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IMPACT OF DIGOXIN AND SODIUM VALPROATE ON THE MECHANISMS OF NEUROINFLAMMATION AND NEUROAPOPTOSIS UNDER EXPERIMENTAL EPILEPTOGENESIS

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Abstract. Impact of digoxin and sodium valproate on the mechanisms of neuroinflammation and neuroapoptosis under experimental epileptogenesis. Tsyvunin V.V., Shtrygol' S.Yu., Lytkin D.V., Shtrygol' D.V. *Cardiac glycoside digoxin may be an effective adjuvant to classical antiepileptic drugs (AEDs) in the drug-resistant epilepsy treatment. However, the mechanisms of digoxin's anticonvulsant effect, in particular ability of digoxin itself and its combination with classical AED sodium valproate to influence neuroinflammation as well as counteracting neuronal damage, remains unexplored. Thus, the aim of the study is to elucidate the role of individual markers of neuroinflammation and neuronal apoptosis, in particular interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), interleukin-4 (IL-4), Fas ligand (FasL), heat shock protein 70 (HSP_{70}), 5-lipoxygenase (5-LOX) and nitric oxide synthase (NOS) in the realization of the anticonvulsant potential of digoxin and sodium valproate under experimental epileptogenesis. The model of chronic epileptogenesis, kindling induced pentylenetetrazole (PTZ) in mice, has been used. 40 animals were divided into 5 groups of 8 mice each: vehicle control (receiving solvent – water), positive control (receiving only PTZ), sodium valproate (150 mg/kg intragastrically), digoxin (0.8 mg/kg subcutaneously), and valproate+digoxin combination. Medicines – both per se and in combination – were administered 30 min before PTZ (30 mg/kg intraperitoneally). After 16 days, IL-6, TNF-α, IL-4, FasL, HSP_{70}, 5-LOX and NOS have been identified in the whole brain. It was confirmed that the combination of digoxin with sodium valproate more effectively prevents the development of seizures than monotherapy. It was proven that sodium valproate and digoxin exhibit pronounced anti-inflammatory properties, restoring the content of TNF-α (but not IL-6) and increasing the expression of IL-4, and in combination they also normalize the level of 5-LOX in the brain. Also, digoxin more clearly than sodium valproate counteracts neuroapoptosis and neurodegeneration by affecting FasL and HSP_{70}. Cerebral NOS, however, is not involved in the development of experimental seizures, nor in the anticonvulsant effect of sodium valproate and digoxin. The obtained results expand the understanding of the mechanisms of anticonvulsant action of digoxin and sodium valproate and may be important in the development of new strategies for drug-resistant epilepsy treatment.*

Реферат. Вплив дигоксину та вальпроату натрію на механізми нейрозапалення та нейроапоптозу за експериментального епілептогенезу. Цивунін В.В., Штриголь С.Ю., Литкін Д.В., Штриголь Д.В. *Серцевий глікозид дигоксин може бути ефективним ад'ювантом до класичних протиепілептичних препаратів (ПЕП) у лікуванні фармакорезистентної епілепсії. Однак механізми протисудомної дії дигоксину, зокрема здатність самого дигоксину та його комбінації з класичним ПЕП вальпроатом натрію впливати на нейрозапалення, а також протидіяти пошкодженню нейронів, залишаються невивченими. Таким чином, метою дослідження було з'ясування ролі окремих маркерів нейрозапалення та апоптозу нейронів, зокрема інтерлейкіну-6 (IL-6), фактора некрозу пухлини α (TNF-α), інтерлейкіну-4 (IL-4), Fas-ліганду (FasL), білка теплового шоку 70 (HSP_{70}), 5-ліпоксигенази (5-LOX) та синтази оксиду азоту (NOS) у реалізації протисудомного потенціалу дигоксину та вальпроату натрію за умов експериментального епілептогенезу. Було використано модель хронічного епілептогенезу – кіндлінгу, індукованого пентилентетразолом (ПТЗ), у мишей. 40 тварин були розподілені на 5 груп по 8 мишей у кожній: інтактний контроль (отримували розчинник – воду), позитивний контроль (отримували тільки ПТЗ), вальпроат натрію (150 мг/кг внутрішньошлунково), дигоксин (0,8 мг/кг підшкірно) та комбінація вальпроату з дигоксином. Препарати – як окремо, так і в комбінації – вводили за 30 хвилин до ПТЗ (30 мг/кг внутрішньочеревно). Через 16 днів у мозку визначали вміст IL-6, TNF-α, IL-4, FasL, HSP_{70}, 5-LOX та NOS. Підтверджено, що комбінація дигоксину з вальпроатом натрію ефективніше запобігає розвитку судом, аніж монотерапія препаратами. Доведено, що вальпроат натрію та дигоксин проявляють виражені протизапальні властивості, відновлюючи вміст TNF-α (але не IL-6) та підвищуючи експресію IL-4, а в комбінації вони також нормалізують рівень 5-LOX у мозку. Також дигоксин більш виражено, ніж вальпроат натрію, протидіє нейроапоптозу та нейродегенерації, впливаючи на FasL та HSP_{70}. Однак церебральна NOS не бере участі ані в розвитку експериментальних судом, ані в протисудомній дії вальпроату натрію та дигоксину. Отримані результати розширюють розуміння механізмів протисудомної дії дигоксину й вальпроату натрію та можуть бути важливими в розробці нових стратегій лікування фармакорезистентної епілепсії.*

The prevalence of epilepsy, particularly its refractory forms, is becoming increasingly important in medical and social terms. Cases of the disease resistant to classical first-line antiepileptic drugs (AEDs) occur in 30% of patients [1]. The International League Against Epilepsy (ILAE) defines drug-resistant epilepsy (DRE) as one in which it is impossible to achieve sustained remission of seizures with the use of two well-tolerated, correctly selected and prescribed drugs in adequate doses (in monotherapy or in combination) [2].

Current strategies for treating DRE include surgical resection or destruction of epileptogenic brain areas (which is limited exclusively to focal forms of the disease), stimulation of the vagus nerve, deep nucleus of the thalamus or cerebral cortex, and the use of a ketogenic diet, but polytherapy remains the most common option [3, 4]. Rational selection of combinations of AEDs (preferably with different mechanisms of action to ensure a more effective impact on the development of spontaneous seizures) is one of the most important tasks in the treatment of DRE. It is

important to consider the high risks of developing undesirable effects, which are exacerbated by the simultaneous use of several AEDs, as well as the consequences of possible drug interactions. At the same time, even with polytherapy, a significant proportion of patients with DRE do not achieve complete control over seizures [5]. Therefore, further research and development of new approaches are needed to improve the treatment outcomes of such patients.

One of the promising way for improving the pharmacocorrection of DRE is the use of medicines from other pharmacological groups, in particular cardiac glycosides (primarily digoxin) in subcardiotonic doses in addition to classical AEDs [6]. We have previously proven that digoxin is able to significantly enhance the anticonvulsant potential of subeffective doses of the main first and second line AEDs due to a favorable pharmacodynamic interaction [7]. The cardiac glycoside realizes such properties due to its positive impact on neuroinflammation (mainly on the cyclooxygenase pathway of the arachidonic acid cascade in the brain), which has been proven under chemoinduced kindling [8]. The same study demonstrated the ability of digoxin in combination with valproate to counteract neuronal death by normalizing cerebral levels of the marker enzyme neuron-specific enolase. However, the ability of both digoxin itself and its combination with classical AED to influence other mechanisms of neuroinflammation (in particular, the cytokine and lipoxygenase pathways of the arachidonic acid cascade), as well as counteracting damage (apoptosis and necrosis) of neurons, remains unexplored.

The aim of this study is to elucidate the role of individual markers of neuroinflammation and neuronal apoptosis, in particular tumor necrosis factor α, interleukins-6 and 4, 5-lipoxygenase, NO synthase, soluble Fas ligand, and heat shock protein 70 in the realization of the anticonvulsant potential of digoxin and sodium valproate under conditions of experimental epileptogenesis.

MATERIALS AND METHODS OF RESEARCH

The study was conducted on 40 outbred male albino mice weighing 20-24 g obtained from the Educational and Scientific Institute of Applied Pharmacy of the National University of Pharmacy (Kharkiv, Ukraine). Animals were kept in a well-ventilated room on a standard vivarium diet (laboratory rodent chow) with free access to water under controlled conditions (20-24°C and 50% humidity with a 12 h light/dark cycle) [9].

Experiment was 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 National University of Pharmacy Bioethical Committee (Protocol No. 3, September 10, 2020).

Murine model of chronic epileptogenesis, kindling induced pentylenetetrazole (PTZ), has been chosen [8].

For the experiment all animals were randomly divided into 5 groups 8 mice in each: 1 – vehicle control (VC); 2 – positive control (PC), PTZ-induced kindling without treatment; 3 – PTZ-induced kindling + sodium valproate; 4 – PTZ-induced kindling + digoxin; 5 – PTZ-induced kindling + sodium valproate + digoxin.

For kindling modeling, PTZ was used in a dose of 30 mg/kg intraperitoneally for 16 days. All studied medicines were administered 30 min before PTZ. Control mice (groups 1 and 2 – vehicle control and positive control, respectively) received intragastrically and subcutaneously solvent (water) in a volume of 0.1 ml/10 g body weight for 16 days. Digoxin was used subcutaneously at a subcardiotonic dose of 0.8 mg/kg ($^1/_{10}$ LD_{50}), which showed an anticonvulsant effect previously. Sodium valproate was administered intragastrically at a dose of 150 mg/kg.

Medicines have been used in the form of officinal drugs: digoxin (Digoxin-Zdorovie® 0.25 mg/ml solution for injection, DNCLZ / Zdorovie, Ukraine) and sodium valproate (Depakine® 57.64 mg/ml syrup, Sanofi Aventis, France). Pentylenetetrazole (PTZ) has been used in the form of a substance (Sigma, USA).

Number of mice with convulsions (clonic/tonic) in the groups on day 16 of the kindling was determined.

After 16 days of PTZ-induced kindling, animals were euthanized by dislocation of the cervical vertebrae, their brains were removed and shockly frozen with liquid nitrogen. Biological samples stored in a freezer at -70°C. Before examining tissue samples were homogenized with standard phosphate-buffered saline (buffer-to-material ratio is 10:1) at a tempetature of -4°C and thrice centrifugated at 6000×g for 10 min.

Interleukin-6 (IL-6), tumor necrosis factor α (TNF-α), interleukin-4 (IL-4), Fas ligand (FasL), heat shock protein 70 (HSP_{70}), 5-lipoxygenase (5-LOX) and nitric oxide synthase (NOS) have been identified in the whole brain homogenates. Species-specific enzyme immunoassay kits (MyBioSource, USA) have been used.

For statistical processing of the results, the licensed program Statistica 10.0 (StatSoftInc., serial number STA999K347156-W) was used. The results were expressed as mean ± standard error of the mean (M±m) as well as median and lower and upper quartiles (Me [Q_{25}–Q_{75}]). Normality of distributions was assessed with the Shapiro-Wilk test. The Kruskal-Wallis test is used to verify the null hypothesis. Following a significant overall effect ($p<0.05$), post hoc pairwise comparisons were conducted using the

ranking Mann-Whitney U test with Holm correction. Differences between qualitative (categorical) data such as the number of mice with seizures in groups were assessed using Fisher's exact test. In such cases, the results are given in %. Spearman's correlation coefficient (ρ) was used to establish the relationship between indicators. The levels of statistical significance were considered as $p<0.05$ [10].

RESULTS AND DISCUSSION

By the end of the experiment (day 16), all animals in the PC group had seizures (Table 1). In particular, severe tonic paroxysms were recorded in 75% of mice. Both valproate and digoxin *per se* significantly reduced the number of mice with seizures. Thus, clonic seizures against the background of classical AED were observed in 62.5% ($p<0.01$) of mice in the group, while with the use of cardiac glycoside – in 75% ($p<0.05$) of animals. In terms of the effect on the tonic component of seizures, both drugs were equally effective, highly reliably reducing the percentage of animals in which such seizures were observed to 12.5% ($p<0.01$). The combination of valproate with digoxin completely prevents the development of spontaneous seizures – there were no clonic or tonic paroxysms in this group of animals on day 16 of PTZ-induced kindling (Table 1).

Table 1

Number of mice with seizures on day 16 of the pentylenetetrazole-induced kindling simulation under the influence of valproate, digoxin and their combination

Group	Number of mice, %		
	with seizures	with clonic seizures	with tonic seizures
Positive control, PTZ (n=8)	100	100	75
Valproate (n=8)	62,5**	62,5**	12,5**
Digoxin (n=8)	75*	75*	12,5**
Valproate + Digoxin (n=8)	0**##^^	0**##^^	0**##^^

Notes: statistically significant differences * – $p<0.05$; ** – $p<0.01$ – compared with positive control, PTZ; ## – $p<0.01$ – compared with valproate; ^^ – $p<0.01$ – compared with digoxin.

The effect of digoxin, sodium valproate and their combination on kindling-induced changes in cerebral cytokines content is given in Table 2.

It was found that chronic epileptogenesis is associated with activation of cerebral inflammation: a statistically significant induction of pro-inflammatory TNF-α expression – by 1.5 times, as well as a expressive, but statistically insignificant (apparently due to the high dispersion of the indicator) increase in IL-6. In addition, in the positive control group, a tendency to depletion of the cerebral pool of anti-inflammatory IL-4 – by 1.3 times ($p>0.05$) compared with vehicle control animals was observed.

Table 2

Impact of valproate, digoxin and their combination on the cytokine expression in the brain of mice under the pentylenetetrazole-induced kindling, M±m; Me [Q_{25}–Q_{75}]

Group		IL-6, pg/g	TNF-α, pg/g	IL-4, pg/g
Vehicle control		9.29±1.92 8.07 [4.05; 15.01]	60.44±7.83 55.54 [44.36; 72.29]	2709±351 2896 [1881; 3332]
Pentylenetetrazole-induced kindling	Positive control	13.68±2.72 11.58 [7.69; 19.05]	91.08±5.25 * 89.57 [78.32; 103.95]	2108±252 2096 [1653.5; 2565.5]
	Valproate	14.81±2.59 13.64 [10.18; 20.04]	73.19±6.48 74.18 [55.72; 89.62]	3307±173 ## 3220 [2853.5; 3809]
	Digoxin	19.56±2.70 * 20.40 [11.58; 24.37]	55.55±7.52 ## 55.54 [34.36; 74.41]	3025±108 ## 3037.5 [2796.5; 3276]
	Valproate + Digoxin	13.26±2.42 14.67 [6.96; 18.72]	91.19±7.29 *^^ 85.91 [76.23; 105.50]	3032±555 3009.5 [2358; 3843.5]

Notes: IL-6 – interleukin-6, TNF-α – tumor necrosis factor α, IL-4 – interleukin-4. Statistically significant differences: * – $p<0.05$ – compared with vehicle control; ## – $p<0.01$ – compared with positive control; ^^ – $p<0.01$– compared with digoxin.

Regarding the classical AED sodium valproate, its ability to counteract neuroinflammation was noted: the drug significantly increases the expression of anti-inflammatory IL-4 – by 1.6 times compared with positive control ($p<0.01$); while it does not affect the content of IL-6 (which comparable to the group of untreated kindling mice), it normalizes the content of TNF-α – almost to the level of the group of healthy animals.

The impact of digoxin on the cytokine pathway of neuroinflammation is somewhat different: although against the background of cardiac glycoside, a statistically significant more than two-fold induction of IL-6 content is observed compared with vehicle control, at the same time only digoxin causes a significant decrease in TNF-α expression – by 1.6 times compared with animals from the positive control group. Moreover, digoxin, like valproate, highly reliably ($p<0.01$) increases the content of anti-inflammatory IL-4 – by 1.4 times compared with kindled mice.

The combined use of valproate with digoxin did not show any advantages in contrast to monotherapy with both medicines. Thus, the combination did not affect the content of pro-inflammatory cytokines: the values of both IL-6 and TNF-α were at the level of similar indicators of the positive control group. At the same time, the ability to stimulate the anti-inflammatory cytokine brain pathway by inducing IL-4 expression by 1.4 times ($p>0.05$) is preserved with the combined use of drugs.

Table 3 shows the results of the study of the effect of digoxin, sodium valproate and their combination on the cerebral content of soluble FasL, heat shock protein HSP_{70}, 5-lipoxygenase and nitric oxide synthase.

Table 3

Impact of valproate, digoxin and their combination on the expression of Fas ligand, heat shock protein 70, 5-lipoxygenase and nitric oxide synthase in the brain of mice under the pentylenetetrazole-induced kindling, M±m; Me [Q_{25}–Q_{75}]

Group		FasL, ng/g	HSP_{70}, pg/g	5-LOX, ng/g	NOS, ng/g
Vehicle control		**3.18±0.93 2.58 [1.13; 5.17]**	**1267±98 1367 [1038; 1430]**	**0.73±0.11 0.69 [0.51; 1.03]**	**2.15±0.13 2.21 [1.95; 2.42]**
Pentylenetetrazole-induced kindling	**Positive control**	**9.11±2.22 * 7.90 [4.34; 13.21]**	**2998±169 ** 2963.5 [2591; 3449.5]**	**1.82±0.25 ** 1.72 [1.28; 2.29]**	**2.61±0.22 2.49 [2.09; 3.01]**
	Valproate	**12.66±1.41 ** 13.10 [10.79; 15.54]**	**1962±200 *# 1858.5 [1489; 2401]**	**0.72±0.14 ## 0.85 [0.33; 0.97]**	**2.42±0.18 2.61 [2.09; 2.77]**
	Digoxin	**5.73±1.28 §§ 7.30 [1.67; 8.40]**	**1187±225 ##§ 1058 [711.55; 1612]**	**1.46±0.20 **§§ 1.25 [1.06; 1.82]**	**2.16±0.11 2.21 [1.95; 2.37]**
	Valproate + Digoxin	**10.53±2.28 ** 8.01 [5.83; 16.04]**	**2768±189 **§^^ 2746 [2490; 3204]**	**0.80±0.10 ## ^^ 0.82 [0.66; 1.00]**	**2.67±0.23 2.84 [2.33; 3.08]**

Notes: FasL – Fas ligand, HSP_{70} – heat shock protein 70, 5-LOX – 5-lipoxygenase, NOS – nitric oxide synthase. Statistically significant differences: * – $p<0.05$; ** – $p<0,01$ – compared with vehicle control; # – $p<0.05$; ## – $p<0.01$ – compared with positive control; § – $p<0.05$; §§ – $p<0.01$ – compared with valproate; ^^ – $p<0.01$– compared with digoxin.

It was found that FasL, a marker of apoptosis, plays a significant role in chronic epileptogenesis, as evidenced by an almost threefold increase ($p<0.05$) in its content in the positive control group compared with intact mice. Interestingly, sodium valproate does not normalize but additionally increases the cerebral level of FasL – on average to 12.66 versus 3.18 ng/g in the group of healthy animals ($p<0.01$) and 9.11 ng/g in the untreated kindled mice. Meanwhile, against the background of digoxin, a significant reduction of FasL is observed – almost to the level of the vehicle control group (5.73 versus 3.18 ng/g), which indicates the ability of the cardiac glycoside to counteract neuronal apoptosis during experimental pentylenetetrazole-induced kindling. However, the combination of valproate with digoxin did not have a normalizing effect on FasL, which remained 3.3 times (10.53 vs. 3.18 ng/g, $p<0.01$) higher compared with intact animals.

HSP_{70} can be considered as an another marker of the neurotoxic effect of chronic administration of pentylenetetrazole: the content of HSP_{70} is highly significant ($p<0.01$) – by 2.4 times compared with healthy animals – increases (apparently compensatory) in the positive control group. Sodium valproate has a moderate normalizing effect on the expression of HSP_{70}, reducing the content of this marker on average to 1962 pg/g, which is statistically significantly different from both the positive control

(2998 pg/g) and the vehicle control (1267 pg/g). Digoxin reduces HSP_{70} more significantly – by 2.5 times compared with kindled animals ($p<0.01$), to a level even lower than the indicator in intact mice group (1187 versus 1267 pg/g). The combination of valproate with digoxin, as in the case of FasL, did not contribute to the restoration of normal cerebral HSP_{70} content, the value of which remained approximately at the level of the positive control – 2768 versus 2998 pg/g, statistically significantly exceeding the indicator of the vehicle control group ($p<0.01$).

Experimental kindling is characterized by a significant increase in cerebral 5-LOX levels, by 2.5 times higher than in intact animals. Both valproate *per se* and its combination with digoxin restore this level to the vehicle control group, significantly reducing brain 5-LOX levels from 1.82 ng/g to 0.72 and 0.80 ng/g, respectively, compared with positive control ($p<0.01$). Digoxin monotherapy, however, was relatively ineffective against 5-LOX, as the enzyme levels in the brain of animals remained closer to those in untreated kindled mice and were significantly higher than those in the vehicle control group (1.46 vs. 0.73 ng/g).

No statistically significant changes in cerebral NO synthase content were detected either in the positive control group or against the background of the studied medicines and their combination.

The correlation coefficients between all the studied indicators – IL-6, TNF-α, IL-4, FasL, HSP_{70}, 5-LOX and NOS – are given in Table 4. Statistically significant positive correlations were established in the pairs HSP_{70} – 5-LOX and 5-LOX – NOS in the group of intact animals, as well as a strong negative relationship in the pair TNF-α – FasL against the background of digoxin, which indicates a deep conjugation of these indicators in maintaining brain functioning, as well as in the implementation of the central effects of cardiac glycoside. Interestingly, although chronic epileptogenesis, as well as therapy with valproate *per se* and a combination of AED with digoxin are not characterized by reliable correlations between the studied indicators, for most pairs a change was established not only in the strength, but also in the nature (positive / negative) of the relationship.

Table 4

Spearman's correlations between indicators, *ρ*

Pairs of indicators	Vehicle control	Pentylenetetrazole-induced kindling			
		positive control	valproate	digoxin	valproate + digoxin
IL-6 – TNF-α	– 0.53	+ 0.36	– 0.01	– 0.11	+ 0.22
IL-6 – IL-4	+ 0.67	– 0.13	+ 0.43	+ 0.28	+ 0.36
TNF-α – IL-4	– 0.60	+ 0.44	– 0.44	+ 0.27	– 0.49
FasL – HSP_{70}	– 0.17	– 0.20	+ 0.37	+ 0.45	– 0.07
FasL – 5-LOX	– 0.07	– 0.17	+ 0.12	+ 0.08	+ 0.24
FasL – NOS	+ 0.00	+ 0.01	– 0.10	+ 0.15	– 0.05
HSP_{70} – 5-LOX	+ 0.80*	– 0.17	+ 0.31	+ 0.16	– 0.31
HSP_{70} – NOS	+ 0.50	– 0.39	+ 0.51	+ 0.46	– 0.45
5-LOX – NOS	+ 0.79*	+ 0.22	+ 0.02	– 0.17	– 0.30
TNF-α – 5-LOX	+ 0.24	– 0.19	+ 0.59	+ 0.44	+ 0.12
TNF-α – FasL	– 0.14	+ 0.57	– 0.23	– 0.73*	+ 0.13
TNF-α – HSP_{70}	+ 0.37	– 0.11	– 0.13	– 0.02	+ 0.00
TNF-α – NOS	+ 0.49	– 0.62	+ 0.37	+ 0.14	– 0.14
IL-6 –5-LOX	+ 0.23	– 0.33	+ 0.67	+ 0.63	+ 0.04
IL-6 – FasL	– 0.47	– 0.05	+ 0.12	+ 0.69	– 0.05
IL-6 – HSP_{70}	+ 0.12	+ 0.51	+ 0.71	+ 0.52	+ 0.05
IL-6 – NOS	+ 0.02	– 0.26	+ 0.07	+ 0.10	+ 0.21
IL-4 – 5-LOX	+ 0.21	– 0.11	– 0.07	+ 0.35	+ 0.38
IL-4 – FasL	– 0.18	+ 0.97*	– 0.40	+ 0.12	+ 0.02
IL-4 – HSP_{70}	+ 0.39	– 0.16	+ 0.10	– 0.17	+ 0.19
IL-4 – NOS	– 0.34	+ 0.07	– 0.02	+ 0.07	– 0.36

Note. * – $p<0.05$.

The results obtained confirm the known information about the presence of central anti-inflammatory activity in sodium valproate, associated, in particular, with its ability to normalize the seizure-induced content of pro-inflammatory IL-1β, TNF-α and IL-6 [11, 12]. This property of classical AED may be due to the inhibition of HDAC (histone deacetylases), which is manifested in epigenetic changes that affect the expression of genes of these cytokines [13]. On the other hand, the effect on the level of cerebral cytokines may be mediated by GABA-ergic properties, since GABA itself, the release of which is affected by valproate, has a proven anti-inflammatory effect, reducing the secretion of cytokines by microglia [14].

The anti-inflammatory potential of digoxin has been studied quite extensively. Experiments have shown the ability of the cardiac glycoside to inhibit the release of pro-inflammatory cytokines [15] as well as to counteract TNF-α-induced inflammation [16]. In a clinical study involving patients with rheumatoid arthritis, digoxin exhibits pronounced immunomodulatory and anti-inflammatory properties, which indicates the therapeutic value of the cardiac glycoside as an effective adjuvant agent in addition to traditional pharmacotherapy with targeted basic antirheumatic drugs [17].

The obtained data complement the known information about the pronounced ability of digoxin to counteract neuroinflammation, which we established earlier in the model of experimental chronic epileptogenesis [8]. It has been proven that digoxin (especially in combination with valproate) has a positive effect not only on the cyclooxygenase, but also on the lipoxygenase pathways of the arachidonic acid cascade; the antagonism of the cardiac glycoside to the pro-inflammatory cytokine TNF-α has also been confirmed – now in terms of the central mechanisms of its anticonvulsant action. Of value is the ability of digoxin (both in monotherapy and in combination with valproate) to increase the cerebral level of the anti-inflammatory IL-4, the protective role of which in epileptogenesis is well known [18].

Thus, the multitargeted anti-inflammatory potential of both digoxin and valproate is crucial in their ability to counteract neuroinflammation and, as a consequence, to exert anticonvulsant effects.

Of importance is the established ability of digoxin to significantly reduce cerebral FasL content, which apparently indicates the ability of the cardiac glycoside to counteract neuroapoptosis [19] – in contrast to the lack of such an effect in valproate and the combination. A similar trend is observed with regard to the effect on HSP_{70} expression in the brain of animals – digoxin normalizes this indicator, while valproate reduces it very moderately, and the combination of drugs has almost no effect at all.

HSP_{70} is known to be highly expressed in the CNS and is considered cytoprotective by inhibiting apoptosis. Although HSP_{70} is not a direct marker of apoptosis, it plays an important role in the regulation of neuronal death and indirectly indicates stress processes, including those accompanying apoptosis [20]. Its role in epileptogenesis has been proven: for example, inhibition of HSP_{70} suppresses neuronal hyperexcitability and attenuates seizure activity by enhancing A-type potassium current [21]. Significantly higher levels of HSP_{70} have been found in children with epilepsy and febrile seizures than in healthy children, making this marker potentially useful for confirming the diagnosis of epilepsy [22].

The multiple effects of arachidonic acid, its metabolic enzymes and intermediates in epileptogenesis have recently attracted considerable interest from the scientific community [23]. However, relatively little is known about the role of the lipoxygenase pathway, in contrast to the cyclooxygenase pathway. We have confirmed the role of 5-LOX both in the pathogenesis of experimental epilepsy and in the realization of the anticonvulsant potential of valproate and digoxin – both when used separately and in combination. Moreover, correlation analysis indicates a deep interaction of the key enzyme of the lipoxygenase pathway of the brain arachidonic acid cascade with other signaling pathways, in particular, HSP_{70}- and NOS-mediated ones.

There is now a sufficient amount of scientific evidence indicating a significant role of neuronal NO synthase in epileptogenesis, which contributes to oxidative stress and neurodegeneration. In particular, it has been proven that during pentylenetetrazole kindling, a significant increase in NO levels in the brain is observed, which is due to the activation of neuronal NO synthase (especially pronounced in the hippocampus, cerebral cortex and hypothalamus) [24]. In our study, neither the neurotoxic effect of chronic administration of pentylenetetrazole, nor the use of sodium valproate, digoxin and their combination, which had a pronounced anticonvulsant effect, were associated with changes in the cerebral content of NO synthase. Such results are probably explained by the fact that we determined the level of the enzyme in the homogenate of the whole brain, and not in its individual structures.

The lack of a positive synergistic effect of digoxin and valproate on the content of individual cerebral markers, in particular IL-6, TNF-α, FasL and HSP_{70}, taking into account the pronounced potentiation of the anticonvulsant effect when they are used in combination, may be associated with different molecular

mechanisms of action of the medicines at the epigenetic level and requires additional research.

Thus, it can be assumed that central anti-inflammatory properties (in particular, the impact on the content of pro- and anti-inflammatory cytokines and 5-lipoxygenase), as well as neuroprotective effects (impact on soluble Fas ligand and heat shock protein 70, but not on NO synthase) are an important component of the antiepileptic mechanism of action of valproate and digoxin.

CONCLUSIONS

1. The anticonvulsant efficacy of digoxin, sodium valproate, and their combination under pentylenetetrazole-induced kindling, as well as the role of individual markers of neuroinflammation and neuronal apoptosis, in particular TNF-α, IL-6 and IL-4, 5-LOX, NOS, FasL, and HSP_{70}, in the realization of the anticonvulsant potential of these drugs have been investigated.
2. Under the pentylenetetrazole-induced kindling model in mice, it was confirmed that the combination of digoxin (0.8 mg/kg) with sodium valproate at a dose of 150 mg/kg exhibits a more pronounced anticonvulsant effect than monotherapy with these medicines.
3. It was proven that sodium valproate and digoxin exhibit pronounced anti-inflammatory properties, restoring the content of TNF-α (but not IL-6) and increasing the expression of IL-4, and in combination they also normalize the level of 5-LOX in the brain.
4. It was determined that digoxin more clearly than sodium valproate counteracts neuroapoptosis and neurodegeneration by affecting FasL and HSP_{70}.
5. It was established that cerebral NO synthase is not involved in the development of experimental seizures, nor in the anticonvulsant effect of sodium valproate and digoxin.
6. Correlation analysis indicates a complex interaction between different signaling pathways in the brain of animals during experimental epileptogenesis, which may be important in the development of new strategies in the treatment of epilepsy, primarily its refractory forms.

Contributors:

Tsyvunin V.V. – methodology, investigation, resources, data curation, formal analysis, writing – original draft;

Shtrygol' S.Yu. – conceptualization, methodology, supervision, writing – review & editing;

Lytkin D.V. – investigation, resources, data curation, validation;

Shtrygol' D.V. – investigation, resources.

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