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IMMUNOHISTOCHEMICAL NEUROINFLAMMATORY MARKERS IN THE HIPPOCAMPUS OF PTZ-KINDLED RATS UNDER CONDITIONS OF RAPAMYCIN AND AXITINIB TREATMENT

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The aim of the study *is to determine the level of HIF-1α, TNF-α, and NF-kB in the hippocampus of kindled rats treated with rapamycin and axitinib.*

Materials and methods. *Kindling was produced in 29 rats by administration of three-week pentylenetetrazole (PTZ, 35.0 mg/kg, i.p.). Treatment with rapamycin (0.5 mg/kg, i.p.) and axitinib (2.5 mg/kg, i.p.) was performed for ten days in fully kindled rats. The avidin-biotin-peroxidase method was used for hippocampal slice staining. For negative control, staining was performed using only secondary antibodies.*

Results. *The HIF-1α expression increased in kindled rats raised by 1.77 times compared to the control ($p<0.001$). Axitinib treatment resulted in of HIF-1α level of 16.7 % ($p<0.05$) compared with kindled animals, while combined treatment with rapamycin and axitinib reduced HIF-1α by 33.8 % ($p<0.01$). In kindled rats, TNF-α expression was 3.74 times greater than in control ($p<0.001$). Rapamycin treatment reduced TNF-α by 31.0 % ($p<0.01$). Axitinib treatment caused a reduction of TNF-α by 21.1 % ($p<0.05$). Combined treatment with rapamycin and axitinib reduced TNF-α by 48.0 % ($p<0.001$) but still exceeded the TNF-α in control by 1.95 times ($p<0.01$). NF-kB level in kindled rats exceeded the control by three times ($p<0.001$). Rapamycin caused a reduction of 19.3 % ($p>0.05$), while axitinib – by 26.5 % ($p<0.05$) compared with kindled rats. Combined treatment with rapamycin and axitinib resulted in NF-kB reduction by 56.7 % compared with kindled rats ($p<0.001$).*

Conclusions. *PTZ-kindling resulted in an increase in the immunoreactivity of HIF-1α, TNF-α, and NF-kB in the hippocampus. Combined treatment with rapamycin and axitinib engendered prevention of generalized seizures and normalized the level of HIF-1α and NF-kB with a significant reduction of TNF-α. Effects of treatment favours of synergy action of rapamycin and axitinib*

Keywords: *experimental epileptic syndrome, kindling, pentylenetetrazol, rapamycin, axitinib, HIF-1α, TNF-α, NF-kB, mTOR, tyrosine kinase B*

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1. Introduction

Epilepsy is among the most severe and resistant to treat brain disease, which causes fundamental and self-sustainable pathology in all omics - fields of brain activity [1]. The drastic decline in social activity, narrowing of the spectrum of professional engagement, and shortage of lifespan are characteristics of patients with epilepsy [2]. Contemporary pharmacology control of epileptic manifestations is effective in 70 % of patients [3]. Hence, the new approaches/options of epilepsy treatment working out is, first of all, actual for the rest 30 % of patients, which couples the group of 20 million people all over the World [4, 5].

Earlier, we demonstrated that combined administration of rapamycin-blocker of mTOR and axitinib-blocker of tyrosine kinase B caused synergy antiseizure action [6]. Such a synergy developed in parallel with the pronounced antioxidant effects precipitation [6]. Oxidative stress possesses a pathogenetic role in both brain epileptisation and neuroinflammation [7]; activating the brain antiepileptic system resulted in antioxidant effects [8, 9]. Hence, specific inflammatory signalling pathways might be primarily involved in establishing chronic brain epileptisation as the first step toward systemic antioxidant potential dropping. Also, they might be first-line triggers for the antiseizure synergy of rapamycin and axitinib, which can impact many cellular signalling pathways and fundamentally modify cellular activity [10, 11].

The anticonvulsant activity of newly synthesized perspective compounds is evaluated on PTZ-induced seizures as a classic test system [12, 13]. PTZ-induced kindling as a model of chronic epileptic syndrome is characterized by the stable long-term heightening of brain excitability with comorbid behavioural deteriorations [14]. Primary epileptic foci start from limbic structures, and the hippocampus is regarded as a principal place for focal epileptogenesis, determining the development of seizures during PTZ administrations [6, 8]. Neuroinflammation is among the principal mechanisms of PTZ-kindled seizure establishment [7, 15, 16].

The **aim** of the present work was to determine the level of expression of markers of neuroinflammation – HIF-1α, TNF-α, and NF-kB in the hippocampal tissue of PTZ-kindled rats treated with rapamycin and axitinib.

2. Planning (methodology) of the research

Based on the results of previous studies [6], the drugs with different mechanisms of action - mTOR inhibitor rapamycin and tyrosine-kinase B inhibitor axitinib were selected for the investigation of their antiseizure action upon immunohistochemical markers of PTZ-induced kindling seizures. Namely, such markers of chronic epilepsy as HIF-1α, TNF-α, and NF-kB levels in the dorsal hippocampus of fully kindled animals were measured following the earlier described method [17, 18]. Mentioned drugs possessed antiseizure activity and demonstrated synergy upon behavioural seizures [6].

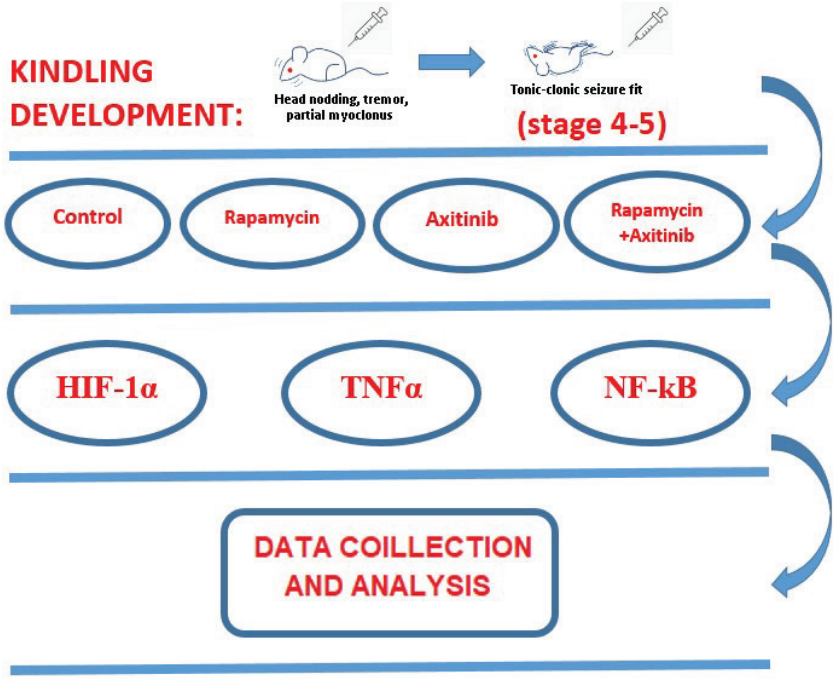

Fig. 1. Algorithm of the research

Stages of the study:

1) analysis of the state-of-the-art research data;

2) modeling of fully developed kindled seizures with repeated PTZ administrations;

3) treatment of kindled animals with Rapamicyn and Axitinib in separative groups and the group with combined drug administration;

4) measurement of seizure manifestation after administration of a testing dosage of PTZ performed after treatment;

5) performing immunohistochemical investigation;

6) processing and analysis of the obtained results;

7) identification of perspectives for further research.

3. Material and methods

Investigations were performed during the 2020–2022 years at Danylo Halytsky Lviv National Medical University, Odesa National Medical University, Yozgat Bozok University, Bozok, Turkey, and the University of Health Sciences, Istanbul, Turkey.

Experimental animals. Experiments were performed on 29 male Wistar rats (two to four months old) with an initial body weight of 180–270 g. Animals were kept in standard conditions (constant temperature 23 °C, relative humidity 60 %, 12 h dark/light cycles; standard diet and tap water were given ad libitum) and were acclimatized to laboratory conditions at least seven days before the experiment. All experiments were carried out following the National Institutes of Health Guidelines for the care and use of laboratory animals and the European Council Directive on 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC). The experiments were approved by the Odesa National Medical University Bioethics Committee (UBC) (approval No. 3 dated 17/03/2020) before the study.

Epilepsy model. Kindled seizures were induced, as described previously [16]. PTZ (Sigma Aldrich, St. Louis, MO, USA) was given intraperitoneally (i.p.) daily at a dose of 35.0 mg/kg for 21 days. The severity of seizures was evaluated according to the following criteria: 0 – absence of symptoms of seizures; 1 – facial tremor and separate myoclonic jerks; 2 – whole-body clonic seizures; 3 – clonic seizures of the whole body with rearings; 4 – generalized clonic-tonic seizures with falling; 5 – repeated seizures as at stage 4 or lethal outcome as a result of seizures. Rats that demonstrated generalized seizures after two last PTZ injections were taken for further observations and evaluation effects of compounds.

Study design and experimental groups. According to the study design, the protocol with four groups of kindled animals was undertaken (Fig. 1). DMSO i.p. administration was used as a control group (Fig. 1, A).

Earlier, we established that the median effective dose (ED_{50}) for axitinib and rapamycin, which prevented generalized, was 4.97 and 0.93 mg/kg, respectively [6]. For synergy verification, we treated one group of kindled rats (n=7) with axitinib (Sigma Aldrich, USA; 2.5 mg/kg, i.p.) (Fig. 1, B). Another group (n=7) was treated with rapamycin (Pfizer, USA; 0.5 mg/kg, i.p.) (Fig. 1, C). Combined treatment – axitinb (2.5 mg/kg, i.p.) and rapamycin (0.5 mg/kg, i.p.) was performed in 7 kindled rats (Fig. 1, D). Treatment started within 24 h after the last PTZ administration and was performed for ten days. In 24 h after the 10^{th} administration, testing trials with PTZ (35.0 mg/kg, i.p.) were performed, and behavioural seizures were evaluated. At 24 h after testing PTZ injection, animals were euthanized, and their brains were collected for immunohistochemical investigations (Fig. 2).

Immunohistochemical analysis.

Brain tissues were fixed in 10 % formaldehyde, washed in tap water (running water for one night), and kept in increasing alcohol series to perform the water recovery procedure. Tissues were made transparent by waiting in xylol for 30 minutes. Then tissues were embedded in paraffin in the appropriate orientation. 5 µm thick sections taken from blocks embedded in paraffin using a rotary microtome were placed on slides coated with poly-L-lysine for immunohistochemistry analysis [17].

Avidin-biotin peroxidase complex method was used to determine differences in HIF-1α, TNF-α and p-NF-κB expressions in brain tissue [18]. For this purpose, 5–6 µm sections were kept on slides at 60 °C overnight. They were deparaffinized and rehydrated by passing them through xylene and through graded alcohol series (100 %, 95 %, and 70 %). Then the slides were washed three times for 5 min with phosphate buffer solution (PBS). The antigen retrieval step was performed by boiling the tissue sections five times, 3 min each at 600 W in a microwave oven with 5 % citrate buffer (pH 6.0; Thermo Fisher Scientific, UK, AP-9003-500) for 5 min and cooled for 15 min at room temperature. Then, the slides were placed in 3 % hydrogen peroxide (H_2O_2) for 10 min to prevent endogenous peroxidase. Ultra V Block solution (Thermo Fisher Scientific, UK, TA-125-UB) was applied to prevent non-specific staining. Sections were then incubated overnight at 4 °C with Kir6.2 (1:50 dilution rate; Alomone labs, APPC-020), HIF-1α (1:200 dilution rate; Bioss Antibodies, bs-0737R), TNF-α (1:100 dilution rate; Elabscience, E-AB-22159) and p-NF-κB (1:50 dilution rate; Bioassay Technology Laboratory, BT-MCA291). After several rinses in PBS, a biotinylated goat anti- polyvalent secondary antibody (Thermo Fisher Scientific, UK, TP-125-BN) was applied for forty minutes at 37 °C in the oven. Following several rinses in PBS, the slides were incubated in streptavidin peroxidase (Thermo Fisher Scientific, UK, TS-125-HR) for thirty minutes at 37 °C in the oven. The antibody complex was visualized by incubation with diaminobenzidine (DAB) chromogen (Thermo Fisher Scientific, UK, TA-125-HD), and then slides were counterstained with hematoxylin solution modified acc. to Gill III (Merck, Germany, 105175). Finally, the tissue sections were dehydrated with increasing alcohol series and passed through xylene before covering with Entellan mounting medium and coverslipped. Images were obtained under the Olympus BX53 light microscope and analyzed performed with Image J Version 1.46 (National Institutes of Health, Bethesda, Maryland) (Fig. 3). Data were evaluated depending on the intensity of the staining compared to the control. To quantify the immunohistological staining for each protein, jpeg images were imported into the ImageJ software. The threshold function was applied to separate the signal from the background.

Average signal intensity was measured with the 'measure' function [19]. The mean intensity of the hippocampus background was obtained by averaging the values of negative control images that had been treated with secondary antibody only [19]. The immunohistological staining intensity level value was calculated by dividing the average signal intensity above the background for a minimum of 10 images per rat for a minimum 6 rats per experimental group.

Statistical procedures.

The SPSS program for Windows (SPSS Inc., version 24.0, Chicago, USA) was used for the statistical processing of the obtained results. Data were presented as mean values with standard error of mean ($M\pm$SEM). An unpaired One-way ANOVA analysis of variance and Tukey's multiple comparison tests was applied to compare immunohistochemistry data between the control and PTZ-induced kindling groups. Kruskal-Wallis followed with a *post hoc* test was used for seizure severity values comparing, and "z" criteria for comparing two proportions. Differences between groups were accepted as significant at $p<0.05$.

Negative control – brain slices of rats treated with 0.9 % NaCl solution and painted only with secondary antibodies.

Notes: the black arrow indicates increased immunoreactivity of the cells.

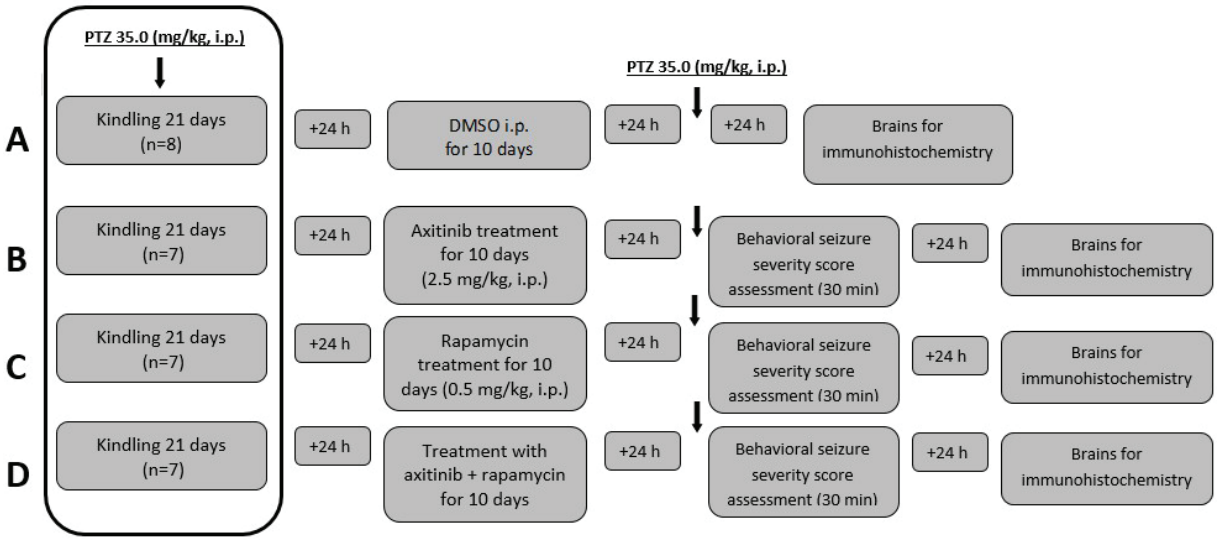

Fig. 2. Design of investigation

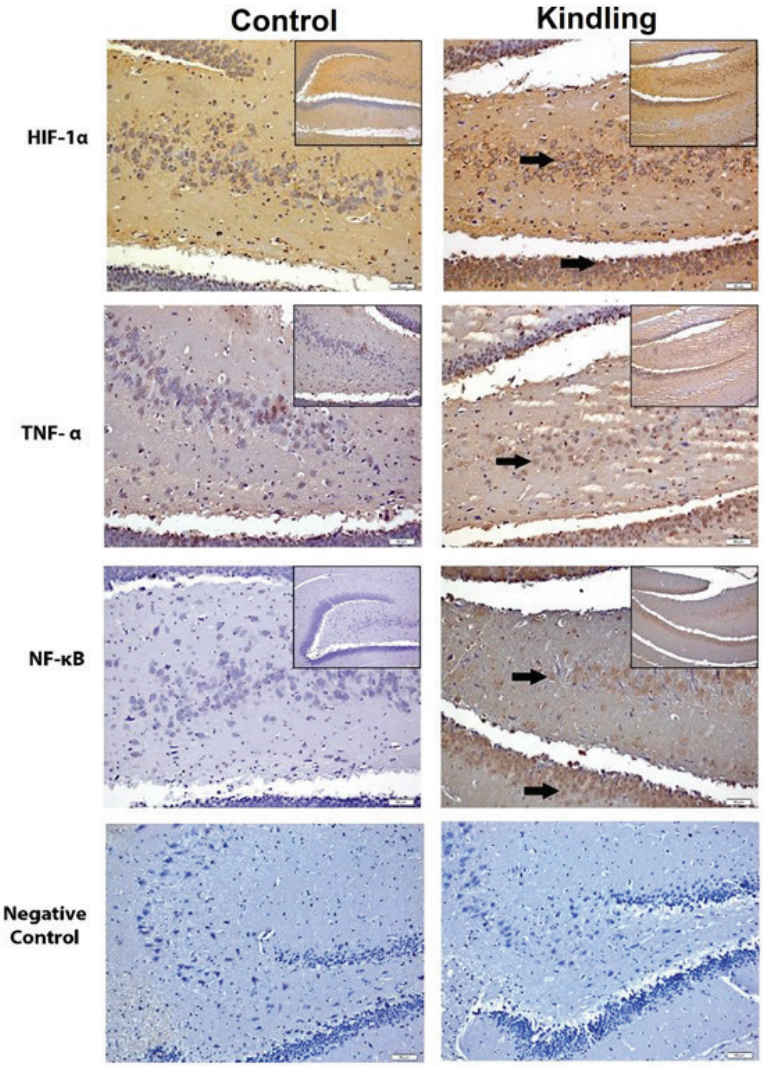

Fig. 3. Representative images of HIF-1α, TNFα, and NF-kB in the dorsal hippocampus of control rats and PTZ-kindling rats

The pictures were taken at a magnification of ×200. Smaller images (right upper corner) were taken at a magnification of ×40. Scale calibration (lower right corner of the images – white rectangle): 50 μm.

4. Results

The precipitation of tonic-clonic seizures characterized by fully kindled PTZ seizures fits with losing balance, falling animals, and developing post-seizure depression. 8 out of 29 rats manifested with repeated seizure fits. The average score of seizure severity was 4.28±0.45. The severity of seizures in each group of observation was 4.29±0.49 and was not differ from control rats (4.25±0.46) (p>0.05).

Treatment with rapamycin (0.5 mg/kg, i.p.) prevented generalized seizures in 2 out of 7 fully kindled rats. In comparison, treatment with axitinib (2.5 mg/kg, i.p.) prevented generalized tonic-clonic fits in 3 out of 7 rats. Combined drug usage blocked generalized seizures in 5 out of 7 rats (z=2.378, p=0.017).

The average seizure severity was reduced after treatment with axitinib by 16.0 % compared with kindled rats (H=4.923, p=0.027) (Fig. 4).

The difference between the combined usage of rtapamycin (0.5 mg/kg, i.p.) and axitinib (2.5 mg/kg, i.p.) was 26.1 % (H=7.806, p=0.005) (Fig. 4).

Kindled rats demonstrated a net increase of HIF-1α expression, which raised by 1.77 times when compared with the control data (p<0.001) (Fig. 5).

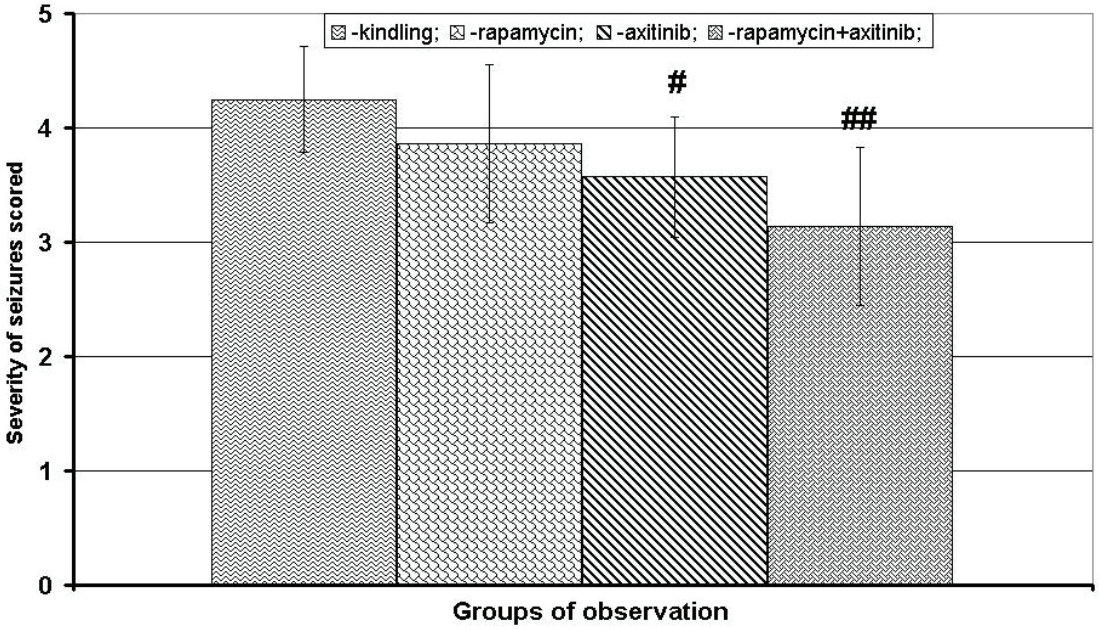

Fig. 4. Kindled seizure severity estimated after ten days of treatment with rapamycin and axitinib. The data average is expressed as M±SEM. Kriskal-Wallis test, followed by Dunn test, was used for comparison # – P<0.05 and ## – P<0.01 vs. kindled rats

Histogram plot showing the immunoreactivity intensity of the antibodies analyzed. The data average is expressed as $M\pm$SEM. One-way ANOVA analysis of variance and Tukey's multiple comparison tests was applied (** – $p<0.01$; *** – $p<0.001$ vs. control; # – $p<0.05$, ## – $p<0.01$ vs. kindling). Abscissa – groups of observation, ordinate – relative units of colour intensity.

Rapamycin treatment resulted in a non-significant reduction of the investigated index (11.1 %, $p>0.05$) compared to the kindled rats. Axitinib treatment resulted in a significant decrease of the HIF-1α level – by 16.7 % ($p<0.05$) when compared with kindled animals but exceeded the value in control by 31.2 % ($p<0.01$). The reduction of HIF-1α in rats treated with rapamycin and axitinib was less than in kindled animals by 33.8 % ($p<0.01$) (Fig. 5).

TNF-α expression was 3.74 times greater than in control rats ($p<0.001$). Rapamycin treatment resulted in the reduction of TNF-α by 31.0 % ($p<0.01$) while exceeding the control value by 2.57 times ($p<0.001$) (Fig. 6).

Histogram plot showing the immunoreactivity intensity of the antibodies analyzed. The data average is expressed M±SEM. One-way ANOVA analysis of variance and Tukey's multiple comparison tests was applied (** – $p<0.01$, *** – $p<0.001$ vs. control; # – $p<0.05$, ## – $p<0.01$; ### – $p<0.001$ vs. kindling). Abscissa – groups of observation, ordinate – relative units of colour intensity.

Axitinib treatment caused a reduction intensity of TNF-α expression by 21.1 % ($p<0.05$). The investigated index exceeded the control value by 2.95 times ($p<0.001$). Combined treatment with rapamycin and axitinib showed a significant decrease in TNF-α level by 48.0 % ($p<0.001$) compared to the kindled rats. The expression still was more prominent than in control animals by 1.95 times ($p<0.01$) (Fig. 6).

The level of NF-kB in kindled rats exceeded control data by three times ($p<0.001$) (Fig. 7).

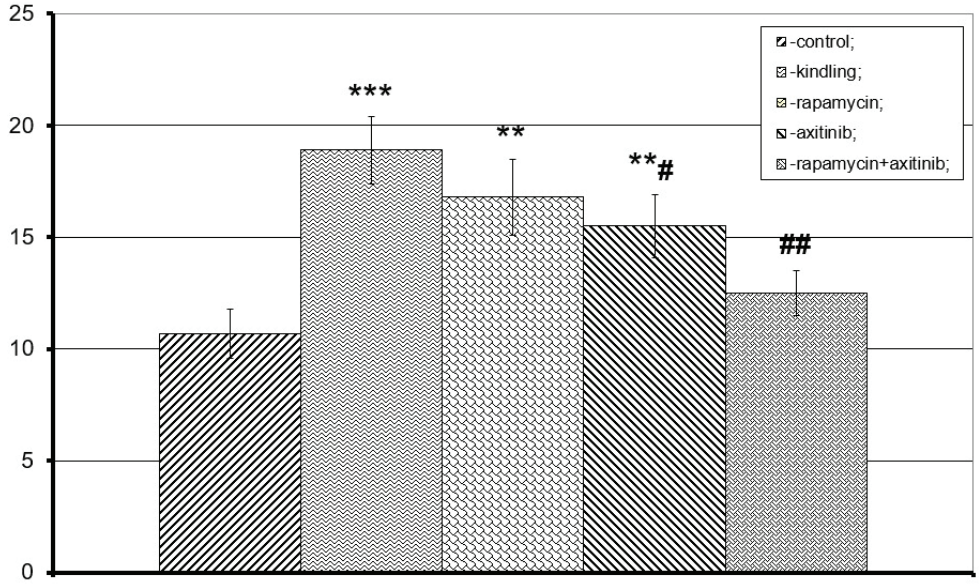

Fig. 5. Effects of treatment with rapamycin and axitinib upon HIF-1α expression in the hippocampus of kindled rats

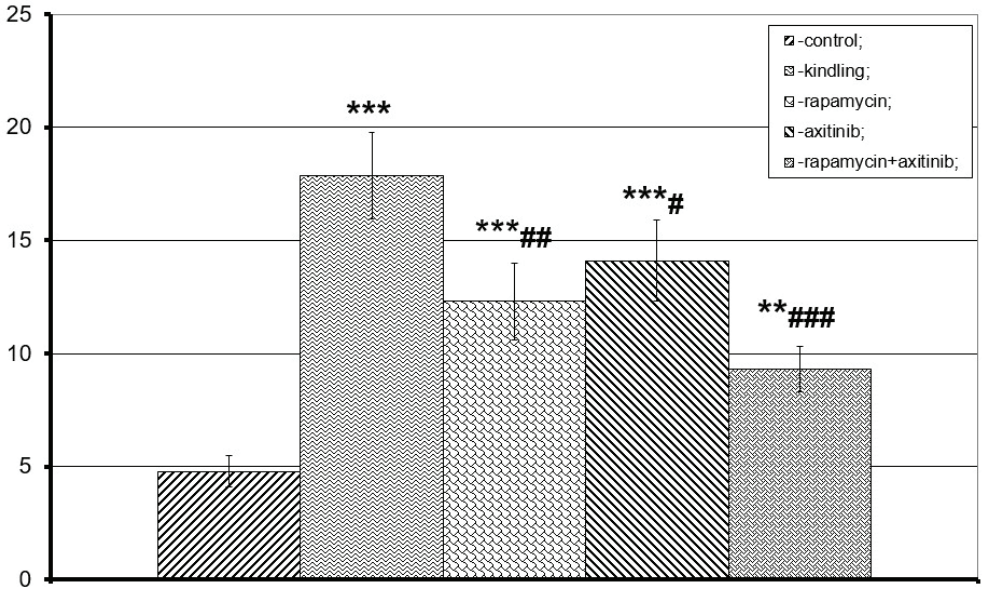

Fig. 6. Effects of treatment with rapamycin and axitinib upon TNF-α expression in the hippocampus of kindled rats

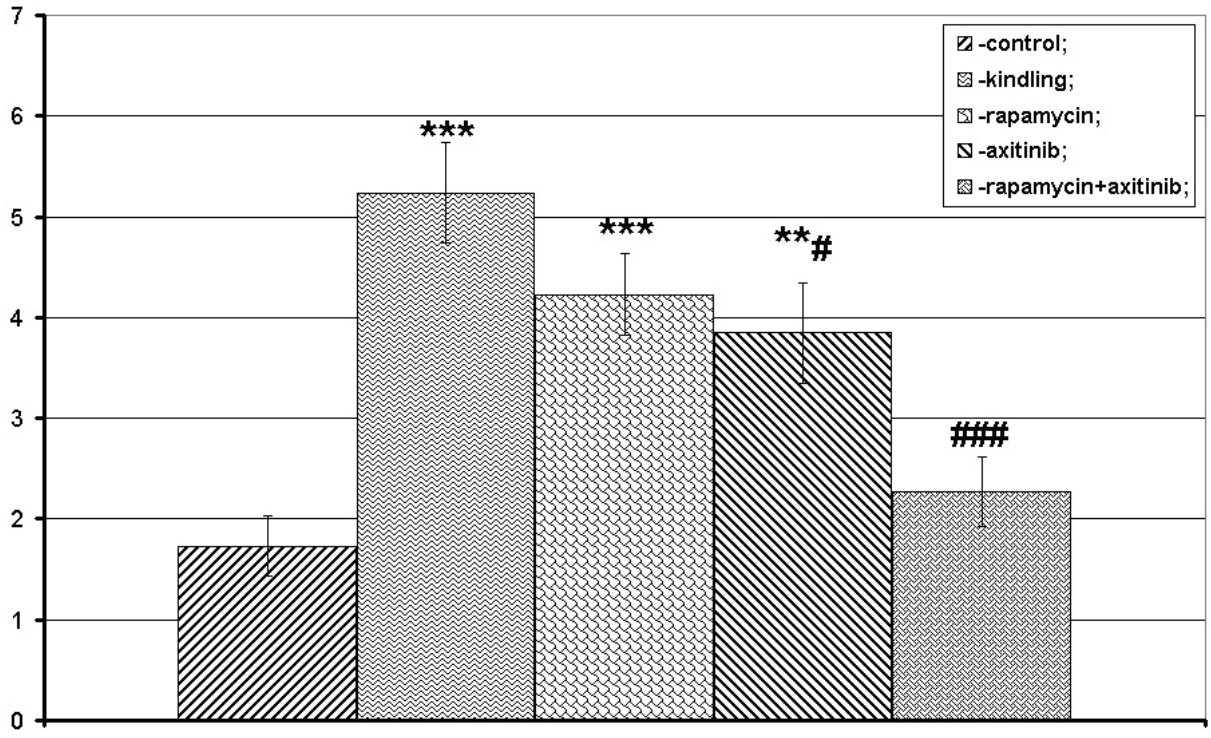

Fig. 7. Effects of treatment with rapamycin and axitinib upon NF-kB expression in the hippocampus of kindled

Histogram plot showing the immunoreactivity intensity of the antibodies analyzed. The data average is expressed $M\pm$SEM. One-way ANOVA analysis of variance and Tukey's multiple comparison tests was applied (** – $p<0.01$; *** – $p<0.001$ vs. control; # – $p<0.05$; ### – $p<0.001$ vs. kindling). Abscissa- groups of observation, ordinate – relative units of colour intensity.

Rapamycin caused a reduction by 19.3 % ($p>0.05$), while treatment with axitinib – by 26.5 % ($p<0.05$) when compared with kindled rats. Both were significantly greater than in control animals. Combined treatment with rapamycin and axitinib caused NF-kB reduction by 56.7 % compared with the kindled rats ($p<0.001$) (Fig. 7).

5. Discussion

Hence, gained data showed that the level of expression of HIF-1α, TNF-α, and NF-kB increased in the hippocampus of rats kindled with PTZ administrations. Such a result is in correspondence with data on the prominent role played by mechanisms of neuroimmune deterioration in the pathogenesis of PTZ–kindling [7, 15]. Besides, delivered data favours a net reduction of HIF-1α, TNF-α, and NF-kB expression in the hippocampal tissue of PTZ-kindled rats caused by treatment with rapamycin and axitinib. Such effect was most pronounced under the combined administration of investigated drugs and developed along with the protection of generalized seizure attacks induced with a testing dosage of PTZ. It should stress that antiseizure protection was also of synergy type as separately administrated drugs failed to demonstrate it.

The actual data on the reduction of neuroinflammatory markers expression in the hippocampal tissue of kindled rats are the first in a chain-like mechanism responsible for synergy manifestations, including antioxidant effects caused by rapamycin and axitinib.

The possible neuropharmacological mechanisms of action of the rapamycin and axitinib synergy should note. Thus, rapamycin reduces the production of HIF-1α [20, 21] and abolishes insulin-induced HIF-1 activation in retinal pigment epithelial cells [22]. Hence, mTOR upstream is considered a way of HIF-1α activation and HIF-1-dependent gene expression [20]. Besides, HIF-mediated transcription of angiogenic factors [21] causes vascularization-targeted mTOR signalling [23]. It should stress that tyrosine kinase inhibitors decrease VEGF expression by hypoxia-inducible factor (HIF)-1-independent and HIF-1-dependent mechanisms [24, 25]. Also, we have shown that axitinib suppressed neoangiogenesis in PTZ–kindled brain [26]. Such an effect was observed in parallel with the prevention of generalized seizure attacks [26].

Rapamycin is an autophagy activator, and its anti-inflammatory action is explained by the inhibition of the NF-κB proinflammatory signalling pathway [27, 28]. Also, tyrosine kinase inhibitor (imatinib) has also been recognized as a potent inhibitor of NF-κB signalling and inflammation in vivo and in vitro [29, 30].

Proinflammatory cytokines also might be the target for mTOR inhibitory influences and inhibition of tyrosine kinase. The suppression of TNF-α release [31], as well as destabilization of TNF-α mRNA [32], are in favour of additional potential for NF-kB blockade caused by rapamycin. Similar relationships were observed in glucose-induced inflammation [33]. Also, tyrosine kinase inhibitor (imatinib) suppresses TNF-α production in vitro and prevents TNF-α-dependent neuropathic pain syndrome [34]. In obese mice, imatinib reduced TNFα-gene expression in peritoneal and liver macrophages

and systemic lipid levels [35]. Neuropathic pain might be lessened with mTOR blockers by affecting neuropeptide synthesis and nociceptors regulation [36]. The last one is valid for a family of antinociceptive peptides, including kyotorphin, which demonstrates antiseizure activity [37]. Thus, the effects of mTOR and tyrosine kinase inhibitory effects upon inflammation are realized via the interruption of multiple intrinsic links between investigated modulators of signalling pathways.

Positive interaction between NF-kB and HIF [38, 39] on the one side and activation of NF-kB with TNF-α on the other [40, 41] points to the tight involvement of all investigated markers in the mechanisms of neuroinflammation development. Besides, numerous proinflammatory cytokines – depended pathways also include HIF-1α induction caused by TNFα [42, 43]. Hence, the prevalence of mutually positive interaction between investigated inflammatory markers is characteristic of their role in both neuroinflammation and chronic brain epileptisation [27].

Hence, such numerous target points for rapamycin and axitinib at divergent and powerful signalling pathways might serve for the realization of their seizure-suppressive synergy. It should stress that synergy is observed under the condition of the involvement of neuroinflammatory mechanisms as the main component of the pathogenesis of chronic brain epileptisation as it takes place in fully developed PTZ-induced kindled seizures.

Study limitation: This study did not determine the effects of combined treatment upon seizures with different pathogenesis, and the combination of different dosages of drugs was not investigated.

Further research prospects: Determining the antiseziure effectiveness on other models of epilepsy, dose dependence, and neurochemical, neurophysiological mechanism of action, as well as effects of coadministration with modern antiepileptic drugs.

6. Conclusions

1. PTZ-kindling resulted in an increase in the immunoreactivity of HIF-1α, TNF-α, and NF-kB in the hippocampal tissue.

2. Treatment with rapamycin (50 % from ED_{50}, ten days) reduced the expression of TNF-α, while treatment with axitinib (50 % from ED_{50}, ten days) reduced the level of expression of all investigated markers. In both cases, the level of markers exceeded the control value, and the treatment did not prevent generalized seizures.

3. Combined treatment with rapamycin and axitinib engendered prevention of generalized seizures and normalized the level of HIF-1α and NF-kB, which favours their action's synergy.

Conflict of interest

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this article.

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Data availability

The data used and analyzed can be obtained from the corresponding author under a reasonable request.

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