mg/kg, i.p.) combined with pioglitazone results in almost complete blocking of generalized kindled seizures and ictal discharges as well. Hence, observed effects favor of synergy of antiepileptic action of investigated drugs.

Considering the mechanisms of a synergy effect, it is worth noting that the H3 receptor antagonist/inverse agonist pitolisant, as well as other pharmacological modulators of H3 receptors structurally related, cause anticonvulsant action [2, 3]. This action can be explained by the activation of heteroreceptors located on both GABAergic presynaptic terminals and the terminals of the excitatory amino acid system [2, 3]. Additionally, the H3 receptor is a presynaptic heteroreceptor located at the terminals of mediator systems that can control neuronal excitation. Modulation of H3 receptor activity provides numerous direct and indirect effects on the wake-sleep cycle and neuronal excitability [15, 19, 20].

Pioglitazone's antiseizure action is also complex and may be due to modulation of the nitric oxide (NO) level, indirect influence on seizure activity by modulating the mammalian target of rapamycin (mTOR) pathway, and affecting glial cell function [1, 11, 12]. The last one is of special significance for chronic kindled seizures as they are underlain by neuroinflammation, strongly supported by glial involvement [8, 19].

Hence, the strengthening of inhibition might be achieved via different pathways involving H3- and PPAR-γ-dependent systems, which may be responsible for the synergistic strengthening of inhibitory control over epileptic activity.

One of the important pathogenetic mechanisms of chronic epileptic activity is the activation of angiogenesis, a characteristic feature of chronic neuroimmune inflammation [5, 8, 18]. The present study found an increase in the levels of collagen IV and HIF-1α, markers of newly created capillaries [5, 8, 15]. Accordingly, against the background of the onset of kindling seizures, the use of pitolisant (5.0 mg/kg) and pioglitazone (50.0 mg/kg) prevented the occurrence of such changes. It is worth noting that the activation of histaminergic mechanisms initiates angiogenesis [7, 14]. Therefore, the effect of pitolisant in this regard should be considered as a weakening of histaminergic effects realized by H1-, H2-, and H4-receptors.

Such an effect also prevents the characteristic consequences of histamine action, including the increase in capillary permeability, which allows for the emigration of immunocompetent blood cells, as well as the penetration of immunoglobulins into the area of pathogen presence in the inflammation zone [4, 14]. Additionally, preventing the effect on endothelial cells, which are also capable of migration and subsequently participate in angiogenesis, is significant [7]. These features of histamine are valuable for

neuroinflammation as one of the fundamental mechanisms of the pathogenesis of chronic epileptic syndrome.

Until recently, the investigation of pathogenesis in modeling chronic epileptic syndrome, which is contributed to by PARP-γ receptors that interact closely with the histaminergic system, has not been studied. Meanwhile, it is important to note the prospects of using PARP-γ receptor agonists and H3 receptor modulators as anticonvulsants [1, 5, 11, 12]. The results obtained support the potential of combining histaminergic H3 modulators and PPAR-γ receptor agonists for further study of the pathogenesis of chronic epileptic syndrome.

Conclusions:

1. Separate use of pitolisant and pioglitazone prolongs the latency period of PTZ-induced kindled seizures, while the combined use of drugs reduces the severity and prevents generalized tonic-clonic seizures, and reduces the duration of ictal potentials by three times.
2. The combined use of pitolisant and pioglitazone halves angiogenesis - the density of microvessels in the structures of the anterior cerebral cortex of kindled rats, and also reduces the level of HIF-1α in the dentate gyrus of the hippocampus by 38.6%.
3. The combined use of pitolisant and pioglitazone causes a synergistic anticonvulsant effect in the model of PTZ-induced chronic epileptic syndrome.

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