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Anticonvulsant activity of *Fumaria schleicheri* dry extract and sodium valproate: Role of neurotrophin and cytokine pathways

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ABSTRACT

Introduction: Cytokine and neurotrophin pathways can be potential targets for principally new anticonvulsant drugs which in perspective will be probably able to solve the problem of pharmacological resistance for people sick with epilepsy and to avoid polypragmasy which considerably increases the risk of developing side effects. **Objective:** The role of neurotrophin and cytokine link in anticonvulsant activity of *Fumaria schleicheri* Soy.-Will. dry extract and sodium valproate was assessed by the change in the cerebral level of NGF and IL-1β, TNF-α, IL-4. **Materials and Methods:** A total of 51 mice were used. The animals were divided into 4 groups: intact control, pentylenetetrazole, pentylenetetrazole + *F. schleicheri* dry extract, pentylenetetrazole + sodium valproate. *F. schleicheri* dry extract (100 mg/kg) and sodium valproate (300 mg/kg) were administered into the stomach once a day for 3 days. Pentylenetetrazole (80 mg/kg) was administered subcutaneously on the 3rd day for 30 minutes after the introduction of the tested drugs. After 18 hours the content of cerebral NGF and neurotropic cytokines was determined in the surviving animals. **Results:** Both *F. schleicheri* dry extract and sodium valproate provide an expressed anticonvulsant activity, resulting in a significant increase in the latency of the first seizure and reducing the number of clonic-tonic convulsions in 1 mouse, % of mice with tonic seizures and the duration of the convulsive period in the group. Pentylenetetrazole causes considerable changes in both neurotrophin NGF, and cytokines with the proved neurotropic properties – IL-1β, TNF-α и IL-4. *F. schleicheri* dry extract as sodium valproate, decreased pentylenetetrazole-associated NGF overexpression, normalized the content of IL-1β, TNF-α and increased the level of IL-4 compared not only with the group of animals that were getting pentylenetetrazole, but also compared with intact control. The significant correlation relationship was marked only in the seizure control between TNF-α and IL-4. **Conclusion:** Thus, the experimental evidence supports a role of NGF and IL-1β, TNF-α and IL-4 on anticonvulsant activity of F. schleicheri dry extract and sodium valproate and suggests potential targets for perspective anticonvulsant drugs.

Keywords: Cytokines, *Fumaria schleicheri*, neurotrophins, seizures, valproate

INTRODUCTION

Multidrug-resistant epilepsy is a serious problem affecting around 20–30% of patients.[1,2] Despite the wide range of antiepileptic drugs with different mechanisms of anticonvulsant activity, there are still many patients in the world population do not react to all existing medicines including the newest ones.[3-5] Hence, it is still topical to search for new drugs mainly with multiple mechanisms of activity influencing fragments of epilepsy pathogenesis.

The end of the 20th century was marked with the extremely important discovery of the role of cytokines including interleukin-1β (IL-1β) and its receptor antagonist IL-1ra, tumor necrosis factor-α (TNF-α), IL-6, and also the group of neurotrophins (nerve growth factor [NGF], BDNF, and NF-4) in the pathogenesis of epilepsy and experimental seizures.[6,7] Many cytokines, especially IL, have direct neurotrophic effects and they can also stimulate production of certain dedicated neurotrophic factors. IL-1 and IL-6 have both been shown to stimulate the production of NGF under various conditions. Increased neurotrophic factors production has been demonstrated in several experimental seizure models.[8-11]

Hence, cytokine and neurotrophin pathways can be potential targets for principally new anticonvulsant drugs which in perspective will be probably able to solve the problem of pharmacological resistance for people sick with epilepsy and to avoid polypragmasy which considerably increases the risk of developing side effects.

The data about the role of cytokine and neurotrophin pathways in implementing of anticonvulsant effect of the known anticonvulsant medicines (including frequently used antiepileptic drug sodium valproate) are very limited, controversial and demand further studying.[12]

The influence on cytokine link of pathogenesis is inherent for many herbal medicines among which there are still no antiepileptic drugs in the pharmaceutical market. In the previous research, we established the clear anticonvulsant properties of *Fumaria schleicheri* dry extract, outlined the range and potential mechanisms of its activity.[13-15] Still the molecular targets of extract influence including the role of neurotrophins and cytokines in implementing anticonvulsant effect demand further verification.

The aim of the present study was researching the role of neurotrophin and cytokine pathways in anticonvulsant activity of *F. schleicheri* Soy.-Will. dry extract and sodium valproate.

MATERIALS AND METHODS

Plant Material

Above-ground parts of *F. schleicheri* Soy.-Will. (Fumariaceae) were gathered during the flowering season (in full bloom) in Kharkiv region, Ukraine. The herbal material was cleaned, washed, and dried. After complete drying, the dry herbs were stored at room temperature. Then, herb samples were powdered and used for further research.

Extraction and Standardization

A 100 g of the air-dried and powdered herb of *F. schleicheri* were placed into a percolator, and extraction was allowed to run using water as a solvent in a ratio 1–20 at 80 C for 2 h. Then, the extract was filtered and concentrated in a vacuum evaporation apparatus at 50–60°C and at 80–87 kPa to a thick consistency. Finally, the extract was dried under the vacuum in the desiccators to yield 11.28 g of the dry extract with a residual moisture content of 5%.

The prepared dry extract was standardized by the content of flavonoids, alkaloids, and individual alkaloid protopine according to the European Pharmacopoeia (Strasbourg 2009) methods.

Animals and Treatment

A total of 51 adult random-bred male albino mice weighing 20–26 g were used in the present study. The animals were obtained from the vivarium of the Central Scientific-Research Laboratory, National University of Pharmacy (Kharkiv, Ukraine) and maintained at 19–24°C and at 50% humidity in a well-ventilated room with a 12 h light/dark cycle. Mice were housed in standard polypropylene cages with free access to food (standard laboratory rodent chow) and water. However, food – not water – was withdrawn 12 h before the experiment. All of the experimental protocols were approved by the Committee of Bioethics of the National University of Pharmacy. The animals were cared for in accordance with Directive 2010/63/EU of the European Parliament and of the Council of September.

The common model of pentylenetetrazole (PTZ)-induced seizures was used.[16]

The animals were randomly divided into four groups:
Group I: Intact control, $n = 7$
Group II: PTZ, $n = 20$
Group III: PTZ + *F. schleicheri* dry extract, $n = 12$
Group IV: PTZ + sodium valproate, $n = 12$

F. schleicheri dry extract was dissolved in distilled water and administered into the stomach at a dose that possesses anticonvulsant activity (100 mg/kg) once a day for 3 days.[13] Sodium valproate at a standard dose (300 mg/kg) administered in a similar mode. Groups I and II were treated with distilled water at a dose of 0.1 ml/10 g intragastrically. PTZ (80 mg/kg) was administered subcutaneously on the 3rd day for 30 min after the introduction of the tested samples. Then, the animals were placed into individual transparent plastic boxes and observed for 1 h for seizures.

Assessment of anticonvulsant action was conducted according to time (latency, duration of the convulsive period, and lifetime), conventional indicators (number of clonic-tonic convulsions in one mouse and severity of seizures), and alternative indicators (% of mice with clonic and tonic convulsions and lethality).

If convulsions had not occurred for 1 h, then the latency period would equal 60 min. The severity of seizures was evaluated according to a scale ranging from 1 to 6, where: 1 –trembling; 2 – circus movement; 3 – clonic seizures; 4 – clonic-tonic seizures with a lateral position; 5 – tonic extension; and 6 – tonic extension leading to the animal's death.[17]

NGF and Cytokines Determination

After 18 h, the surviving animals (Group I – 7; Group II – 7; Group III – 8; and Group IV – 7) were taking out of the experiment by dislocation of the cervical vertebrae and decapitated. Mice brain was immediately retrieved, frozen with liquid nitrogen, and placed into the freezer at a temperature of –70°C before use. The content of NGF, IL-1β, TNF-α, and IL-4 in homogenates of brain was determined

by the *in vitro* enzyme-linked immunosorbent assay (ELISA) using commercially available kits on the Stat Fax® 303 Plus ELISA Analyzer (USA).

Ethics Approval and Consent to Participate

All the experimental protocols were approved by the Bioethics Commission of the National University of Pharmacy (protocol No. 2, February 17, 2016). Experiments were conducted in accordance with "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes."

Chemicals and Reagents

PTZ was purchased from Sigma-Aldrich (USA).

Sodium valproate was used in the form of syrup 57.64 mg/1 ml (trade name Depakine, Sanofi-Aventis, France).

Commercially available kits from RayBiotech, Inc., USA (β-NGF), Vector-Best, Russian Federation (IL-1β), and cytokines, Russian Federation (TNF-α and IL-4) were used for ELISA.

Statistical Analysis

STATISTICA 8.0 for Windows has been used. Results are expressed as mean ± standard error of mean. The level of statistical significance was considered as $P < 0.05$. Statistical differences between groups were analyzed using the Mann–Whitney *U*-test and the Fisher's angular transformation (with Yates correction, if necessary). Relationship between the individual parameters was determined using the Spearman's correlation coefficient of *P* and multiple correlation coefficient of r.

RESULTS

In mice under PTZ -induced seizures, *F. schleicheri* dry extract at a dose of 100 mg/kg has shown an expressed anticonvulsant activity, resulting in a significant increase in the latency of the first seizure and reducing the number of clonic-tonic convulsions in 1 mouse, % of mice with tonic seizures, and the duration of the convulsive period in the group [Table 1]. Sodium valproate (300 mg/kg) at the same time significantly increased the latency period of the first attack, reduced the number of clonic-tonic seizures in 1 mouse, % of mice with clonic and tonic convulsions, and also the severity of seizures. Furthermore, *F. schleicheri* dry extract (but not sodium valproate) has shown a significant influence on the integral protective index – reduction of lethality – by decreasing animals' death from 65 to 33% ($P < 0.05$) compared with PTZ control group [Table 1].

The results obtained in surviving mice, which brain has been used for NGF and cytokine determination, are presented at Table 2. It was found the similar modality of *F. schleicheri*

Table 1: The influence of *Fumaria schleicheri* dry extract and sodium valproate on the pentylenetetrazole-induced seizures in mice, M±m

Indicators		Pentylenetetrazole-induced seizures		
		Pentylenetetrazole (*n*=20)	*Fumaria schleicheri dry* extract (*n*=12)	Sodium valproate (*n*=12)
Latency, min		5.99±0.44	14.54±4.23*	21.42±6.75*
Number of clonic-tonic seizures in 1 mouse		4.00±0.40	2.00±0.35*	2.17±0.51*
% of mice with convulsions	Clonic	100	92	75*
	Tonic	100	83*	58*
Severity of seizures, points		5.40±0.20	4.25±0.49	3.58±0.70*
Duration of the convulsive period, min		13.72±1.53	6.97±2.11*	10.25±3.12
Lifetime of the animal to death, min		18.44±1.75	15.82±1.51	25.54±3.84
Lethality, %		65	33*	42

* - $P<0.05$ when compared with PTZ

Table 2: The influence of *Fumaria schleicheri* dry extract and sodium valproate on the pentylenetetrazole-induced seizures indicators in surviving mice, M±m

Indicators		Pentylenetetrazole-induced seizures		
		Pentylenetetrazole (*n*=7)	*Fumaria schleicheri* dry extract (*n*=8)	Sodium valproate (*n*=7)
Latency, min		6.37±0.83	16.27±6.33*	31.38±10.16*
Number of clonic-tonic seizures in 1 mouse		4.57±0.95	2.00±0.42*	1.14±0.51*
% of mice with convulsions	Clonic	100	88	57*
	Tonic	100	75*	38*
Severity of seizures, points		4.28±0.18	3.38±0.50	2.00±0.72*
Duration of the convulsive period, min		15.42±3.53	7.47±2.84*	4.66±3.14*

* - $P<0.05$ when compared with PTZ

dry extract and sodium valproate action – they both shown statistically significant changes in latency period of the first convulsion, number of clonic and tonic seizures in 1 mouse, and duration of the convulsive period. Sodium valproate opposite to *F. schleicheri* dry extract also decreased the % of mice with clonic convulsions and the severity of seizures [Table 2].

In the brain of mice that were getting PTZ, one could observe considerable changes in all studied immunochemical indicators: Both neurotrophin NGF and cytokines with the proved neurotropic characteristics – IL-1β, TNF-α, и IL-4 [Table 3].

Two-fold increasing the level of NGF [Table 3] in the experimental animals' central nervous system (probably, compensatory) in 18 h after administering a convulsing agent compares with the research data outlining the similar effect on humans with epileptiform activity[18] and on animals on the models of seizures with different pathogenesis.[19]

F. schleicheri dry extract similar to the comparison drug sodium valproate, considerably decreased PTZ -associated NGF overexpression [Table 3], moreover, the extract significantly decreased the level of cerebral NGF in 1.8 times even compared with intact control ($P < 0.001$).

Besides, both *F. schleicheri* dry extract and sodium valproate normalized the content of pro-neurotoxic cytokines – IL-1β and TNF-α [Table 3], the level of which rose in the group of animals that were getting PTZ.

PTZ produced a not considerable but statistically significant influence on the level of neuroprotective IL-4 increasing its level in mice brain by 15% ($P < 0.05$) [Table 3]. At the same time, *F. schleicheri* dry extract increased the level of IL-4 in 4 times compared with the group of animals that were getting PTZ and 4.7 times compared with intact control.

The significant correlation relationship was marked only in the second group of animals (seizure control) – between the levels of neurotoxic TNF-α and neuroprotective IL-4 ($P = +0.82$) [Table 4]. Otherwise, both in the group of intact control and in the groups of animals that were getting *F. schleicheri* dry extract and sodium valproate, there were no statistically significant relationships between NGF and cerebral cytokines [Tables 4 and 5].

Multiple correlations, which show the relationship between three cytokines in mice brain, also found a strong significant positive dependence between TNF-α and IL-4 (with unchangeable IL-1β content) under the PTZ administration (seizure control group): r = +0.80 [Table 6]. Cumulative correlation coefficient (IL-1β – TNF-α – IL-4) showed more than two-fold increase in PTZ group compared with intact control. At the same time, *F. schleicheri* dry extract opposite to sodium valproate normalized this ratio to the intact control value [Table 6].

DISCUSSION

In the central nervous system for the 1st time, cytokines were discovered in glial cells.[20] Together with neurotrophins,

Table 3: The influence of *Fumaria schleicheri* dry extract and sodium valproate on brain NGF and cytokines levels in mice after pentylenetetrazole administration, M±m (n=7–8)

Indicators	Intact control	Pentylenetetrazole-induced seizures		
		Pentylenetetrazole	*Fumaria schleicheri* dry extract	Sodium valproate
NGF, pcg/mg	4.82±0.07	9.58±0.11###	2.62±0.09*** ### ^ ^ ^	6.02±0.07*** ###
IL-1β, pcg/mg	1.23±0.03	1.79±0.02###	1.49±0.03*** ### ^ ^ ^	1.59±0.03*** ###
TNF-α, pcg/mg	0.35±0.01	1.02±0.01###	0.44±0.01*** ### ^ ^ ^	0.69±0.02*** ###
IL-4, pcg/mg	0.065±0.004	0.075±0.003#	0.303±0.005****### ^	0.104±0.002******##

– P<0.05 when compared with Group I, ## – P<0.01 when compared with intact control, ### – P<0.001 when compared with intact control, * – P<0.05 when compared with PTZ, ** – P<0.01 when compared with PTZ, *** – P<0.001 when compared with PTZ, ^ – P<0.05 when compared with sodium valproate, ^ ^ – P<0.01 when compared with sodium valproate, ^ ^ ^ – P<0.001 when compared with sodium valproate. Nerve growth factor

Table 4: Spearman's coefficients of correlation (P) between cytokines in mice brain

Indicators	Intact control	Pentylenetetrazole-induced seizures		
		PTZ	*Fumaria schleicheri* dry extract	Sodium valproate
IL-1β – TNF-α	–0.05	–0.43	–0.30	–0.27
IL-1 β – IL-4	–0.29	–0.68	+0.08	+0.43
TNF-α – IL-4	+0.20	+0.82 *	–0.26	+0.29

* – P<0.05. PTZ: Pentylenetetrazole, IL-1β and IL-4: Interleukin-1β and interleukin-4, TNF-α: Tumor necrosis factor-α

Table 5: Spearman's coefficients of correlation (P) between NGF and cytokines in mice brain

Indicators	Intact control	Pentylenetetrazole-induced seizures		
		PTZ	*Fumaria schleicheri* dry extract	Sodium valproate
NGF – IL-1β	–0.68	+0.50	+0.38	–0.59
NGF – TNF-α	+0.49	+0.18	–0.53	+0.70
NGF – IL-4	+0.11	+0.18	+0.55	–0.29

PTZ: Pentylenetetrazole, NGF: Nerve growth factor, IL-1β and IL-4: Interleukin-1β and interleukin-4

Table 6: Coefficients of multiple correlations (r) between cytokines in mice brain

Indicators	Intact control	Pentylenetetrazole-induced seizures		
		PTZ	*Fumaria schleicheri* dry extract	Sodium valproate
IL-1β – TNF-α (IL-4)	–0.11	+0.31	–0.49	–0.45
IL-1 β – IL-4 (TNF- α)	–0.31	–0.63	+0.004	+0.55
TNF-α – IL-4 (IL-1β)	+0.19	+0.80 *	–0.30	+0.47
IL-1β – TNF-α – IL-4	0.30	0.72	0.30	0.60

* – $P<0.05$. IL-1β and IL-4: Interleukin-1β and interleukin-4, TNF-α: Tumor necrosis factor-α, PTZ: Pentylenetetrazole

they probably can fulfill the ordinary characteristic for them intercellular signaling function of secondary messengers which determining the immune activity and inflammatory response of glia, and considerably, they can influence on the neuronal excitability.[21-23] The role of cytokines in neuronal excitability dues to NMDA receptor-mediated Ca^{2+} influx into neurons enhanced by IL1β,[24] increasing the glutamate release and, as a result, promoting excitotoxicity and possibly in seizure generation.[25-29] Apart from increasing of glutamatergic transmission, IL1β also inhibits the GABA-mediated Cl^- influx, directly suppressing $GABA_A$ receptors and thus possibly reducing inhibitory transmission.[30] Besides, TNF-α induces endocytosis of $GABA_A$ receptors.[31,32]

Trophic function and axon growth stimulation by NGF have particular meaning, including the occasion of epileptic seizures.[18,19]

Correction of PTZ -associated changes in the cerebral content of NGF and IL-1β, TNF-α, and IL-4 under *F. schleicheri* dry extract and sodium valproate administering proves the role of neurotrophins and cytokines in the mechanism of anticonvulsive effect of these drugs. In the previous research, we investigated the considerable influence of *F. schleicheri* dry extract on the neurotransmitter amino acids exchange in the central nervous system[14] which indirectly indicates the suppression of cytokine-mediated glutamate releasing activation and reducing GABA inhibitory effects.[32-35]

What should specifically be mentioned is the general similar influence of *F. schleicheri* dry extract and sodium valproate on the content of NGF and cytokine profile of CNS. It is proved by practically identical changes in the level of cerebral excitatory and inhibitory amino acids under administering of these medicines in the previous research.[14] However, at the same time, *F. schleicheri* dry extract leads to hyper expression of neuroprotective anti-inflammatory cytokine – IL-4 and positively distinguishes it from sodium valproate which inducing a less pronounced increase of this indicator in the group.

Deep correlation analysis using as traditional Spearman's coefficient, as rarely used multiple correlation with cumulative correlation coefficient, shows strong impairment of cytokine transmission in the CNS under the experimental seizures. From the other hand, *F. schleicheri* dry extract and sodium valproate not only affect the NGF and cerebral cytokines content but also diminish the negative PTZ impact on the relationships between them.

Responsibility of cerebral cytokine profile and content of NGF toward the seizure effect of convulsive agent and also normalization of the level of marker indicators under administering of investigated drugs (both herbal and typical anticonvulsants) enables to assume potentially new targets for perspective anticonvulsant medicines – cytokine and neurotrophin pathways.

CONCLUSIONS

Experimental studies have found that *F. schleicheri* dry extract provides a potent anticonvulsant activity not inferior to sodium valproate.

The role of cytokine and neurotrophin pathways in mechanisms of anticonvulsive activity of perspective herbal anticonvulsant drug *F. schleicheri* dry extract and sodium valproate has been investigated.

It has been established that the convulsive agent PTZ provokes considerable changes of cerebral cytokine and neurotrophin profile including compensatory overexpression of the trophic factor NGF and neuroprotective IL-4 and also the increase in the level of neurotoxic cytokines IL-1β and TNF-α. *F. schleicheri* dry extract as the reference drug sodium valproate normalizes the content of NGF, IL-1β, and TNF-α in the CNS, at the same time increasing the level of IL-4.

The experimental evidence supports a role of neurotrophic factors (NGF) and cytokines (IL-1β, TNF-α, and IL-4) on PTZ-induced seizures and suggests potential targets for perspective anticonvulsant drugs.

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AUTHORS' CONTRIBUTIONS

VT and SS conceived and designed the research. VT, TG, and DS conducted experiments. VT and SS analyzed data. VT and DS wrote the manuscript. All authors read and approved the manuscript.

CONFLICTS OF INTEREST

We wish to confirm that there are no known conflicts of interest associated with this publication.

ETHICAL CONSIDERATIONS

All the experimental protocols were approved by the Bioethics Commission of the National University of Pharmacy (protocol No. 2, February 17, 2016). Experiments were conducted in accordance with "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes."

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