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Effect of digoxin, sodium valproate, their combination and celecoxib on neuroactive amino acids content and cerebral Na⁺, K⁺-ATPase activity in pentylenetetrazole-kindled mice

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Epilepsy is the third most common cause of global disease burden among neurological disorders [1], affecting 65 million people worldwide [2]. In approximately 30% of cases, epilepsy is drug-resistant [3, 4], which significantly complicates seizure control, impairs the quality of life of patients and increases economic costs. This explains the relevance of finding new methods of pharmacotherapy of epilepsy.

One of the ways to solve this problem is to use non-antiepileptic drugs – medicines, that are not classical antiepileptic drugs (AEDs), but belong to other pharmacological groups and can be used as adjuvants. It was found that the vast majority of antiarrhythmic drugs such as sodium channel blockers (lidocaine), as well as calcium channel blockers (nifedipine, amlodipine, cinnarizine, diltiazem, verapamil) and β -blockers (propranolol, metoprolol, pindolol) have an anticonvulsant effect, which was revealed in several models of seizures in animals, and for individual drugs – in the clinic [5–7]. Anticonvulsant properties are also inherent in ivabradine as a blocker of I_f-channels of the sinus node, used in the treatment of angina pectoris [8]. A

pronounced clinically verified anticonvulsant effect is exerted by the anti-gout medicine, the xanthine oxidase inhibitor allopurinol, which indirectly indicates a separate role in the disturbance of purine metabolism in epileptogenesis [9]. The pleiotropic effects of statins (including atorvastatin, simvastatin, and pravastatin) have been successfully complemented by anticonvulsant properties, which were founded in experiment and clinic [10, 11]. The presence of anticonvulsant action in selective phosphodiesterase-5 inhibitors – erectile dysfunction correctors sildenafil and tadalafil [12, 13], as well as antidiabetic medicine – inhibitor of sodium-glucose cotransporter (SGLT2) dapagliflozin [14] has been experimentally proven. Given the importance of neuroinflammation in the pathogenesis of epilepsy [15, 16], the anticonvulsant potential of anti-inflammatory drugs is being explored [17, 18]. The presence of anticonvulsant action in nonsteroidal anti-inflammatory drugs (both selective – celecoxib, etoricoxib, nimesulide, and non-selective – indomethacin, acetylsalicylic acid), which further indicates the role of glia inflammation in the development of epileptic seizures [17, 19–22]. In addition, the experimental data showed the presence of anticonvulsant properties in the COX-3 inhibitor paracetamol [23]. In our previous

studies, the selective COX-2 inhibitor celecoxib exhibited moderate anticonvulsant properties [24, 25]. On the other hand, drugs that can affect neuronal Na^+ , K^+ -ATPase attract attention. The role of this enzyme in the processes of maintaining the membrane potential of neurons and in the pathogenesis of neurological diseases, including epilepsy [26, 27] has been proven. A well-known group of drugs that modulate the activity of Na^+ , K^+ -ATPase are cardiac glycosides, drugs which are divided into hydrophilic and lipophilic medicines. Digoxin is a lipophilic drug and, unlike hydrophilic drugs (such as strophanthin), is able to cross the blood-brain barrier [28, 29], which is important in the context of central neurotropic properties. Our previous studies have demonstrated the ability of digoxin at safe subcardiotonic doses to potentiate the anticonvulsant effects of standard antiepileptic drugs, including sodium valproate at a subeffective dose ($1/2 \text{ED}_{50}$) [30, 31]. Given the long-term (often lifelong) use of AEDs, which have serious side effects, the use of digoxin in a subcardiotonic dose in combination with classical anticonvulsants in low doses should reduce the frequency of adverse reactions. In addition, this approach may be useful in overcoming drug-resistant seizures.

Therefore, in-depth study of the effects of celecoxib and digoxin on the neurochemical mechanisms of seizures, as well as the mechanisms of digoxin potentiation of the anticonvulsant action of sodium valproate, a widely used AED, is needed. Since the imbalance of excitatory (glutamate, aspartate) and inhibitory (GABA, glycine) amino acids plays a significant role in the pathogenesis of seizures, it is important to study the effect of the above-mentioned drugs on the level of these neurotransmitters in the brain.

Na^+ , K^+ -ATPase activity is useful for maintaining membrane potential and normal excitability of neurons, which is important in the context of convulsive conditions. It is known that a decrease in the activity of Na^+ , K^+ -ATPase increases the susceptibility to paroxysms, as it hinders the repolarization of membranes. For example, a mutation in the gene of α -subunit of Na^+ , K^+ -ATPase has been detected in mice and in humans with epilepsy or similar disorders [32–34]. It is also known that in the cerebral cortex of patients with epilepsy and rats in the model of acute pentylene-tetrazole (PTZ)-induced seizures the activity of Na^+ , K^+ -ATPase is significantly reduced [35, 36]. Thus, the activity of cerebral Na^+ , K^+ -ATPase is an substantial marker of neuronal excitability.

Kindling models of epilepsy are of great importance at the stage of pre-clinical studies, when the excitatory factor in the subthreshold dose repeatedly affects the motor neurons of animals, after which the brain is able to generate epileptic discharges without stimulation. As a result, after a while, seizures appear spontaneously without the influence of a provoking factor. Such animal models of chronic epileptogenesis are as close as possible to human clinical pathology [37].

The aim of this study is to investigate the effect of digoxin, sodium valproate, combination of these drugs, as well as celecoxib on the content of neurotransmitter amino acids (GABA, glycine, glutamate, aspartate) and Na^+ , K^+ -ATPase activity in brain of PTZ-kindled mice.

Material and methods. The present work is a part of scientific project «Rationale for improving the treatment of multidrug-resistant epilepsy through the combined use of classical anticonvulsant medicines with other drugs» (No. 0120U102460, 2020/2022) sup-

ported by the Ministry of Health of Ukraine and carried out at the expense of the State Budget of Ukraine.

A total of 56 adult random-bred female albino mice weighing 20–24 g have been chosen. Animals were kept in the vivarium of the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy (Kharkiv, Ukraine) in plastic boxes on a standard diet with free access to water at constant humidity 60% and temperature + 20–22 °C, 12-hour light/dark cycle.

The study protocol does not contradict the provisions of the Helsinki Declaration on the Humane Treatment of Animals (2000) and the Council of the European Union Directive on the Protection of Animals Used for Scientific Purposes (2010) and approved by the Local Bioethical Committee of the National University of Pharmacy, Kharkiv, Ukraine (protocol No. 3, September 10, 2020).

Pentylenetetrazole (Sigma-Aldrich, USA), Celecoxib (Celebrex, Pfizer, USA – capsules 100 mg), Sodium valproate (Depakine, Sanofi Aventis, France – syrup for oral administration 57.64 mg/1 ml), Digoxin (DNCLZ/Health, Ukraine – solution for injection, 0.25 mg/ml) have been used. All other chemicals were of analytical grade. Diagnostic kits of ELISA were used for biochemical estimation.

To simulate kindling, 50 animals were randomized to the following equivalent groups (n = 10): 1) control pathology (untreated mice); 2) kindled mice, which were receiving sodium valproate; 3) kindled mice, which were receiving digoxin; 4) kindled mice, which were receiving a combination of sodium valproate with digoxin; 5) kindled mice, which were receiving celecoxib. Another 6 mice were used as vehicle control (VC).

PTZ-induced kindling was simulated by the use of PTZ at a dose of 30 mg/kg intraperitoneally (i.p.) [38] for 16 days. The convulsant was administered daily at the same time once a day in the morning. The classic AED sodium valproate has been used at a subeffective dose (1/2 ED₅₀) of 150 mg/kg intragastrically (i.g.) [39]. This dose usually does not provide the maximum protective effect, which enables to identify possible modulation of anticonvulsant action – either attenuation and enhancement. Digoxin was administered subcutaneously (s.c.) at a previously determined effective anticonvulsant dose of 0.8 mg/kg which is equal to 1/10 LD₅₀ [30–40]. Animals of the combination group of digoxin and sodium valproate received drugs in the above-mentioned doses. Celecoxib as an anti-inflammatory agent capable of exhibiting anticonvulsant properties [41] was administered at a dose of 4 mg/kg i.g. as a suspension stabilized with Tween-80. The dose of celecoxib was selected based on literature data [41, 42]. Sodium valproate and celecoxib were administered 30 minutes and digoxin – 15 min before PTZ administration. The volume of fluid for each route of administration was 0.1 ml per 10 g of body weight. Mice of the control pathology (CP) group received i.p. and s.c. solvent (0.9% NaCl) in the appropriate volume (0.1 ml/10 g) and administration regimen. The animals of VC that received neither the studied drugs, nor PTZ, which were administered daily for 16 days i.p. and s.c. solvent (0.9% NaCl) in the appropriate volume (0.1 ml/10 g) and administration regimen.

After each PTZ administration, the animals were observed for 1 hour. The latency of the first seizures, the number of days with and without convulsions, the number of animals with seizures, as well as the nature of paroxysms (clonic

or tonic) have been determined. At the end of the kindling, the brains of mice were examined by biochemical methods. This required another group of animals (VC) that did not receive the studied drugs. On day 16th of the experiment, 1 h after the last administration, the kindled and VC animals were euthanized by dislocation of the cervical vertebrae [43]. The brain was immediately removed, frozen in liquid nitrogen, stored until analysis in a freezer at $-70\text{ }^{\circ}\text{C}$, and homogenized immediately before the sample was analyzed. The brains of 6 animals were taken from each group (if the animals had convulsions, the brains of such mice were necessarily examined).

The level of excitatory (glutamate, aspartate) and inhibitory (GABA, glycine) amino acids was determined in the brain homogenate. The content of GABA, aspartate, glutamate was determined by high-voltage electrophoresis [44]. Separation was performed for 3 h in pyridine-acetic acid buffer under voltage 600 V. A portion of the frozen brain was ground to a powder. The extraction was performed in 96% ethanol in a boiling water bath (ethanol-tissue ratio 10:1) for 19 minutes. Glycine content was determined by thin layer chromatography on Silufol plates using n-butanol : glacial acetic acid : water solvents in a ratio of 90 : 10 : 25 [45]. Glycine substance (Sigma, USA) was used as a standard.

To determine the activity of Na^+ , K^+ -ATPase in the synaptosomal membranes of the mice brains a 10% homogenate on 0.3 M sucrose prepared on 50 mM Tris-HCl (pH 7.4) has been used. Synaptosomes were isolated by the Hajos method [46]: the homogenate was centrifuged for 10 minutes at $2\text{ }^{\circ}\text{C}$ and 20 000 g; the precipitate was resuspended in 3 ml of separation medium, layered on 0.8 M sucrose solution,

centrifuged for 20 min at $2\text{ }^{\circ}\text{C}$, 20 000 g. The supernatant (synaptosomes in 0.8 M sucrose) was diluted to a sucrose concentration of 0.32 M (2.5 times).

Synaptosome precipitation was performed by centrifugation at 20 000 g, $2\text{ }^{\circ}\text{C}$ for 30 minutes. To obtain synaptosomal membranes used osmotic shock – the precipitate was diluted with distilled water in a ratio of 1:10 v/v, kept for 1 hour. Membranes of synaptosomes were precipitated by centrifugation (20 000g, $2\text{ }^{\circ}\text{C}$, 30 min). The precipitate was resuspended in distilled water, stored frozen. The activity of Na^+ , K^+ -ATPase was determined by the method [47] in the Gorbach author's modification. Incubation mixture: 0.1 M Tris-HCl buffer (pH 7.4) – 2 ml, 1 mM EDTA, 1 mM ATP, 120 mM NaCl, 60 mM KCl, 0.4 ml of membrane suspension. Incubation for 15 minutes at $37\text{ }^{\circ}\text{C}$. The enzyme activity was determined by the content of inorganic phosphate in the incubation medium. To avoid nonspecific ATPase activity, a control assay with ouabain (1 mM), which inhibits specific Na^+ , K^+ -ATPase, has been used. Protein in the synaptosome fraction was determined by the Lowry method.

Statistical processing of the results was performed using the software package STATISTICA 12.0. The results are given as the mean value and its standard error ($M \pm m$). Significance of intergroup differences was assessed by the parametric Student's t-test in cases of normal distribution and non-parametric Mann-Whitney U-test in its absence. For the results in the alternative form (days with and without seizure, percentage of mice with clonic and tonic convulsions etc.) the Fisher's angular transformation was used. Differences were considered significant at $p < 0.05$ [48].

Results and discussion. The results of the course of kindling are shown in

Table 1. On the day 4th of the experiment, subthreshold doses of PTZ led to a gradual increase in convulsive activity: in the groups of CP, digoxin and celecoxib, the appearance of the first clonic seizures was observed. In the sodium valproate group, the first paroxysms were recorded on the day 7th of convulsant administration. However, in group of the combination of valproate + digoxin, a complete protective effect was observed: there was no seizure during all 16 days.

In the CP, digoxin and celecoxib groups the total number of days without seizures was 3. In the group of sodium valproate – 8 (after the first paroxysms of clonic seizures in one mouse on the day 7th for days 8 and 9 there were no seizures, they resumed from the day 10th), which significantly exceeded the rate of the other three groups ($p < 0.05$). In the combination of sodium valproate with digoxin, the number of days without seizures was 16, which is statistically significantly more than in all other groups ($p < 0.05$). The number of days with seizures in these groups was

13 (CP), 8 (sodium valproate), 13 (digoxin), 0 (valproate + digoxin) and 13 (celecoxib), respectively (Table 1).

The number of animals with seizures gradually increased in all groups except the group valproate + digoxin, in which seizures did not occur. On day 14th, the indicator reached a maximum and amounted to 80% in the CP group, 50% in the sodium valproate group, 30% in the digoxin group ($p < 0.05$ relative to the CP and celecoxib groups), 70% in the celecoxib group. In the group of the valproate + digoxin combination, as already mentioned, seizures did not occur in any mouse, which is significantly different from all other groups ($p < 0.05$).

All animals of the CP and celecoxib groups, which developed convulsive syndrome, after 16 days of observation had both clonic paroxysms (clonus of the head, limbs without lateral position) and more severe tonic convulsions (tonic extension of the limbs in the lateral position). However, only clonic seizures occurred in mice of the sodium valproate and digoxin groups (Table 1),

Table 1

The course of pentylenetetrazole kindling in mice under the influence of digoxin, sodium valproate, their combination and celecoxib (n = 10)

Group	Latency, days	Days		Number of mice, %		
		without seizures	with seizures	with seizures	with clonic convulsions	with tonic convulsions
Control pathology	3	1–3 (3)	4–16 (13)	80	80	80
Sodium valproate	6	1–6, 8, 9 (8 [#] , °, ^)	7, 10–16 (8 [#] , °, ^)	50	50	0 [#] , ^
Digoxin	3	1–3 (3)	4–16 (13)	30 [#] , ^	30 [#] , ^	0 [#] , ^
Sodium valproate + Digoxin	16	1–16 (16 [#] , °, §, ^)	(0 [#] , °, §, ^)	0 [#] , °, §, ^	0 [#] , °, §, ^	0 [#] , ^
Celecoxib	3	1–3 (3)	4–16 (13)	70	70	70

Note. n – number of animals in the group, the number of days is given in parentheses, [#] $p < 0.05$ compared with control pathology, ° $p < 0.05$ – compared with digoxin, § $p < 0.05$ – compared with sodium valproate, ^ $p < 0.05$ – compared with celecoxib.

and the number of animals with tonic seizures was 0% ($p < 0.05$ relative to the CP and celecoxib groups).

Therefore, sodium valproate at 150 mg/kg in the model of PTZ-induced kindling has a moderate protective effect, which is manifested by a twofold increase in the latency period of the first seizures, a decrease in the number of days with convulsions ($p < 0.05$) and the number of animals with paroxysms 1.6 times (compared with CP), as well as complete protection against tonic convulsions. Digoxin at 0.8 mg/kg also has a moderate protective effect in the form of reducing the number of animals with seizures by 2.7 times ($p < 0.05$) compared with CP and complete protection against tonic paroxysms without affecting the latency period of seizures and the number of days with convulsion. At the same time digoxin clearly potentiates the anticonvulsant activity of sodium valproate, providing a complete protective effect. The anticonvulsant effect of celecoxib is tendentious and is

manifested only by a slight decrease in the number of animals with convulsions.

The results of the content of neuroactive amino acids study in the brains of PTZ-kindled mice are given in Table 2. In the CP group a long-term administration of PTZ led to the typical changes in the level of inhibitory and excitatory amino acids. As expected, the content of inhibitory amino acids decreased relative to VC: GABA – by 2.2 times ($p < 0.001$), glycine – by 1.6 times ($p < 0.001$). The concentration of glutamate significantly increased relative to VC by 2.2 times ($p < 0.001$) and aspartate – by 1.5 times ($p < 0.001$).

In the sodium valproate group, the severity of changes in the content of neuroactive amino acids relative to the indicators of the CP group was less ($p < 0.001$), but significant differences from the indicators of the VC group remained.

The content of inhibitory amino acids relative to CP significantly increased: GABA by 1.8 times ($p <$

Table 2

The effect of digoxin, sodium valproate, their combination and celecoxib on the content of GABA, glutamate, aspartate, glycine in the brain of mice under the model of pentylenetetrazole kindling (n = 6)

Group	GABA, $\mu\text{mol/g}$	Glycine, $\mu\text{mol/g}$	Glutamate, $\mu\text{mol/g}$	Aspartate, $\mu\text{mol/g}$
Vehicle control	7.43 \pm 0.10	1.39 \pm 0.05	13.20 \pm 0.20	3.40 \pm 0.14
Control pathology	3.38 \pm 0.15***	0.88 \pm 0.03***	28.98 \pm 0.76***	5.02 \pm 0.09***
Sodium valproate	6.09 \pm 0.12***, ###, ^^	1.04 \pm 0.04***, ##	20.49 \pm 0.48***, ###, ^^	3.95 \pm 0.10***, ###, ^^
Digoxin	6.82 \pm 0.12***, ###, \$\$, ^^	1.45 \pm 0.03###, \$\$\$, ^^	15.92 \pm 0.21***, ###, \$\$\$, ^^	3.73 \pm 0.11###, ^^
Sodium valproate + Digoxin	7.87 \pm 0.06**, ###, \$\$\$, °°, ^^	1.67 \pm 0.05**, ###, \$\$\$, °°, ^^	13.10 \pm 0.11###, \$\$\$, °°, ^^	3.41 \pm 0.10###, \$\$\$, ^^
Celecoxib	3.86 \pm 0.15***, #	1.01 \pm 0.03***, #	25.17 \pm 0.57***, ##	4.78 \pm 0.14***

Note. n – number of animals in the group, the content of GABA, glutamate, aspartate, glycine in the brain were expressed as $M \pm m$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ – compared with vehicle control, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ – compared with control pathology, ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$ – compared with digoxin, \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$ – compared with sodium valproate, ^ $p < 0.05$, ^^ $p < 0.05$, ^^° $p < 0.05$ – compared with celecoxib.

0.001), and glycine by 1.18 times ($p < 0.001$). The level of glutamate decreased by 28.3% ($p < 0.001$), and the content of aspartate by 21.3% ($p < 0.01$).

In the digoxin group, the content of neuroactive amino acids was normalized significantly more than in sodium valproate group. Regarding CP, the level of GABA increased by 2.02 times ($p < 0.001$), glycine – by 1.64 times ($p < 0.001$); the content of glutamate decreased by 45.1% ($p < 0.001$), aspartate – by 25.7% ($p < 0.001$). It is noteworthy that the content of aspartate and glycine was restored to VC values.

In the group of combination of digoxin with sodium valproate, a synergistic normalizing effect on amino acid imbalance was observed, which significantly outweighed the effects of these drugs *per se* (Table 2). Regarding CP parameters, the concentration of GABA significantly increased by 2.33 times ($p < 0.001$), glycine – by 1.89 times ($p < 0.001$); the content of glutamate decreased by 54.8% ($p < 0.001$), aspartate – by 32.1% ($p < 0.001$). The concentration of excitatory amino acids decreased to the level of VC, and inhibitory was even higher than the values of intact animals ($p < 0.01$).

Celecoxib as a drug with insignificant anticonvulsant properties less than sodium valproate reduced the PTZ-kindled changes: relative to CP, the content of GABA significantly increased by only 14.2% ($p < 0.05$), glycine – by 14.8% ($p < 0.05$); the concentration of glutamate decreased by 13.1% ($p < 0.01$), aspartate – insignificantly by 4.8% ($p > 0.05$).

The results of the study of the activity of Na^+ , K^+ -ATPase in the brain of mice with PTZ-induced kindling are given in Table 3. In the CP group, long-term administration of PTZ led to a significant reduction in the activity of cerebral Na^+ , K^+ -ATPase by 45.9% ($p < 0.001$). In the group of sodium valproate, the activity of Na^+ , K^+ -ATPase was higher than in CP by 44.0% ($p < 0.001$), but lower than in VC by 22.1% ($p < 0.001$). Although digoxin contributed to a slight but typical for cardiac glycosides significant decrease in Na^+ , K^+ -ATPase activity against CP by 6.0% ($p < 0.01$), its combined use with sodium valproate potentiated an increase in Na^+ , K^+ -ATPase activity in the brain relative to CP by 59.8% ($p < 0.001$), which exceeded the effect of sodium valproate *per se* by 10.9% ($p < 0.001$).

Table 3

The effect of digoxin, sodium valproate, their combination and celecoxib on the activity of Na^+ , K^+ -ATPase in the brain of mice under the model of pentylenetetrazole kindling ($n = 6$)

Group	Na^+ , K^+ -ATPase, $\mu\text{mol inorganic phosphate/mg protein} \times \text{hour}$
Vehicle control	31.65 ± 0.50
Control pathology	17.12 ± 0.23***
Sodium valproate	24.66 ± 0.19***, ###, ^^
Digoxin	16.09 ± 0.10***, ##, \$\$\$, ^^
Sodium valproate + Digoxin	27.35 ± 0.26***, ###, \$\$\$, °°, ^^
Celecoxib	22.75 ± 0.23***, ###

Note. n – number of animals in the group, the activity of Na^+ , K^+ -ATPase in the brain was expressed as $M \pm m$, *** $p < 0.001$ – compared with vehicle control pathology, ### $p < 0.001$ – compared with control, °° $p < 0.001$ – compared with digoxin, §§ $p < 0.001$ – compared with sodium valproate, ^^ $p < 0.05$ – compared with celecoxib.

In the celecoxib group, the activity of Na^+ , K^+ -ATPase was higher than in CP by 32.9% ($p < 0.001$), but remained significantly lower than in the VC group ($p < 0.001$).

Thus, 16-day use of PTZ led to a marked imbalance of inhibitory and excitatory neuroamino acids and a significant decrease in the activity of Na^+ , K^+ -ATPase, while all the studied drugs improved these indicators, bringing them closer to the values of VC.

Both digoxin at 0.8 mg/kg and sodium valproate at 150 mg/kg had a significant restorative effect on the pool of studied neurotransmitters, but only their combined use completely eliminated the negative effect of PTZ on the level of excitatory and inhibitory amino acids (the content of the last ones even significantly exceeds the indicators of VC). Although the moderate inhibitory effect of digoxine on the Na^+ , K^+ -ATPase activity, that is typical for cardiac glycosides [49], the combination of digoxine and sodium valproate showed a significant increase in the activity of this enzyme which promotes repolarization of membrane and reduces its excitability. The results obtained indicate the complex nature of the influence of digoxin on the activity of cerebral Na^+ , K^+ -ATPase under the convulsive syndrome, which depends on the conditions of use (*per se* or in combination with sodium valproate). Obviously, of paramount importance in the mechanism of anticonvulsant action, the isolated use of cardiac glycoside has an effect on the metabolism of neurotransmitter amino acids. Although digoxin *per se* does not promote the activation of Na^+ , K^+ -ATPase, it exhibits anticonvulsant activity in a model of PTZ-induced kindling. It is likely that the reduced activity of the ion pump is compensated to a certain extent by the normalization of the content of neuro-

active amino acids, which can help to reduce the neurons' excitability.

Celecoxib moderately, although statistically significantly, affected the level of amino acids (except aspartate) and the activity of Na^+ , K^+ -ATPase in the brain of PTZ-induced kindling. In this model, celecoxib reduces the severity of neuroinflammation [42], which apparently may contribute to the normalization of neurotransmission and excitability of neurons by reducing their damage, although the mild clinical anticonvulsant potential of the drug.

The most effectiveness valproate + digoxin combination needs to be discussed. Considering the importance of the balance of excitatory and inhibitory neurotransmission in the pathogenesis of epilepsy, the restoration of this balance to normal values or even with a significant shift towards inhibition (increased levels of GABA and glycine relative to VC) is likely to be a crucial factor in anticonvulsant activity of standart AED and adjuvant drug. It is known that the normal functioning of the glutamate and aspartate transporter (GLAST) – one of the most important transporters of excitatory amino acids [28, 49] – depends on sufficient activity of Na^+ , K^+ -ATPase. Impaired Na^+ , K^+ -ATPase activity adversely affects the functioning of the glutamate transporter (GluT) and causes an increase in the extracellular content of glutamate, which causes increased excitability [50, 51]. In addition, an increase in intracellular Ca^{2+} levels due to inhibition of Na^+ , K^+ -ATPase activity reduces the amplitude of the Cl^- current directed to the cell [52], which may allow modulating the activity of GABA receptors [53]. Thus, this suggests that one of the main mechanisms of pronounced anticonvulsant action of the combination of sodium valproate

with digoxin is the restoration of Na^+ , K^+ -ATPase activity, which probably prevents the imbalance of neurotransmitters by affecting the relevant transporters and receptors. Finding out the correctness of this assumption may be one of the areas of further research. It is advisable to determine the specific mechanisms that cause the restoration of the normal level of neuroamino acids with the combined use of digoxin and sodium valproate in experimental seizures. In particular, it requires further study of changes in the functional state of receptors and the activity of transporters when using a combination of drugs.

Our studies confirm the ability of digoxin to potentiate the anticonvulsant activity of the AEDs in a subeffective dose. A better understanding of the mechanisms of this interaction will allow further development with promising medical implementation. Moreover, elucidation the Na^+ , K^+ -ATPase role in the restoring of «excitation-inhibition» balance will make it possible to conduct studies using other selective inhibitors of this ion pump, which would make it possible to potentiate the action of AEDs in subeffective doses even more efficiently and with a lower frequency of side reactions.

Conclusions

1. The effect of digoxin, sodium valproate, the combination of sodium valproate with digoxin and celecoxib on the course of pentylenetetrazole kindling in mice, as well as on the content of neurotransmitter amino acids (GABA, glycine, glutamate, aspartate) and Na^+ , K^+ -ATPase activity in the brain of animals has been studied.
2. Sodium valproate and digoxin *per se* moderately reduce the severity of convulsive syndrome in the model of

pentylenetetrazole kindling, and when combined with each other cause the maximum protective effect, completely preventing the seizures. Celecoxib has minimal influence on the clinical signs of kindling, only tending to reduce the number of animals with seizures.

3. In untreated animals with simulated pentylenetetrazole kindling, there is a significant decrease in the cerebral content of inhibitory (GABA, glycine) and an increase in the content of excitatory (glutamate, aspartate) amino acids, as well as an almost twofold decrease in Na^+ , K^+ -ATPase activity in the brain. Both sodium valproate and especially digoxin *per se* reduce the severity of neuroactive amino acid imbalances, but sodium valproate also normalizes the reduced Na^+ , K^+ -ATPase activity, while digoxin further inhibits this enzyme. The combination of sodium valproate with digoxin affects the level of excitatory amino acids significantly reducing it to the values of healthy animals, and the content of inhibitory amino acids even exceeds the corresponding values. The activity of cerebral Na^+ , K^+ -ATPase increases to the maximum when using this combination of drugs, which can reduce the excitability of neurons both directly and by affecting the content of neuroactive amino acids. Celecoxib slightly reduces the changes in the content of neuroactive amino acids and the activity of Na^+ , K^+ -ATPase.
4. The results substantiate the prospects of the combined use of sodium valproate with low doses of digoxin in epileptic convulsions and the expediency of further research into the mechanisms of realization of the anticonvulsant action of this combination.

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V. V. Tsyvunin, S. Yu. Shtrygol', T. V. Gorbach
Effect of digoxin, sodium valproate, their combination and celecoxib on neuroactive amino acids content and cerebral Na⁺, K⁺-ATPase activity in pentylenetetrazole-kindled mice

Our previous studies have shown moderate anticonvulsant efficiency of celecoxib as well as high efficiency of the combination of digoxin at subcardiotonic dose with widespread antiepileptic drugs at a sub-effective doses in various models of primary-generalized chemoinduced seizures.

The aim of the study was to determine the efficiency of these medicines in the model of chronic epileptogenesis and the possible neurochemical mechanism of anticonvulsant action of digoxin and celecoxib *per se* as well as the combination of digoxin with sodium valproate. For this purpose, the effect on the course of pentylenetetrazole (PTZ) kindling, on the content of neurotransmitter amino acids (GABA, glycine, glutamate, aspartate) and the activity of Na⁺, K⁺-ATPase in the mice brain has been determined. PTZ kindling was chosen to its maximal pathogenetic similarity to human epilepsy.

A total of 56 adult random-bred female albino mice weighing 20–24 g have been chosen. PTZ-induced kindling was simulated by the use of PTZ at a dose of 30 mg/kg intraperitoneally for 16 days. The classic anti-epileptic drug sodium valproate has been used at a subeffective dose (1/2 ED₅₀) of 150 mg/kg intragastrically. Digoxin was administered subcutaneously at a previously determined effective anticonvulsant dose of 0.8 mg/kg which is equal to 1/10 LD₅₀. Animals of the combination group of digoxin and sodium valproate received drugs in the above-mentioned doses. Celecoxib as an anti-inflammatory agent capable of exhibiting anticonvulsant properties was administered at a dose of 4 mg/kg intragastrically. Sodium valproate and celecoxib were administered 30 minutes and digoxin – 15 min before PTZ administration. The content of GABA, aspartate, glutamate in the brain homogenate was determined by high-voltage electrophoresis. Glycine content was determined by thin layer chromatography. The activity of Na⁺, K⁺-ATPase was determined in the synaptosomal membranes.

For 16 days, celecoxib did not affect the clinical course of kindling, when sodium valproate and digoxin *per se* showed moderate efficacy, and the combination of valproate + digoxin had a complete protective effect against spontaneous seizures. It has been found a statistically significant decrease in the content of GABA, glycine and increase in the content of glutamate, aspartate, as well as almost twofold decrease in Na⁺, K⁺-ATPase in the brains of untreated animals. All studied medicines (to a lesser extent – celecoxib, most pronounced – valproate + digoxin) contributed to the normalization of the balance of neurotransmitter amino acids. All drugs, except digoxin *per se*, significantly increased the activity of Na⁺, K⁺-ATPase (valproate + digoxin maximally). Therefore, the combination of sodium valproate with digoxin in terms of the effectiveness of seizure control and the influence on neurochemical mechanisms of neuronal excitability control significantly exceeded the effect of these drugs *per se* and celecoxib. The pronounced potentiation of the anticonvulsant activity of sodium valproate with digoxin determines the feasibility of further in-depth study of the mechanisms of action of this combination as a promising approach to the epilepsy treatment.

Key words: pentylenetetrazole kindling, sodium valproate, digoxin, celecoxib, neuroactive amino acids, Na⁺, K⁺-ATPase

В. В. Цивунін, С. Ю. Штриголь, Т. В. Горбач

Вплив дигоксину, вальпроату натрію, їхньої комбінації та целекоксибу на вміст нейроактивних амінокислот і активність церебральної Na^+ , K^+ -АТФази в мишей з пентилентетразоловим кіндлінгом

У наших попередніх дослідженнях встановлено помірну протисудомну активність целекоксибу, а також високу ефективність комбінації дигоксину в субкардіотонічній дозі з широким живаними проти-епілептичними препаратами в субефективних дозах на різних моделях первинно-генералізованих хеміндукованих судом.

Мета дослідження – визначити ефективність цих препаратів на моделі хронічного епілептогенезу, можливий нейрохімічний механізм протисудомної дії дигоксину та целекоксибу *per se*, а також комбінації дигоксину з вальпроатом натрію. З цією метою визначено їхній вплив на перебіг пентилентетразолового (ПТЗ) кіндлінгу, на вміст нейромедіаторних амінокислот (ГАМК, гліцину, глутамату, аспартату) та активність Na^+ , K^+ -АТФази в мозку мишей. ПТЗ кіндлінг обраний через максимальну патогенетичну схожість з епілепсією людини.

У дослідженні використано 56 дорослих білих мишей-самок масою 20–24 г. Кіндлінг моделювали уведенням ПТЗ у дозі 30 мг/кг внутрішньоочеревинно протягом 16 днів. Класичний протиепілептичний засіб вальпроат натрію використовували в субефективній дозі ($1/2 \text{ED}_{50}$) 150 мг/кг внутрішньошлунково. Дигоксин вводили підшкірно в попередньо визначеній ефективній протисудомній дозі 0,8 мг/кг, що дорівнює $1/10 \text{LD}_{50}$. Тварини групи комбінації дигоксину та вальпроату натрію отримували препарати в зазначених вище дозах. Целекоксиб як протизапальний засіб, що має протисудомні властивості, вводили внутрішньошлунково в дозі 4 мг/кг. Вальпроат натрію та целекоксиб вводили за 30 хв, дигоксин – за 15 хв до ПТЗ. Уміст ГАМК, аспартату, глутамату в гомогенаті головного мозку визначали методом високовольтного електрофорезу, гліцину – методом тонкошарової хроматографії. Активність Na^+ , K^+ -АТФази вимірювали в синаптосомальних мембранах.

Протягом 16 днів целекоксиб не впливав на клінічний перебіг кіндлінгу, у той час як вальпроат натрію та дигоксин показали помірну ефективність, а комбінація вальпроат + дигоксин чинила повний захисний ефект щодо судом. Встановлено статистично значуще зниження вмісту ГАМК, гліцину та підвищення вмісту глутамату, аспартату, а також майже дворазове зниження активності Na^+ , K^+ -АТФази в мозку нелікованих тварин. Усі препарати (меншою мірою – целекоксиб, найбільшою – комбінація вальпроат + дигоксин) сприяли нормалізації балансу нейромедіаторних амінокислот. Водночас усі препарати, окрім дигоксину *per se*, значно підвищували активність Na^+ , K^+ -АТФази (максимально – комбінація вальпроат + дигоксин). Таким чином, комбінація вальпроату натрію з дигоксином за протисудомною ефективністю, а також за впливом на нейрохімічні механізми контролю збудливості нейронів значно перевершувала ефект цих препаратів *per se* та целекоксибу. Виразне потенціювання протисудомної активності вальпроату натрію з дигоксином визначає доцільність подальшого поглибленого вивчення механізмів дії цієї комбінації як перспективного підходу до лікування епілепсії.

Ключові слова: пентилентетразоловий кіндлінг, вальпроат натрію, дигоксин, целекоксиб, нейроактивні амінокислоти, Na^+ , K^+ -АТФаза

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