

# Effect of digoxin, sodium valproate, and celecoxib on the cerebral cyclooxygenase pathway and neuron-specific enolase under the pentylenetetrazole-induced kindling in mice

## Vliv digoxinu, valproátu sodného a celecoxibu na mozkovou cyklooxygenázovou dráhu a neuron-specifickou enolázu při pentylenetetrazolem podnícené záchvaty u myší

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### Summary

Neuroinflammation plays an important role in the pathogenesis of epilepsy, so it is necessary to clarify the influence of standard antiepileptic drugs as well as adjuvant agents (e.g., cardiac glycoside digoxin, which previously showed a clear anticonvulsant potential) on cyclooxygenase pathway and neuron-specific enolase under the conditions of chronic epileptogenesis. The aim of the article is to determine the effect of digoxin, sodium valproate, and celecoxib *per se*, as well as the combination of digoxin with sodium valproate on the content of cyclooxygenase 1 and

2 types, prostaglandins  $E_2$ ,  $F_{2\alpha}$ ,  $I_2$ , thromboxane  $B_2$ , 8-isoprostane and neuron-specific enolase in the brain of mice in the pentylenetetrazole-induced kindling model. It was found that only the combination of sodium valproate with digoxin provides a complete protective effect (absence of seizures) and shows the clearest influence on neuroinflammation markers and neuronal damage than monotherapy with each of these drugs and celecoxib, which appeared to be an ineffective anticonvulsant. The obtained results indicate that digoxin is a promising adjuvant drug to classical antiepileptic drugs (mostly sodium valproate) in epilepsy treatment.

**Key words:** pentylenetetrazole-induced kindling • digoxin • valproate • celecoxib • cyclooxygenase • neuron-specific enolase

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### Souhrn

Neurozánět hraje důležitou roli v patogenezi epilepsie, a proto je nutné objasnit vliv klasických antiepileptik i adjuvantních látek (např. srdečního glykosidu digoxinu, který již dříve vykazoval jasný antikonvulzivní potenciál) na dráhu cyklooxygenázy a neuron-specifické enolázy v podmínkách chronické epileptogeneze. Cílem článku bylo objasnit vliv digoxinu, natrium-valproátu a celecoxibu samostatně, ale i kombinace digoxinu s natrium-valproátem na obsah cyklooxygenázy 1. a 2. typu, prostaglandinů  $E_2$ ,  $F_{2\alpha}$ ,  $I_2$ , tromboxanu  $B_2$ , 8-isoprostanu a neuron-specifické enolázy v mozku myší na modelu pentylenetetrazolem podnícených záchvatů. Bylo zjištěno, že pouze kombinace natrium-valproátu s digoxinem poskytuje úplný ochranný účinek (absence záchvatů) a vykazuje nejzřetelnější vliv na markery neurozánětu a poškození neuronů ve srovnání s monoterapií každým z těchto léčiv a cele-

koxibem, který se ukázal jako neúčinné antikonvulzivum. Získané výsledky naznačují, že digoxin je slibným adjuvantním lékem ke klasickým antiepileptikům (především natrium-valproátu) v léčbě epilepsie. **Klíčová slova:** pentylenetetrazolem podněcené záchvaty • digoxin • natrium-valproát • celecoxib • cyklooxygenáza • neuron-specifická enoláza

## Introduction

According to the World Health Organization, the prevalence of epilepsy is extremely high – the incidence in the population is about 0.5–1%<sup>1</sup>. In 30 % of cases, it is impossible to achieve seizure control with standard treatments<sup>2,3</sup>, which significantly affects patients' quality of life and countries' economic losses. It determines the urgency of the search for new targets of influence on epileptogenesis as well as the discovery of new combinations of anticonvulsant drugs with other medicines.

In previous studies, we found the presence of a pronounced anticonvulsant effect of digoxin *per se*, as well as its potentiating effect on classical commonly used antiepileptic drugs (AEDs) in acute primarily generalized models of seizure induced by pentylenetetrazole (PTZ), picrotoxin, thiosemicarbazide, strychnine, camphor and maximal electroshock in mice. It has been verified that low-dose digoxin clearly increases the antiepileptic potential of sodium valproate, carbamazepine, lamotrigine, levetiracetam, topiramate, clonazepam, and phenobarbital, supplying a pronounced pharmacological effect of their sub-effective doses even under incomplete seizure control – the equivalent of drug-resistant epilepsy<sup>4–7</sup>.

As for the multifaceted epileptogenesis, it becomes even more critical when researching a new potential anticonvulsant medicine to investigate its effect on various links of epilepsy pathways. It is known that neuroinflammation plays an important role in the pathogenesis of brain diseases, including epilepsy<sup>8</sup>. Cyclooxygenase (COX) and prostaglandins (PG) are the main targets for reducing neuroinflammation. Both isoforms of COX, COX-1, and COX-2, are expressed in the CNS<sup>9</sup>. The COX-1 isoform is found mainly in microglia, while COX-2 is found mainly in neurons<sup>10</sup>. The presence of COX-2 is restricted to cell bodies and dendritic areas of neurons in the hippocampus, cerebral cortex, and amygdala<sup>11</sup>. The expression of COX-1 in the brain increases with aging, which indicates not only the homeostatic role of this enzyme<sup>12</sup>. In addition, there are studies indicating the constitutive expression of the COX-2 isoform in the brain<sup>13</sup> and its participation in synaptic plasticity<sup>14</sup>. The fact that the COX-2 enzyme is expressed throughout the CNS and can be increased under the influence of any pathophysiological stimuli determines the researchers' interest in studying its role in seizure development.

Besides, one of the triggering factors of neuroinflammation is oxidative stress, which initiates

the widespread death of neurons. Certain damage to neurons contributes to the formation of excitatory neurotransmitters, a deficiency of macroergic substances, the accumulation of calcium currents, nitric oxide, pro-inflammatory cytokines, and other cerebral substance systems, which collectively contribute to strengthening the lipoperoxidation process<sup>15, 16</sup>. The active radicals destabilize cell membranes' work and accelerate lipids' degradation, contributing to the excess influx of glutamate, Ca<sup>2+</sup> ions, and other altering components through micro-defects inside the cell<sup>17</sup>.

Isoprostanes are a group of prostaglandin isomers, while 8-isoprostane (8-iso-PGF<sub>2α</sub>) is an isomeric prostaglandin F<sub>2α</sub>. It is used as a marker of oxidative stress since the content of 8-isoprostane is directly proportional to the amount of free radicals<sup>16, 18</sup>. With a number of diseases, including neurological ones, the level of isoprostanes in biological samples increases significantly<sup>19, 20</sup>. An increase in the content of 8-isoprostane in the brain is observed under experimental seizure models, in particular, those induced by kainate<sup>21</sup>.

NSE (neuron-specific enolase) is an enzyme of the glycolytic chain, the activity of which increases significantly in case of neurons being damaged. Therefore, an increase in the level of NSE can be considered a highly specific and sensitive biochemical indicator reflecting neuronal damage<sup>22</sup>.

Therefore, the cerebral content of COX-1, COX-2, different prostaglandins, and 8-isoprostane and NSE can be considered targets for the mechanism of action of AEDs. Taking into account the previously studied antiepileptic activity of digoxin<sup>4–7</sup> as well as the recently discovered anti-inflammatory properties of cardiac glycosides<sup>23</sup>, we wanted to investigate its effect in combination with classical AED sodium valproate on the pro-inflammatory factors in the brain of mice under the pentylenetetrazole (PTZ)-induced kindling model.

The aim of the present study is to determine the effect of digoxin in a subcardiotonic dose *per se* and in combination with sodium valproate as well as celecoxib *per se* on the content of cyclooxygenase 1 and 2 types (COX-1, COX-2), PG E<sub>2</sub>, F<sub>2α</sub>, I<sub>2</sub>, thromboxane (TX) B<sub>2</sub>, NSE and 8-isoprostane in the mice brain under the condition of the PTZ-induced kindling. This animal model of kindling and increasing the state of epileptic readiness of the brain with long-term use of subthreshold doses of a convulsant approximates the natural conditions of long-term formation of the epileptic brain<sup>24</sup>. Therefore, this model is relevant when studying the effect of drugs on various links of epileptogenesis, including the neuroinflammatory pathway.

## Experimental part

### Materials

Medicines were used in the form of officinal drugs: digoxin (Digoxin-Zdorovie® 0.25mg/ml solution for injection, DNCLZ/Zdorovie, Ukraine); sodium valproate

(Depakine® 57.64 mg/ml syrup, Sanofi Aventis, France) and celecoxib (Celebrex® 200 mg capsules, Pfizer, USA).

The convulsive agent pentylenetetrazole (PTZ) was used as a substance (Sigma, USA).

Cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), prostaglandin F<sub>2</sub>α (PGF<sub>2α</sub>), prostacyclin (PGI<sub>2</sub>), thromboxane B<sub>2</sub> (TXB<sub>2</sub>), 8-isoprostane and neuron-specific enolase (NSE) species-specific enzyme immunoassay kits (MyBio-Source, USA) were used.

## Methods

### Study design

Control mice (groups 1 and 2 – vehicle control and positive control, respectively) received intragastrically and subcutaneously the solvent (water) in a 0.1 ml/10 g body weight for 16 days.

For kindling modeling, PTZ was used in a dose of 30 mg/kg intraperitoneally<sup>25)</sup> for 16 days.

All studied medicines were administered 30 min before PTZ.

Digoxin was used subcutaneously at a subcardiotonic dose of 0.8 mg/kg ( $1/10$  LD<sub>50</sub>)<sup>4–7)</sup>, which showed an anticonvulsant effect previously.

The classical AED sodium valproate was administered intragastrically at a dose of 150 mg/kg<sup>26)</sup>. This dose usually does not provide the maximum protective effect, which allows for detecting both the weakening and strengthening of the anticonvulsant activity<sup>4)</sup>.

Celecoxib was administered intragastrically at a dose of 4 mg/kg<sup>27)</sup>. Celecoxib as a reference drug was chosen based on the assumption that COX-2 (rather than COX-1) plays a key role in neuroinflammation. Additional use of non-selective inhibitors of COX-1/2 (in particular, acetylsalicylic acid and other agents) as reference drugs were not advisable for bioethical reasons, since the main aim of the study was to determine the neurochemical mechanisms of digoxin – both *per se* and in combination with classical AED, but not non-steroidal anti-inflammatory drugs.

Observation was carried out live daily, in the morning, by three identical researchers for all days. At the same time, the researchers were “blinded” as to which medicine (or combination) was administered to the experimental groups of animals before.

Observation of animals after PTZ injection lasted 1 hour. A latency period of the first seizures, number of days with convulsions, number of mice with seizures as well as seizure severity were observed. Seizure severity was evaluated using a modified Racine scale<sup>4, 28)</sup> in points where 0 means no seizures; 1 – ear and facial jerks, sporadic head nodding; 2 – clonus of one forelimb without upright position; 3 – bilateral forelimb clonus with/without rearing; 4 – bilateral fore- and hindlimb clonus and falling with/without rearing; 5 – tonic extension. Days with convulsions is calculated by the number of days where at least 1 mouse with convulsions in the experimental group

has been detected. This indicator, together with the daily number of mice with seizures, characterizes the degree of a protective effect of the studied drugs in the dynamics of kindling, taking into account even the most sensitive animals.

After PTZ-induced kindling, the brains of mice were examined by immunochemical methods. On the 16th day of the experiment, 1 hour after the last drug administration (in groups VC and PC – after solvent administration), animals were euthanized by dislocation of the cervical vertebrae<sup>25)</sup>. The brains of 6 animals from each group were used. The brain was immediately removed and frozen with liquid nitrogen, stored in a freezer at –70 °C. Before examination, the samples were homogenized with standard phosphate-buffered saline (buffer-to-material ratio is 10 : 1) at a temperature of –4 °C and thrice centrifugated at 6000 × g for 10 min.

### Experimental animals and grouping

The study was conducted on 56 outbred female albino mice weighing 20–24 g kept in a well-ventilated room on a standard vivarium diet (commercial laboratory rodent chow) with free access to food and water under controlled conditions (20–24 °C and 50% humidity with a 12 h light/dark cycle).

The animals were obtained from the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy (NUPh), Kharkiv, Ukraine.

The experiments were carried out in accordance with the principles and requirements of the EU Directive 2010/63/EU (2010) on the protection of animals used for scientific purposes and approved by the NUPh Bioethical Committee (Protocol No. 3, September 10, 2020).

- The animals were randomly divided into 6 groups:
- Group 1 (n=6): vehicle control (VC) – for neurochemical studies.
  - Group 2 (n = 10): positive control (PC), PTZ-induced kindling without treatment;
  - Group 3 (n = 10): PTZ-induced kindling + sodium valproate;
  - Group 4 (n = 10): PTZ-induced kindling + digoxin;
  - Group 5 (n = 10): PTZ-induced kindling + sodium valproate + digoxin;
  - Group 6 (n = 10): PTZ-induced kindling + celecoxib.

### Statistical Analysis

The results were expressed as mean ± standard error of the mean ( $M \pm m$ ). Statistical differences between groups were analyzed using a parametric one-way ANOVA test as well as a non-parametric Kruskal-Wallis test (for mean seizure severity score). Fisher's angular transformation was used for the results in the alternative form (% of mice with seizures, days with convulsions, highest seizure severity). The levels of statistical significance were considered as  $p < 0.05$ , 0.01, and 0.001.

**Results**

**Beneficial effects of digoxin and sodium valproate on the course of PTZ-induced kindling**

The results of the influence of studied drugs on the PTZ-induced kindling process are shown in Figures 1–2 and Table 1.

Repeated administration of PTZ at a sub-convulsive dose (30 mg/kg) every subsequent day (for 16 days in a row) in all groups (except the valproate + digoxin

group) led to the onset and gradual increase in the severity of seizures.

In the control, digoxin, and celecoxib groups, the latency period lasted three days. The first clonic seizures were observed in 1 mouse (10% of the number of animals in the group) on the 4th day of the experiment. In the sodium valproate group, the latency period made 6 days – the first paroxysms were registered in 1 mouse (10% of the number of animals in the group) on the 7th day of the convulsant administration (Fig. 1).

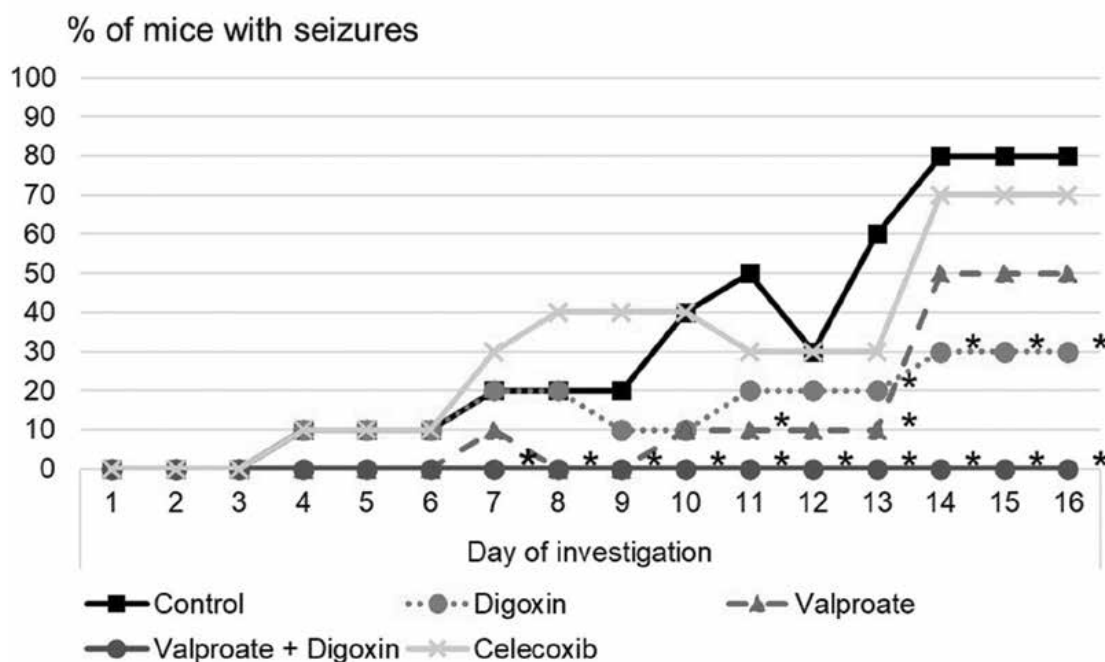


Fig. 1. Effects of digoxin, sodium valproate, their combination, and celecoxib on the course of pentylenetetrazole-induced kindling in mice

\* statistically significant difference ( $p < 0.05$ ) compared with control

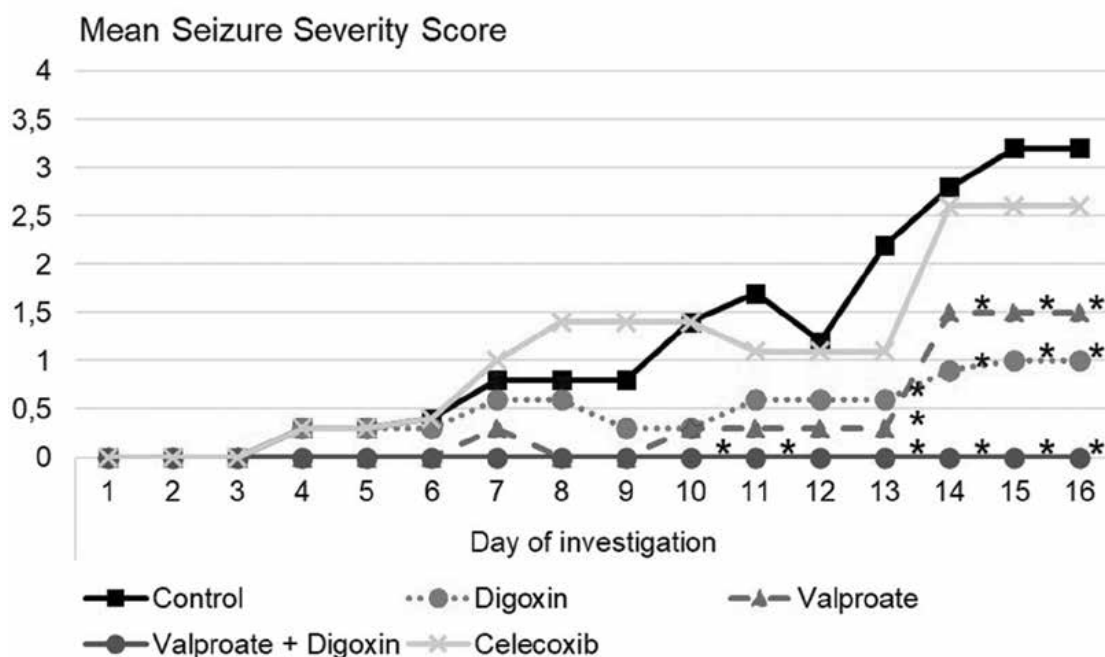


Fig. 2. Effects of digoxin, sodium valproate, their combination, and celecoxib on the seizure severity in the dynamics of pentylenetetrazole-induced kindling in mice

\* statistically significant differences ( $p < 0.05$ ) compared with control

Table 1. Days with convulsions and the highest seizure severity in the course of pentylenetetrazole-induced kindling in mice under digoxin, sodium valproate, their combination, and celecoxib

Experimental Group	Days with convulsions	Highest seizure severity
Control	13/16	4/5
Digoxin	13/16	4/5
Valproate	8/16 <sup>*#^</sup>	3/5
Valproate + Digoxin	0/16 <sup>*#§^</sup>	0/5 <sup>*#§^</sup>
Celecoxib	13/16	4/5

Statistically significant differences ( $p < 0.05$ ) compared with:

\* – control

# – digoxin

§ – valproate

^ – celecoxib

The total number of days marked by seizures in the control, digoxin, and celecoxib groups was 13, while under sodium valproate, the number of such days was 8 (Table 1), which was significantly lower compared to the three previous groups ( $p < 0.05$ ).

However, the combination of valproate and digoxin provided a complete protective effect: there were no seizures during all 16 days of observation, which is statistically significant compared to all other experimental groups ( $p < 0.05$ ). The same effect of the combination has been verified in relation to the severity of seizures – the combined use of digoxin with valproate statistically significantly ( $p < 0.05$ ) reduced the highest severity score on days 10, 11, and 13 compared to the control group as well as on days 14–16 – compared to other experimental groups (Table 1).

The number of animals with seizures gradually increased in the control, sodium valproate, digoxin, and celecoxib groups. It reached a maximum on the 14th day. It was, as we can see in Figure 1, 80 % in the control group, 50% on the background of sodium valproate, and 30% under the influence of digoxin ( $p < 0.05$  compared to the indicators of the control and celecoxib groups), 70% on against celecoxib. The absence of animals with convulsions under the valproate + digoxin combination reliably distinguished this group from all others ( $p < 0.05$ ).

The effect of digoxin, sodium valproate, and, especially, a combination of these drugs (but not celecoxib) is also manifested in a decrease in the progression of the seizure severity compared to control in the dynamics of PTZ-induced kindling. At the same time, the mean seizure severity score in the digoxin and valproate groups from days 13 to 16 was statistically significantly lower ( $p < 0.05$ ) than in the control and celecoxib groups (Fig. 2). The specificity of the course of the convulsive syndrome was that the clonic attack was not limited to one forelimb: in the case of onset from separate forelimb, the convulsions also covered the contralateral one. As can be seen from Table 1, the severity of seizures in the valproate group was limited to bilateral clonic convulsions of the forelimbs without loss of postural control (3 points).

In the control, digoxin, and celecoxib groups, the most severe seizures involved the hindlimbs and were accompanied by falling, corresponding to 4 points. The severe tonic extension (5 points), which usually results in the death of animals with seizure models, was not observed in any case.

Therefore, the protective effect of sodium valproate and digoxin on the PTZ-induced kindling model has certain differences. Sodium valproate doubles the latency period of the first attacks, reduces the number of days with seizures ( $p < 0.05$ ), and tends to increase the number of mice with convulsions by 1.6 times. Digoxin significantly reduces the number of animals with seizures by 2.7 times ( $p < 0.05$ ) but does not affect the latency period of convulsions and the number of days with seizures. However, digoxin potentiates the anticonvulsant activity of sodium valproate, which provides a full protective effect. In contrast to sodium valproate, digoxin, and their combination, celecoxib shows only a weak tendency toward anticonvulsant activity, consisting of only a slight reduction in the number of animals with seizures.

#### **Influence on cerebral cyclooxygenase pathway**

The results of the influence of studied drugs on the cyclooxygenase pathway indicators of the arachidonic acid cascade and 8-isoprostane are presented in Tables 2–3.

In the PC group, pronounced changes in the studied markers, which indicate the development of a neuroinflammatory reaction against the background of long-term PTZ administration, were observed. Compared with the indicators of the VC group, the content of COX-1 statistically significantly increased ( $p < 0.001$ ) by 12.81%, while the expression of COX-2 increased by 1.64 times ( $p < 0.001$ ) (Table 2), the content of  $\text{PGF}_{2\alpha}$  increased by 27.68% ( $p < 0.001$ ),  $\text{TXB}_2$  doubled ( $p < 0.001$ ), and the levels of  $\text{PGE}_2$  and  $\text{PGI}_2$  decreased by 42.33% and 49.76%, respectively ( $p < 0.001$ ) (Table 3). A significant increase in the content of 8-isoprostane by 2.75 times ( $p < 0.001$ ) (Table 3) also has been verified.

In the digoxin group, the level of COX-1 and COX-2 decreased by 11.99% and 59.19%, respectively

Table 2. The influence of digoxin, sodium valproate, and their combination, as well as celecoxib on the expression of cyclooxygenase-1 and 2 in the mice brain under the pentylenetetrazole-induced kindling model ( $M \pm m$ )

Indicators	Vehicle Control	Pentylenetetrazole-induced kindling				
		Positive Control	Digoxin	Valproate	Valproate + Digoxin	Celecoxib
COX-1, pg/g	794.23 ± 5.90	895.99 ± 9.45 ***	788.59 ± 5.96 ### <sup>SS</sup>	750.62 ± 5.62 *** <sup>###^</sup>	767.30 ± 5.77 *** <sup>o</sup>	787.22 ± 9.48 ###
COX-2, ng/g	136.18 ± 2.63	223.61 ± 7.09 ***	91.26 ± 1.37 *** <sup>###^^^</sup>	105.20 ± 4.76 *** <sup>###^^^</sup>	76.50 ± 1.40 *** <sup>###<sup>SS</sup>ooo^^^</sup>	210.29 ± 2.87 ***

VC – vehicle control, PC – positive control

Statistically significant differences:

\*  $p < 0.05$ ; \*\*\*  $p < 0.001$  – compared with VC

###  $p < 0.001$  – compared with PC

<sup>S</sup>  $p < 0.05$ ; <sup>SS</sup>  $p < 0.01$ ; <sup>SSS</sup>  $p < 0.001$  – compared with sodium valproate

<sup>o</sup>  $p < 0.05$ ; <sup>ooo</sup>  $p < 0.001$  – compared with digoxin

<sup>^</sup>  $p < 0.05$ ; <sup>^^^</sup>  $p < 0.001$  – compared with celecoxib

Table 3. The effect of digoxin in a subcardiotonic dose, sodium valproate, and their combination, as well as celecoxib, on the content of prostaglandins and 8-isoprostane in the mice brain under the pentylenetetrazole-induced kindling model ( $M \pm m$ )

Indicators	Vehicle Control	Pentylenetetrazole-induced kindling				
		Positive Control	Digoxin	Valproate	Valproate + Digoxin	Celecoxib
PGE <sub>2</sub> , pg/g	706.29 ± 4.93	407.30 ± 3.59 ***	543.19 ± 13.70 *** <sup>###<sup>SS</sup>^^^</sup>	658.45 ± 7.36 *** <sup>###^^^</sup>	725.12 ± 6.32 *** <sup>###<sup>SS</sup>ooo^^^</sup>	611.88 ± 4.06 *** <sup>###</sup>
PGF <sub>2α</sub> , pg/g	983.62 ± 12.22	1255.97 ± 20.56 ***	1035.04 ± 6.54 *** <sup>###<sup>SS</sup>^^^</sup>	1106.79 ± 4.99 *** <sup>###</sup>	974.53 ± 5.69 ### <sup>SS</sup> ooo^^^	1133.49 ± 18.62 *** <sup>###</sup>
PGI <sub>2</sub> , ng/g	6.13 ± 0.04	3.08 ± 0.07 ***	3.54 ± 0.03 *** <sup>###<sup>SS</sup>^^^</sup>	4.19 ± 0.02 *** <sup>###^^^</sup>	5.81 ± 0.07 *** <sup>###<sup>SS</sup>ooo^^^</sup>	2.91 ± 0.06 ***
TXB <sub>2</sub> , pg/g	132.76 ± 1.52	263.24 ± 1.43 ***	136.37 ± 2.86 ###^^^	142.49 ± 1.35 *** <sup>###^^^</sup>	100.95 ± 2.85 *** <sup>###<sup>SS</sup>ooo^^^</sup>	180.98 ± 2.62 *** <sup>###</sup>
8-Isoprostane, μmol/g	19.73 ± 0.21	54.35 ± 1.89 ***	22.98 ± 0.33 *** <sup>###<sup>SS</sup>^^^</sup>	32.20 ± 0.45 *** <sup>###^^^</sup>	18.92 ± 0.07 *** <sup>###<sup>SS</sup>ooo^^^</sup>	40.37 ± 0.63 *** <sup>###</sup>

VC – vehicle control, PC – positive control

Statistically significant differences:

\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  – compared with VC

## –  $p < 0.01$ ; ###  $p < 0.001$  – compared with PC

<sup>SSS</sup>  $p < 0.001$  – compared with sodium valproate

<sup>ooo</sup>  $p < 0.001$  – compared with digoxin

<sup>^^</sup>  $p < 0.01$ ; <sup>^^^</sup>  $p < 0.001$  – compared with celecoxib

( $p < 0.001$ ) (Table 2). It should be noted that the amount of COX-1 and COX-2 in the brain instead of measuring their activities has been determined based on the assumption of the nuclear impact of digoxin and its influence on enzymes' expression. The content of prostaglandins also changed significantly ( $p < 0.001$ ): PGE<sub>2</sub> increased by 33.36%, PGF<sub>2α</sub> decreased by 17.59%, PGI<sub>2</sub> increased by 14.94%, and TXB<sub>2</sub> decreased almost twice (Table 3). Reduction in the intensity of oxidative stress is evidenced by a decrease in the level of 8-isoprostane by 57.72% (Table 3).

Sodium valproate shows a statistically significant ( $p < 0.001$ ) influence on all investigated markers of the arachidonic acid cascade: the level of COX-1 decreased by 16.22%, COX-2 – by 52.95% (Table 2), PGE<sub>2</sub> content increased by 61.66%, PGF<sub>2α</sub> decreased by 11.88%, PGI<sub>2</sub> increased by 36.04%, and TXB<sub>2</sub> decreased by 45.87%

(Table 3). Sodium valproate also showed pronounced antioxidant and neurocytoprotective properties in the PTZ-kindled brain, significantly ( $p < 0.001$ ) reducing the level of 8-isoprostane by 40.75% (Table 3) compared to the PC group.

In the framework of combining digoxin with sodium valproate, the expression of inflammatory enzymes was suppressed (Table 2): the level of COX-1 decreased by 14.36%, and COX-2 – by 65.78%. The concentration of PGE<sub>2</sub> increased by 1.78 times ( $p < 0.001$ ), the content of PGF<sub>2α</sub> decreased by 22.41% ( $p < 0.001$ ), and PGI<sub>2</sub> increased by 1.89 times ( $p < 0.001$ ), while TXB<sub>2</sub> decreased by 2.61 times ( $p < 0.001$ ) (Table 3). The content of 8-isoprostane decreased by 2.87 times ( $p < 0.001$ ) (Table 3) compared with PC. Almost all studied markers came as close as possible to the indicators of the VC under the combination use. While comparing the

combination's and sodium valproate's effects *per se*, a significantly more pronounced effect on all markers, except COX-1, attracts attention. Almost all studied markers came as close as possible to the indicators of the VC under the combination use.

Despite lipophilic properties that allow the capability to penetrate through the blood-brain barrier and belonging to highly selective COX-2 inhibitors, celecoxib had almost no effect on the increased level of COX-2, which remained 1.54 times higher than VC ( $p < 0.001$ ). But the increased content of COX-1 under the influence of celecoxib decreases to the level of VC, which was statistically significantly different from the level of PC ( $p < 0.001$ ), the content of PGE<sub>2</sub> increased by 50.22% ( $p < 0.001$ ), PGF<sub>2α</sub> decreased by 9.75% ( $p < 0.01$ ), the level of PGI<sub>2</sub> almost did not change, and TXB<sub>2</sub> decreased by 31.25% ( $p < 0.001$ ) compared to the corresponding indicators of PC. The level of 8-isoprostane decreased by 25.72% ( $p < 0.001$ ) against PC, which indicates the moderate antioxidant properties of celecoxib, for which it was significantly ( $p < 0.001$ ) inferior to sodium valproate, digoxin, and especially their combination.

#### Influence on neuronal damage

The results of the influence of studied drugs on the content of NSE – an indicator of neuronal damage – are presented in Table 4.

Significant growth of NSE level by 17.46 times ( $p < 0.001$ ) compared with the VC group in the PC animals indicates the pronounced damage to neurons under the influence of PTZ. Neurocytoprotective properties of digoxin have been verified by a decrease in the cerebral content of NSE by 65.29% ( $p < 0.001$ ) compared with the PC group. Sodium valproate also significantly reduces the PTZ-induced growth of NSE level – by 50.60% ( $p < 0.001$ ). The combination of digoxin with sodium valproate shows the most significant protection against neuronal damage, decreasing NSE's content by 8.40 times ( $p < 0.001$ ) compared to PC – much closer to the

VC level than the drugs *per se*. Celecoxib practically did not show neurocytoprotective properties, as it reduced the cerebral content of NSE by only 3.20% compared with PC ( $p > 0.05$ ).

#### Discussion

According to the experimental data, the seizure syndrome on the chronic model of epilepsy (PTZ-induced kindling), celecoxib practically did not affect the course of the experimental seizure syndrome. However, on the model of acute PTZ-induced seizures, this highly selective COX-2 inhibitor exerts an anticonvulsant effect, which is obviously related to the anti-inflammatory properties of celecoxib and explained by a facilitatory role for the COX-2/PGE<sub>2</sub> pathway in the acute seizures, induced by PTZ<sup>27</sup>. In the case of PTZ-induced kindling, the effect of celecoxib was insufficient to reduce the severity of seizures. Instead, not only sodium valproate but also digoxin at a subcardiotonic dose significantly improve the course of the convulsive syndrome in this model of chronic epileptogenesis. At the same time, digoxin and sodium valproate, as can be seen from Figs. 1–2, statically significantly reduce the number of animals with convulsions and the severity of seizures (13–16 days). Also, digoxin significantly reduces the number of mice with seizures in the final phase of kindling (13–16 days), when this marker rapidly increases in the control group, while sodium valproate also prolongs the latency period of seizures by 2-fold, significantly reduces the total number of days with convulsions and prevents loss of postural control (Fig. 2, Table 1).

An important result of the experiment is the detection of synergism of the anticonvulsant effect of digoxin and valproate used together. This consists of the complete absence of seizures throughout the experiment. Literature data, which are confirmed by our results, show that this is largely due to the anti-inflammatory properties of digoxin.

Table 4. The effect of digoxin in a subcardiotonic dose, sodium valproate, and their combination, as well as celecoxib on the expression of neuron-specific enolase in the brain of mice under the pentylenetetrazole-induced kindling model ( $M \pm m$ )

Experimental Group	NSE, ng/g
Vehicle Control	4,04 ± 0,05
Positive Control	70,52 ± 0,96***
Digoxin	24,48 ± 1,01***###\$\$\$^^^
Valproate	34,84 ± 0,67***###^^^
Valproate + Digoxin	8,40 ± 0,35***###\$\$\$°°°^^^
Celecoxib	68,26 ± 0,49***

VC – vehicle control, PC – positive control

Statistically significant differences:

\*\*\*  $p < 0.001$  – compared with VC

###  $p < 0.001$  – compared with PC

\$\$\$  $p < 0.001$  – compared with sodium valproate

°°°  $p < 0.001$  – compared with digoxin

^^^  $p < 0.001$  – compared with celecoxib

Indeed, the last decade has seen the discovery of previously unknown properties of cardiac glycosides, in particular, their effect on inflammation<sup>23</sup>. It was found that digoxin provides its anti-inflammatory activity impairing the overproduction of pro-inflammatory cytokines<sup>29</sup>. The beneficial action of ouabain has been established on lipopolysaccharides-activated astrocytes, which indicates its effectiveness in neuroinflammation<sup>23, 30</sup>. On the other hand, proven anti-inflammatory properties, including under conditions of neuroinflammation, are inherent to classical AEDs – in particular, valproates<sup>31–33</sup>. However, how significant is the role of anti-inflammatory properties of cardiac glycosides and AEDs in the anticonvulsant activity? And also, how correlated are the anti-inflammatory and anticonvulsant activity of known anti-inflammatory drugs?

Experimental and clinical studies have shown that inflammatory molecules (cytokines, chemokines, and prostaglandins) are released by the brain and endotheliocytes of cerebral capillaries, affecting the excitability and functional state of neurons<sup>34</sup>. We investigated the effect of the cardiac glycoside digoxin in a subcardiotonic dose, classic AED sodium valproate, the selective COX-2 inhibitor celecoxib *per se*, and the combination of digoxin with sodium valproate on the cyclooxygenase pathway of the arachidonic acid cascade, the content of 8-isoprostane and NSE in the brain of mice under PTZ-induced kindling.

The course of PTZ-induced kindling varies significantly depending on the studied drugs. Under the influence of sodium valproate, the non-antiepileptic drug digoxin, and a combination of these agents, a distinct anticonvulsant effect was observed, while celecoxib, on the contrary, almost did not improve the course of seizures in mice. Thus, in the model of chronic epileptogenesis, the highly selective COX-2 inhibitor did not show efficacy as an anticonvulsant, and the high level of 8-isoprostane and NSE in the mice brain indicates the absence of a clear protective effect on oxidative stress and neuronal destruction under long-term exposure to PTZ. Thus, although celecoxib exerts a moderate anticonvulsant impact in an acute model of PTZ-induced seizures<sup>27, 35</sup>, its protective effect is insufficient under the model of chronic epileptogenesis.

It is known that both isoforms of COX can be induced in the brain under the influence of various pathophysiological factors: convulsions, craniocerebral trauma, or the process of epileptogenesis<sup>36</sup>. The results of the study on the kindling model prove that COX-1 and COX-2 are involved in the process of epileptogenesis: foamy and branched microglial cells expressing COX-1 increase, and the content of this enzyme progressively increases<sup>37</sup>. Under normal conditions, COX-2 is expressed at extremely low basal levels in the hippocampus of mice, but the level of COX-2 begins to increase 1 h after the induction of status epilepticus and reaches a maximum within 4–24 h, almost 60 times the basal level<sup>38</sup>. In our study, PTZ provides the

increase in the cerebral content of COX-2 more than 1.6 times, which was not observed against the background of the use of drugs with high anticonvulsant activity (sodium valproate, digoxin, and their combination), while the content of COX-1 increased slightly (by 1.13 times). Predominant inhibition of COX-2 by these drugs may indicate that with their long-term use, the risk of side effects due to inhibition of constitutive COX-1 will decrease.

Activation of COX-2 initiates the synthesis of all five types of prostanoids in the brain: PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2α</sub>, PGI<sub>2</sub>, and TXA<sub>2</sub>, which can activate a total of nine receptors associated with G protein-coupled receptors (GPCRs), which mediates multiple deleterious and beneficial effects after prolonged seizures<sup>39, 40</sup>. Experimental evidence suggests the role of PGE<sub>2</sub> as a key mediator in COX-2 signaling<sup>41</sup>. Moreover, administration of exogenous PGE<sub>2</sub>, but not PGD<sub>2</sub> or PGF<sub>2α</sub>, increases seizure frequency and amplitude of excitatory postsynaptic potentials<sup>42</sup>. In addition, PGE<sub>2</sub> increases glutamate release from astrocytes<sup>43</sup>, suggesting a role for PGE<sub>2</sub> in excitatory transmission in the brain. Recent experimental studies suggest that PGE<sub>2</sub> is an important mediator of inflammation in chronic seizure syndrome through the activation of four GPCRs, namely EP1-EP4, which regulate a wide range of physiological and pathological functions in the brain<sup>44</sup>. For example, the EP2 receptor is constitutively expressed in the CNS under normal conditions to regulate important physiological brain functions such as synaptic plasticity, cognition, and emotion<sup>45, 46</sup>.

In our study, in the PTZ-induced kindling model, PGE<sub>2</sub> expression in the PC group decreases by 42%, while in the seizure-treated groups, it increases and approaches the VC level, especially under the influence of the combination valproate + digoxin. It is probably due to COX-2 suppression and reduced neuronal damage due to prolonged exposure to the convulsant, as evidenced by a marked decrease in NSE levels.

PGF<sub>2α</sub> expression is known to increase epilepsy<sup>47, 48</sup>. Experimentally, PGF<sub>2α</sub> levels begin to increase in rat brains within 10 min of subcutaneous injection of kainic acid<sup>47</sup>, possibly by conversion from PGE<sub>2</sub> or PGD<sub>2</sub>. In an immunohistochemical study of the rat brain 30 min after kainic acid-induced seizures, PGF<sub>2α</sub> was detected in hippocampal CA3 neurons and dentate gyrus granule cells, suggesting that these populations of hippocampal neurons are the main source of PGF<sub>2α</sub> in the brain after rope seizure induction, again still, probably due to PGE<sub>2</sub> isomerization<sup>48</sup>. Taken together, these data demonstrate that PGF<sub>2α</sub> is rapidly synthesized in response to seizure induction and is involved in the acute phase of seizure activity. It can be assumed that the effect of the activation of PGF<sub>2α</sub> receptors on the survival of neurons and the intensity of seizures depends on the duration of the seizure syndrome, the functional characteristics of tissues, and signaling pathways involved in asynchronous excitation, etc. In the context of these data, the increase in the content



of  $\text{PGF}_{2\alpha}$  in the brain of animals of the PC group and the decrease in its level against the background of the protective effect of anticonvulsants indicate the benefit of the proconvulsant value of  $\text{PGF}_{2\alpha}$  in the conditions of chronic epileptogenesis. Only the combination of digoxin with sodium valproate provided a full protective effect and reduced the cerebral content of  $\text{PGF}_{2\alpha}$  to the level of VC, in contrast to monotherapy with these drugs, the anticonvulsant effect of which was less and was not accompanied by a complete normalization of this prostaglandin content.

The role of  $\text{PGI}_2$  in epileptogenesis is not studied sufficiently. In our study, the level of  $\text{PGI}_2$  increased statistically significantly ( $p < 0.001$ ) compared to the PC under the influence of all studied groups except for celecoxib. Against the background of the use of a combination of digoxin and sodium valproate, the expression of  $\text{PGI}_2$  was most pronounced, which is evidence of a positive effect under conditions of chronic epileptogenesis.

The prostacyclin antagonist is  $\text{TXB}_2$ , which stimulates platelet aggregation and causes vasoconstriction. Its elevated level is dangerous in view of the development of epileptic brain ischemia<sup>49</sup>. In our study, a two-fold increase in the content of  $\text{TXB}_2$  in the brains of animals of the PC group and its decrease to an almost normal (sodium valproate), normal (digoxin), and even subnormal level (valproate + digoxin) proves the pathogenetic value of  $\text{TXB}_2$  in the mechanisms of chronic epileptogenesis and its antithromboxan effect as one of the links of the mechanism of AEDs' neuroprotective activity. Special attention is drawn to the lowest antithromboxan effect of celecoxib, which corresponds to its insufficient effectiveness as an anticonvulsant.

Thus, chronic administration of low-dose PTZ is associated with the activation of neuroinflammation. PTZ-induced kindling is associated with an increase in the cerebral expression of COX-1 and 2 and a pronounced imbalance in the components of the cyclooxygenase pathway of the arachidonic acid cascade. At the same time, the pathogenetic role of individual prostaglandins as well as thromboxane in epileptogenesis requires further clarification. Also, the decrease in the cerebral content of  $\text{PGE}_2$  and  $\text{PGI}_2$  (but not  $\text{PGF}_{2\alpha}$  and  $\text{TXB}_2$ ) against the background of an increase in the expression of the enzymes producing them requires in-depth study. Digoxin, valproate, and especially their combination normalize the PTZ-induced changes of the COX-1/2 content (digoxin, probably due to its steroid structure, penetrates into the nucleus of glial cells and regulates the gene expression of enzymes), as well as other cyclooxygenase pathway components in the brain, which, obviously, is one of the possible mechanisms for implementing their anticonvulsant action. Interestingly, celecoxib does not cause a significant reduction of COX-2 and this entire pathway, although it is a highly selective inhibitor of this isoenzyme and penetrates into the central

nervous system through its lipophilic properties<sup>27, 50</sup>. Obviously, the anti-inflammatory effect of celecoxib is limited by the absence of pronounced influence on the cerebral COX-2 isoform. The weak influence of celecoxib on neuroinflammation corresponds to the lack of anticonvulsant activity in the experiment. At the same time, the verified significant effect of digoxin and valproate (especially when used in combination) on the cyclooxygenase pathway corresponds to the previously established anti-inflammatory properties of cardiac glycosides as well as AEDs. These results suggest that the effect on neuroinflammation is one of the components of successful seizure control.

Neuroinflammation and oxidative stress are closely related phenomena that may reinforce each other<sup>51</sup>. Studies on animals have shown that inhibition of reactive oxygen species production improves survival and cognitive deficits as well as reduces neuronal damage after status epilepticus<sup>52</sup>. In the model of electrically induced epileptic status in rats, it was shown that the redox-sensitive protein HMGB1 links oxidative stress with neuroinflammation in epilepsy<sup>53</sup>. In rats with a status epilepticus, the antioxidant drug N-acetyl-cysteine, a precursor of glutathione, and sulforaphane, which activates the transcription factor NRF2, blocked the generation of reactive oxygen species and prevented the cytoplasmic translocation of HMGB1 in hippocampal neurons and glia. These effects inhibited the progression of seizures. During treatment, the drug did not affect the course of epileptic status. Still, after the withdrawal, the onset of epileptic seizures was delayed, and the frequency of chronic seizures was significantly reduced<sup>53</sup>. These data prove that oxidative stress is an important pathogenetic factor in the development of convulsions. We found a 2.75-fold increase in the cerebral content of 8-isoprostane – an indicator of an oxidative stress – in the animals of the PC group and its decrease in the groups of effective anticonvulsants to full normalization under the influence of the combination of valproate + digoxin, confirming the significant role of oxidative stress in brain damage during kindling as well as the antioxidant effect of anticonvulsants as a component of their protective effect mechanism.

NSE is a biomarker associated with microglial activation, which enables the assessment of the degree of damage to brain neurons, which occurs in ischemia and various metabolic, inflammatory, and neurodegenerative diseases, including epilepsy<sup>54</sup>. As a result of brain cell damage, the level of neuro-specific enzymes and their isozymes in the extracellular environment increases. Therefore, the degree of severity of structural and functional disorders of the CNS biomembranes can be determined by the degree of NSE increase in brain tissue. Due to the destruction of brain cells, the amount of NSE in the blood increases also. In studies of ischemic strokes in adult patients, a correlation was found between increased levels of NSE in the blood and the severity of neurological deficits<sup>22</sup>.

A 17.46-fold increase in the level of NSE in the PC group compared to the VC indicator specifies serious damage of mice neurons. The combination of valproate + digoxin effectively reduced NSE concentration the most (by 8.4 times). It proves these drugs' neuroprotective effects and correlates with their high anticonvulsant activity. Due to their pronounced anticonvulsant activity, digoxin, valproate, and their combinations apparently contribute to the reduction of neuronal damage and, accordingly, to less induction of cerebral COX-2. The underlying causes and subtle mechanisms of these differences in effects on the inflammatory pathway require further clarification. Unlike sodium valproate and digoxin, celecoxib did not affect NSE content. This corresponds to the absence of a pronounced effect on the course of PTZ-induced kindling and, together with the condition of the cyclooxygenase pathway, indicates a limitation of the anti-inflammatory and antioxidant properties of celecoxib in the CNS without its neuroprotective effect.

Along with the pharmacodynamic interaction of digoxin with sodium valproate, which probably leads to an increase in the anticonvulsant effect, the role of possible pharmacokinetic interaction cannot be excluded. The pharmacokinetic interaction of several antiepileptic drugs with digoxin is known<sup>55–57</sup>. For example, phenytoin, carbamazepine, and topiramate can decrease plasma concentrations of digoxin. However, neither digoxin nor valproate are P-glycoprotein inhibitors, which excludes this mechanism in possible CNS accumulation of drugs. Certainly, to clarify the possibility of changing the concentration of sodium valproate in the blood and, especially, in the brain under the influence of digoxin, special studies are needed, which are to be assigned to the next stage of our study.

## Conclusions

The results of the study of the effect of digoxin, sodium valproate, their combination, and celecoxib on the cyclooxygenase pathway of the arachidonic acid cascade, the content of 8-isoprostane and NSE in the brain of mice under the PTZ-induced kindling are presented. Only the combination of sodium valproate with digoxin provides a complete protective effect (absence of seizures) and shows the most pronounced effect on markers of neuroinflammation and neuronal damage than monotherapy with each of these drugs and celecoxib, which appeared to be an ineffective anticonvulsant. Against the background of the combination of valproate + digoxin, the increased level of cerebral COX-1, COX-2, PGF<sub>2α</sub>, and TXB<sub>2</sub> was most effectively reduced, while the reduced content of PGE<sub>2</sub> and PGI<sub>2</sub> increased. A statistically significant decrease in the level of 8-isoprostane in the valproate + digoxin group indicates a beneficial effect on the course of oxidative stress caused by long-term administration of PTZ. Distinct neuroprotective properties of the

valproate + digoxin combination were revealed, as indicated by a statistically significant decrease in the elevated level of NSE in the animal brain. The obtained results indicate digoxin is a promising agent for further study as an adjuvant component to classical antiepileptic drugs (mostly sodium valproate) in epilepsy treatment.

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