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MOLECULAR MECHANISMS UNDERLYING THERAPEUTIC ACTION OF VITAMIN B_6

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The aim of the study was to analyze the molecular mechanisms that determine the possibility of using vitamin B_6 in clinical practice for the correction of various pathological conditions.
Materials and methods. Information retrieval (Scopus, PubMed) and library (eLibrary) databases were used as research tools. In some cases, the ResearchGate application was used for a semantic search. The analysis and generalization of the scientific literature on the topic of research, covering the period from 1989 to the present, has been carried out in the work.
Results. It has been shown that all chemical forms of vitamin B_6 are able to penetrate the membranes of most cells by free diffusion, while forming phosphorylated forms inside. Pyridoxal phosphate is a biologically important metabolite that is directly involved as a cofactor in a variety of intracellular reactions. Requirements for this cofactor depend on the age, sex and condition of the patient. Pregnancy and lactation play a special role in the consumption of vitamin B_6. In most cases, a balanced diet will provide an acceptable level of this vitamin. At the same time, its deficiency leads to the development of a number of pathological conditions, including neurodegenerative diseases, inflammations and diabetes. Negative manifestations from the central nervous system are also possible with an excessive consumption of B_6.
Conclusion. Replenishment of the vitamin B_6 level in case of its identified deficiency is a necessary condition for the successful treatment of the central nervous system diseases, diabetes and correction of patients' immune status. At the same time, it is necessary to observe a balanced intake of this cofactor in order to avoid negative effects on metabolism in case of its excess.
Keywords: pyridoxine; pyridoxal phosphate; metabolism; vitamin B_6
Abbreviations: PN – pyridoxine; PM – pyridoxamine; PL – pyridoxal; PNP – pyridoxine phosphate; PMP – pyridoxamine phosphate; PLP – pyridoxal phosphate; PNG – pyridoxine glycoside; PDXK – pyridoxalkinase; PNPO – pyridoxine(amine) phosphate oxidase; ALP – tissue non-specific alkaline phosphatase; PDXP – pyridoxal(pyridoxine/pyridoxamine)phosphatase; AT – aminotransferase; DH – aldehyde dehydrogenase; PNGH – pyridoxine glycoside hydrolase; LPH – lactase-phloretin hydrolase; AOX – aldehyde oxidase; E-PLP – enzyme-linked pyridoxal phosphate; E-PMP – enzyme-bound pyridoxamine phosphate; RDM – recommended daily maintenance; POX – L-pipecolate oxidase; PYRC – Δ1-pyrroline-5-carboxylate reductase; AASA – α-aminoadipate-6-semialdehyde; AASDH – antiquitin (α-aminoadipate-6-semialdehyde dehydrogenase); AADAT – α-aminoadipate aminotransferase; P6C – L-Δ1-piperidine-6-carboxylate; P5C – L-Δ1-pyrroline-5-carboxylate; KYN – kynurenine; KYNA – kynurenic acid; XA – xanthurenic acid; PUFAs – polyunsaturated fatty acids; AGEPs – advanced glycation end product; ROS – reactive oxygen species; CRP – C-reactive protein; TNF-α – tumor necrosis factor-α; IL-1b – interleukin-1b; IL-6 – interleukin-6; WBCs – number of white blood cells; ATP- adenosine triphosphate; GABA – gamma aminobutyric acid; CNS – central nervous system; MMT – mitochondrial membrane transporter; BBB – blood-brain barrier.

МОЛЕКУЛЯРНЫЕ МЕХАНИЗМЫ, ЛЕЖАЩИЕ В ОСНОВЕ ТЕРАПЕВТИЧЕСКОГО ДЕЙСТВИЯ ВИТАМИНА B_6

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ОБЗОРЫ

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Цель. Анализ молекулярных механизмов, определяющих возможность использования витамина B_6 в клинической практике для коррекции различных патологических состояний.

Материалы и методы. В качестве инструментов проведения исследования использовались информационно-поисковые (Scopus, PubMed) и библиотечные (eLibrary) базы данных. В ряде случаев для семантического поиска использовалось приложение ResearchGate. В работе осуществлялся анализ и обобщение научной литературы по теме исследования, охватывающей период с 1989 по настоящее время.

Результаты. Показано, что все химические формы витамина B_6 способны проникать через мембраны большинства клеток путем свободной диффузии, при этом внутри формируют фосфорилированные формы. Пиридоксальфосфат является биологически важным метаболитом, непосредственно участвующим в качестве кофактора во множестве внутриклеточных реакций. Потребности в данном кофакторе зависят от возраста, пола и состояния пациента. Особую роль в потреблении витамина B_6 играет беременность и период лактации. В большинстве случаев сбалансированное питание позволяет обеспечить приемлемый уровень данного витамина. В то же время его дефицит приводит к развитию целого ряда патологических состояний, включающих нейродегенеративные заболевания, воспаление и диабет. Также возможны негативные проявления со стороны центральной нервной системы при избыточном потреблении B_6.

Заключение. Восполнение уровня витамина B_6 при его выявленном дефиците является необходимым условием успешной терапии заболеваний центральной нервной системы, диабета и коррекции иммунного статуса пациентов. При этом необходимо соблюдать сбалансированный прием данного кофактора во избежание негативных эффектов на метаболизм при его переизбытке.

Ключевые слова: пиридоксин; пиридоксальфосфат; метаболизм; витамин B_6

Список сокращений: PN – пиридоксин; PM – пиридоксамин; PL – пиридоксаль; PNP – пиридоксинфосфат; PMP – пиридоксаминфосфат; PLP – пиридоксальфосфат; PNG – пиридоксингликозид; PDXK – пиридоксалькиназа; PNPO – пиридоксин(амин)фосфатоксидаза; ALP – тканенеспецифическая алкалинфосфотаза; PDXP – пиридоксаль(пиридоксин/пиридоксамин)фосфатаза; AT – аминотрансфераза; DH – альдегиддегидрогеназа; PNGH – пиридоксингликозидгидролаза; LPH – лактаза-флоретингидролаза; AOX – альдегидоксидаза; E-PLP – связанный с ферментом пиридоксальфосфат; E-PMP – связанный с ферментом пиридоксаминфосфат; РСП – рекомендуемая суточная потребность; POX – L-пипеколатоксидаза; PYRC – Δ1-пирролин-5-карбоксилатредуктаза; AASA, α-аминоадипат-6-полуальдегид; AASDH – антиквитин (α-аминоадипат-6-полуальдегиддегидрогеназа); AADAT – α-аминоадипатаминотрансфераза; P6C – L-Δ1-пиперидин-6-карбоксилат; P5C – L-Δ1-пирролин-5-карбоксилат, KYN – кинуренин; KYNA – кинурениновая кислота; ХА – ксантурениновая кислота; ПНЖК – полиненасыщенные жирные кислоты; КПГ – конечные продукты гликирования; АФК – активные формы кислорода; CRP – С-реактивный белок; TNF-α – фактор некроза опухоли-α; IL-1b – интерлейкин-1b; IL-6 – интерлейкин-6; WBC – число белых кровяных телец; ATP – аденозинтрифосфат; ГАМК – гамма аминомаслянная кислота; ЦНС – центральная нервная система; MMT – митохондриальный мембранный транспортер; ROS – реактивные формы кислорода; ГЭБ – гематоэнцефалический барьер.

INTRODUCTION

Vitamin B_6 is one of the vital water-soluble vitamins. Its discovery dates back to the beginning of the 20th century; the discovery occurred as a result of the search for a pellagra cure [1]. In solution, this vitamin is present in the form of 3 main chemical forms (vitamers) – alcohol, aldehyde and amine. B_6 vitamers are stable in an acidic environment, but become extremely unstable in a neutral or alkaline environment, especially when heated or exposed to light [2]. Although the chemical forms of vitamin B_6 are relatively diverse, only the phosphorylated form of the aldehyde functions as a coenzyme in mammalian organisms. The main metabolism of this vitamin occurs in the liver; however, other tissues have a corresponding metabolic activity. In this case, the catabolism product of vitamin B_6 is pyridoxic acid (4-pyridoxic acid, PA).

Among the biochemical reactions directly involved in the vitamin B6 metabolism, it is necessary to highlight several key enzymes (Fig. 1), which include:

• pyridoxalkinase (PDXK, pyridoxal kinase, EC 2.7.1.35), catalytic activity cofactors: divalent metal ions (Mg^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}); the corresponding reaction is:

$$ATP + PL/PN/PM \rightarrow ADP + H^+ + PLP/PNP/PMP$$

• pyridoxine (amine) phosphate oxidase (PNPO, pyridoxine-5'-phosphate oxidase, EC 1.4.3.5), catalytic activity cofactor: flavin mononucleotide (FMN); the corresponding reaction is:

$$H_2O + O_2 + PNP/PMP \rightarrow H_2O_2 + NH_4^+ + PLP$$

• tissue-nonspecific alkaline phosphatase (ALP, tissue non-specific alkaline phosphatase, EC 3.1.3.1), catalytic activity cofactors: Mg^{2+}, Zn^{2+}; the corresponding reaction is:

$$PLP + H_2O \rightarrow PL + phosphate$$

• pyridoxal (pyridoxine/pyridoxamine) phosphatase (PDXP pyridoxal (pyridoxine/pyridoxamine) phosphatase, EC 3.1.3.74), a catalytic activity cofactor: Mg2+; the corresponding reaction is:

$$PLP + H_2O \leftrightarrow PL + phosphate$$

• aminotransferase (AT aminotransferase, EC 2.6.1.54); the corresponding reaction is:

$$PMP + 2\text{-}Oxoglutarate \leftrightarrow PLP + D\text{-}Glutamate$$

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• pyridoxine glycoside hydrolase (PNGH, PNG hydrolase, EC 3.2.1.62) and/or LPH – lactase-phloretin hydrolase (lactase-phloretin hydrolase, EC 3.7.1.4); the corresponding reaction is:

PN-5'-β-D-glucoside + H_2O → pyridoxine + D-glucose

• aldehyde oxidase (AOX, aldehyde oxidase, EC 1.2.3.1), catalytic activity cofactors: [2Fe-2S] clusters, FAD, Mo-molybdopterine; the corresponding reaction is:

$$PL + H^+ + H_2O_2 \leftrightarrow PA + H_2O + O_2$$

The combination of the enzymes listed above, forms pools of intracellular vitamin B_6 derivatives and predetermines the corresponding levels of the metabolic processes activity directly associated with the participation of pyridoxal phosphate. It is noteworthy that certain types of bacteria of the human intestinal microflora are able to synthesize vitamin B_6 in the form of pyridoxal phosphate from deoxyxylulose 5'-phosphate and 4-phosphohydroxythreonine, as well as from glyceraldehyde-3-phosphate and D-ribulose 5'-phosphate. The following bacteria have these biosynthetic pathways: bacteroids *(Bacteroides fragilis* and *Prevotella copri),* actinobacteria *(Bifidobacterium longum* and *Collinsella aerofaciens),* and proteobacteria *(Helicobacter pylori)* [3]. Despite rather extensive information on the impact on the biochemical processes of this representative water-soluble vitamins group, today, there is no fully substantiated idea of its use possibility in various pathological conditions and molecular processes that underlie the alleged positive effects.

THE AIM of the study was to analyze the molecular mechanisms that determine the possibility of using vitamin B_6 in clinical practice for the correction of various pathological conditions.

MATERIALS AND METHODS

Information retrieval (Scopus, PubMed) and library (eLibrary) databases were used as research tools. In some cases, the ResearchGate application was used for a semantic search. The analysis and generalization of the scientific literature on the topic of research, covering the period from 1989 to the present, has been carried out in the work.

The following keywords and word combinations were used in the search: pyridoxal phosphate, recommended daily maintenance for vitamin B_6, vitamin B_6 deficiency, pyridoxal phosphate, pyridoxine, PLP, vitamin B_6 metabolic pathways, PLP-dependent enzymes, pyridoxal phosphate-dependent reactions, vitamin B_6 daily intake, vitamin B_6 deficiency, pyridoxal phosphate and oxidative phosphorylation, PLP-dependent epilepsy, pyridoxal phosphate and diabetes, vitamin B6 therapy, pyridoxine toxicity.

The BRENDA database (https://www.brenda-enzymes.org) was used to describe the B_6 derivatives metabolism using the appropriate classification of enzymes, the reactions they catalyze, and the cofactors involved. When detailing the processes of intake and distribution of vitamin B_6 in the body, metabolic pathways maps of the KEGG information database (https://www.kegg.jp) were used. To build chemical formulas and illustrations, the Corel Draw 2018/2022 software package was used.

RESULTS AND DISCUSSION

Intake and distribution of vitamin B_6 in the body

A distinctive feature of pyridoxal (PL) and its derivatives is the difference between the biologically significant active form of the vitamin and the form that is able to penetrate into cells.

Phosphorylated forms of B_6 vitamers and pyridoxine glycoside are not absorbed in the intestine, so they are subject to dephosphorylation by intestinal phosphatases (PDXP) and deglycosylation by hydrolases (PNGH/LPH) [5]. B_6 vitamers have been shown to be absorbed in the intestine *via* passive diffusion [6, 7]. It is possible that there is transport of B_6 vitamers by thiamine transporters (THTR), which belong to the SLC19A2 and SLC19A3 families and function in the acidic environment of the small intestine or other tissues [8]. The absorption of vitamin B_6 increases with an increase in the level of carriers transcription (with a deficiency of pyridoxal phosphate), as well as under the action of protein kinase A. In turn, with an increase in the intracellular level of cAMP, there is a significant absorption inhibition of vitamin B_6, which is also sensitive to the action of the diuretic amiloride [7].

After the absorption by the small intestine cells, B_6 vitamers are rephosphorylated by the corresponding kinases (PDXK) and converted to pyridoxal phosphate or delivered to the liver with the blood flow. The liver also rephosphorylates and converts pyridoxine phosphate (PNP) and pyridoxamine phosphate (PMP) to pyridoxal phosphate (PLP) by pyridoxine (amine) phosphate oxidase (PNPO). Although this process occurs primarily in the liver, PNPO is also expressed in many other tissues. PLP is exported from the liver *via* sinusoidal capillaries in a bound state with a lysine 190 residue of albumin [9].

If the intake of B_6 vitamers exceeds the required level, PLP is dephosphorylated by pyridoxal phosphatase (PDXP) and oxidized by aldehyde oxidase (AOX) to pyridoxic acid (PA). It has also been shown that aldehyde dehydrogenase (ALDH, EC 1.2.1.4) and pyridoxal oxidase (PO, EC 1.2.3.8) can be responsible for the process of PL oxidation. Pyridoxic acid is excreted from the body in the

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urine, which accounts for more than 90% of the total excreted vitamin B_6 [10].

The penetration through the cell membrane of most tissues is carried out by passive diffusion. The exception is penetration through the membrane of mitochondria and erythrocytes, after dephosphorylation by tissue-nonspecific alkaline phosphatases (ALP) – ecto-enzymes sewn to cell membranes with glycophosphatidylinositol (phosphoglyceride anchors, GPI Anchors – glycosylphosphatidylinositol anchors). PL, possibly, crosses the blood-brain barrier *via* facilitated diffusion using a carrier, and is "deposited" inside brain or choroid plexus (CP) cells *via* PDXK phosphorylation [7]. Apart from the liver, the choroid plexus is the only organ capable of rapidly mobilizing PLP, which explains its high proportion in cerebrospinal fluid (CSF) relative to the total amount in the body, which is 38% in humans [11]

A possible participation of carriers in the process of PL transport into mitochondria, erythrocytes or *via* the blood-brain barrier in humans has been shown, although the corresponding proteins and genes encoding their synthesis have not been identified yet. However, in yeast, the Tpn1 protein, which is a member of the purine-cytosine permease family, is responsible for the transport of pyridoxine across the cytoplasmic membrane, while the Mtm1p protein is responsible for the transport of PLP into mitochondria [12].

In some cells, in addition to the liver, catabolism of PLP to pyridoxamine phosphate under the action of aminotransferase (AT) is possible, and the subsequent reverse process under the action of PNPO, the so-called salvage pathway, is also possible [11].

PLP homeostasis inside cells

To avoid "undesirable" reactions of aldehyde or carbonyl stress, a free intracellular PLP concentration is maintained at a low level of 1 μM. For this, there are PLP-binding proteins, such as glycogen phosphorylase in muscles, hemoglobin in erythrocytes, and albumin in blood plasma [6].

The action of PLP synthesizing enzymes, i.e. PDXK and PNPO, is inhibited by the reactions product. Moreover, it has been shown that there is a system for transferring the synthesized pyridoxal phosphate directly to the target pyridoxal phosphate dependent enzymes [11].

In the cellular homeostasis of pyridoxal phosphate, the protein PROSC, which binds it, plays the role, the dysfunction of which leads to the accumulation of pyridoxine phosphate inside the cells [13].

Inside mitochondria, PLP is also present in a protein-bound form: E-PLP – enzyme bound PLP; E-PMP, enzyme bound PMP, where it enters *via* the mitochondrial membrane transporter (MMT) SLC25A39/40 [11]. The spatial distribution of the transformation processes and transport of vitamers in tissues should be also notified. In most cases, there is no specific carrier to carry the dephosphorylated vitamin B_6 form. This certainly makes the use of this coenzyme as an active component of the dosage form very promising. The main routes of transport and metabolic transformations of B_6 vitamers are shown in Fig. 2.

Reactive properties of vitamin B_6

All the reactive properties of pyridoxal phosphate listed below, are manifested only in the composition of the corresponding enzyme [14]. At rest, PLP is covalently bound to the enzyme (Fig. 3). In this case, the aldehyde group of pyridoxal phosphate and the ε-amino group of the lysine residue in the active site of the enzyme form a Schiff base.

This condition is called Internal aldimine. Substrate binding to the enzyme leads to the ε-amino group replacement of the lysine residue by the substrate amino group; the process is called transaldimination, and the product is called substrate-PLP. This condition is called external aldimine. The state formed after breaking one of the three bonds of the substrate α-carbon atom is a transition state, and it is called Quinonoid. The detachment of the H+ proton from the substrate α-carbon atom corresponds to the reactions of transamination, β-elimination or racemization, the detachment of the COO- carboxyl group corresponds to decarboxylation, and the detachment of the R side chain corresponds to retroaldol hydrolysis (cleavage). During the reactions, PLP acts as an electron acceptor, stabilizing carbanion. Stabilization is possible due to the redistribution of the negative charge within the system of π-bonds formed by the Schiff base and the PLP pyridine ring. In this case, it is necessary that the corresponding bond be located perpendicular to the PLP pyridine ring, and the corresponding p-orbitals be parallel. This makes it possible for the negative charge of the substrate α-carbon atom to be most optimally stabilized within the system of π-bonds [15].

Along with the above-mentioned properties, the PLP participation in the antioxidant defense system can be notified. Due to its high reactivity, PLP is characterized by a significant rate of $^1O_2$ quenching, comparable to the action of vitamins C and E [16]. In addition, the aldehyde B_6 vitamer is necessary to ensure the glutathione synthesis, since PLP-dependent enzymes synthesize

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about 50% of cysteine, one of the components of this important antioxidant [17].

Daily maintenance for vitamin B_6

The daily maintenance for vitamin B_6 varies quite a lot depending on the patient's condition, age and gender (Table 1). With full confidence, the reason for the differences in the daily maintenance for vitamin B_6 can be considered an individual variability in the content and activity of enzymes that use it as a substrate or cofactor, as well as an increased consumption of B_6 during pregnancy [18], breastfeeding [19] and with age-related changes.

Vitamin B_6 intake may vary depending on the age of the patients. A recent clinical study of the quantitative B_6 vitamers content in the blood when taken orally [20] showed the following. In the older age group (70.1+2.7 years, 10 men and 10 women) there was a reduced level of pyridoxine and pyridoxal phosphate in the blood plasma, but elevated levels of pyridoxic acid, compared with the data of the younger age group (24.2+2.8 years, 10 men and 10 women). The total amount of B_6 vitamers taken was in line with the recommended daily maintenance of Harvard Medical School: 1.3 mg for both sexes in the younger age group; 1.5 mg and 1.7 mg for women and men, respectively, in the older age group.

The time of maximum plasma concentration of pyridoxal after the administration of vitamin B_6 in the form of pyridoxine hydrochloride in the pharmacokinetic studies is 1 hour, while for pyridoxal phosphate the time of the maximum concentration reaches 10 hours [10], and reflects the time required for the metabolism of pyridoxal by liver cells.

From 40% to 60% of the total vitamin B_6 intake is excreted in the urine [10]. According to the clinical study in the younger age group, there is a greater excretion of pyridoxine in the urine compared to the older group after a single intake of the vitamin-mineral complex and breakfast. At the same time, there is no noticeable difference in the level of urinary excretion of other B_6 vitamers in these age groups [20]. That indicates a greater bioavailability of pyridoxine for the people in the older age group.

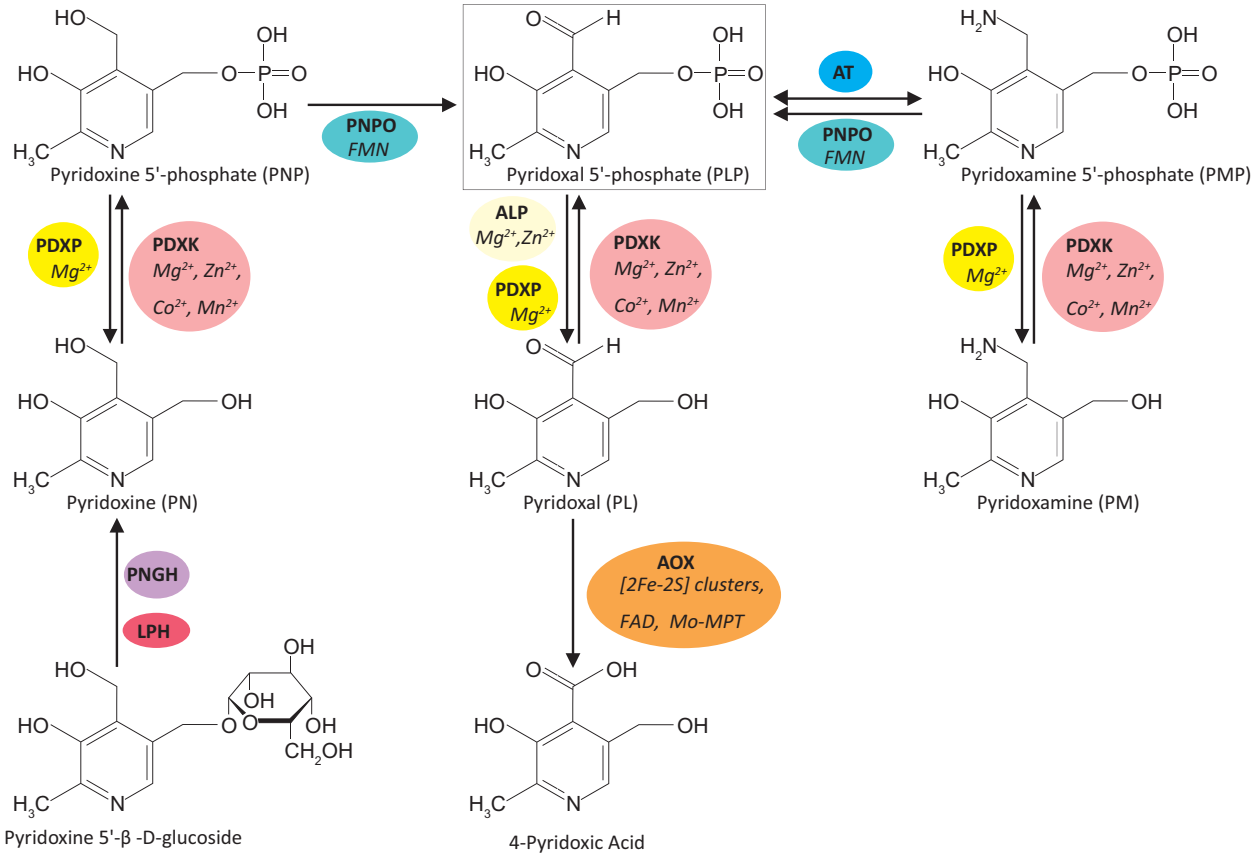

Figure 1 – Mutual conversion of three B_6 vitamers

Note: Three possible forms are involved in the metabolic cells processes. They are: alcohol (pyridoxine, pyridoxine, PN), amine (pyridoxamine, pyridoxamine, PM) and aldehyde (pyridoxal, pyridoxal, PL). Each of the vitamers can be in a phosphorylated form: pyridoxine phosphate (pyridoxine 5'-phosphate, PNP), pyridoxamine phosphate (pyridoxamine 5'-phosphate, PMP), and pyridoxal phosphate (pyridoxal 5'-phosphate, PLP), respectively [4]. In the case of an alcohol vitamer, a glycosylated form may also exist: pyridoxine glycoside (pyridoxine-5'-β-D-glucoside, PNG) [5].

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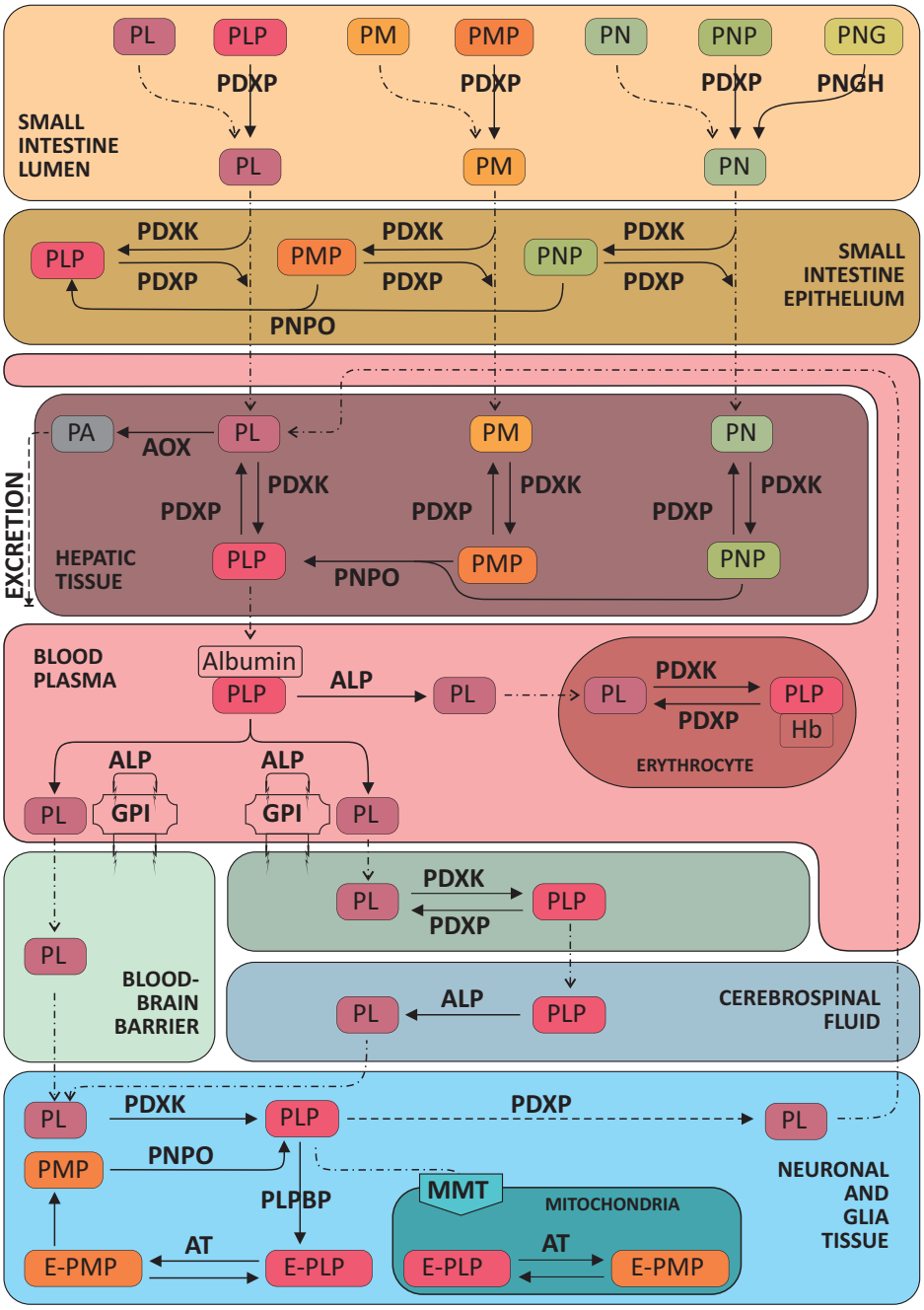

Figure 2 – Main ways of transport and tissue distribution of B_6 vitamers

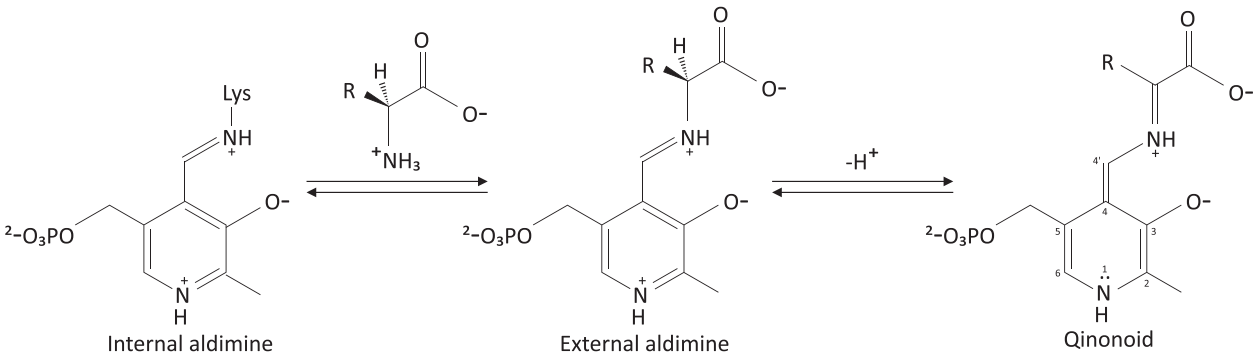

Figure 3 – Generalized representation of PLP states in composition of active protein center, taking into account transition of internal aldimine to external, followed by quinonoid formation

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Table 1 – Recommended Daily Maintenance for (RDM) for vitamin B_6, proposed by European Food Safety Authority, in accordance with a certain life stage (according to Ali MA et al., 2022 [19])

Life stage		RDM, mg	Significant processes and effects on the body	Deficiency symptoms
Newborns	0–6 months	0.1	Vital for growth, development and weight gain. Lifelong therapy is necessary for newborns with congenital glutamate decarboxylase deficiency.	Deficiency can lead to treatment-resistant polymorphic seizures.
	7–12 months	0.3		
Children	1–3 years old	0.5–0.6	Required for thymidine biosynthesis and immunity formation. Effective in treatment of behavioral disorders symptoms associated with autism, hyperactivity and schizophrenia. Adjuvant to antiepileptic drugs. Has a beneficial effect on stressful conditions that accompany puberty.	
	4–9 years old	0.6		
Adolescents	Girls	1–1.2		
	Boys	1–1.3		
Adults	Men	1.3	Extremely effective against colorectal cancer in adult men. Reduces cholesterol level in blood plasma.	Microcytic hypochromic anemia, lymphopenia, convulsions.
	Women			
	Not pregnant	1.3	Essential for estrogen metabolism. Indicated for women with breast cysts. Effective during PMS.	
	Pregnant	5.5–7.6	Pregnancy stabilization, prevention of miscarriage. Correction of hyperemesis manifestations in pregnant women. Necessary for heme and porphyrin synthesis, as well as for proper exploitation of iron by red blood cells. Maintaining natal and postnatal development within the norm range.	Hyperemesis of pregnancy, anemia, nausea, vomiting, spontaneous miscarriages.
	Lactating	5.5–7.6	The same as in the previous paragraph. Mood swings. Reducing anemia risks.	The same as in the previous paragraph.
Elderly		0.5–1.7	Reducing irritable bowel syndrome risk.	Deficiency can cause irritable bowel syndrome.

Table 2 – Mechanisms leading to dysfunction of PLP-dependent enzymes and subsequently forming clinical or biochemical disorders

Clinical and/ or biochemical manifestations in violation of vitamin B_6 metabolism	PLP-dependent enzymes associated with clinical and/or biochemical manifestations of disorders	Mechanism	References
Epilepsy (Seizures)	Branched-chain amino acid aminotransferase, BCAT1 + 2, EC 2.6.1.42	Inhibition of glutamate synthesis in brain by disrupting transamination of branched chain amino acids valine, leucine, isoleucine and α-ketoglutarate to the corresponding α-ketoacid and glutamate.	[24]
	Glutamate decarboxylase, GAD, EC 4.1.1.15	Dysregulation of GABA/glutamate interconversion and, consequently, neuronal excitability, due to inhibition of one of the processes: synthesis of inhibitory neurotransmitter GABA by transamination of α-ketoglutarate to glutamate (an excitatory neurotransmitter), which, in turn, is decarboxylated back to GABA.	[25]
	GABA-transaminase, GABA-T, EC 2.6.1.19		
Hypotension, movement disorders (gaze palsy, dystonia, hypokinesia), vegetovascular dystonia	Aromatic L-amino acid decarboxylase, AADC, EC 4.1.1.28	Inhibition of final step catalysis in synthesis of dopamine and serotonin and, subsequently, norepinephrine and epinephrine.	[26]

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Clinical and/ or biochemical manifestations in violation of vitamin B_6 metabolism	PLP-dependent enzymes associated with clinical and/or biochemical manifestations of disorders	Mechanism	References
Defects in neurotransmission, synaptogenesis, long-term potentiation of synaptic transmission, CNS development, and excitotoxicity of L-serine racemase	Serine racemase, SRR, EC 5.1.1.18	Catalysis inhibition L-serine racemization reaction to D-serine, the most important co-agonist of N-methyl-D-aspartate receptor (NMDA-R), with subsequent CNS dysfunction.	[27]
Anemia and lactic acidosis	Δ-Aminolevulinic acid synthase, ALAS1 + 2, EC 2.3.1.37	Violation of heme and Fe-S-clusters synthesis.	[28, 29]
	Cysteine desulfurase, NFS1, EC 2.8.1.7		
Hypoglycemia	Aspartate transaminase, AST or Glutamate oxaloacetate transaminase, GOT, EC 2.6.1.1	Disruption of the malate-aspartate shuttle mechanism when aspartate aminotransferase is inhibited; inhibition of pyruvate synthesis by alanine aminotransferase and L-serine non-hydratase. Distribution of many aminotransferases in tissues is used in tissue damage diagnosis. Thus, an increase in the number of these aminotransferases in the blood plasma is a sign of a liver violation.	[6, 30, 31]
	Aspartate transaminase, AST or Glutamate oxaloacetate transaminase, GOT, EC 2.6.1.1		
	l-Serine dehydratase, SDH, although BRENDA gives the more accurate name L-serine ammonia-lyase, SDS, EC 4.3.1.17		
	Glycogen phosphorylase, GP, EC 2.4.1.1	Glycogen phosphorylase catalyzes one of the gluconeogenesis steps. Vitamin B_6 deficiency leads to inability to mobilize sufficient glucose from glycogen stored in the liver.	[32]
Hyperammonemia, girate atrophy	Ornithine aminotransferase, OAT, EU 2.6.1.13	Deficiency of ornithine aminotransferase is characterized by an increase in the concentration of ornithine in the blood and urine and is accompanied by progressive degeneration of choroid and retina of eyes and hyperammonemia.	[33]
Changes in the amount of serine, threonine and glycine in blood plasma and cerebrospinal fluid.	Serine hydroxymethyltransferase, SHMT, EC 2.1.2.1	These enzymes are essential for biosynthesis and catabolism of serine, threonine and glycine. Significant deficiency of vitamin B6 leads to an increase in concentrations of these amino acids in blood plasma and cerebrospinal fluid.	[11, 34]
	Glycine dehydrogenase, GLDC, EC 1.4.4.2		
	Serine/threonine deaminase, SDS, EC 4.3.1.17		
	Phosphoserine aminotransferase, PSAT1, EC 2.6.1.52		
	Glycine C-acetyltransferase, GCAT, EC 2.3.1.29		
Increased levels of xanthurenic acid in urine.	Kynureninase, KYNU, EC 3.7.1.3	Tryptophan metabolism disorder	[35]
	Kynurenine aminotransferase, KYAT1 & 2, EC 2.6.1.7		

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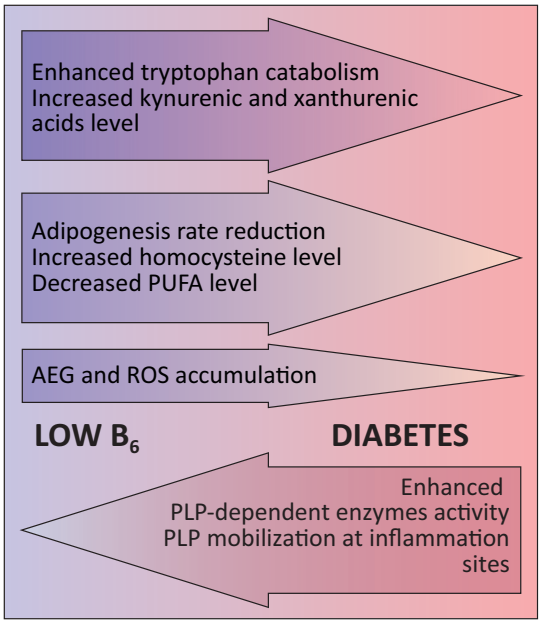

Figure 4 – Scheme of mutual influence processes leading to vitamin B_6 deficiency and formation of diabetes mellitus

Vitamin B_6 deficiency

Deficiency of vitamin B_6 alone is rare in developing countries, although low levels of circulating PLP have been reported with oral contraceptives and some other drugs, smoking and alcoholism, celiac disease, and diabetes [4]. Despite the reduced bioavailability of plant B_6 vitamers (the predominance of pyridoxine glycoside) relative to vitamers from animal food, vegetarians do not have this vitamin deficiency [21].

From a biochemical point of view, vitamins B have a clear relationship, playing a significant role in key metabolic pathways in the human body. B_6 deficiency combined with other vitamins has other clinical manifestations. For example, the manifestation of pellagra is usually caused by a deficiency of pyridoxine, niacin and riboflavin [22]. Clinical and/or biochemical manifestations of vitamin B_6 metabolism disorders are characterized by inhibition of the PLP-dependent enzymes functions, and can be recorded by measuring the concentration levels of the corresponding metabolites in blood plasma, urine, or cerebrospinal fluid [6, 23] (Table 2).

As Table 2 shows, it is obvious that PLP is closely related to the metabolism of neurotransmitters and the state of the central nervous system. Changes in the mutual conversion of GABA, glycine, and glutamic acid can lead to an imbalance in the processes of excitation and inhibition in neurons [36].

At the same time, the process of PLP synthesis is ATP-dependent, which, in turn, requires energy costs and activation of mitochondrial oxidative phosphorylation in neurons and astrocytes. An increase in the content of PLP, which activates glycine dehydrogenase (a part of the glycine cleavage system), will lead to its decrease in plasma and cerebrospinal fluid and, possibly, affect microcirculation [37] and the supply of nervous tissue with key metabolites, in particular glucose [38, 39].

Clinical manifestations of vitamin B_6 deficiency

The cofactors of the methionine cycle and the tricarboxylic acid cycle are thiamine (B_1) and pyridoxine (B_6). Violation of the methionine cycle is associated with cognitive impairment and is accompanied by low levels of pyridoxine (B_6) and cobalamin (B_{12}). Therefore, a combined use of thiamine, pyridoxine, and cobalamin, even without a proven deficiency of one of them, can improve the clinical picture in neuropathy, motor dysfunction, nociceptive and neuropathic pain [40].

Metabolism of homocysteine depends on several cofactors, including PLP (B_6), folate (B_9) and cobalamin (B_{12}). Their deficiency leads to the accumulation of homocysteine [41]. Its excess – hyperhomocysteinemia – is one of the stages in the development of increased blood clotting, accompanied by ischemic cerebrovascular and cardiovascular disorders, and is one of the causes of migraine [42].

Vitamin B_6 deficiency is directly associated with the development of hypertension [43] and an increased risk of a cardiovascular disease, stroke and venous thrombosis [44]. In systemic inflammation, accompanied by an increased level of C-reactive protein, changes in plasma pyridoxal phosphate serve as a method for diagnosing myocardial infarction [4].

Low levels of vitamin B_6 are detected in some types

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of cancer: brain ventricular cancer, colorectal cancer, lungs, breast and kidney cancer. An increase in the level of pyridoxal phosphate in the blood plasma of patients with kidney cancer is associated with a decrease in the mortality rate [10].

Vitamin B_6 deficiency is observed in rheumatoid arthritis, and its plasma level is inversely proportional to the severity of the disease. It is noteworthy that with a low level of pyridoxal phosphate in the blood plasma, such patients have a normal level of erythrocyte PLP. This phenomenon is not explained by a low intake of B_6 vitamers, inborn defects in B_6 metabolism, or its deficiency. In the rat models, low plasma levels of pyridoxal phosphate corresponded to low levels in the liver, while the amount of this cofactor in the muscles, which have the largest pool of vitamin B_6 in the body, remained unchanged. An increase in the level of vitamin B_6 catabolism was not detected either in rats or in humans, since urinary excretion of pyridoxic acid was not increased [45].

Convulsive disorders (epileptic episodes) associated with pyridoxine deficiency, are among the first described genetic disorders [46]. Clinical manifestations can be already observed in the first 24–48 hours after birth, in some situations, epileptic episodes can be detected in the perinatal period, and cases of manifestation of the disease months and years after birth are not uncommon. In a mild form, such disorders are accompanied by excessive excitability, irritability, trembling, abnormal crying, frequent startling in response to a sound or touch. Severer clinical manifestations of convulsive disorders are usually accompanied by encephalopathy, which is more likely a precursor to epilepsy than its consequence, and systemic disorders: hyper- or hypothermia, abdominal distension, vomiting (possibly with bile impurities), hepatomegaly, shortness of breath with hypoxemia, and metabolic acidosis. Therapy with high doses of vitamin B_6, preferably intravenously, or orally, in the case of older age, gives a positive trend, although a case of a buccal administration of a pyridoxine solution by a nursing mother has been described [47].

In some cases, PLP deficiency can lead to epileptic disorders. Pyridoxine-dependent epilepsy is often accompanied by a mental retardation and requires immediate therapy, which should include not only pyridoxine, but also other drugs, since the underlying mechanisms of a mental retardation are unique. Indeed, various gene mutations lead to the accumulation of different reactive components: ALDH7A and MOCS2 mutations lead to the accumulation of α-aminoadipic semialdehyde (AASA) and L-Δ1-piperidine-6-carboxylate (L-Δ1-piperideine-6-carboxylate, P6C), and the ALDH4A1 mutation – to γ-glutamyl semialdehyde (γ-glutamyl semialdehyde, GGSA) and L-Δ1-pyrroline-5-carboxylate (L-Δ1-pyrroline-5-carboxylate, P5C) [48]. These components are the most pathogenic factors in the manifestation of mental retardation, as they accumulate in brain tissues and their amount is not necessarily reduced during the pyridoxine therapy [49].

Due to their aldehyde groups, AASA and GGSA can interact non-enzymatically with glutathione and other key body macromolecules, and alter their functionality. These macromolecules include DNA, RNA, proteins and phospholipids, as well as molecules containing –SH groups. These interactions lead to the accumulation of glycation end products [48]. P6C and P5C form a complex with PLP, which reduces the level of bioavailable PLP and manifests as an epileptic disorder in patients [6, 50].

Antiquitin, α-aminoadipate semialdehyde dehydrogenase (AASDH), is responsible for the AASA synthesis. Antiquitin deficiency, in addition to these features, is accompanied by oxidative stress, which is one of the main causes of brain cell death in epilepsy; therefore, the diagnosis of pyridoxine-dependent epilepsy, monitoring of the disease dynamics, and correction of the therapeutic plan can be carried out by measuring the metabolites associated with oxidative stress [48]. Clinical studies of high-dose vitamin B_6 therapy in combination with a diet restricting lysine intake have shown a reduction in neurotoxic effects due to the accumulation of pyridoxine-dependent enzymes substrates that cause a developmental delay and cognitive impairment, compared with monotherapy [51]. The current standard is triple therapy, which includes high doses of vitamin B_6 (15–30 mg/kg/day, in 3 doses), lysine restriction, and arginine support (150 mg/kg/day, in 3 doses) [52].

In the case of a late manifestation of pyridoxine-dependent epilepsy with antiquitin deficiency, an unexpressed reaction to therapy with high doses of pyridoxine is possible. In this case, therapy is supplemented with high doses of folic acid (3–5 mg/kg/day) [53].

Vitamin B_6 deficiency and diabetes are strongly associated. According to the literature sources, vitamin B_6 deficiency can be both a consequence and a cause of diabetes (Fig. 4). The effect of vitamin B_6 on type 1 diabetes differs from its effect on type 2 diabetes due to the difference in pathophysiological processes [54].

In the case of type 1 diabetes mellitus, in the context including pregnancy and obesity, an increased need for vitamin B_6 for specific PLP-dependent enzymes, as well as triggering inflammatory pathways, may reduce its availability. In the case of type 2 diabetes mellitus, a decrease in vitamin B_6 levels can lead to an aggravation of the clinical diabetes manifestations, affecting insulin secretion or its biological activity. At the same time, mechanisms including increased tryptophan catabolism

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via the kynurenine pathway reduced the adipogenesis (lipogenesis) rate, impaired lipid metabolism, or a reduced ability to resist the formation of advanced glycation end products, can contribute to the development of the disease. Moreover, a violation of the vitamin B_6 antioxidant activity can also contribute to the development of complications in diabetes and cancer [55].

Possibilities of vitamin B_6 therapeutic use

Taking vitamin B6 in appropriate dosages is undoubtedly justified in case of its confirmed deficiency.

Pyridoxine can affect not only the central nervous system functions, but also the physiological processes performed by the peripheral nervous system [56]. A B_6 participation in the synthesis of the neurotransmitters serotonin and GABA (Table 2), which have an inhibitory effect on the transmission of pain nerve impulses in the spinal cord and brain, indicates the possibility of the alleviating pain in carpal tunnel syndrome. In the scientific literature, cases of this positive effect from taking pyridoxine at the doses of 50 to 200 mg per day have been described, toxic side effects have been recorded at higher doses, but the topic requires a further study [57].

It should be also notified that taking vitamin B_6 could lead to mixed results. As a cofactor for aromatic amino acid decarboxylase (EC 4.1.1.28), PLP promotes increased dopamine synthesis in hypothalamic neurons, which leads to the inhibition of the prolactin action. This mechanism underlies the decrease in the effectiveness of Parkinson's syndrome therapy with levodopa when taken together with vitamin B_6. However, according to the recent analysis of relevant clinical studies, lactation inhibition by pyridoxine is controversial and unsubstantiated and requires further research [58]. However, in a number of countries, a combination of doxylamine and pyridoxine hydrochloride is used as a drug in the treatment of nausea and vomiting in pregnant women [59–61].

There are prerequisites for the use of pyridoxine and magnesium high doses in autism spectrum disorders. However, a blind placebo-controlled study did not reveal statistically significant differences in the effects of pyridoxine and magnesium from the placebo effect [62]. Nevertheless, a combined use of pyridoxine and cations, as well as the inclusion of cation salts in the composition of the dosage form, seems to be quite reasonable, as well as the previously analyzed possible combined use of the amino acid glycine and zinc compounds [63].

At present, there are prerequisites for the use of pyridoxine as a prevention of hand-foot syndrome (a side effect of chemotherapy) [64].

Vitamin B_6 is also used as an antidote for acute intoxications with isoniazid (as an anti-tuberculosis drug), gyromitrin from *Gyromitra esculenta*), monomethylhydrazine (a component of rocket fuel), and an exposure to hydrazine (a corrosion inhibitor) [65].

Vitamin B_6 deficiency has a strong effect on cellular immunity and, to a lesser extent, on humoral immunity. A marked decrease in the level of lymphocyte proliferation, T-cell cytotoxicity, delayed-type hypersensitivity, allograft rejection, and an altered cytokine profile have been demonstrated in B_6-deficient rats. In humans, a similar situation is observed, and therapy with vitamin B_6 doses exceeding the recommended daily maintenance, improves this situation [4]. Replenishment of PLP deficiency can be also recommended for inflammation. In the case of cardiovascular diseases and cancer, it is the inflammatory process that is considered to play a key role in the pathological course or progression of diseases. It has been shown that vitamin B_6 deficiency accompanies inflammatory bowel diseases [4].

Inflammation is characterized by a decrease in serum albumin concentration and an increase in tissue-specific alkaline phosphatase (ALP). In general, this enhances the process of PLP mobilization, reducing the proportion of the albumin-bound state and increasing the rate of its dephosphorylation to PL. Such a change in the distribution of vitamin B_6 may not be limited to areas of inflammation, but may also affect other intact tissues and cells [45].

C-reactive protein (CRP) is a marker of the IL-1b/TNF-α/IL-6 inflammatory pathway. IL-6 – interleukin-6 – causes an increase in ALP. Interleukin-1b (IL-1b) and interleukin-6 (IL-6) are among the activators of the hypothalamic-pituitary-adrenal (HPA) axis, in which cortisol plays a key role. Cortisol has multiple effects on the body and is a major regulator of the physiological stress response, which includes increased gluconeogenesis and protein breakdown in muscles and connective tissue. The released amino acids can then be utilized for the energy production, immunomodulating protein synthesis, immune cell proliferation, and tissue repair. All these processes require the PLP participation, and therefore, the cellular demand for this cofactor increases [10].

In blood plasma, the PLP level demonstrates an inverse relationship with the content of clinical markers of inflammation – CRP, IL-6 receptor, α-1-antichymotrypsin, serum amyloid A, white blood cell count (WBC), kynurenine/tryptophan ratio (KTR), neopterin; overall inflammatory summary score and summary scores representing different inflammatory modalities) [45].

Moreover, an increase in the intracellular PLP level is associated with a change in the cellular response to

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the action of glucocorticoids during the prednisone therapy. During such therapy, there is an increase in the concentration of PLP, PL and PM in the blood plasma. This process is accompanied by an increased activity of pyridoxal phosphate-synthesizing enzymes – PDXK, PMPO/PNPO – and suppression of the PDXP activity in the liver, while the effect of ALP in plasma remains unchanged [11].

Toxic effect of vitamin B_6

Excessive intake of vitamin B_6 (from 2 to 6 g per day) leads to the development of severe sensory neuropathy, although cases of motor neuropathy have also been described. At the same time, the abolition of B_6 leads to a marked improvement in clinical manifestations. This effect is similar to the clinical manifestation of a hereditary mutation of the gene encoding PDXK, since high amounts of circulating pyridoxine can inhibit the action of this enzyme [66]. However, for many years, the therapeutic dose of B_6, which is 200–500 mg per day, has not given either clinical symptoms or electrophysiological evidence of peripheral neuropathy in homocysteinuria [67]. It should be notified that despite the positive effect of taking pyridoxine in various disorders of the peripheral nervous system, it is necessary to take into account the possible risk of its toxic effect, which undoubtedly depends both on the dose and on the administration duration [65].

CONCLUSION

Thus, replenishing the B_6 vitamers deficiency in case of their identified deficiency is a necessary condition for the successful treatment of the central nervous system diseases, inflammatory processes, and diabetes; it is also necessary for correcting the immune status of patients. In most cases, the improvement in the condition will be due to the normalization of the balance of redox reactions involving PLP. It is advisable to consider a combined use of vitamin B_6 derivatives with various metabolites. At the same time, it should be taken into account that dephosphorylated forms of pyridoxine derivatives are necessary for the absorption, as well as the fact that a combined intake of metabolites will entail a shift in the cell biochemical processes, which can lead to the manifestation of negative effects on metabolism in conditions of its excess.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTION

OAZ – references collection, sources analysis, article writing; YRN – review idea and concept, references collection, sources analysis, article writing.

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ОБЗОРЫ

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FEATURES OF QUALITY CONTROL STRATEGY FOR DRUGS BASED ON VIABLE SKIN CELLS

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The aim of the study was to research the international experience in quality assurance of the products based on skin cells in order to identify the features of the quality control strategy in the development, production, as well as during an expert quality assessment as a part of the state registration procedure in the Russian Federation.

Materials and methods. The article provides an analysis of the materials presented in the assessment reports of the USA and Japanese regulatory authorities, as well as on the official websites of manufacturers, in review and scientific papers on the study of the structure and properties of tissue-engineered skin analogs.

Results. The manufacture of products containing human skin cells is associated with such risks as the possibility of contamination of the preparation with infective agents transmitted by materials of the animal origin, feeder cells, donor cells, or during the manufacturing process; a small amount of biopsy materials; a complexity of a three-dimensional product structure when combining cells with a scaffold; continuity of the manufacture process and a short product expiry date. The raw materials and reagents control, the creation of cell banks, using animal feeder cells only from qualified cell banks, an in-process control and release testing in accordance with the requirements of the finished product specification, make it possible to obtain a preparation with a reproducible quality. The specification should contain information about the identity, safety and potency of the product. For each preparation, the choice of approaches for assessing the quality is individual and depends on its composition and mode of action.

Conclusion. The features of the quality control strategy for the drugs based on human skin cells, consist in the implementation of control measures in order to obtain a proper quality of cellular (viability, sterility, identity, potency, et al) and non-cellular (physico-chemical scaffold properties) components or the whole graft (bioburden, barrier properties). The approaches and methods for determining the potency should be selected individually for each product and reflect the number, viability and identity of cells, a proliferative activity and secretable ability of the cellular component.

Keywords: skin substitute; keratinocytes; skin fibroblasts; organic and synthetic scaffolds; product quality control; quality attributes

Abbreviations: SS – skin substitute (complete full-thickness skin replacement); ES – epidermal substitute; DS – dermal substitute; MSC – mesenchymal stem cell; FDA – U.S. Food and Drug Administration; PMDA – Pharmaceuticals and Medical Devices Agency; MCB – Master Cell Bank; WCB – Working Cell Bank; PCR – Polymerase Chain Reaction; VEGF – Vascular Endothelial Growth Factor.

ОСОБЕННОСТИ СТРАТЕГИИ КОНТРОЛЯ КАЧЕСТВА ПРЕПАРАТОВ НА ОСНОВЕ ЖИЗНЕСПОСОБНЫХ КЛЕТОК КОЖИ

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Цель. Изучение международного опыта обеспечения качества препаратов на основе клеток кожи с целью выявления особенностей стратегии их контроля качества при разработке, производстве, а также при экспертной оценке качества в рамках процедуры государственной регистрации в РФ.
Материалы и методы. В статье приведен анализ материалов, представленных в экспертных отчетах регуляторных органов США и Японии, а также на официальных сайтах производителей, в обзорных и научных работах по исследованию структуры и свойств тканеинженерных аналогов кожи.
Результаты. Производство препаратов, содержащих клетки кожи человека, сопряжено с такими рисками, как возможность загрязнения продукта инфекционными агентами при использовании материалов животного происхождения, фидерных клеток, клеток донора или в процессе производства; небольшой объем биопсийного материала; сложность трехмерной структуры препаратов при комбинировании клеток с носителем; непрерывность процесса производства и небольшой срок хранения продукта. Контроль сырья и материалов, создание банков клеток, использование фидерных клеток животных только из аттестованных банков, внутрипроизводственный контроль и тестирование препарата при выпуске в соответствии с требованиями спецификации на готовый продукт позволяют получить продукт с воспроизводимым качеством. Спецификация должна содержать сведения о подлинности, безопасности и активности продукта. Для каждого препарата выбор подходов для оценки качества индивидуален и зависит от его состава и механизма действия.
Заключение. Особенности стратегии контроля качества препаратов на основе клеток кожи человека заключаются в проведении контрольных мероприятий с целью получения надлежащего качества клеточного (жизнеспособность, стерильность, подлинность, активность и другие) и неклеточного (физико-химических свойств носителя) компонентов или целого графта (бионагрузка, барьерные свойства). Подходы и методы для определения активности должны выбираться индивидуально для каждого продукта и отражать число, жизнеспособность и подлинность клеток, пролиферативную и секреторную способность клеточного компонента.
Ключевые слова: эквиваленты кожи; кератиноциты; фибробласты кожи; органические и синтетические носители; контроль качества препарата; показатели качества
Список сокращений: ЭК – эквивалент кожи (полнослойный); ЭЭ – эпидермальный эквивалент; ДЭ – дермальный эквивалент; МСК – мезенхимальные стволовые клетки; FDA – Управление по санитарному надзору за качеством пищевых продуктов и медикаментов (США); PMDA – Агентство по фармацевтической продукции и медицинским приборам (Япония); МБК – мастер банк клеток; РБК – рабочий банк клеток; ПЦР – полимеразная цепная реакция; VEGF – фактор роста эндотелия сосудов.

INTRODUCTION

Skin substitutes (SSs) are tissue-engineered analogs of the skin, which are three-dimensional constructs based on *in vitro* cultured skin cells and various synthetic or organic carriers (scaffolds, matrices, matrixes) used in medicine for temporary or permanent replacement of damaged epidermal, dermal or full-layer skin areas [1-3]. For the same purpose, it is possible to use cultured skin cells, for example, fibroblasts or keratinocytes, without creating a three-dimensional structure on their basis using a carrier in the form of a cell suspension [4].

The use of SSs and skin cells without a carrier is aimed at restoring the structure and functions of the skin, primarily the barrier one (protecting the body from infection by pathogens from the environment and preventing the loss of water and mineral salts by the body through the wound surface). It is also important to note the acceleration of healing processes and the reduction of pain in burns, acute and chronic wounds, scars, diabetic ulcers, nevi, skin structure disorders as a result of genetic and other diseases [5–9].

A key step in the development and production of SSs is the isolation of cells certain types from a donor skin, followed by the cultivation of these cells *in vitro* in order to obtain the amount of cellular material necessary for a therapeutic effect. All the SSs currently approved for a clinical use in the world, contain only two types of cells (either individually or in tandem): keratinocytes and fibroblasts. The use of keratinocytes able of forming a layer of outer cornified epithelium underlies the creation of epidermal grafts (epidermal tissue-engineeed equivalent, epidermal substitute – ES), and the use of fibroblasts in combination with organic or synthetic carriers makes it possible to create an analogue of the dermis – a dermal graft (dermal tissue engineered equivalent, dermal substitute – DS), which increases the probability of the subsequent successful engraftment from 15 to 45–75% [10]. There are products given a permission for a clinical use in different countries of the world. They combine both ESs and DSs, and are composite two-layer SSs (full-layer SSs) [11]. Full-thickness skin equivalents are an alternative to skin grafts obtained both from healthy skin areas of the patients themselves (autografts) and from healthy donors (allografts).

The developments were carried out to improve the functionality of tissue-engineered structures and achieve greater similarity with healthy human skin through the use of other cell types: endothelial cells, Langerhans cells, melanocytes [12–14]. The inaccessibility and complexity of cultivating keratinocytes and fibroblasts led to attempts to use mesenchymal stem cells (MSCs) of various origins. They have a high proliferative potential and the ability to differentiate, either alone or in tandem with a carrier [15]. However, all these studies are under development or clinical trials: currently, there are no registered SSs in the world based on the use of these cell types.

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Among the preparations containing the skin cells that have been approved for a clinical use, there are products based on the cells of both autologous (more often ESs) and allogeneic origin (more often DSs and full-thickness SSs) [16]. In this case, the product can be a suspension of cells and be applied to the wound surface by spraying (in the form of a spray), or in the form of a tissue-engineered graft to cover the wound as a result of an application [4].

In a number of preparations, intradermal injections of cells using a needle and syringe are used. To create a graft, SSs cells are placed on a carrier (matrixes, scaffolds, matrices). This is most often used as collagen of the animal origin (bovine, porcine, murine) and synthetic carriers (silicone, hyaluronic acid, and others). This makes it possible to obtain a multilayer structure with a well-defined barrier function, a biomechanical stability, stratification of keratinocytes, formation of intercellular interactions, synthesis of the basement membrane and important components of the extracellular matrix [17, 18].

All SSs can be used as temporary bioactive barrier dressings, however a number of products, mostly containing biodegradable carriers, can be used as a permanent replacement for a damaged skin area.

During the production and implementation of the registration procedure for SSs, the question of an adequate and comprehensive assessment of their quality arises. This is complicated by a composite of these preparations, which include both a component of viable cells and, often, a non-cellular component – a carrier.

THE AIM of the study was to research the international experience in quality assurance of the products based on skin cells in order to identify the features of the quality control strategy in the development, production, as well as during an expert quality assessment as a part of the state registration procedure in the Russian Federation.

MATERIALS AND METHODS

To analyze the characteristics of quality control strategies for the products based on viable skin cells, the basis of the study was the materials presented in assessment reports of the regulatory authorities of the United States (Food and Drug Administration – FDA) and Japan (Pharmaceuticals and Medical Devices Agency – PMDA) for the following products that had received approval for a clinical use by Fda.gov and Pmda.go.jp. They were: Apligraf and GINTUIT (Organogenesis, Inc., USA)[1]; LAVIV (Fibrocell Technologies, Inc., USA)[2]; STRATAGRAFT (Sratatech Corporation, USA)[3]; Epicel (Genzyme Biosurgery, USA)[4]; Invitrx (Ortec International, Inc., USA)[5]; JACE (Japan Tissue Engineering Co., Ltd., Japan)[6].

Information on the other products used in the countries of the European Union (EU), the Republic of Korea, the Russian Federation (RF) and other countries of the world, was obtained from the official websites of manufacturers, as well as from review and scientific papers on the study of the structure and properties of tissue-engineered skin substitutes.

The following electronic resources were used to conduct the study: PubMed, Scopus, Google Scholar, eLibrary, Ema.europa.eu., Fda.gov, Pmda.go.jp. The queries were conducted on combinations of the following keywords: “skin substitutes”, “scaffolds for skin repair and regeneration”, “skin tissue engineering”, “skin cells products quality control”, “skin cells products quality attributes”, as well as on the trade names of the products, approved for a medical use. The search was carried out for the period from Oct 2021 to Ap 2022.

The logical methods of the system analysis and modeling were used in the work.

RESULTS AND DISCUSSION

Currently (dated Apr 2022), more than two dozen products containing viable human skin cells have been approved for clinical use in the EU, USA, Australia, Japan, the Republic of Korea and the Russian Federation (for some products, the license term expired has expired and has not been renewed).

Table 1 presents preparations based on human skin cells used for skin resurfacing (ESs, DSs, full-thickness SSs and the preparations based on skin cells without a carrier) that have received a clinical approval in different countries of the world. Information on them is presented on the manufacturers’ official websites and in assessment reports of regulatory authorities (see “Materials and methods”).

[1] Food and Drug Administration (FDA). Gintuit – Summary Basis for Regulatory Action, 2012. Available from: http://wayback.archive-it.org/7993/20170723023240/https://www.fda.gov/downloads/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/UCM297753.pdf

[2] Food and Drug Administration (FDA). LAVIV – Summary Basis for Regulatory Action, 2011. Available from: https://wayback.archive-it.org/7993/20170723023939/https://www.fda.gov/downloads/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/UCM262780.pdf

[3] Food and Drug Administration (FDA). SRTATAGRAFT – Package Insert, 2021. Available from: https://www.fda.gov/media/150129/download

[4] Food and Drug Administration (FDA). Epicel – Summary of safety and probable benefit, 1998. Available from: https://www.fda.gov/media/103308/download

[5] Food and Drug Administration (FDA). Invitrx – Summary of safety and probable benefit, 1998. Available from: https://www.accessdata.fda.gov/cdrh_docs/pdf/H990013B.pdf

[6] Pharmaceuticals and Medical Devices Agency (PMDA). JACE – Review Report, 2007. Available from: https://www.pmda.go.jp/files/000223079.pdf

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Table 1 – Examples of products based on human skin cells that have received approval for clinical use in the world

Product name	Manufacturer and country of registration	Cellular component	Carrier type	Origin of cells	Intended diseases for treatment
EPIDERMAL SUBSTITUTES AND PREPARATIONS CONTAINING KERATINOCITES					
Epicel (Cultured epidermal autografts)	Genzyme Biosurgery, USA	Keratinocytes layer (2–8 cells thick) cultured in the presence of non-dividing mouse fibroblasts (feeder layer)	–	Autologous	Deep burns (more than 30% of the body surface)
JACE (Human autologous epidermal cell sheet)	Japan Tissue Engineering Co., Ltd. (J-TEC), Japan	Keratinocytes layer (several cells thick) cultured in the presence of irradiated 3T3-J2 cells derived from a mouse embryo (feeder layer	–	Autologous	Extensive (more than 30% of the body surface) burns, the 2nd-3rd degree
KeraHeal	Biosolution Co., Ltd., Republic of Korea	Suspension of cultured keratinocytes	–	Autologous	Deep burns, the 2nd degree (more than 30% of the body surface), and the 3rd degree (more than 10% of the body surface)
KeraHeal-Allo	Biosolution Co., Ltd., Republic of Korea	Suspension of keratinocytes	Thermosensitive hydrogel	Allogeneic	Deep burns, the 2nd degree
Holoderm	Tego Science, Inc., Republic of Korea	Cultured keratinocyte progenitors with epidermal flap formation	–	Autologous	Burns, the 3rd degree (more than 50% of the body surface)
Kaloderm	Tego Science, Inc., Republic of Korea	Cultured keratinocytes with epidermal flap formation	–	Allogeneic	Deep burns, the 2nd degree; Diabetic foot ulcers
EpiDex	Modex Therapeutiques, Switzerland	Cultured keratinocytes	Not available	Autologous	Not available
EPIBASE	Laboratoires Genevrier, France	Keratinocytes cultured to a confluent monolayer	–	Autologous	Not available
MySkin	CellTran Ltd., United Kingdom	Keratinocytes cultured to a subconfluent monolayer	Surface Coated Silicone Matrix	Autologous	Neuropathic, decubitus, diabetic foot ulcers; Burns
Laserskin (Vivoderm)	Fidia Advanced Biopolymers, Italy	Keratinocytes cultured to a confluent monolayer	Microperforated hyaluronic acid membrane	Autologous	Diabetic and venous ulcers of the lower extremities; Shallow burns; Vitiligo
Bioseed-S	BioTissue Technologies GmbH, Germany	Keratinocytes cultured to a subconfluent monolayer	Matrix on fibrin sealant	Autologous	Treatment-resistant chronic venous ulcers of the lower extremities
CellSpray	Clinical Cell Culture (C3), Australia	Keratinocytes uncultivated / cultured to a subconfluent monolayer	–	Autologous	Not available
Multi-layered sheet of keratinocytes	Institute of Cytology RAS, Russia	Cultured keratinocytes	Not available	Not available	Burns of varying severity, including critical and supercritical; Ulcers of different etiology; Wounds resulting from trauma; Fistulas, bedsores
DERMAL SUBSTITUTES AND PREPARATIONS CONTAINING FIBROBLASTS					
LAVIV (Azficel-T)	Fibrocell Technologies, Inc., USA	Cultured fibroblasts	–	Autologous	Medium-deep and deep wrinkles of the nasolabial folds

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Product name	Manufacturer and country of registration	Cellular component	Carrier type	Origin of cells	Intended diseases for treatment
TransCyte (DermagraftTC)	Advanced BioHealing, Inc., USA	Cultured neonatal fibroblasts	Silicone film backing, porcine collagen nylon mesh	Allogeneic	Superficial and deep burns; Chronic ulcers of the lower extremities, including diabetic and venous ones; bedsores
Dermagraft	Shire Regenerative Medicine, Inc., USA	Cultured neonatal fibroblasts	Bioresorbable collagen on polyglactin or polyglactin-910 sponge	Allogeneic	Chronic diabetic ulcers of the lower extremities, affecting the dermis, but not reaching the tendons, muscles and bones; Chronic and infected wounds
CureSkin Inj.	S.Biomedics Co., Ltd., Republic of Korea	Cultured fibroblasts	–	Autologous	Post-acne depressive scars
Rosmir	Tego Science, Inc., Republic of Korea	Cultured fibroblasts	–	Autologous	Reduction of the nasolacrimal trough
Hyalograft 3D	Fidia Advanced Biopolymers, Italy	Cultured fibroblasts	Microperforated hyaluronic acid membrane	Autologous	Diabetic and venous ulcers of the lower extremities; Shallow burns; Vitiligo
Cell technology SPRG therapy	Human Stem Cell Institute	Cultured fibroblasts	–	Autologous or allogeneic	Correction of age-related skin changes; Recession and mucosal deficiency in the area of teeth and dental implants
Dermal equivalent	Institute of Cytology RAS, Russia	Cultured fibroblasts	Collagen gel	Allogeneic	Burns of varying severity, including critical and supercritical; Ulcers of different etiology; Wounds resulting from trauma; Fistulas, bedsores
			FULL-THICKNESS HUMAN SKIN EQUIVALENT		
Apligraf	Organogenesis, Inc., USA	Layers of cultured keratinocytes and fibroblasts	Matrix of bovine collagen and extracellular matrix proteins	Allogeneic	Soft tissue wounds of the oral cavity
GINTUIT (Allogeneic Cultured Keratinocytes and Fibroblasts in Bovine Collagen)	Organogenesis, Inc., USA	Layers of cultured keratinocytes and fibroblasts	Type I bovine collagen	Allogeneic	Defects and lesions of the mucous membrane of the gums
STRATAGRAFT (Allogeneic Cultured Keratinocytes and Dermal Fibroblasts in Murine Collagen)	Sratatech Corporation, USA	Stratified epithelial layer containing differentiated keratinocytes deposited on a dermis-like structure formed by fibroblasts	Type I mouse collagen	Allogeneic	Thermal burns with intact dermal elements (deep burns, the 3rd degree.)
Invitrx (Composite Cultured Skin)	Ortec International, Inc., USA	Layers of cultured keratinocytes and fibroblasts	Type I bovine collagen sponge with a thin gel-like layer of bovine collagen	Allogeneic	Dystrophic epidermolysis bullosa (after arm reconstruction surgery)
OrCel	Ortec International, Inc., USA	Layers of cultured keratinocytes and fibroblasts	Type I bovine bollagen sponge	Allogeneic	Dystrophic epidermolysis bullosa (after hand reconstruction surgery); Burns and wounds
PolyActive	HC Implants BV, Netherlands	Cultured keratinocytes and fibroblasts	Synthetic matrix from terephthalate derivatives	Autologous	Shallow wounds

Note: Laserskin and Hyalograft 3D preparations can be used together. Such a complex preparation has the trade name TissueTech Autograft System; SPRG – Service for Personal Regeneration of Gum.

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Table 2 – Some qualification of keratinocytes and fibroblasts production cell banks

Indicator	Approach to testing	Methods of analysis
Virus safety	In vitro and in vivo testing for viruses (including retroviruses) specific to humans, pigs, cattle	Transmission electron microscopy; Reverse transcription
Sterility, mycoplasma contamination	Detection of all types of microbiological contaminants: bacteria and fungi	Microbiological methods; PCR
Tumorigenicity	In vitro studies of cell genome stability; In vivo studies of tumor formation	Karyotyping; Isoenzyme analysis; Cell culture aging tests; Tumors formation tests
Identity	Expression of involucrin by keratinocytes; Biosynthesis of collagen by fibroblasts	Isoenzyme analysis; Immunochemical methods; PCR
Activity	Viability; Cell culture growth parameters	Cell counting with an automatic counter or with a hemocytometer; Flow cytometry; Morphological analysis
Comparability (with previously characterized cells)	In vitro tests, including confirmation of cellular purity, degree and intensity of percutaneous absorption, cytokine profile analysis and VEGF quantitation; In vivo studies on immunodeficient animals of the involucrin expression level, engraftment, integration, morphology and deformation of the graft, reduction of the wound surface through a wound contraction	Histology; Immunochemical methods; MTT test

Note: PCR – polymerase chain reaction; VEGF – Vascular Endothelial Growth Factor; MTT – Mitochondrial Tetrazolium Test (colorimetric method using tetrazolium salt).

It should be notified that not all skin cell products had been originally registered as drugs. A number of products, such as Epicel (Genzyme Biosurgery) and Invetrx (Ortec International), received the FDA approval for a clinical use as medical devices (Humanitarion Device Examption, HDE), but then were reclassified as biological tissue based products -"Tissue & Tissue Products"- regulated by the FDA's Center for Biologics Evaluation and Research (CBER). In the Russian Federation, there is currently no experience of products state registration containing viable human skin cells as biomedical cell products within the framework of the Federal Law dated Jun 23, 2016, No. 180-FZ "On Biomedical Cell Products" currently in force in the Russian Federation, or as advanced therapy medicinal products within the framework of the Decision of the Eurasian Economic Commission Council dated Nov 3, 2016, No. 78 "On the Rules of marketing authorization and assessment of medicinal products for human use". Previously, three tissue-engineered products (SPRG cell technology from the Institute of Human Stem Cells, a dermal substitute and multilayer keratinocyte layer from the Institute of Cytology of the Russian Academy of Sciences) [6] received a permission from the Federal Service for Surveillance in Healthcare (Roszdravnadzor) for clinical use in medical practice as cell technologies for a personalized skin treatment[7]. Now, the validity of permits for the use of cellular technologies and registration certificates for medical devices have expired, or their commercial use has been suspended due to the changed legislative framework of the Russian Federation in the field of development and registration of the products based on viable human cells.

Currently, only expert reports on the products registered by the FDA and PMDA often containing incomplete information regarding the conduct of a quality assessment during an in-process control and at the release control of the product are available in the public domain. Based on the available data, the following features of the quality control strategy for SSs and the products based on viable skin cells as well as the main problems associated with their production, can be identified.

Input control of raw materials and reagents

The use of raw materials of the human and animal origin (cell culture serum, bovine pituitary extract, collagen for matrix) is associated with such a safety problem for the product use as the risk of microbiological and viral contamination, and in case of the materials obtained from cattle, there is an additional risk transmission of transmissible spongiform encephalopathy. In order to reduce these risks, materials and reagents of the animal origin are tested for sterility (the presence of bacteria, fungi, mycoplasma), the presence of the viral contamination

[7] Federal Service for Surveillance in Healthcare of Russian Federation. List of medical technologies approved for use in medical practice as of December 30, 2011. Available from: https://roszdravnadzor.gov.ru/documents/12545.

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and bacterial endotoxins. All the materials from cattle should be obtained from the countries where cases of transmissible spongiform encephalopathy have not been reported [22].

In case of the most preparations production, cell donors are tested for the presence of pathogens of infectious diseases. However, for the autologous product Epicel (Genzyme Biosurgery), donors of cellular materials are not subjected to such testing, which leads to the risk of a possible infection of the personnel working with the biomaterial and product. This requires special precautions in the production[8].

In addition, the materials used to create cell carriers and form a three-dimensional structure of the product must be tested for physical and chemical properties.

Creation of cell banks

Another feature of the products production based on human cells intended for an allogeneic use is the need to create cell banks.

For the production of ESs, DSs and full-thickness SSs for an allogeneic use, human keratinocytes and fibroblasts are used. They are obtained from the biopsy materials of healthy donors, cultured in order to develop the cellular material necessary for the therapeutic effect. Obtaining a required number of cells, which can be used for one or even several patients from a small donor area of the skin, would be difficult without a creation of the production cell banks: a master cell bank (MCB) and a working cell bank (WCB). As a rule, a number of passages between MCB and WCB is small9. The cell banks should be cvalificated and the banked cell lines should be characterized by quality attributes such as sterility, absence of mycoplasma contamination and introduced viral agents, a cytogenetic stability, tumorigenicity, purity, potency and identity, a proliferative activity and viability. The features of testing cell lines for some quality attributes, given in the assessment reports of the regulatory authorities that have issued the permission for the clinical products use (section “Materials and methods”), are shown in Table 2.

Risk of using animal cell feeder layer

For the cultivation of human skin cells in the production of a number of preparations, for example, Epicel (Genzyme Biosurgery), STRATAGRAFT (Stratatech Corporation) and JACE (Japan Tissue Engineering Co.), a mouse cell feeder layer, which may be present in a residual amount in the finished product, is used. The FDA classifies these products as xenografts, and despite the possibility of using feeder cells only from qualified cell banks and recognizing a low risk of transmitting infectious agents through these cells, it recommends product recipients to refuse to donate blood, plasma, tissues, eggs, breast milk and other biomaterials.

In-process control

The main characteristic of the production of preparations containing viable human cells is the implementation of all processes under aseptic conditions. The impossibility of sterilizing the resulting finished product leads to the need to control the product for sterility and the absence of mycoplasma as an in-process control.

The structural features of the preparations[10,11] based on skin cells, which are multilayer structures on a carrier, lead to the need to track the morphology of the product using a visual assessment and histological examination, an investigation of physical properties after the graft washing stage, a proliferative activity of keratinocytes, and viable cells counting [23, 24]. Reporting of cell morphology in the form of photographs, control of stratification in the cell culture, and characterization of other cell types (other than fibroblasts) present in the cell culture, was also requested by the FDA advisory committee for Laviv containing skin fibroblasts without a carrier.

The results of testing for the above-listed quality indicators, along with some others, such as “bioburden”, may be included in the specification for the finished product, but obtained during the production process. This is due to the short shelf life of the finished product (without cryo-freezing) [25], which does not allow long-term tests during the drug release. Therefore, the shelf life of Apligraf and Gintuit (Organogenesis, Inc.) after thawing is 15 days[13], and the shelf life of STRATAGRAFT (Stratatech Corporation) is 4 h.

Release testing of finished products

All the products currently registered in the world and containing viable human skin cells are obtained as a result of a continuous production process, and therefore, have only one final specification for the finished product. Depending on the structure of the product, the presence of a multilayer structure, or carrier, the specifications may differ in the set of quality attributes by which the product is release tested. Such testing may be performed partly before cryopreservation the finished product and

[8] Food and Drug Administration (FDA). Epicel – Summary of safety and probable benefit, 1998.
[9] Food and Drug Administration (FDA). Gintuit – Summary Basis for Regulatory Action, 2012.
[10] Ibid.
[11] Pharmaceuticals and Medical Devices Agency (PMDA). JACE – Review Report, 2007.

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partly after packaging on thawed samples, as outlined in the assessment report for LAVIV (Fibrocell Technologies, Inc.).

In general, the specification for a finished product containing viable human skin cells includes the following quality attributes:

– Visual assessment of the product appearance (description);
– Viability and total number of cells;
– Sterility;
– Mycoplasma;
– Bacterial endotoxins;
– Identity (for fibroblasts and keratinocytes);
– Purity;
– Activity (efficiency);
– Container closure integrity.

The quality attribute "Description" should reflect such parameters as color, transparency of the product, the appearance of ESs or Dss (if applicable): the presence of irregularities on the surface of the structure, wrinkling, deformation, changes in the thickness of the layers[12].

The assessment of sterility is mainly carried out using a standard microbiological test, followed by Gram staining of microbiological preparations[13,14]. The test for mycoplasma at the release control of JACE (Japan Tissue Engineering Co., Ltd.) was carried out by a cytochemical method by staining DNA with a fluorescent dye (using indicator cell lines). It is assumed that the results of testing for sterility and mycoplasma can be obtained after the clinical use of the product, due to the excess of the duration of the test over the expiration dates of some preparations[15]. The possibility of the product clinical use without results of the sterility assessment and mycoplasma contamination leads to the requirement to provide information for the doctor with a treatment plan for the patient in case of infection with product-contaminating agents on the package insert of the finished product.

The assessment report on the JACE preparation (Japan Tissue Engineering Co., Ltd.) indicates the need for the content control of the residual amount of bovine serum albumin, determine the residual number of feeder cells and the physical properties of the flap (possibly at the stage of in process control). Such tests can be attributed to the quality attribute "purity" and prove the absence of impurities in the finished product resulting from the technological process.

One of the most difficult and controversial points in the selection of adequate methods of analysis in assessing the quality of products used to heal wounds and skin lesions is the confirmation of their potency. Most often, the mechanism of products action is not demonstrated (not established) or is not given in the regulatory documentation for the product. A number of assessment reports on products indicate the possibility of wound healing due to the activation of a recipient cell division during the secretion of biologically active substances by the cells of the product (platelet, fibroblast, vascular endothelial, epidermal growth factors, cytokines, type IV collagen, tenascin, fibronectin, and others). A similar mechanism of action has been notified for Apligraf, GINTUIT, STRATAGRAFT, KeraHeal-Allo, OrCel, TransCyte and Dermagraft. The ability of fibroblasts to accelerate the mechanisms of tissue regeneration due to the secretion of biologically active substances by them is considered proven [26, 27]. At the same time, such products are temporarily on the wound surface, with a gradual elimination of cells and a resorption of non-cellular material (if applicable). For another group of products (KeraHeal, TissueTech Autograft System, CellSpray), a decrease in the wound surface was shown due to the proliferation and differentiation of the cells of the product itself with the formation of the skin structures necessary to perform a barrier function. These preparations are used as permanent applications to close the wound surface due to the possible proliferation, migration and differentiation of cells with the formation of a stratified epithelium [28].

The choice of methods and approaches for assessing the potency (which is a measure of the effectiveness of a product in clinical use) of each preparations containing human skin cells occurs on an individual basis and is agreed by the product manufacturers with the regulatory authorities of the countries where their clinical use will be carried out. For example, the effectiveness of LAVIV (Fibrocell Technologies, Inc.) was concluded based on the determination of the total cells number in the preparation, the confirmation of the fibroblasts identity, and the analysis of the collagen secreted by the cells. To evaluate the effectiveness of GINTUIT (Organogenesis, Inc.), a histological examination of the graft was performed. However, the advisory committee accepted this approach only as the evidence of the structural product integrity, and suggested that an additional analysis of cell-secreted cytokines be carried out.

Besides, if necessary, additional quality attributes can be added to the specification for the finished product: pH, bioburden[16], barrier properties of grafts, which

[12] Food and Drug Administration (FDA). SRTATAGRAFT – Package Insert, 2021.
[13] Food and Drug Administration (FDA). Epicel – Summary of safety and probable benefit, 1998.
[14] Food and Drug Administration (FDA). LAVIV – Summary Basis for Regulatory Action, 2011.
[15] Food and Drug Administration (FDA). Gintuit – Summary Basis for Regulatory Action, 2012.
[16] Ibid.

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confirm the formation of the outer cornified epithelium and are examined on the basis of a histological analysis[17]. The barrier function confirmation and studies of graft permeability are carried out using different approaches, for example, by determining the thickness of the epidermis and studying the lipid profile [29]. A formation of the correct basement membrane is possible by confirming the presence of type IV collagen at the dermo-epidermal junction.

CONCLUSION

Due to the continuity of the technological production process, a short shelf life and the impossibility of carrying out sterilization procedures for the finished product containing viable human skin cells, as well as the complexity of combined preparations (applicable to grafts), which consists in the presence of a non-cellular component-carriers (matrixes, scaffolds, matrices) in the finished product) which is often difficult to separate from cells, it is of great importance to comply with a number of requirements for a technological process and take into account the peculiarities of the quality control strategy for such drugs. These requirements are as follows: creation of a qualification program for raw materials and reagents, which would include the risks of their use; a control of materials of the animal origin; creation of skin cell banks used for the production of allogeneic products; the use of feeder cells only from qualified banks; conducting a number of studies on quality attributes during in process control with the inclusion of the results of these studies in the specification for the finished product. In addition to standard tests (description, viability, total number of cells, sterility, mycoplasma, bacterial endotoxins, identity, purity, potency) characteristic of a quality control of any preparations containing viable human cells, a quality control of preparations containing skin cells includes additional studies of physical and chemical properties of the carrier and/or the whole graft (bioburden, barrier properties of the graft).

Due to the difficulty of demonstrating the potency of products used for wound healing, and the incomplete knowledge of the nature of their action, approaches and methods for determining this quality attribute should be selected individually for each product and reflect such properties of the cellular component as the number, viability and identity of cells, as well as their proliferative and/or secretory capacity.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Olga A. Rachinskaya – information and analytical search on the investigation topic, data processing, article writing;
Ekaterina V. Melnikova – aim and objectives setting, text correction;
Vadim A. Merkulov – concept planning, consulting for legal acts regulating drugs circulation.

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[17] Food and Drug Administration (FDA). SRTATAGRAFT – Package Insert, 2021.

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DEVELOPMENT OF MURINE STEM CELLS WITH CONDITIONAL KNOCKOUT OF HUMANIZED *SNCA* GENE

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α-synuclein is one of the key molecular links in the pathogenesis of Parkinson's disease. The accumulated data indicate that pathogenic mutations in the *Snca* gene are associated with the development of neurodegenerative brain damage, indicating the relevance of studying the synuclein neurobiological role.

The aim of the study was to create a genetically modified clone of mouse stem cells with a conditional knockout of humanized α-synuclein, which can be used for the reinjection into mouse blastocysts, as well as for basic and applied *in vitro* research in the field of pathophysiology and neuropharmacology.

Materials and methods. To create mouse stem cells with a conditional knockout of the humanized *Snca* gene, a previously obtained clone with the first *Snca* exon flanked by LoxP sites, was used. The CRISPR/Cas9-mediated homologous recombination system with donor DNA oligonucleotides of the human sites of the corresponding gene sites was used to humanize the fourth and fifth exons. Cas9 nuclease, single guide RNA, and donor DNA were transfected into mouse cells.

Results. An approach to obtaining clones of mouse genetically modified stem cells expressing pathological humanized α-synuclein, has been proposed and implemented. The resulting clones were plated on Petri dishes for propagation and a further genetic analysis. Clone 126-2F4 was found out carrying the necessary genetic modifications. The results obtained are fundamentally important not only for understanding the development of the pathological process in α-synucleinopathies, but which is more important, for the development of new therapeutic approaches that will stop the extension of the human α-synuclein aggregation pathology throughout the nervous system, and the validation of these approaches in preclinical trials.

Conclusion. As a result of the study, a strategy for CRISPR/Cas9-assisted homologous recombination in the genome of mouse embryonic stem cells has been developed to create a fully humanized *Snca* gene encoding α-synuclein, and the clone genome of mouse embryonic stem cells has been edited using a CRISPR technology. The RNA and DNA oligonucleotides necessary for the creation of RNP complexes that carry out a directed homologous recombination in the *Snca* locus of the mouse genome have been synthesized. The developed cell clone can serve to create a line of genetically modified mice that serve as a test system for pathophysiological and neuropharmacological studies associated with synucleinopathies. Herewith, before the induction of the Cre-dependent recombination, this line is a representative model for studying a biological role of mutant *Snca*. At the same time, after a Cre-dependent knockout activation, it is possible to imitate the pharmacological inhibition of α-synuclein, which is of particular interest for applied research in neuropharmacology.

Keywords: α-synuclein; neurodegeneration; Parkinson's disease; conditional knockout; CRISPR/Cas9

Abbreviations: NDs – neurodegenerative diseases; sgRNA – single-guide RNA; NAC – non-amyloid-β component; RNP – ribonucleoprotein; PCR – polymerase chain reaction; PD – Parkinson's disease.

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СОЗДАНИЕ КЛОНА ЭМБРИОНАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК МЫШЕЙ С КОНДИЦИОННЫМ НОКАУТОМ ГУМАНИЗИРОВАННОГО ГЕНА *SNCA*

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Одним из ключевых молекулярных звеньев в патогенезе болезни Паркинсона является белок α-синуклеин. Накопленные данные свидетельствуют о том, что патогенные мутации в гене *Snca* ассоциированы с развитием нейродегенеративного повреждения головного мозга, тем самым указывая на актуальность изучения нейробиологической роли α-синуклеина.

Цель. Создание генетически-модифицированного клона стволовых клеток мышей с кондиционным нокаутом гуманизированного α-синуклеина, который может быть использован для реинъекции в мышиные бластоцисты, а также для фундаментальных и прикладных *in vitro* исследований в области патофизиологии и нейрофармакологии.

Материалы и методы. Для создания мышиных стволовых клеток с кондиционным нокаутом гуманизированного гена *Snca* был использован прежде полученный клон с фланкированным LoxP-сайтами первым экзоном *Snca*. Для гуманизации IV и V экзона была использована система CRISPR/Cas9-опосредованной гомологичной рекомбинации с донорными ДНК олигонуклеотидами человеческих сайтов соответствующих генов. Нуклеаза Cas9, гидовые РНК и донорная ДНК были трансфецированы в клетки мыши.

Результаты. Нами был предложен и реализован подход к получению клонов мышиных генетически-модифицированных стволовых клеток, экспрессирующих патологический гуманизированный α-синуклеин. Полученные клоны были высеяны на чашки Петри для размножения и дальнейшего генетического анализа. Был обнаружен клон 126-2F4, несущий необходимые генетические модификации. Результаты проведенного исследования принципиально важны не только для понимания развития патологического процесса при α-синуклеинопатиях, но и, что ещё важнее, для разработки новых терапевтических подходов, которые позволят остановить распространение агрегационной патологии человеческого α-синуклеина по нервной системе и валидации этих подходов в доклинических испытаниях.

Заключение. В результате проведенного исследования разработана стратегия CRISPR/Cas9-ассистированной гомологической рекомбинации в геноме эмбриональных стволовых клеток мыши для создания полностью гуманизированного гена *Snca*, кодирующего α-синуклеин. Выполнено редактирование генома клона эмбриональных стволовых клеток мыши с использованием CRISPR технологии. Синтезированы РНК и ДНК олигонуклеотиды, необходимые для создания рибонуклеопротеиновых комплексов, осуществляющих направленную гомологическую рекомбинацию в *Snca* локусе генома мыши. Разработанный клон клеток может служить для создания линии генетически-модифицированных мышей, служащих тест-системой для патофизиологических и нейрофармакологических исследований, связанных с синуклеинопатиями. При этом до индукции Cre-зависимой рекомбинации данная линия является репрезентативной моделью для исследования биологической роли мутантного *Snca*. В то же время, после Cre-зависимая активация нокаута позволяет имитировать фармакологическое ингибирование α-синуклеина, что представляет особый интерес для прикладных исследований в нейрофармакологии.

Ключевые слова: α-синуклеин; нейродегенерация; болезнь Паркинсона; кондиционный нокаут; CRISPR/Cas9

Список сокращений: НЗ – нейродегенеративные заболевания; огРНК – одиночная гидовая рибонуклеиновая кислота; РНП – рибонуклеопротеины; ПЦР – полимеразная цепная реакция; NAC – неамилоидный компонент; БП – болезнь Паркинсона.

INTRODUCTION

A heterogeneous group of pathologies, united by the concept of neurodegenerative diseases (NDs), continues to acquire an increasing medical and social significance. In view of the increase in life expectancy, the burden of NDs, classically associated with an old age, is becoming one of the most relevant biomedical problems [1]. Herewith, despite high rates of the neurobiology development, many aspects of NDs pathogenesis remain disclosed only fragmentarily. One of these aspects is the role of α-synuclein in the main processes associated with the degenerative death of neurons.

As the main component of protein aggregates, α-synuclein has been found out in a number of NDs, which are combined into a group of synucleinopathy, including Parkinson's disease, dementia with Lewy

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bodies, a Rapid Eye Movement sleep behavior disorder, and a pure autonomic failure [2].

α-synuclein is a product of the *Snca* gene located on chromosome 4 at position q22.1 [3], and is a small protein (140 amino acids) expressed mainly in neurons, as well as in some tumor cells [4]. Its structure is represented by three main domains. They are: an N-terminal domain (1–60) containing a conserved motif of several repeating amino acid sequences (a consensus sequence of XKTKEGVXXXX); the central domain (61–95), known as the non-amyloid-β component (NAC), which is highly hydrophobic and is involved in the aggregation of α-synuclein during the formation of the β-sheet structure; C-terminal domain (96–140) enriched in negatively charged residues and a proline which provides polypeptide flexibility [5].

Although an α-synuclein physiological function remains understudied, its localization in presynaptic terminals [6], the association with the synaptic vesicle reserve pool [7], and observed deficiencies in the synaptic transmission in response to the gene knockdown or overexpression, suggest that α-synuclein participates in the regulation of the neurotransmitters release, as well as in neuroplasticity [8].

A possible role of α-synuclein in the regulation of synaptic homeostasis is associated not only with its direct interaction with synaptic vesicles: it interacts with synaptic proteins that control vesicle exocytosis, such as phospholipase D and the Rab family of small guanosine triphosphatase [9]. The accumulated evidence suggests that α-synuclein can act as a chaperone, control the degradation, and influence the assembly, maintenance, and distribution of the presynaptic SNARE protein complex, which is involved in the release of neurotransmitters, including dopamine [10]. Taken together, these observations indicate that α-synuclein plays an important role in the movement and exocytosis of vesicles [8].

In this paper, a procedure for creating a clone of mouse embryonic stem cells with CRISPR/Cas9-mediated humanization of the *Snca* gene with the first exon flanked by LoxP sites, have been described.

THE AIM of the study was to create a genetically modified clone of mouse stem cells with a conditional knockout of humanized α-synuclein, which can be used for the reinjection into mouse blastocysts, as well as for basic and applied *in vitro* research in the field of pathophysiology and neuropharmacology.

MATERIALS AND METHODS

Ethics review of study

The experiments were carried out at the Research Institute of Pharmacology of Living Systems (Belgorod State National Research University) in compliance with the ethical standards regulated by the ARRIVE management. The experimental studies were approved by the Bioethical Commission of Belgorod State National Research University (protocol No. 08/21 dated February 8, 2021).

Obtaining cell clone with flanked first exon of *Snca* gene

A mouse clone of embryonic stem cells carrying identically oriented LoxP sites flanking the first coding exon of the *Snca* gene (Clone 126) had been obtained in the previous laboratory studies and was used to generate mice with a conditional knockout of this gene. The obtained line of mice and the evidence for the depletion of α-synuclein encoded by the *Snca* gene in the nervous system after the induction of the LoxP/Cre recombination have been described in the published articles [11–13]. The clone was used for a further genomic editing in order to obtain stem cells with the humanized *Snca* gene.

The surfaces of all plastic Petri dishes, flasks and plates used for the cultivation of mouse embryonic stem cells, had been preliminarily coated with a layer of gelatin: a 0.1% gelatin solution (Merk, Germany) was layered on the working surface of the plastic and aspirated after 15–30 min of incubation at room temperature. Immediately afterwards, the surface was covered with a layer of a culture medium.

The clone cells stored in liquid nitrogen with a flanked first exon of α-synuclein were thawed, washed with ESGRO Complete Basal Medium (Sigma-Aldrich, USA), resuspended in 4 ml of the medium with GSK3 by the ESGRO Complete Plus Clonal Grade Medium (Sigma-Aldrich, USA) inhibitor and plated on plastic Petri dishes 6 cm in diameter (Nunc, Denmark). After 16 h of the incubation at 37°C in the atmosphere of 5% CO_2, the medium was changed for the fresh ESGRO Complete Plus Clonal Grade Medium, pre-washing the dishes with the ESGRO Complete Basal Medium. After 48 h, the cells concentration in the Goryaev chamber was calculated, and 200,000 cells were seeded on each of the prepared plastic Petri dishes using ESGRO Complete Accutase (Merk, Germany).

Preparation of cells for nucleofection

48 hours after the passage, the cells were treated with an Accutase solution as described above. 2 aliquots of 200 000 cells were taken, centrifuged; the supernatant was carefully removed, and each pellet was resuspended in 20 µl of Complete P3 buffer prepared immediately before use, by mixing 34.2 µl of the Nucleofector TM Solution and 7.6 µl of P3 Primary Cell 4D-Nucleofector® X kit S (Lonza, Switzerland).

The recombinant Cas9 protein, single-guide

ribonucleic acid (sgRNA), as well as single-stranded DNA oligonucleotides for the homologous recombination carrying nucleotide substitutions corresponding to the sequence of the human *Snca* gene, were used to introduce directed breaks into the edited regions of the *Snca* gene.

Ribonucleoprotein (RNP) complexes were formed by mixing 1 µl of 100 µM sgRNA5 solution, 1 µl of 100 µM sgRNA5 solution, and 1 µl of 10 mg/ml Cas9 solution. Incubated for 10 min at 20°C, 0.4 µl of a freshly prepared mixture of donor DNA solutions (ssODN4 and ssODN5) was added at the concentration of 250 µM for each of the oligonucleotides, and 20 µl of the cells resuspended in Complete P3 buffer, were immediately added as described above.

The CRISPR/Cas9-assisted homologous recombination strategy in the mouse embryonic stem cell genome to generate a fully humanized *Snca* gene expressing a human α-synuclein variant with an increased propensity for the aggregation associated with the development of a hereditary form of Parkinson's disease is shown in Fig. 1 and 5.

Delivery of RNP complexes into cells by nucleofection

The cell suspension was transferred into Nucleocuvette™ (Lonza, Switzerland) and a nucleofection was performed in a 4D-Nucleofector™ device (Amaxa, Ukraine) using a CA-120 program. At the end of the cells were transferred into 5 ml of ESGRO Complete Plus Clonal Grade Medium, resuspended to obtain a monocellular suspension. The concentration of survived cells in the Goryaev chamber was counted, and 200, 400, 600, 800, and 1000 cells were seeded for each of five prepared Petri dishes with a diameter of 10 cm in 10 ml of the same medium, an aliquot for the isolation of genomic DNA being previously taken.

The cells were grown until the appearance of separate colonies originating from one cell, separated by accutase in the well of a 96-well plate, incubated for 3 min at room temperature, 0.2 ml of ESGRO Complete Plus Clonal Grade Medium was added, resuspended, and the cells were grown until reaching a 30–50-percent monolayer. At this stage, the cells were subcultured into wells of 4-well plates in triplets. The last of the three parallel dishes was used to isolate the genomic DNA.

Genomic DNA isolation and exon editing analysis of *Snca* gene

After the medium removal, the cells were lysed directly on the wells surface and DNA was isolated using a Wizard Mammalian Cell DNA Extraction kit (Promega, USA) according to the manufacturer's instructions. DNA was used for the PCR amplification of DNA fragments containing mouse *Snca* exon sequences using a GenPak PCR Core kit (Isogen, Russia), according to the manufacturer's instructions.

The presence of homologous recombination with donor DNA was assessed using the allele-specific PCR and restriction analysis. The reaction mixture was incubated with restriction endonucleases specific to the mutant sequence and electrophoretically separated to assess the presence of the homologous recombination with donor DNA. The reaction products were analyzed in a 1.5% agarose gel.

Table 1 – Components of mixture transfected into clone 126 of embryonic stem cells for humanization of mouse *Snca* gene

Component	Sequence	Molecule type
sgRNA4 (Alt® CRISPR-Cas9 sgRNA for humanization of *Snca* gene exon IV)	5'- mG*mU*mC*CUUCUUGACAAAGCCAGGUUUUAG AGCUAGAAAUAGCAAG UUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC mU*mU*mU*U -3'	RNA
sgRNA5 (Alt® CRISPR-Cas9 sgRNA for humanization of *Snca* gene exon V)	5'- mG*mG*mG*UGAGGAGGGGUACCCACGUUUUAGAGCUArGAAAUAGCAAG UUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGC mU*mU*mU*U -3'	RNA
ssODN4 (Alt-RTM HDR Donor single-stranded oligonucleotide for humanization of *Snca* gene exon IV)	5'- /Alt-R-HDR1/G*T*T ATT ACT GAG CAT AAA ACA GGC AGC CAT ACC TTG CCC AAC TGG TCC TTC TTG ACA AAG CCA GTT GCA GCA GCT ATG CTC CCA GCT CCC TCC ACT GTC TTC TGA GCG ACA GCT GTC* A*C/Alt-R-HDR2/ -3'	DNA
ssODN5 (Alt-RTM HDR Donor single-stranded oligonucleotide for humanization of *Snca* gene exon V)	5'- /Alt-R-HDR1/A*A*A ACA CTC TCT TAT TGT GCT TTC TCT TCC CTC TCT GTA GAA TGA GGA GGG GGC CCC ACA AGA AGG AAT CCT GGA AGA CAT GCC TGT GGA TCC TGA CAA TGA GGC TTA TGA AAT GCC TTC AGA GGT AAA TGC CTG TA*T* A/Alt-R-HDR2/ -3'	DNA
Cas9 nuclease	–	Protein

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Nucleotide sequence of exon IV and flanking regions of mouse *Snca* gene
Green background: exon IV
Blue background: direct primer
Yellow background: reverse primer
Bold type: CRISPR-Cas9 crRNA and PAM sequence (underlined)
Red font type: nucleotides to be changed for exon IV humanization

```
TGTTATAATTAAACTCCCAATATTTTCTAATTTCTGCACTAAATTCATATTCAGTGTTTGGCTGAAACATGTCTCTTCTACCTTGCTGTCTTG
TTTCTTCAGACTCCTGTTACCTATGATATATGTGTCTATAGAAGTTGACAGCTGCTAGAAGTGGAATTATTAAAGTCTCTGTCACACCATCAT
CTTTTACTCTGTTGTCACTCTTGATTTTCTTAAGTGGCTGAGAAGACCAAAGAGCAAGTGACAAATGTTGGAGGAGCAGTGGTGACTGGTGTG
                                  V  A  E  K  T  K  E  Q  V  T  N  V  G  G  A  V  V  T  G  V
ACAGCAGTCGCTCAGAAGACAGTGGAGGGAGCTGGGAATATAGCTGCTGCCACTGGCTTTGTCAAGAAGGACCAGATGGGCAAGGTATGGCTG
 T  A  V  A  Q  K  T  V  E  G  A  G  N  I  A  A  A  T  G  F  V  K  K  D  Q  M  G  K
CCTGTTTTATGCTCAGTAATAACCCTGGACACCATGTCCTTGCATGCATCATAGAGCATGCACATGATGCACACTGTGGGGAACACTGCCTTT
AAAGGGCTCTTATTTTGATGCACTGATGTCCTTGGGAAATGTCATGCACACAATAACCCTGATTGTTTTAGTTTCTGGAAGAAAGATATAGAA
```

Nucleide sequence homologous to ssODN4 (Alt-R™ HDR Donor a single-stranded oligonucleotide for exonIV humanization of *Snca* gene
Italic font type: "mismatch" primer
Red lowercase type: Nucleotide substitutions leading to IV exon humanization or creating new restriction endonuclease recognition sites

```
                                                                                                GTG
ACAGCtGTCGCTCAGAAGACAGTGGAGGGAGCTGGGAgcATAGCTGCTGCaACTGGCTTTGTCAAGAAGGACCAGtTGGGCAAGGTATGGCTG
   PvuII
CCTGTTTTATGCTCAGTAATAAC
```

Nucleotide sequence of exon V and flanking regions of mouse *Snca* gene
Green background: exon V
Blue background: direct primer
Yellow background: reverse primer
Bold type: CRISPR-Cas9 crRNA and PAM sequence (underlined)
Red font type: nucleotides to be changed for exon V humanization

```
TAGATTGGTAACCCATGCATGCACAATGTTTTTTCCAGTGGTTTGGTACACTTAGAATCCATCAATAATACAGAAGAATGCACTTCTGATAAC
ACTTCGTGCAGCACCTTGAAGATAAGGTGTCTTTTTCAAGCTGGTTTTCAGAAGTTAAAACACTCTCTTATTGTGCTTTCTCTTCCCTCTCTG
TAGGGTGAGGAGGGGTACCCACAGGAAGGAATCCTGGAAGACATGCCTGTGGATCCTGGCAGTGAGGCTTATGAAATGCCTTCAGAGGTAAAT
    G  E  E  G  Y  P  Q  E  G  I  L  E  D  M  P  V  D  P  G  S  E  A  Y  E  M  P  S  E
GCCTGTATAAAGAAAACTAAGCAAAACACTTTAGGTGTTTAATTTGGAACACATACCATCAAAACCCTGCCACTATCAGATCTCTCTCACATT
ATGGTTGGCATAGTTCAATCAAGAAAATATTTTAGAGCAAATGATTTTAATCTTTGTGGGAGAGGGTAAGGGATATAGTAGGTCAAAATTAAA
```

Nucleide sequence homologous to ssODN4 (Alt-R™ HDR Donor a single-stranded oligonucleotide for exon V humanization of *Snca* gene
Italic font type: "mismatch" primer
Red lowercase type: Nucleotide substitutions leading to V exon humanization or creating new restriction endonuclease recognition sites

```
                                                                AAAACACTCTCTTATTGTGCTTTCTCTTCCCTCTCTG
TAGaaTGAGGAGGGGgcCCCACAaGAAGGAATCCTGGAAGACATGCCTGTGGATCCTGaCAaTGAGGCTTATGAAATGCCTTCAGAGGTAAAT
            ApaI
GCCTGTATA
```

Figure 1 – CRISPR/Cas9-assisted homologous recombination strategy in mouse embryonic stem cell genome to generate fully humanized *Snca* gene
Note: for some primers and CRISPR-Cas9 crRNA, their positions on the given DNA strand are shown.
The actual sequences can be found in the text.

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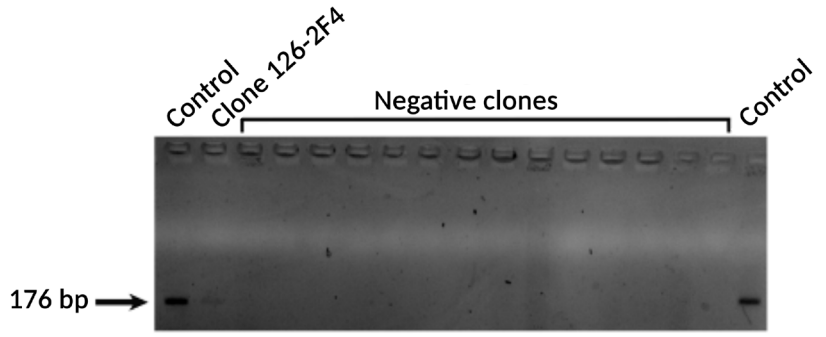

Figure 2 – DNA amplification analysis of PCR products of analyzed clones after cells nucleofection of clone 126 of mouse embryonic stem cells with RNP complexes

Note: Amplification of 176-nucleotide fragment using primers mexVfor-out and exV-rev-mism indicates successful homologous recombination and, as a result, humanization of exon V in the genome of subclone 126-2-F4 cells. As a positive control, amplification with the same template primers of a synthetic fragment corresponding to the humanized mouse V exon with flanking sequences was used.

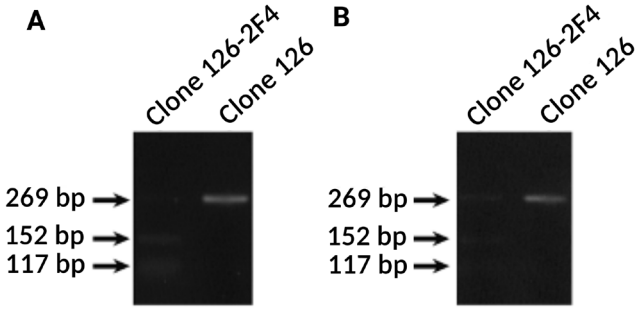

Figure 3 – Analysis of two clones in which homologous recombination was detected in exon V of *Snca* gene during primary screening by treating a 269-nucleotide PCR amplification product with restriction endonuclease ApaI

Note: complete cleavage of this fragment into fragments of 152 and 117 bps in size indicates that clone 126-2-F4 is homozygous for the humanization of exon V (A), and only partial cleavage in the case of clone 126-3-B6 indicates that that only one of the two allelic copies (B) of the *Snca* gene was humanized in this clone.

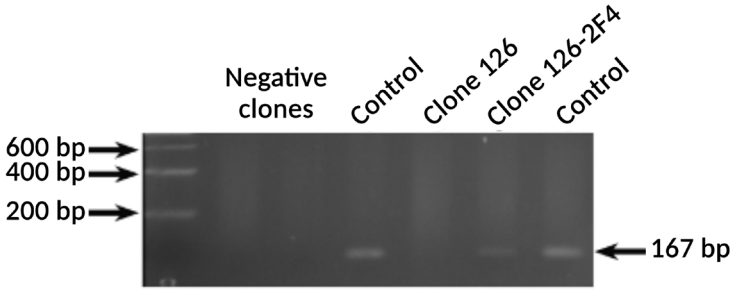

Figure 4 – Analysis of exon IV humanization in genome of clone 126-2-F4 cells. DNA of maternal clone 126, clone 126-2-F4

Note: Amplification with the same primers from the template of the synthetic fragment corresponding to the humanized mouse exon IV with flanking sequences was used as a positive control. The detection of a 167-nucleotide fragment in the DNA analysis of clone 126-2-F4 indicates that exon IV of the *Snca* gene was humanized in the cell genome of this clone.

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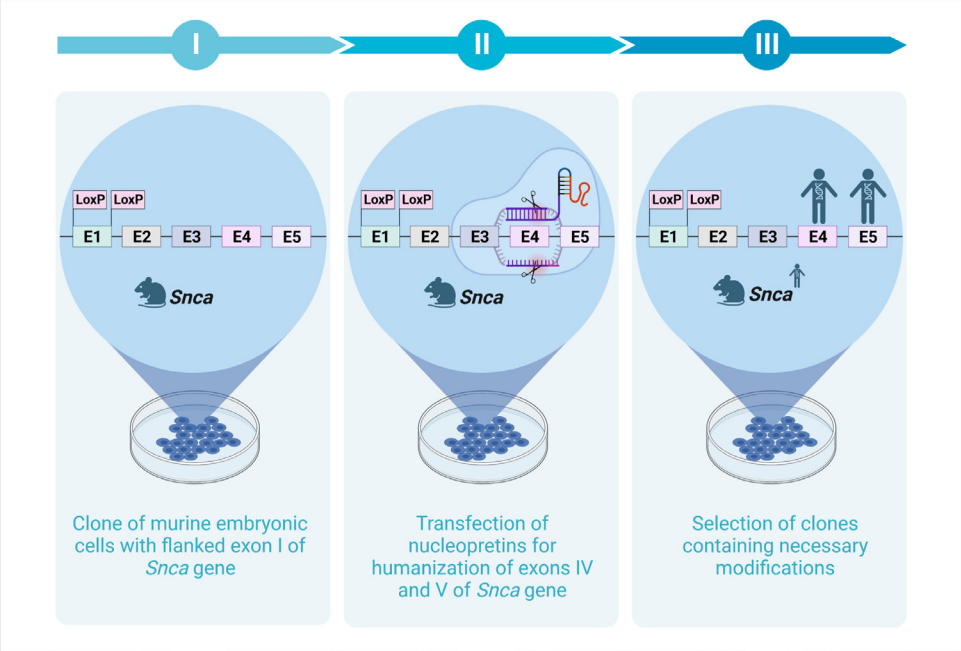

Figure 5 – Strategy for creating clone of mouse embryonic stem cells with conditional knockout of humanized *Snca* gene

Note: I – to create a clone of mouse embryonic stem cells with a conditional knockout of the humanized *Snca* gene, the cells with the first *Snca* exon flanked by LoxP sites, were taken; II – by transfection of CRISPR/Cas9, guide RNAs and fragments of exons IV and V of human *Snca* for homologous repair, the mouse *Snca* gene was humanized; III – after the modification by the allele-specific PCR and restriction analysis, the selection of clones carrying the necessary modification, was carried out.

The list of the primers used for the PCR analysis of the homologous recombination at the *Snca* locus of the mouse embryonic stem cell of clone 124 is as follows:

mexIV-for: 5'-GTCTCTGTCACACCATCATC-3'
mexIV-rev: 5'-AGTGTGCATCATGTGCATGC-3'
exIV-rev-mism: 5'-CAGTaGCAGCAGCTATgc-3'
mexVfor-out: 5'-CCAGTGGTTTGGTACACTTAG-3'
mexVfor-ins: 5'-CTGATAACACTTCGTGCAGC-3'
mexVrev-ins: 5'-TAGTGGCAGGGTTTTGATGG-3'
mexVrev-out: 5'-CTATGCCAACCATAATGTGAG-3'
exV-rev-mism: 5'-tTGTGGGgcCCCCTCCTCAtt-3'

RESULTS

Primary screening of clones for humanized exon V

162 clones were selected for the presence of a 176-bp PCR amplification product analysis using mexVfor-out and exV-rev-mism primers after nucleofection. The two 3'-terminal nucleotides in exV-rev-mism corresponded to the nucleotides present in exon V of the human *Snca* gene, while the mouse gene contains 2 other nucleotides at these positions. In addition to those indicated, in the 5'-terminal part of this primer, there are 3 more nucleotides that are characteristic for only a human gene. Thus, an amplification product with this primer is formed only when the template is human DNA, or mouse DNA humanized for this gene. When the template is native mouse DNA, no amplification products are formed. An example of such an analysis of PCR amplification products of DNA isolated from the cells 15 selected clones is shown in Fig. 2.

As Fig. 2 shows, 1 out of 15 clones tested for the presence of a 176-nucleotide PCR amplification product gave a positive result, i.e. a homologous recombination occurred in exon V in the DNA cells of this clone. That was the evidence that in the clone designated 126-2-F4, in accordance with its position in the well of one of the initial 96-well plates, this exon turned out to be humanized.

Test for homozygosity of modification in clone 126-2-F4

The DNA of clone 126-2-F4 and maternal clone 126 were amplified using primers mexVfor-ins and mexVrev-ins corresponding to the sequences flanking mouse V exon. As expected, the same amplification product, a 269-nucleotide fragment, was detected in both cases. The treatment of the reaction mixture with the restriction endonuclease ApaI did not lead to a cleavage of the parent clone 126 fragment amplified from the DNA template, since in the mouse genome in the analyzed region, there is no recognition site for this enzyme. However, the point substitutions used in the humanization of exon V resulted in the appearance of such a site. The fragment amplified from the DNA template of clone 126-2-F4, was cut with the restriction endonuclease ApaI into 152-nucleotide and 117-nucleotide fragments (Fig. 3A). It is important to notify that in this case, the original 269-nucleotide fragment completely disappeared, which

indicated that in the genome of clone 126-2-F4 cells, the homologous recombination and, consequently, the exon V humanization, occurred in both alleles of the *Snca* gene. In another clone, 126-3-B9, selected in the primary screening, only a partial cleavage of the 269-nucleotide fragment by the restriction endonuclease ApaI, was observed (Fig. 3B). It indicated that in this clone genome, the homologous recombination occurred in only one allelic copy gene or that this clone had originated not from one, but from two cells, in the genome of one of which the DNA sequences of exon V had not been edited.

Verification of humanized exon IV presence in genome of clone 126-2-F4 cells

The DNA of clones 126-2-F4, maternal clone 126 and two negative clones from the above screening were amplified using primers mexIVfor and exIV-rev-mism. As a positive control, amplification with the same template primers of a synthetic fragment corresponding to the humanized mouse exon IV with flanking sequences was used. The analysis result of the PCR amplification products is shown in Fig. 4.

A fragment of the expected size (167 bps) was detected only when the DNA of clone 126-2-F4 cells was used as a template, which indicated that this clone had also a homologous exon IV recombination in the mouse *Snca* gene. Testing for homozygosity of this exon modification was carried out according to the same scheme as had been used for exon V using primers mexIV-for and mexIV-rev and the amplification products treatment with the restriction endonuclease PvuII. It was found out that the 280-nucleotide fragment, the product of the DNA amplification of the clone 126-2-F4 cells, had been completely cut by this enzyme into fragments of 164 and 116 base pairs (Fig. 4). That indicates that in the genome of the clone 126-2-F4 cells, the homologous recombination and hence the exon IV humanization occurred in both alleles of the *Snca* gene. As expected, in the absence of the PvuII recognition site, in the studied fragment of the mouse genome, the 280-nucleotide PCR amplification product of maternal clone 126, the DNA cells were not cleaved by this enzyme. A partial cleavage was observed for the 280-bp PCR DNA amplification product of the clone 126-3-B9 cells, which supports the earlier assumption that this clone had originated from not one, but two cells.

DISCUSSION

Due to the progressive aging of the population, the incidence and prevalence of Parkinson's disease (PD) have increased significantly and will continue to grow - thus, this is a serious medical and social problem. The search for effective therapeutic approaches requires the use of optimal models for the development of the pathological process in sporadic (~90% of cases) PD. The models currently used, do not correspond to this task (not humanized - or incorrectly humanized, there is no possibility of regulation) and cannot be used in experimental neuropharmacology.

The most important role of α-synuclein in the degenerative cell death has been shown in the whole spectrum of neurodegenerative diseases. Moreover, mutations (A53T and A30P) were among the first discovered genetic correlates of the disease [14, 15]. This finding has intensified the molecular mechanisms study of α-synuclein-induced neuropathology. It is now known that under pathological conditions, α-synuclein tends to form the structures rich in β-sheets including oligomers, protofibrils, and insoluble fibrils, which are eventually accumulated to form Lewy bodies. Although the disease has traditionally been associated with insoluble forms of aggregated α-synuclein, it is the soluble intermediate oligomers that are characterized by neurotoxic effects. Oligomers have been found out to mediate aberrant calcium signaling, lipid peroxidation, oxidative stress, mitochondrial dysfunction, and neuronal death [16–18]. *In vivo* studies have shown that oligomer-prone and fibril-inhibiting forms of α-synuclein lead to the death of dopaminergic neurons. On the contrary, fibril-producing forms do not lead to the loss of these neurons [19].

In general, the molecular cascades associated with the aberrant function of α-synuclein, continue to be the most important topic of the study for modern neurobiology [20, 21].

In this regard, an approach to obtaining genetically modified mice expressing pathological humanized α-synuclein has been proposed and implemented. To obtain this line, the strategy of creating genetically modified animals through CRISPR/Cas9-mediated editing of embryonic stem cells has been used. The resulting clone of stem cells can be used for the reinjection into blastocysts, which will then be transplanted into recipient mice to carry genetically modified embryos.

The genetic model described in this work makes it possible to carry out the studies aimed at a precise assessment of the role of pathological α-synuclein in mice.

Thus, the exons IV and V humanization will make it possible to evaluate the phenotypic effects of pathogenic human α-synuclein on a representative test system. In addition, the presence of LoxP sites flanking the first exon allows spatial and temporal control of the humanized *Snca* expression due to the possibility of Cre-induced gene knockout. This feature makes it possible to precisely study the effects of a tissue-specific impairment of the protein expression, providing the information about its role in a specific cell population [22–25]. Moreover, the possibility of inducing knockout in adulthood eliminates the effect of the antenatal adaptation to the genetic modification.

A Cre-dependent knockout induction is intended to mean that crossing a line containing a gene region

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flanked by LoxP sites with transgenic animals expressing Cre recombinase leads to the deletion of this region and the loss of this gene functional activity [18]. To date, the Cre-mice repertoire is characterized by a great diversity, and the variety between different strains lies in the tissue-specific recombinase expression. Moreover, there are lines in which the penetration of Cre-recombinase into the nucleus and, accordingly, its activity, depend on tamoxifen. In this type of mice, a site-specific recombination between the two LoxP sites occurs only after the treatment with tamoxifen, which makes the gene expression regulation over time possible [19].

Alongside the creation of a genetically modified clone of embryonic stem cells, the authors' team is also implementing a direct editing approach of mouse blastocytes. In other words, a mixture containing a DNA template for a homologous recombination, Cas9 mRNA, and guide RNAs, was microinjected into fertilized eggs of CBA×C57Bl6J mice. After a 24 h incubation, the survived embryos were transplanted into the oviducts of female recipients, who had served as surrogate mothers for the mutants. At present, the primary offspring of mutant mice has already been obtained in a similar way, which is undergoing a genetic analysis for the presence of the desired nucleotide substitutions.

The results obtained are fundamentally important not only for understanding the development of the pathological process in α-synucleinopathies, but what is more important, for the development of new therapeutic approaches that will stop the extension of human α-synuclein aggregation pathology throughout the nervous system, and the validation of these approaches in preclinical trials.

CONCLUSION

As a result of the study, a strategy for CRISPR/Cas9-assisted homologous recombination in the genome of mouse embryonic stem cells has been developed to create a fully humanized *Snca* gene encoding α-synuclein, and the clone genome of mouse embryonic stem cells has been edited using a CRISPR technology.

RNA and DNA oligonucleotides necessary for the creation of RNP complexes that carry out directed homologous recombination in the *Snca* locus of the mouse genome, have been synthesized.

Clones screening obtained by maternal clone 126 nucleofection of mouse embryonic stem cells with RNP complexes, made it possible to identify clone 126-2-F4 that meets the primary criteria for the successful humanization of both alleles of the endogenous *Snca* gene in the mouse embryonic stem cell genome.

Thus, the developed cell clone can serve to create a line of genetically modified mice that serve as a test system for pathophysiological and neuropharmacological studies associated with synucleinopathies. At the same time, before the induction of the Cre-dependent recombination, this line is a representative model for studying the biological role of mutant *Snca*. At the same time, after a Cre-dependent knockout activation, it is possible to imitate the pharmacological inhibition of α-synuclein, which is of particular interest for the applied research in neuropharmacology.

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CONFLICT OF INTEREST

Authors declare about no conflict of interest.

AUTHORS' CONTRIBUTION

Evgeniy A. Patrakhanov – DNA isolation, PCR analysis, restriction analysis, article writing; Vladimir M. Pokrovsky – article writing, cell cultivation and nucleofection; Anastasia Yu. Karagodina – list of references formalization, graphic materials preparation; Anastasia M. Krayushkina – list of references formalization, graphic materials preparation; Nikita S. Zhunusov – PCR analysis, article writing; Alexey V. Deykin – consultation on research methodology; Mikhail V. Korokin – consultation on research methodology, experiment design; Mikhail V. Pokrovsky – consultation on research methodology, experiment design; Oxana B. Altukhova - experiment design, article writing.

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RESEARCH ARTICLE

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HYPOGLYCEMIC EFFECT OF SITAGLIPTIN AND AMINOGUANIDINE COMBINATION IN EXPERIMENTAL DIABETES MELLITUS

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The aim of the work was to determine the antidiabetic effect of a sitagliptin and aminoguanidine combination in rats with experimental diabetes mellitus.

Materials and methods. The study was carried out on male Wistar rats and C57BL/KsJ-db/db mice. According to the models used, it was divided into 4 series, in which alloxan, steroid-induced (dexamethasone) and streptozotocin-nicotinamide-induced diabetes mellitus (DM) were formed, respectively, in rats, and in the 4 series, obese C57BL/KsJ-db/db mice were used. In the 1 and 2 series, the treatment was started prophylactically – 3 h after the alloxan administration and simultaneously with the dexamethasone administration, in the 3rd and 4th series, the treatment was carried out after the pathology had developed – 7 days after the streptozotocin with nicotinamide administration, and in the obese mice – immediately after their distribution according to the groups. The treatment was carried out with sitagliptin (10 mg/kg), aminoguanidine (25 mg/kg), or a combination thereof. The treatment was continued till the end of the experiment, which was completed with an oral glucose tolerance test (OGTT) after 4 h of fasting. The obtained data were subjected to statistical processing.

Results. In the course of the experiments, it was found out that the prophylactic administration of a sitagliptin and aminoguanidine combination, unlike each of the components, prevented the development of alloxan DM. More effectively than the administration of sitagliptin alone, it reduced the severity of steroid-induced DM, which was expressed in a significantly lower level of fasting glycemia (after 4 h of fasting) and postprandial glycemia (during OGTT). Under the conditions of streptozotocin-nicotinamide-induced DM, the studied combination slowed down the progression of the pathology, and in the obese mice, the course therapeutic administration of sitagliptin and its combination reduced the severity of carbohydrate metabolism disorders (fasting glycemia) and increased the rate of glucose utilization.

Conclusion. As an iNOS blocker, aminoguanidine enhances the antidiabetic effect of sitagliptin, preventing the development of alloxan diabetes and reducing the severity of steroid-induced DM when administered prophylactically. When administered therapeutically, it reduces the severity of streptozotocin-nicotinamide-induced DM in rats and type 2 DM in mice with a predisposition to obesity.

Keywords: DPP-4 inhibitors; sitagliptin; preclinical studies; diabetes; alloxan; streptozotocin

Abbreviations: eNOS – endothelial nitric oxide synthase; iNOS – inducible nitric oxide synthase; nNOS – neuronal nitric oxide synthase; NO – nitric oxide (II); GLP-1 – glucagon-like peptide-1; DPP-4 inhibitors – inhibitors of dipeptidyl peptidase-4; OGTT – oral glucose tolerance test; DM – diabetes mellitus; SDM – steroid-induced diabetes mellitus.

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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ГИПОГЛИКЕМИЧЕСКОЕ ДЕЙСТВИЕ КОМБИНАЦИИ СИТАГЛИПТИНА С АМИНОГУАНИДИНОМ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ САХАРНОМ ДИАБЕТЕ

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Цель. Определить эффективность противодиабетического действия комбинации ситаглиптина с аминогуанидином у крыс с экспериментальным сахарным диабетом.

Материалы и методы. Исследование проведено на крысах-самцах линии Wistar и мышах линии C57BL/KsJ-db/db. Согласно используемым моделям, оно было разделено на 4 серии, в которых формировали аллоксановый, стероид-индуцированный (дексаметазоновый) и стрептозотоцин-никотинамид-индуцированный сахарный диабет (СД) у крыс. В 4 серии использовали склонных к ожирению мышей линии C57BL/KsJ-db/db. В 1 и 2 сериях лечение начинали профилактически – через 3 ч после введения аллоксана и одновременно с введением дексаметазона; в 3 и 4 сериях лечение проводили после сформировавшейся патологии – через 7 сут после введения стрептозотоцина с никотинамидом и у мышей с ожирением сразу после их распределения по группам. В качестве лечения вводили ситаглиптин (10 мг/кг), аминогуанидин (25 мг/кг) или их комбинацию. Лечение проводили до конца эксперимента, который завершали пероральным тестом на толерантность к глюкозе (ПТТГ) после 4 ч голодания. Полученные данные подвергались статистической обработке.

Результаты. В ходе проведенных экспериментов было установлено, что профилактическое введение комбинации ситаглиптина с аминогуанидином, в отличие от каждого из компонентов, предотвращало развитие аллоксанового СД, а также более эффективно, чем введение только ситаглиптина снижало выраженность стероид-индуцированного СД, что выражалось в значительно более низком уровне гликемии натощак (через 4 ч голодания) и постпрандиальной гликемии (в ходе проведения ПТТГ). В условиях стрептозотоцин-никотинамид-индуцированного СД исследуемая комбинация замедляла прогрессирование патологии, а у мышей с ожирением терапевтическое курсовое введение ситаглиптина и его комбинации снижало тяжесть нарушения углеводного обмена (уровень гликемии натощак) и увеличивало скорость утилизации глюкозы.

Заключение. Аминогуанидин как блокатор iNOS усиливал противодиабетическое действие ситаглиптина, предотвращая развитие аллоксанового диабета и уменьшая выраженность стероид-индуцированного СД при профилактическом введении, а при лечебном курсовом введении снижал тяжесть течения стрептозотоцин-никотинамид-индуцированного СД у крыс и СД 2 типа у мышей с предрасположенностью к ожирению.

Ключевые слова: ингибиторы ДПП-4; ситаглиптин; доклинические исследования; сахарный диабет; аллоксан; стрептозотоцин

Список сокращений: eNOS – эндотелиальная синтаза оксида азота; iNOS – индуцибельная синтаза оксида азота; nNOS – нейрональная синтаза оксида азота; NO – оксид азота (II); ГПП-1 – глюкагоноподобный пептид-1; иДПП-4 – ингибиторы дипептидилпептидазы-4; ПТТГ – пероральный тест на толерантность к глюкозе; СД – сахарный диабет; ССД – стероид-индуцированный сахарный диабет.

INTRODUCTION

The number of diabetes mellitus (DM) patients registered in Russia at the beginning of 2021, was almost 4.8 million people [1]. While in the world, according to the International Diabetes Federation (IDF), the number of diagnosed and undiagnosed cases exceeded 536 million people in 2021 and, according to its forecasts, by 2045, this number will increase by 46%, reaching 783.2 million people [2]. A low availability of modern hypoglycemic drugs significantly limits the effectiveness of the endocrinological service and medical and social measures aimed at curbing DM and related diseases. Modern guidelines for the treatment of DM indicate the feasibility of an early treatment using rational combinations of drugs, and note the importance of preventing vascular DM complications [3, 4].

Steroid-induced diabetes mellitus (SDM) is also a common and potentially dangerous problem in clinical

practice, affecting almost all medical specialties but often difficult to detect in the clinical setting. Glucocorticoids are widely used as potent anti-inflammatory and immunosuppressive drugs for the treatment of a wide range of diseases. However, they are also associated with a number of side effects, including new onset hyperglycemia in patients without a history of DM or severe uncontrolled hyperglycemia in the patients diagnosed with DM [5]. The mechanism of the steroid-induced diabetes mellitus (SDM) development includes a decrease in insulin sensitivity (respectively, glucose utilization) in target tissues, followed by an increase in catabolic processes (proteolysis and lipolysis) and an increase in the glucose production by the liver (due to the stimulation of gluconeogenesis and glycogenolysis), as well as the suppression of insulin synthesis, including the ones due to the direct damaging effect of steroids on pancreatic β-cells [5].

Currently, scientifically substantiated and reliably tested methods for the prevention of SDM are inconsiderable in number. As with other types of diabetes, the risk factors principles of the early identification and modification are used. SDM screening should be performed in all patients receiving moderate to high doses of glucocorticoids. Problems in the management of SDM are associated with large fluctuations in postprandial hyperglycemia and the lack of well-defined treatment protocols. Along with lifestyle changes, hypoglycemic drugs with an insulin-sensibilizing action are indicated [6, 7]. However, the insulin therapy is often unavoidable, so insulin can be considered the treatment of choice. In the SDM treatment, the degree and nature of hyperglycemia, as well as the type, dose and regimen of glucocorticoids, should be taken into consideration. In addition, it is important to instruct patients and/or their families on how to make the necessary adjustments. Prospective studies are needed to answer the remaining questions regarding SDM. The hyperglycemia that occurs during the use of glucocorticoids, is believed to disappear after their withdrawal, but this is not always confirmed in practice. A reverse situation occurs more often, especially in individuals with risk factors such as obesity or prediabetes, which is confirmed by the data from the patients who have undergone COVID-19 [8].

Dipeptidyl peptidase-4 (DPP-4) inhibitors have a moderate hypoglycemic activity and are often used to create rational combinations of hypoglycemic drugs. The drugs of this pharmacotherapeutic group including the ones created by leading pharmaceutical companies around the world [9] have been developed for more than 30 years. A distinctive feature of the drugs with an incretins activity, which also include DPP-4 inhibitors, is a number of pleiotropic effects associated with a decrease in the risk of developing cardiovascular DM complications [10]. In Russia, DPP-4 inhibitors are actively used in the DM combination therapy. The characteristics of the domestic market for DPP-4 inhibitors are summarized in Fig. 1.

Aminoguanidine is a nitric oxide synthase inhibitor with a high (50-fold) specificity for its inducible isoform (iNOS), as well as an inhibitor of the formation of advanced glycation end products [11]. Under the experimental conditions, the administration of aminoguanidine delayed the formation of autoimmune DM, the rate of plaque formation in a diet with excess cholesterol, and improved the course of alloxan DM [12–14]. A number of studies testify in favor of a significant role of nitric oxide in the development of autoimmune diabetes, and indicate the advisability of using selective iNOS inhibitors to reduce the disease states associated with the nitric oxide expression and its increased production [13–15]. Nitric oxide can be considered as one of the targets for the DM treatment and its complications, since its role in the modulation of insulin secretion and its signaling pathways has been proven [16]. The attention of many researchers is focused on studying the role of iNOS in the pathogenesis of many diseases, including the formation of insulin resistance and death of β-cells in DM. It is known that this enzyme can be induced by many inflammatory cytokines, the increased expression of which accompanies DM and obesity [14, 15, 18].

As a part of the search for means to prevent vascular DM complications, aminoguanidine was previously studied as an antiglycating agent and a drug for the treatment of diabetic nephropathy [19]. However, clinical trials of aminoguanidine were discontinued in phase III due to safety concerns and lack of efficacy. Nevertheless, the therapeutic potential of aminoguanidine is of interest in the development framework of new pathogenetic approaches to the treatment of DM and its complications by creating rational combinations. To do this, it is advisable to study the combination potential of an agent with an incretin activity that improves the function of β-cells and an inhibitor of iNOS aminoguanidine, which is able to reduce the autoaggression of the immune system against β-cells in DM, and has the properties of an antiglycation agent.

THE AIM of the work was to determine the hypoglycemic effect of a sitagliptin and aminoguanidine combination under the conditions of various experimental diabetes mellitus models.

MATERIALS AND METHODS

Model objects

All experiments were performed in accordance with the legislation of the Russian Federation and the technical standards of the Eurasian Economic Union for Good Laboratory Practice (GOST R 53434-2009, GOST R 51000.4-2011). The study design was approved by the Regional Independent Ethics Committee (the registration number: IRB 00005839 IORG 0004900 (OHRP), as evidenced by the extract from Protocol No. 132 dated 20 May 2019 of the meeting of the Commission for Expertise of the Study of the Ethics Committee at Volgograd State Medical University.

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The work was performed on 150 male Wistar rats (aged 6 months, body weight was 300–350 g, Rappolovo Nursery of Laboratory Animals), and 40 male mice of the C57BL/KsJ-db/db line (aged 4–5 months, body weight was 50–60 g), characterized by severe obesity and spontaneously developing severe diabetes, causing necrosis of β-cells, nephro-, neuro-, retinopathy and other complications. The mice of this line carry an autosomal recessive mutation in the leptin receptor gene (linkage group 8, chromosome 4). In the homozygous state, it causes hyperleptinemia, hyperinsulinemia, dyslipidemia, hyperglycemia, obesity and diabetes, which is difficult to correct with the drugs from the DPP-4 inhibitors in monotherapy [20]. As controls, 10 mice of the C57BL/KsJ–db+/+m line were used (*m* gene – misty, a recessive marker of the opposite chromosome that brightens the color and does not carry the db gene); the mice were without obesity and without diabetes (aged 4–5 months, body weight was 20–25 g) [21, 22]. All the mice had been obtained from the nursery of laboratory animals "Stolbovaya" (Scientific Center for Biomedical Technologies of Federal Medical and Biological Agency of Russia). After the arrival from the nursery, the animals were quarantined for 14 days. in the vivarium of Volgograd State Medical University, where they were kept throughout the experiment at 20±2°C under the conditions of 40–60% humidity of an alternating day/night cycle (12/12 h) with an unlimited access to food and water.

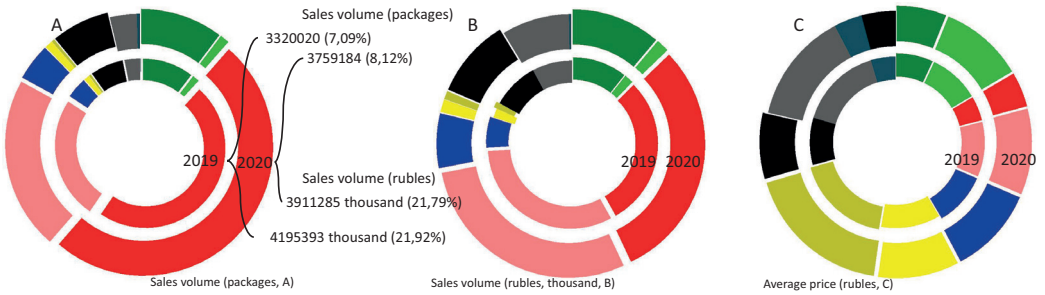
Pathology Modeling

In rats, disturbances in carbohydrate metabolism were induced by multiple intraperitoneal dexamethasone injections (injection solution – 4 mg/ml; KRKA, Slovenia) at the dose of 20 mg/kg/day (within 7 days) or a single intraperitoneal alloxan injection (130 mg/kg; Sigma-Aldrich, USA) [23] or streptozotocin (65 mg/kg, 15 min after 230 mg/kg nicotinamide; Sigma-Aldrich, USA) [24]. Alloxan and streptozotocin were administered to the animals after 36 h of fasting, which provided a better reproducibility of these models due to a decrease in the level of glycemia to the lower limit of the reference range and a decrease in the variability of its level among the animals [24]. The mechanism of the diabetogenic action of these substances is schematically shown in Fig. 2. Due to the ability to selectively interact with the glucose transporter GLUT2, alloxan and streptozotocin accumulate in pancreatic β-cells and have a selective, but different in mechanism, cytotoxic effect. The administration of alloxan leads to the development of an oxidative stress and subsequent death of the β-cells, and causes a state of severe hyperglycemia, which may correspond to type 1 diabetes. In this case, damage to liver and kidney cells is possible due to a slight expression of GLUT2. The cytostatic effect of streptozotocin develops as a result of alkylation of nucleic acids and an increase in the generation of reactive oxygen species. DNA alkylation leads to repair errors, which, when accumulated, lead to the cell death through the activation of apoptotic mechanisms. At the same time, a combined administration of nicotinamide and streptozotocin makes it possible to reduce the activity of PARP-1, which reduces the activity of the SOS repair and somewhat reduces the intensity of apoptosis processes. The administration of streptozotocin and nicotinamide in certain doses (and in certain ratios) makes it possible to achieve a partial decrease in β-cell mass with the development of a moderate hyperglycemia state, which may correspond to one of the forms of type 2 diabetes, characterized by an impaired insulin secretion without insulin resistance [25].

Study design

The overall design of the study is presented below on Figure 3. The study was performed in 4 series: in the first series, the treatment was started 3 h after the alloxan administration, in the second – simultaneously with the first injection of dexamethasone, in the third – after 7 days after the streptozotocin administration, and in the fourth – after quarantine and distribution into groups. Sitagliptin (Januvia, 10 mg/kg/day, *per os*) or aminoguanidine (Sigma, 25 mg/kg/day, i.p.) or their combination, were administered as a treatment (the dose, regimen, and an administration route for each drug remained unchanged). In the 1 and 2 series, in which the treatment had been started before the pathology development, the animals were divided into the groups randomly before the administration of the study drugs. In the 3 series (streptozotocin-nicotinamide-induced DM) and in the 4 series (the obese mice), the level of glycemia was preliminarily measured in the animals with developed pathology. The rats and mice with fasting glycemia levels of more than 11 mmol/l were subjected to the randomization to the experimental groups. The treatment was started immediately after the division into groups. The effectiveness of the therapy was assessed by measuring the concentration of glucose (glucometer Kontur TC, Bayer, Germany) in the blood after 4 h of fasting and/or during an oral glucose tolerance test (OGTT). During that test, the level of glycemia was recorded before and after 60 and 120 min after the oral administration of an aqueous 40% glucose solution at the dose of 4 g/kg, followed by the calculation of the area under the "glycemic level-time" curve ($AUC_{0\text{-}120}$).

In each series of the studies, the animals were divided into equal (n=10) groups: intact, diabetes + placebo (0.9% NaCl) – "placebo", diabetes + sitagliptin – "Sit", diabetes + aminoguanidine – "AMG", diabetes + sitagliptin + aminoguanidine – "Sit + AMG". The doses of the substances were selected taking into account the literature data [19, 26].



	Sales volume (packages, A)		Sales volume (rubles, thousand, B)		Average price (rubles, C)	
Alogliptin	344901 (10,39%)	386596 (10,28%)	415665 (10,63%)	468604 (11,17%)	1090,31 (6,95%)	1027 (6,15%)
Alogliptin + metformin	45844 (1,38%)	43585 (1,16%)	70568 (1,80%)	67604 (1,61%)	1494,12 (9,53%)	1732,54 (10,38%)
Vildagliptin	1584690 (47,73%)	1876337 (49,91%)	1150815 (29,42%)	1267789 (30,22%)	723,23 (4,61%)	719 (4,31%)
Vildagliptin + metformin	824156 (24,82%)	814582 (21,67%)	1259694 (32,21%)	1214527 (28,95%)	1623,7 (10,36%)	1774,82 (10,64%)
Linagliptin	140976 (4,25%)	174160 (4,63%)	235270 (6,02%)	285992 (6,82%)	1576,83 (10,06%)	1799,83 (10,78%)
Saxagliptin	36965 (1,11%)	37853 (1,01%)	69293 (1,77%)	67458 (1,61%)	1754,28 (11,19%)	1669,22 (10,00%)
Saxagliptin + metformin	14636 (0,44%)	14084 (0,37%)	46093 (1,18%)	43739 (1,04%)	2826,98 (18,03%)	3037,6 (18,20%)
Sitagliptin	222278 (6,7%)	272118 (7,24)	357402 (9,14%)	414779 (9,89%)	1395,99 (8,90%)	1355,08 (8,12%)
Sitagliptin + metformin	104762 (3,16%)	126716 (3,37%)	305884 (7,82%)	354919 (8,46%)	2464,31 (15,72%)	2291,16 (13,73%)
Gosogliptin	812 (0,02%)	11675 (0,31%)	599 (0,02%)	8697 (0,21%)	729,67 (4,65%)	578,31 (3,47%)
Evoligliptin	–	1478 (0,04%)	–	1284 (0,03%)	–	703,72 (4,22%)

Figure 1 – Some indicators of iDPP-4 domestic market (according to DSM Group data)[1]

Notes: The data are presented in Russian rubles, dated 1 August 2022, 1 US dollar (USD) corresponded to 61.3 Russian rubles (RUB).

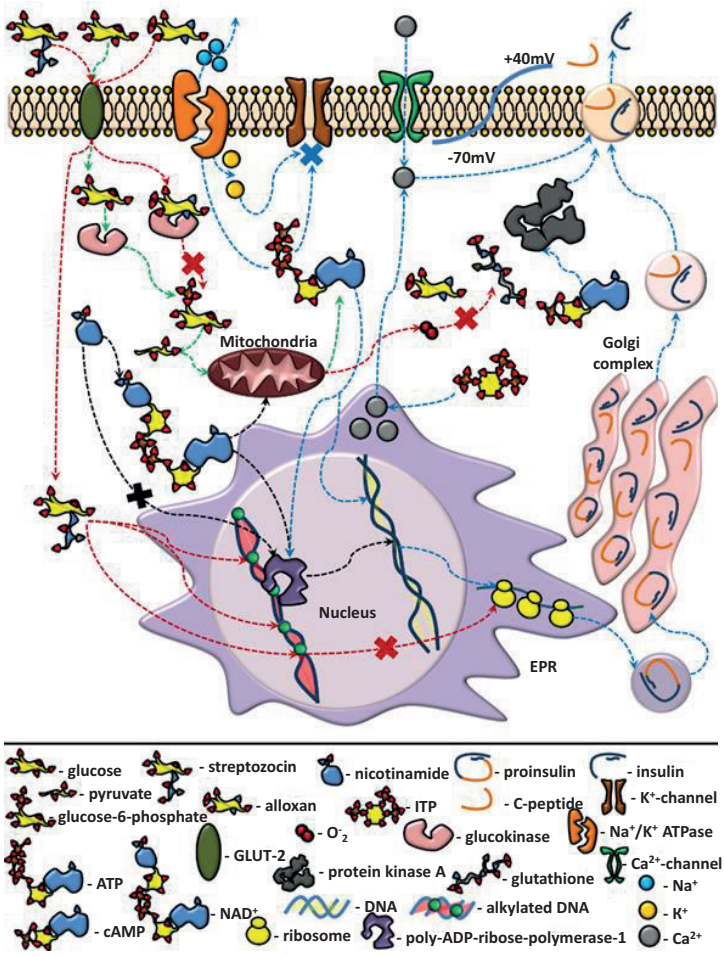


Figure 2 – Mechanism of streptozotocin and alloxan diabetogenic action, adapted from [25]

Note: ADP, adenosine diphosphate; ATP – adenosine triphosphate; GLUT-2, glucose transporter, type 2; DNA – deoxyribonucleic acid; ITP, inositol 1,4,5-triphosphate; mV – millivolt; NAD+, nicotinamide adenine dinucleotide (oxidized); cAMP – cyclic adenosine monophosphate; EPR – endoplasmic reticulum.

[1] The data were officially acquired from the DSM Group company, the calculations were made and the diagrams were presented on their basis.

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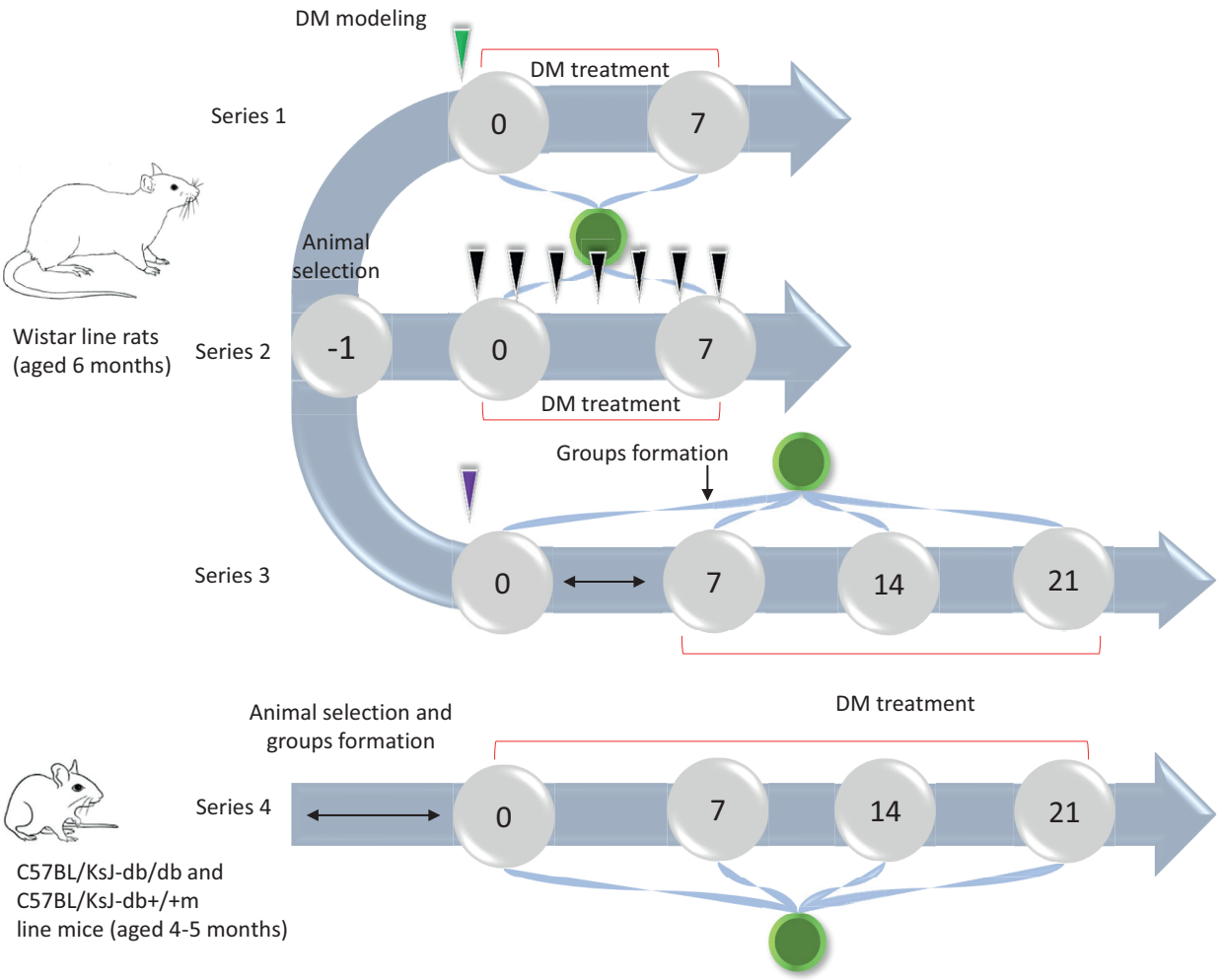

Experimental groups (n=10):
Intact
Diabetes + placebo
Diabetes + Sitagliptin (Sit)
Diabetes + Aminoguanidine (AMG)
Diabetes + Sit + AMG

Determination of glucose in blood
DM treatment
DM development without therapy

Alloxan (130 mg/kg) and test substances
Dexamethasone (20 mg/kg) and test substances
Nicotinamide (230 mg/kg) + streptozotocin (65 mg/kg)

Figure 3 – Study design
Note: AMG – aminoguanidine; DM – diabetes mellitus; Sit – sitagliptin.

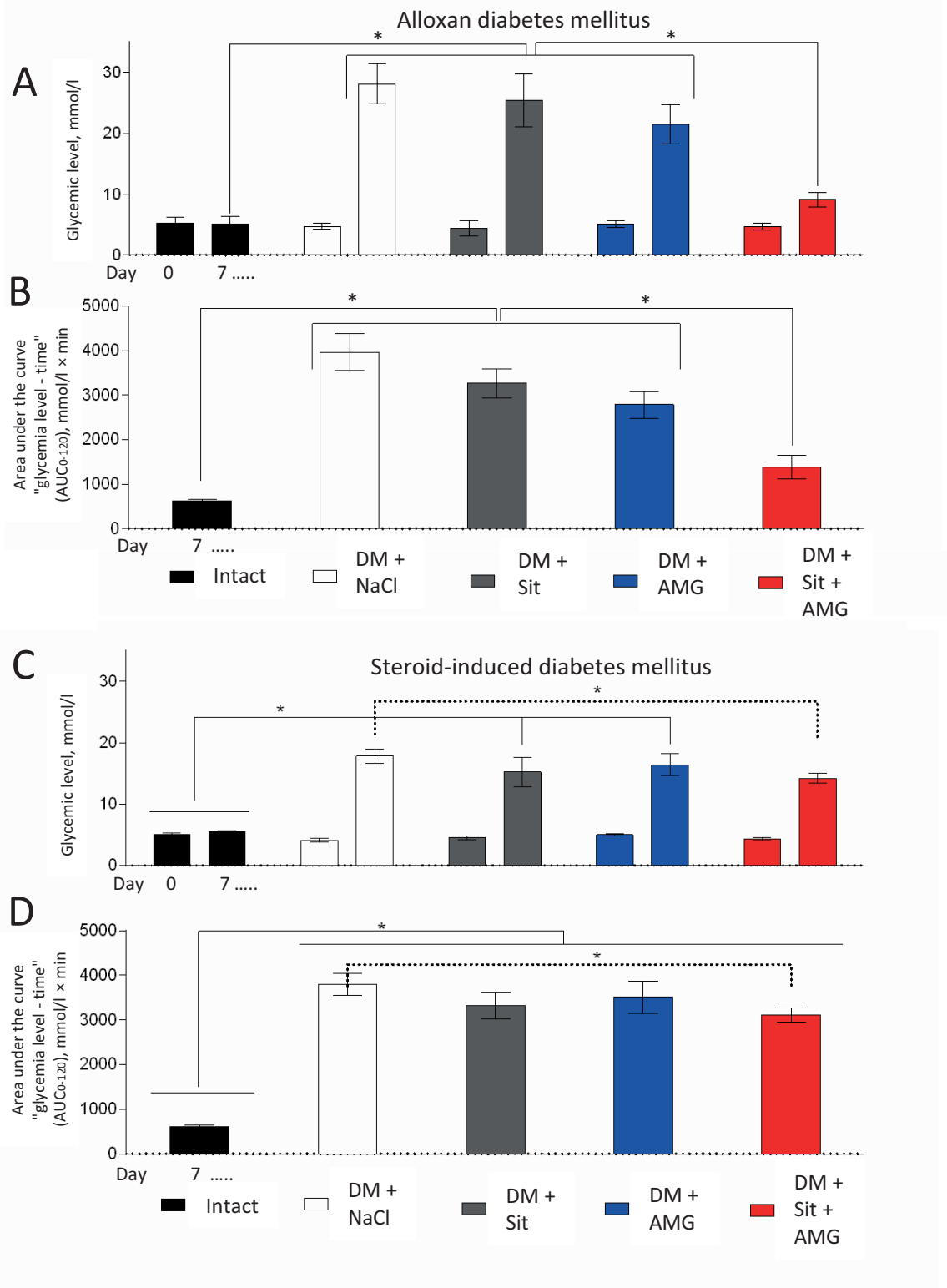

Figure 4 – Influence of sitagliptin, aminoguanidine and their combination in prophylactic administration to rats with alloxan (A, B) and steroid-induced (C, D) DM on fasting blood glucose levels (mmol/l; A, C) and its utilization during oral glucose tolerance test (AUC_{0-120}, mmol/l*min; B, D)

Note: A – the first column – the values before modeling DM (alloxan, day 0), the second – after treatment (day 7); B – area under the curve "glycemic level – time" (AUC_{0-120}) 7 days after treatment of diabetes mellitus caused by the alloxan administration; C – the first column – values before modeling DM (dexamethasone, day 0), second – after treatment (day 7); D – AUC_{0-120} 7 days after treatment for dexamethasone-induced DM; * – $p<0.05$ one-way analysis of variance with Newman-Keuls post-hoc test; compared samples are indicated by lines.

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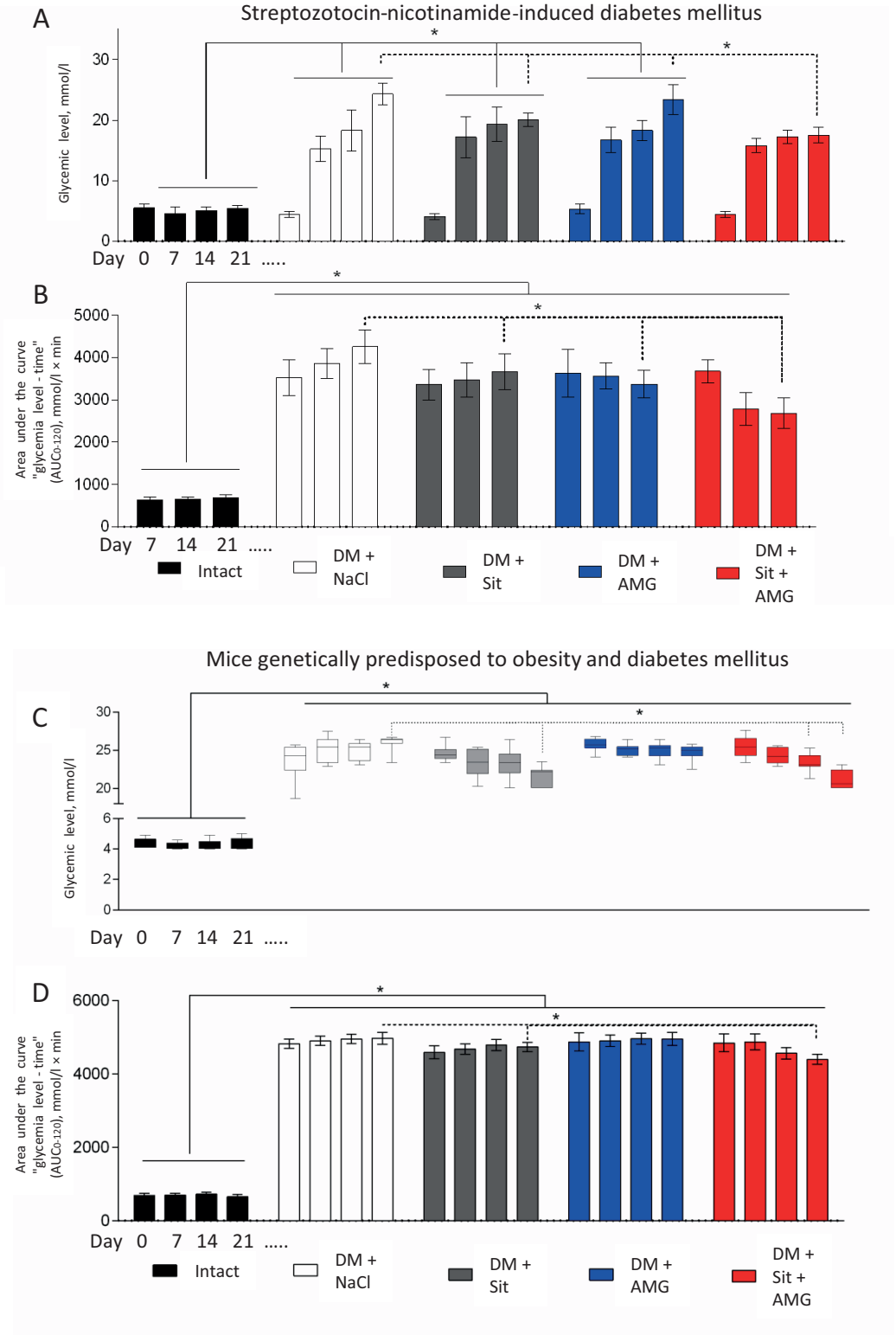

Figure 5 – Effect of sitagliptin, aminoguanidine and their combination in therapeutic administration to rats with streptozotocin-nicotinamide-induced DM (A, B) and in C57BL/KsJ-db/db mice with genetic predisposition to DM (C, D) on glucose levels in fasting blood (mmol/l; A, C) and its utilization during oral glucose tolerance test (AUC_{0-120}, mmol/l*min; B, D) before and after treatment

Note: A – the first column – the values before modeling DM (streptozotocin, day 0), the second – before treatment (day 7), the third and fourth, respectively, after treatment; B – the first column – before treatment (day 7), the second, third columns – AUC_{0-120}, respectively, after treatment; C – the first column – the values before treatment (day 0), the second, third and fourth – days 7, 14 and 21 after treatment, respectively; D – the first column – the values before treatment (day 0), the second, third and fourth – 7, 14 and 21 days, respectively, after treatment; * – $p < 0.05$ one-way analysis of variance with Newman-Keuls post-test; compared samples are indicated by lines.

Statistical processing

Statistical processing of the obtained results was carried out by methods of descriptive and analytical statistics using Prism 6 software (GraphPad Software Inc., USA). The distribution of quantitative indicators was assessed using the Shapiro-Wilk test. Intergroup differences were assessed using a one-way analysis of variance with the Newman-Keuls post-hoc test. The differences were considered significant at $p<0.05$. The numerical values were presented as histograms using the arithmetic mean and a standard error of the arithmetic mean (M+SD), as well as range plots with the median of 25th and 75th percentile.

RESULTS

The alloxan administration causes a pronounced toxic effect on pancreatic β-cells: 7 days after the administration, in the animals that were simultaneously injected with saline (placebo), the concentration of glucose, in the blood was maximum and amounted to 28.1±3.3 mmol/l (the rate of glucose utilization during OGTT in this group was minimal; Fig. 4A and B). This indicates a pronounced violation of carbohydrate metabolism due to the destruction of pancreatic β-cells.

A simultaneous administration of sitagliptin or aminoguanidine with alloxan did not significantly affect the severity of glucose metabolism disorders, the level of hyperglycemia in the animals was 25.4±4.3 mmol/l or 21.5±3.2 mmol/l, respectively. A combined administration of sitagliptin and aminoguanidine had a pronounced protective effect, since in the animals of this group, the glucose concentration was 9.1±1.2 mmol/l (the rate of glucose utilization during OGTT was also significantly higher than in the animals of other groups, $p\leq0.05$; Fig. 4A and B). Thus, a prophylactic administration of a sitagliptin (DPP-4 inhibitors) and aminoguanidine (iNOS inhibitor) combination prevented the development of alloxan diabetes mellitus. This may be due to the combination of the protective incretins action and the inhibitory effect of aminoguanidine against iNOS: an enzyme that, by causing the secretion of a large amount of NO, significantly enhances apoptosis and, accordingly, potentiates the cytotoxic effect of alloxan, and, accordingly, its blockade prevents the occurrence of the oxidative stress and reduces damage to the pancreas β-cells.

In contrast to the mechanism of DM development after the use of alloxan, the course administration of glucocorticosteroids causes an increase in catabolic processes in insulin target tissues, as well as a decrease in the function and death of β-cells. Depending on the administration duration, the initial state of carbohydrate metabolism and other individual characteristics, glucocorticosteroids can cause minor reversible changes, as well as persistent and progressive disorders of carbohydrate metabolism [5]. In the study, a course administration of dexamethasone led to the development of severe hyperglycemia (Fig. 4C and 4D). In the animals treated simultaneously with dexamethasone, sitagliptin (15.2±2.4 mmol/l), aminoguanidine (to a lesser extent, 16.4±1.8 mmol/l) or their combination (to a greater extent, 14.2±0.8 mmol/l, $p<0.05$), the blood glucose concentration was significantly lower than in those who were administrated with dexamethasone and placebo (17.8±1.2 mmol/l).

Like alloxan, the administration of streptozotocin with nicotinamide caused less, but at the same time, pronounced disturbances in glucose metabolism due to incomplete damage to the β-cell mass, which is preferable when conducting the studies lasting more than 14 days, due to less general somatic depletion of the animals. In this series of experiments, the test substances were administered to the animals starting from the 7th day after streptozotocin. In the animals that had been injected with saline (placebo), the glucose concentration increased from 15.2±2.1 (7 days) to 24.3±1.8 mmol/l (21 days), which indicates a progressive destruction of β-cells of the pancreas (Fig. 5A and B). In the animals treated with sitagliptin or aminoguanidine for 14 days after streptozotocin, the glucose concentration increased from 17.2±3.4 and 16.7±2.1 mmol/l to 20.1±1.1 and 23.4±2.5 mmol/l, respectively. A minimal progression of hyperglycemia was notified in the animals that had received a combination of sitagliptin and aminoguanidine for 14 days (from 15.8±1.2 to 17.5±1.3 mmol/l, $p<0.05$), which may also be due to blocking iNOS and reducing the number of factors promoting apoptosis.

The mice on which the last series of studies were carried out, were kept on a standard diet and characterized by extremely high values of hyperglycemia (Fig. 5C and 5D), which is probably a feature of this DM model. The administration of sitagliptin, aminoguanidine or their combination to the animals had a moderate hypoglycemic effect. Glycemic levels in the mice that had been administered with the listed drugs for 3 weeks, was 21.6±1.2 mmol/l, 24.7±0.9 mmol/l, 21.1±1.2 mmol/l, respectively, while in the animals treated with placebo, the concentration of glucose in the blood was 26±0.96 mmol/l.

DISCUSSION

The islet of Langerhans consists of approximately 1000 β-cells, which contain from 10 to 13 thousand granules with 106 insulin molecules each. All NOS isoforms are expressed in pancreatic cells. The production of nitric oxide by inducible synthase (iNOS) significantly exceeds that of other isoforms. Moreover, the iNOS expression is increased at high concentrations

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(≥10 mM) of glucose in the cytoplasm. Nitric oxide is involved in the early phase of insulin secretion, increases the level of cyclic guanosine monophosphate and intracellular Ca^{2+}, promotes insulin synthesis, stimulates the activity of the insulin gene promoter and its expression, increases the blood flow in the pancreas and prevents apoptosis through S-nitrosylation, modulates the dynamic association of glucokinase with secretory granules which, in combination, promotes insulin secretion. In addition to the protective effect of NO at high concentrations, created mainly by iNOS, it has a pronounced negative effect on many body systems, including the insular apparatus.

Physiological (produced by nNOS or eNOS) concentrations of NO, cause positive effects that stop functioning when iNOS is activated and an NO production is too high, which enhances the islet dysfunction. In response to inflammatory stimuli, pancreatic cells increase the iNOS expression, which is accompanied by an increase in NO concentration to cytotoxic levels, causing damage, dysfunction, and death of β-cells, which is important in the onset and progression of DM. The suppression of iNOS has a protective effect on pancreatic cells, which are considered particularly sensitive to damage by free radicals of various origins due to the low level of enzymes of the antioxidant system – superoxide dismutase, catalase, and glutathione peroxidase [16–18].

The activity of inducible NOS is regulated at all stages of the expression, including protein stability, and largely depends on the functional state of the cell. Taking into account the toxicity of excess NO, blockade of iNOS under the conditions of inflammation is a promising experimental approach to control a number of pathological processes. Many attempts have been made to normalize the NOS activity for various diseases: blocking the upstream ROS production, the BH4 administration, the folic acid administration to recycle BH2 to BH4, arginase inhibitors, resveratrol, calcium dobesilate, cavnoxin, NOS transcription enhancers (AVE3085 and AVE9488), L-arginine, blockers and activators of various NOS [11].

Metabolic protection of pancreatic β-cells is a promising approach to the treatment of diabetes mellitus and reduction of its complications. A combination of several drugs with multidirectional effects and, accordingly, influencing various pathogenetic mechanisms is a promising approach in the development of modern drugs. From this perspective, aminoguanidine (an inhibitor of iNOS and an inhibitor of glycation end products formation [19]) and sitagliptin (which increases the level of endogenous incretins that have a protective effect on β-cells [27]) are reasonable candidates for the consciousness of a combined antidiabetic drug able of protecting β-cells and slowing down the progression of diabetes of various etiologies.

In this study, an attempt to evaluate the antidiabetic effect of the combination of the common drug sitagliptin and an agent that exhibits a pronounced iNOS inhibitory effect was made. The models used in this work are characterized by different mechanisms of induction of carbohydrate disorders: complete or partial death of β-cells due to a specific toxin, the formation of steroid-induced DM, and the genetic model of DM2. With respect to the pathology formation moment, the time of therapy initiation differed: a prophylactic administration (alloxan DM with the induction of β-cells complete death and steroid-induced DM with a complex disorder of carbohydrate metabolism) and a therapeutic administration (streptozotocin-nicotinamide-induced DM and a genetic model of DM2), which are characterized, respectively, by the death of most β-cells, and obesity with the primary insulin resistance).

In the course of the work, it was found out that the protective effect of the combination significantly exceeds the effects of individual substances. This fact indicates the synergy of the components, which, in turn, can be explained, among other things, by the diversity of their action. The therapeutic effect was especially pronounced during the prophylactic administration in the model of alloxan DM, which is characterized by a complete death of β-cells, which obviously did not happen against the background of the administration of an iNOS inhibitor and an incretin mimetic (iDPP-4) combination. The protective effect of aminoguanidine on the alloxan diabetes model has been previously stated in the literature, herewith, its prophylactic administration and monotherapy were focused. However, for the development of a pronounced effect, its administration was required for 6 weeks, while the gradually developing protective effect is associated with the restoration of the antioxidant defense system, inhibition of the formation of advanced glycation end products, the reduction of inflammation and restoration of islets [14]. In the current study, only in the group treated with the combination (sitagliptin+aminoguanidine, but not the individual components), the level of glycemia compared with the negative control group was significantly (several times) lower, as early as 1 week after the administration of alloxan. That indicates a protective effect of the combinations in relation to a cellular toxin, which realizes its action through an oxidative stress with a subsequent death of β-cells. Given the nature of experimental diabetes induced by the administration of cytotoxic substances similar in the mechanism of action (alloxan and streptozotocin), i.e., the key role of apoptosis in damage to β-cells and the participation of iNOS in this

process, it can be assumed that the hypoglycemic and protective effect on β-cells in sitagliptin (realizing its action mainly through incretins) is complemented by the anti-apoptotic (antioxidant and anti-inflammatory due to iNOS inhibition) effect of aminoguanidine, which leads to a decrease in the intensification of apoptosis and, accordingly, a lower loss of β-cells. To elucidate the exact mechanism of the combination effect on the β-cell death process, additional research is required to identify the expression of factors that affect apoptosis (e.g., transcription factors, cytokines, effectors and regulators of apoptosis, etc.).

In the second series of the studies, it was found out that the combination of sitagliptin and aminoguanidine simultaneously (prophylactically) administrated with dexamethasone, significantly limits the hyperglycemic effect of the latter (progression of carbohydrate metabolism disorders). The revealed effect was less pronounced compared to the first series, which is possibly due to the complex effect of glucocorticosteroids on carbohydrate metabolism, with a limited number of application points of the studied combination, primarily the protection of β-cells. However, this approach may be of interest for the correction of persistent carbohydrate metabolism disorders after the withdrawal of glucocorticosteroids.

In the third series of the experiments, the therapeutic administration of a sitagliptin and aminoguanidine combination managed to significantly slow down the progression of streptozotocin-nicotinamide DM. That can be also associated with a decrease in the intensity of apoptosis and an improvement in the functional state of the remaining β-cells due to the combination of the hypoglycemic and protective effects of incretins (DPP-4 inhibitors) and the anti-inflammatory effect of aminoguanidine.

In the animals with obesity and DM, a certain hypoglycemic effect of sitagliptin and its combination with aminoguanidine, was also notified. It is known that obesity and insulin resistance cause a cascade of reactions, the common link of which is systemic inflammation with an increase in the secretion of pro-inflammatory cytokines. That accelerates damage to β-cells and the transformation of type 2 diabetes into type 1. The use of aminoguanidine in combination with sitagliptin can significantly affect various pathogenetic links initiated by hyperglycemia, in particular, reduce the level of systemic inflammation by inhibiting the activity of iNOS and its role in the destruction of β-cells. In the further work with this model, due to the severity of the pathology, it is advisable to use more effective incretin mimetics – agonists of the glucagon-like peptide-1 receptor.

CONCLUSION

A combined application of sitagliptin and aminoguanidine increases the antidiabetic effect of individual components, preventing the development of diabetes mellitus after the prophylactic alloxan administration, reducing the severity of carbohydrate disorders after the course administration of dexamethasone. When administered medically, it slows down the progression of streptozotocin-nicotinamide DM and reduces the level of glycemia in the animals with a genetic predisposition to diabetes mellitus and obesity. The sitagliptin and aminoguanidine combination can become a basis for the development of a new promising approach to the treatment of diabetes mellitus and its complications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Denis V. Kurkin, Andrey V. Strygin – study idea and planning, graphic material design, approval of manuscript final version; Tamara M. Andriashvili, Alina A. Sokolova, Nikita S. Bolokhov, Vladislav E. Pustynnikov, Evgeny A. Fomichev – pathology modeling, experimental work; Evgeny I. Morkovin – statistical data processing, graphic material design, text editing; Dmitry A. Bakulin, Yuliya V. Gorbunova – literature data collection and analysis, manuscript writing.

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DF-5 COMPOUND DELAYS DEVELOPMENT OF DIABETIC NEPHROPATHY IN RATS

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Advanced glycation end-products play an important role in the development of diabetic complications, so slowing down of glycated proteins' cross-links formation have been suggested as a potential therapeutic option for the treatment of vascular diabetic complications and preventing their progression.

The aim of the work was to assess the influence of novel anticrosslinking agent DF-5 on the renal advanced glycation end-products and collagen contents, body weight, blood glucose and glycated hemoglobin levels and the development of early renal disease in streptozotocin-induced diabetic rats.

Materials and methods. 40 male Sprague-Dawley rats were used in the study. Two months after inducing diabetes, the study substance was administered intragastrically once a day for 28 days (12.5 mg/kg). Measurements included the assessment of blood glucose and HbA1c levels, the evaluation of the renal function, and the results of histology and immunohistochemical staining of kidneys.

Results. A repeated intragastric administration of DF-5 for 30 days significantly reduced the level of HbA1c in the blood, but did not affect the level of fasting blood glucose. DF-5 compound significantly reduced proteinuria and prevented kidney damage in experimental animals by limiting damage of the glomeruli and tubules. It was found that DF-5 inhibits the progression of an early renal dysfunction in rats with streptozotocin-induced diabetic nephropathy. This was associated with a decreased accumulation of advanced glycation end-products in the kidney, accompanied by the improvement of both renal morphology and function.

Conclusion. The results obtained provide investigators with additional therapeutic options for the treatment of diabetic nephropathy and possibly other complications of diabetes.

Keywords: advanced glycation endproducts; cross-links; ALT-711 (alagebrium); diabetes mellitus; diabetic kidney disease (nephropathy); streptozotocin-induced diabetes.

Abbreviations: BSA – bovine serum albumin; ECM – extracellular matrix; GBM – glomerular basement membrane; DMSO – dimethyl sulfoxide; IR – infrared spectroscopy; ELISA – enzyme-linked immunosorbent assay; AGEs – advanced glycation end products; m.p. – melting point; NMR – nuclear magnetic resonance; IDF – International Diabetes Federation; HbA1c – glycated hemoglobin.

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СОЕДИНЕНИЕ ДФ-5 ЗАМЕДЛЯЕТ РАЗВИТИЕ ДИАБЕТИЧЕСКОЙ НЕФРОПАТИИ У КРЫС

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Конечные продукты гликирования играют важную роль в развитии осложнений сахарного диабета. По этой причине замедление образования поперечных сшивок гликированных белков, как предполагается, является потенциальным терапевтическим подходом к лечению и профилактике осложнений заболевания, связанных с поражением сосудов.

Цель. Оценка способности нового антисшивающего соединения ДФ-5 влиять на количество конечных продуктов гликирования и коллагена в почках, на массу тела, уровни глюкозы и гликированного гемоглобина, а также на развитие ранних проявлений поражения почек у крыс со стрептозотоциновым сахарным диабетом.

Материалы и методы. Работа проведена на 40 самцах крыс Sprague-Dawley. Через 2 месяца после индукции диабета исследуемое вещество вводили внутрижелудочно (12,5 мг/кг) 1 р/сут в течение 28 дней с помощью зонда. Определяли уровень глюкозы и гликированного гемоглобина в крови, оценивали функцию почек, а также проводили гистологическое и иммуногистохимическое исследования тканей почек.

Результаты. Регулярное внутрижелудочное введение ДФ-5 в течение 30 сут статистически значимо снижало уровень HbA1c в крови, но не влияло на уровень глюкозы в крови натощак. Соединение ДФ-5 существенно уменьшало протеинурию и предотвращало повреждение почек у экспериментальных животных за счет ограничения повреждений клубочков и канальцев. Было установлено, что соединение ДФ-5 замедляет повреждение почек на ранней стадии диабетической нефропатии, что сопровождается снижением количества конечных продуктов гликирования в ткани почек, улучшением их морфологической картины и функции.

Заключение. Полученные результаты открывают возможность для разработки дополнительного терапевтического подхода к лечению диабетической нефропатии и, возможно, других осложнений сахарного диабета.

Ключевые слова: конечные продукты гликирования; поперечные сшивки; ALT-711 (алагебриум); сахарный диабет; диабетическая болезнь почек (нефропатия); стрептозотоцин-индуцированный диабет

Список сокращений: БСА – бычий сывороточный альбумин; ВКМ – внеклеточный матрикс; ГБМ – гломерулярная базальная мембрана; ДМСО – диметилсульфоксид; ИК – инфракрасная спектроскопия; ИФА – иммуноферментный анализ; КПГ – конечные продукты гликирования; $T_{пл}$ – температура плавления; ЯМР – ядерный магнитный резонанс; IDF – Международная диабетическая федерация; HbA1c – гликированный гемоглобин.

INTRODUCTION

Diabetes is a serious chronic disease affecting an increasing number of people worldwide because of its prevalence, costs, and health effects [1]. According to estimates from the World Health Organization (WHO)[1] [2] in 2014, a total of 422 million adults had diabetes and in 2019, an estimated 1.5 million deaths were directly caused by this illness. The International Diabetes Federation (IDF) estimates that there were about 537 million people with diabetes worldwide in 2021, which indicates a rapid spread of the disease.

Diabetes and its complications are rapidly becoming the world's most significant cause of morbidity and mortality. In the case of chronic hyperglycemia, glucose and other reducing sugars react with proteins by a series of non-enzymatic reactions to form a class of

[1] World Health Organization (WHO). Diabetes. Available from: https://www.who.int/news-room/fact-sheets/detail/diabetes

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heterogeneous compounds that are called advanced glycation end products (AGEs) [2, 3]. Some of these AGEs form irreversible cross-links throughout the lifetime of many large proteins (such as collagen and elastin), covalently modifying their structure and damaging their function. AGEs formation in the long-lived connective tissue and extracellular matrix components is a causal factor in the development of long-term diabetic complications and ageing-related diseases, according to many studies [4–10]. AGEs are important in the pathogenesis of such diabetes complications as nephropathy, neuropathy, retinopathy, cataract, cardiomyopathy. AGEs are also involved in the formation of immunopathologies, neoplastic diseases and atherosclerosis. [2, 11, 12].

In case of a diabetic kidney disease (nephropathy), AGEs could accumulate in glomerular basement membrane (GBM), mesangial cells, endothelial cells and podocytes in patients with diabetes. Therefore, AGEs play an important role in the development and progression of nephropathy leading to the formation of glomerulosclerosis and tubulointerstitial fibrosis in the renal tissues [7]. In addition, heavily glycated proteins are more resistant to digestion by the proteasomal – as well as the lysosomal proteolytic systems [13]. The ability of anti-AGE agents to decrease tissue AGEs is believed to be a potential effective therapeutic approach to restore the elasticity of vascular extracellular matrix (ECM) as well as to treat vascular diabetic complications and prevent their progression.

In the present study, the improved procedure of the synthesis of a new representative of anti-crosslinking agents, 9-benzyl-2-biphenylimidazo[1,2-a] benzimidazole (DF-5 compound, **I**) is described, its *in vivo* activity, namely, the influence on glycated hemoglobin (HbA1c) levels, body weight, blood glucose and the development of early renal disease in streptozotocin-induced diabetic rats, is evaluated. The *in vivo* activity of this novel anti-crosslinking agent was compared with that of well-known AGE cross-link breaker ALT-711 (alagebrium) [14].

THE AIM of the work was to assess the influence of novel anti-crosslinking agent DF-5 on the renal advanced glycation end-products and collagen contents, body weight, blood glucose and glycated hemoglobin levels and the development of early renal disease in streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Materials

All the chemicals used in the present experiments were of analytical grade and commercially available. Streptozotocin and 3,3′,5,5′-tetramethylbenzidine were purchased from Sigma-Aldrich (USA); glucose was purchased from Agat-Med (Russia); bovine serum albumin (BSA), fraction V was purchased from Biowest (France); dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (USA); rabbit polyclonal anti-BSA antibody and goat anti-rabbit IgG secondary antibody were purchased from Cloud-Clone (USA); 10% (w/v) neutral buffered formalin, Masson's trichrome stain and hematoxylin were purchased from Moditech (Russia); homogenized paraffin media was purchased from Bio Vitrum (Russia); rabbit polyclonal anti-AGE antibody was purchased from Abcam (USA); ALT-711 was purchased from Kailu Xingli Pharmaceutical Co., Ltd. (China).

Synthesis of Benzyl-2-biphenylimidazo[1,2-a] benzimidazole (DF-5)

The synthesis of DF-5 compound (**I·HCl**) was carried out in the Institute of Physical and Organic Chemistry at Southern Federal University (Rostov-on-Don, Russia) from 2-amino-1-benzylbenzimidazole (**II**).

IR spectra (v/cm^{-1}) of new compounds were recorded on a Varian Excalibur 3100 FT-IR spectrophotometer (Varian, USA) for powdered samples using the method of attenuated total reflection; ^{1}HNMR spectra were recorded on a Bruker Avance 600 (Germany) spectrometer. Chemical shifts for ^{1}H were given as δ values in parts per million (ppm) relative to the signals of residual protons of a deuterated solvent DMSO-d_6 and $CDCl_3$ (δ 2.49 and 7.24, respectively) and coupling constants were in hertz (Hz).

Melting points were measured on a Fisher-Johns Melting Point Apparatus (Fisher Scientific, USA). The elemental analysis was carried out using a classical method [15]. The reaction progress and purity of the obtained compounds were monitored by thin layer chromatography (TLC) (plates with Al_2O_3 III degree of activity, eluent $CHCl_3$, visualization with iodine vapors in a moist chamber).

2-Imino-3-[(2-biphenyl-4-yl)-2-oxoethyl)]-1-benzylbenzimidazoline hydrobromide (III). 4-(bromoacetyl)biphenyl (2.75 g,10 mmol) was added to a hot solution of 2.23 g (10 mmol) of 2-aminobenzimidazole **II** in 65 mL of MeCN. The mixture was stirred until dissolving of the quaternizating reagent, then heated until the completion of the precipitate formation and kept for 6–8 h at room temperature. After that, the precipitate of salt **III** was filtered off and thoroughly washed with acetone. The yield was 94%, the m.p. was 254–256°C. The IR, v/cm^{-1}, was: 3207, 3240 (NH_2), 1687 (C=O). The following data were established: C, 67.47; H, 4.85; Br, 16.03; N, 8.43%. The ultimate

analysis for $C_{28}H_{24}BrN_3O$ was as follows: C, 67.45; H, 4.88; Br, 16.00; N, 8.39%. ^{1}HNMR (600 MHz, DMSO-d_6), δ: 5.57 (s, 2H, CH_2CO); 6.07 (s, 2H, $N_{Bzm}CH_2$); 7.30–7.36 (m, 5H, 5,6-H_{Bzm} + 3H_{Ph}); 7.40–7.48 (m, 3H, 2H_{Ph} +1H_{Biph}); 7.51–7.55 (m, 3H, 7-H_{Bzm} +2H_{Biph}); 7.67–7.68 (m, 1H, 4-H_{Bzm}); 7.81 (d, 2H, 2H_{Biph}, *J* = 7.2 Hz); 7.97 (d, 2H, H_{Biph}, *J* = 8.4 Hz); 8.19 (d, 2H, H_{Biph}, *J* = 8.4 Hz); 9.06 (s, 2H, N^+H_2).

9-Benzyl-2-(biphenyl-4-yl)-9*H*-imidazo[1,2-*a*] benzimidazole (I). A mixture of bromide **II** (2 g, 4.0 mmol), finely powdered Na_2CO_3 (0.89 g), EtOH (40 mL) and water (9 mL) was refluxed until the reaction completion (20–25 h). Then, the alcohol was distilled off from the reaction mixture, the water (25–30 mL) was added and the product was filtered off and dried in air. The obtained base was purified by column chromatography on Al_2O_3, $CHCl_3$ was used as eluent collecting a fraction with R_f 0.9. After the solvent evaporation, the residue was recrystallized from DMF to obtain 1.3 g (81%) of **I.**

A mixture of 2.0 g (4 mmol) of amine **III** and freshly melted sodium acetate 0.33 g (4 mmol) was boiled in glacial acetic acid (5 hr), controlling the completeness of the reaction by thin layer chromatography. Then the reaction mass was cooled to room temperature, the precipitate of imidazo[1,2-a]benzimidazole **I** was filtered off, washed with water and dried at 85°C. The yield was 1.5 g (92%), the m.p. was 228–229 °C. The IR, ν/cm^{-1}, was 1513, 1617 (C=C), 1664 (C=N). The following data were established: C, 84.21; H, 5.38; N, 10.46%. The ultimate analysis for $C_{28}H_{21}N_3$• HCl was as follows: C, 84.10; H, 5.30; N, 10.52%. ^{1}HNMR (600 MHz), δ: 5.76 (s, 2H, CH_2); 7.29–7.53 (m, 10H, 5H_{Ph}, 6,7-H_{Bzm} +3H_{Biph}), 7.76 (d, 2H, H_{Biph}, *J* = 7.9 Hz), 7.84 (d, 2H, 5,8-H_{Bzm}, *J* = 8.3 Hz), 7.97 (d, 1H, H_{Ar}, *J* = 7.8 Hz), 8.03 (d, 2H, H_{Ar}, *J* = 8.3 Hz); 8.64 (s, 1H, 3-H).

9-Benzyl-2-(biphenyl-4-yl)-9*H*-imidazo[1,2-*a*] benzimidazole hydrochloride (DF-5). This compound was prepared by treatment of imidazo[1,2-*a*]benzimidazole **I** with conc. HCl. The yield was 93%, the m.p. was 237–238°C. The IR, ν/cm^{-1}, was 1512, 1614 (C=C), 1663 (C=N). The following data were established: C, 77.11; H, 5.13; Cl, 8.08; N, 9.58%. The ultimate analysis for $C_{28}H_{21}N_3$• HCl was as follows: C, 77.14; H, 5.09; Cl, 8.13; N, 9.64%.

Cleavage of AGE-BSA-collagen complexes pre-formed *in vitro*

The ability of DF-5 to cause cleavage of pre-formed AGE-BSA-collagen complex was tested by an enzyme-linked immunosorbent assay (ELISA) technique [16] with minor modifications detalized in Patent RU 2627769 C1, Russia (2017) [17].

Collagen was extracted from rat tails by a 0.1% (w/v) solution of acetic acid for seven days, centrifuged (8000 g, 10 min) to precipitate the debris, and used to coat plate wells. The BSA solution (50 mg/mL) was incubated with glucose (0.5 M) in phosphate-buffered saline for three months. The AGE-BSA was added to plate wells and the incubation was carried out at 37°C for 4 h to form cross-links (AGE-BSA-collagen complexes). A solution of DF-5 or ALT-711 was added to the plate wells after the incubation and the plate was incubated at 37°C for 16 h. After washing, 80 µL/well of rabbit anti-BSA antibodies (1:500) were added to the plate wells and the plate was incubated for 50 min at 37°C. After the next washing, 80 µL/well of horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibodies were added (1:1000) to the plate wells and the plate was incubated for 50 min at 37°C. Then 100 µL/well of substrate 3,3′,5,5′-tetramethylbenzidine was added. After the 20-min incubation in darkness at room temperature, the reaction was stopped with 2M H_2SO_4. The absorbance of plate wells was measured at 450 nm with an Infinite M200 Pro microplate reader (Tecan, Austria).

When evaluating the activity of each compound, 4 groups of data were formed: 1) glycated BSA + collagen, 2) glycated BSA + collagen + test compound, 3) non-glycated BSA + collagen, 4) non-glycated BSA + collagen + test compound. To minimize the interference due to the non-specific adhesion of BSA to the collagen matrix, the results of the samples containing glycated BSA, were subtracted from the samples results of the appropriate composition containing non-glycated BSA.

Animals

Animal experiments were conducted in accordance with animal research standards defined by the Russian Federation law (GOST R 33044-2014 and GOST R 33647-2015) and EASC technical standards for Good Laboratory Practice. The study was performed after approval by the Local Research Ethics Committee, Volgograd, Russia [registration number: IRB 00005839 IORG 0004900 (OHRP)] dated June 5, 2015 (Protocol No. 2016-2015).

Male Sprague-Dawleyrats (5–6 weeks old, weighing 200–230 g) were purchased from LLC “NIC BMT”, Moscow, Russia. The animals were housed at Volgograd State Medical UniversityAnimal Care Unit. The animals were acclimated to the housing environment in a room with a 12/12-hour light/dark cycle at an ambient temperature of 25°C for 2 weeks. The animals had a free access to food and water before the study.

Diabetic model of rats

Diabetes was induced in rats by a single intraperitoneal injection of streptozotocin (65 mg/kg in citrate buffer, pH 4.5) after an overnight fast. The

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procedure was performed in 40 animals. Additionally, non-diabetic animals were injected with a citrate buffer only (n=10). Three days after the streptozotocin injection, only rats with blood glucose levels exceeding 15 mmol/L measured in fasting conditions were classified as diabetic and included in the study (n=30). The animals that had failed to reach this criterion were excluded from the current experiment (n=10). Five diabetic animals died during the next two months after the streptozotocin injection (before the treatment and groups formation).

The choice of the effective dose (12.5 mg/kg) for ALT-711 and DF-5 was based on the experimental studies results of ALT-711 in various animal models of diabetic complications described in the scientific literature [18–20]. Two months after inducing diabetes, the animals with diabetes (n=24) were randomly divided into three groups: the untreated diabetic control group (distilled water, 5 mL/kg) and two diabetic treatment groups, receiving either DF-5 or ALT-711 (12.5 mg/kg, dissolved in distilled water, 5 mL/kg). Three animals were excluded from the experiment at the beginning of the treatment: for achieving equal groups' volume – 8 animals per group – 2 non-diabetic rats and 1 diabetic rat. During a 4-week treatment period, the administration was performed *via* intragastral administration once a day in the morning (1 h after the lights were on). The untreated non-diabetic (ND) group of 8 healthy animals habituated to the same regiment and administration (distilled water, 5 mL/kg intragastric) was also included in this study. The animals' blood glucose level and body weight were monitored periodically. The study was carried out for over 12 weeks.

Biochemical analysis

The blood glucose level was measured using a blood glucose meter (Glucocard, Arkray, Japan) after the collection of blood samples from the tail vein. A HbA_{1c} concentration was quantified at the end of the study using a Diabet-Test Assay Kit (HbA_{1c}) (LLC Fosfosorb, Russia) according to the manufacturer's instruction).

Renal function

The renal function was assessed at the end of the study by measuring a daily urine output, a urinary total protein concentration and excretion. During the material collection, the rats were housed in the metabolic cages (Open Science, Russia) for a 24 h urine collection. Total urinary protein was measured using an assay kit (Total Protein-PK-Vital Cat. No. B 06.03, Vital Development Corporation, Russia) according to the manufacturer's instruction.

Kidney histology and immunohistochemistry

The animals were sacrificed *via* decapitation at the end of the experiment (the animals had been anesthetized with chloral hydrate, 400 mg/kg i.p.). The kidneys were removed and the renal cortex and medulla specimens were fixed for 24 h in 10% (w/v) neutral buffered formalin and embedded in paraffin. For the assessment of the injury, 3–5 µm thickness sections were stained with Masson's trichrome to evaluate glomerulosclerotic changes and a connective tissue deposition. Other formalin-fixed kidney sections were mounted on slides and stained with rabbit polyclonal anti-AGE antibodies according to the manufacturer's instructions. After the immunological reaction, the tissues were stained with hematoxylin. Imaging was performed with the Axiostar Plusmicroscope (Carl Zeiss Microscopy GmbH, Germany) and a digital camera Axiocam 105 color (Carl Zeiss Microscopy GmbH, Germany). The images were analyzed with Zeiss Zen Pro 2012 software (Carl Zeiss Microscopy GmbH, Germany). The results were expressed as the percentage of area with positive staining.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., USA). The data were analyzed by ANOVA followed by a Tukey post hoc test used for multiple comparisons versus the control group. The data normality test was carried out using Shapiro-Wilk test. A $p \leq 0.05$ was considered statistically significant. The data are presented as M ±SD.

RESULTS

Chemical synthesis

Compound **I** was synthesized from 2-amino-1-benzylbenzimidazole (**III**) by the reaction sequence depicted in Fig. 1 as described before [21] but with the additionally improved Stage 2. In the first stage, amine **III** was quaternized by an equimolar amount of 4-(bromoacetyl)biphenyl (MeCN, room temperature, 6–8 h) and a nearly quantitative yield of salt **III** was obtained. Then, in the final stage, this salt was cyclized in MeCOOH (reflux, 5 h) in the presence of sodium acetate. The yield of compound **I** was 92%, which was much more than for cyclization of salt **III** in aq. EtOH [21], while the reaction time was much shorter in this case. It has also been notified that in refluxing DMF, the cyclization proceeds was even faster (15–20 min), but with a low yield.

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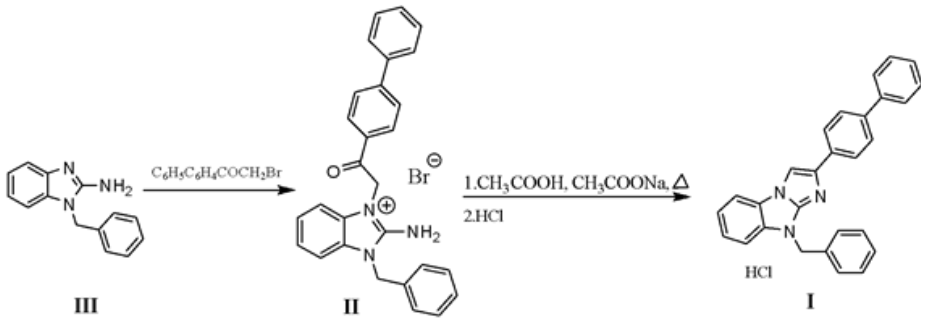

Figure 1 – Synthesis scheme for preparation of 9-benzyl-2-biphenylimidazo[1,2-a]benzimidazole hydrochloride (DF-5)

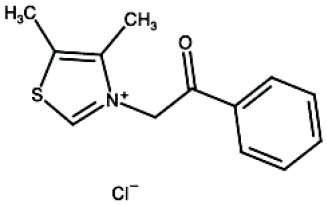

Figure 2 – Chemical structure of ALT-711 (alagebrium)

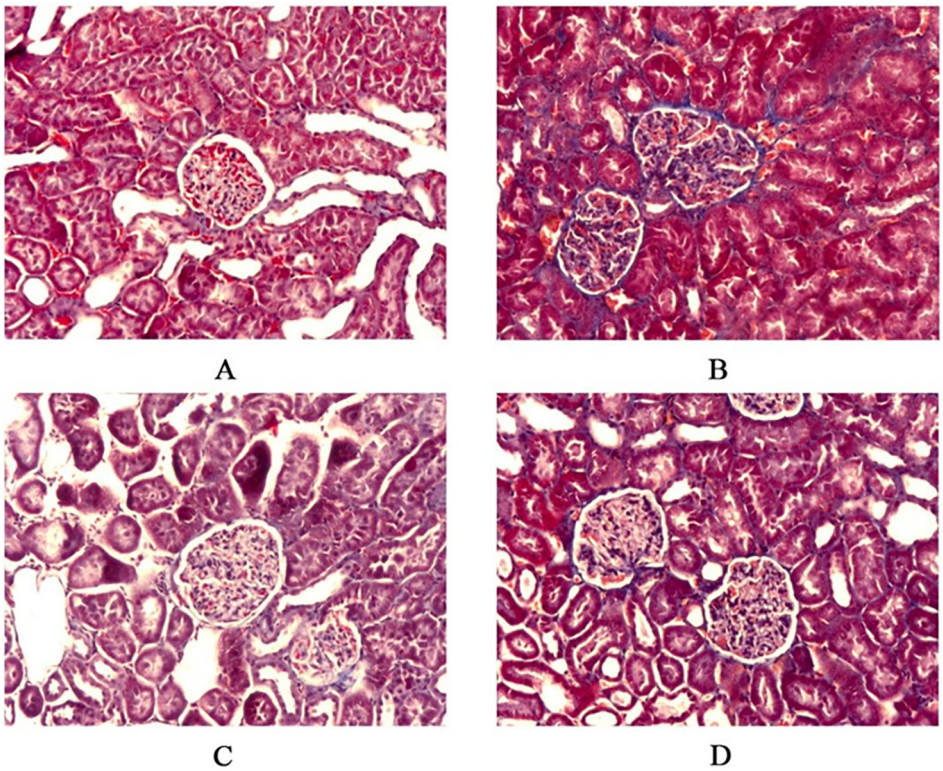

Figure 3 – Glomerular histopathology. Effect of diabetes and either DF-5 or ALT-711 on kidney tissues
Note: Masson trichrome staining: (A) non-diabetic control, (B) diabetic control, (C) diabetic + ALT-711 and (D) diabetic + DF-5. Magnification ×100.

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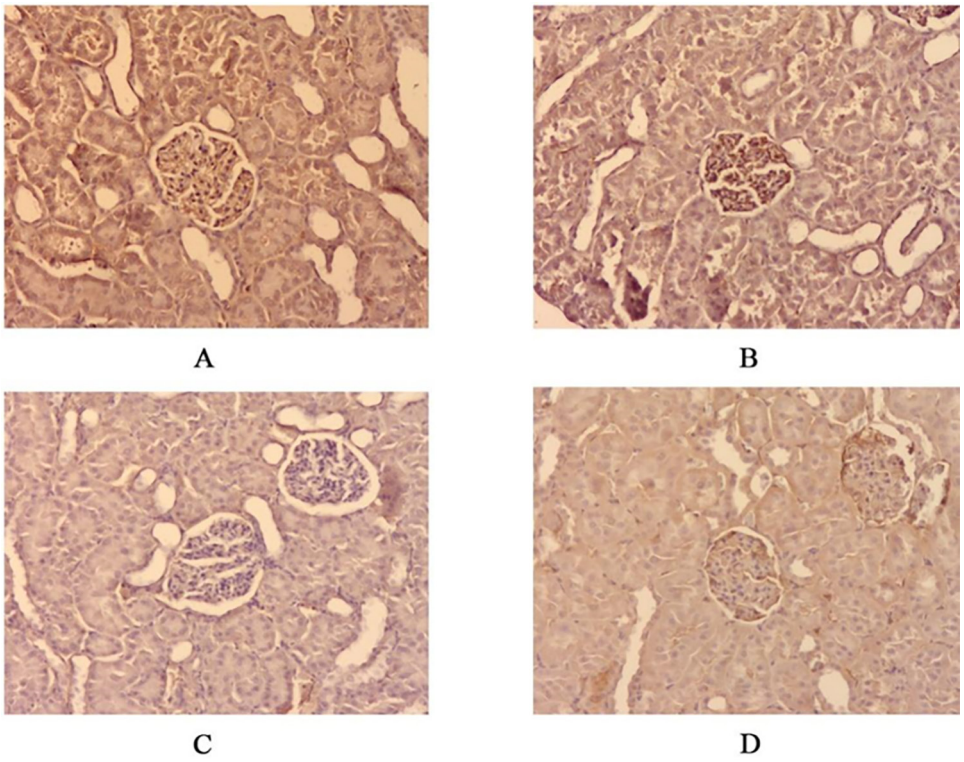

Figure 4 – Effect of diabetes and either DF-5 or ALT-711 on renal AGEs accumulation
Note: Immunohistochemical staining for AGEs in kidneys: (A) non-diabetic control, (B) diabetic control, (C) diabetic + ALT-711 and (D) diabetic + DF-5. Magnification ×100.

Table 1 – Body weight, blood glucose, HbA1c levels and mortality levels in non-diabetic (ND) and diabetic (D) rats treated with or without DF-5 or ALT-711 at the end of the study

Group	Final body weight (g)	Blood glucose (mmol/L)	HbA_{1c} (%)	Mortality
ND + vehicle	455.25±17.82	5.37±0.18	6.42±0.49	0/8
D + vehicle	323.80±18.75a	26.83±1.14[a]	19.77±0.08[a]	3/8
D + DF-5 (12.5 mg/kg)	358.00±32.35	27.93±0.95[a]	11.46±1.39[ab]	2/8
D + ALT-711 (12.5 mg/kg)	335.83±20.80	25.78±0.43a	12.30±1.88ab	2/8

Note: [a] – $p \leq 0.05$ vs. ND rats; [b] – $p \leq 0.05$ vs. D rats.

Table 2 – Renal function and urinary protein excretion in non-diabetic (ND) and diabetic (D) rats treated with or without DF-5 or ALT-711 at the end of the study

Group	Urine output (mL/24 h)	Urinary total protein concentration (g/L)	Urinary total protein excretion (mg/24 h)
ND + vehicle	15.00 ± 1.54	0.017 ± 0.004	0.26 ± 0.08
D + vehicle	23.20 ± 1.62[a]	0.058 ± 0.005[a]	1.32 ± 0.08[a]
D + DF-5 (12.5 mg/kg)	21.83 ± 1.58[a]	0.025 ± 0.007[ab]	0.59 ± 0.21[ab]
D + ALT-711 (12.5 mg/kg)	22.83 ± 4.15[a]	0.033 ± 0.005[ab]	0.72 ± 0.13[ab]

Note: [a] – $p \leq 0.05$ vs. ND rats; [b] – $p \leq 0.05$ vs. D rats.

Table 3 – Morphometric analysis of glomerular damage in Masson trichrome-stained kidney sections in non-diabetic (ND) and diabetic (D) rats treated with or without DF-5 or ALT-711 at the end of the study

Group	Total glomerular area (μm²)	Glomerular connective tissue area (μm²)	Relative area of glomerular connective tissue (%)
ND + vehicle	14885.17±1932.23	695.92±195.25	4.6
D + vehicle	12572.63±1015.25	3356.46±1871.64[a]	26.7[a]
D + DF-5 (12.5 mg/kg)	14753.21±975.23	2397.29±832.43	16.2[b]
D + ALT-711 (12.5 mg/kg)	13968.94±856.26	1640.38±543.19	11.7[b]

Note: [a] – $p \leq 0.05$ vs. ND rats; [b] – $p \leq 0.05$ vs. D rats.

Table 4 – AGEs staining in kidneys of non-diabetic (ND) and diabetic (D) rats treated with or without DF-5 or ALT-711 at the end of the study

Group	Total glomerular area (μm2)	Positive AGE-stained glomerular area (μm2)	Relative AGE-stained glomerular area (%)
ND + vehicle	15287.35 ±1759.13	1634.04 ± 759.17	10.68
D + vehicle	12121.19 ±1234.57	3746.13 ±1098.73	30.90[a]
D + DF-5 (12.5 mg/kg)	12982.17 ±971.56	2471.09± 658.81	17.48[b]
D + ALT-711 (12.5 mg/kg)	13106.82 ± 1346.49	2117.85 ± 732.43	19.03[b]

Note: [a] – $p \leq 0.05$ vs. ND rats; [b] – $p \leq 0.05$ vs. D rats.

Cleavage of AGE-BSA-collagen cross-links pre-formed *in vitro*

It was found that DF-5 exhibits a significant anti-crosslinking activity on AGE-BSA-collagen pre-formed cross-links (half-maximal inhibitory concentration, IC_{50}=0.31 mM), which is 6-fold more potent than the activity of ALT-711 (the structure is shown in Fig. 2), a well-known cross-link breaker (IC_{50}=1.89 mM). The ability of the ALT-711 compound to break cross-links has been described, although the mechanism of action has not yet been established. A more detailed description is given in Patent RU 2627769 C1, Russia (2017) [17]. It has been notified that based on the present experiment, the exact mechanism of the decrease in crosslinking cannot be assumed and that is why it is called "an anti-crosslinking agent" instead of "a cross-link breaker".

The design of the experiment takes into account the correction for interference associated with non-specific adhesion of BSA to the surface of the collagen matrix and the plate plastic. For this reason, compounds DF-5 and ALT-711 are believed to act on a certain product formed as a result of the interaction of albumin glycation products with amino acid residues of collagen, and attach albumin to the collagen matrix.

Cyclic beta-keto iminium cations are well suited for the role of products that react on the albumin side and quickly form a cross-link. They are the result of the cyclization of Lederer's glucosone or pentosone. Reacting with the free guanidine group of arginine, they are able to form glucosepane or pentosidine in several stages. It is possible that Lederer's glucosone and pentosone, as well as Amadori products that precede them, are also important for the formation of complexes that fix BSA on the collagen matrix, but their contribution remains to be established.

Animal body weight

At the end of the study, the mean final body weights of the diabetic animals were significantly lower than those of non-diabetic rats (Table 1; $p \leq 0.05$). DF-5 and ALT-711-treated diabetic rats showed a slight increase in weight compared to their untreated counterparts, but the difference was statistically significant only in case of DF-5-treated diabetic rats (Table 1; $p \leq 0.05$).

Glycemic control

Diabetic rats had significantly increased blood glucose and HbA_{1c} levels compared with the non-diabetic rats (Table 1; $p \leq 0.05$). The elevated HbA_{1c} contents observed in diabetic animals, were significantly decreased by almost 40% with the treatment of either DF-5 or ALT-711 (Table 1; $p \leq 0.05$). The treatment of diabetic rats with DF-5 or ALT-711 only slightly, not significantly, reduced a blood glucose concentration.

Renal function and histology

Diabetes was associated with an increased urinary volume output and a urinary total protein excretion ($p \leq 0.05$ vs. non-diabetic control; Table 2). At the end of the study, the diabetic rats had an elevated proteinuria

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(Table 2). The treatment of diabetic rats with DF-5 or ALT-711 significantly prevented an increase in a urinary total protein concentration (≈50%, $p \leq 0.05$), but not in a daily urine output, compared to the untreated diabetic rats (Table 2).

A histopathological examination showed an increased kidney tissue damage in the diabetes group. Diabetes is typically accompanied by a progressive glomerular and tubular damage. At the end of the study, the kidneys of rats in the diabetes group showed significant morphological changes compared with the non-diabetic control group (Fig. 3). The GBM thickening and mesangial matrix expansion in the glomeruli were observed in the diabetic rats. GBM thickening and a range of mild to moderate mesangial matrix expansion causing capillary luminal narrowing were consistently observed. Compared to the non-diabetic control animals, the mean glomerular area was slightly decreased, but the difference was not statistically significant, and the mean glomerular connective tissue area and the relative area of the glomerular connective tissue were significantly ($p \leq 0.05$) 4.8-fold and 5.8-fold increased, respectively, in the diabetes group (Table 3). The treatment of diabetic animals with either DF-5 or ALT-711 significantly reduced the extent of the glomerular damage (Fig. 3). The mean relative areas of the glomerular connective tissue observed in the streptozotocin-diabetic rats treated with either DF-5 or ALT-711, were significantly ($p \leq 0.05$) 1.6-fold and 2.3-fold lower, respectively, than in the vehicle-treated diabetic animals (Table 3).

Kidney AGEs level

Immunohistochemical staining for AGEs in the rats' kidneys demonstrated that there was a widespread staining for AGEs in the diabetic rats glomeruli compared to the non-diabetic control rats. This increased staining was attenuated by treatment of diabetic rats with either DF-5 or ALT-711 (Fig. 4). The mean relative AGE-stained glomerular areas observed in the streptozotocin-diabetic animals treated with either DF-5 or ALT-711, were significantly ($p \leq 0.05$) 1.6-fold and 1.8-fold lower, respectively, than in the diabetic vehicle-treated group (Table 4).

DISCUSSION

Protein glycation and formation of AGEs play an important role in the pathogenesis of long-term diabetes mellitus complications like retinopathy, nephropathy, neuropathy, cardiomyopathy along with some other diseases such as rheumatoid arthritis, osteoporosis and ageing [2]. Glycation and cross-linking of proteins not only lead to a decrease in the elasticity of blood vessels, but also affect the structural integrity and physiological functions of internal organs.

Glycation is a stepwise process involving the formation of hemiaminal, Schiff base, enaminol, Amadori product, enediol, Lederer's glucosone (or pentosone). Then it undergoes an intramolecular condensation between the amino group and the terminal aldehyde function, which affords the positively charged ring compound named a cyclic beta-keto iminium ion (6 or 7 membered) [2, 22]. The latter, reacting with the guanidine group of arginine, forms complex and stable cross-links, such as glucosepane or pentosidine. Early products also decompose into carbonyl compounds (methylglyoxal, glyoxal, etc.), which are capable of forming other types of crosslinking AGEs, such as MOLD and GOLD [22]. All AGEs are characterized by a high stability.

Diabetic nephropathy is defined as a diabetes-associated progressive decline in the glomerular filtration rate, accompanied by proteinuria and other kidneys complications [2]. In relation to the kidneys, glycation and activation of the complement cascade *via* recognition of glycated proteins by mannan-binding lectin and/or dysfunction of glycated complement regulatory proteins [23], activation of signaling cascades of receptors for AGEs [24] etc. are noted. Key events in GBM, mesangial and tubulointerstitial matrix, such as an increase in collagen IV and VI, an increased glycation of collagen IV, an increased cross-linking of collagen IV, an increase in laminin and fibronectin, an increase in collagen type I etc., are directly related or indirectly associated with glycation [25]. The expansion of the mesangial matrix and thickening of the GBM, podocyte injury, glomerulosclerosis and renal fibrosis are observed [26–28].

Cleavage of pre-formed AGEs within the kidney by a cross-link breaker, such as ALT-711, is believed to confer renoprotection in diabetes. Forbes J.M. et al. (2001) believed that ALT-711 might provide a new kind of therapy for the treatment of diabetic nephropathy. The intervention with ALT-711 from weeks 16–32 of the study had the capacity to not only improve functional parameters, such as albuminuria, but also markers of structural injury, including glomerulosclerosis, a tubulointerstitial injury, and an oxidative stress [29].

The mechanism of a cross-link breaking activity is not yet entirely understood. According to the data originally proposed for the ALT-711 analogue, phenylthiazolium bromide [16], it involves the cleavage of the C–C bond between two adjacent carbonyl groups. AGEs of this 1,2-dicarbonyl structural type, however, have not yet

been experimentally identified, although the cross-links' rupture has been immunologically proved by experiments with antibodies to one or another of the two cross-linked proteins [30-33]. It should be noted that such a C–C bond between two adjacent carbonyl groups is presented in some early glycation products [22]. In addition, there is compelling evidence from *in vivo* experiments that demonstrate the positive effect of ALT-711 on diabetic nephropathy [34, 35]. The concept of a direct destruction of AGE cross-links in the whole organism is controversial, [36] despite convincing evidence of the activity of AGE cross-link breakers in diabetic nephropathy. The anti-crosslinking mechanism can theoretically be realized by hydrolysis, through the cleavage of immature cross-links (an anti-crosslinking activity), formed on early stages of the cross-linking process, and through the inactivation of early products of glycation.

It can be assumed that in the whole organism, the inactivation of intermediate pre-crosslinked electrophilic products, glucose-derived adducts or free derivates of glucose, are possible mechanisms of action. Their structures are similar to the structure of a theoretical dicarbonyl cross-link which has never been found out [14, 16]. Such products may include Schiff bases, carbonyl compounds, α-oxoaldehyde protein adducts etc. As for α-oxoaldehydes, their participation in the formation of glycation cross-links has been proved for 2-ammonio-6-({2-[(4-ammo-nio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1H-imidazol-5-ylidene}amino) hexanoate (GODIC), 2-ammonio-6-({2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene}amino) hexanoate (MODIC), glucosepane [37, 38], and many more. The next important product, cyclic b-keto iminium ion, possesses electrophilic properties [22] and, when interacting with the guanidine group, forms pentosinane at one stage, pentosidine at two stages (if it is a 6-member ring) or glucosepane (if it is a 7-member ring).

The possibility of the early α-oxoaldehyde adducts destruction by cross-link breakers is described in [39], using glycation of apolipoprotein A-I by methylglyoxal in the presence of ALT-711. This is discussed as a possible action mechanism for cross-link breakers in [40], and a direct possibility of methylglyoxal binding (representative of α-oxoaldehydes) by ALT-711 was considered in [40].

Thus, the hypothesis of the action mechanism assumes the ability of cross-link breakers to produce nucleophilic attacks on electrophilic glycation intermediates and early products of cross-linking (an anti-crosslinking activity). A detailed molecular mechanism of the likely reaction requires clarification, but considering nucleophilicity of DF-5 and ALT-711, this mechanism is possible.

An *in vivo* study was conducted to confirm whether the DF-5 compound could prevent the development of an early diabetic kidney disease in streptozotocin-induced diabetic rats. The test compound or the reference compound ALT-711, were administered intragastrically to the experimental animals for 30 days. Throughout the whole duration of the experiment, the blood glucose level of diabetic rats was not less than 15 mmol/L, which contributed to the development of diabetic vascular complications. At the end of the study, the animals' body weight, blood glucose and HbA_{1c} levels as well as renal function and histology in the groups of non-diabetic, diabetic rats treated with vehicle, DF-5 or ALT-711, were assessed and compared.

Being administered intragastrically on a regular basis for 30 days, DF-5 significantly reduced HbA_{1c} levels in blood, but demonstrated the absence of significant influence on fasting blood glucose levels. This fact likely points to the ability of DF-5 to act as assumed above – a breaker of early-stages products, including the so called Amadori products (HbA_{1c} has a structure of products of such a type) [41]. According to the above discussed hypothesis on the mechanism of the AGE cross-link breakers action, they could detach reversible glucose from its reversible adducts with proteins (Schiff bases and/or Amadori products) [42].

The current study has demonstrated the possibility of preventing diabetic nephropathy in streptozotocin-induced diabetic rats by using DF-5. As expected, diabetes induced an increase in proteinuria over time. The test compound DF-5 significantly reduced proteinuria and prevented a kidney damage in the experimental animals by limiting glomerular and tubular injuries. A similar effect has also been described for ALT-711 [43]. The observed phenomena, such as a reduced increase in the amount of the connective tissue and a reduced content of AGEs in the kidneys when compared with a control group of animals, are probably characteristic properties of the anti-crosslinking agents activity.

The accumulation of AGEs is associated with a renal production of extracellular matrix components in diabetes. An early intervention in this process can ameliorate long-term functional and structural features of diabetic nephropathy. In this study, the AGEs accumulation within the kidney was increased by diabetes and attenuated by the treatment with DF-5 compound.

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CONCLUSION

To sum up, the novel anti-crosslinking agent DF-5 has been identified. However, the elucidation of the exact action mechanism of the compound requires further research. DF-5 (12.5 mg/kg) inhibits the progression of an early renal dysfunction in streptozotocin-induced diabetic rats. This compound improves both functional and morphologic damages of experimental diabetic nephropathy. These changes were associated with a decreased accumulation of AGEs in the kidney. These findings provide investigators with additional therapeutic options for the treatment of diabetic nephropathy and possibly other diabetes complications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Alexander A. Spasov – supervision, conceptualization; Olga N. Zhukovskaya, Haider S.A. Abbas, Anatoliy S. Morkovnik – DF-5 synthesis and structure elucidation; Andrey I. Rashchenko, Anasthasia A. Brigadirova, Roman A. Litvinov, Natalia A. Gurova – methodology, investigation, software, article writing; Alexey V. Smirnov, Nikolay G. Pan'shin – investigation, visualization; Andrey I. Rashchenko, Anasthasia A. Brigadirova, Natalia A. Gurova – data processing; Roman A. Litvinov – hypothesis development of the action mechanism not associated with a direct breaking of mature glycated proteins' cross-links. All the authors have read and approved of the final manuscript version.

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RESEARCH ARTICLE

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BIOEQUIVALENCE STUDY OF GENERIC MOLNUPIRAVIR IN HEALTHY VOLUNTEERS

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Molnupiravir is one of the drugs for the etiotropic therapy of a new coronavirus infection COVID-19. It has confirmed its clinical efficacy in the treatment of patients with mild and moderate COVID-19, including those who are at high risk of progressing to severe disease.

The aim of the study was to evaluate bioequivalence of the generic drug molnupiravir ALARIO-TL and the original drug Lagevrio with a single oral administration in healthy volunteers.

Materials and methods. This bioequivalence study was an open, randomized, two-period crossover study. In each of the two periods, volunteers received a single dose of the test drug, or reference drug molnupiravir, in the form of capsules at the dose of 200 mg. The washout period between the doses was 3 days. To determine pharmacokinetic (PK) parameters and bioequivalence, the concentration the concentration of N-hydrozycytidine (NHC), the main molnupiravir metabolit in the blood plasma of volunteers was evaluated. The blood plasma sampling was carried out in the range from 0 to 16 hours in each of the study periods. Bioequivalence was assessed by comparing 90% confidence intervals (CIs) for the ratio of geometric means of $AUC_{(0–16)}$ and C_{max} of the test drug and reference drugs with the established equivalence limits of 80.00 – 125.00%.

Results. A total of 28 healthy male volunteers were included in the study. According to the results of the statistical analysis, after the administration of the test and reference drugs, the 90% CIs for the ratio of the geometric means of $AUC_{(0–16)}$ and C_{max} were 96.31% – 113.64% and 91.37% – 114.8%, respectively. These intervals fit within the established limits of 80.00–125.00%, which confirms the bioequivalence of the drugs. When comparing the frequency of the individual adverse events registration, no significant differences were found out after the administration of the test and reference drugs.

Conclusion. Based on the results of this study, it can be concluded that the test and reference drugs of molnupiravir are bioequivalent. In addition, the data obtained indicate that the drugs have similar safety profiles.

Keywords: COVID-19; molnupiravir; bioequivalence; pharmacokinetics; N-hydroxycytidine

Abbreviations. COVID-19 – a novel coronavirus infection caused by the SARS-CoV-2 virus; NHC, N-hydroxycytidine; CI – confidence interval; AUC – area under the concentration-time curve; $AUC_{0–t}$/$AUC_{0–16}$ – area under the concentration-time pharmacokinetic curve from zero to the last blood withdrawal at which the drug concentration is equal to or higher than the lower limit of quantitation; $AUC_{0–\infty}$ – area under the concentration-time pharmacokinetic curve, starting from zero time, extrapolated to infinity; C_{max} – the maximum concentration of the drug in blood plasma; NHC-TP – N-hydroxycytidine triphosphate; T_{max} – time to reach the maximum concentration; HPLC-MS/MS – high performance liquid chromatography with tandem mass spectrometry; GLP – Good Laboratory Practice; AE/SAE – undesirable/serious adverse event; CDKT – comparative dissolution kinetics test; BMI – body mass index.

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ИССЛЕДОВАНИЕ БИОЭКВИВАЛЕНТНОСТИ ВОСПРОИЗВЕДЕННОГО ПРЕПАРАТА МОЛНУПИРАВИРА У ЗДОРОВЫХ ДОБРОВОЛЬЦЕВ

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Молнупиравир – один из препаратов этиотропной терапии новой коронавирусной инфекции COVID-19, который подтвердил свою клиническую эффективность в терапии пациентов с лёгким и среднетяжёлым течением, в том числе с факторами риска развития тяжёлого течения.

Цель. Оценка биоэквивалентности воспроизведенного препарата молнупиравира АЛАРИО-ТЛ и оригинального препарата Лагеврио при однократном пероральном применении у здоровых добровольцев.

Материалы и методы. Данное исследование биоэквивалентности представляло собой открытое рандомизированное двухпериодное перекрестное исследование. В каждом из двух периодов добровольцы принимали однократно исследуемый или референтный препарат молнупиравира в виде капсул в дозе 200 мг. Отмывочный период между приемами препаратов составил 3 сут. Для определения фармакокинетических параметров и биоэквивалентности оценивали концентрацию основного метаболита молнупиравира N-гидроксицитидина (NHC) в плазме крови добровольцев. Отбор образцов плазмы крови производили в интервале от точки 0 до 16 ч в каждом из периодов исследования. Биоэквивалентность оценивали, сравнивая 90% доверительные интервалы (ДИ) для отношения средних геометрических значений $AUC_{(0–16)}$ и C_{max} исследуемого и референтного препаратов с установленными пределами эквивалентности, равными 80,00–125,00%.

Результаты. Всего в исследование было включено 28 здоровых добровольцев мужского пола. По результатам проведенного статистического анализа, 90% ДИ для отношения средних геометрических показателей $AUC_{(0–16)}$ и C_{max} после приема исследуемого и референтного препаратов составили 96,31% – 113,64% и 91,37% – 114,8%, соответственно. Данные интервалы укладываются в установленные пределы 80,00–125,00%, что подтверждает биоэквивалентность препаратов. При сравнении частоты регистрации отдельных нежелательных явлений не было выявлено достоверных различий после приема исследуемого и референтного препаратов.

Заключение. По результатам данного исследования можно заключить, что исследуемый и референтный препараты молнупиравира биоэквивалентны. Кроме того, полученные данные указывают на то, что препараты обладают сходными профилями безопасности.

Ключевые слова: COVID-19; молнупиравир; биоэквивалентность; фармакокинетика; N-гидроксицитидин

Список сокращений: COVID-19 – новая коронавирусная инфекция, вызванная вирусом SARS-CoV-2; NHC – N-гидроксицитидин; ДИ – доверительный интервал; AUC – площадь под кривой «концентрация–время»; $AUC_{0–t}$/$AUC_{0–16}$ – площадь под фармакокинетической кривой «концентрация–время» от нуля до последнего отбора крови при котором концентрация препарата равна или выше нижнего предела количественного определения; $AUC_{0–\infty}$ – площадь под фармакокинетической кривой «концентрация–время», начиная с нулевого значения времени, экстраполированная до бесконечности; C_{max} – максимальная концентрация препарата в плазме крови; NHC-TP – N-гидроксицитидин трифосфат; T_{max} – время достижения максимальной концентрации; ВЭЖХ-МС/МС – высокоэффективная жидкостная хроматография с тандемной масс-спектрометрией; GLP – надлежащая лабораторная практика; НЯ/СНЯ – нежелательное/серьезное нежелательное явление; ТСКР – тест сравнительной кинетики растворения; ИМТ – индекс массы тела.

INTRODUCTION

The novel coronavirus infection (COVID-19) pandemic has significantly increased the burden on healthcare systems around the world and required decisive measures, in particular, an active search for effective treatments [1–4]. Currently, there are 3 main areas of therapy for the treatment of COVID-19: etiotropic (antiviral), pathogenetic and symptomatic.

Molnupiravir is an antiviral drug that is effective against SARS-CoV-2. It is a prodrug that is chemically a 5'-isobutyrate ester of the ribonucleoside analog of N-hydroxycytidine (NHC). Once in the bloodstream, molnupiravir is hydrolyzed to NHC, which, upon penetrating into the cell, is transformed to pharmacologically active N-hydroxycytidine triphosphate (NHC-TP). NHC-TP, in turn, is inserted into viral RNA by viral RNA polymerases and generates errors in the genetic code of the virus. Genome errors caused by NHC-TP, accumulate, disrupting viral replication. Thus, the antiviral effect of molnupiravir is realized [5–7].

In clinical studies, molnupiravir has demonstrated efficacy in the treatment of the novel coronavirus infection COVID-19 and a favorable safety profile [8–12]. The recommended dosage regimen of molnupiravir for COVID-19 is 800 mg twice/daily, regardless of food intake for 5 days.

At the end of 2021, molnupiravir was approved for the use in adult patients with mild to moderate COVID-19 with risk factors for developing a severe disease in various countries, incl. the US, Europe and the UK. In addition, it is included in the Interim Guidelines of the Ministry of Health of Russia "Prevention, diagnosis and treatment of a novel coronavirus infection (COVID-19)", starting with version 14 dated 27 December, 2021.

The original drug of molnupiravir is Lagevrio, which was registered in Russia in 2022 by "MSD Pharmaceuticals" LLC and is presented in the form of capsules, 200 mg. LLC "Technology of Medicines" has developed a generic drug molnupiravir – ALARIO-TL. To confirm the bioequivalence of the developed generic and original drugs, this bioequivalence study has been conducted.

THE AIM of the study was to evaluate bioequivalence of the generic drug molnupiravir ALARIO-TL and the original drug Lagevrio with a single oral administration in healthy volunteers.

MATERIALS AND METHODS

Study design

The bioequivalence of drugs was assessed as the 1st stage of a clinical trial with a combined two-stage design (No. CJ051025138). The first stage of this study, i.e. the bioequivalence study, was a randomized two-period crossover study with a single oral fasting dose of 200 mg of test and reference drugs in healthy volunteers.

The study design was developed taking into account both Russian recommendations for conducting bioequivalence studies[1] and international guidelines[2]. The WHO recommendations[3] on conducting bioequivalence studies of drugs based on molnupiravir, were also taken into account when planning the design.

Prior to the start of the study protocol No. CJ051025138 was approved by the Russian Ministry of Health and the Ethics Council under it (Permission No. 294 dated 20 Apr, 2022), as well as by the local ethics committees of the study site (Protocol No. 236 dated 28 Apr, 2022). The study was conducted in full compliance with the requirements of Good Clinical Practice of the International Council for Harmonization (ICH GCP) E6 (R2), the rules of good clinical practice of the Eurasian Economic Union, the ethical principles of the Declaration of Helsinki of the last revision and other applicable legislative acts of the Russian Federation and the Eurasian Economic Union.

The clinical stage of the bioequivalence study was conducted on the basis of Eco-safety Research Center LLC from Apr 28 to May 18, 2022.

Before starting the study, a *in vitro* equivalence dissolution test and a comparative quantitation were performed using the same series that were subsequently used in the bioequivalence study. *In vitro* equivalence dissolution test was carried out using a paddle stirrer type device and a device for immersion under the conditions of a stirrer rotation of 75 rpm, the temperature of 37±0.5°C, and the medium volume of 900 ml. Three dissolution media were used for testing: a buffer solution pH 1.2; an acetate buffer solution pH 4.5; a phosphate buffer solution pH 6.8. The samples were analyzed at points 10, 15, 20, 30, 45, and 60 min by UV spectrophotometry. As a research result of both the test and the reference drugs, in all media, the release of more than 85% of the active substance was observed

[1] Decision of Council of the Eurasian Economic Commission of November 3, 2016 No. 85 "About approval of Rules of carrying out researches of bioequivalence of medicines within the Eurasian Economic Union".

[2] Committee for Medicinal Products for Human Use (CHMP). Guideline on the investigation of bioequivalence. Doc. Ref.: CPMP/EWP/QWP/1401/98 Rev. 1/ Corr **, 2010. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-bioequivalence-rev1_en.pdf

[3] WHO, Guidance Document 15 November 2021, Notes on the design of bioequivalence study: Molnupiravir. Available from: https://extranet.who.int/pqweb/sites/default/files/documents/BE_molnupiravir_Nov2021.pdf

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within 15 min, which made it possible to consider the dissolution kinetics equivalent without mathematical evaluations. The quantification showed that the content of the active substance in the preparations differed by no more than 5%, and the release profiles of molnupiravir *in vitro* equivalence dissolution test were equivalent, which confirmed the correctness of the series choice of the test and reference drugs.

Study population

In total, 31 healthy volunteers were screened, and 28 volunteers of them were successfully screened and randomized (14 volunteers in each group). The randomization was carried out using the envelope method. The main inclusion criteria were: a male gender; an age of 18–45 years; a body mass index 18.5–30 kg/m^2, a verified diagnosis "healthy" according to standard clinical, laboratory and instrumental methods of examination. The volunteers with a positive test for SARS-CoV-2, an aggravated allergic history, hypersensitivity to the components of the study products, as well as chronic diseases of various organ systems, were not allowed to participate in the study. The criteria for an discontinuation from the study were: withdrawal of an informed consent, the occurrence of adverse events (AEs) or serious adverse events (SAEs) in volunteers, in which a further participation in the study was undesirable, death, use of the prohibited therapy, and significant protocol violations. No replacing of the retired volunteers had been provided.

Administration of study products

In this research, the test (T) was a generic drug of molnupiravir – ALARIO-TL ("R-Pharm" JSC, Russia), in the dosage form of capsules, 200 mg. As a reference drug (R), the original drug Lagevrio, capsules, 200 mg (Patheon Pharma Services, Thermo Fisher Scientific Inc, USA) was used. The volunteers were randomized to one of the groups with a different sequence of dosing (TR or RT). The drugs were administrated twice with an interval of 3 days at the dose of 200 mg (1 capsule). In group No. 1 (TR), in the 1st period, the volunteers were administrated with the test drug once, and in the 2nd period – with the reference drug; in group No. 2 (RT) – vice versa. The duration of the washout period was chosen to exclude a possible effect of the molnupiravir administration in the 1st period of the study on the pharmacokinetic (PK) parameters of the drug in the 2nd period. The average half-life of the main molnupiravir NHC metabolite is about 3.3 h. Accordingly, to guarantee a decrease in the concentration of molnupiravir below the lower limit of quantitation in the volunteers at the beginning of the 2nd period of the study, the interval between the drugs doses should be at least 5 half-lives, i.e., at least 16.5 h.

Drugs were administrated in the morning on an empty stomach after refraining from eating for at least 10 hours with 200 ml of non-carbonated drinking water at the room temperature. The volunteers were to be in the "sitting" position for 4 hours after taking the drug (it was permissible to get up and walk, the "lying" position was not allowed). If during the first 4 h after taking the drug in any of the periods of the study, a volunteer experienced vomiting or diarrhea, he dropped out of the study.

Sampling and sample preparation

To assess the NHC concentration in plasma, time points for biosampling were chosen in such a way that the most complete data for each fragment of the pharmacokinetic curve could be obtained. In order to achieve this target, frequent sampling near T_{max} (~1 h when taking molnupiravir at the dose of 200 mg) was envisaged, as well as at least 3–4 points during the terminal phase. Thus, biosampling was carried out at the following points: before taking the test/reference drug and then after 15, 30, 45, 60 min, 1 h and 15 min, 1 h and 30 min, 1 h and 45 min, 2 h, 2 h and 15 min , 2 h and 30 min, 2 h and 45 min, 3 h, 3 h and 30 min, 4 h, 5 h, 6 h, 8 h, 10 h, 12 h and 16 h after dosing in each of the 2 study periods (the total of 21 sampling points in a period). To determine the molnupiravir concentration, timing postponement of blood sampling was not allowed in the first 2 h; it was permitted for no more than 5 min in the period from 3 to 16 h.

Venous blood in the volume of at least 6 ml was taken into special vacutainer tubes containing the anticoagulant K_2EDTA. Blood plasma was separated by centrifugation at 2000 g for 10 min. Then the test tubes were frozen and stored at the temperature not exceeding –65°C. The time interval between blood sampling, centrifugation and freezing did not exceed 30 min.

Analytical method

NHC, the main molnupiravir metabolite, was chosen as the analyte in this study, since molnupiravir undergoes hydrolysis to NHC before it reaches the systemic circulation, as a result of which unchanged molnupiravir is practically not detected in the blood.

All sample preparation was carried out under cooling conditions in an ice bath, since at room temperature, molnupiravir hydrolysis by plasma enzymes continued after sampling. The extraction of NHC from blood plasma was carried out by precipitation of blood plasma proteins with chilled methanol containing 0.1% formic acid.

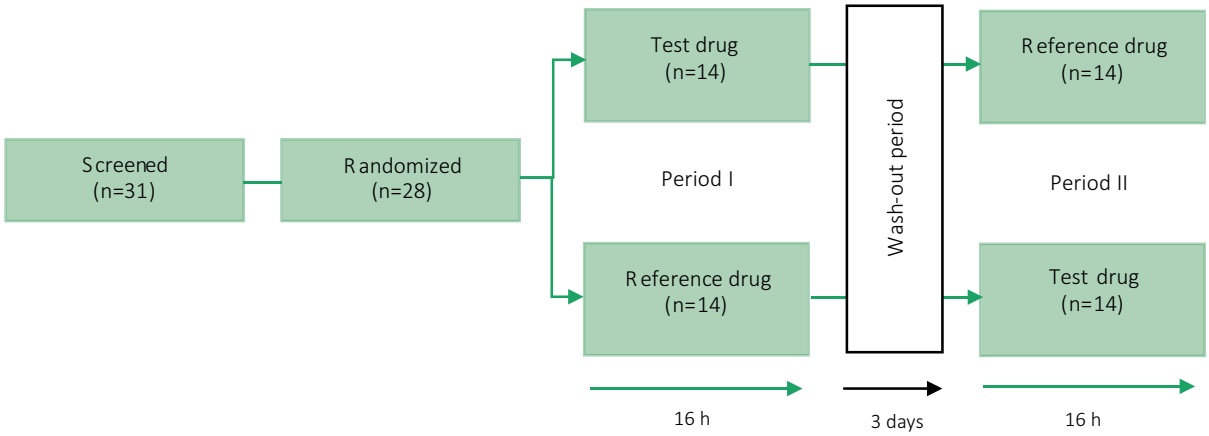

Figure 1 – Bioequivalence study design

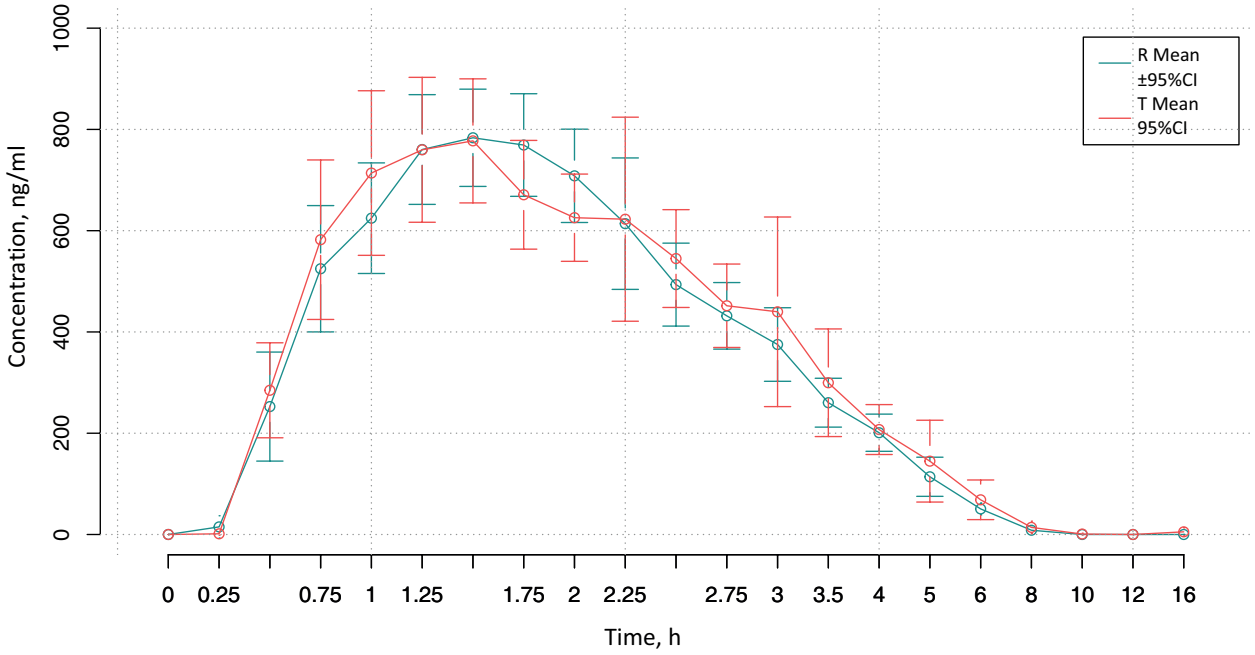

Figure 2 – Dynamics of NHC metabolite concentrations (mean and 95% CI) after taking test and reference drugs (N = 28)
Note: R – reference drug, T – test drug.

Table 1 – Obtained values of pharmacokinetic parameters after taking test and reference drugs (n = 28)

Pharmacokinetic parameters	Test drug (n = 28)	Reference drug (n = 28)
$AUC_{(0-16)}$, (ng/ml)*h	2229.66 (±963.99)	2083.21 (±656.45)
C_{max}, ng/ml	1028.12 (±503.31)	972.68 (±317.16)
$AUC_{(0-\infty)}$, (ng/ml)*h	2327.01 (±984.49)	2154.71 (±673.22)
T_{max}, h	1.3 (0.8–2.5)	1.5 (0.8–2.3)
$T_{1/2,}$ h	1.7 (±0.42)	1.58 (±0.26)

Notes: n – a number of observations; C_{max} – the maximum concentration of the drug in the volunteers' blood; T_{max} – time to reach C_{max}; $T_{1/2}$ – half-life; $AUC_{(0-16)}$ – the total area under the curve "concentration – time" in the time interval from 0 to 16 hours; $AUC_{(0-\infty)}$ – the area under the "concentration-time" curve in the time interval from 0 to infinity. The values of the indicators are presented as an arithmetic mean (standard deviation), except T_{max}, which is presented as a median (min – max).

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Table 2 – Calculated 90% CI values for ratios of NHC pharmacokinetic parameters of geometric means after test and reference drugs administration

Parameter	Ratio of geometric T/R means	Calculated values of 90% CI	CV_{intra} [1]
$AUC_{(0–16)}$	104.6%	96.31% – 113.64%	18.30%
C_{max}	102.4%	91.37% – 114.80%	25.44%

Notes: [1] – CV_{intra} – intra-individual coefficient of variability; CIs – confidence intervals; T – test drug, R – reference drug.

Table 3 – Summary table of AEs frequency after taking each of the drugs

Adverse effect	Test drug (N=28)	Reference drug (N=28)	P[1]value
Cardiovascular disorders			
Increase in diastolic blood pressure	1 (3.6%)	0 (0.0%)	1.000
Laboratory and instrumental data			
Increase in leukocytes number	0 (0.0%)	1 (3.6%)	1.000
Decrease in leukocytes number	1 (3.6%)	0 (0.0%)	1.000
Increase in lymphocytes number	1 (3.6%)	0 (0.0%)	1.000
Increase in creatine phosphokinase levels	1 (3.6%)	0 (0.0%)	1.000

Note: [1] – McNemar's criterion with Edwards' correction. All adverse effects given in the table refer to grade 1 severity.

The plasma NHC concentration was determined using validated high performance liquid chromatography with a tandem mass spectrometry (HPLC-MS/MS) technique. The determination method was developed and validated in accordance with the standards of good laboratory practice (GLP) and the recommendations of Appendix No. 6 to the "Rules for Conducting Bioequivalence Studies of Medicinal Products" within the Eurasian Economic Union. Validation was carried out according to the main characteristics of the methods: extraction efficiency from plasma and the matrix effect; a lower level of quantitation (LLOQ); a calibration range; accuracy and precision; selectivity (specificity); a sample transfer; stability.

The obtained samples were analyzed on an Infinity 1290 high performance liquid chromatograph (Agilent) equipped with a Triple Quad 5500+ (AB Sciex Pte. Ltd., Singapore) mass spectrometric detector with a triple quadrupole and electrospray ionization. A chromatographic separation was carried out on a Phenomenex Kinetex EVO C18 chromatographic column (100A 50×2.1 mm, 2.6 µm) in a gradient elution mode at a flow rate of 0.4 ml/min. The volume of the injected sample was 2 µl. A combination of ammonium acetate and methanol solutions was used as the mobile phase. Under these conditions, the retention time for N-hydroxycytidine was 0.5 min, the internal standard tolbutamide was 1.8 min. The total analysis time was 4 min.

For a selective and sensitive detection of the studied compounds, the optimal conditions for ionization in an electrospray and registration of negatively charged ions were chosen (MRM transitions for NHC were 258.2/126.1, for tolbutamide – 269.1/170.2).

Quantitative data processing was carried out with Analyst 1.7.2 program (AB Sciex Pte. Ltd., Singapore) using the internal standard method (solution of tolbutamide at the concentration of 1 mg/mL).

The analyte concentration was calculated from the calibration dependence of the chromatographic peak of the analyte to the area of the peak of the internal standard area on the nominal analyte concentration. The calibration curves were linear functions, the linear concentration range for NHC was 20–5000 ng/mL.

Safety assessment

For the purpose of a safety analysis, periodic assessments of physiological (blood pressure, pulse rate, body temperature), hematological (erythrocytes, hemoglobin, platelets, a leukocyte formula, an erythrocyte sedimentation rate) and biochemical (alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, creatine phosphokinase, uric acid, glucose, total bilirubin, creatinine) indicators, urinalysis, as well as adverse events (AEs) and serious adverse events (SAEs) registrations, were carried out. Laboratory and instrumental examinations were performed at screening as well as face-to-face visits on days 1, 4, and 6 from the start of the study. A telephone visit on day 10±1 was chosen as an additional point for safety monitoring. The selected safety endpoints were: the incidence and severity of all AEs and SAEs, the incidence of NCI CTCAE 5.0 Grade 3–5 AEs, and the incidence of an early withdrawal from the study associated with AEs/SAEs.

Statistical analysis

The statistical analysis was carried out using the software package for a statistical analysis R, version 4.2.0. (R Foundation, Austria).

The sample size was calculated taking into account the selected level of statistical significance α equal to 0.05, power – 0.8 (80%), as well as an intra-individual coefficient of variation for C_{max} and AUC 22% molnupiravir – 0.22 (22%).

Pharmacokinetic and bioequivalence analyzes were performed in the population of all volunteers who had missed no more than 2 blood samplings in each of the periods, and no more than 2 consecutive samplings. A safety analysis was performed in the population of all participants who had received at least one dose of test or reference drugs.

Based on the obtained values of NHC concentrations in the volunteers' blood plasma, the main pharmacokinetic (PK) parameters were calculated in the time intervals provided for in this protocol: C_{max} – the maximum concentration of the drug in the volunteers' blood; T_{max} – time to reach C_{max}; $AUC_{(0-t)}$ – total area under the concentration-time curve in the time interval from 0 to 16 hours; $T_{1/2}$ – half-life; $AUC_{(0-\infty)}$ is the area under the "concentration-time" curve in the time interval from 0 to infinity.

All of the above parameters were presented using the arithmetic mean + standard deviation (M+SD), with the exception of T_{max}, for which the median, minimum, and maximum had been used.

The drugs bioequivalence was assessed by comparing the boundaries of confidence intervals (CIs) for the ratio of the $AUC_{(0-16)}$ and C_{max} geometric means after administrating the test and reference drugs with established equivalence limits equal to 80.00–125.00%. To establish bioequivalence, a analysis of variance (ANOVA) of logarithmically transformed NHC parameters necessary for assessing bioequivalence (AUC and C_{max}), was used. Based on the residual variation of the dispersion models, the variation coefficients of the studied parameters and the corresponding CIs (on a logarithmic scale) were calculated to search for the differences between the compared drugs. The resulting CIs were inversely transformed to construct the desired CIs for the ratio of means in the original (non-transformed) units.

A statistical analysis took into account the sources of variability that could affect the variable under study. The fixed factors were used in the analysis of variance models: the sequence of drugs, the subject of the study nested in the sequence, the period and the drug. Analysis of variance was used to test hypotheses about the statistical significance of each of these factors contribution to the observed variability. The assessment of the residual variation obtained by applying the analysis of variance, was used to calculate a 90% confidence interval for the ratio of the corresponding pharmacokinetic parameter means.

RESULTS

Population

A total of 28 male volunteers were included in the study. All randomized volunteers were white people. The height and weight of the study participants were within the normal range of body mass index (BMI). The mean age was 26.46 (±4.74) years, the body weight was 77.55 (±9.20) kg, and BMI was 23.71 (±2.07) kg/m^2. All volunteers completed the study according to the protocol, and therefore were included in the population for the evaluation of pharmacokinetics and bioequivalence.

Assessment of pharmacokinetics and bioequivalence

Based on the results of the NHC concentration analysis in the volunteers' blood plasma, the main parameters of the calculated molnupiravir pharmacokinetics, are presented in Table 1.

After taking the test and reference drugs, the pharmacokinetic parameters of molnupiravir were similar to each other. Thus, after taking the test drug, the maximum concentration of C_{max} NHC was 1028.12±503.31 ng/ml, while after taking the reference drug, this parametr was equal to an mean of 972.68±317.16 ng/ml. The area under the curve measured up to 16 h post-dose $AUC_{(0-16)}$, was 2229.66±963.99 ng*h/ml; and 2083.21±656.45 ng*h/ml – after taking the reference drug.

Fig. 2 shows a graph of changes in the NHC concentrations after taking the test and reference drugs.

After calculating the PK parameters, a statistical assessment of bioequivalence was carried out. Since the calculation results showed that $AUC_{(0-16)}$ was more than 80% of the $AUC_{(0-\infty)}$ value, $AUC_{(0-16)}$ values were used to establish bioequivalence.

According to the results of the statistical analysis, 90% CI for the ratio of the geometric mean $AUC_{(0-16)}$ of the test drug and reference drug, was 96.31% – 113.64% for NHC. For the ratio of geometric mean C_{max} of the studied drugs, 90% CI was 91.37% – 114.8%.

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The intervals obtained correspond to the established equivalence limit for $AUC_{(0–16)}$ and C_{max} – 80.00–125.00%, which indicates the studied drugs bioequivalence (Table 2). The results of the ANOVA showed that sources of variation, such as differences between the drugs, between the subjects (intersubject differences), and the administration sequence and study periods, did not significantly affect the variables assessed.

Safety

Throughout the study, both study and reference drugs were well tolerated by the volunteers. Hematological and biochemical blood tests, as well as urinalysis and physiological parameters in most volunteers remained normal throughout the study. Any deviations from the norm of laboratory and instrumental parameters were recorded as AEs. In total, 5 AEs were registered in the study: an increase in the diastolic blood pressure, an increase or decrease in the number of lymphocytes, an increase in the number of lymphocytes, an increase in the level of creatine phosphokinase. The registered deviations had a random multidirectional character. All AEs were grade 1 according to Common Terminology Criteria for Adverse Events (CTCAE) 5.0. The list of AEs is presented in Table 3.

According to the investigators' conclusion, all registered AEs were not associated with the study products, i.e., the degree of association was regarded as "doubtful".

When comparing the frequency of individual AEs registration, there were no significant differences ($p>0.05$) after taking the test drug and the reference drug, on the basis of which it can be concluded that the drugs are similarly tolerated.

DISCUSSION

Molnupiravir is a low molecular weight ribonucleoside prodrug that is hydrolyzed in the blood to N-hydroxycytidine. Molnupiravir has an antiviral activity against SARS-CoV-2 variants [13–15] and other RNA-containing viruses such as influenza, Ebola and respiratory syncytial viruses [16–20]. During preclinical development *in vitro* and *in vivo* trials, molnupiravir showed a high efficacy against SARS-CoV-2, as well as a low toxicity to animals [21, 22]. In clinical studies conducted for the original drug, molnupiravir demonstrated efficacy in the treatment of mild and moderate of a new coronavirus infection COVID-19 [11, 12, 23, 24]. In the most extensive research of MOVe-OUT study (NCT04575597), phase 3, which included 1,433 patients, it was found out that molnupiravir at the dose of 800 mg 2 twice a day for 5 days, significantly reduced the risk of hospitalization or death compared with placebo [12]. In the mITT population, which was represented by randomized patients who had been administrated with at least one dose of molnupiravir or placebo and had not been hospitalized prior to the therapy initiation, the rate of hospitalization or death was 6.8% (48 out of 709 patients) in the molnupiravir group and 9.7% (68 out of 699 patients) in the placebo group. The difference between the groups was 3% [95% CI –5.9; –0.1]. In addition, the drug showed a favorable safety profile. The proportion of patients who had experienced at least one AE was similar in both groups (30.4% in the molnupiravir group and 33.0% in the placebo group). Given the wide spread of the new coronavirus infection COVID-19, the administration of a bioequivalent generic drug molnupiravir to the market will increase the availability of the effective and safe treatment for this disease.

As a result of the bioequivalence study, it was found out that the PK parameters of the generic drug molnupiravir are comparable to the parameters of the reference (original) drug. In addition, the PK parameters of the test drug were comparable to the data obtained as a result of the 1st phase study of the original drug Lagevrio. Thus, the geometric mean C_{max} obtained in the study EIDD-2801-1001-UK [25] of the original drug with a single dose of 200 mg was 926 ng/ml, while the geometric mean of this parameter for the test drug in this bioequivalence research was 950 ng/ml. Similar trends can be observed in terms of $AUC_{0-\infty}$: the geometric mean value in the study of the original drug was 1830 ng*h/ml, and the value of the test drug in this bioequivalence research was 2189 ng*h/ml.

After calculating the PK parameters of the test and reference drugs in this research, a statistical determination of bioequivalence was carried out. According to its results it was found out that the obtained 90% CI fully fit into the required range of 80.00–125.00% for $AUC_{(0–16)}$ and C_{max}, which was established in accordance with the protocol and the "Rules for Conducting Bioequivalence Studies of Medicinal Products" within the Eurasian Economic Union, approved by the Decision of the Council of the Eurasian Economic Commission No. 85 dated November 03, 2016, as well as the international guidelines on bioequivalence of the European Medicines Agency (EMA).

The spectrum of the reported AEs was consistent with the safety profile of the original drug molnupiravir.

The frequency of AEs registration did not differ after taking the test and reference drugs. Thus, it can be concluded that in the framework of the clinical trial, the test and reference drugs demonstrated similar safety characteristics.

CONCLUSION

The results of bioequivalence study fully meet all criteria for the drugs established in generally recognized by international guidelines. Thus, it can be concluded that the test and reference molnupiravir products are bioequivalent. Based on the results of the study, it can also be concluded that the drugs have similar safety profiles.

Based on the results of the bioequivalence and safety research (July 2022), the drug ALARIO-TL was registered in the Russian Federation under the registration procedure for the drugs to be used under the threat conditions of occurrence and liquidation of emergency situations[4].

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CONFLICT OF INTEREST

The clinical trial was organized by the sponsor R-Pharm group of companies. The authors of the article V.G. Mozgovaya, O.V. Filon, A.V. Petkova, V.G. Ignatiev, M.Yu. Samsonov, I.S. Kozlova, E.K. Khanonina are employees of R-Pharm group of companies.

AUTHORS' CONTRIBUTION

Vasily B. Vasilyuk – research conducting; Anna Yu. Boroduleva, Pavel D. Sobolev, Aiyyna G. Nikiforova – development, validation of the analytical part, biosamples analysis; Valentina G. Mozgovaya, Olga V. Filon – development of study design, text writing and editing; Irina S. Kozlova, Elizaveta K. Khanonina – results analysis, text writing and editing; Anna V. Zinkovskaya – statistical processing of study results; Vasily G. Ignatiev, Mikhail Yu. Samsonov – aim setting, development of study design.

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EFFICACY AND SAFETY OF ORIGINAL DRUG BASED ON HEXAPEPTIDE SUCCINATE IN COMPLEX COVID-19 THERAPY IN ADULTS HOSPITALIZED PATIENTS

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Currently, there are data that that make it possible to speak about a high clinical efficacy of the use of succinic salt of tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine (hexapeptide succinate) for the COVID-19 treatment. This article is devoted to the results of clinical trials of the original Russian drug based on it.

The aim of the study was to evaluate a clinical efficacy, safety and tolerability of intramuscular and inhalation use of hexapeptide succinate in complex therapy in comparison with standard therapy in patients with moderate COVID-19.

Materials and methods. The research was conducted from February 28, 2022 to November 22, 2022 based on 10 research centers in the Russian Federation. The study included hospitalized patients (n=312) over 18 years of age with moderate

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COVID-19 who had undergone a screening procedure and were randomized into 3 groups: group 1 received standard therapy in accordance with the Interim Guidelines in force at the time of the study, within 10 days; group 2 received hexapeptide succinate (Ambervin® Pulmo) intramuscularly at the dose of 1 mg once a day for 10 days; group 3 received hexapeptide succinate (Ambervin® Pulmo) 10 mg once a day by inhalation for 10 days.

Results. According to the results of the study, therapy with the drug hexapeptide succinate, both intramuscular and inhaled, provided an acceleration of recovery up to the complete absence of the disease signs in more than 80% of hospitalized COVID-19 patients. By the end of the therapy course with the drug, more than 60% of patients had met the criteria for discharge from hospital and could continue the treatment on an outpatient basis. About 70% of patients in the inhalation group and 80% in the intramuscular hexapeptide succinate injection group had concomitant diseases (hypertension – 28%, obesity – 14%), which indicates the effectiveness of this drug use in comorbid patients. The use of the drug contributed to the restoration of damaged lung tissues, normalization of oxygenation, the disappearance of shortness of breath and a decrease in the duration of the disease symptoms compared with standard therapy. As a result of a comparative analysis of adverse events in terms of their presence, severity, causal relationship with the therapy and outcome, there were no statistically significant differences between the treatment groups.

Conclusion. Thus, the results of the clinical study of the succinate hexapeptide efficacy and safety showed the feasibility of using the drug in pathogenetic therapy COVID-19 regimens.

Keywords: ambervine; hexapeptide succinate; acute respiratory distress syndrome; “cytokine storm”; COVID-19; tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine succinate

Abbreviations: AE – adverse events; SAE – serious adverse events; IG – Interim guidelines “Prevention, diagnosis and treatment of a new coronavirus infection”; ALT – alanine aminotransferase; AST – aspartate aminotransferase; LDH – lactate dehydrogenase; CRP – C-reactive protein; GFR – glomerular filtration rate; PIS – patient information sheet; HFO – high-flow oxygen; NIVL – non-invasive lung ventilation; ALV – artificial lung ventilation; ECMO – extracorporeal membrane oxygenation; ARDS – acute respiratory distress syndrome; SARS-CoV-2 – coronavirus, the causative agent of COVID-19; CTs – clinical trials, SD – standard deviation; LPO – lipid peroxidation; RR – respiratory rate.

ЭФФЕКТИВНОСТЬ И БЕЗОПАСНОСТЬ ОРИГИНАЛЬНОГО ПРЕПАРАТА НА ОСНОВЕ СУКЦИНАТА ГЕКСАПЕПТИДА В КОМПЛЕКСНОЙ ТЕРАПИИ COVID-19 У ВЗРОСЛЫХ ГОСПИТАЛИЗИРОВАННЫХ ПАЦИЕНТОВ

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На сегодняшний день имеются данные, позволяющие говорить о высокой клинической эффективности применения янтарнокислой соли тирозил-D-аланил-глицил-фенилаланил-лейцил-аргинина (гексапептида сукцинат) для лечения COVID-19. Настоящая статья посвящена результатам клинических исследований оригинального российского лекарственного препарата на его основе.

Цель. Оценить клиническую эффективность, безопасность и переносимость внутримышечного и ингаляционного применения препарата гексапептида сукцината в комплексной терапии в сравнении со стандартной терапией у пациентов со среднетяжелым течением COVID-19.

Материалы и методы. Исследование проводилось с 28 февраля 2022 г. по 22 ноября 2022 г. на базе 10 исследовательских центров на территории РФ. В исследование были включены госпитализированные пациенты (n=312) старше 18 лет со среднетяжелым течением COVID-19, которые прошли процедуру скрининга и были рандомизированы на 3 группы: группа 1 получала стандартную терапию в соответствии с Временными методическими рекомендациями, действующими на момент проведения исследования в течение 10 сут; группа 2 получала препарат гексапептида сукцинат (Амбервин® Пульмо) внутримышечно по 1 мг 1 раз/сут в течение 10 дней; группа 3 получала препарат гексапептида сукцинат (Амбервин® Пульмо) ингаляционно по 10 мг 1 раз/сут в течение 10 дней.

Результаты. По результатам исследования терапия лекарственным препаратом гексапептида сукцинат как при внутримышечном, так и при ингаляционном введении обеспечивала ускорение выздоровления вплоть до полного отсутствия признаков заболевания более, чем у 80% госпитализированных пациентов с COVID-19. К окончанию курса терапии препаратом более 60% пациентов соответствовали критериям выписки из стационара и могли продолжить лечение в амбулаторных условиях. Около 70% пациентов в группе ингаляционного введения и 80% в группе внутримышечного введения гексапептида сукцинат имели сопутствующие заболевания (гипертензию – 28%, ожирение – 14%), что говорит об эффективности применения указанного лекарственного препарата у коморбидных пациентов. Применение препарата способствовало восстановлению поврежденных тканей легких, нормализации оксигенации, исчезновению одышки и уменьшению продолжительности симптомов заболевания по сравнению со стандартной терапией. В результате сравнительного анализа нежелательных явлений по их наличию, степени тяжести, причинно-следственной связи с терапией и исходу не было выявлено статистически значимых различий между группами терапии.

Заключение. Таким образом, результаты проведенного клинического исследования эффективности и безопасности гексапептида сукцинат показали целесообразность применения препарата в схемах патогенетической терапии COVID-19.

Ключевые слова: амбервин; гексапептида сукцинат; острый респираторный дистресс-синдром; «цитокиновый шторм»; COVID-19; тирозил-D-аланил-глицил-фенилаланил-лейцил-аргинина сукцинат

Список сокращений: НЯ – нежелательные явления; СНЯ – серьёзные нежелательные явления; ВМР – Временные методические рекомендации «Профилактика, диагностика и лечение новой коронавирусной инфекции»; АЛТ – аланинаминотрансферазы; АСТ – аспартатаминотрансфераза; ЛДГ – лактатдегидрогеназа; СРБ – С-реактивный белок; СКФ – скорость клубочковой фильтрации; ИЛП – информационный листок пациента; ВПО – высокопоточная оксигенотерапия; НИВЛ – неинвазивная вентиляция легких; ИВЛ – искусственная вентиляция легких; ЭКМО – экстракорпоральная мембранная оксигенация; ОРДС – острый респираторный дистресс-синдром; SARS-CoV-2 – коронавирус, возбудитель COVID-19; КИ – клинические исследования, СО – стандартное отклонение; ПОЛ – перекисное окисление липидов.

INTRODUCTION

Since the mid-1970s, an era of research into endogenous substances that activate the same receptors as opiates began. Subsequently, these studies led to the discovery of the first endogenous opioid peptide. In 1975, two classes of endogenous peptides were discovered – methionine-enkephalin (met-enkephalin) and leucine-enkephalin (leu-enkephalin). Since then, more than 20 opioid peptides have been discovered. Each of these peptides binds with different affinity to three types of opioid receptors (μ, δ, or k) [1–5]. Currently, endogenous opioid peptides are divided into four families: enkephalins, dynorphins, endorphins, and nociceptin/orphanin FQ [6, 7].

Tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine is the world's first synthetic opioid peptide created on the basis of endogenous leucine-enkephalin by the standard replacement of Gly2 with D-Ala2 and the addition of a highly charged arginine residue to the C-terminal part of the molecule in order to obtain a peripheral effect and stability of the peptide. This modification of the leucine-enkephalin molecule contributed to the leveling of some side effects characteristic of other opiates: it did not cause addiction, physical dependence [8]. Tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine was previously used in the treatment of patients with peptic ulcer of the stomach and duodenum, resistant to the therapy and with an insufficient effect from the treatment with other drugs. Then the drug began to be used to treat acute and chronic pancreatitis [8]. Further studies revealed cardioprotective properties of tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine in the patients operated on under cardiopulmonary bypass [9]. Subsequent studies have demonstrated a protective effect of the drug on the lungs [10].

Immune system cells are ones of the main opioid peptides targets due to the detection of the corresponding receptors on the surface of immunocytes of the lymph nodes, bone marrow, and spleen. Endorphins, dynorphins, and enkephalins are involved in the development and pathogenesis of a number of autoimmune disorders and, therefore, can alter the antiviral and antimicrobial response [11–14]. Taking into account a wide range of an opioid peptides therapeutic action, their high safety profile and good tolerability due to the fact that they are mainly composed of natural amino acids and have a high selectivity of action, no interest in them has faded, and the search for their possible use in various diseases continues [15, 16].

Enkephalins work as delta receptor agonists, suppressing excessive synthesis of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) and thus reducing the consequences of a systemic hyperimmune reaction (cytokine storm) [17–20]. Cytokine storm is the main cause of the acute respiratory distress syndrome (ARDS) development, in particular with COVID-19, which requires the transfer of a patient to the artificial lung ventilation due to severe hypoxia [21-23].

Delta receptor agonists, which include tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine, stimulate regeneration and healing processes; normalize microcirculation in the area of damage, contribute to the maintenance of structural homeostasis [13–15]. Hexapeptide has an immunomodulatory effect, regulates the activity of cells of innate and adaptive immunity, enhances the activity of the phagocytic link of immunity (macrophages and neutrophils). Tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine increases the activity of natural killer cells (NK cells), the availability of which decreases with severe infections caused by RNA viruses (influenza, Ebola virus, COVID-19, SARS, MERS). Hexapeptide stimulates the production of endogenous interferons, increases the body's resistance to viral infections [24, 25]. In completed preclinical studies was shown that tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine and his derivates have a positive effect on the course of acute respiratory distress syndrome (ARDS), significantly reducing animal mortality, inflammation and swelling of the lung tissue , as well as suppressing the "cytokine storm" [25].

The study by Ukrainskaya L.A. et al. (2002) showed that the use of tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine succinate in experimental stress-induced lung alteration reduced lipid peroxidation (LPO) hyperactivation, surfactant breakdown, and the severity of edema and leukocyte infiltration of the alveoli and increased the gas exchange area. Limiting the altering stress effects by a hexapeptide administration has an effective pulmonoprotective action [26]. To date, a number of experimental studies have shown an immunomodulating effect of tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine hexapeptide [24, 27].

In 2022, the drug Ambervin® Pulmo was developed and registered (RU No. LP-008604 dated October 07, 2022; Patent No. EA038010). It contained tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine succinate (hexapeptide succinate) in dosages of 1.16 mg and 5.8 mg. Ambervin® Pulmo has an anti-inflammatory effect by inhibiting the synthesis in the lungs and

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inhibiting the entry into the systemic circulation of one of the main pro-inflammatory mediators of the cytokine storm – IL-6, as well as other pro-inflammatory cytokines (in particular, IL-1, TNF-α, HMGB1). It also increases the formation of IL-10 and VEGF, which have an anti-inflammatory effect and increase the body's defenses. Being an analogue of leu-enkephalin, the drug[1] has a vasoprotective effect, reducing the permeability of the vascular wall and preventing the destruction of the endothelium, increases tolerance to hypoxia, prevents and reduces the severity of acute lung injury, reduces the risk of an oxygenation decrease and the development of secondary bacterial complications.

Due to the succinic acid fragment included in the structure of the hexapeptide, the drug[2] under consideration exhibits antioxidant, antihypoxic properties, including the ones in the alveolar cells of the lung tissue, in the epithelial cells of the middle and upper parts of the respiratory system. It inhibits lipid peroxidation, improves the structure and function of cell membranes, reduces the inhibition degree of oxidative processes in the Krebs cycle under hypoxic conditions, and increases the body's resistance to various damaging factors.

Hexapeptide succinate[3] stimulates regeneration and healing processes, promotes the damaged tissues restoration. It includes alveolar epithelial cells, reduces the severity of interstitial edema in the lower respiratory tract (alveoli, bronchi, bronchioles), normalizes microcirculation in the area of damage, helps maintain structural homeostasis, has anti-inflammatory, detoxification, antioxidant, reparative and immunomodulatory effects, increasing the effectiveness of ongoing antiviral and antibacterial therapy.

This article is devoted to the clinical study results of this drug use in the treatment of COVID-19 patients.

THE AIM of the study was to evaluate a clinical efficacy, safety and tolerability of intramuscular and inhalation use of hexapeptide succinate in complex therapy in comparison with standard therapy in patients with moderate COVID-19.

MATERIALS AND METHODS

The efficacy, safety, and tolerability of tyrosyl-D-alanyl-glycyl-phenylalanyl-leucyl-arginine succinate, or succinate hexapeptide, compared with standard therapy in patients hospitalized with COVID-19 was studied in an open-label, randomized, multicenter, comparative, phase III clinical trial (CCT the Ministry of Health No. 100, dated 2022 Feb 14).

The research was conducted from February 28, 2022 to November 22, 2022 on the basis of 10 research centers in the Russian Federation:

1. National Research Ogarev Mordovia State University,
2. Regional Clinical Hospital;
3. Municipal clinical hospital No. 24, Moscow City Health Department
4. Voronezh Regional Clinical Hospital No. 1;
5. Ryazan State Medical University named after academician I.P. Pavlov;
6. City Clinical Hospital named after S.I. Spasokukotsky, Moscow City Health Department;
7. Smolensk Clinical Hospital No. 1;
8. Infectious Clinical Hospital No. 1, Moscow City Health Department;
9. City Hospital No. 40, St. Petersburg, Kurortny District;
10. Emergency Hospital, Cheboksary, Chuvash Republic.

Study design

The hospitalized male and female patients (n=313) aged 18 to 80 years inclusive, with moderate COVID-19, were screened and randomized into 3 groups in a 1:1:1 ratio. The drug choice for patients was carried out in accordance with the randomization number assigned to patients at the time of randomization.

Randomization of study subjects into groups

Male and female patients (at least 312 people) aged 18 to 80 years inclusive, hospitalized with COVID-19, meeting the inclusion criteria and not meeting the exclusion criteria, were randomized into 3 groups in a 1:1:1 ratio (Fig. 1).

The randomization was carried out according to the following algorithm: each patient who had met all the inclusion criteria and had not meet any of the exclusion criteria, was assigned a three-digit randomization number using the IWRS system. A patient's randomization number and other relevant data were entered by the investigator into the Subject Screening/Randomization Journal. If a patient discontinued participation in the

[1] Russian State Register of Medicines. Instructions for Ambervin® Pulmo. Available from: https://grls.rosminzdrav.ru/Grls_View_v2.aspx?routingGuid=1f912539-dd59-4a95-adeb-31621b26fb0b
[2] Ibid.
[3] Ibid.

study prematurely, their randomization number was not reused.

This study was open, so both the patient and the investigator knew what therapy the patient was receiving.

Group 1 (n=104) received standard therapy in accordance with the BMPs[4] in force at the time of the study for 10 days;

Group 2 (n=104) received hexapeptide succinate (Ambervin® Pulmo, PROMOMED RUS LLC) intramuscularly at the dose of 1.16 mg once a day for 10 days;

Group 3 (n=104) received hexapeptide succinate (Ambervin® Pulmo, PROMOMED RUS LLC) by inhalation using a nebulizer, 11.6 mg once a day for 10 days.

As concomitant therapy, patients in groups 2 and 3 received standard therapy, presented in the BMPs, valid at the time of the study. Intramuscular and inhalation uses of the study drug was carried out in a hospital setting. The design of the study is shown in Fig. 2. The total duration of a patient's participation in the study was no more than 30 days.

Selection of subjects for analysis

Primary and secondary efficacy outcomes were analyzed using a dataset of study participants selected according to the protocol compliance, i.e. all the patients who had completed the study in accordance with the Study Protocol. A participant was excluded from the data set if they had met the exclusion criteria.

The safety data set included all randomized patients who had been exposed to the study drug, regardless of the degree of adherence to the Protocol during the study.

Inclusion criteria

Availability of a signed and dated Informed Consent Form (ICF) by the patient, male and female, aged 18 to 80 years inclusive at the time of signing the ICF; a confirmed case of COVID-19 at the time of screening based on the results of the analysis for the determination of SARS-CoV-2 RNA by the nucleic acid amplification method (NAAM); hospitalization due to the COVID-19 disease; a moderate course of SARS-CoV-2 infection (presence of at least 2 of the following criteria: body temperature >38°C; respiratory rate (RR) >22/min; dyspnea on exertion; changes on computed tomography (CT), typical for viral damage; SpO_2<95%; Serum C-reactive protein (CRP)>10 mg/l.); the volume of the lungs damage is minimal or medium (CT 1-2); a patient's consent to use reliable methods of contraception throughout the study and for 3 weeks after the end of the study. The reliable means of contraception are sexual abstinence, the use of a condom in combination with spermicide. The study could also include women who are unable to bear children (history: hysterectomy, tubal ligation, infertility, menopause for more than 2 years), as well as men with infertility or a history of vasectomy.

Noninclusion criteria

Noninclusion criteria are as follows: hypersensitivity to the components of the study drug; obstacles or inability to perform intramuscular injections and/or inhalations; the inability to perform a CT procedure (for example, a plaster cast or metal structures in the study area); arterial hypotension (a decrease in blood pressure (BP) below 100/60 mm Hg) at the time of screening and/or a history of hypotensive crises; the need to use drugs from the list of prohibited therapies; the presence of criteria for severe and extremely severe course of the disease at the time of screening; the presence of a probable or confirmed case of COVID-19 moderate course within 6 months prior to screening; the presence of a probable or confirmed case of severe and extremely severe COVID-19 in history; vaccination less than 4 weeks prior to screening; the need for treatment in the intensive care unit at the time of screening. There are some more noninclusion criteria: an abnormal liver function (AST and / or ALT ≥3 ULN and/or total bilirubin ≥1.5 ULN) at the time of screening; an impaired renal function (GFR<60 ml/min) at the time of screening; positive for HIV, syphilis, hepatitis B and/or C at the time of screening; a chronic heart failure of FC III–IV according to the functional classification of the New York Heart Association (NYHA); a history of malignant neoplasms, except in patients who have not been observed for the disease within the last 5 years, patients with completely healed basal cell skin cancer or completely healed carcinoma *in situ*; a history of alcohol, pharmacological and/or drug dependence and/or at the time of screening; a history of epilepsy; schizophrenia, schizoaffective disorder, bipolar disorder, or other psychiatric disorder in history or suspected of having them at the time of screening; severe, decompensated or unstable somatic diseases (any diseases or conditions that threaten

[4] Interim guidelines "Prevention, diagnosis and treatment of novel coronavirus infection (COVID-19)". Version 16 (2022 Aug 18). Available from: https://static-0.minzdrav.gov.ru/system/attachments/attaches/000/060/193/original/%D0%92%D0%9C%D0%A0_COVID-19_V16.pdf

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the patient's life or worsen the patient's prognosis, and also make it impossible for him to participate in a clinical trial); any history data that, in the opinion of the investigator, may complicate the interpretation of the results of the study or create additional risks for the patient as a result of his participation in the study; unwillingness or inability of the patient to comply with the procedures of the Protocol (in the opinion of the investigator); pregnant or lactating women, or women planning a pregnancy; participation in another clinical trial within 3 months prior to the enrollment in the study; other conditions that, in the opinion of the investigator, prevent the inclusion of a patient in the study.

Exclusion criteria

A decision to exclude a subject from the study was made by the investigator.

A patient was withdrawn from the study immediately if any of the following situations had occurred:

1. Negative SARS-CoV-2 RNA NAAT selected at screening (for patients with a probable case of COVID-19 at the time of screening).
2. The appearance of any diseases or conditions that worsen the patient's prognosis, and also make it impossible for the patient to continue participating in the clinical trial during the study.

If it was necessary to transfer the patient to high-flow oxygen (HFO), non-invasive lung ventilation (NILV), the therapy provided for by the Protocol continued, the patient was not excluded from the study. The inhalation use of the hexapeptide succinate preparation was carried out through the apparatus circuit while maintaining the specified oxygenation parameters.

If it was necessary to transfer a patient to the artificial lung ventilation (ALV), extracorporeal membrane oxygenation (ECMO), the patient was excluded from the study and prescribed therapy in accordance with the clinical practice of the research center.

3. Taking drugs of prohibited therapy or the need to prescribe them.
4. Pregnancy of a patient.
5. Erroneous inclusion of a patient who does not meet the inclusion criteria and/or meets the non-inclusion criteria.
6. Other violations of the Protocol, which, in the opinion of the investigator, are significant.
7. Patient refusal to participate in the study.
8. Other administrative reasons.

Criteria for efficacy evaluation

Primary criteria for efficacy:

- Frequency of achieving category 0-1 on the categorical ordinal scale of clinical improvement at Visit 4 (Table 1).

Secondary criteria for efficacy:

- Frequency of patients with clinical status fewer than 4 points on the categorical ordinal scale of clinical improvement at Visits 3 and 4;
- Frequency of improvement in clinical status on the categorical ordinal clinical improvement scale of 2 or more categories at Visits 3 and 4;
- Time (in days) to improve clinical status on a categorical ordinal scale of clinical improvement by ≥1 point;
- The rate of patients meeting discharge criteria for a continued outpatient treatment according to IGs at Visits 2 and 3.

Discharge criteria (meeting all the criteria, however, a patient could continue to stay in hospital after reaching the discharge criteria if the investigator considered it necessary or it was required for social reasons):

- persistent improvement of the clinical picture;
- level of blood oxygen saturation in air ≥95%;
- body temperature <37.5℃;
- CRP level <10 mg/l;
- level of blood lymphocytes >1.2×109/l.

- Rate of patients with RR <22/min at Visits 2 and 3. The evaluation was performed only for patients who had a RR >22/min at Visit 1;
- Incidence of patients with CRP levels <10 mg/l at Visits 2 and 3;
- Evaluation was performed only for patients who had a CRP level >10 mg/l at Visit 0;
- Incidence of patients with blood lymphocytes >1.2×109/l at Visits 2 and 3. The evaluation was limited to the patients who had a blood lymphocyte count <1.2×109/l at Visit 0;
- Assessment of the lung damage degree according to CT data for Visit 4;
- Incidence of patients with SpO_2≥95% for 2 consecutive days at Visits 2, 3 and 4. The evaluation was performed only for patients who had an SpO_2 <95% at Visit 1;
- The frequency of transfers of patients to the intensive care unit;
- The frequency of cases of the use of HFO, NIVL, ALV, ECMO;
- Incidence of acute respiratory distress syndrome (ARDS);
- Incidence of patient deaths.

Additional research parameters

- Frequency of patients reaching reference levels at Visits 2, 3 for each of the following: IL 6, D dimer, ferritin, fibrinogen, CRP, lymphocytes, leukocytes, platelets, triglycerides, LDH;
- Change (%) to Visits 2, 3 for each of the following: IL-6, D-dimer, ferritin, fibrinogen, CRP, lymphocytes, leukocytes, platelets, triglycerides, LDH.

Criteria for safety assessment

- Total number of AEs stratified by severity and frequency;
- Frequency of adverse reactions;
- Frequency of SAEs, including those associated with the study drug/standard therapy;
- Proportion of patients with at least one AE;
- Proportion of patients who interrupted treatment due to AE/SAEs.

Statistical analysis

For a statistical analysis, software with validated algorithms for performing statistical analyzes and a proper documentation was used (StatSoft Statistica 10.0., IBM SPSS Statistics 22 (current version, GPL-2/GPL-3 license).

Continuous (quantitative) data are presented using the number of observations, arithmetic mean, 95% confidence interval (CI) for the mean, standard deviation, median, interquartile range (25^{th} and 75^{th} centiles), minimum and maximum.

Qualitative data (ordinal, nominal) are presented using absolute frequencies (a number of observations), relative frequencies (percentage) and 95% CI.

Checking for the normality of the distribution was carried out by one of the generally accepted methods (Shapiro-Wilk test, Kolmogorov-Smirnov test). In the case of a Non-Gaussian distribution, non-parametric evaluation methods were used to compare efficacy and safety indicators.

Significance levels and confidence intervals were calculated as two-tailed, and the statistical significance of differences was two-tailed by default and referred to a significance level of 0.05 (unless otherwise indicated).

For the analysis of the primary criterion for efficacy, it is assumed to use an intergroup comparison of shares using a one-sided version of Fisher's exact test or $\chi 2$ (the chi-square) test, if all the expected values in the cells of the contingency table for this analysis are 5 or more. The proportion of patients achieving grade 0–1 on a categorical ordinal scale of the clinical improvement at Visit 4 is presented with a two-sided 95% confidence interval (CI) by treatment groups. The difference in proportions between the treatment groups and the 95% two-sided CI for the difference in proportions calculated by the Newcomb-Wilson method, are shown. Secondary criteria for efficacy and additional study parameters are presented descriptively for each group.

Safety population: the patients who received at least one dose of the study drug and for whom there is an assessment of the condition and/or AE for at least one time point after application. If the study drug was not taken by the volunteer/patient, their data were not included in the statistical analysis, but were presented in the final report of the study.

RESULTS AND DISCUSSION

Baseline Patient Characteristics

313 patients underwent the screening and randomization procedure, 312 were included in the study, one patient was excluded from the study before taking the drug due to meeting the exclusion criterion "Patient refusal to participate in the study": 104 patients received standard therapy in accordance with current IGs, 104 patients – hexapeptide succinate intramuscularly (IM) and 104 patients – hexapeptide succinate by inhalation. The groups were comparable in terms of demographic, anthropometric, and clinical characteristics (Table 2).

The average age of all the patients included in the study was 58.21 years (from 18 to 80 years), the number of women was slightly more – 53.21% (n=166) than men – 46.79% (n=146). The average body mass index (BMI) was 27.55 kg/m^2 (from 15.30 to 51.42 kg/m^2), which corresponds to the overweight according to the WHO classification. In 242 patients (77.56%), comorbidities were identified. The most common comorbidities were hypertension 28% (n=173) and obesity 14% (n=88). Other comorbidities/conditions that occurred with a frequency of 2 to 5% were atrial fibrillation (3.2%), chronic heart failure (2.4%), myocardial ischemia (2.6%), angina pectoris (2.6%), osteochondrosis (2.1%), type 2 diabetes mellitus (4.8%), menopause (2.4%). In 163 (52.24%) patients, ECG abnormalities were detected. The groups were comparable in terms of sex, age and comorbid status of patients.

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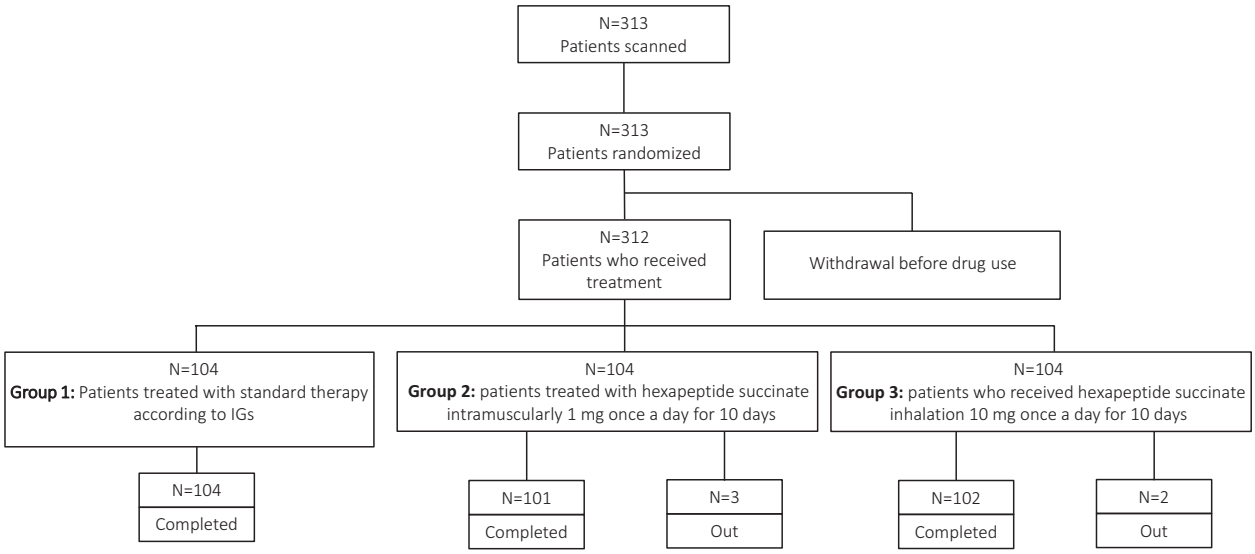

Figure 1 – Groups' allocation

Note: IGs – Interim guidelines "Prevention, diagnosis and treatment of novel coronavirus infection (COVID-19)". Version 16 dated 2022 Aug 18 (here and Fig. 2).

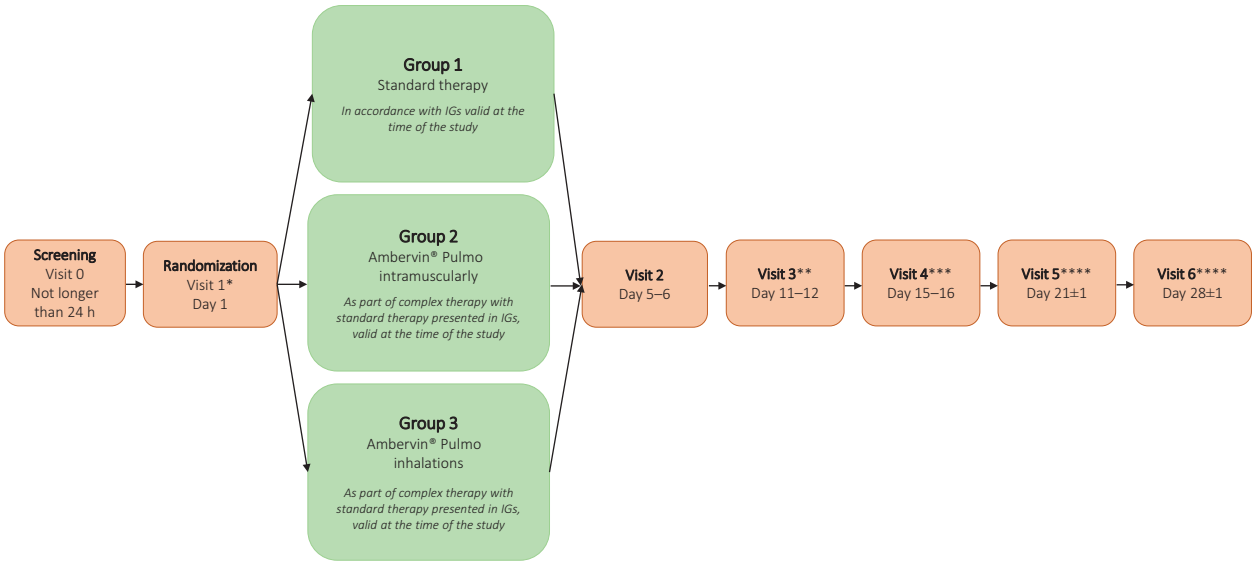

Figure 2 – Study design

Note: *Visit 1 could coincide with Visit 0. If Visit 1 and Visit 0 were the same, then a physical examination, vital signs assessment, registration of concomitant therapy, pulse oximetry with SpO_2 measurement were not repeated, evaluation of inclusion and non-inclusion criteria was performed immediately before randomization, and exclusion criteria were assessed after drug use. **For patients in group 1: if a patient was discharged from hospital earlier, at the time of discharge the patient was undergoing procedures of Visit 3, CT of the lungs and the assessment of changes in the lungs using an "empirical" visual scale (according to CT of the lungs). If discharge from hospital was carried out earlier than day 7, then CT of the lungs and the assessment of changes in the lungs using an "empirical" visual scale (according to CT of the lungs) was carried out at the discretion of the researcher. *** If discharge from hospital was carried out on the 13th or 14th day from the therapy start, at the time of discharge, a carried out visit corresponded to the volume of procedures provided for an in-person Visit. All patients who were discharged earlier than day 15 received a Visit on days 15–16 corresponding to the scope of procedures provided for a Visit conducted by a phone call. ****If a discharge the hospital was carried out earlier, then, instead of a face-to-face Visit, it was made *via* a phone call.

Table 1 – Categorical ordinal scale for clinical improvement in COVID-19

Patient Status	Description	Category
Outpatient	No clinical and virological signs of infection	0
	No activity restrictions	1
	Activity restrictions	2
Hospitalized:	Hospitalized, no oxygen therapy	3
– mild disease	Oxygenation with a mask or nasal cannula	4
– severe disease course	Non-invasive ventilation or high-flow oxygenation	5
	Intubation or artificial lung ventilation	6
	Ventilation + additional organ support – vasopressors, renal replacement therapy, extracorporeal membrane oxygenation (ECMO)	7
Dead	Death	8

Table 2 – Baseline demographic, anthropometric and clinical characteristics of patients

Characteristics	Standard therapy, n=104	Hexapeptide succinate IM, n=104)	Hexapeptide succinate inhalation, n=104
Age, years (M ±SD)	57.64±16.44	57.54±16.02	59.46±16.46
Male, n (%)	50 (48.08)	53 (50.96)	43 (41.35)
BMI, kg/m^2 (M ±SD)	27.87±5.72	26.91±5.90	27.86±5.30
Following comorbidities/conditions *			
Hypertension, n (% of all FCs)	60 (29.56)	49 (25.13)	64 (29.22)
Obesity, n (% of all FCs)	34 (16.75)	21 (10.77)	33 (15.07)

Note: *in addition to those indicated in the table, the following comorbidities/conditions (FCs) were identified with a frequency of 5% or less: atrial fibrillation, chronic heart failure, myocardial ischemia, angina pectoris, osteochondrosis, type 2 diabetes mellitus, menopause.

Table 3 – Summarized data on comparative evaluation for hexapeptide succinate efficacy

Check point	Groups		
	Standard therapy	Hexapeptide succinate (IM)	Hexapeptide succinate (inhalation)
Primary criterion			
Achievement of category 0–1			
Visit 4 (Day 15)	66.35% (69/104)	85.15% (86/101)	83.33% (85/102)
Secondary criteria for efficacy			
Clinical status fewer than 4 points			
Visit 3 (Day 11)	69.23% (72/104)	87.13% (88/101)	83.33% (85/102)
Visit 4 (Day 15)	94.23% (98/104)	99.01% (100/101)	99.02% (101/102)
Improvement in clinical status by 2 or more categories			
Visit 3 (Day 11)	52.88% (55/104)	58.42% (59/101)	59.80% (61/102)
Visit 4 (Day 15)	90.38% (94/104)	98.02% (99/101)	96.08% (98/102)
Time till improvement in clinical status by ≥1 point			
Median time, days	7	6	6
Eligibility for discharge to continue treatment on outpatient basis in accordance with the IGs			
Visit 2 (Day 5)	13.46% (14/104)	16.83% (17/101)	17.65% (18/102)
Visit 3 (Day 11)	52.88% (55/104)	67.33% (68/101)	67.65% (69/102)
RR<22/min			
Visit 2 (Day 5)	60.98% (25/41)	71.43% (25/35)	85.71% (36/42)
Visit 3 (Day 11)	92.68% (38/41)	100.00% (35/35)	100.00% (42/42)
CRP<10 mg/l			
Visit 2 (Day 5)	52.78% (38/72)	59.46% (44/74)	55.88% (38/68)
Visit 3 (Day 11)	79.17% (57/72)	83.78% (62/74)	92.65% (63/68)
Blood lymphocytes >1.2×109/l at Visits 2 and 3			
Visit 2 (Day 5)	55.26% (21/38)	67.86% (19/28)	69.23% (27/39)
Visit 3 (Day 11)	71.05% (27/38)	75.00% (21/28)	76.92% (30/39)
Lung damage degree according to CT			
Visit 4 (Day 15) CT-0	30,77% (28/91)	33,33% (33/99)	33,33% (34/102)
CT-2	9,89% (9/91)	6,06% (6/99)	5,88% (6/102)
SpO_2≥95% for 2 consecutive days			
Visit 2 (Day 5)	64,29% (36/56)	72,41% (42/58)	74,14% (43/58)
Visit 3 (Day 11)	87,50% (49/56)	96,55% (56/58)	96,55% (56/58)
Visit 4 (Day 15)	91,07% (51/56)	100,00% (58/58)	98,28% (57/58)

Note: RR – respiratory rate; CRP – C-reactive protein; CT – computer tomography.

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Table 4 – Description of total number of AEs registered in patients in study groups AE (RT according to MeDRA)*

AE (RT according to MeDRA)*	Number of AEs, absolute value (% of AEs total number)			
	Hexapeptide succinate (IM) group, n=104	Hexapeptide succinate (IM) inhalation group, n=104	Standard therapy group, n=104	Total, n=312
Arrhythmia	0 (0%)	0 (0%)	1 (8.33%)	1 (3.33%)
Hyperglycemia	0 (0%)	0 (0%)	1(8.33%)	1(3.33%)
Headache	0 (0%)	1 (12.5%)	0 (0%)	1 (3.33%)
Diarrhea	0 (0%)	0 (0%)	1 (8.33%)	1 (3.33%)
Respiratory failure	1 (10%)	2 (25%)	0 (0%)	3 (30%)
Urinary tract infection	1 (10%)	0 (0%)	0 (0%)	1 (3.33%)
Concrement in urinary tract	0 (0%)	1 (12.5%)	0 (0%)	1 (3.33%)
Increase in ALT level	3 (30%)	1 (12.5%)	2 (16.67%)	6 (20%)
Increase in AST level	1 (10%)	2 (25.%)	2 (16.67%)	5 (16.67%)
Increase in blood glucose	2 (20%)	1 (12.5%)	1 (8.33%)	4 (13.33%)
Increase in blood creatinine level	1 (10%)	0 (0%)	0 (0%)	1 (3.33%)
Nausea	0 (0%)	0 (0%)	1 (8.33%)	1 (3.33%)
Prolongation of activated partial thromboplastin time	0 (0%)	0 (0%)	1 (8.33%)	1 (3.33%)
Prolongation of prothrombin time	0 (0%)	0 (0%)	2 (16.67%)	2 (6.67%)
Heart failure	1 (10%)	0 (0%)	0 (0%)	1 (3.33%)
Total:	10 (100%)	8 (100%)	12 (100%)	30 (10%)

Note: *PT (preferterm) – the level of the international dictionary of medical and therapeutic terms MeDRA; ALT – alanine aminotransferase; AST – aspartate aminotransferase.

Results of efficacy evaluation

Summarized comparative analysis data on efficacy criteria are presented in Table 3.

Primary criterion for efficacy

In the succinate hexapeptide intramuscular group, the proportion of patients who achieved category 0–1 on the categorical ordinal scale of the clinical improvement at Visit 4 was 85.15% (86/101), in the succinate hexapeptide inhalation group it was 83.33% (85/102), in the standard therapy group – 66.35% (69/104). The 95% CI for the proportion of patients achieving category 0–1 on the categorical ordinal scale of the clinical improvement at Visit 4 was 95% CI [0.7637; 0.9118] for hexapeptide succinate intramuscularly, and 95% for hexapeptide succinate inhalation CI [0.7437; 0.8972], in the standard therapy group – 95% CI [0.5634; 0.7514]. The difference in proportions between the succinate hexapeptide intramuscular group and the standard therapy group was 0.188 (18.80%), a 95% CI for the difference in proportions between the groups was -95% CI [0.0638; 0.3049]. The difference in proportions between the succinate hexapeptide inhalation group and the standard therapy group was 0.1699 (16.99%), a 95% CI for the difference in proportions between the groups was -95% CI [0.0443; 0.2886].

As a result of the analysis, statistically significant differences were found in the frequency of achieving category 0–1 on the categorical ordinal scale of the clinical improvement by Visit 4 both between the group of the drug hexapeptide succinate, intramuscular administration, and the standard therapy group (p=0.0017), and between the hexapeptide succinate group, the inhalation administration, and the standard therapy group (p=0.0050).

Thus, it was shown that, in contrast to standard hexapeptide therapy, succinate, both intramuscular and inhaled, provided an acceleration of recovery up to the complete absence of signs of the disease in more than 80% of hospitalized COVID-19 patients.

Moreover, since there were patients with concomitant diseases among the study participants, it can be concluded that hexapeptide succinate therapy is highly effective both in patients without concomitant diseases and in patients with comorbid pathology who have risk factors for the progression of COVID-19 to a severe course, regarding the acceleration of recovery and discharge from hospital, as well as reducing the risk of a aggravated course of COVID-19 and transfer to the ICU, which confirms the clinical efficacy and pharmacoeconomic feasibility of using the studied treatment regimens.

The course of therapy with the drug hexapeptide succinate helped to accelerate the recovery and discharge from hospital, prevent the progression of COVID-19 to a severer course, which indicates a high efficacy and substantiates the introduction of studied therapy regimens into the clinical practice.

Secondary criteria for efficacy

At Visit 3, as a result of a comparative analysis of the patients' frequency with a clinical status of fewer than 4 points on a categorical ordinal scale of clinical improvement, statistically significant differences were revealed between the succinate hexapeptide group (IM) and the standard therapy group (p=0.0020), and also between the succinate hexapeptide group (inhalation) and the standard therapy group (p=0.0175). The data obtained indicate a more effective, compared with standard therapy, effect of succinate hexapeptide on the dynamics of symptoms in COVID-19 patients, leading to a pronounced improvement in the clinical condition of patients. The treatment with succinate hexapeptide, both intramuscularly and by inhalation, by the end of therapy, 10 days after its start, ensured the absence of restrictions on daily activities in more than 80% of patients with a coronavirus infection. These data confirm the efficacy of therapy in relation to the course of the disease, improving the quality of life of patients.

As a result of a comparative frequency analysis of the improvement in a clinical status on a categorical ordinal scale of a clinical improvement by 2 or more categories, statistically significant differences were found between the hexapeptide succinate (IM) group and the standard therapy group at Visit 4 (p=0.0334). Thus, it has been shown that, compared with standard therapy, the use of hexapeptide succinate leads to a more pronounced, rapid and significant improvement in the condition of COVID-19 patients.

As a result of a comparative frequency analysis of the of patients meeting the criteria for discharge to continue treatment on an outpatient basis in accordance with the BMRs, there were statistically significant differences between the succinate hexapeptide (inhalation) group and the standard therapy group at Visit 3 (p=0.0305), and between the hexapeptide succinate (IM) group and the standard therapy group (p=0.0348). Thus, it was shown that, in contrast to the standard therapy in the main group, by the end of therapy with hexapeptide succinate, both intramuscularly and inhaled, more than 60% of patients met the discharge criteria and could continue treatment on an outpatient basis, which reduces the burden on the healthcare system and indicates the appropriateness of the study therapy.

As a result of a comparative frequency analysis of patients with a RR<22/min by the end of the therapy, statistically significant differences (p=0.01) were revealed between the group of the patients who had received hexapeptide succinate (the inhalation administration) and standard therapy: 85.7% (36/42) and 60.9% (25/41), respectively. That indicates an improvement in the condition of patients, the disappearance of shortness of breath and a respiratory failure, which helps to reduce the risk of developing COVID-19 complications.

As a result of a comparative frequency analysis of patients with a level of CRP<10 mg/l at Visits 2 and 3, there were no statistically significant differences between the study groups. It should be notified that, in contrast to the patients receiving standard therapy, more than 50% of the patients treated with hexapeptide succinate showed a decrease in CRP<10 mg/l by the 5^{th} day of therapy. It should be emphasized that by the end of therapy, more than 90% of patients who had received the drug in the inhalation form, achieved a decrease in CRP to normal values. That indicates the anti-inflammatory effect of the drug, reducing the consequences of a systemic hyperimmune reaction, reducing the severity of the acute tissue damage, and reducing the risks of developing COVID-19 complications and improved the disease prognosis.

According to the CT data in the succinate hexapeptide (IM) group at Visit 4, the mean value (Mean±SD) of the lung injury degree was 0.73±0.57; in the group of hexapeptide succinate (inhalation) – 0.73±0.57; in the standard therapy group – 0.79±0.61.

According to the CT data, the assessment of the lung damage degree showed that therapy with hexapeptide succinate leads to a significant improvement in the condition of the lungs up to a complete disappearance of the disease symptoms. It should be notified that, according to the results of the intragroup analysis of the lung damage degree, in contrast to the standard therapy, in both succinate hexapeptide groups, a statistically significant difference was found out between the moment of screening patients and days 15-16 of therapy (p<0.0001). That indicates the presence of positive

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dynamics in the course of the disease – a decrease in the lung damage degree in the study drug group, both with the intramuscular and inhalation administrations. Therefore, the study drug use contributes to the restoration of damaged lung tissues, including alveolar epithelial cells.

As a result of a comparative frequency analysis of the patients with SpO_2≥95% for 2 consecutive days before Visit 4, statistically significant differences were found between the hexapeptide succinate (IM) group and the standard therapy group (p=0.0260). It should be notified that by the end of therapy, in the study drug group, more than 90% achieved normalization of the oxygenation index, which indicates a decrease in the risk of developing COVID-19 complications and an improvement in prognosis. Thus, the use of hexapeptide succinate reduces the severity of diffuse alveolar damage to the lung tissue, which helps prevent the development of pulmonary fibrosis and normalizes a ventilation lungs function.

Additional research parameters

As a result of comparing the biochemical blood test parameters, statistically significant differences were revealed between groups 1 and 3 at Visit 2 in terms of "LDH" (p=0.016).

In the group of the studied drug, a decrease in LDH was observed in the hexapeptide succinate inhalation administration at Visit 2, and the values of this enzyme were lower compared to the standard therapy group. That may indicate a more damage reduction and recovery, restoration of damaged tissues, including alveolar epithelial cells, improving energy metabolism in the cells and the function of cell membranes. In addition, in the groups treated with the test compound, there was a decrease in such indicators as ESR, CRP, IL-6, D-dimer, lactate, triglycerides. These factors also confirm its anti-inflammatory effect.

Safety assessment

The frequency of patients with reported cases of AE/SAE was 7.69% (24/312). A total of 24 patients had 30 AEs (Table 4).

A comparative analysis in terms of their presence, severity, causal relationship with therapy and the outcome, no statistically significant differences were found out between the treatment groups. In the study drug groups, the majority of AEs were transient, and there were no cases of discontinuation of therapy or dose changes due to the development of AEs in the study drug groups. The study physicians assessed that the study drug had been well tolerated by the patients.

As a result of a comparative frequency analysis of the patients' transfer cases to the intensive care unit, the use of HPE, NIVL, ALV, ECMO, the development of ARDS, no statistically significant differences were found out between the study groups.

There were no serious adverse events associated with the study drug. Thus, the assessment of the ongoing therapy safety indicates a positive benefit/risk profile in relation to the drug Ambervin® Pulmo.

CONCLUSION

Thus, the results of the clinical study "Open randomized multicenter comparative study to assess the efficacy, safety and tolerability of the use of Ambervin® Pulmo, a lyophilisate for the preparation of a solution for intramuscular injection and a solution for inhalation in patients hospitalized with COVID-19" showed that therapy study drug, both intramuscular and inhaled, provided an acceleration of recovery up to the complete absence of signs of the disease in more than 80% of hospitalized COVID-19 patients. By the end of the therapy course with hexapeptide succinate, more than 60% of the patients met the criteria for discharge from hospital and could continue treatment on an outpatient basis, which reduces the burden on the healthcare system and confirms the feasibility of using the study therapy. It is important to notify that 70% of patients in the inhalation group and 80% in the intramuscular group of the study drug had comorbidities (mainly hypertension and obesity), which are risk factors for the progression of COVID-19 to a severe course. The use of the drug contributed to the restoration of damaged lung tissues, including alveolar epithelial cells, the normalization of oxygenation, the disappearance of shortness of breath and a decrease in the duration of symptoms of the disease compared with standard therapy. As a result of a comparative analysis of adverse events in terms of their presence, severity, causal relationship with therapy and the outcome, there were no statistically significant differences between the treatment groups. According to the investigators, the study drug is characterized by a high safety profile and good tolerability.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Larisa A. Balykova – development and implementation of research design, text writing and editing; Olga A. Radaeva – study design development, results analysis, text editing; Kira Ya. Zaslavskaya – research design development, text editing, analysis of literary sources; Petr A. Bely – study design development, results analysis, text editing; Vera F. Pavelkina – sources collecting, data processing, article writing; Nikolai A. Pyataev – sources collecting, data processing, article writing; Anastasia Yu. Ivanova – study design implementation, data processing; Grigory V. Rodoman – study design implementation, data processing; Natalya E. Kostina – study design implementation, data processing; Viktor B. Filimonov – research design implementation, data processing; Elena N. Simakina – study design implementation, data processing; Dmitry A. Bystritsky – study design implementation, data processing; Alina S. Agafyina – study design implementation, data processing; Ksenia N. Koryanova – sources collecting, data processing, article writing; Dmitry Yu. Pushkar – development and implementation of research design, data processing

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EXPERIMENTAL PARTICIPATION OF PHARMACOLOGICAL SUBSTANCES IN MECHANISMS OF LEAD ACETATE TOXICITY

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The aim of the work is to study pharmacological substances that play a role of eNOS expression regulators in the modification of lead intoxication effects in the experiment.

Materials and methods. In the experiment, linear male rats of the same age were used: intact and with lead intoxication (120 heads). The study design was the following: group 1 – control; group 2 – intoxication with a lead acetate solution; group 3 – intact + L-nitroarginine methyl ester; group 4 – lead acetate + L-nitroarginine methyl ester; group 5 – intact + L-arginine; group 6 – lead acetate + L-arginine. The research carried out the study state of the redox reactions, the content of nitric oxide (NOx) stable metabolites, a lipid profile, the level of NO-synthase (eNOS) expression in the vascular endothelium, the main processes of urination and the activity of Na^+/K^+-ATPase in the renal tissue layers, as well as in the liver. The results were subjected to statistical processing.

Results. Saturnism caused the oxidative stress development, a decrease in the NO_x content in blood plasma, a violation of the L-arginine for eNOS bioavailability, and an endothelial dysfunction. Indicators of the impaired renal function were a decrease in the glomerular filtration rate (GFR), the tubular reabsorption of water, sodium, and the Na^+/K^+-ATPase activity. The damage to hepatocytes was evidenced by changes in the activity of organ-specific enzymes in the blood and Na^+/K^+-ATPase. L-arginine exhibited antioxidant properties, increased the NO_x content and the level of eNOS expression. The eNOS L-nitroarginine methyl ester inhibitor showed the effects opposite to L-arginine.

Conclusion. Biochemical markers of damage to kidney and liver cells during saturnism are indicators of the oxidative stress, NO_x deficiency and hemodynamic disturbances in them. These mechanisms involved the following pharmacological substances: an eNOS inhibitor, L-nitroarginine methyl ester, which caused a decrease in the expression level of the enzyme, and an eNOS inducer, L-arginine, which increased this indicator severity. The lead toxicity mechanisms have been implicated in the impaired cholesterol metabolism, contributing to the L-arginine reduced availability for eNOS and the NO_x production. Therefore, the use of L-arginine can be recommended as a regulator of the oxidative stress and an NO-producing endothelial function in other pathologies.

Keywords: lead acetate; lipid peroxidation; antioxidant system; total nitric oxide metabolites; endothelial dysfunction; L-arginine; L-NAME; kidney function; cholesterol; hepatocytes

Abbreviations: MPC – maximum permissible concentration; LPO – lipid peroxidation; AOS – antioxidant system; NO – nitric oxide; NO_x – total nitric oxide metabolites; eNOS – endothelial NO synthase; iNOS – inducible NO synthase; IP – inorganic phosphorus; Na^+/K^+-ATPase – sodium-potassium adenosine triphosphatase; L-NAME – L-nitroarginine methyl ester; TNF-α – tumor necrosis factor-α; iL-1β – interleukin 1β; iL-10 – interleukin 10; MDA – malonic dialdehyde; SOD – superoxide dismutase; CP – ceruloplasmin; TC – total cholesterol; LDL – low density lipoproteins; HDL – high density lipoproteins; GFR – glomerular filtration rate; ADMA – asymmetric dimethylarginine; ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGTP – gamma-glutamyl transpeptidase; AORs – active oxygen radicals; AOMs – active oxygen metabolites; FRO – free radical oxidation.

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УЧАСТИЕ ФАРМАКОЛОГИЧЕСКИХ ВЕЩЕСТВ В МЕХАНИЗМАХ ТОКСИЧНОСТИ АЦЕТАТА СВИНЦА В ЭКСПЕРИМЕНТЕ

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Цель. Изучение фармакологических веществ, играющих роль регуляторов экспрессии eNOS, в модификации эффектов свинцовой интоксикации в эксперименте.

Материал и методы. В эксперименте были использованы линейные крысы-самцы одного возраста: интактные и со свинцовой интоксикацией (120 голов). Дизайн исследования: группа 1– контроль; группа 2 – интоксикация раствором ацетата свинца; группа 3 – интактные + L-нитроаргинин метиловый эфир; группа 4 – ацетат свинца + L-нитроаргинин метиловый эфир; группа 5 – интактные + L-аргинин; группа 6 – ацетат свинца + L-аргинин. В исследовании проводилось изучение состояния окислительно-восстановительных реакций, содержания стабильных метаболитов оксида азота (NO_x), липидного профиля, уровня экспрессии NO-синтазы (eNOS) в эндотелии сосудов, основных процессов мочеобразования и активности Na^+/K^+-АТФ-азы слоёв почечной ткани, а также в печени. Результаты подвергались статистической обработке.

Результаты. Сатурнизм вызвал развитие окислительного стресса, снижение содержания NO_x в плазме крови, нарушение биодоступности L-аргинина для eNOS и дисфункцию эндотелия. Показателями нарушения функции почек были снижение скорости клубочковой фильтрации (СКФ), канальцевой реабсорбции воды, натрия и активности Na^+/K^+-АТФ-азы. О повреждении гепатоцитов свидетельствовало изменение активности органоспецифических ферментов в крови и Na^+/K^+-АТФ-азы. L-аргинин проявлял антиоксидантные свойства, повышал содержание NO_x и уровень экспрессии eNOS. Ингибитор eNOS – L-нитроаргинин метиловый эфир показал противоположные L-аргинину эффекты.

Заключение. Биохимическими маркерами повреждения клеток почек и печени при сатурнизме являются показатели окислительного стресса, дефицит NO_x и нарушение гемодинамики в них. В этих механизмах участвовали фармакологические вещества: ингибитор eNOS – L-нитроаргинин метиловый эфир, вызывавший снижение уровня экспрессии энзима, и индуктор eNOS – L-аргинин, повышавший степень выраженности этого показателя. В механизмах токсичности свинца участвовало нарушение обмена холестерина, способствующее сниженной доступности L-аргинина для eNOS и продукции NO_x. Следовательно, применение L-аргинина можно рекомендовать как регулятора окислительного стресса и NO-продуцирующей функции эндотелия при других патологиях.

Ключевые слова: ацетат свинца; перекисное окисление липидов; антиокислительная система; суммарные метаболиты оксида азота; дисфункция эндотелия; L-аргинин; L-NAME; функция почек; холестерин; гепатоциты

Список сокращений: ПДК – предельно допустимая концентрация; ПОЛ – перекисное окисление липидов; АОС – антиокислительная система; NO – оксид азота; NO_x – суммарные метаболиты оксида азота; eNOS – эндотелиальная NO-синтаза; iNOS – индуцибельная NO-синтаза; Рн – неорганический фосфор; Na^+/K^+-АТФ-аза – натрий-калиевая аденозинтрифосфатаза; L-NAME – L-нитроаргинин метиловый эфир; TNF-α – фактор некроза опухоли-α; iL-1β – интерлейкин 1β; iL-10 – интерлейкин 10; МДА – малоновый диальдегид; СОД – супероксиддисмутаза; ЦП – церулоплазмин; ОХС – общий холестерин; ЛПНП – липопротеины низкой плотности; ЛПВП – липопротеины высокой плотности; СКФ – скорость клубочковой фильтрации; АДМА – ассиметричный диметиларгинин; АЛТ – аланинаминотрансфераза; АСТ – аспартатаминотрансфераза; ГГТП – гамма-глутамилтранспептидаза; АРК – активные радикалы кислорода; АМК – активные метаболиты кислорода; СРО – свободно-радикальное окисление.

INTRODUCTION

Experimental and clinical studies conducted in recent years have shown the negative role of ecopathogenic factors and their participation in the development of vascular complications, an endothelial dysfunction and pathology of internal organs – kidneys and liver. In this aspect, heavy metals, which quite often exceed the maximum permissible concentration (MPC) in the environment, make their negative contribution. Scientists are also interested in the effect of lead on metabolism and the function of internal organs. When evaluating the toxicity of lead for the body, one should take into account its persistence and cumulative capacity for biological media in humans and animals [1–3]. Lead ions at low levels, which were previously considered safe, cause toxic effects [4–6]. Being a polytropic poison,

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lead is capable of disrupting the structure and function of the internal organs' cells. Lead nephropathy and hepatopathy are an integral part of the toxic effect; almost all elements of the nephron and hepatocyte are damaged [7, 8].

By changing the heme structure of blood hemoglobin, lead is the cause of anemia, hypoxia of the organ cells and the activation of lipid peroxidation (LPO) in erythrocytes and tissues [9-14]. Being a necessary component of body systems under physiological conditions, free radical oxidation (FRO) can be a factor in the development of a pathological process. However, it should be noted that there are very few published data that indicate the ability of lead to activate the lipid peroxidation (LPO) process in the blood and cells of the internal organs. Moreover, the increased activity of free radical reactions (FRRs) can cause not only negative phenomena, but also play the role of a pathogenetic link in a number of pathological processes in various nosologies. This process can be interpreted as a multifunctional stress response of the body to toxic effects. Developing an oxidative stress inhibits the production of nitric oxide (NO), which acts as an intracellular messenger and is involved in the implementation of response reactions from the cells of organs and tissues [15]. NO is formed by the enzymatic oxidation of L-arginine with NO synthase (eNOS). LPO reactions cause changes in lipoproteins in biological membranes, while increasing their hydrophilicity, permeability and disruption of lipid-protein interactions, including the participation of enzymes.

A few pieces of information in the literature are devoted to the activity analysis of Na^+/K^+-ATPase erythrocytes during the lead intoxication in the metallurgical industry workers [16]. Experimental studies on rats have shown that a systematic exposure to lead acetate is accompanied by its accumulation in the nephron structures and the impairment of the functional kidneys state. The contact duration with lead and the amount of cumulated substance can lead to chronic nephropathy, characterized by the development of inflammation and apoptosis of kidney cells [17].

Pro-inflammatory cytokines are involved in the mechanisms of nephropathy development: tumor necrosis factor-α (TNF-α), interleukin 1β (iL-1β), etc., with an insufficient level of anti-inflammatory cytokines – iL-10 in the nephron. At the same time, it should be noted that complex studies on changes in redox reactions and their role in the disturbance of NO metabolism, the activity and expression of NO synthase, a kidney and liver function in case of systematic poisoning of the body with lead acetate are insufficiently represented in the available literature. There are no literature data on the pharmacological drugs that play a role of the eNOS expression regulators in the lead intoxication; on the participation of L-arginine and its modified derivative L-NAME (L-nitroarginine methyl ester) in these processes. That was the basis for this experimental study.

THE AIM of the work is to study pharmacological substances that play a role of eNOS expression regulators in the modification of lead intoxication effects in the experiment.

MATERIALS AND METHODS

The studies were carried out on linear male rats of the same age group (10–14 months), weighing 200–280 grams: intact – control (n=20) and with a chronic lead intoxication caused by a daily administration of a lead acetate solution at the dose of 5 mg/kg of an animal body weight subcutaneously for 30 days (n=60). Against the background of the lead intoxication, the intact animals (n=40) were administered pharmacological substances: L-arginine (10 mg/kg) and L-NAME (25 mg/kg). During the experiments, the animals obtained from the vivarium of the Institute of Institute of Biomedical Research – branch of Vladikavkaz Scientific Center of the Russian Academy of Sciences were on a standard diet with a free access to water, and a natural light regime.

The experimental animals were divided into the following series: the 1st control group (n=20) – the studies on intact rats; the 2nd experimental group (n=20) with a systematic intoxication caused by a subcutaneous injection of a lead acetate solution (5 mg/kg of an animal body weight) for 30 days; the 3rd group (n=20) – intact rats + modified L-arginine (L-NAME); group 4 (n=20) – the rats intoxicated with lead acetate + L-NAME; group 5 (n=20) – the intact rats with the L-arginine administration; group 6 (n=20) – the rats intoxicated with lead acetate + L-arginine. All series of the experiments had a duration of 30 days. The experiments were carried out in accordance with the requirements for the work using animals in the experiment. The compliance with the international requirements for working with experimental animals, including humane treatment of them, is confirmed by the decision of the Institute of Biomedical Research – branch of Vladikavkaz Scientific Center of the RAS Ethics Committee (December 26, 2018, Protocol No. 6). The eNOS expression regulators, L-arginine (Dia-m LLC, Ajinomoto, Japan) and L-NAME (Etalon LLC, Cat. No. 5757 Sigma-Aldrich, USA) were used.

At the end of the experiments, the chest in intact and experimental rats was opened up under roush anesthesia, the blood was taken with sodium citrate anticoagulant from the left ventricle of the heart through a catheter, centrifuged in a laboratory centrifuge TsLMN-P10-01-ELEKON, ELEKON-M, Russia, for 10 min at 1500 rpm, the blood plasma was collected. The erythrocyte mass was washed twice with saline, then a certain volume was lysed. At the same time, the samples of kidney and liver tissues were taken, homogenized at +4°C and homogenates were obtained. The effectiveness of modeling was assessed by the content of lead in the blood and the development of intoxication syndrome by the intensity of the LPO-AOS system. The state of the antioxidant system (AOS) of the body was assessed by

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the activity of its enzymes – catalase in blood plasma spectrophotometrically according to the method of M.A. Korolyuk (1988) [19], and superoxide dismutase (SOD) – by the adrenaline oxidation method in erythrocyte hemolysate (Sirota TV, 1999) [20], the concentration of ceruloplasmin (CP) – by Ravin's method [21]. Cholesterol metabolism (CM) was determined according to the total cholesterol (TC), LDL-C, HDL-C and TAG in the blood plasma using kits (Vital Development Corporation, Russia). The degree of peroxidation was determined according to the change in the concentration of the end product of lipid peroxidation – malonic dialdehyde (MDA) in the hemolysate of erythrocytes, in the homogenates of the renal and hepatic tissues by the colorimetric method with thiobarbituric acid according to the method of Asacawa T. [18]. The state of the antioxidant system (AOS) of the body was assessed by the activity of its enzymes – catalases in blood plasma spectrophotometrically according to the method of M.A. Korolyuk (1988) [19] and superoxide dismutase (SOD) – by the method of adrenaline oxidation in erythrocyte hemolysate (Sirota TV, 1999) [20], the concentration of ceruloplasmin (CP) – by Ravin's method [21]. Cholesterol metabolism (CH) was determined according to the total cholesterol (TC), LDL-C, HDL-C and TAG in blood plasma using kits (Vital Development Corporation, Russia).

At the same time, the activity of enzymes – alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGTP) and alkaline phosphatase in blood plasma – was studied using the kits of the "Vital" company. For the analysis and determination of stable nitric oxide (NO_x) metabolites in the blood plasma, the method developed by V.A. Metelskaya was used. [22].

The study of the expression level of endothelial NO-synthase (eNOS) was carried out by Western blotting in the aorta endothelium in experimental animals, including the samples treatment in liquid nitrogen. The resulting content was placed in a centrifuge tube, washed with phosphate buffer 3 times, and the precipitate was centrifuged for 10 min at 1000 g. The precipitate obtained was subjected to the action of a lysis buffer in the volume of 100 µl. To determine the effect of the used pharmacological preparations, a comparative analysis of the eNOS expression level was used in comparison with control marker proteins. The data were expressed in arbitrary units, calculating the ratio of the studied band X in relation to the control data on each film. The determination of this indicator was carried out jointly with the biochemical laboratory of the National Medical Research Center for Therapy and Preventive Medicine. The toxicity of lead acetate in the experimental animals was determined according to the content of this heavy metal in the animals' blood. The metal was extracted with concentrated nitric acid, after which the lead content was determined spectrophotometrically on a Hewlett-Packard ICH-VSHP 4500 mass spectrophotometer (USA).

To study the functional nephron state, the conditions of spontaneous diuresis were used, the main processes of urine formation were evaluated: glomerular filtration rate (GFR) and tubular reabsorption of sodium (R_{Na}%) and water (R_{H2O}%), filtration charges of potassium and sodium. The value of glomerular ultrafiltration was determined by the clearance method by the ratio of urinary creatinine to its content in blood plasma, taking into account hourly diuresis. All kidney function indicators were calculated according to the formulas developed by Academician Natochin Yu.V. [23]. The activity of Na^+/K^+-ATPase in the homogenates of the cortical and medulla of the kidneys, as well as the liver, was determined by the method of Scow J.C. (1957). A specific activity was calculated per mg of protein per hour (µmolRn/mg protein/h). The protein in the samples was determined by the Lowry O.H. method. (1951) [24].

The results were statistically processed using the Microsoft Excel 2006 program and the Statistica 6.0 Package. The data obtained are characterized by a normal distribution in accordance with the Shapiro-Wilk test, and a parametric statistical method was used in their processing. The data were presented as mean (M) and error of mean (±m). The significance of differences was determined by Student's t-test, the statistical significance of differences between the groups was checked, and $p<0.05$ was considered the level of the statistical significance. Correlation coefficients were determined according to Pearson.

RESULTS AND DISCUSSION

Getting into the body of animals and humans, lead inhibits the synthesis of hemoglobin and causes the development of anemia, followed by tissue hypoxia. Violation of the oxygen transport blood function contributes to the formation of reactive oxygen species (ROSs) and activation of the LPO process. Being necessarily present in cell membranes FRO is an important factor in the normal functioning of cells, providing renewal of cell membrane phospholipids and the regulation of metabolism. However, an increased CPP activity can be the cause of negative manifestations and pathological processes. Nevertheless, it should be noted that the literature provides insufficient information on the ability of lead to activate FRO in the blood and internal organs as a pathogenetic link in vascular complications, its relationship with other metabolic processes, in particular with the regulation of the NO content, NO synthase activity, interaction with enzyme systems and participation in these processes of eNOS expression regulators – L-arginine and L-NAME. Systematizing and analyzing the results obtained on the nature of changes in redox reactions during the lead intoxication, it should be noted that there is an activation of lipid peroxidation according to a significant increase in erythrocytes in the MDA concentration, in the cells of the renal and hepatic tissues (Table 1). The MDA concentration in the hemolysate of erythrocytes increases by an average

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of 33.3% ($p<0.02$), in the cortical and medulla of the kidneys with a parenteral administration of the heavy metal – by 74.2% ($p<0.001$) and 25.4% ($p<0.001$), respectively, as well as in hepatocyte – by 94.1% ($p<0.001$).

In the interconnection with the FRO processes is the AOS of the organism, which limits lipid peroxidation in almost all of its links. The state of the AOS was judged by the activity of superoxide dismutase (SOD), catalase, and the concentration of ceruloplasmin (CP). The data showed an imbalance in the AOS – a decrease in the SOD activity and an increase in the level of catalase and CP (Table 1).

The analysis of the AOS activity data showed the SOD inhibition in erythrocytes by 62.2%, while the catalase activity and CP content in the blood serum increased by 69.5% and 27.6%, respectively. To understand the lack of unidirectionality in the change activity of AOS enzymes, the difference in their molecular structure is taken into account; while the catalase enzyme is more protected than SOD due to the presence of four heme molecules and 4 NADPH (nicotinamide adenine dinucleotide phosphate).

The intoxication syndrome development in the parenteral administration of lead acetate is characterized by a significant increase in the lead content in blood plasma, urine, kidney and liver tissues. A strong correlation was found out between the administered lead acetate dose and its concentration in the blood serum ($r=+0.95$; $p<0.001$). These results are consistent with the literature data [12]. Any xenobiotic, as a rule, is oxidized in the microsomal fraction of the hepatocyte, exerts its damaging effect and is excreted by the kidneys. In accordance with the data obtained, it was noted that during the lead intoxication, reactive oxygen radicals (RORs) induce the development of a systemic oxidative stress, which is accompanied by a decrease in the concentration of NO_x in the blood plasma (Table 1).

An analysis of the relationship between an increase in the MDA blood concentration in the NO_x level decrease showed a strong negative relationship between these indicators ($r=–0.69$; $p<0.001$). It should be notified that a disruption of the L-arginine-NO-synthase-NO signaling pathway plays a decisive role in the NO regulation of the vasodilator action. LPO products can disrupt the interaction between the oxygenase and reductase domains of NO synthase, as a result of which the enzyme begins to produce ROS and, accordingly, less NO is formed. In discussing these results, the causal relationship of reduced NO_x levels, the intensity of lipid peroxidation, the presence of the L-arginine substrate, its modified derivatives, the activity and the eNOS expression level should be notified. In this aspect, it should be assumed that in the development of L-arginine deficiency as an eNOS expression inducer, its use in the ornithine cycle might also play a role.

The literature data indicate an increased content of urea in the blood serum against the background of the lead intoxication, due to its production [25, 26]. Therefore, many researchers use the arginase enzyme inhibitors of the urea synthesis cycle to increase the concentration of the L-arginine substrate [27]. The reason for the L-arginine deficiency may be its conversion into asymmetric dimethylarginine (ADMA) and L-NAME, i.e., modified derivatives that play a competitive role with L-arginine and reduce its bioavailability for eNOS. In this regard, the effect of these amino acids on the content of LPO products, NOx, and eNOS expression was studied. A participation of the increased LPO activity, the MDA concentration in erythrocytes, the cells of the cortical and medulla of the kidneys and hepatocytes in the disruption of the NO_x production was shown in this study. Their relationship is confirmed by the presence of a negative correlation between these indicators. To establish the fact of the violation of the NO-producing endothelium function and the insufficient NO_x formation, the involvement of the expression level and the eNOS functional activity were studied. Another cause of the L-arginine deficiency is an increase in the modified L-arginine derivative level – ADMA and its analogue – L-NAME. The data obtained showed that the eNOS expression inhibitor caused the activation of the LPO process and, at the same time, an even more pronounced decrease in the NO_x level (Table 1). In contrast to this result, L-arginine showed its ability to increase the NO concentration during saturnism and at the same time provide a decrease in the LPO activity according to the MDA content (Table 2). The adaptive system under conditions of the reduced FRO activity under the influence of L-arginine showed an increase in the SOD activity and positive dynamics in relation to catalase and CP (Table 2).

In the regulation of the eNOS expression level and its activity, an important role is played by coenzymes, which lose their reduced state under an oxidative stress, as well as in the presence of an expression inhibitor – eNOS, an analogue of ADMA, L-NAME. To confirm this assumption, a study of the eNOS expression against the background of L-NAME during the lead acetate intoxication was undertaken. The data showed that in saturnism, L-NAME inhibited the eNOS expression by 23.9%, while L-arginine stimulated this expression by 29.05%. By normalizing redox reactions, L-arginine simultaneously increased the production and content of NO_x, herewith promoting vasodilation. The data obtained indicate the participation of L-NAME and L-arginine in the regulation of the expression level of the endothelial NO synthase. The results of the study confirm that the iNOS activation under the oxidative stress conditions is accompanied by a decrease in the eNOS expression level, which is responsible for the basic level of the NO_x production as the main vasodilator. The very fact of an increase in the level of the eNOS expression under the influence of the eNOS inducer L-arginine and a decrease in this indicator against the background of a modified amino acid derivative, L-NAME, gives priority to the results.

Table 1 – Influence of expression inhibitor eNOS – L-NAME on changes nature in indicators of oxidative stress and lipid metabolism during saturnism in the experiment

Indicators	Units of measurement	Control	Lead acetate	Intact + L-NAME	Lead acetate + L-NAME
MDA, erythrocytes	nmol/ml	4.74±0.16	6.32±0.015[a]	4.82±0.043[b]	6.59±0.03[b,c]
MDA, cortex	nmol/mg protein	3.18±0.22	5.54±0.02[a]	3.24±0.01[b]	5.67±0.02[b,c]
MDA, medulla	nmol/mg protein	4.25±0.059	5.33±0.009[a]	4.55±0.042[b]	5.48±0.015[b,c]
MDA, hepatocyte	nmol/mg protein	1.73±0.05	3.36±0.007[a]	1.77±0.07[b]	3.52±0.013[b,c]
SOD	c.u.	88.05±0.07	54.94±0.081[a]	82.1±1.67[b]	51.97±0.318[b,c]
Catalase	mkat/l	225.56±29.09	382.36±0.313[a]	285.6±4.63[b]	395.41±3.01[b,c]
CP	mg/l	339.14±6.59	432.29±1.14[a]	360.2±1.15[b]	448.6±3.18[b,c]
NO	µmol	50.95±0.65	29.38±0.029[a]	48.13±0.57[b]	28.33±0.32[b,cc]
TC	mmol/L	1.88±0.03	4.67±0.009[a]	2.08±0.01[b]	5.02±0.014[b,c]
LDL cholesterol	mmol/L	1.09±0.01	4.15±0.02[a]	1.11±0.01[b]	4.54±0.005[b,c]
HDL cholesterol	mmol/L	0.673±0.01	0.27±0.006[a]	0.65±0.03[b]	0.205±0.009[b,c]
TAG	mmol/L	0.246±0.011	0.55±0.009[a]	0.25±0.01[b]	0.61±0.007[b,c]

Note: MDA – malondialdehyde; SOD – superoxide dismutase; CP – ceruloplasmin; NO – nitric oxide; TC – total cholesterol; LDLs – low density lipoproteins; HDLs – high density lipoproteins; TAG – triacylglycerides; L-NAME – L-nitroarginine methyl ester; a – p<0.001 – significance of lead acetate relative to control; b – p<0.001, significance of lead acetate + L-NAME relative to intact + L-NAME; c – p<0.001 – significance of lead acetate + L-NAME relative to control; cc – p<0.01 – significance of lead acetate + L-NAME vs lead acetate.

Table 2 – Influence of eNOS expression inductor – L-arginine – on the nature of changes in oxidative stress and lipid metabolism in saturnism during the experiment

Indicators	Units of measurement	Control	Lead acetate	Intact + L-NAME	Lead acetate + L-NAME
MDA, erythrocytes	nmol/ml	4.74±0.16	6.32±0.015[a]	4.18±0.10[b]	6.05±0.1[b,cc]
MDA, cortex	nmol/mg protein	3.18±0.22	5.54±0.02[a]	2.8±0.05[b]	5.26±0.005[b,c]
MDA, medulla	nmol/mg protein	4.25±0.059	5.33±0.009[a]	3.9±0.06[b]	5.19±0.009[b,c]
MDA, hepatocyte	nmol/mg protein	1.73±0.05	3.36±0.007[a]	1.67±0.03[b]	3.22±0.013[b,c]
SOD	c.u.	88.05±0.07	54.94±0.081[a]	88.8±1.37[b]	57.18±0.38[b,c]
Catalase	mkat/l	225.56±29.09	382.36±0.313[a]	221.72±2.97[b]	370.17±3.12[b,c]
CP	mg/l	339.14±6.59	432.29±1.14[a]	336.4±6.39[b]	416.3±3.71[b,c]
NO	µmol	50.95±0.65	29.38±0.029[a]	53.25±0.08[b]	32.07±0.29[b,c]
TC	mmol/L	1.88±0.03	4.67±0.009[a]	1.84±0.02[b]	4.32±0.009[b,c]
LDL cholesterol	mmol/L	1.09±0.01	4.15±0.02[a]	1.03±0.03[b]	3.77±0.015[b,c]
HDL cholesterol	mmol/L	0.673±0.01	0.27±0.006[a]	0.69±0.03[b]	0.39±0.011[b,c]
TAG	mmol/L	0.246±0.011	0.55±0.009[a]	0.23±0.03[b]	0.49±0.007[b,c]

Note: MDA – malondialdehyde; SOD – superoxide dismutase; CP – ceruloplasmin; NO – nitric oxide; TC – total cholesterol; LDLs – low density lipoproteins; HDLs – high density lipoproteins; TAG – triacylglycerides; L-NAME – L-nitroarginine methyl ester; a – p<0.001 – significance of lead acetate relative to control; b – p <0.001, significance of lead acetate + L-NAME relative to intact + L-NAME; c – p<0.001 – significance of lead acetate + L-NAME relative to control; cc – p <0.01 – significance of lead acetate + L-NAME vs lead acetate.

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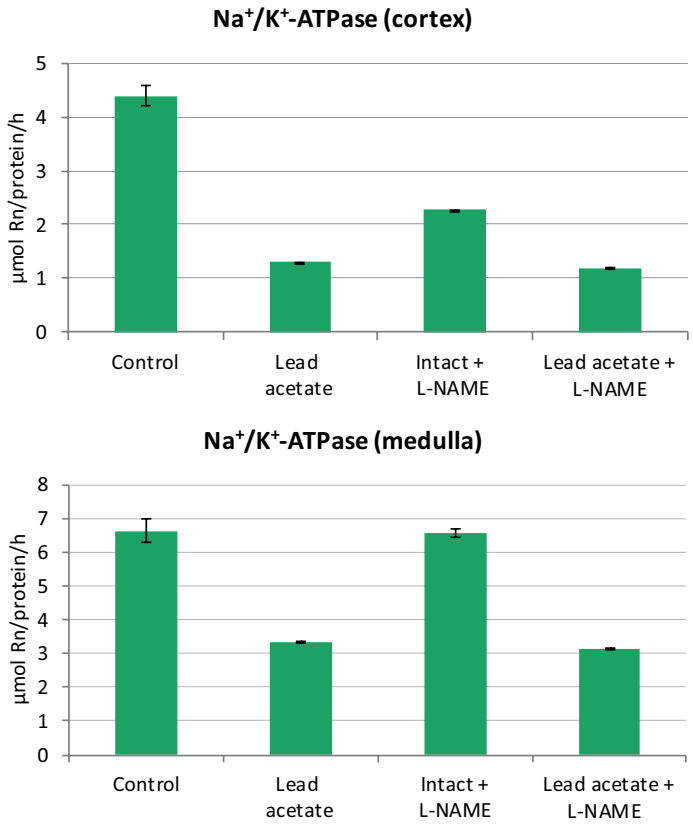

Figure 1 – Dynamics of changes in Na^+/K^+-ATPase activity during saturnism and inhibitor effect of eNOS expression – L-NAME

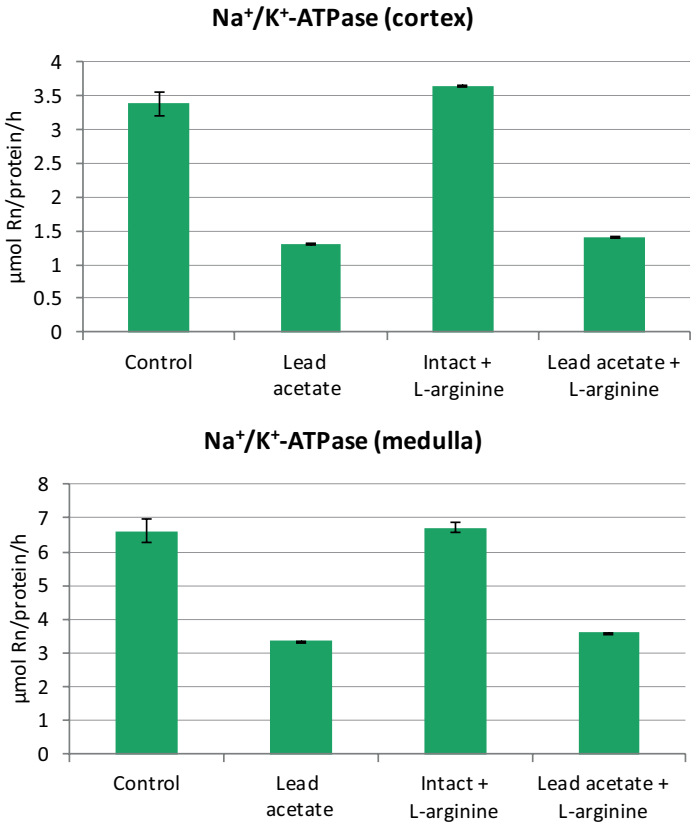

Figure 2 – Dynamics of changes in Na^+/K^+-ATPase activity during saturnism and influence of eNOS expression inducer – L-arginine

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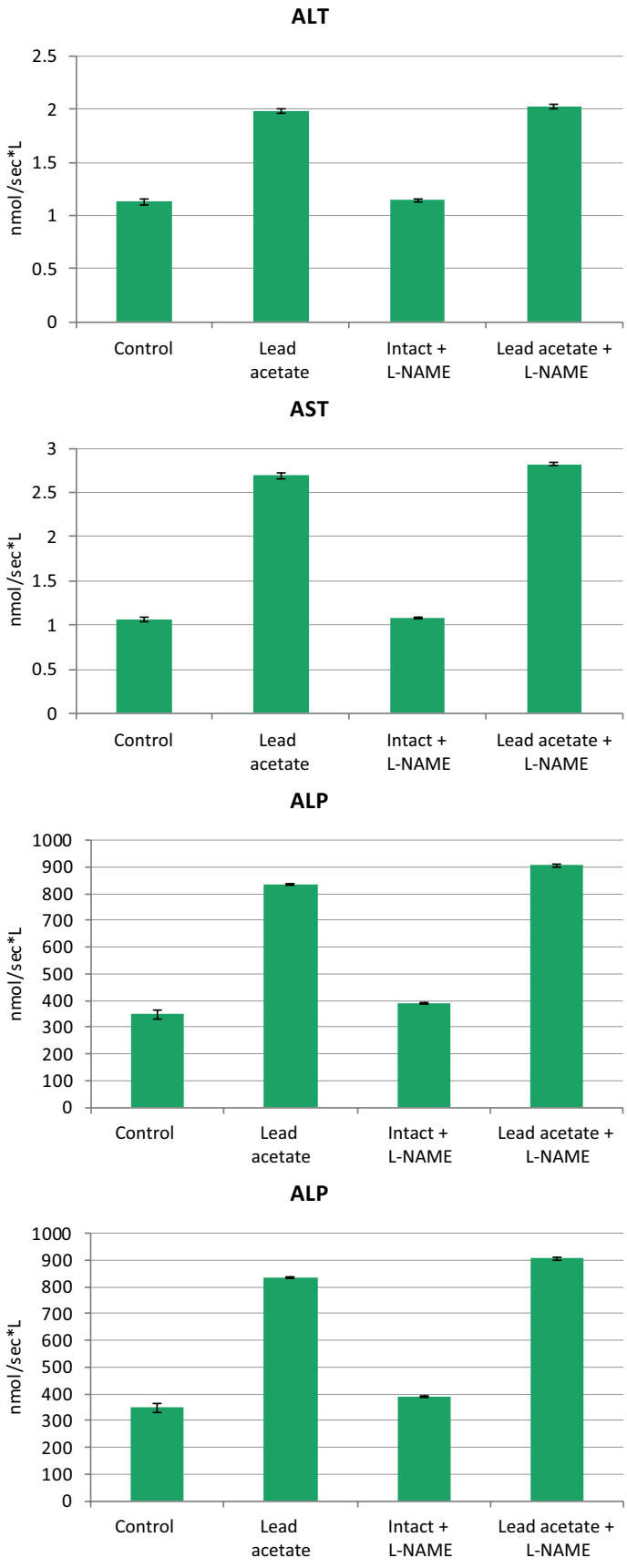

Figure 3 – Data on changes in the activity of organ-specific enzymes under the influence of an inhibitor of eNOS expression – L-NAME

Note: ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGTP – gamma-glutamyl transpeptidase; ALP – alkaline phosphatase.

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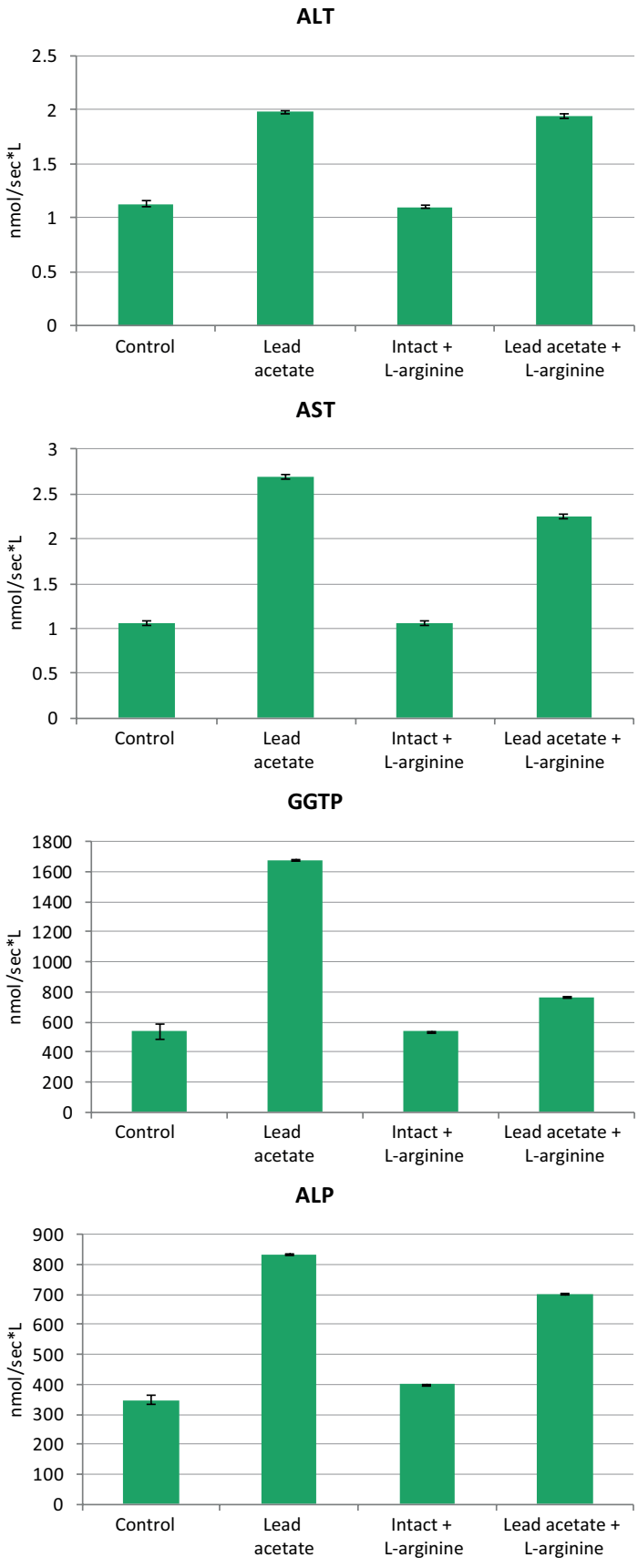

Figure 4 – Data on changes in the activity of organ-specific enzymes under the influence of the inducer of eNOS expression – L-arginine

Note: ALT – alanine aminotransferase; AST – aspartate aminotransferase; GGTP – gamma-glutamyl transpeptidase; ALP – alkaline phosphatase.

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A concomitant role may be played by an impaired bioavailability of L-arginine to NO-synthase due to the inadequacy of the transport mechanism for the amino acid. In this regard, changes in cholesterol metabolism – hypercholesterolemia and hyper-β-lipoproteinemia – can contribute to the disruption availability of the L-arginine substrate. Our data on the lead intoxication showed an increase in the content of total cholesterol, LDL cholesterol in the blood serum and a decrease in HDL cholesterol (Tables 1, 2). Moreover, the results showed that under the oxidative stress conditions, the oxidative modification of LDL occurs, not only with the lipid component, but also with the β-apolipoprotein protein – apo B_{100}. The affinity of β-globulin for LDL receptors is impaired, their uptake by tissue cells is reduced, and the cholesterol content in the blood plasma is increased. Such changes in the metabolism of cholesterol lead to the interaction of lipoproteins with the receptors-"scavengers" of phagocytes, and their absorption. Lipids in the vascular endothelium accumulate; "foamy" cells – risk factors for atherogenesis – are formed. Under these conditions, the endothelium structure of the vascular wall changes, which leads to a disruption in the availability of L-arginine for NO synthase and a decrease in the production of NO_x as the main vasodilator. NOx deficiency is accompanied by hemodynamic changes, in particular in the nephron. A violation of the adequate interaction between the vascular tone of the afferent and efferent arterioles of the kidneys glomerulus causes a change in their functional state.

The analysis of the data obtained revealed a decrease in the GFR by 17.09% in the lead intoxication. At the same time, there was an increase in diuresis due to a significant decrease in the tubular reabsorption of water and sodium. Calculations of these indicators revealed that the levels of the tubular reabsorption of water and sodium are significantly reduced compared to the control by 0.26% and 3.01%, respectively.

To determine the cause-effect relations of sodium metabolism disorders in the nephron and a decrease in the level of the ion tubular reabsorption, the activity of the ATPase enzyme activated by Na and K was determined in the homogenates of the cortical and medulla of the renal tissue. The data showed the activity inhibition of Na^+/K^+-ATPase in both layers of the kidneys in the lead intoxication, as well as an even more significant change in the enzyme data when it was combined with L-NAME (Fig. 1). Conformational changes in the enzyme molecule as a result of the LPO process, which changes the molecular structure of phospholipids in renal tubular cells, were accompanied by a decrease in the ATPase activity (Fig. 1). The phospholipid structure violation of cell membranes is also confirmed by the literature data [16, 28].

Damage indicators of hepatocytes during the lead intoxication against the background of L-NAME were the data indicating an increase in the MDA content in them, a decrease in the function of Na^+/K^+-ATPase, as well as significant changes in the activity level of blood plasma enzymes: ALT, AST, GGTP and alkaline phosphatase (Fig. 1, 3). In contrast to these data, the administration of L-arginine to experimental animals caused the FRO suppression in the cells of the renal and hepatic tissues, the restoration of the phospholipid structure of the organs cell membranes, and an increase in the activity of Na^+/K^+-ATPase in them (Fig. 2). In blood plasma, under the influence of L-arginine, the data showed a decrease in the activity of organ-specific enzymes: ALT, AST, GGTP, and alkaline phosphatase (Fig. 4).

Therefore, biochemical markers of the hepatocyte membrane hydrophilicity and the increased permeability are the activity level of organ-specific enzymes in blood plasma, the modulation activity of Na^+/K^+-ATPase in liver cells.

Discussing these results in the lead intoxication, it was notified that lipid peroxidation contributes to the dysfunction of the endothelium and microcirculation, as well as nephropathy and hepatopathy. Comparing these biochemical data with the literature data, the activation of mitogen-activated protein kinase was notified, which triggers a certain sequence of reactions for the formation of pro-inflammatory proteins, an increase in the vascular tone and blood pressure [29].

An auxiliary role in the bioavailability disruption of L-arginine for eNOS is played by changes in cholesterol metabolism. The changes in cholesterol metabolism were accompanied by the development of pre-atherogenic changes in the vascular endothelium and were an obstacle to the availability of L-arginine to eNOS, which is also confirmed by the literature data [30].

Microcirculatory hemodynamic disorders in the nephron led to functional changes in the kidneys, as well as in the liver. Against the background of metabolic changes, functional disorders developed both in nephrons and in hepatocytes, including indicators of the main processes of urination, a decrease in the activity of the membrane enzyme Na^+/K^+-ATPase, a violation of cholesterol metabolism in the liver, and an increase in the activity of organ-specific enzymes in the blood. In these mechanisms, the regulatory role was played by pharmacological substances: the amino acid L-arginine and its modified derivative, L-NAME. Thus, the data obtained in the study with the lead intoxication on changes in the LPO-AOS system, the NO-producing endothelial function, including the level of the eNOS expression, as well as impaired renal and liver functions in one study, are priorities.

CONCLUSION

A direct correlation was found between the administered dose of lead acetate, the metal content in blood plasma, and the activity of lipid peroxidation in erythrocyte hemolysate. The POL activation is

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also notified in the homogenates of the cortical and medulla of the renal tissue, as well as in the liver, as evidenced by an increase in the concentration of the secondary product of the lipid peroxidation – MDA. A decrease in the AOS – SOD activity is responsible for the development of the oxidative stress. There is a direct positive correlation between the indicators characterizing the lead acetate dose, its content in the blood, organs and the intensity of the lipid peroxidation. The oxidative stress is accompanied by a decrease in the NOx content in blood plasma. Since eNOS is the main NO producer, the effect of the NO synthase expression level regulators was studied: the L-NAME inhibitor and L-arginine inducer. The data confirmed the participation of the NO-synthase inhibitor L-NAME, against the background of which LPO increases, the NO_x content and the level of eNOS expression decrease. In contrast, L-arginine promoted an increase in NO_x content, inhibition of FRO intensity, and an increase in the level of the eNOS expression. In the mechanism of the reduced NO_x level, a violation of the availability of L-arginine for the NO synthase also plays a role due to the changes in cholesterol metabolism – hypercholesterolemia and hyper-β-lipoproteinemia. In the vascular endothelium, pre-atherogenic structural changes develop; they are involved in reducing the availability of L-arginine for eNOS. The oxidative stress causes a decrease in NOx levels and a change in the functional state of the kidneys: polyuria, natriuresis, a decrease in the level of tubular reabsorption of water, sodium, and the Na^+/K^+-ATPase activity. At the same time, there is an activation of lipid peroxidation in hepatocytes, an increase in the permeability of the cell membrane, the activity level of the organ-specific enzymes in the blood serum. L-arginine caused an increase in the NO_x level, an increase in microcirculatory hemodynamics and an increase in the activity of Na^+/K^+-ATPase in the tissues of the kidneys and liver. It is important to note the unidirectional changes in the LPO process in erythrocytes, in the cells of the renal, hepatic tissues, which is the basis for recommendations for the use in clinical conditions of determining the FRO activity in erythrocytes to assess the dysfunction of internal organs in lead poisoning.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Sergey G. Dzugkoev – idea, planning and scientific guidance of the study, article writing; Fira S. Dzugkoeva, Olga I. Margieva – participation in the study design development, information search and analysis, analysis and interpretation of the data obtained, preparation of the final article version; Irina V. Mozhaeva, Olga I. Margieva, Anna E. Khubulova – pathology modeling, biochemical research, calculations.

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