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RESEARCH ARTICLE

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Effectiveness assessment of sulfur-containing amino acids in rats with experimental “alcohol withdrawal syndrome” with modified zoosocial interaction methods

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The aim of the work was to compare the effects of ademethionine, acetylcysteine and taurine on the zoosocial behavior of rats in a post-intoxicated state after an acute ethanol poisoning.

Materials and methods. The study was conducted on male rats of the Wistar line. The post-intoxication state was modeled by a single injection of ethanol (3 g/kg, intraperitoneally). 30 min after awakening, the animals were injected with a physiological solution, acetylcysteine (1 g/kg), ademetionine (100 mg/kg) or taurine (40 mg/kg). A zoosocial interaction test was performed 30 min after the administration.

Results. Among the behavioral indicators investigated, the following were validated: the number of acts of freezing, their duration, the number of acts of sniffing in front, the number of acts of avoidance and the number of vertical stances without support (p <0.05 between the values of the negative and positive control groups in all cases). The administration of acetylcysteine, ademetionine and taurine reduced the number of freezing acts by 53.64, 7.27 and 24.51%, respectively (p <0.05 when compared with the indicator index in the animals from the positive control group in all cases). The administration of acetylcysteine and taurine reduced the number of avoidance acts by 50 and 10%, respectively (p <0.05 when compared to that of the animals from the positive control group in both cases). All amino acids normalized the communicative performance, although it did not differ from that of the animals from the positive control group (p >0.05). Alcoholization reduced the number of vertical stances by 65% (p <0.001 when compared with that in the animals from the negative control group), and when followed by the administration of ademetionine and taurine, the reduction was 38 and 36%, respectively (p <0.05 when compared to that in the animals from the negative control group).

Conclusion. According to the data obtained, sulfur-containing amino acids, primarily those that had central effects, normalized neuronal functions, positively influencing a complex behavior of rats. Taking into account the results of the previous studies, it was possible to conclude that the therapeutic effect of ademetionine and taurine in the context of a post-intoxication state is mediated by their central effects, which are not so pronounced in comparison with acetylcysteine.

Keywords: ethanol; acetylcysteine; taurine; ademetionine; preclinical studies

Abbreviations: AB – alcoholic beverage; AWS – alcohol withdrawal syndrome; LPO – lipid peroxidation; mNSS – modified Neurological Severity Scores.

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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Оценка эффективности серосодержащих аминокислот у крыс с экспериментальным «алкогольным похмельем» с помощью методики зоосоциального взаимодействия

В.И. Петров[1], Н.А. Осадченко[1], А.С. Тарасов[1,2], А.М. Доценко[1,2], Е.И. Морковин[2]

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Цель. Сравнить влияние адеметионина, ацетилцистеина и таурина на зоосоциальное поведение крыс, находящихся в постинтоксикационном состоянии после острого отравления этанолом.

Материалы и методы. Исследование проведено на крысах-самцах линии Wistar. Постинтоксикационное состояние моделировали однократным введением этанола (3 г/кг, внутрибрюшинно). Через 30 мин после пробуждения животным вводили: физиологический раствор, ацетилцистеин (1 г/кг), адеметионин (100 мг/кг) или таурин (40 мг/кг). Через 30 мин после введения проводили тест зоосоциального взаимодействия.

Результаты. Среди исследуемых показателей поведения были приняты во внимание: количество актов замирания, длительность следования, количество актов обнюхивания спереди, количество актов избегания и количество вертикальных стоек без опоры (p <0,05 между показателями групп отрицательного и положительного контроля во всех случаях). Введение ацетилцистеина, адеметионина и таурина снижало количество актов замирания на 53,64, 7,27 и 24,51% соответственно (p <0,05 при сравнении с показателем у животных из группы положительного контроля во всех случаях). Введение ацетилцистеина и таурина снижало количество актов избегания на 50 и 10% соответственно (p <0,05 при сравнении с показателем у животных из группы положительного контроля в обоих случаях). Все аминокислоты нормализовали показатели коммуникативности, несмотря на то, что они не отличались от показателей у животных из группы положительного контроля (p >0,05). Алкоголизация снижала количество вертикальных стоек на 65% (p <0,001 при сравнении с показателем у животных из группы отрицательного контроля), а при последующем введении адеметионина и таурина снижение составило 38 и 36% соответственно (p <0,05 при сравнении с показателем у животных из группы отрицательного контроля).

Заключение. Согласно полученным данным, серосодержащие аминокислоты, в первую очередь те, что обладали центральными эффектами, нормализовали функции нейронов, положительно влияя на сложное поведение крыс. Учитывая результаты предыдущих исследований, удалось заключить, что терапевтическое действие адеметионина и таурина в контексте постинтоксикационного состояния опосредовано их центральными эффектами, не столь выраженными в сравнении с ацетилцистеином.

Ключевые слова: этанол; ацетилцистеин; таурин; адеметионин; доклинические исследования

Список сокращений: АН – алкогольные напитки; ПС – похмельный синдром; ПОЛ – перекисное окисление липидов; mNSS – модифицированная шкала неврологического дефицита (modified Neurological Severity Scores).

INTRODUCTION

In the modern society, alcohol abuse is common due to an increased stressor load as well as the availability of alcoholic beverages (ABs)[1,2]. The consumption of ABs in excessive amounts leads to the formation of an alcohol withdrawal syndrome (AWS). This condition is defined as “a set of mental and physiologic symptoms that a person experiences after a single episode of an alcohol consumption in large quantities, developing against the background of the absence of ethanol in the blood” [1]. According to WHO’s 2018 Global Status Report on Ethanol Consumption, 18.2% of the world population consumes ABs in the sufficient quantity to develop AWS[3]. In their systematic review, Gunn C. et al. (2018) presented the evidence supporting the negative impact of AWS on the performance of tests of short-

[1] Clinical Guidelines. Alcoholic liver disease in adults. Clinical guidelines rubric. Available from: https://cr.minzdrav.gov.ru/recomend/711_1. Russian

[2] Federal State Statistics Service of Russian Federation. Available from: https://rosstat.gov.ru/folder/210/document/13218. Russian

[3] WHO (World Health Organization). Global status report on alcohol and health 2018. Available from: https://www.who.int/publications/i/item/9789241565639. Russian

and long-term memory, the attention concentration, and a psychomotor reaction speed [2]. Later, this observation was confirmed by Palmer et al. (2020) in a study involving students taking a test to assess memory performance and other cognitive functions the day after taking NA in large quantities [3]. Later, this observation was confirmed by Palmer E.O.C. et al. (2020) in a study involving students taking a test to assess the memory performance and other cognitive functions the day after taking ABs in large quantities [3].

AWS requires research and the development of therapy, as it is a widespread condition that reduces many aspects of quality of life and creates a risk of injury both for the person experiencing it and for the people around him. Against the background of this condition, the risk of traumatization when driving a car or at work increases [4, 5], and the quality of sleep decreases [6].

The state of AWS in humans is characterized by a possible presence of 47 symptoms [7], therefore, when modeling this pathology in animals for the subsequent assessment of the effect of drugs on this state, a significant number of behavioral tests should be used, in which various aspects of motor, emotional and cognitive spheres of the psyche are evaluated [3]. Since the postintoxication state in animals is characterized by an impaired locomotor function [8], the Combs and D'Alecy neurological deficit scales [9] and modified Neurological Severity Scores (mNSS) [10, 11] are used to assess its severity. The Morris Water Maze test [12] and the Open Field test [12] are suitable for assessing the effect of AWS and the effect on cognitive functions (mnestic, an exploratory activity) [12] and "Open Field" [10, 13]. The "Open Field" test is also aimed at studying an anxiety activity [13], i.e. to assess the emotional sphere of the psyche, which is influenced by AWS.

In Russia, for the therapy of AH, drugs and dietary supplements based on acetylsalicylic and/or succinic acids, sorbents to be used during the alcohol consumption to prevent the occurrence of AWS, and symptomatic drugs other than those mentioned above – arginine glutamate drugs, herbal drugs are used. Thus, the hangover therapy to date has been limited to symptomatic and prophylactic approaches[4].

AWS is a special intoxication case, which is accompanied by a decrease in glutathione stores and a toxic liver damage, leading to neurological disorders. The hypothesis of the study is that sulfur-containing amino acids, which are participants in the glutathione metabolism, can be used to treat AWS. Repurposing of known and studied drugs for new indications is a promising way to find solutions for the treatment of common pathologies.

[4] State Register of Medicines of Russian Federation. Available from: https://grls.minzdrav.gov.ru/Default.aspx. Russian

For acetylcysteine, in addition to mucolytic action, antioxidant properties [14] and an NO-ergic activity [15] have been proven; however, the results of a clinical trial on the efficacy of acetylcysteine in AWS were mixed [16]. Taurine had a positive effect on the course of experimental neurological deficits caused by poisoning [17], ischemia [18], brain injury [19], and hemorrhage [20]. The role of this sulfonic acid in the course of neurologic deficits has been confirmed in clinical studies [21]. Ademethionine exerts various pharmacological effects on the central nervous system, in particular, it affects the metabolism of monoamine neurotransmitters and receptor systems [22, 23], which is also reflected in the behavioral performance. The metabolism of these sulfur-containing amino acids involves the formation of glutathione, the deficiency of which is a central link in the pathogenetic chain of alcohol withdrawal syndrome. On this basis, the administration of sulfur-containing amino acids was hypothesized to prevent a glutathione depletion and an alleviate oxidative stress leading to the impaired metabolic activity of the liver and impaired neuronal functioning.

THE AIM of the study was to compare the effects of ademethionine, acetylcysteine, and taurine on the zoosocial behavior of rats in a postintoxicated state after acute ethanol poisoning.

MATERIALS AND METHODS

Experimental animals

The study was performed on 40 male Wistar rats with a body weight of 300–450 g, obtained from the laboratory animal nursery Rappolovo (Russia). The animals were kept in the standard vivarium conditions under a light-dark cycle of 12/12 h, the temperature of 20±2°C and the humidity of 40-60%. The animals received water and food *ad libitum*.

Ethical approval

All experimental studies were conducted in accordance with the Rules of Laboratory Practice approved by the order of the Ministry of Health of Russia No. 708n dated 23 August 2010, in strict compliance with the European Convention for the Protection of Vertebrate Animals Used for Experiments or Other Scientific Purposes (Directive 2010/63/EC). The protocol was approved by the Regional Independent Ethical Committee at Volgograd State Medical University (IRB 00005839 IORG 0004900 (OHRP) protocol No. 132 dated 20 May 2019).

Study design

A total of 80 animals were used; 40 of them were intact and the other 40 were used in the experimental series. Five groups of 8 animals each were formed. The animals from the positive control and experimental

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groups were injected with a 20% aqueous ethanol solution at a dose of 3 g/kg, once intraperitoneally, and immediately after awakening (a sleep duration was 5±0.5 h), they were administered one of three drugs: acetylcysteine (1 g/kg, intragastrically once), ademethionine (100 mg/kg, intragastrically once) or taurine (40 mg/kg, intragastrically once), or a saline solution in the appropriate volume (in the positive and negative control groups). The doses for the administration had been chosen based on the literature data and according to the results of the experiments conducted previously [24–27]. The animals from the negative control group were injected with a physiologic solution in an appropriate volume instead of ethanol, and the physiologic solution was administered instead of drugs. The scheme of the study design is presented in Figure 1.

Studied compounds

The following drugs in a powder form were used in this work: acetylcysteine (Zambon, Italy), ademethionine butanedisulfonate (VEROPHARM LLC, Russia), taurine (Supptrue Taurine, Russia).

Assessment of zoosocial behavior

The assessment of the zoosocial behavior according to Petrov V.I. methods [28] was started 30 min after the administration of the tested drugs. Since the interaction between two animals is evaluated in the experiment, 40 healthy animals which were pairs for the tested rats, were used for the test. After typing the animals by body weight, animal was matched with a pair for a resident–intruder interaction among the 40 additional animals.

The test setup was a 97×97 cm square open field without burrows, located in a dark room and illuminated with red light [29]. A pair of animals – first intact (resident), then an animal from the experimental group (intruder) – was placed in the center of the setup in turn and observed for 10 min.

The following components of the animal behavior were assessed: anxiety, sociability, negativity, exploratory activity, and aggressiveness. An anxiety was evaluated by the number of acts of freezing and the number of acts of short grooming. A communicative behavior was assessed by the duration of following the intact animal, the number of sniffing acts (front, side, tail, and anus), and the number of allo- and autogrooming acts. The avoidance behavior was assessed by the number of acts of movement away from the resident. The exploratory behavior was assessed by the number of upright stances with and without a wall support. The aggressiveness was assessed by the number of acts of approaching from aside.

Statistical processing

The statistical processing was performed by methods of descriptive and analytical statistics. The distribution of quantitative values was evaluated using the Shapiro–Wilk test. The intergroup differences were assessed by a one-way analysis of variance using the Newman–Keuls post hoc test. All the data were presented as the mean and standard error of the mean (unless otherwise indicated). The differences in the categorical data were evaluated by the chi-square test. The differences between the indicators in the group were considered statistically significant at p <0.05.

The results were processed and analyzed using Microsoft Excel 2019 (Microsoft Corporation, USA) and GraphPad Prism 5 statistical package (Dotmatics, USA).

RESULTS

The obtained results and their statistical processing are presented in Table 1.

Anxiety

The numbers of freezing acts in the animals from the negative and positive control groups were 13.75±2.96 and 22.5±5.21 (p <0.01), respectively. In the animals administrated with acetylcysteine, ademethionine, or taurine after the alcoholization, these values were 6.38±3.16, 12.75±9.59 and 10.38±7.11, respectively (p <0.01 in all cases when compared with the number of animals in positive control group).

The numbers of short grooming acts in the animals from the negative and positive control groups were 4.38±2.07 and 7.88±4.19, respectively (p >0.05). Thus, the alcoholization had no statistically significant effect on the number of acts of short grooming. In the animals from acetylcysteine, ademethionine, and taurine groups, the indices were 2.63±2.13, 3.86±2.85 and 5.5±4.93, respectively. The index in the animals from the acetylcysteine group was statistically significantly lower than in the animals from the positive control group (p <0.05). Since the assessment of this behavioral component did not pass validation (negative and positive controls did not differ), it is not possible to interpret the effect of acetylcysteine as anxiolytic. The results of the anxiety assessment are presented in Figure 2.

Communicativeness

The average duration of following the resident in animals from the negative and positive control groups was 18.38±3.75 and 8.05±4.7 s, respectively (p <0.05). The value in the animals from the acetylcysteine, ademethionine and taurine groups did not differ statistically significantly from that in the animals from the control groups and occupied intermediate values: 9.55±5.54, 14.47±7.72 and 11.06±9.77 s, respectively.

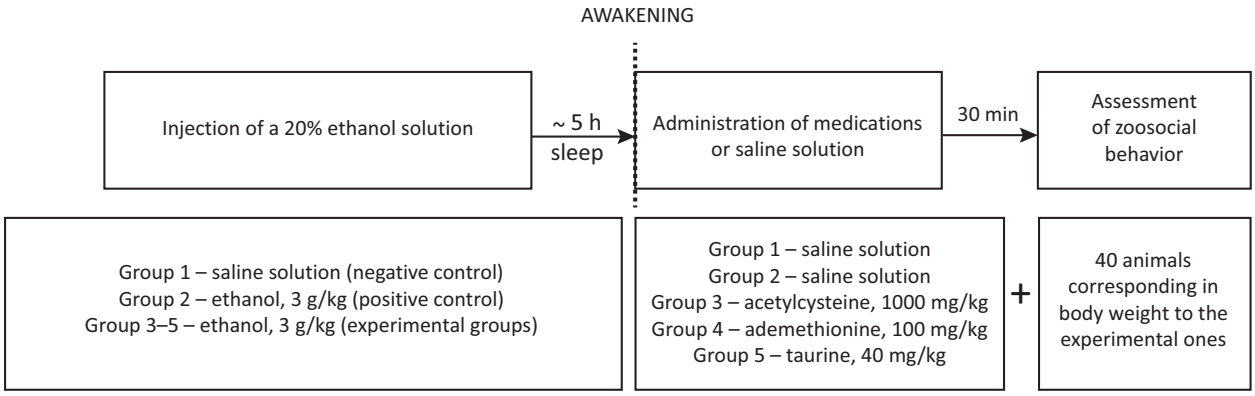

Figure 1 – Schematic study design to assess zoosocial behavior

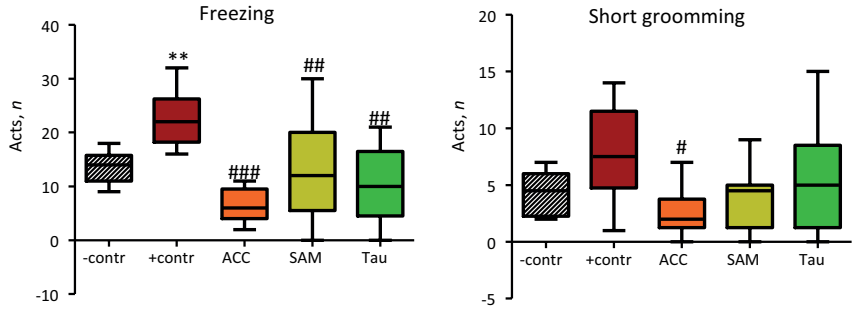

Figure 2 – Effect of acetylcysteine, ademethionine, and taurine administration in post-intoxication phase on anxiety indices in zoosocial interaction in ethanol-intoxicated animals

Note: -contr – negative control group; +contr – positive control group; ACC – animals administered with acetylcysteine; SAM – animals administered with S-ademethionine; Tau –animals administered with taurine; ** – statistically significant difference from the animals from the negative control group at p <0.01; # – statistically significant difference from the animals from the positive control group at p <0.05; ## – statistically significant difference from the animals from the positive control group at p <0.01; ### – statistically significant difference from the animals from the positive control group at p <0.001; the data are presented as median, standard deviation (box) and minimum and maximum values (whiskers).

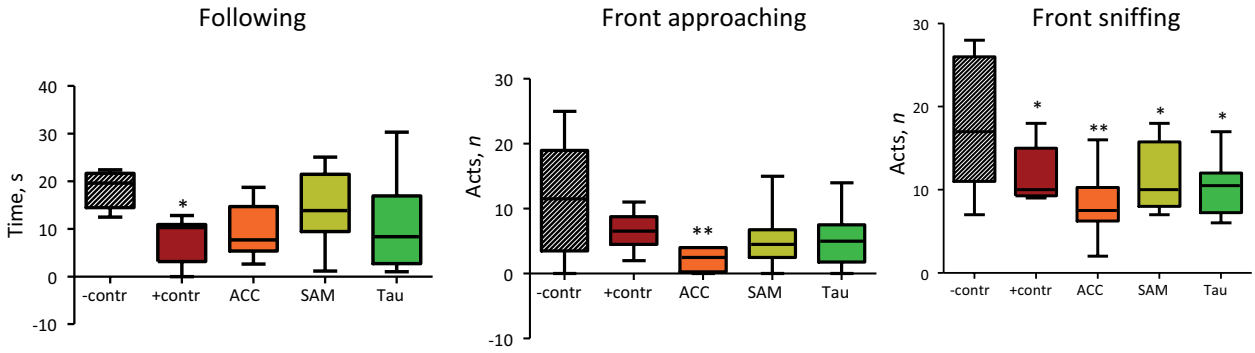

Figure 3 – Effect of acetylcysteine, ademethionine, and taurine administration in post-intoxication phase on communicative indices in zoosocial interaction in ethanol-intoxicated animals

Note: -contr – negative control group; +contr – positive control group; ACC – animals administered with acetylcysteine; SAM – animals administered with S-ademethionine; Tau – animals administered with taurine; * – statistically significant difference from the negative control group animals at p <0.05; ** – a statistically significant difference from the animals from the negative control group animals at p <0.01; the data are presented as median, standard deviation (box) and minimum and maximum values (whiskers).

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Table 1 – Results of measuring behavioral indicators reflecting neurological deficits and impaired zoosocial interaction

Behavioral component	Negative control	Positive control	Acetylcysteine	Ademethionine	Taurine
Anxiety			Acts of freezing, *n*		
	13.75±2.964	22.50±5.210 (+63.64%)**	6.375±3.159 (–53.64%)###	12.75±9.588 (–7.27%)##	10.38±7.110 (–24.51%)##
			Acts of short grooming, *n*		
	4.375±2.066	7.875±4.190 (+80%)	2.625±2.134 (–40%)*	3.875±2.850 (–11.43%)	5.500±4.928 (+25.71%)
Communicativeness			Duration of the following, s		
	18.38±3.752	8.046±4.696 (–56.22%)*	9.548±5.536 (–48.05%)	14.47±7.715 (–21.27%)	11.06±9.771 (–39.83%)
			Acts of front approaching, *n*		
	11.50±8.635	6.625±2.825 (–42.39%)	2.250±1.753 (–80.43%)**	5.375±4.47 (–53.26%)	5.375±4.34 (–53.26%)
			Acts of front sniffing, *n*		
	17.88±7.661	11.75±3.412 (–34.28%)*	8.125±4.051 (–54.56%)**	11.50±4.243 (–35.68%)*	10.38±3.503 (–41.95%)*
			Acts of side sniffing, *n*		
	7.875±2.357	6.000±1.604 (–23.81%)	6.875±2.031 (–12.70%)	6.250±3.196 (–20.63%)	9.250±2.550 (+17.46%)
			Acts of anus sniffing, *n*		
	10.63±6.391	6.375±3.815 (–40.03%)	4.125±1.458 (–61.19%)	5.750±4.950 (–45.91%)	6.625±4.838 (–37.68%)
			Acts of tail sniffing, *n*		
	10.38±8.535	10.13±4.998 (–2.41%)	4.250±3.370 (–59.06%)	7.625±4.470 (–26.54%)	6.750±3.576 (–34.97%)
			Acts of allogrooming, *n*		
	1.000±0.5345	0.3750±0.7440 (–62.5%)	0.8750±0.8345 (–12.5%)	0.6250±0.7440 (–37.5%)	0.6250±0.9161 (–37.5%)
			Acts of autogrooming, *n*		
	3.750±2.493	1.750±1.389 (–53.33%)	3.875±2.031 (+3.33%)	3.250±1.982 (–13.33%)	5.250±5.392 (+40%)
Negativity			Acts of avoidance, *n*		
	2.500±1.195	4.125±1.553 (+65%)*	1.250±1.035 (–50%)###	3.250±1.488 (+30%)	2.250±1.165 (–10%)#
Exploratory behavior			Acts of rearing with support, *n*		
	28.50±6.655	27.63±9.211 (–3.05%)	17.13±7.180 (–39.89%)	23.38±8.959 (–17.96%)	24.63±9.380 (–13.58%)
			Acts of rearing without support, *n*		
	27.00±8.401	9.375±4.749 (–65.28%)***	10.00±8.089 (–62.96%)***	16.63±9.724 (–38.41%)*	17.25±8.172 (–36.11%)*
Aggression			Acts of side approaching, *n*		
	3.125±1.808	1.125±0.991 (–64%)	2.375±1.685 (–24%)	2.375±1.598 (–24%)	1.500±1.309 (–52%)

Note: * – p <0.05 when compared with animals from the negative control group; ** – p <0.01 when compared with animals from the negative control group; *** – p <0.001 when compared with animals from the negative control group; # – p <0.05 when compared with animals from the positive control group; ## – p <0.01 when compared with animals from the positive control group; ### – p <0.001 when compared with animals from the positive control group; the data are presented as an arithmetic mean and a standard deviation (a percentage decrease).

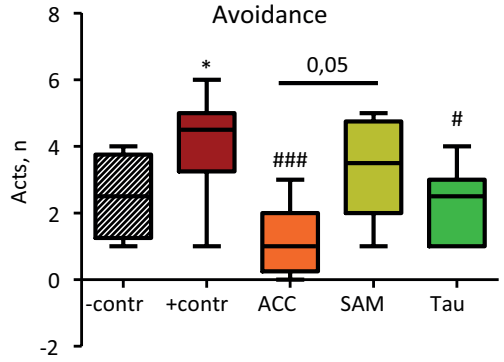

Figure 4 – Effect of acetylcysteine, ademethionine, and taurine administration in the post-intoxication phase on the number of avoidance acts of zoosocial interaction in ethanol-intoxicated animals

Note: -contr – negative control group; +contr – positive control group; ACC – animals administered with acetylcysteine; SAM – animals administered with S-ademethionine; Tau – animals administered with taurine; * – a statistically significant difference from the animals from the negative control group at p <0.05; # – a statistically significant difference from the animals from the positive control group at p <0.05; ### – a statistically significant difference from the animals from the positive control group at p <0.001; compared groups are indicated by a horizontal line; the data are presented as median, standard deviation (box) and minimum and maximum values (whiskers).

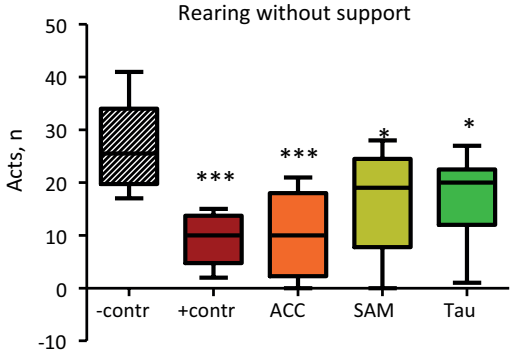

Figure 5 – Effect of acetylcysteine, ademethionine, and taurine administration in post-intoxication phase on the number of rearings without support during zoosocial interaction in ethanol-intoxicated animals

Note: -contr – negative control group; +contr – positive control group; ACC – animals administered with acetylcysteine; SAM – animals administered with S-ademethionine; Tau – animals administered with taurine; * - a statistically significant difference from the animals from the negative control group at p <0.05; *** – a statistically significant difference from the animals from the negative control group at p <0.001; the data are presented as median, standard deviation (box) and minimum and maximum values (whiskers).

The number of front approach acts in the animals from the negative and positive control groups were 11.5±8.64 and 6.63±2.83, respectively (p >0.05). Thus, the alcoholization had no significant or statistically significant effect on approaching from the front. The index in the animals from the acetylcysteine group was even lower than in the animals from the positive control group and amounted to 2.25±1.75 (p <0.01 when compared to the index in the animals from the negative control group). At the same time, the number of front sniffing acts as an anxiety score passed the validation. The values in the animals from the negative and positive control groups were 17.88±7.66 and 11.75±3.41 acts (p <0.05). The scores in the animals from the experimental groups were comparable to the mean score in the animals from the positive control group and were 8.13±4.05 (p <0.01 when compared to the intact animals), 11.5±4.24 and 10.38±3.5 (p <0.05 when compared to the intact animals in both cases) acts for acetylcysteine, ademethionine and taurine, respectively. The results of the communicative assessment are summarized in Figure 3.

Negativity (avoidance behavior)

The number of avoidance acts characterizing the negativity of behavior in the animals from the negative and positive control groups was 2.5±1.2 and 4.13±1.55, respectively (p <0.05). The index in the animals from the acetylcysteine group was lower than in the animals from the control groups and amounted to 1.25±1.04 (p <0.001 when compared to the index in the animals from the positive control group). The index in the animals from the ademetionine group occupied an intermediate position between the index in the animals from both control groups and did not differ statistically significantly from either of them, amounting to 3.25±1.49 acts (p >0.05, but p <0.05 when compared with the index in the animals from the acetylcysteine group). In the animals administered with taurine, the mean was statistically significantly different from that of the animals from

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the positive control group (p <0.05) and amounted to 2.25±1.17 acts. The results of measuring the number of avoidance acts are presented in Figure 4.

Exploratory behavior

The number of unsupported stances in the animals from the negative and positive control groups were 27±8.4 and 9.38±4.75, respectively. In the animals from acetylcysteine, ademethionine and taurine groups, the values were 10±8.09 (p <0.001 when compared to negative control), 16.63±9.72 and 17.25±8.17 (p <0.05 when compared to the negative control). The results are summarized in Figure 5.

Among the indicators assessed in the zoosocial behavior test, the parameters showing the following behavioral components: anxiety (acts of freezing), sociability (following, front sniffing), negativity (acts of avoidance) and exploratory behavior (rearing without support), were validated.

The administration of acetylcysteine, ademetionine, and taurine reduced the number of acts of freezing by 72, 43, and 54%, respectively, when compared with the rate in the positive control group. The duration of following the resident increased by 19, 80 and 38%, respectively. The number of sniffing acts decreased in all treatment groups, but was greatest in the acetylcysteine group.

DISCUSSION

Metabolic therapy agents with antioxidant, hepato- and neuroprotective properties can correct the course of toxic neuropathies [30]. The choice of drugs for the study was conditioned by the presence of sulfur atom in the molecule of ademethionine, acetylcysteine and taurine and their ability to form disulfide bonds.

Acetylcysteine is a derivative of L-cysteine, which is a precursor of the antioxidant tripeptide glutathione. The basis of a mucolytic action of acetylcysteine is its ability to discharge disulfide bonds of mucoproteins, which leads to the liquefaction of sputum. There are reasons to believe that due to its antioxidant properties, acetylcysteine may have an antiapoptogenic effect [31–33]. It has been suggested that acetylcysteine normalizes a glutamate neurotransmission in various brain structures [34]. The use of acetylcysteine to improve the condition after an acute alcoholization may have a dual effect – an improvement of the substance processing in the liver and a protective effect on the nervous system, which helps to reduce the desire to use ABs.

Taurine is a 2-aminosulfonic acid, a sulfonic acid that is widely distributed in living organisms and involved in many metabolic processes. Most mammals, including adult humans, are capable of self-synthesizing taurine. They obtain it directly from food of the animal origin in the amounts sufficient to meet metabolic needs[5]. Taurine deficiency is observed in various diseases, especially in diabetes mellitus and cardiovascular diseases. Against the background of the taurine administration to the animals with experimental diabetes mellitus, an increase in the glycogen content in the liver and an increase in the glucose utilization by muscles were observed [35]. Taurine can bind to lipid hydroperoxides that disrupt the integrity of the vascular epithelium, which prevents cell apoptosis and the development of endothelial dysfunction [36].

S-adenosylmethionine, an intermediate product of taurine synthesis, acts as a carrier of methyl groups in the body, which allows it to be used as a drug for the treatment of both hepatobiliary disorders and some types of depression [37–40].

In previous studies on the effects of sulfur-containing amino acids on biochemical parameters in the post-intoxication state, it was found out that acetylcysteine, ademethionine and taurine restored a liver function by normalizing the levels of aspartate aminotransferase and glutathione. Acetylcysteine had the most pronounced positive effect on these parameters, reducing an aspartate aminotransferase activity by 16% and increasing glutathione reserves by 16% relative to the parameters in the positive control group. Ademethionine and taurine decreased the aspartataminotransferase activity by 9 and 11%, respectively. Both drugs had a clear positive effect on the recovery of glutathione stores, increasing them by 11% in both cases [41].

As a part of the evaluation of neuro- and hepatoprotective effects of sulfur-containing amino acids in the conditions accompanied by a decrease in glutathione reserves, a spectrum of behavioral assessments was previously used: an elevated cruciform maze, an open field, an adhesive test, a test of a conditioned passive avoidance response, Morris water maze test, as well as Combs and D'Alecy scales and mNSS. According to the results of the earlier studies, the use of acetylcysteine, ademetionine and taurine in the rats undergoing a acute alcohol intoxication partially corrects behavioral disorders. The most pronounced effect was exerted by ademethionine, increasing the mean Combs and D'Alecy score relative to the index in the animals with experimental ABs without treatment. A motor activity in the "open field" test among the alcoholized animals was also the highest in the ademetionine group [10].

Since the suppression of neuropsychiatric changes in animals after an acute alcoholization coincided with an increase in the amount of glutathione stores in the

[5] Froger N. Taurine Deficiency and the Eye. Handbook of Nutrition, Diet and the Eye. V.R. Preedy, J. Sahel, S. Picaud editors. Academic Press; London: Elsevier. 2014;51:505–13. DOI: 10.1016/B978-0-12-401717-7.00051-4

liver, it was concluded that the action mechanism of all the studied drugs is mediated by a hepatoprotective action.

However, AWS in humans is a multifactorial condition, so a zoosocial interaction test was performed to assess behavioral components not accounted for by the above-mentioned methods. Despite the fact that only anxiety, sociability, negativity, and exploratory kinds of activity have been validated, the results obtained for these behavioral components correlate with the results of standard behavioral testing, adding an additional clarification to the existing ideas about modeling and treatment of AWS in an *in vivo* experiment.

Among the compounds studied, ademethionine had the most pronounced effect on the communicative behavior, while acetylcysteine worsened the condition, which is presumably due to the central effects of ademethionine and taurine, which acetylcysteine does not possess [23, 42, 43]. Acetylcysteine significantly suppressed the avoidance behavior, reducing the number of avoidance acts by 70% (*vs* 21 and 45% in the ademetionine and taurine groups), and its administration also resulted in a reduction in the number of freezing acts. Taurine most pronouncedly enhanced the exploratory component of behavior. The administration of taurine, ademetionine and acetylcysteine increased the number of unsupported vertical stands by 84, 77 and 7%, respectively.

The post-intoxication state, in addition to the previously detected anxiogenic effect and suppression of the exploratory activity, impaired the animal communication, enhancing the avoidance-related behavior (negativity). According to the results of the previous experimental series, acetylcysteine has a significantly more pronounced effect on the biochemical component of a postintoxication neuropsychiatric failure than ademethionine and taurine.

According to the data obtained in this experimental series, sulfur-containing amino acids, primarily those with central effects, normalized a neuronal function, positively affecting the complex behavior of rats.

Limitations of the study

As mentioned above, AWS in humans is characterized by the presence of 47 symptoms, all of which cannot be modeled and evaluated in animals. Therefore, preclinical studies of the described condition do not allow a direct extrapolation of the findings to humans. Clinical trials are required to develop an effective therapy for AWS.

CONCLUSION

Among the studied compounds, ademethionine had the most pronounced effect on the communicative behavior, while acetylcysteine worsened the condition, which is presumably due to the central effects of ademethionine and taurine, which acetylcysteine does not possess. Acetylcysteine significantly suppressed the avoidance behavior, reducing the number of avoidance acts by 70% (*vs.* 21 and 45% in the ademetionine and taurine groups), and its administration also resulted in a reduction in the number of freezing acts. Taurine most pronouncedly enhanced the exploratory component of behavior. The administration of taurine, ademetionine and acetylcysteine increased the number of unsupported vertical stands by 84, 77 and 7%, respectively.

Taking into account the data obtained in the zoosocial interaction test, it was possible to clarify that the contribution of the central effects of ademethionine and taurine was greater than the metabolic ones in the context of the studied condition.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

All authors have made equal and equivalent contributions to the preparation of the publication. All authors confirm that their authorship complies with the ICMJE international criteria (all authors made a significant contribution to the conceptualization, research and preparation of the article, read and approved the final version before the publication). Vladimir I. Petrov – idea of the experiment, methodology and scientific guidance; Nazar A. Osadchenko, Alexander S. Tarasov, Anna M. Dotsenko – literature review, collection of materials, conducting experiments, writing and editing the article; Evgeny I. Morkovin – search of sources for the literature review, statistical processing of the results.

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Analysis of actual results of drug supply implementation within framework of High-Cost Nosologies Program

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The accessibility and pharmaceutical care coverage are linked to political, economic and managerial decisions. That fact necessitates the evaluation of the state programs results in the field of the drug provision.

The aim of the work was to assess the quantitative results of the implementation of the High-Cost Nosologies program in the Russian Federation from 2008 to 2023 to determine further vectors of its improvement.

Materials and methods. The regulatory base of the work was made up of the Russian Federation legislation in the field of the drug provision. The open sources were used as the research information base for the data collection and analysis: reports of federal and regional executive authorities, materials of specialized conferences, results of published studies.

Results. The drug coverage under the High-Cost Nosologies (VZN) program is provided for 14 nosologies, 11 of which are classified as orphan diseases. Since its implementation, the HCNs program has been expanded twice by including new nosologies in 2019 and 2020. As of 01 October 2023, the number of patients in the Federal Register of VZN was 263 721 people, which was 13.58 times greater compared to 2008. The drug provision is carried out according to the list of 47 INNs. The amount of funding for the program increased from RUB 32 bn in 2008 to RUB 87.96 bn in 2023. The most resource-intensive nosologies include hemophilia, multiple sclerosis and oncohematology.

Conclusion. The main quantitative characteristics of the implementation of the VZN program and the identified vectors for its further improvement have been analyzed in this study. The results obtained can be used to conduct analytical studies, including the ones within nosologies and nosological groups included in the program, in order to optimize a pharmaceutical care. The focus of improving the implementation of the HCNs program is related to the improvement of the legal framework, a patient treatment paradigm and approaches to its financing.

Keywords: drug provision; list of expensive drugs; High-Cost Nosologies Program; evaluation of medical technologies

Abbreviations: VZN – high-cost nosologies; SRP – State Reimbursement Program; MNs – malignant neoplasms; MP – drug provision; FR – federal register; INN – international nonproprietary name; ODs – orphan diseases; VED – list of vital and essential drugs; MS – multiple sclerosis; BOL – budgetary obligations limit; SCs – state contracts.

Анализ фактических результатов реализации лекарственного обеспечения в рамках программы высокозатратных нозологий

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Доступность и охват фармацевтической помощью связаны с принятием политических, экономических и управленческих решений, что обуславливает необходимость оценки результатов государственных программ в сфере лекарственного обеспечения.

Цель. Оценка количественных результатов реализации программы высокозатратных нозологий в Российской Федерации с 2008 по 2023 гг. для определения дальнейших векторов ее совершенствования.

Материалы и методы. Нормативную базу работы составило законодательство Российской Федерации в сфере лекарственного обеспечения. В качестве информационной базы исследования для сбора и анализа данных использовали открытые источники: отчеты федеральных и региональных органов исполнительной власти, материалы профильных конференций, результаты опубликованных исследований.

Результаты. Лекарственное обеспечение в рамках программы высокозатратных нозологий (ВЗН) осуществляется по 14 нозологиям, 11 из которых отнесены к категории орфанных. С момента реализации программа ВЗН расширялась 2 раза за счет включения новых нозологий в 2019 и 2020 гг. По состоянию на 01 октября 2023 года численность пациентов в Федеральном регистре ВЗН составляла 263 721 человек, что в 13,58 раз выше в сравнении с 2008 г. Лекарственное обеспечение осуществляется по перечню из 47 МНН. Объем финансирования программы увеличился с 32 млрд руб. в 2008 г. до 87,96 млрд руб. в 2023 г. К наиболее ресурсоёмким нозологиям относятся гемофилия, рассеянный склероз и онкогематология.

Заключение. В настоящем исследовании проведен анализ основных количественных характеристик реализации программы ВЗН, определены векторы дальнейшего ее совершенствования. Полученные результаты могут быть использованы для проведения аналитических исследований, в том числе внутри нозологий и нозологических групп, включенных в программу, с целью оптимизации фармацевтической помощи. Фокус совершенствования реализации программы ВЗН связан с совершенствованием правового поля, парадигмы лечения пациентов и подходов к её финансированию.

Ключевые слова: лекарственное обеспечение; перечень дорогостоящих лекарственных препаратов; программа высокозатратных нозологий; оценка медицинских технологий

Список сокращений: ВЗН – программа высокозатратных нозологий; ДЛО – программа дополнительного лекарственного обеспечения; ЗНО – злокачественные новообразования; ЛО – лекарственное обеспечение; ЛП – лекарственный препарат; ФР – федеральный регистр; МНН – международное непатентованное наименование; ОЗ – орфанные заболевания; перечень ЖНВЛП – перечень жизненно-необходимых и важнейших лекарственных препаратов; РС – рассеянный склероз; ЛБО – лимит бюджетных обязательств; ГК – государственные контракты.

INTRODUCTION

In the system of the sustainable development, a human capital occupies a central place, and its preservation and growth is a priority task of the social and economic policy of the state [1–3]. One of the qualitative characteristics of the human resource of the state is the population health, the loss of which is measured by the size of the economic burden caused by morbidity, a temporary loss of working capacity, the level of disability and mortality, especially a premature mortality among the economically active population [4–6]. The system of health protection measures is aimed at ensuring the quality and accessibility of a medical care, including a drug provision (DP) [7, 8]. The effectiveness of the health care system is determined by a combined assessment of three components: social, medical and economic. Herewith, social and medical components of the industry efficiency assessment are predominant [9]. From the point of view of the legislation, medicines are a structural element of both the provision of a medical care and a tool for ensuring social guarantees by the state [10, 11]. I.e., the sphere of circulation of medicines and DP can be considered as a mechanism for regulating the achievement of sustainable development goals for the preservation and increase of the human capital [12].

From the position of a medical and social significance and improvement of patients' quality of life, the state program of treatment with expensive drugs "High-Cost Nosologies Program (VZN)". is the most indicative[1] [13], since its creation became a start not only for the treatment of certain categories of citizens with life-threatening and disabling diseases in outpatient settings, but also served as a beginning for the use of additional sources of funding for the drug therapy of the most resource-intensive nosologies.

The VZN program has been implemented in the Russian Federation for 15 years. The basis for its launch in 2008 was Federal Law No. 132-FZ dated 17 July 2007. Before its adoption in 2007, by the decision of the Government of the Russian Federation and in accordance with Order No. 159 of the Ministry of Health and Social Development of the Russian Federation (abolished

[1] Zhuleva Yu. [Quality of life of patients receiving treatment in the 7VZN program]. Forum of the All-Russian Union of Patients. Moscow, 2018. Available from: https://forum-vsp.ru/media/5cjjitck/vserossiiskiiforumzhulevkachestvozhiznipacientovpoluchayushihlecheniepoprogramme7nozologii-rassh.pdf. Russian

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on 21 May 2012) dated 09.03.2007, drugs for the delegation of powers on the DP of VZN to the federal level were made: the procedure for forming a list of patients in need of the expensive drug therapy was developed, a list of 7 diseases was defined, a list of patients was formed, and their number was determined[2]. The program included malignant neoplasms of lymphoid, hematopoietic and related tissues (oncohematology) (ICD 10 codes C 82, 83. 0, 83.1, 83.3, 83.4, 83.8, 83.9, 84, 84.5, 85, 88.0, 90.0, 91.1, 92.1), multiple sclerosis (MS) (ICD-10 Code G 35.0), hemophilia (ICD-10 Code D 66.0, D 67.0, D 68. 0,), pituitary nanism (ICD-10 Code E 23.0), cystic fibrosis (ICD-10 Code E 84.0), Gaucher disease (ICD-10 Code E 75.5) and conditions after organ and tissue transplantations (transplantation)[3]. In 2008, the number of patients in the program amounted to 19 416[4,5], and the amount of the allocated funding was RUB 32 bn [14, 15]. The patients were treated according to the list of 18 INNs.

During the implementation period, the VZN program has been expanded twice. In 2019, 5 orphan diseases (ODs) were included: hemolytic uremic syndrome, juvenile arthritis with a systemic onset, mucopolysaccharidosis type I, mucopolysaccharidosis type II, mucopolysaccharidosis type VI. In 2020, a financial provision of 2 more ODs (aplastic anemia unspecified, hereditary deficiency of factors II (fibrinogen), VII (labile) X (Stuart-Prower)) were transferred from the regional level to the federal one. Thus, at the moment, 14 nosologies are included in the VZN program, 11 of which have been classified as rare[6,7].

Within the framework of the VZN program, the advantages of DP include guaranteed financing from the federal budget, including the provision of the organizational costs for storage and logistics, a centralized procurement of the drugs on a separate list, a personalized registration of patients in the VZN federal register (VZN FR), incl. the availability of algorithms for the provision of newly identified and already included in the VZN FR patients, a protection of the application for the planned period, monitoring of balances and the possibility of their redistribution. However, despite the significant advantages of the VZN program, a number of problematic issues remains. According to the All-Russian Union of Patients, the most pressing issues of the VZN program implementation include the availability of innovative medicines, preservation and optimization of the treatment regimens, as well as an allocation of a commensurate amount of funding for the program[8,9] [13].

Positive medical and social results of the VZN program implementation indicate the need for a further improvement of its main tools. For the process of strategic goal setting of state programs, first, it is necessary to assess the actual results and current tools of their implementation[10].

In this regard, the aim of this study was to assess the actual results of the High-Cost Nosologies program implementation for a further identification of potential vectors for its development. In the course of the work, the following problems were solved:

– an analysis of normative legal acts (NLAs) regulating the VZN program implementation was conducted;

– a systematic search and a data review to determine the key parameters of the VZN program implementation was conducted;

– an actual results analysis of the VZN program implementation according to the selected key parameters was carried out.

THE AIM of the work was to assess the quantitative results of the implementation of the High-Cost Nosologies program in the Russian Federation from 2008 to 2023 to determine further vectors of its improvement.

[2] Order of the Ministry of Health and Social Developmentof Russian Federation dated 03 September 2007 No. 159 “On measures to ensure certain categories of citizens with necessary medicines”. Russian

[3] Order of the Ministry of Health and Social Development of the Russian Federation dated 4 April 2008 No. 162n “On the procedure for maintaining the Federal Register of patients with hemophilia, cystic fibrosis, pituitary dwarfism, Gaucher disease, myeloid leukemia, multiple sclerosis, as well as after organ and (or) tissue transplantation”. Russian

[4] Vlasov YaV. [Diagnostics and dynamics of the number of patients, receiving treatment under the “7 nosologies” Program]. All-Russian forum “10 years of the 7 nosologies program”. Available from: https://forum-vsp.ru/10-let/. Russian

[5] Report “Analysis of procurement procedures for the 14 VZN program”; in 2017–2021. All-Russian Union of Patients. Available from: https://vspru.ru/media/1440603/19072021-predsedatelyu-pravitelstva-rossiiskoifederacii-mv-mishustinu-o-dop-finansirovanii-vzn.pdf. Russian

[6] Federal Law dated 3 August 2018 No. 299-FZ “On Amendments to the Federal Law “On the Fundamentals of Protecting the Health of Citizens in the Russian Federation”. Russian

[7] Federal Law dated 27 December 2019 No. 452-FZ “On Amendments to the Federal Law “On the Fundamentals of Protecting the Health of Citizens in the Russian Federation”. Russian

[8] Vlasov YaV. [Diagnostics and dynamics of the number of patients, receiving treatment under the “7 nosologies” Program]; 2018. Russian

[9] Report “Analysis of procurement procedures for the 14 VZN program”; in 2017–2021. Russian

[10] Federal Law dated 28 June 2014 No. 172-FZ (as amended on February 17, 2023) “On Strategic Planning in the Russian Federation”. Russian

MATERIALS AND METHODS

The study was conducted at the Department of Regulatory Relations in the field of circulation of drugs and medical devices of Sechenov University in the period from 15 January 2023 to 10 November 2023. A set of research methods was used to solve the set tasks: a literature review; data excerpts from the reporting forms of federal and regional executive authorities in the field of healthcare; a content analysis of regulatory documents governing a drug supply to patients with rare and life-threatening diseases. The regulatory base of the study was the legislation of the Russian Federation regulating relations in the field of DP[11,12,13]. The search for normative legal acts was carried out in the Electronic Periodical Reference Book "GARANT System". As sources of information for determining the key parameters of the VZN program implementation, the data from the reports of the Accounts Chamber of the Russian Federation[14], the reports of the Federal Treasury of the Russian Federation on the budget execution for 2008–2021[15], were used. There were also reports on the implementation of the performance indicators of Federal Centre for the Planning and Organization of Drug Provision to Citizens[16], regional reports on the VZN program implementation 14[17]. The search for published research results on this topic was carried out in the e-library.ru [15–17]. The materials of the All-Russian Patients Union[18,19] posted on the Internet were studied, as well as the Reports of experts at specialized industry events[20,21]. The information from the Unified Information System in Procurement Procurement (UIS Procurement) was analyzed. Previously unpublished proprietary data were also used in the study.

To analyze the actual results of the VZN program implementation, a database was created in the MS Excel 2019 format with the following parameters: the information on the dynamics of the number of patients included in the VZN FR, the number of patients included in the application for the drug provision, the amount of financial support and budget execution of the VZN program, the structure of the number of patients included in the VZN FR, the dynamics of the number of patients by nosologies, the number of patients receiving DP by nosologies, the structure of patients' DP within the VZN program by ICD-10 and the share structure of drug purchases in the relevant INNs within nosologies. The results were interpreted and analyzed using StatTech Software v. 4.1.1 (StatTech LLC, Russia).

RESULTS AND DISCUSSION

Regulation of drug supply for patients with rare and life-threatening diseases: background and current status

The transformation of the pharmaceutical market under the influence of the economic and political situation in the Russian Federation in the late 80's-early 90's led to fundamental changes in the institutional environment for the regulation of DP [18, 19]. RSFSR

[11] Federal Law dated 21 November 2011 No. 323-FZ (as amended on 13 June 2023) "On the fundamentals of protecting the health of citizens in the Russian Federation". Russian

[12] Decree of the Government of the Russian Federation dated 26 November 2018 No. 1416 (as amended on 15 February 2023) "On the procedure for organizing the provision of medicines to persons with hemophilia, cystic fibrosis, pituitary dwarfism, Gaucher disease, malignant neoplasms of lymphoid, hematopoietic and related tissues, multiple sclerosis, hemolytic-uremic syndrome, juvenile arthritis with systemic onset, mucopolysaccharidosis types I, II and VI, unspecified aplastic anemia, hereditary deficiency of factors II (fibrinogen), VII (labile), X (Stuart-Prower), persons after organ transplantation and (or) fabrics (with changes and additions)". Russian

[13] Decree of the Government of the Russian Federation dated 28 August 2014 No. 871 (as amended on 3 December 2020) "On approval of the Rules for the formation of lists of medicines for medical use and the minimum range of medicines necessary for the provision of medical care". Russian

[14] Katrenko VS. Report on the results of the expert-analytical event. [Analysis of the effectiveness of the use of public funds allocated for the implementation of the state's obligations to provide medicines to certain categories of citizens in 2011–2012]. Accounts Chamber of the Russian Federation. Available from: https://ach.gov.ru/upload/iblock/f00/fj405se3rz3in7sdmx1f9153sxx6bm96.pdf. Russian

[15] Federal Treasury official website of the Treasury of Russia. Reporting on budget execution. Available from: https://roskazna.gov.ru/ispolneniebyudzhetov/. Russian

[16] Reports on the implementation of performance indicators of the Federal Centre for the Planning and Organization of Drug Provision to Citizens. Available from: https://fcpilo.minzdrav.gov.ru/?page_id=4029. Russian

[17] Ministry of Health of the Udmurt Republic. Reports on the implementation of the 14 VZN program. Available from: https://mzur.ru/activity/support/14vznreports/. Russian

[18] Vlasov YaV. [Diagnostics and dynamics of the number of patients, receiving treatment under the "7 nosologies" Program]; 2018. Russian

[19] Report "Analysis of procurement procedures for the 14 VZN program" in 2017–2021. Russian

[20] Maksimkina EA. [Drug provision for patients suffering from rare (orphan) diseases, as part of the implementation of the program of High-Cost Nosologies (VZN) and the activities of the Circle of Good Foundation. Materials of the Round Table on the topic: "Results and prospects for the development of the organization of medical and social care for patients suffering from rare (orphan) diseases in the Russian Federation"]. State Duma Committee on Health Protection. Available from: http://komitet2-2.km.duma.gov.ru/Novosti-Komiteta/item/28214556/. Russian

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Government Resolution No. 68[22] dated 26 December 1991 classified vital and essential drugs as priority products, and the RSFSR was instructed to form a list of such drugs. Within the framework of this decree, the State Program for the improvement of DP and development of the pharmaceutical industry in the RSFSR (Program) was also approved. First, it was planned to expand the list of privileged categories of citizens with chronic diseases, who receive drugs free of charge. These provisions were enshrined in the Resolution of the Government of the Russian Federation No. 890 dated 30 June 1994 "On State Support for the Development of the Medical Industry and Improvement of the Provision of the Population and Health Care Institutions with Medicines and Medical Devices". In addition, the program laid down the principles of the state regulation of prices for vital and essential drugs (VEDs).

In 1995, Federal Law No. 181-FZ dated 24 November 1995 "On Social Protection of Disabled Persons in the Russian Federation" was adopted, according to which "a qualified medical care for disabled persons, including a drug provision, is provided free of charge or on preferential terms", in accordance with the norms of the law established in the legislation. The adoption of this law was the beginning of the State Reimbursement Program (SRP) implementation. In accordance with Federal Law of No. 178-FZ "On State Social Assistance" dated 17 July 1999, the state social assistance is also guaranteed to certain categories of citizens in the form of a set of social services, which includes medicines as vital goods. Despite the adopted legal acts, due to the shortage of funding, the actual State Reimbursement Program implementation started only in 2005. For the same reason, since 2007, at the level of the RF Government, it was decided to divide the federal budget funds allocated for SRP of privileged categories of citizens, into two streams[23]:

– federal budget funds to finance the centralized purchase of medicines approved by the RF Government Order No. 1328-r dated 02 October 2007 "On the list of medicines to be centrally purchased at the expense of the federal budget";

– federal budget funds in the form of subventions and transfers to constituent entities of the Russian Federation for the implementation of the authority to provide medical treatment to certain categories of citizens who have retained the right to receive medicines.

According to the current legislation, the DP is carried out in accordance with the "standards of medical care in the amount not less than that stipulated by the VEDs list"[24]. The first VEDs list was formed in 1992 in the context of the economic crisis, which required the state to take measures to ensure not only the affordability of medicines, especially for unprotected segments of the population and privileged categories of citizens, but also measures to develop the local pharmaceutical industry. Based on the regulatory legal acts of those years, it can be concluded that the formation of the VEDs list was based on three principles of improving VEDs: production, the principle of realizing the rights of citizens to the social assistance and the principle of the state regulation of prices for drugs that are included in the list. The regulatory legal framework governing the formation of the VEDs list has been developed over 20 years and continues to be improved to this day. During this period, in the regulatory field, there was a qualitative transition from the methodological recommendations of the Formulary Committee on the formation of the VEDs list to the level of the Government Decree, which approved the order and procedure for the formation of several lists within the system of medical treatment of the population [20]. In 2014, Government Resolution No. 871 established uniform principles for the formation of lists of drugs for medical use: the order and procedure for their formation, criteria for the inclusion of medicines in each of the lists, integral scales on which the information about the benefits of the proposed drug for the inclusion is assessed. The VEDs list has become the basis for the formation of the list of expensive drugs[25]. The criteria for the formation of the VEDs program and the list of expensive drugs are not enshrined in the legal framework, therefore, within the framework of this study it was expedient to determine their essential characteristics (Table 1).

[22] Decree of the Government of the RSFSR dated 26 December 1991 No. 68 "On urgent measures to provide the population and health care institutions of the RSFSR with medicines in 1992 and the development of the pharmaceutical industry in 1992–1995" (together with the "State Program of the RSFSR for improving the supply of medicines and developing the pharmaceutical industry in 1992–1995"). Russian

[23] Katrenko VS. Report on the results of the expert-analytical event. [Analysis of the effectiveness of the use of public funds allocated for the implementation of the state's obligations to provide medicines to certain categories of citizens in 2011–2012]. Russian

[24] Federal Law dated 21 November 2011 No. 323-FZ (as amended on 13 June 2023) "On the fundamentals of protecting the health of citizens in the Russian Federation". Russian

[25] Decree of the Government of the Russian Federation dated 28 August 2014 No. 871 (as amended on 3 December 2020) "On approval of the Rules for the formation of lists of drugs for medical use and the minimum range of medicines necessary for the provision of medical care". Russian

Table 1 – Essential characteristics of High-Cost Nosologies program parameters and high cost drugs list

Parameter	Parameter characteristics	Short description
Criteria for the inclusion of nosologies in VZN program	Medical Aspect: – life-threatening, rare (orphan) disease; – availability of pathogenetic therapy; – identifiable patient cohort. Financial aspect: – significant burden on the budget of the Russian Federation subject; – possibility of savings in case of centralization of procurement; – possibility of redistribution of medicines.	The order and procedure for the inclusion of nosologies in the VZN program is not regulated. The decision on the inclusion in the list of diseases related to VZN is made at the level of the Government of the Russian Federation (Clause 21, Article 14, Chapter 3; Clause 21 introduced by Federal Law No. 112-FZ dated 26 April 2016; ed. by Federal Laws No. 299-FZ dated 03 August 2018, and No. 452-FZ dated 27 December 2019; Clause 7, Article 44; Chapter 10, Article 44, Chapter 5 of the Federal Law No 323-FZ dated 21 November 2011 (edited 28 April 2023) "On the basis of health protection of citizens in the Russian Federation"); In accordance with Art. 104 of the Constitution of the Russian Federation, the President of the Russian Federation, the Federation Council, the Government of the Russian Federation, the legislative bodies of the constituent entities of the Russian Federation, and deputies of the State Duma may initiate the legislation on its expansion.
Source of funding	Federal budget funds: – in respect of adults aged 18 and over – at the expense of budgetary appropriations provided for in the federal budget to the authorized federal executive body; – in respect of children aged 0 to 18 – at the expense of budgetary appropriations provided for in the federal budget to the authorized federal executive body, for the needs of the Circle of Goodness Foundation for the Support of Children with Severe Life Threatening and Chronic Diseases, including rare (orphan) diseases.	Starting from 2021, the procurement of drugs on the list of high cost drugs is carried out by the Federal Centre for the Planning and Organization of Drug Provision to Citizens.
Contingent and drug therapy record keeping tools	The VZN Federal Register	The procedure for maintaining the VZN FR is stipulated by Decree of the Government of the Russian Federation No. 1416[26] dated 26 November 2018. Clause 7, Art. 44, No. 323-FZ. The categories of citizens entitled to preferential treatment are specified.
Moment of entitlement to preferential treatment	Entering data about patients in the VZN Federal Register	Clause 8, Article 44, Chapter 5 of Federal Law No. 323-FZ dated 21 November 2011 (ed. 28 April 2023) "On the Fundamentals of Health Protection of Citizens in the Russian Federation".
Nature of procurement	Centralized procurement	In accordance with Resolution of the Government of the Russian Federation No. 1025 dated 26 June 2021 "On Amendments to Certain Acts of the Government" of the Federal Centre for the Planning and Organization of Drug Provision to Citizens performs the functions of organizing and conducting the procurement of drugs for medical use at the expense of the federal budget.
Criteria for inclusion of a drug in the high cost drugs list	– the drug must be included in the VEDs list; – the drug is used for the pathogenetic treatment of a disease which is classified as a high-cost nosology; – the drug must not increase the amount of budgetary allocations provided for in the federal budget for the relevant fiscal year and planning period	The rules and procedure for forming lists of medicines for medical use are regulated by Decree of the Government of the Russian Federation No. 871n dated 28 August 2014: the drug proposed for the inclusion in this list must be registered in the territory of the Russian Federation, included in the VEDs list and have the advantages compared to their counterparts already on this list.

Note: * – adopted due to the expansion of the high-cost technology program beginning in 2019.

[26] Decree of the Government of the Russian Federation dated 26 November 2018 No. 1416 (as amended on 15 February 2023). Russian

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Table 2 – Dynamics of the number of patients that are included in the VZN program for 2010 and from 2018 to 2023, in the context of nosology

Nosology	The annual number of patients included in VZN FR (absolute value, %)						
	2010	2018	2019	2020	2021	2022*	2023*
MNPs of lymphoid, hematopoietic and related tissues	30 754 (42.89%)	85 335 (47.13%)	88 327 (46.43%)	99 886 (46.33%)	109 026 (45.60%)	108 833 (44.49%)	115 698 (43.87%)
MS	25 048 (34.94%)	63 455 (35.05%)	66 493 (34.95%)	75 114 (34.84%)	83 884 (35.08%)	87 287 (35.69%)	95 316 (36.14%)
Transplantation	5 060 (7.06%)	13 810 (7.63%)	15 077 (7.92%)	17 474 (8.10%)	19 706 (8.24%)	20 246 (8.28%)	22 659 (8.59%)
Hemophilia	6 069 (8.46%)	9 434 (5.21%)	9 413 (4.95%)	10 302 (4.78%)	11 139 (4.66%)	11 684 (4.78%)	12 125 (4.60%)
Pituitary anism	2 704 (3.77%)	5 142 (2.84%)	4 914 (2.58%)	6 460 (3.00%)	6 918 (2.89%)	7 411 (3.03%)	8 259 (3.13%)
CF	1 906 (2.66%)	3 496 (1.93%)	3 651 (1.92%)	3 920 (1.82%)	4 246 (1.78%)	4 429 (1.81%)	4 511 (1.71%)
Juvenile arthritis with a systemic onset	–	2 (0.001%)	1 346 (0.71%)	1 414 (0.66%)	1 666 (0.70%)	1 811 (0.74%)	1 927 (0.73%)
Aplastic anemia unspecified	–	–	–	–	1 057 (0.44%)	1 280 (0.52%)	1 483 (0.56%)
Hemolytic uremic syndrome	–	6 (0.003%)	398 (0.21%)	380 (0.18%)	463 (0.19%)	538 (0.22%)	600 (0.23%)
GD	156 (0.22%)	364 (0.20%)	361 (0.19%)	398 (0.18%)	438 (0.18%)	457 (0.19%)	474 (0.18%)
Hereditary deficiency of factors II (fibrinogen), VII (labile), X (Stuart-Prower)	–	–	–	–	253 (0.11%)	313 (0.13%)	374 (0.14%)
Mucopolysaccharidosis type I	–	1 (0.001%)	11 (0.06%)	123 (0.06%)	139 (0.06%)	112 (0.05%)	94 (0.04%)
Mucopolysaccharidosis, type II	–	8 (0.004%)	98 (0.05%)	98 (0.05%)	104 (0.04%)	143 (0.06%)	146 (0.06%)
Mucopolysaccharidosis type VI	–	–	53 (0.03%)	47 (0.02%)	51 (0.02%)	54 (0.02%)	55 (0.02%)
Total	71 697	181 053	190 250	215 615	239 090	244 600	263 721

Note: * – for 2022 and 2023, the data on the number of patients in the VZN FR are given as of October of the respective year. MNPs – malignant neoplasms; MS – multiple sclerosis; GD – Gaucher disease; CF –- cystic fibrosis.

Table 3 – Number of patients without combined pathologies* included in the VZN FR as of 1 October 2023, in the context of nosological groups and ages

Nosology	Number of patients without combined pathologies included in the VZN FR in 2023, absolute value			Proportion of patients without co-morbidities included in the the VZN FR, by age, %	
	Children under 18 years	Adults	Total	Children under 18 years	Adults
MNPs of lymphoid, hematopoietic and related tissues	367	115 275	115 642	0.32%	99.68%
MS	897	93 899	94 796	0.95%	99.05%
Transplantation	2 254	20 412	22 666	9.94%	90.06%
Hemophilia	3 888	8 049	11 937	32.57%	67.43%
Pituitary nanism	6 035	2 493	8 528	70.77%	29.23%
Cystic fibrosis	3 091	1 273	4 364	70.83%	29.17%
Juvenile arthritis with a systemic onset	1 354	598	1 952	69.36%	30.64%
Aplastic anemia unspecified	185	1 354	1 539	12.02%	87.98%
Hemolytic-uremic syndrome	338	209	547	61.79%	38.21%
Gaucher disease	114	346	460	24.78%	75.22%
Hereditary deficiency of factors II (fibrinogen), VII (labile), X (Stuart-Prower)	209	219	428	48.83%	51.17%
Mucopolysaccharidosis, type I	69	18	87	79.31%	20.69%
Mucopolysaccharidosis type II	111	25	136	81.62%	18.38%
Mucopolysaccharidosis, type VI	34	24	58	58.62%	41.38%
Total without combined nosologies	18 946	244 194	263 140	7.20%	92.80%

Note: As of 1 October 2023, 581 patients with combined nosologies were included in the VZN FR. MNPs – malignant neoplasms; MS – multiple sclerosis.

Table 4 – Dynamics of the declared need to meet the requirements for the drug coverage from 2021 to 2023 in the context of nosology

Nosology	2021		2022		2023	
	Expenses, RUB bn	Share, %	Expenses, RUB bn	Share, %	Expenses, RUB bn	Share, %
Hemophilia	20.556	28.07%	23.744	26.99%	25.455	28.94%
MS	17.763	24.26%	24.254	27.57%	24.185	27.50%
MNPs of lymphoid, hematopoietic and related tissues	17.638	24.09%	20.542	23.35%	18.092	20.57%
Hemolytic-uremic syndrome	4.530	6.19%	5.707	6.49%	6.178	7.02%
Mucopolysaccharidosis type II	3.663	5.00%	3.849	4.38%	3.728	4.24%
Transplantation	2.016	2.75%	2.493	2.83%	2.303	2.62%
JRA	1.878	2.56%	1.768	2.01%	2.047	2.33%
CF	1.543	2.11%	1.529	1.74%	1.502	1.71%
GD	1.377	1.88%	1.518	1.73%	1.501	1.71%
Mucopolysaccharidosis type VI	1.375	1.88%	1.518	1.73%	1.405	1.60%
Mucopolysaccharidosis type I	0.565	0.77%	0.694	0.79%	0.737	0.84%
Pituitary anemia	0.183	0.25%	0.188	0.21%	0.206	0.23%
Aplastic anemia unspecified	0.125	0.17%	0.107	0.12%	0.110	0.13%
Hereditary deficiency of factors II (fibrinogen), VII (labile), X (Stuart-Prower)	0.008	0.01%	0.05	0.06%	0.51	0.58%
Total	73.22	100%	87.96	100%	87.96	100%

Note: The summary table was compiled by the authors based on the data from the unified procurement information system; MNPs – malignant neoplasms; MS – multiple sclerosis; JRA – juvenile rheumatoid arthritis; GD – Gaucher disease; CF – cystic fibrosis.

Table 5 – Ranking of nosologies of the HCNs program, taking into account the number and structure of patients and the amount of financial support in 2023

Nosology	Range			
	by the number of patients in the HCNs FR	by the number of patients in the application	by the amount of financial support in the total program costs	by the share of adult patients
MNPs of lymphoid, hematopoietic and related tissues (oncohematology)	1	2	3	1
MS	2	1	2	2
Transplantation	3	3	6	3
Hemophilia	4	4	1	6
Hypophyseal anism	5	5	13	11
CF	6	6	8	12
Juvenile arthritis with a systemic onset	7	7	7	10
Aplastic anemia unspecified	8	8	14	4
Hemolytic-uremic syndrome	9	10	4	9
GD	10	9	9	5
Hereditary deficiency of factors II (fibrinogen), VII (labile), X (Stuart-Prower)	11	11	12	7
Mucopolysaccharidosis, type I	13	13	5	14
Mucopolysaccharidosis, type II	12	12	11	13
Mucopolysaccharidosis, type VI	14	14	10	8

Note: MNPs – malignant neoplasms; MS – multiple sclerosis; GD – Gaucher disease; CF – cystic fibrosis.

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Table 6 – Dynamics of expenditures per 1 beneficiary in the context of nosologies at the level of the constituent entity of the Russian Federation in 2020–2022 (using the example of data on the 14 VZN program implementation in the Republic of Udmurtia)

Nosology	Expenditures per beneficiary, RUB								
	2020			2021			2022		
	Average amount of expenditures	Minimum amount of expenditures	Maximum amount of expenditures	Average amount of expenditures	Minimum amount of expenditures	Maximum amount of expenditures	Average amount of expenditures	Minimum amount of expenditures	Maximum amount of expenditures
Hemolytic uremic syndrome	5 188 601.70	3 003 927.30	7 373 276.10	6 554 023.20	–	6 554 023.20	12 925 990.20	10 377 203.40	18 023 563.80
Mucopolysaccharidosis type II	19 074 627.00	–	19 074 627.00	19 837 612.08	–	19 837 612.08	17 930 149.38	–	17 930 149.38
Juvenile arthritis with a systemic onset	1 026 797.57	57 978.26	6 102 939.00	645 821.25	44 995.32	1 109 625.00	1 314 600.04	674 929.80	1 362 201.60
Oncohematology	443 160.00	12 097.80	476 511.04	461 462.99	12 359.70	486 045.63	575 726.35	182 482.20	586 470.73
CF	327 345.73	272 308.28	412 959.55	350 164.76	331 320.00	381 572.68	458 371.64	397 584.00	500 455.38
Hemophilia	2 134 154.71	192 959.28	2 179 298.79	2 591 382.42	2 020 806.12	2 803 310.76	2 470 798.90	1 787 210.30	2 812 593.20
MS	274 685.31	196 215.04	278 975.57	287 755.52	196 105.88	292 636.50	295 409.96	166 676.68	302 094.54
Pituitary nanism	46 467.77	–	46 467.77	43 006.16	–	43 006.16	31 293.55	–	31 293.55
GD	2 153 779.20		2 153 779.20	2 183 328.00	–	2 183 328.00	2 363 904.00	–	2 363 904.00
Organ and/or tissue transplantation	1 505.05	7 638.58	1 590.67	1 389.29	6 150.21	1 488.38	1 268.24	74 039.21	1 301.68

Note: MS – multiple sclerosis; ; GD – Gaucher disease; CF – cystic fibrosis. Data are adapted by the authors from the source[27].

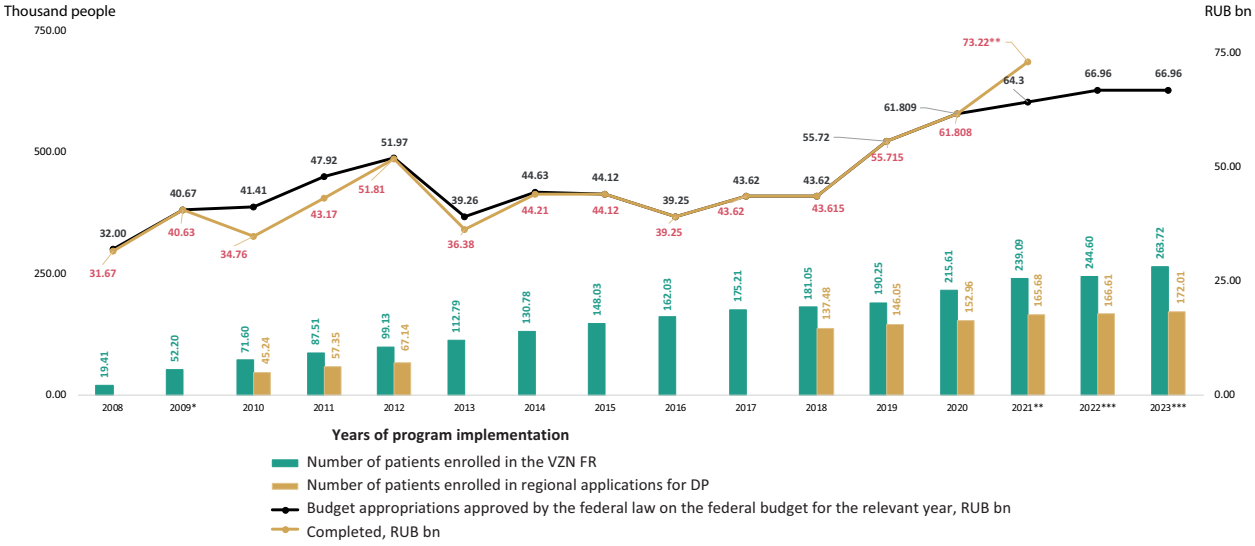

Figure 1 – Dynamics of the number of patients in the VZN program, the amount of budget allocations from the federal budget of the program and its budget execution from 2008 to 2023

Note: * – for 2009 and 2014, data on budget allocations approved by the Consolidated Budget List with account of changes; ** – for 2021 with account of additional RUB 8.90 bn allocated by Federal Centre for the Planning and Organization of Drug Provision to Citizens; *** – for 2022 and 2023, data on the number of patients in the VZN FR are given as of October of the respective year. The figure presented here was compiled by the authors based on data[28,29,30,31] and their own results.

[27] Ministry of Health of the Udmurt Republic. Reports on the implementation of the 14 VZN program. Russian
[28] Federal Treasury official website of the Treasury of Russia. Reporting on budget execution. Russian
[29] Katrenko VS. Report on the results of the expert-analytical event. [Analysis of the effectiveness of the use of public funds allocated for the implementation of the state's obligations to provide medicines to certain categories of citizens in 2011–2012]. Russian
[30] Reports on the implementation of performance indicators of the Federal Centre for the Planning and Organization of Drug Provision to Citizens. Russian
[31] Ministry of Health of the Udmurt Republic. Reports on the implementation of the 14 VZN program. Russian

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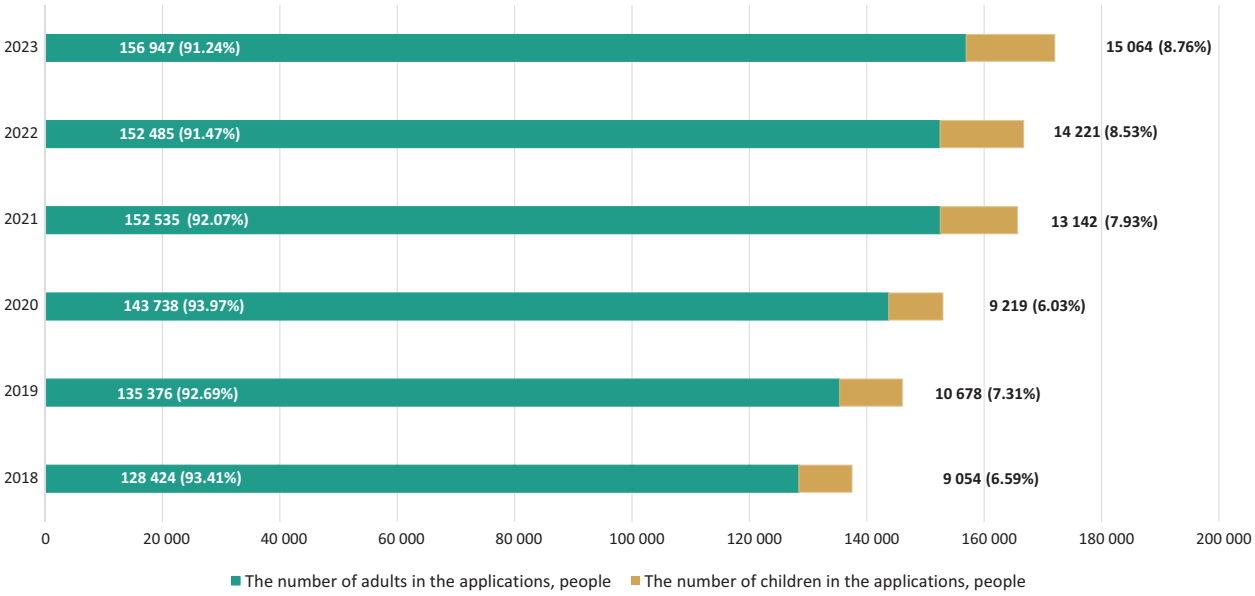

Figure 2 – Numbers of adults and children in absolute values and percentages in the DP application in 2018 and after the inclusion of new nosologies in the program

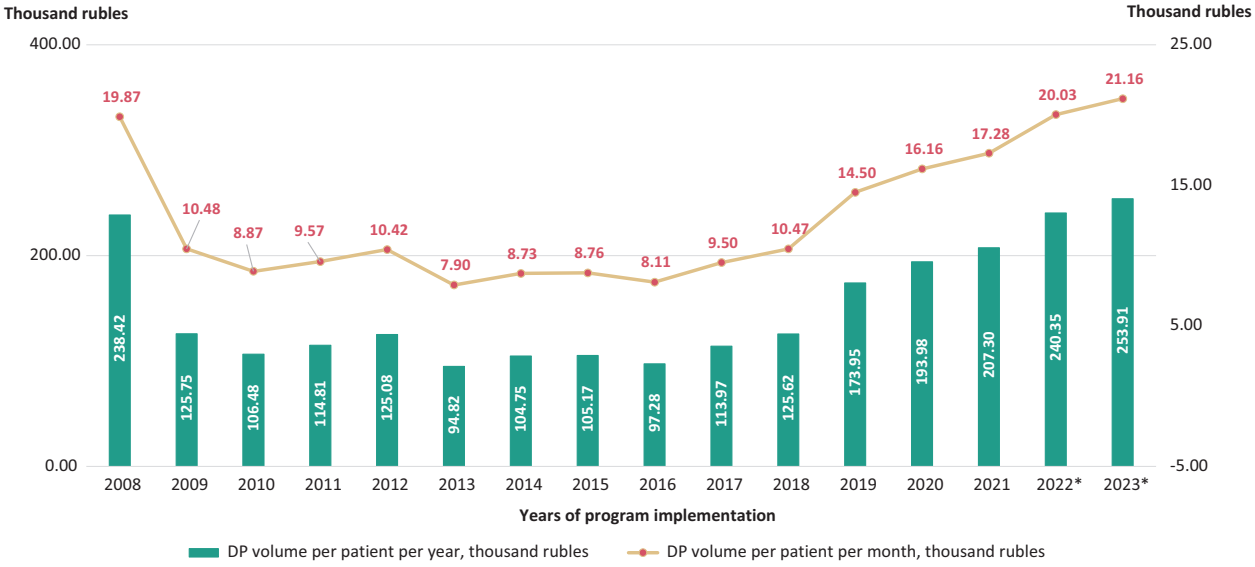

Figure 3 – The volume of drug supply per patient from 2011 to 2023

Note: * – including funds from the Circle of Goodness Foundation; ** the volume of treatment per patient is calculated in 2023 prices, taking into account the discount rate of 13.9% [20].

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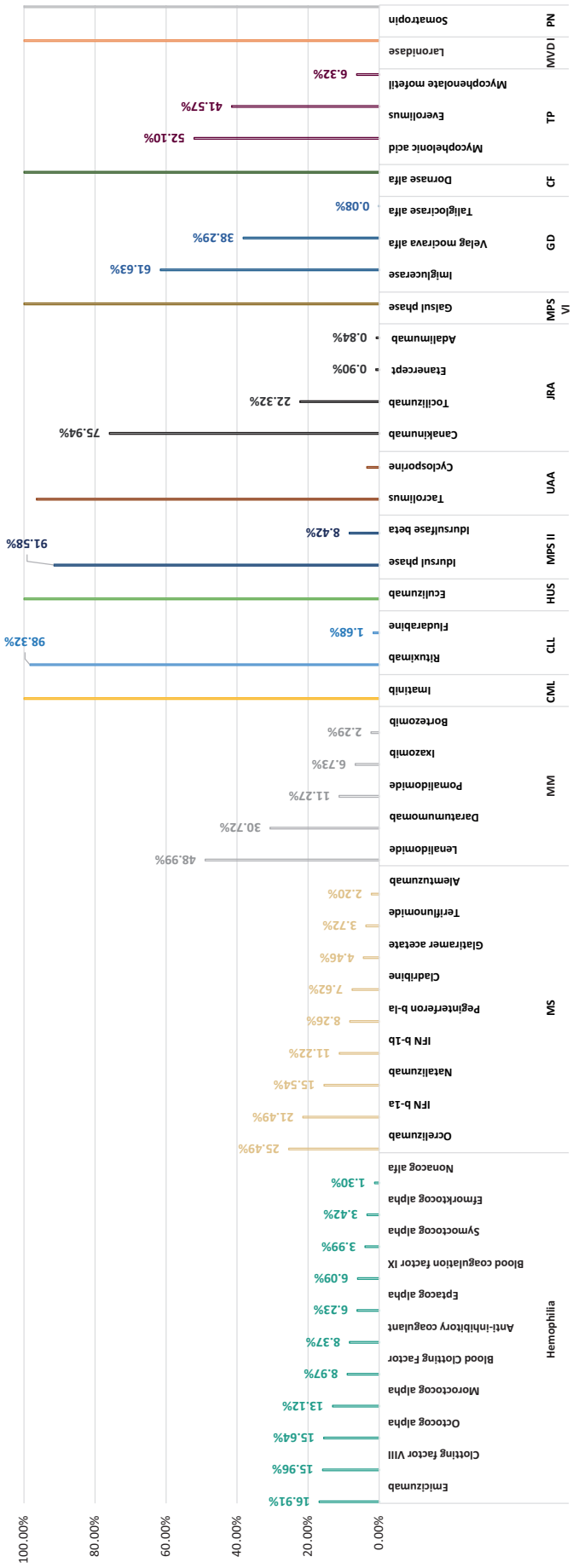

Figure 4 – Share structure of expenditures on patient care in the context of the VZN program in terms of ICD-10 and shares in procurement within the respective INNs in each nosology in 2021–2022

Note: MS – multiple sclerosis; MM – multiple myeloma; CML – chronic myeloid leukemia; CLL – chronic lympholeukemia; HUS – hemolytic uremic syndrome; MPS II – mucopolysaccharidosis type II; UAA – unspecified aplastic anemia; JRA – juvenile rheumatoid arthritis with a systemic onset; MPS IV – mucopolysaccharidosis type IV; GD – Gaucher disease; CF – cystic fibrosis; TP – transplantation; MPS I – mucopolysaccharidosis type I; PN – pituitary nanism. The information is adapted from the source[32].

[32] Orphan zone: how and why the state program "14 VZN" shares wards with the state fund "Circle of Good". Available from: https://vademec.ru/article/orfan-zona_kak_i_pochemu_gosprogramma_-14_vzn_delitsya_podopechnymi_s_gosfondom_-krug_dobra/

Number of patients and volume of budget allocations for drug provision to patients with rare and life-threatening diseases within the framework of the High-Cost Nosologies program 14

In 2008, the volume of budget funds allocated for the treatment of 19.41 thousand patients amounted to RUB 32 bn[33] [14]. By 2021, the volume of financing increased twofold – up to RUB 64.3 bn, and the total number of patients in the VZN FR reached 239.09 thousand people. By 1 October 2022, there were already 244.60 thousand people in the VZN FR, by 1 October 2023 their number increased to 263.72 thousand people (+9.33%), taking into account combined nosologies[34]. From 2022 to 2024, RUB 66.96 bn are annually budgeted to finance the VZN program 14[35]. The dynamics of the number of patients, the volume of budget allocations from the federal budget and the budget execution of the VZN program are shown in Figure 1.

14 VZN program in the context of nosology: population dynamics, age distribution of patients

The cumulative average annual growth rate in the number of patients for the 7 nosologies that had been initially included in the VZN program between 2010 and 2023, was 10.54% and ranged from 6.85% for pituitary dwarfism to 12.22% for transplantation. The major nosologies that accounted for the increase in patients, included hematologic oncology and MS, accounting for more than 80% of the patients in the VZN FR. The cumulative average annual growth rate of the ODs patient population was 8.16% from 2010 to 2023. In 2022, hemophilia (41.39%), hypophysial nanism (26.25%) and cystic fibrosis (15.69%) accounted for 83.32% of the total population of patients with ODs. In 2023, more than 80% (82.85%) of patients with ODs were patients with these rare pathologies. The ratio of orphan and non-orphan diseases in 2022 was 11.54 and 88.46%, in 2023 – 11.39 and 88.61%, respectively, which indicates that this trend will continue in the current year. Table 2 shows the dynamics of the number of patients in the context of nosologies, which are included in the VZN FR.

In 2018, the number of children included in the VZN FR was 9.05 thousand people, or 6.59%. Due to the inclusion of nosologies with a high prevalence rate in the paediatric population in 2019 and 2020, their share in 2023 increased to 15.06 thousand people (8.76%). The increase in the number of children amounted to 6.01 thousand people in absolute terms and +66.38% in percentage terms.

As of 1 October 2023, the total number of children without combined pathologies included in the VZN FR, amounted to 18.94 thousand people, the share of patients with ODs of which amounted to 81.43%, or 15 428 people. Among adults, on the contrary, the share of patients without combined pathologies included in the VZN FR with non-orphan diseases is 94%. Most nosologies are characterized by a high proportion of adult patients receiving DP at the expense of the federal budget. Table 3 shows the number of patients without combined pathologies included in the VZN FR in 2023 in the context of nosological groups and age of beneficiaries.

The numbers of adults and children in absolute values and percentages in the DP application in 2018 and after the inclusion of new nosologies in the program are shown in Figure 2.

From 2018 to 2023, the ratio of adults to children in the program averaged 92.41% (144 924±11 184) and 7.59% (11 897±2 598), respectively.

Considering the age structure of patients included in the DP application, the nosological groups included in the DP program can be classified as follows:

– nosologies with the proportion of adult patients receiving therapy from 10 to 20% of the total number of patients receiving the treatment for mucopolysaccharidosis type II (1);

– nosologies with the proportion of adult patients receiving therapy from 20 to 40% of the total number of patients receiving the treatment for hypophyseal nanism, mucopolysaccharidosis type I, cystic fibrosis juvenile arthritis with a systemic onset (4);

– nosologies with a share of adult patients receiving therapy, from 40 to 70% of the total number of patients, on the preferential treatment – hemophilia, mucopolysaccharidosis type VI, hereditary deficiency of factors II (fibrinogen), VII (labile), X (Stuart–Prower) (3);

– nosologies with a share of adult patients exceeding 70% of the total number of patients receiving DP – MS, oncohematology, Gaucher disease, aplastic anemia unspecified, transplantation (6).

[33] Maksimkina EA. [Drug provision for patients suffering from rare (orphan) diseases, as part of the implementation of the program of High-Cost Nosologies (VZN) and the activities of the Circle of Good Foundation. Materials of the Round Table on the topic: "Results and prospects for the development of the organization of medical and social care for patients suffering from rare (orphan) diseases in the Russian Federation"]. Russian

[34] Ibid.

[35] Federal Treasury official website of the Treasury of Russia. Reporting on budget execution. Russian

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The need for a DP and the structure of centralized procurement in the context of 14 VZN program

Until 2021, the Ministry of Health of the Russian Federation carried out centralized procurement of medicines under the VZN program at the expense of the federal budget on the basis of regional requests. By the order of the Government of the Russian Federation from 2021, this function is assigned to Federal Centre for the Planning and Organization of Drug Provision to Citizens[36]. The budget obligation limits (BOLs) for 2021 amounted to RUB 64.31 bn, RUB 7.129 bn was transferred to Federal Centre for the Planning and Organization of Drug Provision to Citizens and RUB 8.90 bn was additionally allocated. For 2022, BOLs increased to RUB 66.96 bn Federal Centre for the Planning and Organization of Drug Provision to Citizens concluded 147 state contracts (SCs) for the amount of RUB 58.68 bn and 6 additional agreements under multi-year SCs from 2021 for the supply of drugs for 3 INNs in 2022 for the amount of RUB 8.28 bn In order to ensure the uninterrupted treatment of patients suffering from VZN, in the fourth quarter of 2021, Federal Centre for the Planning and Organization of Drug Provision to Citizens procured medicines at the expense of funds and according to the needs for 2022. As in 2022, for the uninterrupted treatment and formation of the carry-over stock of drugs for 2023, in the second quarter of 2022 Federal Centre for the Planning and Organization of Drug Provision to Citizens procured medicines at the expense of funds of 2023 according to the part of the approved needs of 2022. The BOLs for 2023 amounted to RUB 87.96 bn, including BOLs for the provision of the adult population – RUB 66.96 bn and for the provision of children within the framework of the delegation of powers to the Circle of Goodness Foundation – RUB 21 bn[37,38]. Advanced procurement, on the one hand, is due to the shortage of funds allocated for the implementation of the VZN program, on the other hand, it is due to the introduction of the practice of purchasing treatment through Federal Centre for the Planning and Organization of Drug Provision to Citizens within the framework of long-term contracts. Starting from 1 January 2023, DP for children with disabilities is financed from the funds received from the increased tax rate and allocated to the Circle of Goodness Fund. The release of federal budget resources is primarily at the expense of nosologies with high numbers of children, which include hypophyseal nanism, hemophilia, cystic fibrosis, hereditary deficiency of blood factors.

For the period from 2008 to 2023, the increase in the number of persons participating in the VZN program amounted to 244.31 thousand people (+1258%); at the same time, the volume of allocated BOLs per patient per month remained practically unchanged: in 2023 prices, the increase amounted to +6.10% or RUB 1.29 thousand. The median cost per 1 patient per month for the analyzed period amounted to 10 446.12 [Q1 8 846.29; Q3 16 442.29], per year – 125 353.46 [Q1 106 155.47; Q3 197 307.47] (Figure 3).

Three nosologies are among the most financially intensive in the VZN program: hemophilia, MS and oncohematology. In 2023, the cost of an the DP application for patients with hemophilia amounted to RUB 25.45 bn, MS – RUB 24.18 bn, oncohematology – RUB 18.09 bn, or 77% of the total cost of the application (Table 4).

In 2022, 6 nosologies accounted for more than 98% of ICD-10 diagnoses among patients with hematologic oncohematology receiving DP in the context of the VZN program: Diffuse non-Hodgkin lymphoma (C83), 30.85%; lymphoid leukemia [lympholeukemia] (C91), 20.45%; multiple myeloma and malignant plasma cell neoplasms (C90), 19.30%; myeloid leukemia [myeloleukemia] (C92) – 12.20%; other and unspecified types of non-Hodgkin's lymphoma (C85) – 8.02%; follicular [nodular] non-Hodgkin's lymphoma (C82) – 7.48%. Of the hemophilia patients, 60.34% had hemophilia A, 11.11% had hemophilia B, and 27.59% were patients with Willebrandt's disease. Among those who had undergone a transplantation, 63.58% of patients were with a kidney transplantation, 20.14% with a liver transplantation, and 8.25% – with a heart transplantation. All of them received treatment at the expense of the federal budget. The total amount of financing of procurement in 2021–2022, incl. the budget allocated for 2022–2024, amounted to RUB 138.91 bn. More than 45% of funds were spent on the procurement of the drugs for oncohematology – lenalidomide (9.02%) and daratumumab (5.67%), MS – ocrelizumab (6.92%) and natalizumab (4.22%), hemophilia – emicizumab (4.62%), clotting factor VIII (4.36%), octocog alpha (4.27%), and a hemolytic-uremic syndrome – eculizumab (6.16%)[39]. Figure 4 shows the share structure of expenditures on the treatment of

[36] Order of the Government of the Russian Federation dated 28 October 2020 No. 2798-r "On the creation of the federal government institution "Federal Center for Planning and Organization of Drug Provision for Citizens"".

[37] Reports on the implementation of performance indicators of the Federal Centre for the Planning and Organization of Drug Provision to Citizens. Russian

[38] Maksimkina EA. [Drug provision for patients suffering from rare (orphan) diseases, as part of the implementation of the program of High-Cost Nosologies (VZN) and the activities of the Circle of Good Foundation. Materials of the Round Table on the topic: "Results and prospects for the development of the organization of medical and social care for patients suffering from rare (orphan) diseases in the Russian Federation"]. Russian

[39] Federal Treasury official website of the Treasury of Russia. Reporting on budget execution. Russian

patients in the context of the VZN program in terms of ICD-10 and the share of procurement within the respective INNs in each nosology in 2021–2022.

In terms of the number of patients included in the HCNs FR, the number of patients in the application for DP, and the amount of the necessary funding, the most resource-consuming nosologies are: MS, hemophilia, and oncohematology. Table 5 shows the ranking of nosologies included in the HCNs program according to 4 parameters: the number of patients included in the HCNs FR and the application for DP; the amount of financing for DP; the share of adult patients in the morbidity structure.

Guided by the norms of Government Decree No. 1416, Federal Centre for the Planning and Organization of Drug Provision to Citizens conducts an application campaign to organize the purchase of drugs according to the list of the expensive drugs, based on the needs of the constituent entities of the Russian Federation. At the constituent entity level, the application is formed on the basis of clinical recommendations. Herewith, the current standards of medical care, the average course dose and the monthly actual need of a patient in drugs, in accordance with the data of the regional segment of the VZN FR and the need to form a stock for 15 months (1 year plus a carryover balance for 3 months), have been taken into account. Thus, the need for DP is determined by taking into account the projected balances. The application coordinated with Federal Centre for the Planning and Organization of Drug Provision to Citizens supervisors, chief freelance specialists on the profile and experts of federal centers, is defended and approved by the commission of the Ministry of Health of Russia.

The main factors affecting the amount of funding to meet the needs for treatment and the average cost per patient are the composition of nosologies in the subject, a direct number of patients and the therapy scheme used to treat a particular patient. For example, according to the reports of the VZN program implementation 14 in the Republic of Udmurtia in 2020 was provided to 1,161 beneficiaries, the number of serviced prescriptions was 6,550 units, the average cost of 1 prescription – RUB 90 135.52, and the average amount spent per 1 beneficiary – RUB 511 601.11. According to the data for 2021, there were 1 166 beneficiaries on provision, the number of prescriptions served was RUB 5 701, the average cost of 1 prescription increased to RUB 114 321.52, and the average amount spent per 1 beneficiary increased to RUB 57 076.64. In 2022, 1 165 patients received DP, the total number of prescriptions issued amounted to 5 432, the average cost of 1 prescription reached RUB 131 950.35, the average amount spent per 1 beneficiary – RUB 616 828.15. Table 6 shows the dynamics of expenditures per 1 beneficiary in the context of nosology at the level of the constituent entity of the Russian Federation in 2020–2022, using the example of the data on the VZN program implementation in the Republic of Udmurtia[40].

Survey results and their discussion

In 2008, within the framework of the VZN program, the treatment was provided for 7 nosologies according to the list of 18 INNs; at present, the treatment is provided for 14 nosologies according to the list of 47 INNs. The number of patients in the VZN FR as of 01 October 2023, amounted to 263 721 patients. Compared to 2008, their number increased by 13.58 times, which cannot be said about the program financing. The data of earlier analytical studies of the VZN program implementation [22] and the results of the present study show a relative stability of the procurement structure by the main nosological segments. The most resource-intensive nosologies in the VZN program, before and after its expansion, include oncohematology, MS and hemophilia. In this regard, in order to make management decisions, it is necessary to analyze the VZN program implementation within nosologies and nosological groups. It is obvious that the trends of growth in the number of patients in the VZN program and, accordingly, the growth of expenditures on the purchase of medicines will continue in the future. This is due to the increase in the overall life expectancy, as well as to the improvement of diagnostic methods and approaches to the treatment of patients, including the use of innovative therapy schemes. Experts and the patient community have noted the positive results of the state program implementation from the clinical point of view and from the point of view of improving the quality of life of patients, which determines the need for a further VZN program implementation. The increase in the number of beneficiaries, the expansion of the VZN list and the drugs list for their treatment requires revision of not only the amount of funding, but also of other institutional mechanisms to improve the program effectiveness.

Certain difficulties in expanding the list of expensive drugs are associated with the unified rules for the formation of drugs lists for medical use. Since the list includes drugs for the treatment of diseases with high medical and social significance, it is necessary to develop criteria that take into account the specifics of nosologies included in the VZN program and new technologies for their therapy. In many foreign countries, the process of forming reimbursable lists is preceded by the so-called "horizon scanning" stage [23–25]. In this case, horizon

[40] Ministry of Health of the Udmurt Republic. Reports on the implementation of the 14 VZN program. Russian

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scanning means not only the process of a systematic identification of new and emerging technologies, but also the development of new and/or adaptation of existing tools for their subsequent evaluation. The following definition of horizon scanning is used in foreign literature – "early awareness and alert" (EAA), which, in the Russian-language version, can be considered as a system of an early detection and alert (SEDA). SEDA aims to search for and identify promising health technologies or new opportunities for the use of medical technologies already used in clinical practice; to assess or predict their impact on health, the health care system and/or society as a whole; and to further disseminate the results obtained. The implementation of SEDA will make it possible to predict the epidemiological and economic consequences of the use of new drugs within the framework of the VZN program [26].

After the market launch of drugs, studies of their efficacy and safety continue. They include the purpose of expanding the indications for use in new patient groups and lines of therapy. The updated pool of data on drugs, taking into account the data of real clinical practice, should be used for the optimization of patient therapy: the identification of target groups (subgroups) of patients, where the use of drugs brings the greatest clinical and economic effect.

In the absence of a system for monitoring the VZN program implementation, taking into account the outcomes of the treatment, risk sharing agreements not related to the results of the treatment can be used as available innovative methods of payment: price-volume agreements, payment at the expense of funds [27–29]. At present, Federal Centre for the Planning and Organization of Drug Provision to Citizens is already actively using the tool of long-term contacts (price-volume agreements) with manufacturers of original molecules. Financing of children's DP under VZN program implementation 14 at the expense of the Fund "Circle of Good" also refers to new forms of financing. The introduction of various mechanisms of "risk sharing agreements" will make it possible to further optimize the use of budget funds to improve the treatment of patients.

Study limitations

Within the framework of this study, only the main qualitative and quantitative indicators of the VZN program implementation as a whole have been studied. The analysis in nosological segments of the program taking into account regional peculiarities, has not been conducted. Clinical and economic efficiency in the conditions of real clinical practice of drugs that had already been included in the program was not assessed. These areas are promising for a further analysis and forecasting of the need for the expansion and financial support of the VZN program.

CONCLUSION

The present study has identified the main quantitative characteristics of the DP implementation according to the VZN program. The results obtained can be used for further analytical studies, including the ones within nosologies and nosological groups included in the program. The main vectors for improving the VZN program implementation include: the improvement of the legal framework, the drug therapy optimization, the application of new forms of financing the program.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Oksana I. Ivakhnenko – study design, collection and critical analysis of scientific literature and regulatory legal documents, collection and analysis of data, interpretation of results, writing, editing and design of the article, final approval of the manuscript; Vasily V. Ryazhenov – critical analysis of scientific and methodological literature, making remarks of intellectual content, editing of the article; Elena A. Maksimkina – collection of scientific and methodological literature, critical analysis of scientific and methodological literature, making remarks of intellectual content, editing of the article; Victor S. Fisenko – collection and critical analysis of scientific literature and normative legal documents, making remarks of intellectual content, editing of the article; Oleg V. Savoskin – collection, analysis and interpretation of data, editing of the article; Maria M. Kuznetsova – collection and critical analysis of scientific literature and normative legal documents, making remarks of intellectual content, editing of the article. All the authors confirm their authorship compliance with the ICMJE international criteria (all authors made a significant contribution to the conceptualization, research and preparation of the article, read and approved the final version before publication).

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Association of CYP3A4*1B, CYP3A4*22 and CYP3A5*3 polymorphisms carriage with efficacy and safety of tamsulosin in patients with benign prostatic hyperplasia

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Tamsulosin is a first-line drug in the treatment of lower urinary tract symptoms (LUTS) in benign prostatic hyperplasia (BPH). Despite high estimates of its efficacy and safety, it rates may vary due to genetic polymorphisms of genes for the enzymes involved in the drugs metabolism.

The aim of the work was to evaluate the carriage influence of genes polymorphisms of the CYP3A enzymes group of tamsulosin metabolizers on the efficacy and safety of therapy in patients with LUTS in BPH.

Materials and methods. A total of 142 patients with LUTS, with an established BPH diagnosis (N40 according to ICD-10) were included in the study and underwent all stages. All patients received monotherapy with tamsulosin 0.4 mg/day for at least 8 weeks. An IPSS questionnaire with the definition of quality of life, a prostate ultrasound with the determination of the prostate volume and residual urine, as well as uroflowmetry, were used to evaluate the results of the treatment. Controls were performed at 2, 4 and 8 weeks from the start of the therapy. The carriage of polymorphic markers CYP3A4 (*1B, *22) and CYP3A5*3 was determined in patients; HPLC was used to determine drug concentrations in blood plasma and levels of cortisol and its metabolite 6-beta-hydroxycortisol in urine to assess the phenotypic activity of CYP3A.

Results. No statistically significant associations between CYP3A phenotype (defined by CYP3A4 and CYP3A5 genotypes) and clinical parameters of the tamsulosin therapy efficacy and the safety assessment in the studied sample of patients were found (p >0.05). Similar data were obtained for individual variants of CYP3A4*1B, CYP3A4*22, CYP3A5*3 (p >0.05). The comparison of the tamsulosin residual equilibrium concentration values in patients in the study sample with respect to the carriers of CYP3A4 and CYP3A5 gene variants did not reveal the presence of significant differences in either CYP3A phenotypes and carriers and non-carriers of individual CYP3A4*1B (p=0.57), CYP3A4*22 (p=0.37) and CYP3A5*3 (p=0.76) variants. No association was found between the metabolic ratio of 6-beta-hydroxycortisol / cortisol in urine and the CYP3A phenotype encoded by a combination of genotypes of CYP3A4 and CYP3A5 gene variants (p >0.05).

Conclusion. A possible association between the carriage of CYP3A4*1B, CYP3A4*22, CYP3A5*3 variants, a CYP3A activity assessed by the content of an endogenous substrate of this isoenzyme and its metabolite in urine, the level of plasma concentration of the drug, and the efficacy and safety of tamsulosin, has not been confirmed. The contribution of CYP3A4 and CYP3A5 genetic polymorphisms to clinical parameters of the tamsulosin therapy requires a further study.

Keywords: tamsulosin; pharmacogenetics; CYP3A4; CYP3A5; tamsulosin concentration

Abbreviations: LUTS – lower urinary tract symptoms; BPH – benign prostatic hyperplasia; HPLC – high-performance liquid chromatography; ARs – adverse reaction; BPH – benign prostatic hyperplasia; CUA – common urine analysis; GBA – general blood analysis; BBA – biochemical blood analysis; PSA – prostate-specific antigen test; TRUS – transrectal ultrasound; RUV – residual urine volume UFM – uroflowmetry; IPSS – International Prostate Symptom Score; QoLS – Quality of Life scale; EM – “extensive” metabolizers; IM – “intermediate” metabolizers; PM – “poor” metabolizers; NSAIDs – non-steroidal anti-inflammatory drugs; iACEs – angiotensin-converting enzyme inhibitors; OS – IPSS subscale to assess the severity of obstructive symptoms; IS – IPSS subscale to assess the severity of irritative symptoms.

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Ассоциация носительства полиморфизмов CYP3A4*1B, CYP3A4*22 и CYP3A5*3 с эффективностью и безопасностью тамсулозина у пациентов с доброкачественной гиперплазией предстательной железы

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Тамсулозин является препаратом первой линии в лечении симптомов нижних мочевых путей (СНМП) при доброкачественной гиперплазии предстательной железы (ДГПЖ). Несмотря на высокие оценки эффективности и безопасности, показатели могут варьироваться из-за генетических полиморфизмов генов ферментов, участвующих в метаболизме препарата.

Цель. Оценка влияния носительства полиморфизмов генов ферментов группы CYP3A метаболизаторов тамсулозина на эффективность и безопасность терапии у пациентов с СНМП при ДГПЖ.

Материалы и методы. В исследование было включено и прошли все этапы 142 пациента с СНМП при установленном диагнозе ДГПЖ (N40 по МКБ-10). Все пациенты получали монотерапию тамсулозином 0,4 мг/сут на протяжении как минимум 8 недель. Для оценки результатов лечения использовали опросник IPSS с определением качества жизни, ультразвуковое исследование предстательной железы с определением объема простаты и остаточной мочи, а также урофлоуметрию. Контроль осуществляли в сроки 2, 4 и 8 недель от начала терапии. У пациентов определялось носительство полиморфных маркеров CYP3A4 (*1B, *22) и CYP3A5*3, с помощью ВЭЖХ определяли концентрации препарата в плазме крови и уровни кортизола и его метаболита 6-бета-гидроксикортизола в моче для оценки фенотипической активности CYP3A.

Результаты. Статистически значимых ассоциаций между фенотипом CYP3A (определяемого по генотипам CYP3A4 и CYP3A5) и клиническими параметрами оценки эффективности и безопасности терапии тамсулозином в исследованной выборке пациентов установлено не было (p >0,05). Аналогичные данные были получены для отдельных вариантов CYP3A4*1B, CYP3A4*22, CYP3A5*3 (p >0,05). Сравнение значений остаточной равновесной концентрации тамсулозина у пациентов в исследуемой выборке относительно носительства вариантов генов CYP3A4 и CYP3A5 не выявил наличия значимых различий как между фенотипами по CYP3A, так и носителями и неносителями отдельных вариантов CYP3A4*1B (p=0,57), CYP3A4*22 (p=0,37) и CYP3A5*3 (p=0,76). Не было обнаружено связи между метаболическим отношением 6-бета-гидроксикортизол / кортизол в моче и фенотипом CYP3A, кодируемым по сочетанию генотипов вариантов генов CYP3A4 и CYP3A5 (p >0,05).

Заключение. Возможная связь между носительством вариантов CYP3A4*1B, CYP3A4*22, CYP3A5*3, активностью CYP3A, оцениваемой по содержанию в моче эндогенного субстрата данного изофермента и его метаболита, уровнем плазменной концентрации препарата, эффективностью и безопасностью тамсулозина не подтверждена. Вопрос о вкладе генетических полиморфизмов CYP3A4 и CYP3A5 на клинические параметры терапии тамсулозином требует дальнейшего изучения.

Ключевые слова: тамсулозин; фармакогенетика; CYP3A4; CYP3A5; концентрация тамсулозина

Список сокращений: СНМП – симптомы нижних мочевых путей; ДГПЖ – доброкачественная гиперплазия предстательной железы; ВЭЖХ – высокоэффективная жидкостная хроматография; НПР – нежелательные побочные реакции; ОАМ – общий анализ мочи; ОАК – общий анализ крови; БХ – биохимический анализ крови; ПСА – анализ на простат-специфический антиген; ТРУЗИ ПЖ – трансректальное ультразвуковое исследование предстательной железы; ООМ – объем остаточной мочи; УФМ – урофлоуметрия; IPSS – Международная система суммарной оценки симптомов болезней предстательной железы (International Prostate Symptom Score); QoL – шкала IPSS по оценке качества жизни (Quality of Life); ЕМ – «быстрые» метаболизаторы; ІМ – «промежуточные метаболизаторы; РМ – «медленные» метаболизаторы; СОЭ – скорость оседания эритроцитов; НПВП – нестероидные противоовоспалительные препараты; иАПФ – ингибиторы ангиотензинпревращающего фермента; ОС – субшкала IPSS по оценке тяжести обструктивных симптомов; ИС – субшкала IPSS по оценке тяжести ирритативных симптомов.

INTRODUCTION

Benign prostatic hyperplasia (BPH) is one of the most common urologic diseases among men [1]. The classic clinical manifestations of BPH are lower urinary tract symptoms (LUTS), such as pollakiuria, urgency, nocturia and a feeling of an incomplete bladder emptying [2].

According to the European Association of Urology guidelines[1] α1-adrenoblockers are the first-line treatment for LUTS in BPH, and tamsulosin is one of the most commonly used drugs in this group. When using tamsulosin, some patients may experience undesirable adverse reactions (ARs) with vascular reactions being the most dangerous [3]. In addition, the efficacy of the conservative tamsulosin therapy in patients with LUTS for BPH is heterogeneous, and up to one third of patients may claim its ineffectiveness [4]. Thus, the problem of improving the efficacy and safety of the tamsulosin treatment for LUTS in BPH remains relevant.

Tamsulosin is metabolized by cytochrome P450 superfamily enzymes, mainly by CYP3A4 and CYP2D6, with a minor involvement of other CYP isoenzymes [5]. The activity of CYP enzymes is genetically determined and may vary between individuals. Currently, the contribution of carriage of different genetic variants of the cytochrome P450 superfamily enzymes, involved in the metabolism of a huge number of drugs to their efficacy and tolerability, is being actively studied.

The CYP3A subfamily consists of four enzyme isoforms CYP3A4, CYP3A5, CYP3A7, and CYP3A43 [6]. Among human CYP3A enzymes, CYP3A4 and CYP3A5 are considered the most important in the drug metabolism [7]. Both enzymes are abundantly represented in the liver and intestine [8, 9]. The studies have previously characterized the CYP3A4 gene as highly polymorphic. However, most of the variant alleles of the gene cannot explain 10-100-fold differences in the enzyme activity in different populations [10, 11]. The latter may be due to either the limited effect of the CYP3A4 gene polymorphism phenomenon on the enzyme activity or their very low frequency in the population (<0.1%). Another case is with the CYP3A4*22 variant (rs35599367), which encodes an enzyme with a reduced functional activity and for which significant associations with a decreased clearance of a number of drugs (clopidogrel, tacrolimus, cyclosporine, tricyclic antipsychotics, simvastatin, etc.) have been shown, which requires an adjustment of their dosing regimen [12]. The Dutch Pharmacogenetics Working Group (DPWG) has developed recommendations on the prescription and dosing of quetiapine depending on the type of CYP3A4 metabolizers [13].

Another variant of interest to researchers is the CYP3A4*1B variant (rs2740574). Thus, in pharmacokinetic studies, the CYP3A4*1B carriage required increased doses of tacrolimus and cyclosporine in patients after the transplantation because this variant was associated with a decrease in dose-adjusted drug concentrations [14]. In contrast, in patients taking simvastatin, the carriage of the CYP3A4*1B variant was associated with a lower incidence of a drug dose reduction or a need for drug switching [14]. However, controversy remains regarding the encoded effect (a functional activity) of the enzyme in marker carriers [12–14].

CYP3A4 is the major isoform expressed in most humans. However, another CYP3A5 isoform may contribute to the overall CYP3A activity, as these two isoforms have an overlapping substrate specificity. The CYP3A5*3 variant (rs776756, 6986 A >G) carriage is associated with a reduced expression of the enzyme, which is reflected by a decrease in its functional activity [15]. The carriage frequency of this allelic variant is up to 90% in European populations and varies widely in other populations: from 67 to 75% in Asian groups and from 24 to 32% in African groups [16, 17]. The other two alleles, CYP3A5*6 and CYP3A5*7, encoding a non-functional variant of the enzyme, are less common in European and Asian populations with an incidence of <0.5% and are more characteristic of African groups [15]. The scientific literature widely presents the data on the influence of CYP3A5 allelic variants on changes in pharmacokinetics, metabolism, safety and efficacy of drugs of different groups: tamoxifen, atorvastatin, simvastatin, apixaban, dabigatran, and others [18]. The Clinical Pharmacogenetics Implementation Consortium (CPIC) has developed professional recommendations on CYP3A5 for tacrolimus dosing [19].

Despite the widespread use and popularity of tamsulosin preparations in practice, there is currently no accurate information on the effect of CYP3A genetic polymorphisms on the efficacy and safety of the tamsulosin therapy. Based on the evidence that CYP3A enzymes are involved in the excretion of tamsulosin, it was hypothesized that polymorphisms of these enzymes may influence the drug response to the preparation administration.

Therefore, **THE AIM** of this study was to evaluate the contribution of CYP3A4 and CYP3A5 gene marker carriage to the efficacy and safety of the tamsulosin therapy in patients with LUTS for BPH.

[1] EAU Guidelines. Edn. presented at the EAU Annual Congress Paris April 2024. Available from: https://uroweb.org/guidelines/management-of-non-neurogenic-male-luts

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MATERIALS AND METHODS

The study was conducted from December 2021 to May 2023 on the basis of the endoscopic urology department of Municipal Polyclinic No. 7 (Naberezhnye Chelny, Republic of Tatarstan, Russia) and the Research Institute of Molecular and Personalized Medicine of the Russian Medical Academy of Continuing Professional Education (RMA CPE).

Ethical approval

The study was approved by the Ethical Committee of Scientific Research of RMA CPE (Protocol No. 13 dated 27 Dec 2021) and was conducted in accordance with the legislation of the Russian Federation and international regulatory documents (Helsinki Declaration of the World Medical Association, 2013; National Standard of the Russian Federation, GOST R 52379-2005).

Study Design

The authors conducted a single-center prospective observational open-label non-randomized study. A total of 148 male patients (mean age, 65.4) with complaints of LUTS and an established diagnosis of BPH (ICD-10 N40) were included in the study. All patients were followed up for at least 8 weeks and were examined 4 times (day 0, week 2, week 4 and week 8) in dynamics according to the study design (Fig. 1).

All patients were taking tamsulosin (Omnic®, 0.4 mg capsules, Netherlands) 0.4 mg/day. The patients did not receive any other medications for LUTS in BPH during the tamsulosin therapy.

The main part of the study included an 8-week treatment and follow-up, including visit 1 (screening and inclusion) and three follow-up visits after 2, 4 and 8 weeks. At visit 1 (the 1st day), at the inclusion moment of the patient in the study at the initial visit, a patient's medical history was collected, and the patient was examined using a set of clinical assessment of the LUTS manifestation according to the international system IPSS and QoL; instrumental methods (the study of urodynamic parameters: a maximum urine flow velocity (Q_{max}), the determination of residual urine and prostate volumes, according to the ultrasound testing). The routine tests were performed: a general blood analysis, a biochemical blood analysis (creatinine, urea), prostate-specific antigen test (PSA), a general urine analysis, the tamsulosin therapy prescription at a dose of 0.4 mg/day, taking a blood test for genotyping. Not earlier than on the 6th day of the study, after reaching 5 drug half-lives and reaching the equilibrium residual concentration (Css_{min}), the patient was referred for blood plasma before the tamsulosin administration to determine Css_{min} and for a morning urine sample to determine the CYP3A4 activity. At visit 2 (on the 14th day) and 3 (on the 28th day), the dynamics of the prescribed therapy was evaluated using the validated IPSS and QoL questionnaire. At the final visit 4 (on the 56th day), the dynamics of the therapy was evaluated according to the IPSS and QoL questionnaire and instrumental methods (a repeated Q_{max} estimation, the determination of the postvoid residual urine volume and prostate volume according to the ultrasound). The data from 142 patients were included in the outcome analysis, only those who had undergone all the 4 visits. The data from 6 patients were excluded because they had refused to participate in the study.

Eligibility criteria

The inclusion criteria for the study were: a male gender; the age over 18 years; a written informed consent to participate in the study; a confirmed diagnosis of "benign prostatic hyperplasia (N40 ICD10)"; complaints of LUTS moderately or severely pronounced, assessed by the IPSS scale by more than 7 points; a residual urine volume (RUV) less than 100 ml, according to the ultrasound (USG) of the bladder; a prostate volume from 25 to 100 cm^3 according to the transrectal ultrasound (TRUS) of the prostate gland; the absence of prostate cancer, including clinically insignificant (in cases of PSA) increase of more than 4 ng/ml. In accordance with the clinical recommendations of the Ministry of Health of the Russian Federation on the management of patients with benign prostatic hyperplasia (approved by the Ministry of Health of the Russian Federation in 2020)[2], a multifocal prostate biopsy was performed).

The non-inclusion criteria were: complicated BPH; any causes other than BPH that may, in the opinion of the investigator, lead to dysuria or an altered urine flow velocity (e.g., the neurogenic bladder, a bladder neck stricture, the urethral stricture, acute or chronic prostatitis, acute or chronic urinary tract infections); concomitant cancer; concomitant severe cardiovascular (e.g., unstable angina, a recent myocardial infarction, or poorly controlled arterial hypertension) and a cerebrovascular disease (a recent stroke or spinal cord injury); renal and hepatic insufficiency.

The exclusion criteria were: a drug intolerance

[2] Clinical recommendations of the Ministry of Health of the Russian Federation. Benign prostatic hyperplasia, 2020. Available from: https://cr.minzdrav.gov.ru/schema/6_1. Russian

detection; a patient's refusal to take the prescribed therapy; a patient's refusal to participate in the study.

Genotyping

The material for the determination of gene polymorphisms was 4 ml of blood from the veins of the elbow bend, collected using a vacuum system for a venous blood collection VACUETTE (Greiner Bio-One, Austria) into tubes with K3-ethylenediaminetetetraacetate (EDTA). The DNA isolation was performed using the reagent kit "DNA-Extran-1" for a genomic DNA isolation from whole blood (CJSC Syntol, Moscow, Russia).

Genotyping of patients was performed at the Research Institute of Molecular and Personalized Medicine of RMA CPE.

The carriage of CYP3A4*1B (c.-392G >A, rs2740574), CYP3A4*22 (c.522-191C >T, rs35599367) and CYP3A5*3 (c.6986A >G, rs776746) polymorphic markers was determined for all 142 patients.

For genotyping by CYP3A4*1B and CYP3A5*3 allelic variants, SNP-Screen reagent kits (CJSC Syntol, Moscow, Russia) were used according to the manufacturer's instructions. Genotyping by a CYP3A4*22 allelic variant was performed using reagent kits "TaqMan® SNP Genotyping Assays" and TaqMan Universal Master Mix II, without UNG (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions.

The carriage of polymorphic markers was determined by a real-time polymerase chain reaction on a Real-Time CFX96 Touch device (Bio-Rad Laboratories, Inc., USA).

After the inclusion in the study, the blood was collected from all patients for a genetic testing. Depending on the genotyping results, the patients were divided into groups according to the phenotypes of "extensive metabolizers" (EM), "intermediate metabolizers" (IM) and "poor metabolizers" (PM) depending on the carriage of CYP3A4*22 and CYP3A5*3 variants [12, 16].

CYP3A phenotyping

A CYP3A4 activity was determined by estimating the ratio of 6-beta-hydroxycortisol (6b-HC) to cortisol concentrations in the patient urine collected in the morning.

Cortisol is a specific CYP3A4 substrate. By calculating the metabolic ratio of the concentrations of cortisol and its metabolite 6b-HC, the activity of CYP3A4 is determined: high values of the ratio indicate a high activity of the isoenzyme, while low values indicate a low activity. The methodology for determining a CYP3A4 activity is generally accepted [20].

Cortisol and its metabolite were determined by high-performance liquid chromatography (HPLC) with a mass spectrometric detection. Agilent 1200 liquid chromatograph (Agilent Technologies Inc., USA, 2008) and AgilentTripleQuad LC/MS 6410 mass spectrometer were used. The results were processed using Agilient MassHunter Workstation Software LC/MS Data Acquisition for 6400 Series Triple Quadrupole (version B.08.02). To perform a chromatographic determination, the sample preparation technique and conditions of chromatographic analysis presented in the work by Smirnov V.V. et al., were used [20].

Determination of tamsulosin plasma concentration

The tamsulosin plasma concentration was determined by HPLC on an Agilent 1200 liquid chromatograph (Agilent Technologies Inc., USA, 2008). Agilent Polaris 3 C18-A column (length 50 mm; inner diameter 3.0 mm; grain size 3.0 µm) was used. The separation was performed at a column temperature of 40°C. The mobile phase consisted of two components: solution "A" (1 mL of concentrated formic acid was diluted with deionized water to a total volume of 1 L) and solution "B" (1 mL of concentrated formic acid was diluted with acetonitrile to the total volume of 1 L). A chromatographic separation was carried out in a gradient elution mode.

The sample preparation was carried out by the method of blood plasma protein precipitation. The plasma samples were thawed at room temperature. Then 100 µl of plasma was transferred into Eppendorf-type plastic tubes, 250 µl of a methanol mixture with 0.1% hydrochloric acid (in the ratio of components 9:1) was added, mixed on a Vortex shaker (Elmi Ltd., Latvia) and left for 10 min. Then the samples were mixed once again. Next, the obtained samples were centrifuged at 10 000 rpm for 10 min. The supernatant was transferred to chromatographic vials and placed on the autosampler of the chromatograph for the analysis.

For the tamsulosin spectra detection, an AgilentTripleQuad LC/MS 6410 mass spectrometer with an electrospray ionization in the positive ionization mode was used. The tamsulosin spectra were recorded in the multiple molecular reaction mode. The atomizer gas pressure was 35 *psi.* The volume velocity of the drying gas was 10 L/min, and the temperature of the ion source was 350°C. The fragmentation voltage value was

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135 V, and the voltage on the collision cell was –30 V. Under these conditions, the tamsulosin quantification limit was 1 ng/ml.

The results were processed using Agilient MassHunter Workstation Software LC/MS Data Acquisition for 6400 Series Triple Quadrupole (Version B.08.02).

Group analysis

As part of the analysis, comparison groups were formed from the total study sample of 142 patients regarding (1) CYP3A phenotype as determined by CYP3A4*22 and CYP3A5*3 genotype and (2) the carriage of individual allelic variants of CYP3A4*1B, CYP3A4*22 and CYP3A5*3.

Comparisons in (1) were made between the groups of "fast" (Extensive Metabolizers, EM) (n=17), "intermediate" (Intermediate Metabolizers, IM) (n=117) and "slow" (Poor Metabolizers, PM) (n=8) metabolizers (EM *vs* IM *vs* PM).

Comparisons in (2) were made between the groups according to the carriage of genotypes for CYP3A4*1B (AA (n=128) *vs* AG (n=14)), CYP3A4*22 (CC (n=133) *vs* CT (n=9)) and CYP3A5*3 (AA+AG (n=18) *vs* GG (n=124)). For CYP3A5*3, pooling of the AA+AG group was done given a low frequency for the AA genotype (n=1).

Statistical processing

For a statistical processing of the study data, methods of parametric and nonparametric statistics with the help of STATISTICA v10.0 (StatSoft Inc., USA) and Microsoft Excel 2010 program for Windows were used. When selecting the method, the normality of a sample distribution had been taken into account and evaluated using the Shapiro-Wilk's *W*-test and the Kolmogorov-Smirnov criterion.

A sample description for non-normally distributed parameters was performed by calculating the median (Me) and interquartile range as 25th and 75th percentiles (Q1 and Q3), for normally distributed parameters - by determining the mean (M) with a standard deviation (Standart Deviation, SD).

The Student's t-test or Mann-Whitney test (depending on the nature of the distribution of quantitative indicators) was used to compare quantitative indicators.

Depending on the distribution nature, multiple samples of continuous data were compared using either single- or multivariate analysis of variance (for normally distributed data) or the Kruskal–Wallis H-test (for data that do not follow a normal distribution). Correction for multiple comparisons was performed using the Bonferroni.

Frequency characteristics of qualitative indicators were compared using Pearson's χ^2 tests.

To establish the nature and strength of the relationship between the signs, the correlation analysis was used, preliminarily checking the normality of the variables distribution using the Shapiro-Wilk criterion. In case of quantitative variables and their conformity to the law of normal distribution, the Pearson's linear correlation coefficient (r) was calculated; otherwise, the Spearman's rank correlation coefficients (ρ) or the Kendall's correlation coefficients (τ) were used. The critical level of significance was taken as p <0.05. The correlation coefficient r from 0.3 to 0.7 at p <0.05 meant a positive moderate but reliable correlation between the traits; r >0.7 at p <0.05 meant a strong and reliable relationship; a negative value of r corresponded to an inverse correlation.

RESULTS

Study participants

Clinical and epidemiologic characteristics of the patients, who have been included and undergone all phases of the study, are presented in Table 1.

The following information is important with regard to the comorbidities in the study patients. The study group included 108 patients, or 76.1%, who had been diagnosed with at least one comorbidity in addition to BPH. In turn, among these patients, 51 individuals (35.9% of the cohort) had multiple comorbidities in different classes of diseases. Finally, with no comorbidities (other than BPH), 32 individuals were included in the sample, representing 23.9% of the total sample.

The list of drug groups taken by the patients for the comorbid nosology is presented in Table 2.

It should be noted that only 1 patient in the sample was taking a CYP3A inhibitor drug as concomitant pharmacotherapy in the treatment of LUTS associated with BPH.

Primary outcome of the study

Efficacy assessment

In the study sample of 142 patients with LUTS for BPH taking tamsulosin, the distribution of genotypes for the allelic variants studied was as expected and agreed with the Hardy-Weinberg law distribution (p >0.05). This indicated that the frequency distribution of genotypes in this sample of patients reflects their distribution in the population as a whole (Table 3).

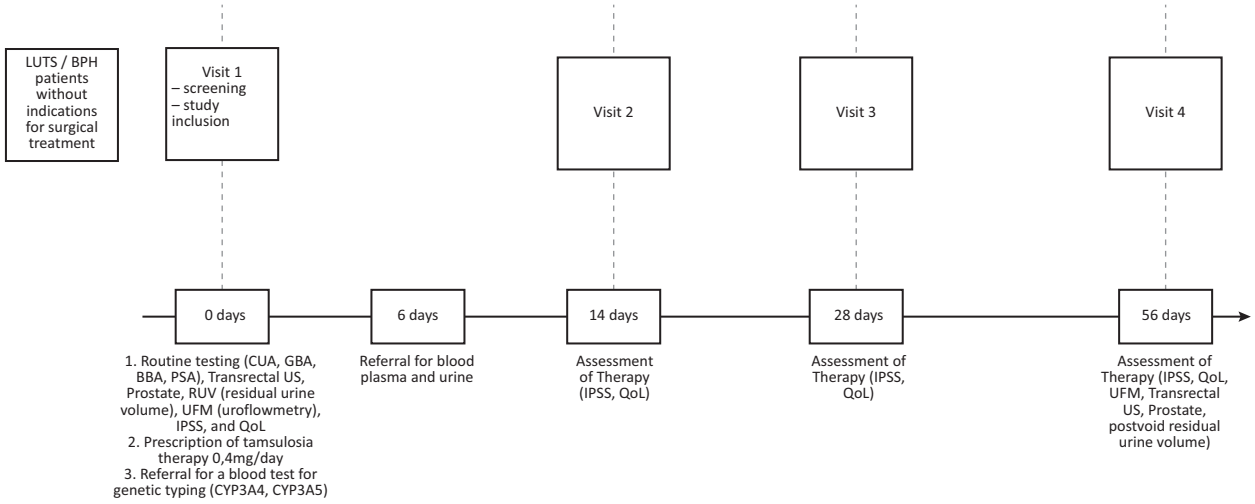

Figure 1 – Study design

Notes: LUTS – lower urinary tract symptoms; BPH – benign prostatic hyperplasia; CUA – common urine analysis; GBA – general blood analysis; BBA – biochemical blood analysis; PSA – prostate-specific antigen test; TRUS – transrectal ultrasound; RUV – residual urine volume; UFM – uroflowmetry; IPSS – International Prostate Symptom Score; QoLS – Quality of Life scale.

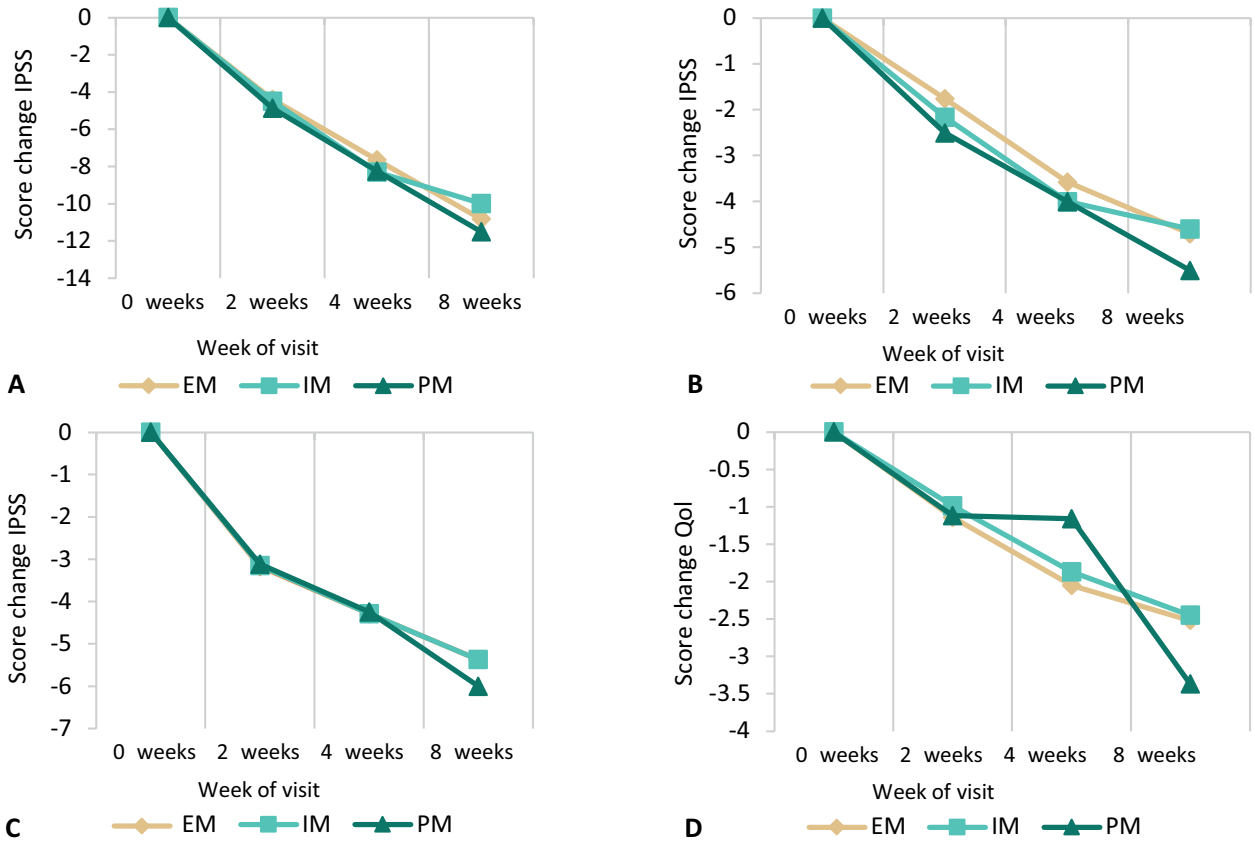

Figure 2 – Dynamics of changes in the sum of IPSS scores

Note: A – total IPSS score; B – obstructive symptoms subscale; C – irritative symptoms subscale; D – IPSS quality of life scale. EM – extensive metabolizers; IM – intermediate metabolizers; PM – poor metabolizers.

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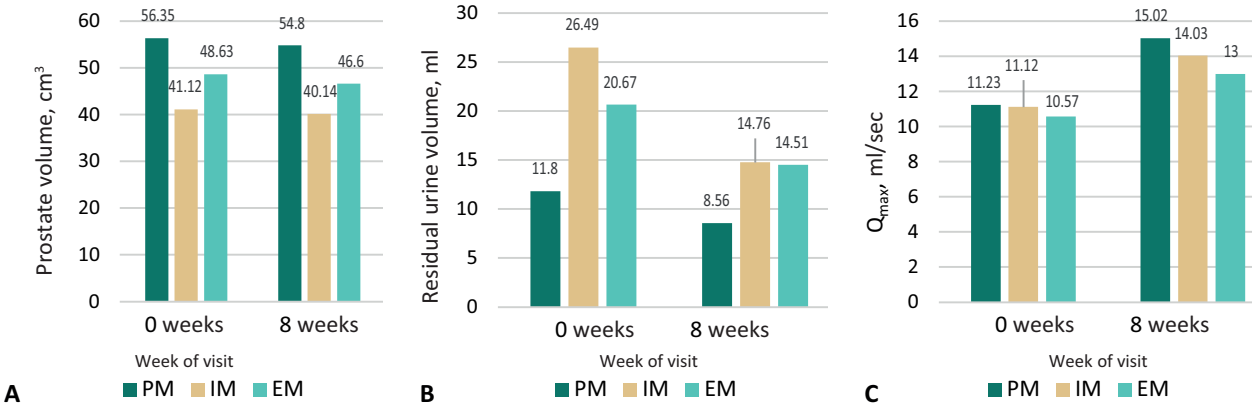

Figure 3 – Data comparison of instrumental assessment of therapy efficacy

Note: A – prostate volume; B – residual urine volume; C – maximum urine stream velocity; EM – extensive metabolizers; IM – intermediate metabolizers; PM – poor metabolizers.

Table 1 – Clinical and epidemiologic characteristics of patients

Indicator	Value	*n*
Mean age (Me [Min; Max]), years	68 [37; 86]	142
Body mass index, kg/m² (M±SD)	26.83±4.31	142
Smoking, *n* (%)	25 (17.6)	142
Alcohol, *n* (%)	77 (54.22)	142
Creatinine, mmol/L (M±SD)	85.4±13.78	142
Urea, mmol/l (M±SD)	5.8±1.41	142
Relative density, g/l (M±SD)	1015.5±8.58	142
Urine pH	5.72±0.7	142
Hemoglobin, g/L (M±SD)	148.5±13.46	142
Erythrocytes, 10^9/L (M±SD)	5.34±3.63	142
Leukocytes, 10^9/l (M±SD)	7.8±2.32	142
Platelets, 10^9/l (M±SD)	258.1±69.88	142
ESR, mm/hour (M±SD)	12.02±10.42	142
PSA, ng/mL (M±SD)	2.59±1.73	142
Comorbidities, *n* (%):		
1. Cardiovascular:	98 (69.0)	
– Hypertensive disease	68 (47.8)	
– Ischemic heart disease	21 (14.7)	
– Others	9 (6.3)	
2. Endocrinologic (type 2 diabetes mellitus – insulin-independent)	6 (4.2)	
3. Pulmonologic (chronic obstructive pulmonary disease, bronchial asthma)	6 (2.4)	
4. Gastroenterological	8 (5.6)	
5. Urologic (urolithiasis, kidney cysts, erectile dysfunction)	7 (4.9)	
6. Neurological (degenerative and dystrophic diseases of the spine, intervertebral hernias)	7 (4.9)	
Total, *n* (%):	108 (76.0)	
Comorbid patients	51 (35.9)	
Without concomitant pathology	34 (23.9)	

Note: ESR – erythrocyte sedimentation rate; PSA – prostate specific antigen test.

Table 2 – Concomitant drug therapy in patients during the follow-up period

Drug group	*n*	Drugs	CYP3A Inhibitors	CYP3A inductors	CYP3A Substrates
Diuretics	10	indapamide spironolactone	–	–	–
Calcium channel blockers	10	amlodipine lercanidipine	nifedipine (*n*=1)	–	–
Angiotensin receptor antagonist	3	candesartan telmisartan valsartan	–	–	–
Diabetics	6	metformin gliclazide empagliflozin manninil insulin	–	–	–
iACEIs	14	perindopril lisinopril enalapril	–	–	enalapril (*n*=4)
Anticoagulants	1	apixaban	–	–	apixaban
β-adrenoblockers	15	bisoprolol nebivolol metoprolol	–	–	–
Statins	11	atorvostatin rosuvostatin simvostatin	–	–	atorvostatin rosuvostatin simvostatin
NSAIDs	1	paracetamol	–	–	–
Antiaggregants	23	acetylsalicylic acid clopidogrel ticagrelor	–	–	–
Others	10	mesalazine isosorbitol dinitrite terbinafine formoterol methotrexate tofizopam phenibut phosphoglyph rebagit	–	–	symbecord inhaled glucocorticosteroids (*n*=3)
Without concomitant drug therapy, *n* (%)	73 (51.4)	–	–	–	–

Notes: NSAIDs – non-steroidal anti-inflammatory drugs; iACEs – angiotensin-converting enzyme inhibitors.

Table 3 – Distribution of genotypes for studied polymorphisms by frequency, conformity of distribution to Hardy-Weinberg law

Allelic variant	Alleles	*n* (%)	Genotypes	*n* (%)	χ^2	*p*–value
CYP3A4*1B (c.-392G >A, rs2740574)	A	270 (95.1)	AA	128 (90.1)	0.3817	0.8262
	G	14 (4.9)	AG	14 (9.9)		
			GG	0 (0)		
CYP3A4*22 (c.522-191C >T, rs35599367)	C	275 (96.8)	CC	133 (93.7)	0.1520	0.9267
	T	9 (3.2)	CT	9 (6.3)		
			TT	0 (0)		
CYP3A5*3 (c.6986A >G, rs776746)	A	19 (6.7)	AA	1 (0.7)	0.2400	0.8869
			AG	17 (11.9)		
	G	265 (93.3)	GG	124 (87.4)		

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Table 4 – Distribution of CYP3A phenotypic variants in study sample

Enzyme	Phenotype	Frequency, *n* (%)	Genotypes
CYP3A	Poor metabolizers (PM)	8 (5,6)	CYP3A4*22/*22 и CYP3A5*3*3
	Intermediate metabolizers (IM)	117 (82,4)	CYP3A4*1/*1 и CYP3A5*3/*3, CYP3A4*1/*22 и CYP3A5*1/*3
	(Extensive Metabolizers, (EM)	17 (12,0)	CYP3A4*1/*1 и CYP3A5*1/*3, CYP3A4*1/*1 и CYP3A5*1/*1

Table 5 – Data of indicators for assessing tamsulosin pharmacotherapy effectiveness in patients under study

Visit	Parameter	CYP3A4*1B genotype p		*p*	CYP3A4*22 genotype		*p*	CYP3A5*3 genotype		*p*
		AA (*n*=128)	AG (*n*=14)		CC (*n*=133)	CT (*n*=9)		AA+AG (*n*=18)	GG (*n*=124)	
1 (day 0)	IPSS, score	19.06±7.22	20.71±6.04	0.41	19.18±7.06	19.77±8.25	0.81	20.05±7.22	19.10±7.12	0.59
	Irritative symptoms subscale	10.54±4.7	10.78 ±4.02	0.83	10.51±4.65	11.33 ±4.44	0.61	10.94 ±4.41	10.51±4.67	0.71
	Obstructive symptoms subscale	8.0 [5.5; 11.0]	9.0 [7.0; 13.0]	0.30	8.0 [6.0; 11.0]	8.0 [5.0; 13.0]	0.78	8.0 [6.0; 14.0]	8.0 [6.0; 11.0]	0.7
	QoL	5.12 ±0.80	5.5 ±0.75	0.09	5.15 ±0.80	5.33 ±0.86	0.51	5.16 ±0.78	5.16 ±0.81	0.97
	Prostate volume, cm^3	35.25 [29.69; 47.3]	48.5 [30.32; 70.0]	0.10	35.66 [30.0; 48.5]	40.0 [33.2; 63.6]	0.92	42.6 [29.08; 63.5]	35.25 [30.0; 48.05]	0.43
	RUV, ml	15.0 [2.5; 31.75]	18.5 [5.0; 40.0]	0.46	15.0 [2.0; 35.79]	9.0 [5.0; 20.14]	0.87	12.5 [0.0; 38.07]	15.0 [3.5; 33.75]	0.65
	Q_{max}, ml/sec	10.9 [8.1; 13.8]	9.45 [7.7; 12.3]	0.30	11.0 [8.5; 13.3]	10.9 [8.8; 14.0]	0.78	10.8 [8.3; 12.7]	10.95 [8.65; 13.45]	0.83
2 (2 weeks)	IPSS, score	–4.42±4.57	–5.28±5.29	0.51	–4.48±4.62	–4.88±5.03	0.79	–4.44±4.0	–4.51±4.73	0.95
	Irritative symptoms subscale	–3.17±3.35	–2.92 ±3.12	0.84	–3.14±3.36	–3.33 ±2.73	0.86	–3.27±3.12	–3.13±3.36	0.86
	Obstructive symptoms subscale	–2.0 [–3.0; 0.0]	–2.5 [–4.0; 0.0]	0.92	–2.0 [–3.0; 0.0]	–2.0 [–4.0; –1.0]	0.80	–1.0 [–3.0; 0.0]	–2.0 [–3.0; –0.5]	0.37
	QoL	–1.03 ±1.11	–1.21 ±0.97	0.55	–1.04 ±1.11	–1.11 ±0.92	0.86	–1.38 ±1.37	–1.0 ±1.05	0.16
3 (4 weeks)	IPSS, балл	–8.10±6.21	–9.35±5.56	0.47	–8.20±6.13	–8.55±6.69	0.86	–7.83±4.21	–8.28±6.39	0.77
	Irritative symptoms subscale	–4.25±3.83	–4.35 ±2.89	0.94	–4.24±3.80	–4.55 ±2.83	0.81	–4.22±2.34	–4.27±3.91	0.95
	Obstructive symptoms subscale	–4.0 [–6.0; –2.0]	–4.0 [–9.0; –2.0]	0.44	–4.0 [–6.0; –2.0]	–3.0 [–6.0; –1.0]	0.76	–3.5 [–5.0; –2.0]	–4.0 [–6.0; –2.5]	0.69
	QoL	–1.84 ±1.25	–2.21 ±1.36	0.29	–1.66 ±1.11	–1.89 ±1.27	0.60	–2.05 ±1.55	–1.85 ±1.22	0.53
4 (8 weeks)	IPSS, score	–9.93±7.14	–12.28±6.26	0.24	–10.08±7.11	–11.44±6.87	0.57	–10.83±5.42	–10.07±7.30	0.67
	Irritative symptoms subscale	–5.40±4.35	–6.35 ±2.70	0.49	–5.45±4.30	–6.11 ±2.80	0.65	–6.16±2.74	–5.40±4.39	0.47
	Obstructive symptoms subscale	–4.0 [–7.0; –2.0]	–6.0 [–10.0; –3.0]	0.29	–4.0 [–7.0; –2.0]	–4.0 [–8.0; –2.0]	0.71	–4.0 [–8.0; –2.0]	–4.0 [–7.0; –2.0]	0.88
	QoL	–2.47 ±1.38	–2.85 ±1.74	0.34	–3.22 ±1.64	–2.46 ±1.39	0.12	–2.5 ±1.65	–2.51 ±1.38	0.96
	Prostate volume, cm^3	36.1 [29.12; 46.9]	37.75 [31.2; 64.0]	0.31	36.5 [29.0; 47.21]	37.9 [32.0; 62.05]	0.48	37.75 [28.09; 59.0]	36.1 [29.62; 47.6]	0.77
	RUV, ml	7.0 [2.5; 19.0]	9.0 [5.0; 20.0]	0.33	7.0 [3.0; 20.0]	7.0 [5.0; 10.0]	0.86	8.0 [3.0; 15.0]	7.0 [2.5; 19.35]	0.77
	ΔRUV	–5.0 [–17.37; 1.0]	–8.39 [–23.0; 0.0]	0.61	–6.0 [–18.0; 1.0]	–2.76 [–17.14; 1.0]	0.53	–7.5 [–14.0; 3.0]	–5.0 [–19.0; 1.0]	0.55
	Q_{max}, ml/sec	13.8 [9.1; 17.2]	12.75 [7.8; 16.7]	0.52	14.0 [10.3; 17.2]	14.7 [13.2; 16.7]	0.45	14.05 [8.7; 16.2]	14.0 [11.0; 17.2]	0.42
	ΔQ_{max}	2.3 [–0.1; 5.0]	2.6 [0.9; 4.4]	0.74	2.8 [0.8; 5.0]	4.3 [2.4; 5.4]	0.40	2.65 [0.9; 3.9]	2.8 [0.8; 5.3]	0.60

Note: IPSS – International Prostate Symptom Score; QoL – IPSS quality of life scale; RUV – residual urine volume; Q_{max} – maximum urine stream velocity according to uroflowmetry results.

Table 6 – ARs distribution in study sample

AR type	*n* (%)
Retrograde ejaculation	8 (22.2)
Orthostatic hypotension	7 (19.5)
Epigastric burning	4 (11.1)
Dizziness	4 (11.1)
Hypertension	3 (8.4)
Dyspepsia	2 (5.5)
Headaches	2 (5.5)
Blurred vision	2 (5.5)
Erectile dysfunction	1 (2.8)
Diarrhea	1 (2.8)
Back pain	1 (2.8)
Rhinitis	1 (2.8)
Total	36

Note: AR – adverse reaction.

Table 7 – Frequency of AR patients with regard to CYP3A metabolic activity classification

Enzyme	Phenotype	*n* (%)	*p*
CYP3A	IM	5 (20,8%)	0,168
	EM	19 (79,2%)	
	PM	0 (0%)	

Note: Pearson's χ^2 test was used for p-value calculations. EM – extensive metabolizers; IM – intermediate metabolizers; PM – poor metabolizers.

Table 8 – Data of descriptive statistics of tamsulosin Css_{min} values in the studied samples

Parameter	Value
Number of samples	75
Mean (M), ng/ml	8,2
SD	7,78
Median (Me), ng/ml	5,9
Q1	2,13
Q3	11,6
Maximum, ng/ml	26,5
Minimum, ng/ml	0,0

Table 9 – Comparison of Css_{min} tamsulosin values in EM, IM and PM groups by CYP3A

Indicator	CYP3A phenotype						*p*
	EM (*n*=11)	min–max	IM (*n*=61)	min–max	PM (*n*=3)	min–max	
Css_{min} (Me [25.75]). ng/ml	7.26[0.0;15.05]	0–23.4	5.88[2.4;11.6]	0–26.5	8.4[0.18;10.19]	0.18–10.19	0.9539

Note: Kruskal–Wallis *H*-test was used to calculate the *p*-value. EM – extensive metabolizers; IM – intermediate metabolizers; PM – poor metabolizers.

Table 10 – Comparison of tamsulosin Css_{min} values between patients regarding carriage of CYP3A4*1B, CYP3A4*22 and CYP3A5*3 variants

Allele	Genotype	*n*	Css_{min} (Me [25,75]), ng/ml	min–max	*p*
CYP3A4*1B	AG	8	7.21[2.25;19.22]	0–26.5	0.57
	AA	67	5.88[2.13;11.3]	0–26.3	
CYP3A4*22	CT	4	9.29[4.29;18.34]	0.18–26.5	0.37
	CC	71	5.88[2.13;11.6]	0–26.3	
CYP3A5*3	AG	11	7.26[0.0;15.05	0.0–23.4	0.76
	GG	64	5.89[2.36;11.45]	0–26.5	

Note: Mann–Whitney test was used for *p*-value calculations.

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Table 11 – Results of HPLC-MS/MS performed for the determination of cortisol and 6b-HC concentrations in urine

Groups (*n*=131)	Values	Cortisol concentration, ng/ml	6b-HC concentration, ng/ml	6b-HC / cortisol (relative units)
EM (*n*=16)	Me	60.6	129.05	1.9
	Q1	43.3	106.2	1.55
	Q3	97.65	217.25	3.85
	max	175.2	325.4.	5.8
	min	18.4	19.2	0.8
IM (*n*=108)	Me	51.65	104.75	2.4
	Q1	28.65	64.45	1.3
	Q3	84.5	178.6	4.1
	max	273.9	1075.5	8.8
	min	1.6	6.1	0.2
PM (*n*=7)	Me	43.12	132.72	2.97
	Q1	36.08	105.25	5.31
	Q3	50.92	289.54	2.55
	max	129.78	80.45	7.97
	min	28.09	344.02	2.23

Note: EM – extensive metabolizers; IM – intermediate metabolizers; PM – poor metabolizers.

Table 12 – Differences in 6-hydroxycortisol / cortisol metabolic ratio in patients with different CYP3A phenotypes

Group of patients by CYP3A phenotypes	Comparison (*p*-value)
EM *vs* IM *vs* PM	0.235
EM *vs* IM	0.902
IM *vs* PM	0.106467
EM *vs* PM	0.076627

Notes: Kruskal–Wallis *H*–test and Mann–Whitney paired *U*–test were used for *p*-value calculations.
EM – extensive metabolizers; IM – intermediate metabolizers; PM – poor metabolizers.

Table 13 – Spearman correlation coefficient values (r_s) reflecting relationship between cortisol concentration, 6b-HC and 6b-HC/cortisol ratio of patients and difference in values of studied clinical parameters before and after therapy

Indicator	Cortisol concentration	*p*	6b-HC concentration	*p*	6b-HC / cortisol	*p*
IPSS	-0.047027	>0.05	0.073377	>0.05	-0.105449	>0.05
OS	-0.004519	>0.05	0.045193	>0.05	-0.059237	>0.05
IS	-0.058387	>0.05	0.084310	>0.05	-0.114064	>0.05
QoL	-0.057905	>0.05	-0.048397	>0.05	-0.019504	>0.05
ΔRUV	-0.098710	>0.05	0.098710	>0.05	0.163890	>0.05
ΔQ_{max}	-0.103879	>0.05	-0.103879	>0.05	0.035049	>0.05

Note: OS – IPSS subscale to assess the severity of obstructive symptoms; IS – IPSS subscale to assess the severity of irritative symptoms; QoL – IPSS scale to assess the quality of life (QoL); RUV – residual urine volume; Q_{max} – maximum urine flow rate according to the results of uroflowmetry.

According to the results of genotyping, depending on the genotype and the encoded phenotypic activity of CYP3A, all patients were divided into groups according to the level of the enzyme activity [16]. The distribution of phenotypic variants of the CYP3A activity is presented in Table 4.

The dynamics of changes in the subjective assessment of LUTS symptomatology by the IPSS scale, subscale and Qol among the patients belonging to different types of CYP3A metabolizers is presented in Fig. 2.

Thus, the obtained data demonstrate the absence of statistically significant (using ANOVA-test) association between the CYP3A phenotype and clinical parameters of the tamsulosin therapy efficacy assessment in the sample of the examined patients with LUTS in BPH (p >0.05).

Figure 3 shows the comparison of the prostate volume, RUV and Q_{max} in patients from PM, IM and EM groups at visits1 and 4.

The analysis shows that there is no statistically significant association between the phenotype determined by the CYP3A4 and CYP3A5 genotype and clinical parameters of the tamsulosin therapy efficacy assessment in the studied sample of patients (p >0.05 by the Mann–Whitney U-test).

Further correlations between the clinical parameters of the therapy efficacy evaluation in patients with LUTS in BPH and the carriage of certain polymorphic markers of the genes CYP3A4*1B, CYP3A4*22, CYP3A5*3, were searched for (Table 5).

When comparing the results of the patients' treatment in the study between the combined group of CYP3A5*3 polymorphism (AA+AG) carriers and non-carriers (GG) during the observation period, no statistically significant data were revealed. Similar results were obtained when analyzing the influence of the CYP3A4*1B and CYP3A4*22 polymorphisms carriage on clinical parameters of the tamsulosin LUTS therapy for BPH.

The analysis of the calculation results showed that in the group of 142 patients no statistically significant associations were found for any of the considered clinical parameters and carriage of CYP3A4 and CYP3A5 variants in the patients.

Safety assessment

Throughout the follow-up of the patients taking tamsulosin for the indication of LUTS for BPH, a total of 36 cases of the adverse reactions (ARs) development were identified in 30 patients (Table 6).

However, 24 patients reported developing 1 AR, and 6 patients developed more than 1 AR. Among all the patients who had developed ARs, none of them was the reason for withdrawal of the prescribed therapy. The distribution of ARs according to CYP3A phenotypes is presented in Table 7.

Evaluation of relationship of tamsulosin equilibrium residual concentration with CYP3A phenotype and CYP3A4 and CYP3A5 allelic variants

Among 142 patients with LUTS for BPH receiving the tamsulosin therapy, plasma was collected from 88 patients to determine the equilibrium residual concentration (Css_{min}) of the drug. Of the 88 samples, 75 sample results were selected for the analysis, and 13 were excluded due to the overestimated absolute Css_{min} values, which might have been due to the patients taking another dose of the drug before their medical prescriptions and before the plasma sample had been collected. The descriptive statistics of the results of the samples included for the analysis are presented in Table 8.

The effect of CYP3A phenotypes on Css_{min} of tamsulosin in patients with LUTS for BPH was evaluated. A statistical calculation was performed for EM (n=11), IM (n=61) and PM (n=3) groups (Table 9).

According to the results of the group comparison, no significant associations between tamsulosin Css_{min} values and CYP3A phenotype type (EM, IM and PM) of patients were revealed (p >0.05).

The comparison of tamsulosin Css_{min} in patients under study regarding the carriage of CYP3A4 and CYP3A5 gene variants revealed no significant differences between carriers and non-carriers of CYP3A4*1B (p=0.57), CYP3A4*22 (p=0.37) and CYP3A5*3 (p=0.76) alleles (Table 10).

Evaluation of the effect of CYP3A isoenzyme activity on efficacy and safety

The metabolic ratio of 6b-HC/cortisol in urine was determined in 131 patients. The results of CYP3A phenotyping of 6b-HC / cortisol in urine from patients with LUTS for BPH genotyped for CYP3A4 and CYP3A5, allelic variants and their descriptive statistics are presented in Table 11 and Fig. 4.

No association was found between the metabolic ratio of 6b-HC / cortisol in urine and the CYP3A phenotype encoded by the combined genotypes of CYP3A4 and CYP3A5 gene variants (Table 12).

The Spearman correlation analysis showed that there was no statistically significant relationship between the concentrations of cortisol, 6b-HC, their ratio and all the studied parameters (Table 13).

DISCUSSION

The biotransformation of tamsulosin in the body occurs under the action of CYP3A4 and CYP2D6 enzymes. In the instructions of tamsulosin preparations in the precautions section, there is information that the drug should not be used in combination with strong inhibitors of CYP3A4 (e.g., ketoconazole) and CYP2D6 (e.g., paroxetine); used with caution with moderate inhibitors of CYP3A4 (e.g., erythromycin) and CYP2D6 (e.g., terbinafine). Clearly, the functional activity of

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metabolizing enzymes plays a key role in the drug response.

Previously, a number of authors have investigated a potential role of genetic markers encoding changes in the activity of CYP3A4, CYP3A5 and CYP2D6 enzymes on the variability of drug pharmacokinetics parameters in healthy volunteers. Thus, Kim K.A. et al. (2018) investigated the effect of allelic variants of CYP2D6 (*2, *4, *5, *10, *14, *21, *41 and *xN) and CYP3A5 (*3) genes on the peak concentration (C_{max}) and area under curve (AUC) of the drug in plasma in 29 volunteers. The authors concluded that a significant effect on C_{max} and AUC values was produced by carriage of CYP2D6*4 and *10 markers, whereas genotypes for CYP3A5*3 had no effect on the studied parameters [21]. In another study by Villapalos-García G. et al. (2021), in a group of 79 healthy subjects, it was shown that the subjects which were slow metabolizers by CYP3A5, had lower clearance rates (Cl/F) of tamsulosin than normal and fast metabolizers, but the associations leveled off after the correction by a multiple comparison correction. Significant correlations were found for CYP2D6 variants: poor (*4/*4 and *4/*5) and intermediate (*1/*4, *1/*5, *4/*15) CYP2D6 metabolizers had higher AUC values (p=0.004), higher T1/2 (*p*=0.008) and lower Cl/F values (*p*=0.006) compared to normal (*1/*1) and extensive(*1/*1×2) metabolizers [22].

It should be noted that the absolute majority of works on tamsulosin pharmacogenetics investigate the effect of CYP2D6 markers on the pharmacokinetics of the drug. In all cases, the studies were conducted on healthy volunteers of a relatively young age [21–24].

The present study was the first attempt to evaluate the carriage contribution of allelic variants of CYP3A4 and CYP3A5 genes to the efficacy and safety of the tamsulosin therapy in patients with LUTS for BPH. The Association for Molecular Pathology (AMP) joint consensus recommendation lists CYP3A4*22 (rs35599367) and CYP3A5*3 (rs776746) variants as tier 1 markers, a minimum sets of alleles to test, if the drug is metabolized by these enzymes. Other alleles, CYP3A5*6 and CYP3A5*7, also belonging to the first level, have not been studied, due to their low prevalence in the European population [13]. This was the reason for the choice of markers for this study.

The analysis of the obtained results shows that the CYP3A phenotype of patients, determined by CYP3A4 and CYP3A5 genotypes, has not played a significant role in modulating IPSS scores used for a subjective assessment of the therapy efficacy and has not affected on the frequency of the ADR. Despite the fact that CYP3A4*22 (rs35599367) and CYP3A5*3 (rs776746) variants encode alternative splicing, lead to the protein shortening and expression of a non-functional protein. In this study, the analysis of their contribution separately did not reveal associations with the parameters of the tamsulosin therapy efficacy assessment (IPSS, QoL, RUV and Q_{max}).

There is increasing evidence that genetic variations in CYP3A4 and CYP3A5 contribute significantly to the interindividual variability of the CYP3A metabolic activity [15, 16]. In particular, the authors have focused their attention on CYP3A4*22 (rs35599367) and CYP3A5*3 (rs776746), for which many studies have identified their effects on the CYP3A activity. In the present study, the joint contribution of these polymorphic markers to the phenotypic activity of CYP3A was investigated and, in turn, was assessed by the level of an endogenous cortisol metabolism. The essence of the method of determining a CYP3A activity is that the ratio of the 6b-HC metabolite to the initial cortisol can be used to judge the enzyme activity. No relationship between CYP3A phenotypes and the difference in 6b-HC/cortisol ratios between the poor, intermediate, and extensive metabolizer groups were found. CYP3A phenotyping by 6b-HC / cortisol is not always a convenient and reliable way to determine the enzyme activity, which had been confirmed by a number of studies [25, 26]. In this case, the results also show that there is no correlation between the metabolic activity of CYP3A, determined by the ratio of endogenous cortisol and its metabolite, and the carriage of alleles encoding a decrease in the functional activity of CYP3A. The analysis also revealed no correlation between 6b-HC / cortisol and the tamsulosin therapy efficacy in patients with LUTS in BPH.

In vitro studies show that the formation of tamsulosin metabolites, AM-1, M-1 and M-2, is catalyzed by CYP3A4, while the formation of M-3 and M-4 is catalyzed by CYP2D6 [27], and the main pharmacological action is due to the parent compound. Considering the metabolic pathway and the fact of potential adverse effects when CYP3A4 inhibitors are co-administered, the influence of CYP3A on the pharmacokinetic parameters of the tamsulosin therapy is undeniable. However, assuming that CYP2D6 variants play a predominant role in the drug metabolism, the effect of CYP3A variants may be masked by the CYP2D6 activity. This may also explain the results obtained in this study.

Study limitations

The study limitation was a relatively small sample size, so some possible clinically significant associations between factors could not be proved by statistical methods. A limited follow-up period, a limited number of candidate genes and allelic variants of CYP3A4 and CYP3A5 in the analysis are also worth mentioning. The contribution of candidate genes and allelic variants of CYP2D6, which is also involved in tamsulosin metabolism, was not analyzed in this work. The study was conducted within outpatient reception hours in a polyclinic, which does not allow minimizing the influence of the daily regimen, lifestyle, diet, possible concomitant pharmacotherapy and other factors on the variability of clinical parameters of efficacy and safety,

values of a measured equilibrium residual concentration of the drug, variability of concentrations of cortisol and its metabolite used to assess the activity of CYP3A enzymes.

CONCLUSION

A possible association between the carriage of CYP3A4*1B, CYP3A4*22, CYP3A5*3 allelic variants, a CYP3A activity estimated by the urine content of the endogenous substrate of this isoenzyme and its metabolite, a plasma concentration, and the tamsulosin efficacy and safety, has not been confirmed.

The issue of the contribution of CYP3A4 and CYP3A5 genetic polymorphisms to clinical parameters of the tamsulosin therapy requires a further study with increasing the sample of patients, with the inclusion of CYP2D6 gene markers in the analysis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All the authors confirm that their authorship meets the ICMJE international criteria (all the authors have made a substantial contribution to the conceptualization, conduct of the study and preparation of the article, read and approved the final version before publication. Shokhrukh P. Abdullaev – idea and concept of the study, conducting the study, systematizing literature data, writing and editing the text of the manuscript, formulating conclusions; Maksim N. Shatokhin – idea and development of the concept of the manuscript, systematization of literary data, text editing, formulation of conclusions, approval of the final version of the manuscript for publication; Oleg L. Sigailo – analysis and interpretation of literature data, participation in the research, analysis and discussion of the results obtained; Sherzod P. Abdullaev – idea and concept of the study, statistical data processing, writing and editing the manuscript, formulation of conclusions; Pavel O. Bochkov – development of methods and quantitative determination of drug concentration in blood plasma, formulation of phenotyping methods in samples, statistical data processing, text editing, formulation of conclusions; Svetlana N. Tuchkova – carrying out genotyping of samples, editing the text of the manuscript; Oleg V. Teodorovich – participation in the development of the concept of the manuscript, editing of individual sections of the manuscript; Oleg B. Loran – critical revision of the manuscript, approval of the final version of sections of the manuscript for publication; Dmitry A. Sychev – development of the research concept, critical analysis of the results obtained, approval of the final version of the manuscript for publication.

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Development of a method for quantitative determination of nitric oxide (NO) in rat tissues based on high-performance liquid chromatography and mass spectrometry

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A quantitative assessment of nitric oxide (NO) production in body tissues is an urgent problem in pharmacology and biochemistry. The study of physiological processes occurring with the participation of NO, as well as the metabolism and pharmacodynamics of pharmacological agents from the group of NO donors, requires the introduction of accurate and reproducible methods for the quantitative determination of this metabolite in biological media.

The aim of the study was to develop the HPLC-MS/MS methods for the quantitative determination of NO in various tissues of rats.

Materials and methods. The indirect NO quantification was based on estimation of the level of more stable metabolites: nitrites and nitrates extracted from rat tissues by homogenization with water. The reduction of nitrates to nitrites was carried out using nitrate reductase. The derivatization of nitrites was based on a reaction with Griess reagent. The resulting azo dye was determined by HPLC-MS/MS using an Agilent InfinityLab Poroshell 120 EC-C18 4.6×100 mm, 2.7 µm analytical column. The total chromatographic analysis time was 12 minutes, and the analyte retention time was 6.1 minutes. The analytical range of the method was 0.1–100.0 nmol (in terms of nitrite) per 1 ml of plasma or tissue homogenate.

Results. The developed a bioanalytical method was validated according to the following parameters: a selectivity, a matrix effect, a recovery degree, a sample transfer, an analytical range linearity, a lower limit of quantification (LLOQ), an intra- and inter-assay accuracy and precision, and a stability at all the stages of the analysis. To test the method, the NO content in the plasma, brain, heart, aorta and lungs of rats was determined.

Conclusion. The developed bioanalytical HPLC-MS/MS methods fully meets the validation requirements. The metrological characteristics of the technique make it possible to highly accurately estimate the NO production in various tissues of rats, which is undoubtedly relevant and in demand in the study of pathological processes as well as the mechanism of action of pharmacological agents from the group of NO donors.

Keywords: HPLC-MS/MS; chromatography; mass spectrometry; endothelial relaxing factor; nitric oxide

Abbreviations: NO – nitric oxide / endothelial relaxing factor; HPLC-MS/MS – high-performance liquid chromatography with mass spectrometric detection; IS – internal standard; AUC – area under the concentration-time curve; NADH – nicotinamide adenine dinucleotide; MRM – multiple reaction monitoring; LLOQ – lower limit of quantitation; EDTA – ethylenediaminetetraacetic acid; NMF – normalized matrix factor; CV – coefficient of variation; SD – standard deviation; DP – declustering potential; EP – entrance potential; CE – collision energy; CXP – collision cell exit potential.

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Разработка методики количественного определения оксида азота (NO) в тканях крыс с применением высокоэффективной жидкостной хроматографии и масс-спектрометрии

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Количественная оценка продукции оксида азота (NO) в тканях организма является актуальной задачей фармакологии и биохимии. Изучение физиологических процессов, протекающих с участием NO, а также метаболизма и фармакодинамики фармакологических средств из группы донаторов NO требует внедрения точных и воспроизводимых методик количественного определения данного метаболита в биологических средах.

Цель. Разработка ВЭЖХ-МС/МС методики количественного определения оксида азота в различных тканях крыс.

Материалы и методы. Непрямое количественное определение NO было основано на оценке уровня более стабильных метаболитов: нитритов и нитратов, которые извлекали из тканей крыс путем гомогенизации с водой. Восстановление нитратов до нитритов осуществляли с помощью нитрат редуктазы. В основе дериватизации нитритов использовали реакцию с реактивом Грисса. Полученный азокраситель определяли с помощью ВЭЖХ-МС/МС с использованием аналитической колонки Agilent InfinityLab Poroshell 120 EC-C18 4,6×100 мм, 2,7 мкм. Общее время хроматографического анализа составило 12 минут, время удерживания аналита составило 6,1 минут. Аналитический диапазон методики составил 0,1–100,0 нмоль (в пересчете на нитрит) на 1 мл плазмы или гомогената ткани.

Результаты. Разработанная биоаналитическая методика была валидирована по следующим параметрам: селективность, матричный эффект, степень извлечения, перенос пробы, линейность аналитического диапазона, нижний предел количественного определения (НПКО), внутри- и межсерийная точность и прецизионность, стабильность на всех этапах анализа. Для апробации методики было проведено определение содержания NO в плазме, головном мозге, сердце, аорте и легких крыс.

Заключение. Разработанная биоаналитическая ВЭЖХ-МС/МС-методика полностью соответствует валидационным требованиям. Метрологические характеристики методики позволяют с высокой точностью оценить продукцию NO в различных тканях крыс, что, несомненно, представляет актуальным и востребованным в исследовании патологических процессов, а также механизма действия фармакологических средств из группы донаторов NO.

Ключевые слова: ВЭЖХ-МС/МС; хроматография; масс-спектрометрия; эндотелиальный релаксирующий фактор; оксид азота

Список сокращений: NO – оксид азота / эндотелиальный релаксирующий фактор; ВЭЖХ-МС/МС – высокоэффективная жидкостная хроматография с масс-спектрометрическим детектированием; IS – внутренний стандарт; AUC – площадь под кривой хроматографического пика; NADH – никотинамидааденинадинуклеотид; MRM – мониторинг множественных реакций; НПКО – нижний предел количественного определения; ЭДТА – этилендиаминтетрауксусная кислота; NMF – нормированный матричный фактор; CV – коэффициент вариации; SD – стандартное отклонение; DP – декластеризующий потенциал; CEP – напряжение на входе в ячейку соударений; CE – энергия столкновений; CXP – выходной потенциал ячейки соударений.

INTRODUCTION

To date, there is a sufficient amount of data on the participation of nitric oxide (NO) in the regulation of a number of biological processes in the body [1, 2]. It is known that NO involved in the regulation of vascular tone and systemic hemodynamic reactions [3, 4], is an inhibitor of a smooth muscle cell proliferation [5, 6]; it plays an important role in the hemostasis system [7, 8] and protection of the mucous membrane of the gastrointestinal tract [9, 10]. In addition, NO has been proven to play a role in the development and progression of many neurological, psychiatric and neurodegenerative disorders [11–13]. A decrease in the NO production, which is one of the causes of an endothelial dysfunction, is one of the early signs of a coronary heart disease and atherothrombosis [14]. At

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the same time, the endothelial dysfunction is important in the development of pathological conditions such as hypercholesterolemia, type 2 diabetes mellitus, arterial hypertension, and a heart failure [15, 16].

The accumulation of data on the NO participation in numerous bioregulation pathways has made it possible not only to study the pathogenesis of many diseases, but also to expand the possibilities of using organic nitrates and other NO donors in the clinical practice. It is known that the kinetics of the NO release from pharmacologically active substances depends on many factors, among which the chemical structure, reactivity, and activity of certain enzymes play a key role [17].

The study of physiological processes occurring with the NO participation, as well as metabolism and pharmacodynamics of pharmacological agents from the group of the NO donors, requires the introduction of accurate and reproducible methods for the quantitative determination of this metabolite in biological media. The assessment of the NO production is mainly carried out by measuring the concentration of its more stable metabolites – nitrites, which, in turn, are determined colorimetrically after the interaction with the Griess reagent [18]. However, this widely used method for the indirect determination of NO has a number of significant drawbacks. First, the Griess reagent can interact chemically with various components of the biological matrix with the formation of colored products that change the optical density of the analyzed samples [19, 20].

Second, this method requires a careful removal of protein components, since their presence in samples ready for the photometric analysis also affects the value of the measured optical density [21]. Third, a photometric detection does not often provide the required level of sensitivity [22]. In addition, this method does not provide for the internal control throughout all stages of analysis (use of an internal standard – IS). The above disadvantages require the improvement of this method for a quantitative determination of the NO production in the direction of increasing the selectivity and sensitivity of the reaction product detection.

One of such solutions is the use of high-performance liquid chromatography in combination with a mass selective detection for this purpose.

The aim of the study was to develop the HPLC-MS/MS methods for the quantitative determination of NO in various tissues of rats.

MATERIALS AND METHODS

Animals

In the process of developing and validating the method, the biological materials obtained from 6 intact male Wistar rats weighing 200–220 g (kennel of SMK STEZAR LLC, Russia), were used. The animals were kept in the vivarium of Tver State Medical University in plastic cages with a mesh lid, equipped with a feeder and drinking bowl. Sterile wood shavings were used as bedding. The rat cages were kept under controlled environmental conditions (temperature of 20–26°C and the relative humidity of 30–70%). In the animal rooms, a 12-hour lighting cycle and 8–10-fold changes in the air volume per hour were maintained. The rats were fed with complete feed PK-120 (Provimi LLC, Russia), food and filtered tap water were given *ad libitum*. The cages were cleaned daily, and water bottles were replaced with new ones every day. Wet cleaning of rooms was carried out daily. The evening before the experiment, the animals were deprived of food.

The animals were removed from the experiment by decapitation using a guillotine (manufacturer – Open Science) under a light general anesthesia using a combination of zolazepam and tiletamine (Zoletil® 100 mg/ml, Virbac, France). The blood was collected into tubes containing EDTA, and plasma was obtained by centrifugation at 3000 g for 10 min. Then fragments of internal organs (brain, heart, aorta, lungs) were selected and then used to obtain homogenates.

Ethical approval

This study was approved by the Local Ethics Committee of Tver State Medical University (Minutes of meeting No. 4 dated 29 May 2019). All the experiments were performed in accordance with the Rules of Laboratory Practice approved by Order of the Ministry of Health of Russia No. 708n dated 23 August 2010 and Directive of the European Parliament and the Council of the European Union dated 2010/63/EU on the protection of vertebrate animals used for scientific purposes.

Description of experimental part

Since NO is a gaseous metabolite with a relatively short half-life in living body tissues, its indirect quantification was based on assessing the level of more stable metabolites – nitrites and nitrates (Fig. 1).

The derivatization of nitrites was based on the well-known reaction with the Griess reagent (Fig. 2). The reduction of nitrates to nitrites was carried out using nitrate reductase.

A sample preparation of rat tissues included several stages: homogenization with deionized water, nitrates reduction, derivatization of the resulting nitrites, and deproteinization. The preparation of homogenates was carried out as follows: a tissue fragment was placed in

a 2 ml Eppendorf test tube pre-tared on an analytical scales VL-124 ("Gosmetr", Russia). After determining the exact mass, water was added at the rate of 400 µl per 100 mg of the tissue using an automatic variable volume dispenser ("Eppendorf Research Plus", Germany). A quartz glass ball with a diameter of 5 mm was placed, and then homogenized in a vibrating mill with a reciprocating frequency of 50 Hz and an amplitude of 30 mm for 15 min. To increase the yield of nitrates and nitrites from the biomaterial, the tubes were additionally kept in an ultrasonic bath (Megeon, China) for 10 min. The separation of the liquid part of the homogenate was carried out by centrifugation at 16 000 g and the temperature of +4°C for 15 min. The supernatant was transferred into separate 0.5 ml Eppendorf tubes, immediately frozen and stored at –40°C until the analysis. At the initial stage, the sample preparation of the blood plasma included a 5-fold waterdilution.

Before the procedure of obtaining the nitrate reduction and derivatization of the nitrite, the necessary solutions and reagents were prepared. The phosphate buffer solution was prepared as follows: 3.75 g of monopotassium phosphate ("PanReac Applichem", Spain), 10 mg of ethylenediaminetetraacetate sodium dihydrate ("PanReac Applichem", Spain), 1.4 g of potassium hydroxide ("Millipore", Germany) were added to a 1000 ml volumetric flask and brought to the mark with deionized water. The resulting solution was stored in the refrigerator at +4°C for no longer than 6 months. The resulting reagent, 1 ml, was transferred into Eppendorf tubes, used immediately or frozen and stored at –20°C for 1 month. The NADH working solution was prepared by diluting 1 ml of the original solution with a phosphate buffer 10 times and used throughout the day. The nitrate reductase stock solution ("R&D Systems", USA) was prepared by reconstituting 1 U of the lyophilized substance in 1 ml of the phosphate buffer and stored on ice for no longer than 8 hours. Immediately before the use, the original solution was 5-fold diluted. The supernatant of tissue homogenates (or diluted plasma) obtained at the previous stage was placed in clean microcentrifuge tubes in the volume of 20 µl, then 10 µl of NADH working solution was added and the reconstituted nitrate reductase was mixed on a V1 plus vortex ("Biosan", Latvia) for 10 seconds, after which it was incubated in a solid-state thermostat TDB-120 ("Biosan", Latvia) at 37°C for 30 min.

The final stage of the sample preparation included the nitrite derivatization using the Griess reagent and subsequent deproteinization. For this purpose, after thermostatting, the samples were transferred to an ice bath, 20 µl of a cooled 1% solution of sulfanilamide ("Merck", Germany) in a 2 N aqueous solution of hydrochloric acid ("Merck", Germany) was added, and left for 5 minutes. Then 20 µl of a 0.1% solution of N-(1-naphthyl)ethylenediamine ("Merck", Germany) in a 2 N aqueous solution of hydrochloric acid was added to the tubes and incubated at room temperature for 15 minutes. Protein precipitation was carried out by adding 200 µl of methanol cooled to –20°C containing IS (4-[4-amino-1-naphthylazo] benzenesulfonamide, 1000 ng/ml) to the samples (Fig. 3). The samples were kept in a thermostated shaker TS-100 C ("Biosan", Latvia) at temperature of 4°C and an oscillation frequency of 1 400 rpm for 5 min, after which the supernatant was separated by centrifugation at the acceleration of 16 000 g and the temperature of 4°C for 15 min. The resulting supernatant was transferred into polyethylene inserts for chromatographic vials and used for the HPLC-MS/MS analysis.

A chromatographic separation was carried out using HPLC 1260 Infinity II ("Agilent Technologies", Germany) in a reverse phase mode using a Poroshell InfinityLab 120 EC-C18 4.6×100 mm, 2.7 µm analytical column ("Agilent Technologies", USA) in combination with precolumn Zorbax Eclipse Plus C18 4.6×12.5 mm, 5 µm ("Agilent Technologies", USA).

The detection of the analyte and IS during the chromatographic analysis was carried out using an AB Sciex QTrap 3200 MD tandem quadrupole mass spectrometer ("Sciex", Singapore), equipped with an ion source with a chemical ionization probe at atmospheric pressure. The selection of the optimal parameters for the mass spectrometric detection was carried out by a continuous injection of individual solutions (100.0 ng/ml) of the azo dyes into the ion source individual solutions (100.0 ng/ml) of azo the dyes (an analyte and an IS) in methanol with the addition of 0.1% formic acid using a syringe pump at the speed of 10 µl/min. At the first stage, the *m/z* of protonated molecules (the first order mass spectrum) was determined for the azo dyes, and the optimal values of the declustering potential (DP) and voltages at the entrance potential to the collision cell (EP) were selected. At the second stage, the mass spectra of product ions were determined for the identified precursor ions; 2 characteristic ions were selected, for which the optimal values of the collision energy (CE) and the collision cell exit potential (CXP) were selected. The obtained values were used for the detection of the azo dyes in the multiple reaction monitoring (MRM) mode and provided the best sensitivity.

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Statistical processing

The primary data processing of the chromatography-mass spectrometric analysis was carried out using built-in Software AB Sciex Analyst 1.3.6; Microsoft Office Excel 365 (Microsoft, USA) Software was used to calculate the values of validation parameters.

The method was validated according to the following parameters: selectivity, a matrix effect, a recovery degree, a sample transfer, a linearity of the analytical range, a lower limit of quantification (LLOQ), the intra- and inter-run accuracy and precision, a stability at all stages of the analysis.

RESULTS AND DISCUSSION

The first- and second-order mass spectra were obtained for the nitrite derivatization product (azo dye) and the IS (Fig. 4). For the analyte and the IS, two characteristic product ions were singled out; herewith, the detection conditions had been selected to ensure the maximum ion current (Table 1).

The chromatographic determination of azo dyes was carried out using a reverse phase column. The use of methanol as a component of the mobile phase with a higher elution force compared to acetonitrile resulted in narrower and higher peaks for both the analyte and the IS. In addition, the use of acetonitrile significantly increased the LLOQ of 4-[4-(2-aminoethylamino)-1-naphthylazo]benzenesulfonamide, which did not allow achieving the required level of sensitivity. Thus, elution was carried out with a mixture of deionized water (A) and methanol (B) and the addition of 0.1% formic acid in a gradient mode (Table 2). The retention times of 4-[4-(2-aminoethylamino)-1-naphthylazo] benzenesulfonamide and 4-[4-amino-1-naphthylazo] benzenesulfonamide (IS) were 6.1 and 6.5 min, respectively (Fig. 5).

To determine the metrological characteristics of the developed method, a series of standard samples with a nitrite content of: 0.1; 0.5; 1.0; 5.0; 10.0; 50.0; 100.0 nmol/ml in terms of homogenate. Due to the lack of a nitrite-free biological matrix, a proof of the feasibility of using deionized water as a substitute is required. To do this, an experiment with the addition of working solutions of nitrite to aliquots of rat tissue homogenates, followed by a sample preparation procedure, including the stages of nitrate reduction and derivatization of the resulting nitrites, was carried out [23]. The calibration curve obtained from the analysis of the samples prepared with deionized water was used to determine the nitrite content in standard samples prepared with homogenates. The difference between the nitrite concentration established by the calibration dependence and the concentration of the standard solutions with the additive without taking into account the endogenous level of the analyte, was determined. The standard solutions were prepared by adding 10 µl of a corresponding 10-fold working solution of nitrite to 90 µl of the deionized water (or tissue homogenate), after which the resulting samples were prepared according to the procedure described above. In addition, the slope coefficients of the calibration curves obtained from the analysis of a series of standard samples prepared in water and in the corresponding homogenates, were compared.

According to the results of the experiment, it was found out that the coefficient of variation of the concentration difference for homogenates of the brain tissue, myocardium, aorta, and rat blood plasma throughout the entire analytical range, were from 7.9 to 10.3%. Only for the lung homogenate, the relative standard deviation was close to 15%. The ratio of the slope coefficients of the calibration curves obtained from the analysis of the deionized water samples (0.0741) and tissue homogenates, ranged from 0.887 to 1.114 (Table 3). Thus, the use of the calibration standard samples prepared with deionized water had not affected the accuracy of determining the concentration of nitrites in biological objects.

The assessment of the matrix effect and the extraction degree was carried out based on the results of the samples analysis with the addition of 4-[4-(2-aminoethylamino)-1-naphthylazo] benzenesulfonamide (analyte) in the quantities equivalent to the conditional nitrite content in low (8.0 nmol/ml) and high (80.0 nmol/ml) concentrations from the analytical range of the technique. The matrix effect was calculated as the ratio of the chromatographic peak area of the analyte in the unextracted sample (*post-spike sample*) to the average signal value of the analyte in deionized water (*solvent-spike sample, n*=6). The normalized matrix factor (NMF) was taken to be the ratio of the analyte peak area value normalized to the internal standard (IS) in the *post-spike sample* to the average value of the ratio of the analyte peak area value normalized to IS in the *solvent-spike sample*.

The extraction degree was determined as the ratio of the chromatographic peaks areas in the extracted sample (*pre-spike sample*) and the unextracted sample (*post-spike sample*), expressed as a percentage. The matrix effect and recovery results are presented in Table 4.

The coefficient of variation values for NMF less

than 15% indicated[1] that the use of 4-[4-amino-1-naphthylazo]benzenesulfonamide as an IS can effectively compensate the influence of the biological matrix components on the signal intensity of the analyte. The average degree of the analyte recovery is in the range of 91.78–94.97% with a maximum coefficient of variation (CV) of 7.79%[2], which indicates an almost quantitative extraction of the nitrite derivatization product from the biological material.

The selectivity of the methods was assessed by comparing the ratios of the chromatographic peak areas of two azo dye product ions (analyte and IS) when analyzing the samples in the deionized water and tissue homogenates. It was found that for high and low nitrite concentrations the difference between these values did not exceed 5% for the analyte and 1% for IS[3].

In addition, a comparative characterization of the chromatograms of biological samples was carried out; this analysis was accomplished without a preliminary procedure of nitrite derivatization and samples to which 4-[4-(2-aminoethylamino)-1-naphthylazo]benzenesulfonamide (analyte) was added in the amount equivalent to the nitrite content at the LDL level. The results of assessing the selectivity of the methods are presented in Table 5.

The analyte response in the intact samples did not exceed 10.6% of the response in the LLOQ samples; for IS, the similar indicator is less than 0.22%, which proves a high selectivity of the developed methods[4].

Based on the results of the analysis of a standard samples series, a calibration graph was constructed. It reflects the ratio dependence of the peak area of the nitrite derivatization product to the peak area of the IS on the concentration of the analyte in the standard sample (Fig. 6). This dependence is presented in the form of a linear regression equation y=0.0735x+0.0123 (normalization 1/x2) with a correlation coefficient of 0.9959. The analytical range of the methods was from 0.1 to 100 nmol/ml of a conditional nitrite content.

Since there is no biological matrix free of NO metabolic products in nature, the LLOQ for nitrites was estimated based on the results of chromatographic analysis of analytical standards prepared on deionized water. The LLOQ was taken to be the minimum conventional nitrite content in 1 ml of tissue homogenate, which can be determined with a relative standard deviation and relative error values of no more than 20%[5], while the signal-to-noise ratio in the chromatogram should be 5:1[6]. The LLOQ value for nitrites was 0.1 nmol/ml. A fragment of a chromatogram of a standard sample with a nitrite content at the LLOQ level is shown in Fig. 7.

The transfer of substances during the analysis was assessed by comparing the chromatograms of blank samples analyzed after six injections of a sample with a nitrite concentration of 100 nmol/ml with chromatograms of LLOQ samples (0.1 nmol/ml). The analysis revealed that the ratio of peak areas in the blank samples to the peak areas in LLOQ samples was below the maximum permissible level (20% for the analyte and 5% for IS), indicating a little transfer of substances from higher to lower concentrations.

The accuracy and precision of the methods was calculated based on the results of the analysis of 5 control samples for each of the four levels of nitrite concentrations: LLOQ (0.1 nmol/ml), a low concentration (LC – 8.0 nmol/ml), a medium concentration (MC – 40.0 nmol/ml), a high concentration (HC – 80.0 nmol/ml) in three independent series. The accuracy was expressed as a percentage, as the ratio of the measured concentration in the control samples to the nominal nitrite content (E). The precision was determined by the coefficient of variation (CV) of the five-fold determination results of the nitrite concentration. The acceptance criterion was the value of the CV and a relative error for the LLOQ level – no more than 20%, for other concentrations – no more than 15%. The intra- and inter-assay accuracy and precision results are presented in Table 6.

The results of the stability assessment confirmed the inalterability of the initial and working nitrite solutions for 3 months. The solutions of sulfonamide and N-(1-naphthyl)ethylenediamine used for the nitrite derivatization remained reactive for 1 year when stored in a refrigerator. An aqueous solution of β-nicotinamide adenine dinucleotide (2 mg/ml) remained stable when frozen at –20°C for 1 month. The reactivity of the reconstituted nitrate reductase was not assessed after storage; a freshly prepared solution was used each time. The IS stock solution in methanol (1 mg/ml) remained stable for 1 year at –20°C.

[1] Requirements for the validation of bioanalytical test methods and analysis of biological samples under study (Decision of the Council of the Eurasian Economic Commission dated November 3, 2016 No. 85 (as amended on February 15, 2023) On approval of the Rules for conducting bioequivalence studies of medicinal products within the framework of the Eurasian Economic Union).
[2] Ibid.
[3] Ibid.
[4] Ibid.
[5] Ibid.
[6] Ibid.

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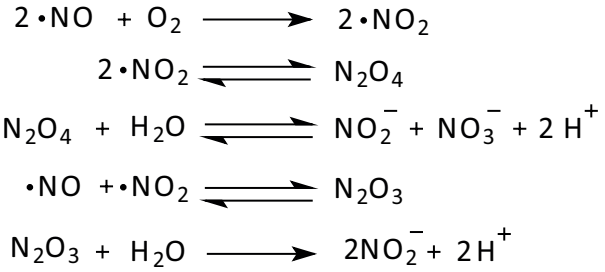

Figure 1 – Equations of chemical reactions for metabolites formation of endothelial relaxing factor (NO)

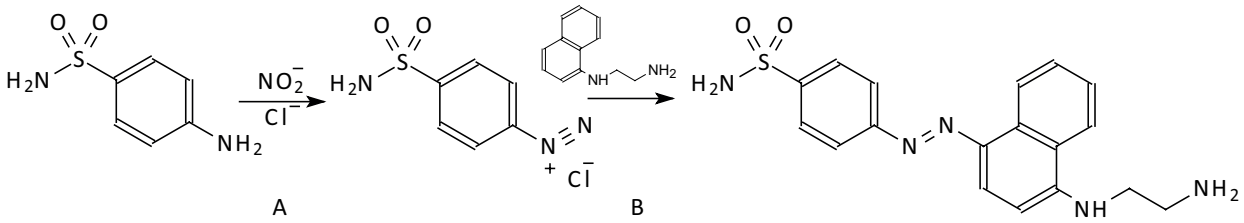

Figure 2 – Reaction of azo dye formation (A – diazotization of sulfonamide, B – azo coupling of diazonium ion with N-(1-naphthyl)ethylenediamine)

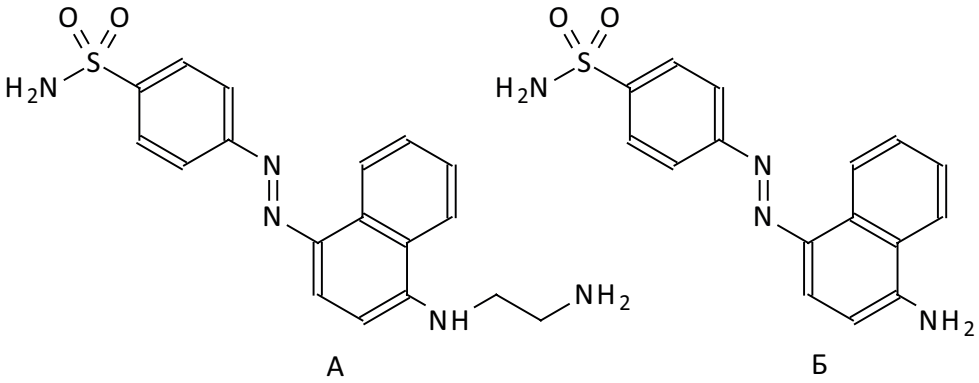

Figure 3 – Chemical structure of analyte (A – 4[4(2aminoethylamino)1naphthylazo]benzenesulfonamide) and internal standard (B – 4-[4-amino-1-naphthylazo]benzenesulfonamide)

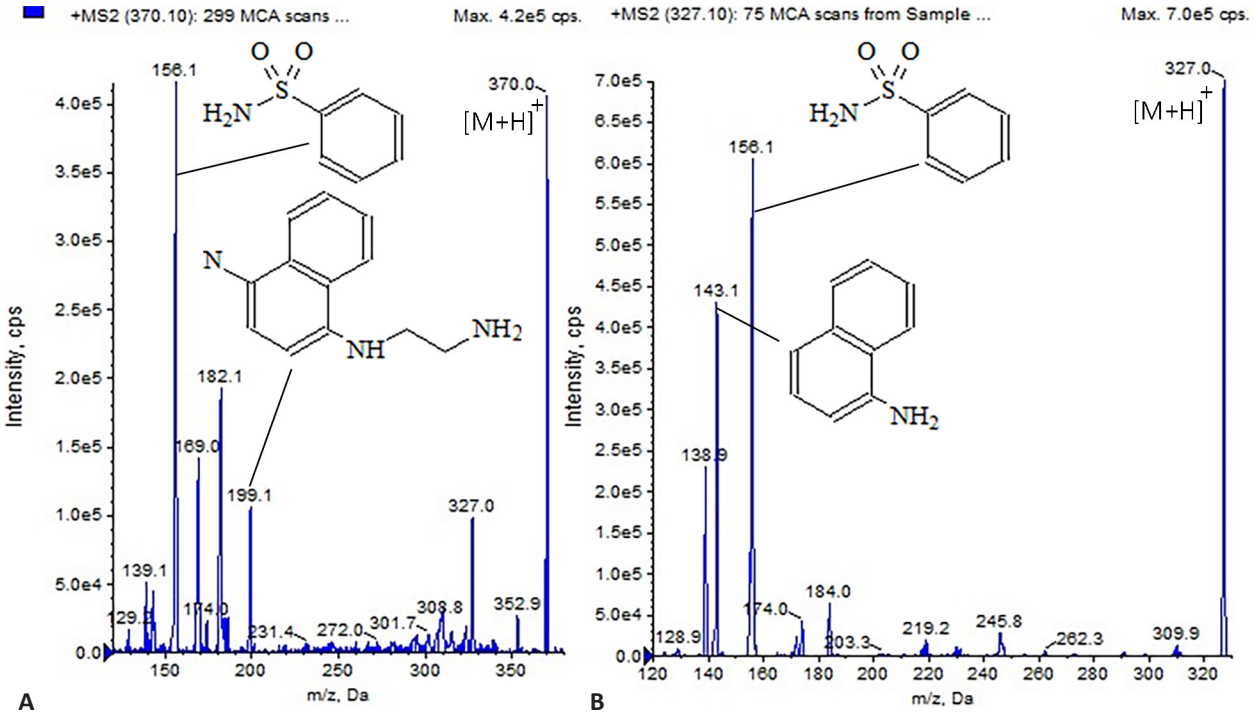

Figure 4 – Fragment ion mass spectra of analyte (A – 4-[4-(2-aminoethylamino)-1-naphthylazo] benzenesulfonamide) and internal standard (B – 4-[4-amino-1-naphthylamino]benzenesulfonamide)

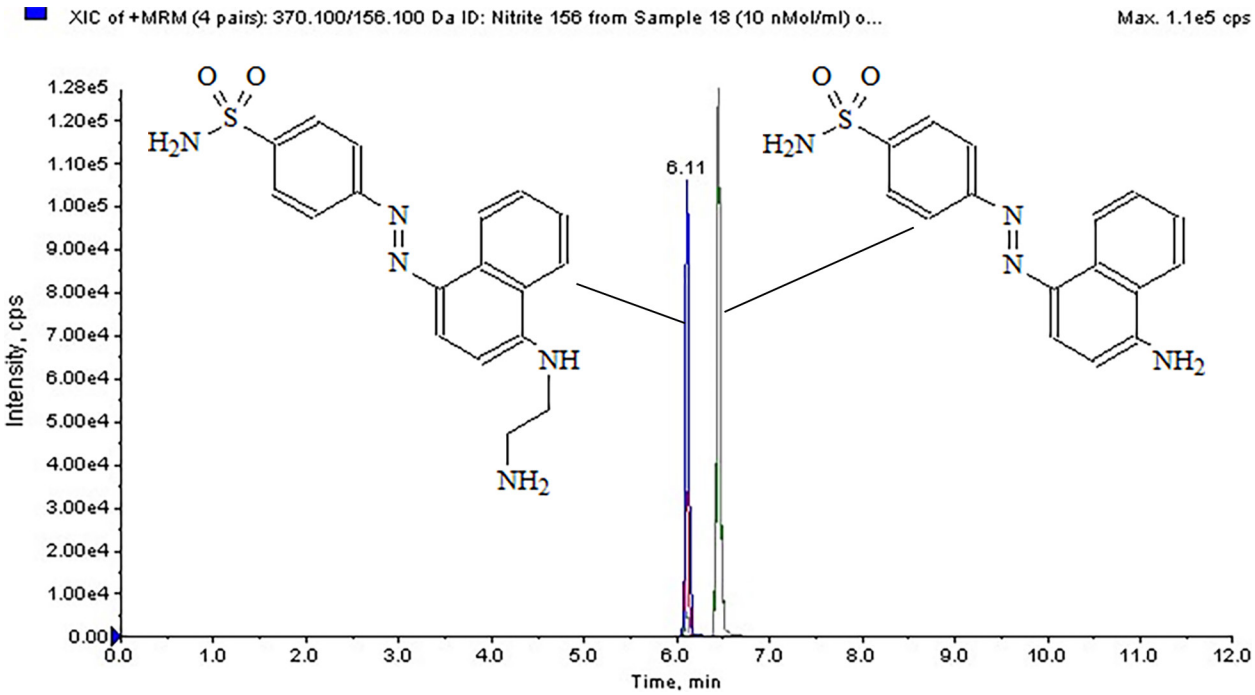

Figure 5 – Chromatogram of standard sample with nitrite concentration of 10.0 nmol/ml

Note: mobile phase – methanol and deionized water with the addition of 0.1% formic acid; elution mode – gradient; chromatographic column – InfinityLab Poroshell 120 EC-C18 4.6×100 mm, 2.7 µm; column temperature – 30°C; aliquot – 5 µl.

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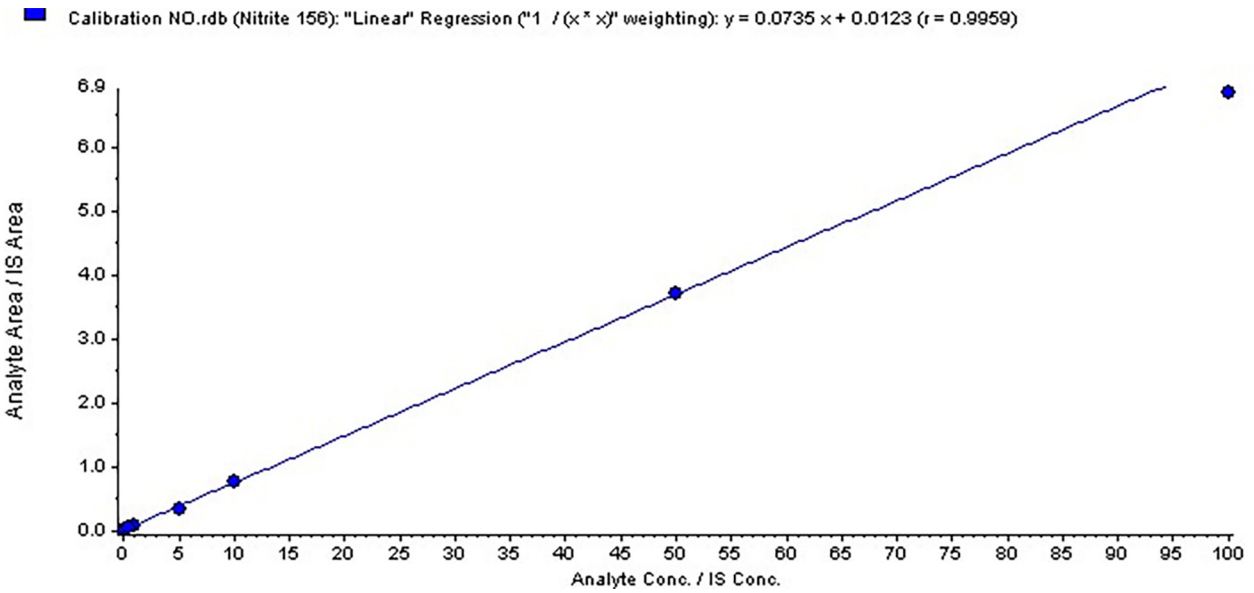

Figure 6 – Calibration curve for quantitative determination of nitrites in rat tissue homogenates
Note: horizontal axis – nitrite concentration in the standard sample, nmol/ml; the vertical axis is the ratio of the area of the chromatographic peak of the analyte to the area of the IS peak).

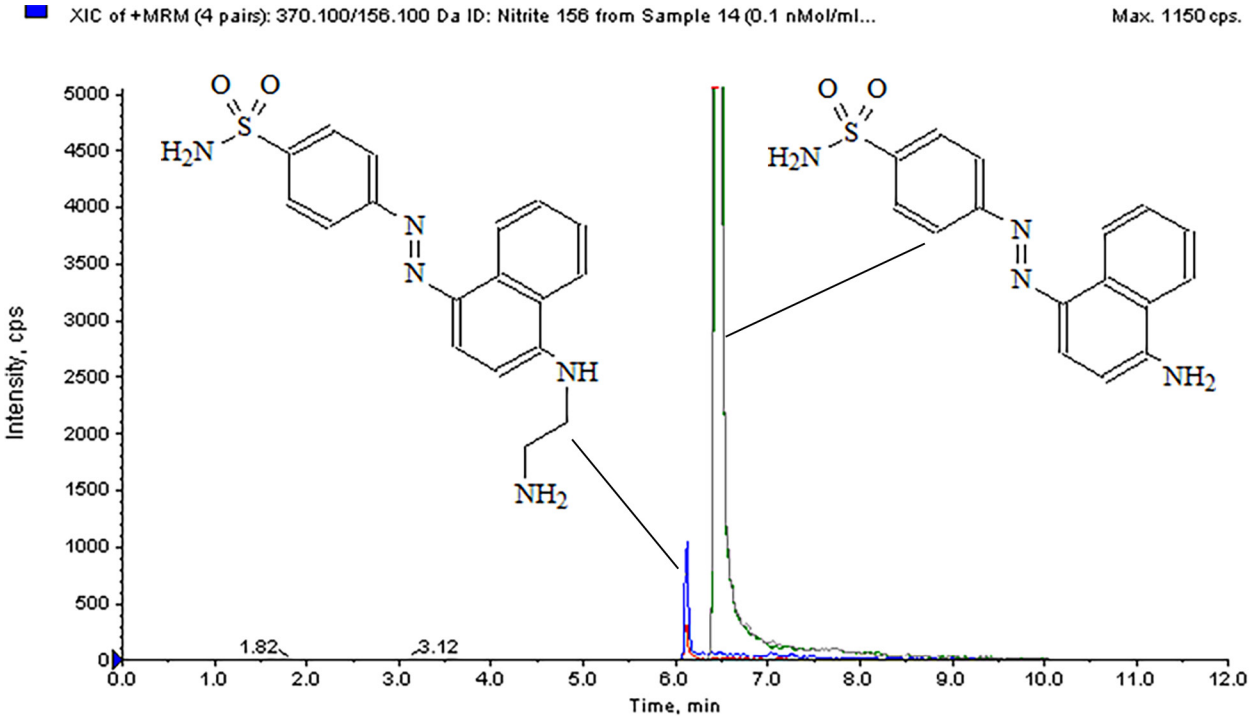

Figure 7 – Fragment of a standard sample chromatogram with a nitrite concentration at the LLOQ of 0.1 nmol/ml
Note: signal-to-noise ratio – 28:1; mobile phase – methanol and deionized water with the addition of 0.1% formic acid; elution mode – gradient; chromatographic column – InfinityLab Poroshell 120 EC-C18 4.6×100 mm, 2.7 µm; column temperature – 30°C; aliquot – 5 µl.

Table 1 – Parameters of mass spectrometric detection of nitrite derivatization product (azo dye) and internal standard

Ion source	Heated Nebulizer				
Ionization method	Atmospheric pressure chemical ionization (APCI)				
Ionization mode	Positive				
Ion source temperature, °C	400.0				
Ion source voltage, V	5500.0				
Gas curtain pressure, *psi*	20.0				
Ion source gas pressure, *psi*	30.0				
Input voltage, V	10				
Dwell time, msec	200				
Product ions	MRM, *m/z*	DP, V	EP, V	CE, eV	CXP, V
Analyte 4-[4-(2-aminoethylamino)-1-naphthylazo]benzenesulfonamide	370.1/156.1 370.1/199.1	67.0	21.0	37.0 28.0	2.5 3.3
Internal standard 4-[4-amino-1-naphthylamino]benzenesulfonamide	327.1/156.1 327.1/143.1	86.0	18.2	32.8 37.0	2.7 2.6

Note: DP – declustering potential; EP – entrance potential to the collision cell; CE – collision energy; CXP – collision cell exit potential.

Table 2 – Chromatographic parameters for detection of nitrite derivatization product

Chromatographic column	Agilent InfinityLab Poroshell 120 EC-C18 4.6×100 mm, 2.7 µm			
Eluent A	Deionized water+0,1% formic acid			
Eluent B	Methanol+0,1% formic acid			
Gradient program	Time, min	Flow rate, µl/min	% A	%B
	0.0	500	90	10
	4.0		0	100
	8.0		0	100
	8.01		90	10
	12.0		90	10
Column thermostat temperature, °C	30			
Sample volume, µl	5			
Total analysis time, min	12			
Injector flushing	Through rinse port, 3 seconds, 50% methanol aqueous solution			

Table 3 – Experiment results of studied homogenates with addition of standards

Nitrite concentration in water, nmol/ml	0.1	0.5	1.0	5.0	10.0	50.0	100.0	Average value, nmol/ml	SD, nmol/ml	CV, %
Blood plasme										
Nitrite concentration with additive, nmol/ml	6.3	6.0	6.8	11.8	16.8	56.4	106.0	–		
Concentration difference, nmol/ml	6.2	5.5	5.8	6.8	6.8	6.4	6.0	6.2	0.5	**7.9**
Calibration curve slope factor	0.0751		Ratio of slope coefficients of calibration curves						**1.014**	
Brain homogenate										
Nitrite concentration with additive, nmol/ml	25.2	24.3	24.9	29.8	32.6	71.3	118.4	–		
Concentration difference, nmol/ml	25.1	23.8	23.9	24.8	22. 6	21.3	18.4	22.8	2.4	**10.3**
Slope of linear regression equation	0.0657		Ratio of slope coefficients of calibration curves						**0.887**	
Heart homogenate										
Nitrite concentration with additive, nmol/ml	20.7	21.3	22.1	26.9	33.1	74.4	125.1	–		
Concentration difference, nmol/ml	20.6	20.8	21.1	21.9	23.1	24.4	25.1	22.4	1.8	**8.0**
Slope of linear regression equation	0.0825		Ratio of slope coefficients of calibration curves						**1.114**	
Aorta homogenate										
Nitrite concentration with additive, nmol/ml	18.6	18.8	19.4	26.4	28.1	69.4	116.2	–		
Concentration difference, nmol/ml	18.5	18.3	18.4	21.4	18.1	19.4	16.2	18.6	1.6	**8.4**
Slope of linear regression equation	0.0711		Ratio of slope coefficients of calibration curves						**0.959**	
Lungs homogenate										
Nitrite concentration with additive, nmol/ml	7.8	7.6	7.8	11.6	16.4	55.3	105.1	–		
Concentration difference, nmol/ml	7.7	7.1	6.8	6.6	6.4	5.3	5.1	6.4	0.9	**14.6**
Slope of linear regression equation	0.0695		Ratio of slope coefficients of calibration curves						**0.938**	

Note: SD – standard deviation; CV – coefficient of variation.

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Table 4 – Results of assessing matrix effect and recovery degree

Values	AUC of analyte *post-spike sample*	AUC IS *post-spike sample*	AUC of analyte/ AUC IS	AUC of analyte *solvent-spike sample*	AUC IS *solvent-spike sample*	AUC of analyte *pre-spike sample*	NMF	Extraction rate, %
Blood plasma (8,0 nmol/ml)								
Average	247 200	397 600	0.62	246 400	391 600	232 600	**0.99**	**94.48**
SD	16 783	8 561	0.04	13 446	5 941	3 781	0.07	7.36
CV, %	6.79	2.15	7.19	5.46	1.52	1.63	7.19	7.79
Brain homogenate (8,0 nmol/ml)								
Average	221 800	372 400	0.60	246 400	391 600	208 600	**0.95**	**94.02**
SD	9 338	8 324	0.03	13 446	5 941	12 915	0.06	3.50
CV, %	4.21	2.24	5.87	5.46	1.52	6.19	5.87	3.72
Heart homogenate (8,0 nmol/ml)								
Average	221 400	375 000	0.59	246 400	391 600	203 200	**0.94**	**91.78**
SD	24 130	18 439	0.06	13 446	5 941	22 286	0.10	1.18
CV, %	10.90	4.92	10.96	5.46	1.52	10.97	10.96	1.29
Aorta homogenate (8,0 nmol/ml)								
Average	229 200	364 400	0.63	246 400	391 600	215 400	**1.00**	**94.11**
SD	13 179	7 893	0.04	13 446	5 941	7 829	0.06	3.76
CV, %	5.75	2.17	6.41	5.46	1.52	3.63	6.41	3.99
Lungs homogenate (8,0 nmol/ml)								
Average	198 600	362 200	0.55	246 400	3 916 00	185 600	**0.87**	**93.48**
SD	10 064	13 311	0.03	13 446	5 941	9 710	0.05	2.58
CV, %	5.07	3.68	6.20	5.46	1.52	5.23	6.20	2.76
Blood plasma (80,0 nmol/ml)								
Average	2 232 000	395 400	5.64	2 268 000	398 800	2 104 000	**0.99**	**94.28**
SD	101 341	6 107	0.19	106 864	10 710	85 029	0.03	0.91
CV, %	4.54	1.54	3.36	4.71	2.69	4.04	3.36	0.96
Brain homogenate (80,0 nmol/ml)								
Average	2 122 000	385 200	5.51	2 268 000	398 800	2 014 000	**0.97**	**94.97**
SD	107 564	13 368	0.31	106 864	10 710	92 897	0.05	3.09
CV, %	5.07	3.47	5.57	4.71	2.69	4.61	5.57	3.25
Heart homogenate (80,0 nmol/ml)								
Average	2 214 000	375 000	2214000	2 268 000	398 800	2 084 000	**1.04**	**93.92**
SD	241 309	18 439	241309	106 864	10 710	290 740	0.11	4.96
CV, %	10.90	4.92	10.90	4.71	2.69	13.95	10.96	5.28
Aorta homogenate (80,0 nmol/ml)								
Average	2 136 000	393 800	5.42	2 268 000	398 800	1 996 000	**0.95**	**93.33**
SD	154 370	16 407	0.32	106 864	10 710	185 687	0.06	2.59
CV, %	7.23	4.17	5.93	4.71	2.69	9.30	5.93	2.78
Lungs homogenate (80,0 nmol/ml)								
Average	1 884 000	387 200	4.87	2 268 000	398 800	1 744 000	**0.86**	**92.47**
SD	59 414	17 922	0.27	106 864	10 710	132 778	0.05	4.16
CV, %	3.15	4.63	5.57	4.71	2.69	7.61	5.57	4.50

Note: AUC – area under the curve of chromatographic peak; IS – internal standard; NMF – normalized matrix factor; SD – standard deviation; CV – coefficient of variation.

Table 5 – Results of assessing methods selectivity

Biomaterial	AUC of analyte peak (average value)		Selectivity, %	AUC of IS peak (average value)		Selectivity, %
	Intact sample	LLOQ Sample		Intact sample	LLOQ Sample	
Blood plasma	204.00	2 105.00	9.72	361.50	374 333,33	0.10
Brain homogenate	188.83	2 333.00	8.19	420.17	372 166,67	0.11
Heart homogenate	188.17	2 308.33	8.32	820.83	365 166,67	0.22
Aorta homogenate	190.33	2 911.67	6.57	381.33	382 666,67	0.10
Lungs homogenate	221.00	2 085.00	10.58	545.33	391 500,00	0.14

Note: AUC – area under the curve of chromatographic peak; IS – internal standard; LLOQ – lower limit of quantitation.

Table 6 – Results of assessing precision and accuracy of the method

Sample	LLOQ 0.1 nmol/ml	LC 8.0 nmol/ml	MC 40.0 nmol/ml	HC 80.0 nmol/ml
Accuracy and precision, batch 1				
Measured concentration, nmol/ml	0.104±0.010	8.67±0.33	38.1±1.0	83.9±2.1
Accuracy, %	104.0	108.4	95.2	104.9
CV, %	9.90	3.84	2.52	2.48
Accuracy and precision, batch 2				
Measured concentration, nmol/ml	0.093±0.009	8.33±0.05	38.1±0.9	82.9±1.5
Accuracy, %	93.4	104.1	95.2	103.7
CV, %	9.41	0.61	2.31	1.84
Accuracy and precision, batch 3				
Measured concentration, nmol/ml	0.092±0.008	8.63±0.29	38.0±1.5	84.6±2.7
Accuracy, %	91.8	104.5	95.1	105.8
CV, %	9.13	3.42	3.84	3.22
Inter-batch accuracy and precision				
Measured concentration, nmol/ml	0.096±0.010	8.45±0.29	38.1±1.0	83.8±2.1
Accuracy, %	96.4	105.7	95.1	104.8
CV, %	10.57	3.38	2.75	2.54

Note: LLOQ – lower limit of quantitation; LC – low concentration; MC – medium concentration; HC – high concentration.

Table 7 – NO content in homogenates in terms of nitrite

Sample	Blood plasma	Brain	Heart	Aorta	Lungs
Content of nitric oxide, nmol/ml	31.8±3.0	122.4±29.5	108.9±14.5	87.9±17.4	35.2±9.4

The results of assessing the post-preparative stability confirmed the safety of the samples ready for a chromatographic analysis within 24 hours (the maximum time for the analysis of all samples in the chromatograph autosampler). The results of studying biological samples after 3 cycles of freezing and thawing showed high values of stability of the metabolic NO products. Thus, the areas of chromatographic peaks of the nitrite derivatization product before and after the procedure did not differ by more than 10%. Storing rat tissue homogenates for 3 months at –40°C did not result in a significant decrease in NO. Thus, the areas of chromatographic peaks of the same samples analyzed 1 day and 3 months after obtaining the biomaterial did not differ by more than 15%.

A 10-fold dilution factor was validated to confirm that the possibility of using this methods could be used to quantify NO metabolites well above the upper limit of quantitation (100 nmol/ml as nitrite). It was found that a 10-fold dilution with water of standard nitrite solutions (1000 and 5000 nmol/ml) prepared on the tissue homogenates does not reduce the precision and accuracy of the analysis.

The developed methods was used to analyze the content of NO metabolites in the tissues of intact Wistar rats (n=6), kept in the vivarium of Tver State Medical University and removed from the experiment in the morning by decapitation. The results of the NO determination are presented in Table 7.

The developed methods can be also used without performing the nitrate reduction stage. In this case, measuring the level of nitrites can play an important role, for example, when studying the mechanisms of cytoprotection under hypoxic conditions using a model of ischemia-reperfusion [24]. In addition, assessing nitrite levels can be useful in studying the replication processes of viruses, for example, SARS-CoV-2 [25]. The use of this methods in the conjunction with determining the level of cyclic guanosine monophosphate [26] in the tissues of the laboratory rats can serve as a useful tool in studying not only physiological and pathological processes occurring with the NO participation [28–31], but also the pharmacodynamics of drug candidates, which is an extremely important stage in the drug development [32–34].

Limitations of the study

According to the authors' data, the following factors may affect the reliability of the analysis results: a decrease in the activity of nitrate reductase and β-nicotinamide adenine dinucleotide, which is a consequence of an improper storage of lyophilisates or ready-made solutions; the presence of sulfonamide drugs in the biomaterial, as well as the intake of nitrates

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and nitrites into the body of laboratory animals in case of non-compliance with feeding requirements.

CONCLUSION

The developed chromatography-mass spectrometric methods for the NO determination in the rat tissues fully met the validation requirements. Compared to the common colorimetric method for assessing the NO production, this method is characterized by a high sensitivity and selectivity, as well as a low influence of interfering components of the biological matrix. The application of this methods makes it possible to accurately estimate the total content of NO metabolic products in the blood plasma, the tissues of the brain, heart, aorta and lungs of rats, which is in demand in the study of pathological processes, as well as the pharmacodynamics and effectiveness of pharmacological agents affecting the NO metabolism.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

All the authors made equivalent and equal contributions to the preparation of the publication. All the authors confirm that their authorship meets the international ICMJE criteria (all the authors have made significant contributions to the development of the concept, conduct of the study and preparation of the article, read and approved the final version before the publication. Nikita S. Popov – development, validation and testing of bioanalytical methods, preparation of the manuscript; Dmitry A. Gavrilenko – analysis of literary sources, conducting the biological part of the study, analysis of the results, preparation of the manuscript; Mikhail S. Baranov – development of the research concept, manuscript preparation; Vadim Yu. Balabanyan – purpose setting, study design development, manuscript preparation.

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Investigator's influence on the muscle strength assessment in animals in experiment: Comparison of automated "inverted grid" test and its classical variant

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The aim of the work was to study the influence of the researcher on the muscle strength assessment in animals in the experiment by comparing the results of the automated "inverted grid" test and its classical variant.

Materials and methods. Male lines (Bla/J, *n*=20; FUS(1-359), *n*=20; Tau P301S$^{+/+}$, *n*=20) and their background controls (C57BL/6J, *n*=20; CD1, *n*=20) were selected for the study. The dynamics of changes in the muscle deficit of the animals was evaluated in the automated and classical variant of the "inverted grid" test.

Results. According to the results of the muscle strength assessment of mice with an edited genome of lines FUS(1-359)$^{+/-}$, Tau P301S$^{+/+}$, B6.ADysf^{prmd}/GeneJ, using the "inverted grid" test in the classical variant and the automated one, it was found that statistically significant differences were not obtained in comparison with the results obtained by the classical variant of the test. The standard error of the mean increases by 23–39% in the classical test compared to the automated one. It was shown that the standard error of the mean in the classical variant of the test in Tau P301S$^{+/+}$ mice was 6.24; 5.94; 5.88; 7.38 at 4 age points; in FUS(1-359)$^{+/-}$ mice, 4.49; 6.8; 6.98 and 4.1; B6.ADysf^{prmd}/GeneJ mice, 7.66; 7.58; 8.3 and 7.92, respectively.

Conclusion. Thus, the value of the standard error of the results study mean of the changes dynamics in the muscle strength when using the automated variant of the "inverted grid" test was reduced in comparison with the results of the classical variant of the test. The results of the study show that the automation of generally recognized behavioral tests is able to increase the accuracy of the obtained data reducing the influence of a human factor on the manipulation.

Keywords: "inverted grid"; automation; behavioral testing; neurodegeneration; transgenic animals

Abbreviations: ALS – amyotrophic lateral sclerosis; HD – Huntington's disease; PD – Parkinson's disease; AD – Alzheimer's disease; MD – motor deficit; PS – inverted grid; MS – muscle strength.

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Влияние исследователя на оценку мышечной силы у животных в эксперименте: сравнение автоматизированного теста «перевернутая сетка» и его классического варианта

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Цель. Изучить влияние исследователя на оценку мышечной силы у животных в эксперименте на примере сравнения результатов автоматизированного теста «перевернутая сетка» и его классического варианта.

Материалы и методы. Для исследования были выбраны самцы линий (Bla/J, *n*=20; FUS(1-359), *n*=20; Tau P301S$^{+/+}$, *n*=20) и их фоновый контроль (C57BL/6J, *n*=20; CD1, *n*=20). Динамику изменения мышечного дефицита животных оценивали в автоматизированном и классическом варианте теста «перевернутая сетка».

Результаты. По результатам оценки мышечной силы мышей с редактированным геномом линий: FUS(1-359)$^{+/-}$, Tau P301S$^{+/+}$, B6.ADysf^{prmd}/GeneJ при помощи теста «перевернутая сетка» в классическом варианте и автоматизированном было установлено, что статистически значимых различий в сравнении с результатами, полученными при проведении классического варианта теста, получено не было. Стандартная ошибка среднего возрастала в классическом тесте по сравнению с автоматизированным на 23–39%. Было показано, что стандартная ошибка среднего в классическом варианте теста на мышах линии Tau P301S$^{+/+}$ составила 6,24; 5,94; 5,88; 7,38 в 4-х возрастных точках; на мышах линии FUS(1-359)$^{+/-}$ – 4,49; 6,8; 6,98 и 4,1; B6.ADysf^{prmd}/GeneJ – 7,66; 7,58; 8,3 и 7,92 соответственно.

Заключение. Таким образом, значение стандартной ошибки среднего результатов исследования динамики изменения мышечной силы при использовании автоматизированного варианта теста «перевернутая сетка» было снижено в сравнении с результатами проведения классического варианта теста. Результаты исследования показывают, что автоматизация общепризнанных поведенческих тестов способна повысить точность получаемых данных снизив влияние человеческого фактора на проведение манипуляции.

Ключевые слова: «перевернутая сетка»; автоматизация; поведенческое тестирование; нейродегенерация; трансгенные животные

Список сокращений: БАС – боковой амиотрофический склероз; БГ – болезнь Хантингтона; БП – болезнь Паркинсона; БА – болезнь Альцгеймера; ДД – двигательный дефицит; ПС – перевернутая сетка; МС –мышечная сила.

INTRODUCTION

The reproducibility and repeatability of experimental results in the biomedical research is a hot topic of discussion among researchers and has been intensely debated over the latest 10 years [1]. In the works by other authors it was shown that from 50 to 90% of the experimental data are non-reproducible and controversial in their conclusions [2]. Several reasons for the poor repeatability and reproducibility of the experimental results are pointed out at once. They are: a bias in the interpretation of the results [3], an incorrect approach to the results statistical processing [4], lack of randomization [5], non-compliance with the rules of the animal housing (different crowding of animals in the cage) [6], neglect of validation of the equipment and auxiliary materials [7]. Working with animals requires a special approach to the study design, randomization of the group and minimization of the experimenter's influence on the results of phenotypic tests [8].

Conducting a behavioral study is an integral part for characterizing neurodegenerative and neuropsychiatric diseases in animal models. Over the past 30 years, behavioral testing has become ubiquitous in neurological and genetic animal studies [9].

From 1940 to 1989, a PubMed library search on "mice" and "behavior" found about 1800 articles, mostly related to behavior genetics, drug testing, and neurobiology, but only one article reported a behavior

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analysis of transgenic mice [10]. From 1990 to 2023, the number of articles on behavior testing of genetically modified mice alone grew to 28 000 [11].

In studying the dynamics of neurological diseases symptoms in transgenic animals, various behavioral tests are used, one of which are the tests aimed at detecting motor disorders that provide a good phenotypic characterization of the disease. In order to interpret the therapy efficacy of a model disease of an experimental animal on humans, it is necessary to obtain, in addition to molecular, also a phenotypic confirmation of the disease identity [12]. This tool is an integral component of modern protocols for studying disease mechanisms using experimental models and genetically modified laboratory animals [13]. Behavioral research provides a quantitative and qualitative marker of human disease symptoms and is a preclinical tool for evaluating the efficacy of new therapies [14].

Creating an animal with an edited genome that mimics the human disease phenotype is important for the study of basic pathophysiological cascades and the development of new methods for the pathology treatment.

There are various genetically modified mouse models for studying pathophysiological aspects of neurodegeneration and myodystrophies. The most commonly used models of these diseases are transgenic mouse models of amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) [15], Parkinson's disease (PD) [16, 17], and Alzheimer's disease (AD) [18]. The motor dysfunction present in these transgenic mice can be categorized as motor deficits (MD), impaired balance, coordination or muscle strength (MS) [19].

The tests that can show phenotypic manifestations of MD are "CatWalk", "Narrowing track", "Staircase", "Staircase with rungs", "Open field". One of the most used tests to assess a motor impairment is the "inverted grid" (IG), which is used to assess the limb strength of mice, their coordination and endurance. The test involves engaging all four of the animal's limbs to fixate to a wire grid, allowing a non-invasive measurement of the animal's MS, showing a sustained limb tension, counteracting the effects of gravity. The stimulus for keeping the animal on the grid is a fear of heights. The parameters of interest are assessed by placing the animal on an ID and determining the duration of its fixation on the grid. The test evaluates the MS, a movement coordination and balance of the animal by measuring how long it can hold on the inverted screen before falling down [20].

In the experiments related to the study of therapy for muscular dystrophies, a number of symptoms of the disease are investigated, such as MD associated with the progression of muscle atrophy in the proximal limb muscles [21]. To study the dynamics of the disease, scientists use behavioral tests that can interpret the clinical improvement of the animal's health from therapy. One of such tests is the «inverted grid».

Creating automated forms of settings for conducting behavioral studies of animals, is able to objectify the study, eliminate the adverse effect of a researcher-animal interaction on the test, optimize the duration of the test and reduce labor costs for its implementation. The protocol automation allows to obtain more data for a statistical comparison between the groups without additional manipulations with the animal [22, 23].

This article describes a comparative study of a classical variant of the "inverted grid" test and its automated form to use it to determine MD and MS dynamics in mice with a confirmed phenotype of human neurodegenarative and myodystrophic diseases.

THE AIM of the work was to study the influence of the researcher on the muscle strength assessment in animals in the experiment by comparing the results of the automated inverted grid test and its classical variant.

MATERIALS AND METHODS

Animals

Males of the lines Bla/J (*n*=20), FUS(1-359)$^{+/-}$ (*n*=20), Tau P301S$^{+/+}$ (*n*=20) and their background controls (C57BL/6J, *n*=20; CD1, *n*=20) were selected for the study and kept in groups according to their age, genetic background and group in the experiment. The animals were kept in the SPF vivarium of Belgorod State National Research University (BSU) under the conditions of artificially regulated daylight hours (12 h of dark and 12 h of light) at a temperature regime from +22 to +26°C, a relative humidity in the housing system from 50 to 65%, had a free access to food and water. The work was guided by ethical principles for the treatment of laboratory animals in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS No. 170). All painful animal manipulations were performed in accordance with the regulatory standards: Directive 2010/63/EU of the European Parliament and of the Council of the European Union dated September 22, 2010 on the protection of the animals used for scientific purposes. Experimental studies were approved by the Bioethical Commission of the BSU (Minutes No. 15/10 of 29.10.2021).

Study design

For a comparative study of the MD registration and MS change dynamics using the classical "inverted grid" test and its automated form in male transgenic animals and their background controls of the B6.ADysf^{prmd}/GeneJ, FUS(1-359)$^{+/-}$, and Tau P301S$^{+/+}$ lines at 4 age points (the manifestation period of clinical disease signs) for each cohort of mice (Fig. 1).

Experimental groups of the B6.ADysf^{prmd}/GeneJ mouse line were established at weeks 50, 51, 52 and 53, respectively.

1) The group for studying the dynamics of MD and MS in the B6.ADysf^{prmd}/GeneJ$^{-/-}$ line (*n*=10) using the classical variant of the IG (Bla/J) test.

2) The group for studying MD and MS dynamics in the C57BL/6J line (*n*=10) using the automated variant of the IG (b/C57BL/6J) test. The group is control to the Bla/J$^{+/+}$ line.

3) The group for studying the dynamics of MD and MS in the line B6.ADys^{fprmd}/GeneJ$^{-/-}$ (*n*=10) using the automated variant of the IG (Bla/J) test.

4) The group for studying MD and MS dynamics in the C57BL/6J line (*n*=10) using the classical variant of the IG (b/C57BL/6J) test. The group is control to the Bla/J line.

Experimental groups of the FUS(1-359) mouse line were established at weeks 11, 12, 13 and 14, respectively.

1) The group for studying the dynamics of MD and MS in the FUS(1-359)$^{+/-}$ line (n=10) using the classical variant of the IG (FUS(1-359)) test.

2) The group for studying the dynamics of MD and MS in FUS(1-359)$^{+/-}$ (*n*=10) using the automated variant of the IG (FUS(1-359)) test.

3) The group for studying the dynamics of MD and MS in the CD-1 line (*n*=10) using the automated variant of the IG (CD-1) test. The group is control to the FUS(1-359)$^{+/-}$ line.

4) The group for studying dynamics of MD and MS in CD-1 line (*n*=10) using the classical variant of the IG (CD-1) test. The group is control to the FUS(1-359)$^{+/-}$ line.

Experimental groups of the Tau P301S$^{+/+}$ mouse line were established at weeks 16, 17, 18 and 19, respectively.

1) The group for studying the dynamics of MD and MS in the Tau P301S$^{+/+}$ line (*n*=10) using the classical variant of the IG (Tau P301S$^{+/+}$) test.

2) Group of studying the dynamics of MD and MS in the Tau P301S$^{+/+}$ line (*n*=10) using the automated variant of the IG (Tau P301S$^{+/+}$) test.

3) The group for studying the dynamics of MD and MS in the C57BL/6J line (*n*=10) using an automated variant of the IG (C57BL/6J) test. The group is control to the Tau P301S$^{+/+}$ line.

4) The group for studying the dynamics of MD and MS in the C57BL/6J line (*n*=10) using the classical variant of the IG (C57BL/6J) test. The group is control to the Tau P301S$^{+/+}$ line.

Three lines of mice exhibiting symptoms of the disease affecting the motor function were used. The FUS line (1-359)$^{+/-}$ has a nervous system with an expressed transgenic sequence encoding an aberrant form of a human FUS protein with a deleted nuclear localization signal under the neuron-specific Thy-1 promoter [24]. The most common disease-associated mutations of the FUS gene affect the nuclear localization signal of the C-terminus of the encoded protein, causing its accumulation in the cytoplasm and at least a partial depletion of its nuclear pool [25]. This mouse line models amyotrophic lateral sclerosis. This neurodegenerative disease is characterized by a loss of neurons in the motor areas of the brain and spinal cord. The FUS(1-359)$^{+/-}$ line is characterized by the manifestation presence of the motor impairment symptoms and MS in a mono-allelic mutant animal at the 15th week of life [26].

The study made use of the Tau P301S$^{+/+}$ transgenic mice that express human Tau (1N4R) with the P301S mutation [27]. The identification of disease-causing mutations in *MAPT*, the *Tau* gene, in cases of frontal temporal dementia, has shown that a dysfunction of the Tau protein is sufficient to cause neurodegeneration and dementia [28]. These mice show a progressive neurofibrillary tangle pathology and neurodegeneration in the brain and spinal cord. In the spinal cord, the Tau pathology leads to the dramatic loss of motor neurons (approximately 50%) and an early, progressive and severe motor impairment [29]. The manifestation of lower limb MD begins at about 3 months of age [30].

A subline of B6.ADysf^{prmd}/GeneJ (Bla/J) mice in which a spontaneous insertion in intron 4 had been detected by chance, was also used. This type of pathology is a phenotypically heterogeneous progressive muscular dystrophy caused by mutations in the *DYSF* gene, which encodes a transmembrane protein dysferlin involved in a sarcolemma repair. It is also involved in the membrane repair, in the intracellular vesicle system and in the development of T-channels in the skeletal muscle [31]. The diseases are characterized by muscle weakness and atrophy that progress slowly and symmetrically in the proximal limb girdle muscles at about the 50th week of the animal's life [32].

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Assessment of muscle strength and motor deficit

The dynamics of changes in the muscle deficit of the animals were assessed in the automated and classical variants of the IG test [33]. In case of using the automated variant, the animal was placed in the center of a 25×25 cm grid with the holes of 5×5 mm width and the thickness of 0.5 mm. Turning the net with the animal was performed at a preset speed using an automated platform. The net was mounted on supports with an adjustable height (from 30 to 100 cm in 10 cm increments), above a cage with a thick layer of bedding. The animal was placed on the net, after the animal had steadily grasped the net with all four paws, the protocol was started on the management controller. After that, the net was turned with the animal by 180° by means of a servo drive. In this case, the weight of the net with the laboratory animal was registered by the corresponding strain gauge sensor, and a jump-like change in the weight of the net was interpreted as a fall of the animal from the net with the registration of the time of the actual presence on it and showing off this result on the display. After the completion of the protocol, the animal was removed from the test by sliding the cage out of the setup.

The automated variant of the test was made on the equipment according to the patent for invention No. 2815584 "Automated device for conducting the behavioral "inverted grid" test ".

When the classical variant of the setup was used, the inverted grid was a 25×25 cm wire mesh of 5×5 mm mesh size with a wire diameter of 0.5 mm, surrounded by a 4 cm partition to prevent the mouse from attempting to climb over to the other side. The test was used to assess a movement coordination and MS of the both pairs of limbs. The mice were placed in the center of a wire mesh that had been inverted and placed 50 cm above a soft surface. The time of the animal's fall was recorded or the animal was removed from the net if the time reached 180 sec [31].

Statistical processing

Statistical processing was performed using GraphPad Prism Software 8.0 program (GraphPad Software Inc., USA). Depending on the type of the traits distribution and equality of variance, the significance of the obtained results was evaluated using parametric (ANOVA) or nonparametric (Mann-Whitney *U*-test). The unpaired Student's *t*-test was used to identify differences in the

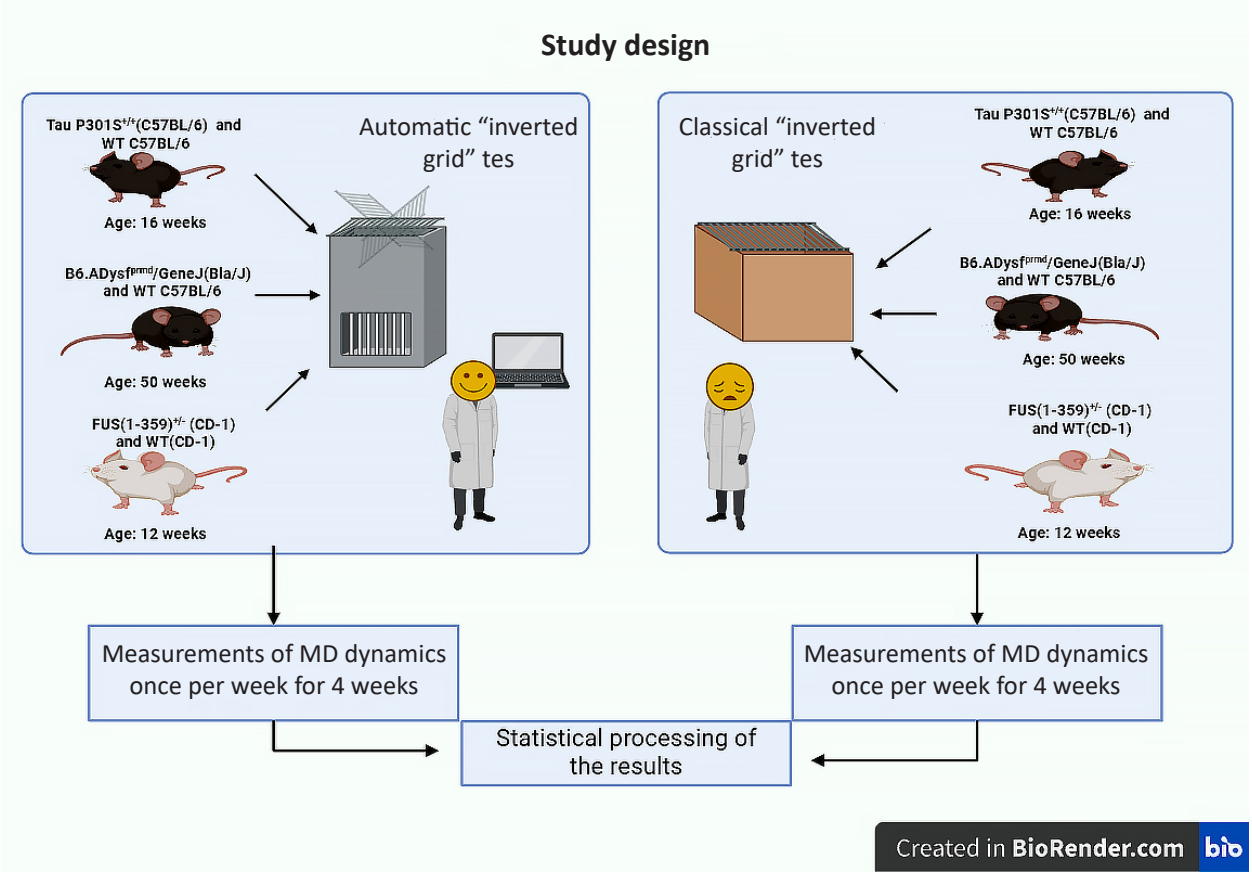

Figure 1 – Study design
Note: Experimental (Bla/J, *n*=20; FUS(1-359)$^{+/-}$, *n*=20; Tau P301S$^{+/+}$, *n*=20) and control (C57BL/6J, *n*=20; CD1, *n*=20) mice.
MD – motor deficits.

intergroup comparisons. The results were considered reliable at $p \leq 0.05$.

RESULTS

In a comparative study of the classical variant of the IG test and its automated form to determine MD and MS dynamics in mice with a confirmed human neurodegenarative and myodystrophy phenotype, the following results were previously reported [21, 34, 35].

As a result of the IG test, the degree of an increase in the MD symptoms and a decrease in the MS in Tau P301S$^{+/+}$ line mice (Fig. 2) compared to the control group (C57BL/6J), in case of the automatic variant of the IG test (Tau P301S$^{+/+}$) was 32.17, 48.67, 67.67 and 82.17% (p <0.001) at 16, 17, 18, 19 weeks of age, respectively. In case of a classical variant test, the group (Tau P301S$^{+/+}$) had 22.17, 39.34, 61.83 and 72.17% (p <0.0001) at 16, 17, 18, 19 weeks of age, respectively, compared to the control group (C57BL/6J). Thus, the difference in the results obtained from the two variants of the IG test were 10, 9.33, 5.84, and 10% at 16, 17, 18, and 19 weeks of age, respectively.

As a result of the IG test, the degree of an increase in MD symptoms and a decrease in MS in the FUS(1-359)$^{+/-}$ mice (Fig. 3) compared to the control group (CD-1), in case of the automatic variant of the test (FUS(1-359)$^{+/-}$ was 21.83, 53.17, 72.67 and 90.5% (p <0.0001) at 11, 12, 13, 14 weeks of age, respectively. In case of the classical variant test, the FUS(1-359)$^{+/-}$ group had 16.17, 40.67, 61.67 and 73.33% (p <0.0001) at 11, 12, 13 and 14 weeks of age, respectively, compared to the control group (CD-1). Thus, the difference in the results obtained from the two variants of the IG test was 5.67, 12.5, 11 and 17.17% at weeks 1–4 of the study.

As a result of the IG test, the degree of an increase in MD symptoms and a decrease in MS in the Bla/J line mice (Fig. 4) compared to the control group (C57BL/6J) in case of the automatic variant of the IG (Bla/J) was 25.83, 33.51, 33 and 58.67% (p <0.0001) at 12, 13, 14 and 15 weeks of age, respectively. In case of the classical variant test, the group (Bla/J) had 41.17, 44.67, 51.83, and 59.33% (p <0.0001) at 50, 51, 52, 53 weeks of age, respectively, compared to the control group (C57BL/6J). Thus, the difference in the results obtained from the two variants of the IG test were 15.34, 11.67, 0.5, and 0.67% at weeks 1–4 of the study.

Using the automation example of the well-known "inverted grid" test, the authors wanted to show the differences in the results obtained when using the automated complex and the classical test on three sublines of transgenic animals (Fig. 5). It was shown that the standard error of the mean in the classical variant of the test on Tau P301S$^{+/+}$ line mice was 6.24, 5.94, 5.88, and 7.38 at 4 age points, and on the FUS(1-359)$^{+/-}$ line mice it was 4.49, 6.8, 6.98, and 4.1; Bla/J was 7.66, 7.58, 8.3, and 7.92, respectively. The analysis of the data from the automated variant of the IG test showed the standard error of the mean as 5.1, 4.93, 3.42, and 2.26 in the Tau P301S$^{+/+}$ line, respectively, and 4.24, 4.52, 5.19, and 2.9 in the FUS(1-359)$^{+/-}$ mice; Bla/J, 5.24, 4.52, 4.7, and 4.85, respectively. Thus, automating the test and reducing the experimenter's influence decreased the standard error of the mean in mice of the Tau P301S+/+ line by 36.6%, FUS(1-359)$^{+/-}$ by 23.1%, B6. ADysf^{prmd}/GeneJ by 38.5%.

It is generally accepted that the experimenter influences the outcome of the experiment and the interpretation of its results, so it is important to automate this variable and minimize the experimenter's influence on both the experiment itself and its interpretation [36, 37].

DISCUSSION

Behavioral testing of animals in experimental studies to determine MD and MS has been used to estimate the efficacy of pharmacological therapies in many disease models. The first experimental article that mentioned the IG test date by 1986[1].

The tests known as "Open field", "Inverted grid", and "Rotating rod", are widely used in the experimental work related to the effectiveness estimation of the diseases therapeutic correction, the main symptoms of which are coordination disorders and motor deficits. Nowadays, there is a possibility for researchers to automate classical tests, which minimizes a human contact with a animal thus making the study more objective and accurate; it also contributes to increasing the throughput capacity of the installation. Strictly speaking, this work shows that it is possible to automate a large number of behavioral tests widely known to the research community, which will increase the intensity of experiments.

The examples include the Cat walk test, the essence of which is to automate the registration of the animal's paws position during its gait by means of the infrared radiation, which had been done before, by applying ink to the subject's paws and placing it on sheets of paper.

[1] Kordower JH, Felten SY, Felten DL, Gash DM. Behavioral sequelae following MPTP administration in mice. A neurotoxin producing a parkinsonian syndrome. Orlando: Academic Press; 1986. P. 413–417.

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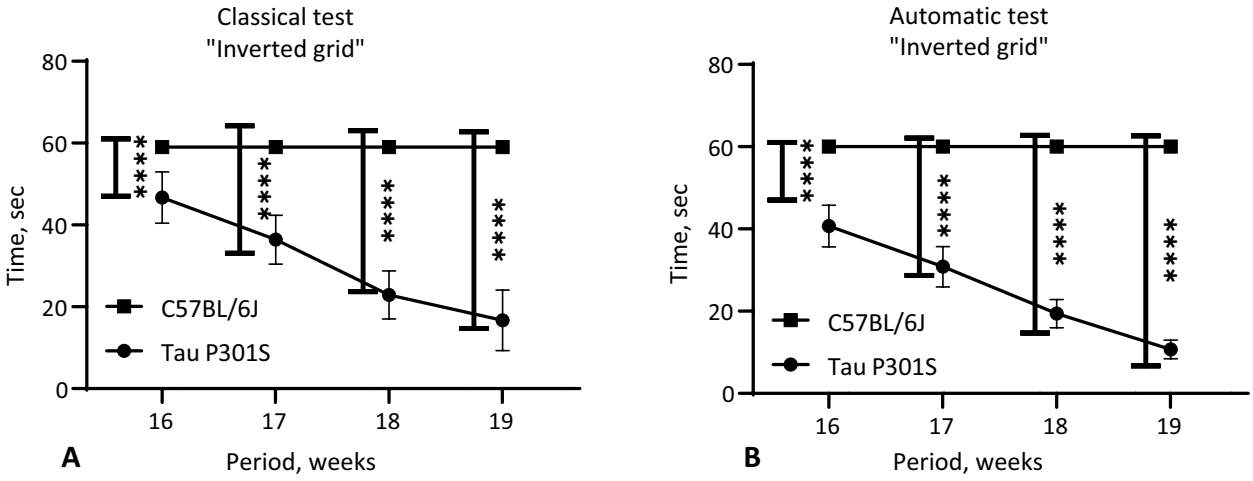

Figure 2 – Examination of motor deficits in Tau P301S$^{+/+}$ line using automated and classical variants of "inverted grid" test

Note: medians and standard error of the mean are presented. Samples were tested for normality, and statistical significance was assessed using the Mann-Whitney *U*-test (****p <0.0001). A – classical IG test, B– automated IG test.

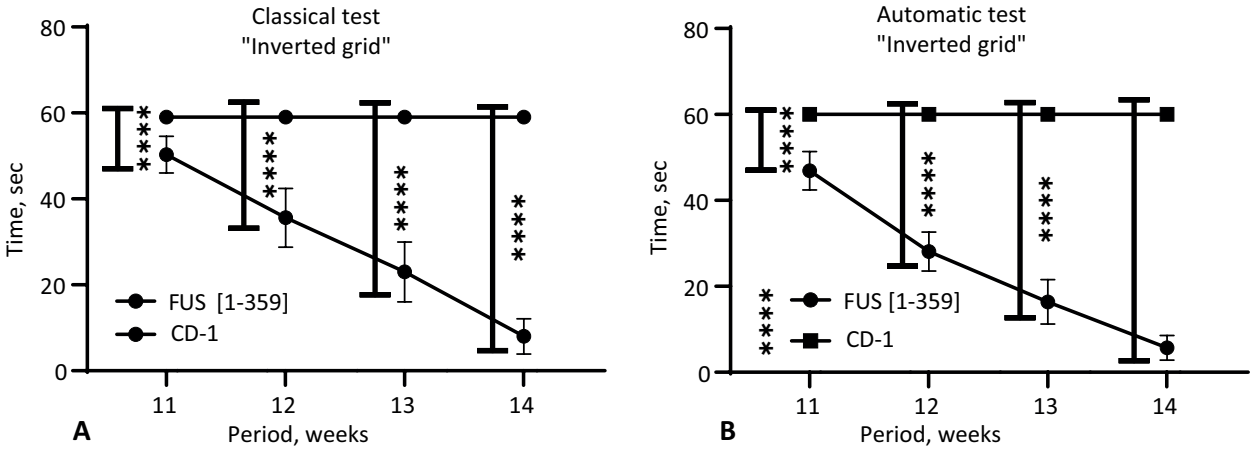

Figure 3 – Examination of motor deficits in the FUS(1-359)$^{+/-}$ line using automated and classical variants of the "inverted grid" test

Note: medians and a standard error of the mean are presented. Samples were tested for normality and the statistical significance was assessed using the Mann-Whitney *U*-test (****p <0.0001). A – classical IG test, B – automated IG test.

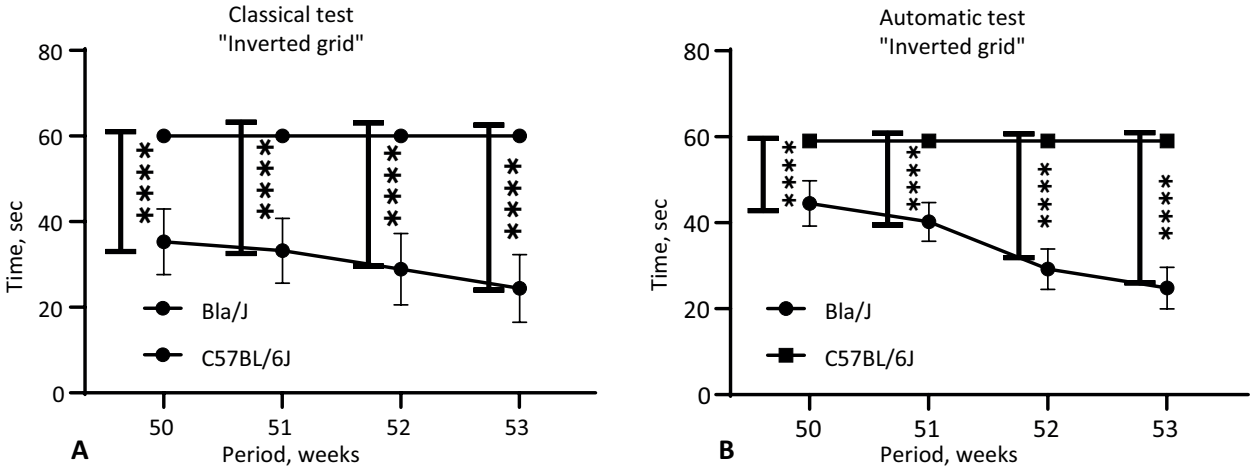

Figure 4 – Examination of motor deficits in the Bla/J line using automated and classical variants of the "inverted grid" test

Note: medians and a standard error of the mean are presented. Samples were checked for normality, and statistical significance was assessed using the Mann-Whitney *U*-test (****p <0.0001). A – classical IG test, B – automated IG test.

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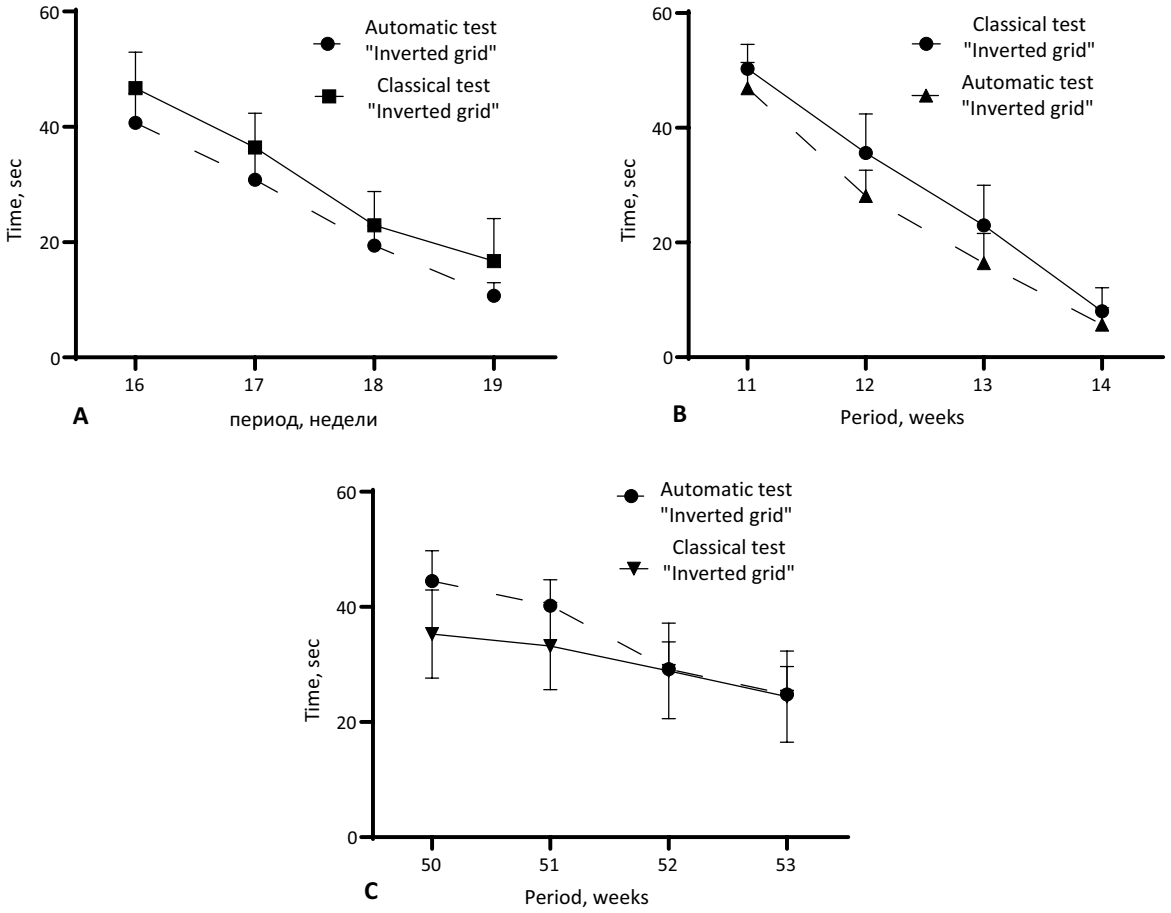

Figure 5 – Comparison of motor deficit study results in line A – Tau P301S$^{+/+}$, B –FUS(1-359)$^{+/-}$, C – Bla/J, using automated and classical variants of "inverted grid" test

Many research groups are working to develop a fully automated system for behavioral testing. For example, the system "IntelliCage" has been developed [11]. Apart from automating the work, it avoids potentially disturbing conditions for animals and has a wide range of different study protocols, but except the considerable advantages, this system cannot completely replace all the known classical behavioral tests. Another prominent example is the complete automation of a home cage for continuous testing of mice [38]. Undoubtedly, this development is a valuable tool for comprehensive phenotyping, but a large amount of data to process and the speed of tests are not suitable for all experimental work.

In some cases, the tests that currently already use automatic tracing of the animal movement (EthoVision and VideoMot) are also being automated and improved. For example, a new tool "Minopontikos" was proposed for the Morris Water Maze test [39]; it allows combining different methods of calculation and interpretation of results.

The carried out automation of the "inverted grid" has shown that in addition to reducing the standard error of the mean compared to the classical test by 23-39%, it also increases the efficiency factor by reducing the time spent on the test. Consequently, thereby the throughput of the test is increasing and the number of new indirect numerical values obtained are increasing, too (e.g., recording the angle at which the animal loses its fixation with the net). On average, a standard manual phenotypic testing of 30–40 mice (including recording of the spontaneous activity and data analysis) takes 20 h of working time. Another important advantage of the setup is the standardization of the mesh rotation speed and height of its installation relative to the cage with a shock-absorbing cover, which allows reducing the difference in the experienced stress among the mice.

Study limitations

The results of the study may be affected by a small sample size, conducting the research on experimental and control animals at different times of the circadian rhythm, unsatisfactory animal housing, including a high stress load on the experimental subject, a close proximity of the experimenter near the device during testing.

CONCLUSION

Nowadays, an integral part of the experimental work with animals is the use of phenotypic tests. However, at the same time, inaccuracies in estimating

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the parameters of the animal behavior sharply reduce the value of the results of the whole experiment and lead to significant financial costs. That is why all over the world, the development of systems of automatic registration and behavior estimation is actively carried out. The work has been done on the "inverted grid" test automation.

During the comparative characterization of the classical test with the automated one, the value of the standard error of the study mean result of the MS dynamics change, was reduced in comparison with the results of the classical variant of the test when using the automated variant of the "inverted grid" test. The results of the study show that the automation of generally recognized behavioral tests is able to increase the accuracy of the obtained data, reducing the influence of the human factor on the manipulation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Petr R. Lebedev – automation of the "inverted grid" test; Elena V. Kuzubova – article writing, research design development; Vladimir M. Pokrovsky – literature analysis, article writing; Alexandra I. Radchenko – evaluation and performance of behavioral tests; Sergey I. Osipyan – preparation of animal cohorts, genotyping; Yulia V. Stepenko – interpretation of results; Alina A. Apostol – observation and care of animals, animal handling; Lyudmila M. Danilenko – study design development; Aleksander A. Dolzhikov – design of graphical material; Tatiana G. Pokrovskaya – consultation on the issues of conducting individual stages of experimental work; Oleg S. Gudyrev – statistical processing of data; Yana S. Kochergina – consultation on the issues of conducting individual stages of experimental work; Olga V. Dudnikova – data analysis. All authors confirm that their authorship complies with the ICMJE international criteria (all authors have made a substantial contribution to the conceptualization, research and preparation of the article, read and approved the final variant before publication).

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REVIEW

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Use of gabapentin for neuropathic pain therapy: A view from perspective of evidence-based medicine

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The aim of the study was to analyze the literature sources for pharmacodynamic and pharmacokinetic features of gabapentin, providing its use in patients with neuropathic pain, as well as a comparative evaluation of its efficacy and safety when used in different doses.

Materials and methods. PubMed, Google Scholar, EMBASE, ResearchGate scientific information network and elibrary.ru databases were used as search resources. The keywords used for the search were "gabapentin", "mechanism of action", "gabapentin targets", "gabapentin pharmacodynamics", "pharmacokinetics", "pharmacokinetic parameters", "neuropathic pain", and "randomized clinical trials". The depth of the search was 26 years (from 1998 to 2024). This review resulted in 87 literature sources.

Results. Neuropathic pain (NeP) is one of the most common types of chronic pain, characterized by a high prevalence among people of the working age. Effective pharmacotherapy aimed at eliminating the pain syndrome is a key tool for improving the quality of life and preserving the work capacity of patients. Heterogeneity of etiologic factors involved in the genesis of NeP indicates the need to use drugs the analgesic effect of which is based on weakening the transmission of pain impulses in the CNS. In clinical trials, gabapentin has demonstrated efficacy in reducing the severity of pain in patients with postherpetic NeP, painful diabetic neuropathy and many other conditions accompanied by NeP. The dose of gabapentin 300 mg/day is the initial dose in the therapy of NeP and requires a further slow titration depending on the patient's response to therapy and tolerability of the drug, especially in elderly and senile patients, as well as in patients with an impaired renal function. According to the published data, the most pronounced analgesic effect is achieved in the patients against the background of the gabapentin administration at a dose of 3600 mg/day.

Conclusion. Gabapentin is the drug of choice in the management of patients with NeP of different etiology and intensity. A satisfactory safety profile and pharmacodynamic effects make gabapentin possible, despite the long history of its use, to remain a relevant drug used by a wide range of physicians, specialties, for pharmacotherapy of NeP patients.

Keywords: gabapentin; gabapentinoids; neuropathic pain; diabetic polyneuropathy; postherpetic neuralgia; chronic pain

Abbreviations: VAS – visual analogue scale; CI – confidence interval; GABA – gamma-aminobutyric acid; NeP – neuropathic pain; RR – relative risk; OR – odds ratio; RCTs – randomized clinical trials; eGFR – estimated glomerular filtration rate; SNRIs – selective norepinephrine reuptake inhibitors; ADP – average daily pain; TCAs – tricyclic antidepressants; CNS – central nervous system; α2δ-1 – alpha2-delta type 1 subunit; AMPA receptors – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors; IMMPACT – Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials; KCNQ2/3 – heteromeric potential-dependent potassium channels; NNT – Number Needed to Treat; LAT-1 – L-type amino acid transporter 1; MD – median deviation; NMDA receptors – N-methyl-d-aspartate receptors; NRS – Numeric Rating Scale.

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Применение габапентина для терапии нейропатической боли: взгляд с позиций доказательной медицины

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Цель. Провести анализ литературных источников на предмет фармакодинамических и фармакокинетических особенностей габапентина, позволяющих использовать его у пациентов с нейропатической болью, а также сравнительную оценку его эффективности и безопасности при применении в различных дозах.
Материалы и методы. В качестве поисковых ресурсов были использованы базы данных PubMed, Google Scholar, EMBASE, научно-информационная сеть ResearchGate и elibrary.ru. В качестве ключевых слов для поиска использовали «габапентин»», «механизм действия», «мишени габапентина», «фармакодинамика габапентина», «фармакокинетика», «фармакокинетические параметры», «нейропатическая боль», «рандомизированные клинические исследования». Глубина поиска составила 26 лет (с 1998 по 2024 гг.). В результате настоящий обзор составили 87 источников литературы.
Результаты. Нейропатическая боль (НБ) является одним из наиболее распространенных видов хронической боли, характеризующимся высокой распространенностью среди лиц трудоспособного возраста. Эффективная фармакотерапия, направленная на устранение болевого синдрома, является ключевым инструментом повышения качества жизни и сохранения работоспособности пациентов. Гетерогенность этиологических факторов, вовлечённых в генез НБ, указывает на необходимость использования препаратов, анальгетический эффект которых основан на ослаблении передачи болевых импульсов в ЦНС. В клинических исследованиях габапентин продемонстрировал эффективность в отношении снижения выраженности боли у пациентов с постгерпетической НБ, болевой диабетической нейропатией и многими другими состояниями, сопровождающимися НБ. Доза габапентина 300 мг/сут является начальной в терапии НБ и требует дальнейшей медленной титрации в зависимости от ответа пациента на терапию и переносимости препарата, в особенности у пациентов пожилого и старческого возраста, а также пациентов с нарушенной функцией почек. Согласно опубликованным данным, наиболее выраженный анальгетический эффект достигается у пациентов на фоне применения габапентина в дозе 3600 мг/сут.
Заключение. Габапентин является препаратом выбора при ведении пациентов с НБ различной этиологии и интенсивности. Удовлетворительный профиль безопасности и фармакодинамические эффекты позволяют габапентину, несмотря на длительную историю его использования, оставаться актуальным препаратом, применяемым врачами широкого круга специальностей для фармакотерапии пациентов с НБ.
Ключевые слова: габапентин; габапентиноиды; нейропатическая боль; диабетическая полинейропатия; постгерпетическая невралгия; хроническая боль
Список сокращений: ВАШ – визуальная аналоговая шкала; ДИ – доверительный интервал; ГАМК – гамма-аминомасляная кислота; НБ – нейропатическая боль; ОР – относительный риск; ОШ – отношение шансов; РКИ – рандомизированные клинические исследования; рСКФ – расчетная скорость клубочковой фильтрации; СИОЗН – селективные ингибиторы обратного захвата норадреналина; ССБ – среднесуточная боль; ТЦА – трициклические антидепрессанты; ЦНС – центральная нервная система; α2δ-1 – альфа-2 дельта субъединица типа 1; AMPA-рецепторы – рецепторы альфа-амино-3-гидрокси-5-метил-4-изоксазол-пропионовой кислоты; IMMPACT – инициатива по методам, измерению и оценке боли в клинических исследованиях; KCNQ2/3 – гетеромерные потенциалзависимые калиевые каналы; NNT – число пациентов, которых необходимо лечить; LAT-1 – система переносчиков L-аминокислот 1 типа; MD – медианное отклонение; NMDA-рецепторы – N-метил-d-аспартатные рецепторы; NRS – числовая рейтинговая шкала боли.

INTRODUCTION

A large number of chronic diseases, in particular metabolic and neurodegenerative ones, are accompanied not only by a decrease in the function of organs and systems, but also by the development of neuropathic pain (NeP), characterized by an increased pain sensitivity and the occurrence of spontaneous pain sensations. The basis of NeP is somatosensory disorders. NeP is usually chronic: persistent or recurrent pain usually lasts longer than 3 months [1].

According to the International Association for the

Study of Pain (IASP, 2019) classification, NeP is divided into peripheral and central [1]. ICD-10 diagnoses related to NeP include trigeminal neuralgia (G50.0), neuralgia after shingles (G53.0, B02.2), and a phantom limb pain syndrome (G54.6). Nevertheless, the spectrum of pathological conditions accompanied by NeP is not limited to the above-mentioned ones. Due to that, NeP is a widespread problem that has a negative impact on the quality of life and work capacity of a significant proportion of the population. The prevalence of NeP in the general population varies from 3 to 17% [2]; in general, the published data indicate that approximately one in twenty people in the Western world suffers from NeP [3].

According to a large-scale epidemiologic study that included the analysis of the UK Biobank (United Kingdom) patient database, the overall prevalence of NeP was 9.2% [4]. The scale of the NeP prevalence in the Russian Federation can be indirectly judged by the results of a 10-year analysis of visits to the Pain Research and Treatment Clinic (the period from 2011 to 2020), which revealed chronic pain in 32% of patients [5]. Among elderly and senile patients, a chronic pain syndrome is found in the absolute majority: according to the survey including 11 regions of the Russian Federation, its overall prevalence in the population ≥65 years of age was found to be 87.2% [6].

As a type of chronic pain, NeP can significantly reduce the quality of life of patients, limit their physical activity and the ability to self-care. This is due to both the pain itself and a number of conditions that it can provoke. It is important to note that NeP is more associated with the development of depression and anxiety disorders than other types of chronic pain. According to a cross-sectional study of NeP patients, 65.6% had depression and 73.7% had anxiety disorders [7]. The relevance of the NeP problem with depression and anxiety disorders in modern healthcare is well illustrated by the analysis results of the Thomson Reuters Web of Science (WoS) publication database: in 2000, the number of published articles on the relevant topic was 8, in 2020 - 106 (the total number of publications for 20 years is 915) [8]. NeP and the associated depression are causes of a sleep quality impairment: the studies have shown that more than 83% of people with NeP may suffer from some forms of insomnia [9].

NeP most often develops in patients of the working age, with significant socioeconomic consequences. According to a multicenter study of NeP outpatients (Turkey, 2021), their mean age was 55.5+14.4 years [10]. According to the US data, NeP most often affects men aged 35 to 44 years (regardless of ethnicity); in the case of women, the following differences were found: in whites, the peak incidence of NeP coincided with that of men, while in Hispanic and African-American women it was observed in the group of 45–54 years [11]. A high prevalence of NeP and associated depressive disorders in the population of people in their early 40s puts them at a significant risk for cardiovascular damage: according to the study conducted in Canada (2011–2012, 1493 patients with a spinal cord injury), NeP increased a cardiovascular risk (the adjusted odds ratio, OR) 2.27-fold (95% confidence interval, CI: 1.21 to 4.60), depression 4.07-fold (95% CI: 2.10 to 7.87) [12]. The economic consequences of NeP are illustrated by the data from a study of real clinical practice in European countries. The minimum amount of total annual direct costs of the health care system per patient with NeP was noted in Italy – 1 939 euros, the maximum – in Spain, 3 131 euros. The cost of the disease (including both direct and indirect costs) had a minimum value also in Italy, 9 305 euros; the maximum value was noted in Germany, 14 446 euros.

A basis for the management of NeP patients is the medications aimed at eliminating a pain syndrome, taking into account the heterogeneity of factors contributing to the development of the main forms of both peripheral and central NeP [14, 15], as well as providing optimal clinical outcomes, taking into account a high frequency of concomitant depression and anxiety disorders. The goals of NeP pharmacotherapy include the following ones [16]: reduction of pain by 30–50%; improvement of a sleep quality; improvement of the quality of life; preservation of the social activity and relationships; preservation of the working capacity; the improvement of organs and systems functioning, as well as the whole organism.

Domestic and international clinical guidelines for the management of NeP patients indicate gabapentinoids (gabapentin and pregabalin) as first-line drugs [17–19]. The efficacy and safety of pharmacotherapy depends on the drug dose and the administration duration.

THE AIM of the study was to analyze the pharmacodynamic and pharmacokinetic features of gabapentin, providing its use in NeP patients, as well as a comparative assessment of its efficacy and safety when used in different doses on the basis of the published data.

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MATERIALS AND METHODS

Abstract databases such as PubMed, Google Scholar, EMBASE, ResearchGate scientific information network and elibrary.ru were used as a source of materials for writing the review article. Each author independently searched the publications to exclude errors. The publications in three areas – pharmacodynamics, pharmacokinetics, and clinical efficacy and safety – were analyzed. In the area of "pharmacodynamics", the following key words and word combinations were used: "gabapentin", "mechanism of action", "gabapentin targets", "gabapentin pharmacodynamics"; in the area of "pharmacokinetics" they were: "gabapentin", "pharmacokinetics", "pharmacokinetic parameters". The analyzed period for these arears was 26 years (from 1998 to 2024), a total of 13 792 publications were found, after excluding duplicates, literature reviews, invalid papers (pharmacokinetics of gabapentin in animals), publications presented only in abstracts, the total number of papers included in the review for these two areas, was 27. In the area "efficacy and safety of gabapentin", the keywords for the search included "gabapentin", "neuropathic pain", "randomized clinical trials". The search was performed on publications from 2014 to 2024. 8 762 publications were found. After excluding duplicates, literature reviews, invalid publications, and publications with unavailable full text, 56 papers were included in this review.

RESULTS AND DISCUSSION

NeP mechanisms and gabapentin targets

Despite the progress in the study of cellular and molecular pathways, there is no unified consensus in understanding the mechanisms of NeP development to date. The complexity lies in the fact that in a single patient, several mechanisms are most often involved in the development of NeP, the most well-known of which [20] are as follows: mechanisms of central sensitization, mechanisms of peripheral sensitization, processes associated with neuroinflammation, dysfunction of descending nociceptive modulatory systems, response to an oxidative stress, and glial cell activation.

The involvement of each of these mechanisms is determined by the type of NeP (peripheral or central), as well as the etiologic factors underlying its development. The main types of peripheral and central NeP, as well as the main etiological factors inducing them, are shown in Fig. 1.

The leading role in the genesis of central NeP is played by damage to the sensory pathways of various parts of the central nervous system (CNS) due to blood flow disorders, strokes, infectious diseases, traumas, and multiple sclerosis [21]. The result of central sensitization is the formation of an increased level of the spontaneous activation of nociceptive sensory neurons, a decrease in the threshold of peripheral stimulation of neurons, and an increase in their response to the suprathreshold stimulation. Two types of neurons with opposite effects on the nociceptive transmission are located in the dorsal horns of the spinal cord: excitatory neurons expressing the vesicular transporter glutamate-2 and inhibitory neurons expressing the vesicular transporter gamma-aminobutyric acid (GABA). The main process underlying the central sensitization is the activation of a type of a glutamate receptor such as N-methyl-d-aspartate receptors (NMDA). They contain 4 subunits (two GluN1 and two GluN2) that form a channel for Na^+, K^+, and Ca^{2+} ions. Normally, this channel is closed due to the blocking action of extracellular Mg^{2+}. The receptor activation accompanied by a channel opening requires the binding of glycine and glutamate to the receptor subunits GluN1 and GluN29 in conjunction with a membrane depolarization to relieve a magnesium blockade. Through the open channel, Ca^{2+} ions rush inside the cell, which increases the cell membrane depolarization promoting an additional calcium influx. Presynaptic NMDA receptors can be activated by endogenous glutamate without removing the Mg^{2+}-block. The activation of most postsynaptic NMDA receptors requires a pronounced depolarization of neurons simultaneously with glutamate binding [22]. The outcome of the NMDA receptor activation is an increase in intracellular calcium, leading to the vesicle exocytosis and neurotransmitter release [22]. The NMDA receptor hyperactivity is a major component of the development and maintenance of chronic NeP [23]. This is due to their contribution to the enhancement of the spinal nociceptive transmission induced by a peripheral nerve damage. The intrinsic activation of presynaptic NMDA receptors in the dorsal horns of the spinal cord characteristic of NeP is accompanied by an enhanced release of glutamate from nociceptive primary afferent terminals [24].

The enzymes that cause their phosphorylation (casein kinase 2, protein kinases A and C, Ca^{2+} / calmodulin-dependent protein kinase II, and tyrosine kinases Src and Fyn) and interaction with such a protein component as the alpha2-delta subunit of potential-dependent

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Ca^{2+} channels type 1 (α2δ-1) play an important role in the hyperactivation of NMDA receptors of dorsal horn neurons [24]. The α2δ-1 subunit forms a complex with NMDA receptors and can also interact with neurexin-1α, thrombospondins (adhesion molecules) and other presynaptic proteins [25]; α2δ-1 can also interact with alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid receptors (AMPA receptors) [26]. The maximum expression of α2δ-1 is observed in dorsal radicular ganglia and dorsal horns of the spinal cord [27].

An overexpression of α2δ-1 is observed in conditions that provoke NeP: a trauma to nerve tissues, chemotherapeutic drugs in oncology, calcineurin inhibitors, opioid-induced hyperalgesia and an acquired tolerance to analgesics, and cerebral blood flow disorders. As a result, an increased formation of α2δ-1-NMDA-receptor complexes (primarily involving presynaptic receptors), leading to the enhanced release of neurotransmitters, was found in NeP [23, 25, 28].

Gabapentin is a structural analog of GABA, but has no significant effect on GABA receptors. It has been shown to act at the level of neurons of the peripheral gray substance, increasing the pain threshold and reducing the intensity of a regional cerebral blood flow in this area [29]. A key element of gabapentin's mechanism of action in NeP involves α2δ-1 binding (Fig. 2). This has important consequences determining the analgesic activity of gabapentin. First, it prevents the formation of α2δ-1-NMDA-receptor complex and allows to stop the process of NMDA receptor activation, which is important for the elimination of NeP [25, 26, 30]. Second, it leads to the modification of neurexin-α-1 effects in synapses, which reduces the rapidly released pool of presynaptic vesicles. Third, α2δ-1 binding by gabapentin promotes the inhibition of astrocyte thrombospondins, which reduces the number of newly formed excitatory synapses and the intensity of their operation (the processes initiated by an injury / inflammation) [25, 27]. The normalization processes of presynaptic and postsynaptic activation of NMDA-receptors of the posterior horns neurons of the spinal cord, accompanied by a reduction / elimination of pain sensations, is a central component of the gabapentin action mechanism.

Gabapentin has also a mechanism (absent in its structural relative, pregabalin) that is independent of α2δ-1 and is associated with a pronounced activating effect on heteromeric potential-dependent potassium channels (KCNQ2/3) responsible for M-currents [31]. Additionally, gabapentin is known to increase the expression of the GABA-A receptor subunit δ (δGABA-A) subspecies responsible for the tonic inhibitory conduction predominantly in the cerebellum and hippocampus [32]. At the early stages of the NeP onset, the analgesic effect of gabapentin is realized in the area of locus coeruleus neurons: it inhibits a presynaptic release of GABA and induces a glutamate release from astrocytes, which increases the neurons activity of this localization and leads to an increase in descending a noradrenergic inhibition [33].

The above pharmacodynamic effects of gabapentin are possible provided that a sufficient level of concentrations in plasma and CNS tissues is formed.

Pharmacokinetics of gabapentin

Gabapentin realizes its action mechanism by penetrating the CNS and neuronal membranes. It was created as a lipophilic analog of GABA, which ensures its entry into various CNS structures. Despite its lipophilicity, gabapentin is transported across cell membranes primarily by facilitated transport via the L-amino acid type 1 (LAT-1) carrier system and only to a minor extent by a passive diffusion; the absorption occurs in the proximal small intestine and is dose-dependent due to the saturation of the carrier system; the absorption profile is described by a hyperbolic function [34]. Its bioavailability reaches 60% for a dose of 900 mg/day and decreases to 27% in patients taking 4800 mg/day[1]. Thus, increasing the dose results in a slight decrease in absorption, which may limit the risk of intoxication when taking ultra-high doses. The absorption and bioavailability of the delayed-release form, gabapentin enacarbil, are somewhat different: it is transported by the monocarboxylate transporter type 1 and sodium-dependent multivitamin transporters (process is unsaturated and, therefore, nondependent); after the absorption, hydrolysis under the action of nonspecific carboxylesterases leads to the formation of active gabapentin. The range of gabapentin bioavailability values varies from 64.8 to 82.9% [35]. Food intake has no effect on the bioavailability of conventional gabapentin, but contributes to its increase for gabapentin enacarbil[2]. The volume of the gabapentin distribution is 0.8 l/kg, it practically does not bind to plasma proteins, its transport across the blood-brain barrier is carried out by LAT-1 [36]. The concentration

[1] DrugBank Online: Gabapentin: Uses, Interactions, Mechanism of Action. Available from: https://go.drugbank.com/drugs/DB00996

[2] Gabapentin | C9H17NO2 | CID 3446 – PubChem. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Gabapentin#section=Absorption-Distribution-and-Excretion)

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of gabapentin in cerebrospinal fluid is 9 to 20% of that in plasma, the concentration in breast milk is almost equal to that in plasma[3]. Gabapentin is not a substrate for cytochromes P450 and has no effect on them; it is not metabolized and excreted with urine unchanged with the participation of the processes of tubule secretion [37]. The clearance of gabapentin varies from 6 to 9 l/h. A renal function is the main factor determining the rate of gabapentin excretion: in norm, the elimination half-life ranges from 5 to 7 h; when creatinine clearance drops below 30 ml/min, it increases to 52 h[4].

Pharmacokinetic parameters of gabapentin in elderly and senile patients were studied in Ahmed G.F. et al. (2017), which included 75 patients (median age, 79 years) [38]. The difference compared to younger patients was a significant decrease in clearance, up to 2.93 l/hour, associated with a decreased renal function in this patient population.

The effect of diseases on the pharmacokinetics of gabapentin was demonstrated in a study including patients with different levels of DM control. In patients with hyperglycemia the apparent volume of distribution was increased by 68% compared to subjects without diabetes. There was also a 36% decrease in the maximum concentration of gabapentin in patients with high glycemia compared to study participants without diabetes (1.6 *vs* 2.5 mcg/mL). Nevertheless, the reliability of the obtained changes was not established by the authors, which suggests an insignificant effect of hyperglycemia on the pharmacokinetic parameters of gabapentin [39].

Changes in gabapentin plasma concentrations are the result of abnormalities in the excretion phase of the drug from the body; factors such as hepatic insufficiency, plasma protein abnormalities, and drug interactions have not shown significant effects. Gabapentin has a wide therapeutic index; its effective plasma concentrations range from 2 to 20 mg/l [40]. At the same time, the risk of toxic effects against the background of high doses is relatively low, which is associated with an absorption limitation, especially pronounced when using doses of more than 4800 mg/day, which was noted in patients who had received the drug for epilepsy therapy [41]. Accordingly, the maximum dose of gabapentin used in the treatment of NeP (3600 mg) is not accompanied by such a significant decrease in absorption.

The pharmacokinetics of gabapentin suggests a minimal risk of drug interactions. The occurrence of some motor disorders when combining gabapentin with losartan and etacrynic acid, a decrease in the anticonvulsant activity of gabapentin when used together with caffeine, were obtained in laboratory experiments involving mice; a clinical significance of these phenomena in humans has not been confirmed [42]. Synergism with regard to an analgesic effect was demonstrated in patients when taken together with tramadol, metamizole [42], celecoxib. When interacting with antacids (magnesium oxide) there was observed a decrease in the maximum concentration of gabapentin by 33%, against the background of proton pump inhibitors (omeprazole) – by 29%, and a significant decrease in its bioavailability was noted exactly for the combination with magnesium oxide, but not with omeprazole [43]. The most clinically significant interactions are observed when gabapentin is used together with opioids [44, 45]. The patients who have to receive gabapentin and opioid analgesics simultaneously require a careful medical monitoring aimed at a timely detection of such side effects as somnolence, sedation and respiratory depression. In combination therapy, the dose of both gabapentin and opioid analgesics should be reduced.

Efficacy and safety of gabapentin in clinical practice

Gabapentin has a long history of use: having been used in the 1970s primarily as an anticonvulsant, now, according to the most current clinical guidelines, it is the first-line drug for the management of patients with NeP [17–19].

The efficacy of gabapentin in NeP has been the subject of a large number of randomized clinical trials (RCTs) as well as systematic reviews and meta-analyses of RCTs. Since 2000, the Cochrane community has published systematic reviews addressing this issue. The latest one is dated 2017 (37 studies, 59 143 patients with NeP receiving gabapentin or gabapentin enacarbil at a dose of 1200 mg/day or more). Based on the definitions laid out in the Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials (IMMPACT), the authors determined that the number of patients with postherpetic NeP to be treated with gabapentin for moderate benefit (pain reduction of ≥30%, NNT30) was 5.7 and for significant benefit (pain reduction of ≥50%, NNT50) was 6.8. Similar parameters for patients with diabetic polyneuropathy were 6.6 and 5.9, respectively [46]. The authors noted that gabapentin was most

[3] DrugBank Online: Gabapentin: Uses, Interactions, Mechanism of Action.
[4] Ibid.

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effective in postherpetic NeP, diabetic polyneuropathy and mixed NeP. The proportion of patients who discontinued the drug for any reason (analysis of 22 studies, *n*=4 617) was 20% for gabapentin (a dose of 1200 mg or more) and 19% for placebo, indicating a satisfactory tolerability profile of therapy.

Gabapentin and pregabalin have demonstrated a comparable efficacy and safety in patients with NeP due to the spinal cord injury. According to a systematic review and meta-analysis of 8 studies, there was no significant difference between the two drugs in reducing pain scores (mean difference, MD=-0.37; 95% CI: -1.67, 0.93; *p* >0.05) [47]. A meta-analysis published one year later (2021) showed a greater efficacy of pregabalin and gabapentin in eliminating NeP against the background of the spinal cord injury compared to carbamazepine, amitriptyline and placebo [48].

According to a meta-analysis by Ko Y.C. et al (2021), gabapentin in patients with diabetic painful neuropathy reduced pain (as measured by a visual analog scale, VAS) equally effectively compared to duloxetine (MD=-1.23; 95% CI: -6.09 to 3.62; *p*=0.62), it was also found to be accompanied by an improvement in patients' functional status [49].

The updated data on the efficacy of gabapentin were obtained in a systematic review and meta-analysis of 50 RCTs devoted to the treatment of NeP (2023): the authors found that NNT30 was 7, NNT50 - 8. The same paper evaluated the similar parameters for pregabalin; they were 8 and 10, respectively, indicating a slightly greater effect of gabapentin [50]. Another meta-analysis in 2023 evaluated the efficacy of gabapentinoids in postherpetic NeP (14 RCTs, *n*=3 545): the standard MD of the Numerical Rating Scale (NRS) score for gabapentin was -2.16 (95% CI: -3.40 to -0.92; *p* <0.05), for pregabalin -0.78 (95% CI: -0.98 to -0.58; *p* <0.05) [51]. In a large meta-analysis of 119 studies on patients with various types of chronic pain, including NeP, 8 studies evaluated gabapentin, analyzing those where a comparison had been made with placebo, the authors noted a significant reduction in pain, MD was -1.49 (95% CI: -2.76 to -0.23; *p* <0.05) [52]. Of interest are the results of a meta-analysis of 30 comparative double-blind RCTs with parallel groups or crossover studies that examined the analgesic effect of at least two first-, second-, and third-line drugs in NeP (*n*=4 087) published in 2024 [53]. 10 RCTs (*n*=920) compared the effect of tricyclic antidepressants (TCAs) with pregabalin or gabapentin; the pooled effect showed no difference in the analgesic effect between TCAs and pregabalin / gabapentin (MD=0.10; 95% CI: -0.13 to 0.32; *p*=0.39), there was no significant difference in the depression severity and drug tolerability either. In 8 RCTs, pregabalin/gabapentin was compared with selective norepinephrine reuptake inhibitors (SNRIs), the pooled effect showed a greater effect of the SNRIs efficacy, but a further group analysis showed no differences. The tolerability of the drugs was also comparable [53].

Since the 2000s, most of the researchers have determined that effective doses of gabapentin for the treatment of NeP are larger than 900 mg/day. One of the first large-scale reviews of RCTs on the efficacy and safety of gabapentin in patients with NeP indicated the following dosing guidelines: start, on average, at a dose of 900 mg/day (300 mg/day on the first day, 600 mg/day on the second day, 900 mg/day on the third day) with a further dose titration up to 1800 and up to 3600 mg/day in patients with severe NeP [54]. Many studies demonstrating the efficacy and favorable tolerability profile of gabapentin in high doses (up to 3600 mg/day) have been published [46, 55, 56].

The analysis of the efficacy and safety of different gabapentin enacarbil doses, performed as part of a randomized, double-blind, placebo-controlled trial including patients with postherpetic NeP, showed the most pronounced reduction in mean daily pain (MDP) compared to placebo when using a dose of 3600 mg/day (MD=-1.07; 95% CI: -1.68 to -0.45; *p*=0.002). A significant reduction in pain was achieved in 76% of patients using the drug at this dose versus 70% in the 2400 mg/day group and 67% in the 1200 mg/day group [55]. Of interest are the comparative evaluation results of efficacy and safety of different gabapentin doses and forms obtained in a systematic review and metaanalysis of 7 RCTs, including 2014 patients in the efficacy evaluation group and 2050 patients in the safety evaluation group (authors searched for all publications of the relevant topics from 1966 to 2017). The results showed the largest reduction in MDP with gabapentin (conventional form) at a dose of 3600 mg/day, with a standardized mean difference in MDP values of -0.86 (95% CI: -1.13 to -0.58; *p* <0.00001), with the smallest reduction in MDP demonstrated for the delayed-release forms (Table 1). The authors have also demonstrated that at doses of 1800 to 3600 mg/day, gabapentin significantly improved the sleep quality and reduced the pain intensity by at least 50% in the majority of patients taking it. The safety analysis of high gabapentin doses revealed such side effects as dizziness, drowsiness and peripheral edema [57]. The MDP reduction against the background of using different doses of gabapentin and gabapentin enacarbil is demonstrated in Table 1.

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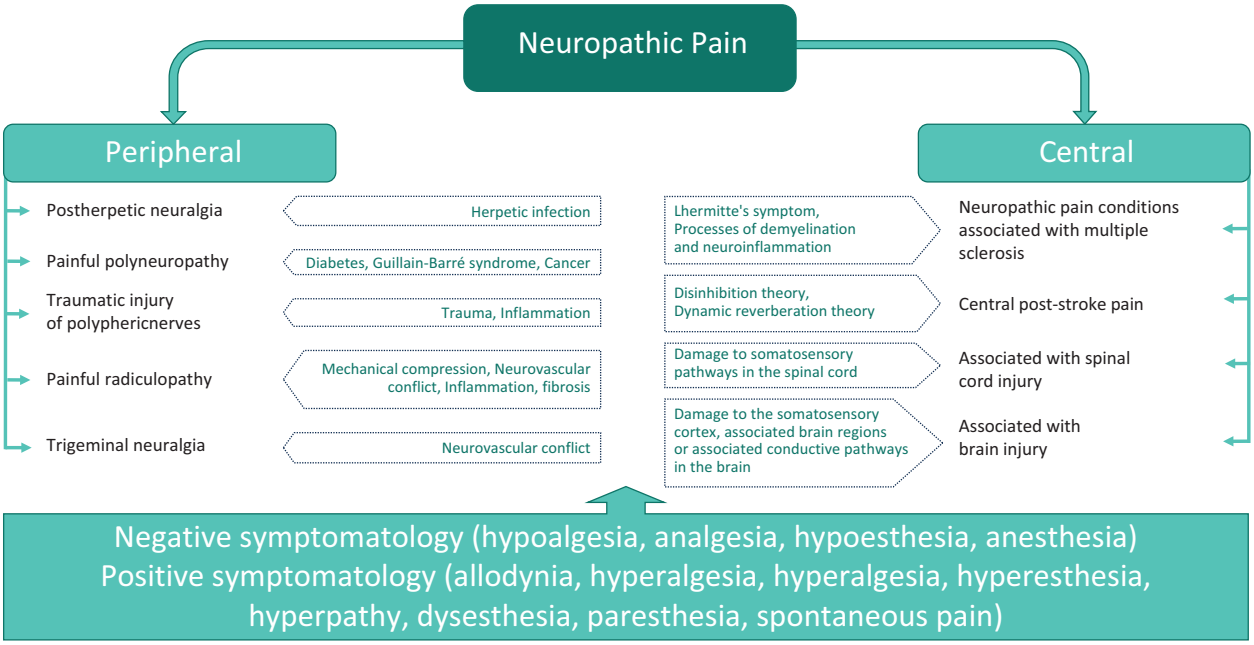

Figure 1 – Varieties of NB and their main etiologic factors

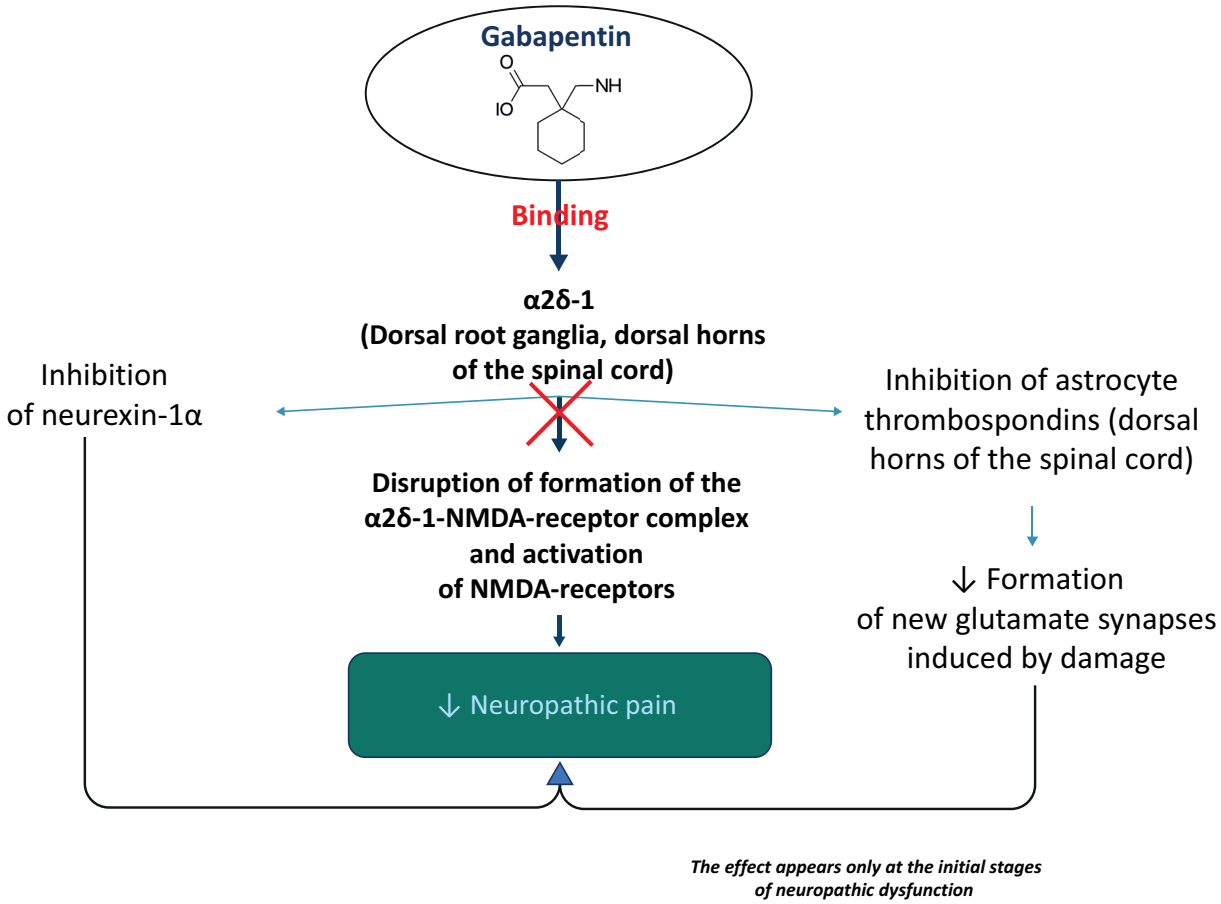

Figure 2 – Action mechanism and main targets of gabapentin underlying the NeP elimination

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Table 1 – ADP reduction rates depending on gabapentin dose

Drug	Dose	MD magnitude of MDP reduction
Conventional form	3600 mg/daily	–1.07; 95% CI: from –1.68 to –0.45; *p*=0.002 [55] –0.86; 95% CI: from –1.13 to –0.58; *p* <0.00001 [57]
Gabapentin enacarbil	3600 mg/daily	–0.50; 95% CI: from –0.79 to –0.20; *p*=0.0009 [57]
Gabapentin enacarbil	2400 mg/daily	–0.70; 95% CI: from –1.33 to –0.07; *p*=0.029 [55] –0.33; 95% CI: from –0.62 to –0.03; *p*=0.03 [57]
Gabapentin enacarbil	1200 mg/daily	–0.81; 95% CI: from –1.40 to –0.23; *p*=0.013 [55] –0.43; 95% CI: from –0.66 to –0.20; *p*=0.0002 [57]
Gabapentin ER	1800 mg once daily	–0.21; 95% CI: from –0.42 to –0.01; *p*=0.04 [57]
Gabapentin ER	1800 mg twice daily	–0.25; 95% CI: from –0.57 to 0.06; *p*=0.12 [57]

Note: MD – median deviation; CI – confidence interval; MDP – mean daily pain.

The array of published RCTs, systematic reviews and meta-analyses is the basis for the development of clinical guidelines for the management of patients. Finnerup N.B. et al. published the results of their own RCTs meta-analysis devoted to the treatment of NeP (the total number was 229) and simultaneously presented the recommendations of the Neuropathic Pain Special Interest Group (NeuPSIG), working as part of the International Association for the Study of Pain (IASP) [58]. The NNT50 for gabapentin was 6.3. Gabapentin at the doses ranging from 1200 to 3600 mg was listed by the authors as a first-line treatment for the management of NeP patients with a high level of evidence (including the conventional form, a slow-release form, and gabapentin enacarbil).

The expert consensus of the Chinese Association for the Study of Pain indicates that gabapentin should be used for an effective NeP control at a dose of 900–1800 mg/day, but does not limit the upper limit of the daily dose [59]. In 2024, an updated consensus on the use of the drugs affecting ion channels for the therapy of chronic pain was published in China. According to its provisions, gabapentin is recommended for the therapy of postherpetic NeP, diabetic polyneuropathy, and many other types of NeP [60]. As the drug of choice for NeP therapy, gabapentin is also noted in the Chinese Guidelines for the Treatment of Chronic Pain Disorders with Non-opioid Analgesics [61]. The consensus of Indian experts on the management of patients with NeP lists gabapentin as the first-line treatment and recommends titrating it to 1800 mg/day [62].

The updated 2022 American Academy of Neurology (AAN) clinical guidelines for the management of patients with painful diabetic polyneuropathy suggest using gabapentin at a dose of 900 to 3600 mg/day for 4–8 weeks [63]. A similar approach was recommended by the American Diabetes Association (ADA) in 2017: the starting dose should be about 300 mg followed by titration to an effective dose of 900 to 3600 mg/day[5]. The updated ADA 2022 guidelines state that a minimum dose of 1800 mg/day should be used in most patients and increased in patients with severe NeP to a maximum dose of 3600 mg/day; lower doses are recommended only for the patients with a reduced estimated glomerular filtration rate (eGFR) [64]. Clinical guidelines for the management of patients with NeP developed in France in 2020, also recommend the use of gabapentin as the first-line treatment for various types of NeP at doses of 1200–3600 mg/day, with gabapentinoid, pregabalin, classified as the second-line treatment [65].

Russian recommendations (Methodical Recommendations on the Diagnosis and Treatment of Neuropathic Pain, Russian Interregional Public Organization for the Study of Pain, Society for the Study of Pain) indicate that gabapentin is effective in doses of 1200–3600 mg/day; it should be slowly titrated in an individual regimen starting at 300 mg/day[6]. According to the clinical recommendations “Chronic pain in elderly and senile patients” developed by the All-Russian public organization “Russian Association of Gerontologists and

[5] Pop-Busui R, Ang L, Boulton AJM, Feldman EL, Marcus RL, Mizokami-Stout K, Singleton JR, and Ziegler D. Diagnosis and Treatment of Painful Diabetic Peripheral Neuropathy. Arlington (VA): American Diabetes Association; 2022. Available from: https://www.ncbi.nlm.nih.gov/books/NBK580224/ DOI: 10.2337/db2022-01

[6] [Guidelines on neuropathic pain diagnosis and treatment]. Yakhno N.N., editor. Moscow. RAMS; 2008. 32 p. Russian

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Geriatrics", in elderly and senile patients, it is necessary to use lower doses of gabapentin, the initial dose should be 300 mg, titrated until the development of the analgesic effect[7].

Consensus clinical guidelines on diagnosis and rational therapy of patients with a painful form of diabetic polyneuropathy developed by leading Russian professional medical communities (2019), give the following dosing regimen of gabapentin as the first-line drug. On the first day 300 mg/day should be taken, on the second day – 600 mg/day, on the third day – 900 mg/day, further on – the titration during 3–8 weeks to reach 1800–3600 mg/day and the administration for at least 2 weeks at the maximum tolerated dose [66].

Many international clinical guidelines specify gabapentin as the first-line treatment in the management of patients with NeP but do not provide doses: UK, NICE guidelines, 2020[8], Germany, 2020 [16] and 2021 [67], Canada, 2021 [68], China, 2023 [69] and 2024 [61]. Similarly, without a dose indication, gabapentin is presented in the practice guideline for the management of patients with trigeminal neuralgia published by Lambru G. et al. (2021). It is listed among the first-line drugs for both idiopathic, classical and secondary forms [60].

Physicians using them are guided by the data in the instructions for the drug and the patient's response to a gradual increase in dose (in most cases, the titration period takes 2 weeks). In general, practice indicates a good tolerability of gabapentin. Risks appear, first of all, when they are used in combination with opioids, as it will be discussed below.

Gabapentin safety

The gabapentin safety is well illustrated by the data from a 2017 Cochrane review that found the occurrence of adverse events in 11% of patients taking gabapentin (1200 mg/day or more) versus 8.2% of patients taking placebo (a risk ratio of 1.4 (1.1 to 1.7)), and the number of patients to be treated for the occurrence of an adverse event was 30 (20 to 66) [46]. The most current safety data on gabapentinoids (gabapentin and pregabalin) are reported in a systematic review and meta-analysis of 50 RCTs (*n*=12 398) published in 2023. Among the side effects of gabapentin, weight gain (relative risk, OR=5.61; 95% CI: 1.04 to 30.22), dizziness (OR=3.33; 95% CI: 2.39 to 4.65), peripheral edema (OR=3.06; 95% CI: 1.25 to 7.48), and somnolence (OR=2.91; 95% CI: 2.10 to 4.03) were the most prominent. Side effects of pregabalin included an impaired coordination (OR=7.21; 95% CI: 1.36 to 38.25), gait disturbances (OR=6.71; 95% CI: 1.57 to 28.71), ataxia (OR=6.02; 95% CI: 2.31 to 31.15), euphoria (OR=6.01; 95% CI: 3.02 to 11.97), and weight gain (OR=4.97; 95% CI: 3.08 to 8.00) [50].

According to the meta-analysis of 8 safety studies of different drugs in NeP patients, the discontinuation rate of gabapentin was similar to that of placebo [48].

The safety profile of gabapentin is quite favorable, it should be noted that side effects are more common in patients using it together with opioids [70–72]. Monotherapy with gabapentin is usually not accompanied by the development of serious adverse drug reactions (ARs); the formation of dependence is not typical either [73].

Acute poisoning associated with gabapentin overdose is not a routine phenomenon in clinical practice either. This may be partly explained by a dose-dependent decrease in absorption and, consequently, bioavailability, against the background of high doses. A clinical case describing an acute overdose with gabapentin (5200 mg administered at once) against the background of a number of other drugs in a 39-year-old man, is available from published works. The clinical picture included severe rhabdomyolysis and acute tubular necrosis, which required renal replacement therapy, after 3 months all parameters returned to normal [74].

A meta-analysis of 11 RCTs (2376 patients with postherpetic NeP, including a gabapentin group (doses of 1200, 1800, 2400 and 3600 mg/day) – 1 424 people, placebo – 952) showed that the risk ratio for ARs with gabapentin compared to placebo was slightly more than one: 1.29 (95% CI: 1.06 to 1.57) [75]. An earlier meta-analysis including 12 RCTs evaluating the efficacy and safety of gabapentin showed the following. The relative risk of discontinuation due to ARs was lower with higher doses: 1.8 (95% CI: 0.82 to 3.8) for the 1800 mg/day, 1.4 (95% CI: 0.91 to 2.0) for the 2400 mg/day, and 1.4 (95% CI: 0.85 to 2.4) for the 3600 mg/day [76].

In general, the published data indicate the following spectrum of adverse events associated with gabapentin: dizziness, confusion, general weakness, impaired coordination of movements, gastrointestinal symptoms, and weight gain. A dependence formation is not characteristic of gabapentin: according to Meaadi J. et al. (2023), among 50 analyzed studies there was not a single one in which the occurrence of euphoria – the main substrate of the dependence formation – had been noted [50].

When prescribing gabapentin for NeP therapy, its tolerability should be taken into account. In case of ARs as dizziness or drowsiness, it is necessary to

[7] Clinical guidelines "Chronic pain in elderly and senile patients", 2020. Available from: https://static-0.minzdrav.gov.ru/. Russian

[8] Neuropathic pain in adults: pharmacological management in non-specialist settings. London: National Institute for Health and Care Excellence (NICE); 2020 Sep 22. (NICE Clinical Guidelines, No. 173.) Available from: https://www.ncbi.nlm.nih.gov/books/NBK552848/

return to the previous dose, slowing down the titration process. During the entire period of selection of an individual effective dose, the patient should be under medical supervision, which is necessary to choose an adequate dosing regimen, an optimal duration of therapy and control the occurrence of side effects. The gabapentin dose titration is one of the fundamentally important factors determining the magnitude of an analgesic effect in NeP patients. The purpose of the titration is to select an individual effective dose within the range studied in clinical trials, which minimizes the risks of occurrence and provides control over potential adverse events. When managing a patient with NeP, it is necessary to carry out a regular monitoring of pain intensity (once every 2–4 weeks) using available tools, a VAS, is most often used in this role. The goal of pharmacotherapy is to reduce the intensity of NeP by 30–50% of the initial value [17–19].

Among the ways to reduce the risk of gabapentin ARs, there are the following ones. The main way is to start with a low dose (300 mg/day) and further on a slow titration until the desired therapeutic effect is achieved. It is important to use doses and dosing regimens that are well studied in clinical trials and correspond to those given in the instructions for medical use or a general characterization of the drug. A strict control of a number of drug prescriptions received by the patient also contributes to reducing the risks of adverse reactions. The phenomenon of polypragmasy is very common among patients receiving drugs affecting the central nervous system and makes a significant contribution to the risks of pharmacotherapy [77, 78]. Clinically unjustified switching from the original drug to generics is also important. A number of studies have demonstrated that there are subpopulations of patients for whom the probability of achieving a comparable bioavailability to the originator when switching to a generic drug is reduced. There is an increased variability of pharmacokinetic parameters of gabapentin in patients with an impaired absorption and a reduced renal function, respectively, there is a high probability that the use of generics in this case will not allow to achieve the required values of the drug concentration in plasma [79]. The original preparation of gabapentin available on the Russian pharmaceutical market is Neurontin®. The use of the original drug is characterized by a greater efficacy and safety compared to generics, which follows from the results of pharmacokinetic studies [79].

Achieving therapeutic efficacy of the drug is impossible without an adequate level of patient adherence to pharmacotherapy. Taking into account the need to titrate the dose of gabapentin from lower to higher, it is worth noting the importance of such a factor as an availability of the drug in various dosages, which allows the patient to take the drug with greater comfort. The original preparation of gabapentin is presented in the form of capsules (300 mg) and film-coated tablets (600 mg). The latter are convenient to use in patients requiring high doses of gabapentin.

CONCLUSION

According to current Russian and international clinical guidelines, gabapentin is the drug of choice in the management of patients with NeP of different etiology and intensity. A satisfactory safety profile and pharmacodynamic effects demonstrated in clinical trials allow gabapentin, despite its long history of use, to remain a relevant drug used by physicians of a wide range of specialties for pharmacotherapy of NeP patients. The data set accumulated in RCTs was obtained primarily for original gabapentin, different dosage forms of which can provide a comfortable process of a dose titration for the patient and achieve an effective pain control. In most studies involving patients with NeP and a normal renal function, the target therapeutic dose of gabapentin, contributing to a maximum analgesic effect against a satisfactory safety profile, was 1800–3600 mg/day (divided into three doses), which allows us to recommend this dose as optimal for the main population of NeP patients.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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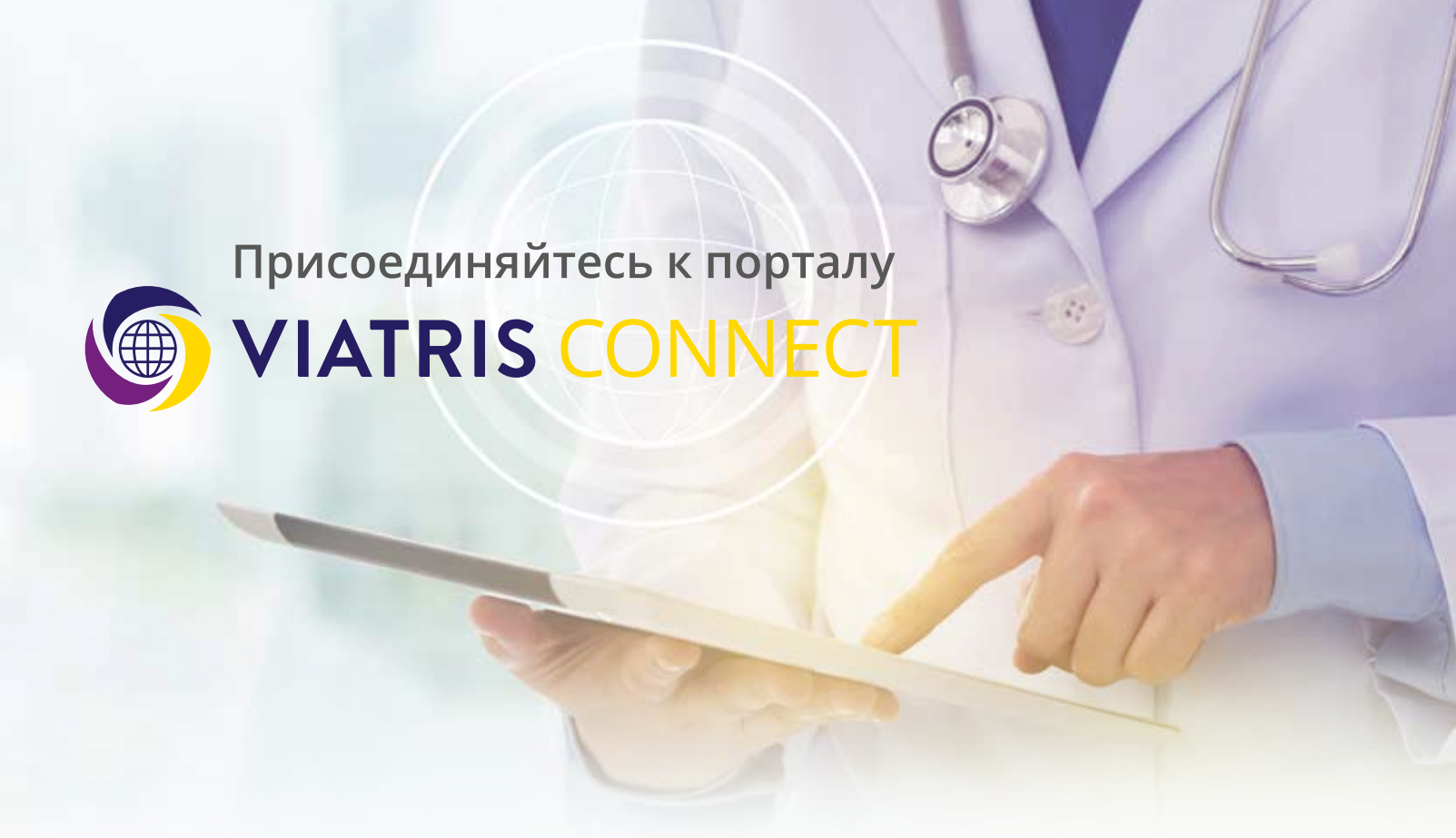

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