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AXITINIB DISPLAYS ANTISEIZURE ACTIVITY ON PENTYLENETETRAZOL – INDUCED KINDLING MODE

Olesya B. Poshyvak[1*], Oleh R. Pinyazhko[1,2], Leonid S. Godlevsky[3]

[1]Pharmacology Department, Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

[2]Department of Civilization Diseases and Regenerative Medicine, WSIiZ, Rzeszow, Poland

[3]Department of Biophysics, Informatics and Medical Devices, Odesa National Medical University,Odesa

*olesya.poshyvak@gmail.com

Abstract

This work aimed to investigate the effects of a specific inhibitor of vascular endothelial growth factor receptor generic axitinib upon pentylenetetrazol (PTZ) - induced kindled and acute convulsions and to compare axitinib effects with diazepam.

Wistar rats kindled with PTZ (30.0 mg/kg, intraperitoneally) during three weeks up to a stage of generalized convulsions stage 5 were observed. The assessment of seizures was performed immediately after completing the kindling procedure, after PTZ-free two weeks, and on acute PTZ-induced convulsions. Axitinib was administered perorally with PTZ injection 60 min later. Diazepam was administered intraperitoneally followed with PTZ in 30 min.

Axitinib prevented generalized convulsions in 5 out of 8 kindled rats in a dose of 2.5 mg/kg, and the pronouncement of effect was comparable with such one caused by diazepam in a dose of 0.1 mg/kg. Axitinib in the dose of 10.0 mg/kg completely protected generalized convulsions. A significant reduction of seizure severity was observed when axitinib (2.5 and 10.0 mg/kg) was administered to kindled rats after two weeks free from PTZ, while diazepam caused the suppression of convulsions only in the highest dose of 1.0 mg/kg. Acute PTZ seizures were suppressed by diazepam in both doses, while axitinib was not effective. Axitinib reduced the density of neomicrovessels in the frontal cortex by 47.0% in early kindling and 43.9% in the postponed period.

The effectiveness of axitinib on the kindling model of epilepsy might be explained by preventing neoangiogenesis as a valid mechanism of chronic brain epileptization development.

Keywords: *tyrosine-kinase inhibitors, anticonvulsants, benzodiazepines, chemical kindling, neoangiogenesis*

Introduction

The pathogenesis of epileptic syndrome develops along with the breaking down blood-brain barrier (BBB) function [1, 2]. Among others, remodeling of blood microcirculation based on angiogenesis makes the first-line contribution to increasing BBB's permeability [3]. In newly developed capillaries, the absence of pericytes is responsible for such increased leakage of their walls [3]. In patients who suffered from chronic intractable temporal lobe epilepsy, significantly higher vessel density in different zones of hippocampi than such one in non-epileptic patients was shown, and a direct positive correlation between vessel density and frequency of seizures established as well [1].

Vascular endothelial growth factor (VEGF) is responsible for neoangiogenesis, and VEGF synthesis is promoted by tyrosine – kinase [4]. Antitumor drug activity was realized via inhibition of tyrosine (tyr)-kinase [5, 6], and their antiseizure effectiveness was proved as well [7, 8]. Thus, it was established that blocking of angiogenesis with repeated sunitinib – a drug that penetrates BBB, and inhibits tyr-kinase, prevented the development of seizures and hippocampal atrophy pilocarpine rat model of mesial temporal lobe epilepsy [9].

Exploring a chemical-genetic approach enabled a conclusion to be reached that inhibitors of tyr-kinase were regarded as a new avenue for epilepsy treatment [8]. Even though VEGF causes persistently inhibitory effects upon neuronal excitability and epileptic syndrome manifestations [10, 11, 12] suppression of VEGF production, the restoration BBB function is the most probable mechanism the antiseizure activity of tyr-kinase inhibitors [8].

To test the effectiveness of tyr-kinase inhibitors, which poorly penetrate BBB, those models of the epileptic syndrome should be explored, characterized by increased BBB permeability. It was shown that pentylenetetrazol (PTZ) induced the leakage of BBB on a timely basis [13], while a more stable effect was evident in PTZ kindling [14]. Preliminary results gained by [15] favored the suppression of the development of PTZ kindling under the condition of peroral tyr-kinase inhibitor axitinib administration.

Earlier the critical role of tyr-kinase in the development of amygdalar ES kindling has been established, and i.c.v. administration of antibodies to tyr-kinase receptor prevented kindling seizures appearance [8]. The deficit of fyn-tyrosine kinase, which critically contributes to the development of long-term potentiation in the hippocampus, resulted in attenuation of the ability to demonstrate a kindling type of seizure development [16]. In contrast, overexpression of fyn-tyrosine kinase accelerated amygdalar kindling in mice [17]. It was also shown that N-Methyl-d-Aspartate Receptor (NMDA), subunit 2B phosphorylation, is responsible for such acceleration, the leading role played by tropomyosin-related kinase B (TrkB) [17], but not by TrkC [18].

One of the most selective VEGF receptor tyrosine kinase inhibitors is axitinib, which engendered its activity against VEGFR-1, VEGFR-2, and VEGFR-3, and in contrast to first-generation inhibitors, axitinib has no substantial inhibitory effect on PDGFRs, B-Raf, c-Kit, and Flt-3 [5, 19]. Axitinib approved as an anti-angiogenic agent for treating renal cell carcinoma, and its potency in vitro is higher than that of the first-generation VEGFR inhibitors such as sunitinib and sorafenib [4, 5, 6].

The study aims at assessing the effects of axitinib upon PTZ-induced kindled convulsions and brain neoangiogenesis. Also, the additional aim comprises the investigations of postponed PTZ-kindled seizures, which are regarded as pharmacologically resistant [20, 21], and comparison with the action of diazepam, which is effective at PTZ-kindled model [22].

Methods

Experimental animals

Experiments were performed on 134 male Wistar rats with initial bodyweight 210-250 g. Animals were kept in standard conditions (constant temperature 23° C, relative humidity 60%, 12 hrs dark/light cycles, standard diet, and tap water were given ad libitum) and were acclimatized to laboratory conditions least seven days before experimentation. All experiments were carried in accordance to the National Institute 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) and approved by Odesa National Medical University Bioethics Committee (UBC) (approval No17 dated 19/03/2013) before the study.

Epilepsy models

Kindled seizures

Kindled seizures were induced, as described previously [21, 23]. PTZ ("Sigma Aldrich") was given intraperitoneally (i.p.) daily at a dose of 30.0 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 rearings and falling; and 5, repeated seizures as at stage 4 or lethal outcome as a result of a seizure fit. As a response to both 20^{th} and 21^{t} PTZ injections, those rats, which failed into generalized fits, were taken for further observations and screening effects of compounds. Some animals with seizures of stages 4 and 5 were left untreated after the completion of kindling for two weeks [21]. Fourteen days after the last kindling trial (21^{t} injection of PTZ), animals were treated again with PTZ (30.0 mg/kg), and the effectiveness of axitinib and diazepam was evaluated.

Acute seizures

PTZ was administered intraperitoneally in suprathreshold dose, 60.0 mg/kg, to induce seizures, which corresponded with their severity to kindled stages 4 and 5 seizures. Animals were observed in individual Plexiglas boxes for 60 min after PTZ injection. The following scale evaluated convulsions; 0, no symptoms of seizures; 1, facial and body tremors, myoclonic jerks; 2, clonic convulsions of the whole body; 3, clonic – tonic seizures with flexion of hindlimbs; 4, clonic – tonic seizures with the extension of hindlimbs or death from seizures.

Histological investigations

After euthanasia brain was removed and fixed in 12% formalin solution on phosphate buffer (pH=7.0-7.2); according to the traditional scheme of an alcohol battery, dehydration was carried out of ascending concentration from 30° up to the absolute. Paraffin-celloidin sections were obtained by using a microtome. The general morphology of the experimental animals' frontal cortex tissue was studied by hematoxylin and eosin staining and silver impregnation. Light microscopy (Olympus C-4100 Zoom) was performed at 40 x, 100 x 200 magnification, and a digital photo (Digital camera DCM 130E, 1.3 Mpixels). The density of microvessels was determined for the total square of 2500 (μm^2). Verification of newly created microvessels was performed on the basis of the next criteria: 1) the presence of rows of actively involved in mitosis endothelial cells (Fig.1) the presence of "buds" of newly created microvessel as an initial stage of microvessel branching (Fig. 2).

Investigated compounds administration

In accordance with the design of investigations (Table 1), axitinib ("Sigma Aldrich") was administered in doses of 2.5 – 10.0 mg/kg, per orally (p.o.), and diazepam ("Calmpose", "Ranbaxy Diagnostics," India) was injected in doses of 0.1 – 1.0 mg/kg, i.p. Both compounds were dissolved in 5.0% methylcellulose (Methocel, "Sigma Aldrich"). Axitinib was administered 60 min before PTZ during the last ten days of kindled PTZ injections, and seizure severity was estimated after 21^{t} PTZ injection. Groups purposed for postponed kindled seizures investigations were treated with axitinib during the second week free from PTZ, and a test with PTZ was performed after the seventh axitinib administration. Diazepam was administered in 30 min before 21^{t} injection of PTZ for early kindled rats and at 14^{th} day free from PTZ in postponed kindled seizures measurement. Acute seizures were observed after ten daily administrations of axitinib (2.5 and 10.0 mg/kg, p.o.), and PTZ was administered in 60 min after the last (10^{th}) axitinib administration, while diazepam was administered in 30 min before testing the usage of PTZ. Control animals were treated with Methocel only.

Statistical analysis

Values were compared using one-way ANOVA and Newman-Keuls test for the density of microvessels; Kruskal-Wallis followed with a post hoc test for seizure severity. The density of microvessels was presented as a mean value (M) ± sigma deviation (SD). P values <0.05 were considered significant. Only observations falling between median ± 3.0 SD of the sample were included in the dataset to avoid outliers' influence.

Results

Kindled seizures

Behavioral characteristics of the seizures in kindled rats

Repeated i.p. administration of PTZ (30.0 mg/kg) resulted in the progressive development of seizure manifestations, starting from the third to sixth injection. At the moment of completion of kindling – after the 21^{t} PTZ administration, the prevalent number of rats (two thirds) demonstrated generalized clonic – tonic fits. Those rats with generalized seizures were used for further investigations.

Effects of axitinib and diazepam

P.o. administration of axitinib in doses of 2.5 and 10.0 mg/kg resulted in a dose-dependent decrease in the severity of kindled seizures. Statistical significance was achieved at a dosage of 2.5 mg/kg (Table 2), at which dose 5 of 8 animals protected against stage 4-5 seizures ($p<0.05$) as compared with controls. When administered in a 10.0 mg/kg dose, axitinib completely prevented the development of stage 4-5 seizures.
I.p. administration of diazepam to kindled animals (0.1 and 1.0 mg/kg) resulted in a dose-dependent seizure-protecting effect that was statistically significant for both two doses (Table 2), $p<0.05$ as compared with controls. Both doses prevented stage 4 seizures in the prevalent majority of rats in each group (7 out of 8 and 6 out of 7 correspondently).

Kindled seizures in two weeks free from PTZ (postponed kindled seizures)

Characteristics of seizures

I.p. administration of PTZ, after a 2-week postkindling interval, resulted in the development of stage 4-5 in all animals with a prevalence of maximal stage of seizures in 6 out of 7 rats (Table 3).

Effects of axitinib and diazepam

Administration of axitinib, in both investigated doses (2.5 and 10.0 mg/kg), to kindled rats, two weeks after kindling completion, prevented stage 4 seizures in 2 out of 7 and 2 out of 6 rats correspondently ($p<0.05$).
Diazepam given to animals with post-kindled PTZ seizures (Table 3) resulted in drastically significant antiepileptic effect only at the maximal dose (1.0 mg/kg), when prevented the stage 4 seizures in 5 out of 7 animals ($p<0.05$) (Table 3).

Acute convulsions

Characteristics of seizures

I.p. administration of PTZ in a dosage of 60.0 mg/kg previously treated with saline (p.o.) resulted in stage 3-4 seizures (Table 4).

Effects of axitinib and diazepam

Administration of axitinib (2.5 and 10.0 mg/kg) to rats in which seizures were elicited acutely by PTZ administration revealed the absence of effectiveness in both doses ($p>0.05$) (Table 4). Generalized seizures were observed in half of the experimental animals when a dose of 2.5 mg/kg axitinib was administered, and in a dose of 10.0 mg/kg, generalized seizures were not prevented in 5 out of 8 rats.

Diazepam given to animals with acute PTZ-induced seizures resulted in a statistically significant antiepileptic effect in a dose of 0.1 mg/kg when only 2 out of 10 rats demonstrated generalized seizures ($p<0.05$). Diazepam administered in a dosage of 1.0 mg/kg completely prevented generalized seizures ($p<0.05$) (Table 4).

Histological data

Gained data revealed that the density of newly created microvessels in the frontal cortex was 21.72 ± 4.56 per 2500 μm^2 and exceeded such one in the control rats by three times ($p<0.05$) (Fig. 3). Significant differences were maintained in the postponed period – two months free from PTZ administrations when microvessels' density exceeded control data by 2.72 times ($p<0.05$). Axitinib treatment of kindled rats caused the reduction of vessel density by 47.0% in early kindling ($p<0.05$) and by 43.9% in postponed period of kindling ($p<0.05$) when compared with the corresponded periods of untreated kindled rats (Fig. 3).

Discussion

Hence, gained data revealed that axitinib caused anticonvulsive action on the PTZ - kindled seizures in rats, which pronounced the prevention of generalized clonic-tonic fits. This effect was also noted in the period of renovation of seizures, after two-weeks free from PTZ injections, but was absent on the model of acute generalized seizures induced by PTZ in a dosage of 60.0 mg/kg.

It should stress that axitinib poorly crosses BBB [24] and BBB is poorly penetrable for other inhibitors of tyr-kinase, depending upon a constellation of transport proteins P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) [25]. That is why gained data showing the effectiveness of axitinib used in relatively low doses upon kindled seizures favor increasing BBB permeability due to PTZ kindling establishment.

Such BBB deteriorations might be connected with different mechanisms of PTZ kindling development. Thus, the appearance of BBB-impermeable contrast agent in the parenchyma of the diencephalon, hippocampus, and cerebral cortex, observed in 15 min after PTZ-induced general seizures in mice, was explained by increased NO production and induction BBB failure [13]. In turn, NO hyper production is induced by increased glutamatergic activity and activates NMDA receptor-coupled neuronal NO synthase. The role played by NO in the regulation of BBB permeability was also supported by other data [26, 27]. It should note that such a chain of events observed under both acute and chronic (kindled) PTZ induced convulsions. That is why it might not be of principal importance for our data explanation, as axitinib did not affect acute PTZ convulsions.

As another mechanism of BBB leakage in kindled rats, angiogenesis is much longer with the time of its manifestation and is not contributive to acute PTZ convulsions. Deregulated angiogenesis could also be beneficial in mitigating seizures, and pharmacological restoring BBB permeability is effective for seizure prevention, especially in combination with antiepileptic drugs [3].

Gained data favored for the increased neoangiogenesis in the frontal cortex of kindled rats. Such an effect was observed both at early and postponed PTZ-induced kindling. This fact corresponds with the data on the pathogenetic significance of neoangiogenesis for chronic epilepsy development [28, 29]. The net prevention of neoangiogenesis caused by axitinib treatment stressed the microvessels' creation's role as significant for heightening brain seizure susceptibility for PTZ – kindling.

Hence, it might assume that, in the course of PTZ kindling, the pronouncement of BBB deterioration progressively increased in parallel to assumed angiogenesis, which explains the effectiveness of axitinib. Such a possibility was supported by [14], in kindled rats, with cortical dysplasia. The authors observed a more pronounced increase of BBB permeability after convulsive dosage of PTZ in animals with kindling syndrome. It should note that VEGF – induced angiogenesis includes NO-depended component as far as VEGF realizes its angiogenic potency, along with reciprocal changes in NO production system [30, 31].

Remarkably, revealing of antiseizure action of tyr-kinase inhibitors needed prolonged administrations [8]. Meanwhile, short-term anticonvulsive

effects, observed after a single administration of axitinib, should not be excluded as well. Thus, an immediate effect of VEGF upon the excitability of the neuronal membrane is known [32, 33]. Hence, direct action of VEGF upon cultured hippocampal neurons results in the net decreasing of Na + channel excitability of hippocampal neurons by negatively shifting the voltage-dependence of steady-state inactivation and slowing down the recovery from inactivation [32]. The effect of VEGF on Na(+) channel steady-state inactivation inhibited by the specific VEGF Flk-1 receptor antagonist SU1498. Besides, local application of VEGF can acutely inhibit I(K) outward delayed-rectifier potassium current in isolated hippocampal neurons from 14-day-old rat brains [33]. For lestaurtinib (CEP-701), which can also diminish TrkB activity, the effectiveness against kainic acid seizures after acute administration to neonates was shown [34]. Hence, tyr-kinase inhibitors can induce rapidly precipitated antiseizure effects, which depend on the functional link of sodium channels with Fyn, and the ability of tyr-kinase inhibitors to modulate Na(V)1.2 channels by tyrosine phosphorylation [35].

Diazepam demonstrated antiseizure activity, both on kindled and acute seizures, induced with PTZ [21, 36, 37]. The pronouncement of the diazepam's antiseizure activity, in the dose of 0.1 mg/kg, corresponded to axitinib's antiseizure efficacy in a 2.5 mg/kg dose upon kindled seizures.

Testing of diazepam upon postponed kindled seizures revealed significant anticonvulsive activity only in the highest dosage (1.0 mg/kg), which might be in favor of the increased resistance of postponed seizures to the action of antiepileptic drugs [20, 21, 38]. The resultant decreasing of the diazepam effects might connect with the deterioration of benzodiazepine receptors, which is characteristic of PTZ kindling development [39]. Meanwhile, axitinib was still effective in both investigated dosages (2.5 and 10.0 mg/kg). The absence of decreasing of axitinib antiseizure effectiveness might be explained by persistent (two weeks) maintenance of morphological microvessels deteriorations, underlying increased BBB permeability, as a result of angiogenesis, and also by the stable role played by tyr-kinase in the regulation of seizure susceptibility of neurons in the kindling model [7, 15].

Interestingly, the expression of VEGF receptors is observed in pharmacologically resistant temporal epilepsy [40]. Hence, gained data on axitinib's antiseizure effectiveness in postponed kindled seizures favors the leading role of neoangiogenesis in chronic epileptogenesis precipitation.

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Table 1.

Design of investigations

N	Groups	Treatment	Seizure testing
1	Control for histological investigations (n=8)	I.p.administration of 0.9% NaCl solution	No
Kindling development			
2	Control to axitinib (n=8)	PTZ (30.0 mg/kg, i.p.) during 11 days + PTZ (30.0 mg/kg, i.p.) during 10 days after methocel (p.o.)	PTZ (30.0 mg/kg, i.p.) in 60 min after last (10^{th}) methocel
3	Axitinib (n=14)	PTZ (30.0 mg/kg, i.p.) during 11 days+ PTZ (30.0 mg/kg, i.p.) during 10 days after axitinib (2.5 and 10.0 mg/kg, p.o.)	PTZ (30.0 mg/kg, i.p.) in 60 min after last (10^{th}) axitinib
4	Control to diazepam (n=7)	PTZ (30.0 mg/kg, i.p.) during 21 days, and methocel (i.p.)	PTZ (30.0 mg/kg, i.p.) in 30.0 min after methocel
5	Diazepam (n=15)	PTZ(30.0 mg/kg, i.p.) during 21 days, and diazepam (0.1 and 1.0 mg/kg, i.p.)	PTZ (30.0 mg/kg, i.p.),in 30.0 min after diazepam
Postponed kindled seizures			
6	Control to axitinib (n=7)	Two weeks free from PTZ, and last 7 days of methocel (p.o.)	PTZ (30.0 mg/kg, i.p.), in 60 min after last (7^{th}) methocel
7	Axitinib (n=13)	Two weeks free from PTZ, and last 7 days of axitinb (2,5 and 10.0 mg/kg, p.o.)	PTZ (30.0 mg/kg, i.p.), in 60 min after last (7^{th}) axitinib
8	Control to diazepam (n=7)	Two weeks free from PTZ, and methocel (i.p.)	PTZ (30.0 mg/kg, i.p.), in 30 min after methocel
9	Diazepam (n=15)	Two weeks free from PTZ, and diazepam (1.0 and 1.0 mg/kg, i.p.)	PTZ (30.0 mg/kg, i.p.) in 30 min after diazepam
Acute seizures			
10	Control to axitinib (n=8)	Ten days of methocel (p.o.)	PTZ (60.0 mg/kg, i.p.) in 60 min after 10^{th} methocel
11	Axitinib (10.0 mg/kg, p.o.) (n=6)	Ten days of axitinib (10.0 mg/kg, p.o.)	PTZ (60.0 mg/kg, i.p.) in 60 min after 10^{th} axitinib
12	Control to diazepam (n=8)	Methocel (i.p.)	PTZ (60.0 mg/kg, i.p.) in 30 min after methocel
13	Diazepam (n=18)	Diazepam (1.0 and 1.0 mg/kg, i.p.)	PTZ (60.0 mg/kg, i.p.) in 60 min after diazepam

Table 2.

Effect of axitinib and diazepam upon 30.0 mg/kg i.p. PTZ-induced kindling

Compounds injected	No. of rats	No/of rats with convulsions of stage						P-Value vs control
		0	1	2	3	4	5	
Control to axitinib	8	0	0	0	0	5	3	
Axitinib (mg/kg, p.o.)								
2.5	8	0	0	3	2	2	1	P=0.023
10.0	6	0	1	2	3	0	0	P=0.001
Control to diazepam	7	0	0	0	0	2	5	
Diazepam (mg/kg, i.p.)								
0.1	8	0	0	4	3	1	0	P=0.001
1.0	7	2	1	3	0	1	0	P=0.002

Notes: statistics derived by Kruscall-Wallis test

Table 3.

Effects of axitinib and diazepam on postponed kindled seizures

Compounds injected	No. of rats	No/of rats with convulsions of stage						P-Value vs control
		0	1	2	3	4	5	
Control to axitinib	7	0	0	0	0	1	6	
Axitinib (mg/kg, p.o.)								
2.5	7	0	0	0	2	3	2	P=0.031
10.0	6	0	0	1	1	3	1	P=0.014
Control to diazepam	7	0	0	0	0	1	6	
Diazepam (mg/kg, i.p.)								
0.1	8	0	0	0	2	3	3	P=0.055
1.0	7	0	1	1	3	1	1	P=0.006

Notes: statistics derived by Kruscall-Wallis test

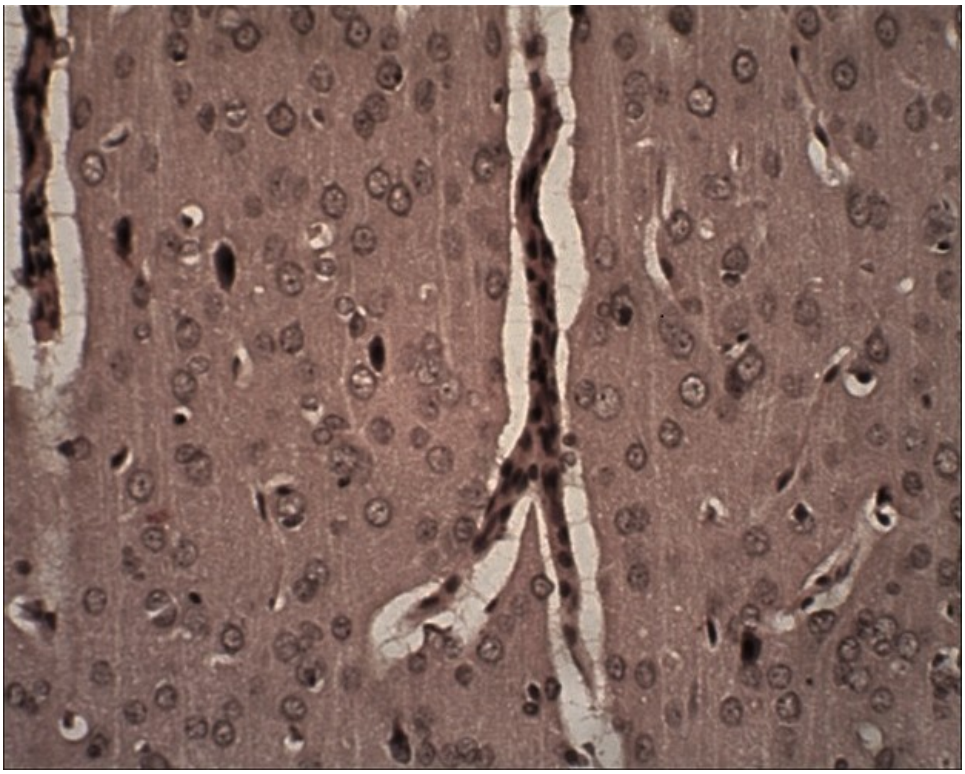
Table 4.

Effect of axitinib and diazepam upon acute PTZ induced convulsions

	No. of rats	No. of rats with convulsions of stage					p-Value vs control
		0	1	2	3	4	
Control to axitinib	8	0	0	0	2	6	
Axitinib (mg/kg, p.o.)							
10.0	6	0	0	0	3	3	P=0.352
Control to diazepam	8	0	0	0	3	5	
Diazepam (mg/kg, i.p.)							
0.1	10	0	0	3	5	2	P=0.038
1.0	8	0	2	4	2	0	P=0.002

Notes: statistics derived by Kruscall-Wallis test

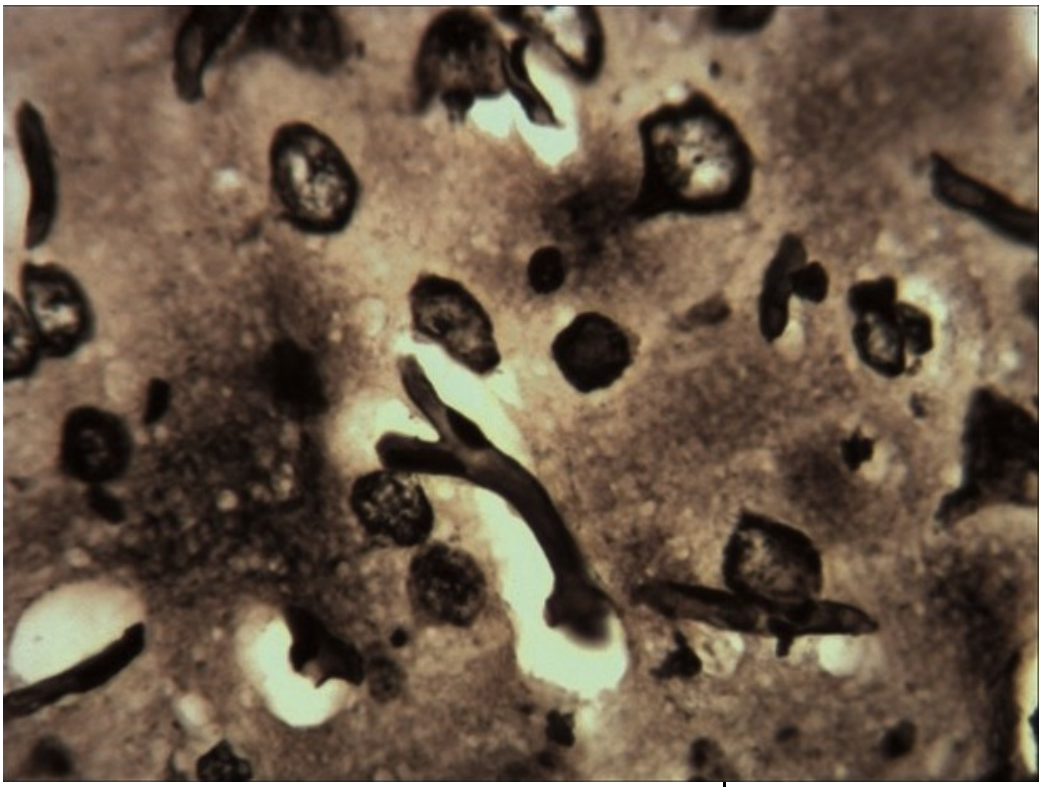
Figure 1.



Creation of the vessel on the basis in the brain cortex. Hematoxylin and eosin, Magnification x 200.

Notes: the number of active endothelial cells is defined. A particular capillary wall composes a "branch" of the vessel, which contains endothelial cell nuclei involved in mitosis (angioblasts). Such cells compose the rows of cells with branches – newly created vessels.

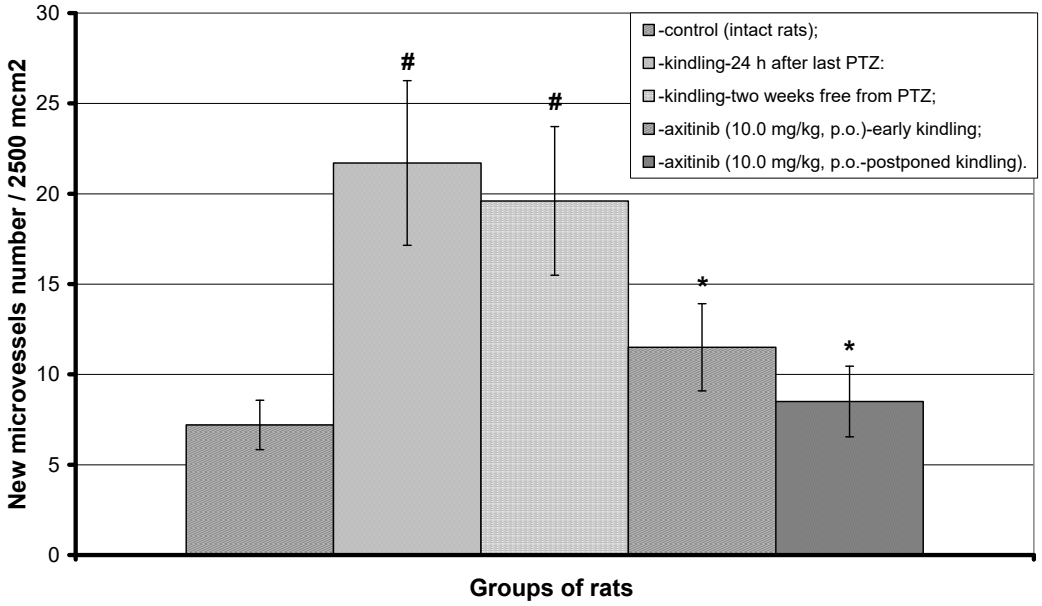
Figure 2.



The frontal cortex of the kindled rat. Magnification x 400. Staining – silver impregnation.
Notes: the vessel's "bud" is defined at the stage of the beginning of bifurcation.

In case of doubts with mentioned criteria identification, greater magnification (x400) on silver impregnate slices were used (Fig.2).

Figure 3.



The density of microvessels in the frontal cortex of kindled rats treated with axitinib

Notes: results presented as M±SD. #-P<0.05 compared with the control group (intact rats treated with 0.9% solution of NaCl); *-P<0.05- compared with corresponded earlier and postponed kindled seizures.

ANOVA+ Newman – Keuls test was appropriate