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ANTITUMOR DRUGS BASED ON INDOLOCARBAZOL DERIVATIVES

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The aim of the work is to generalize the literature data on indolocarbazole derivatives with an antitumor activity.
Materials and methods. The objects of the study were the preparations based on indolocarbazole derivatives with the antitumor activity. To search for materials on the problem under study, the following search and information as well as library databases were used: Ebibrary, PubMed, CyberLeninka, ResearchGate, the State Register of Medicines, clinical trials registries clinline.ru and clinicaltrials.gov. The search for the following words / phrases was performed: indolocarbazoles, indolocarbazole derivatives, staurosporine, rebeccamycin, staurosporine derivatives. The search was conducted from January 11 until March 1, 2021. The compounds with a biological activity which were undergoing or had undergone preclinical and clinical trials, were taken into account. All the materials from 1977 to January 1, 2021, were taken into account.
Results. The materials obtained indicate that indolocarbazole derivatives are promising compounds for the creation of anticancer medicinal preparations due to their properties and peculiarities of the action mechanism. These drugs have a selective action due to the targeted interaction with specific molecular targets: kinases (especially protein kinase C and its isozymes), DNA and DNA topoisomerase. To date, many compounds from the class of indolocarbazoles have been synthesized and investigated. They have shown a high antitumor activity in the treatment of systemic and solid tumors. However, despite this, only one MP based on a staurosporine derivative, registered by the TN of Rydapt® (in the USA and EU countries) and Miticaid® (in the Russian Federation), is approved for use in the clinical practice.
Conclusion. Thus, the basic data from scientific publications on promising anticancer medicinal preparations based on compounds from the class of indolocarbazoles, have been summarized. The information is provided, in particular, on their molecular structure, the origin, classification, the main representatives of the class, which are at various stages of the research and are approved for use in the clinic.
Keywords: indolocarbazoles; indolocarbazole derivatives; antitumor agents; staurosporin derivatives; rebeccamycin derivatives
Abbreviations: P – pharmaceutical; MP – medicinal preparation; DF – dosage form; PKC – protein kinase C; DNA – deoxyribonucleic acid; TN – trade name

ПРОТИВООПУХОЛЕВЫЕ ПРЕПАРАТЫ НА ОСНОВЕ ПРОИЗВОДНЫХ ИНДОЛОКАРБАЗОЛА

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Цель. Обобщение литературных данных о производных индолокарбазола, обладающих противоопухолевой активностью.

Материалы и методы. Объектом изучения являлись препараты на основе производных индолокарбазола с противоопухолевой активностью. Для поиска материалов по исследуемой проблеме использовали следующие поисково-информационные и библиотечные базы данных: Ebibrary, PubMed, CyberLeninka, ResearchGate, а также Государственный реестр лекарственных средств, реестры клинических исследований clinline.ru и clinicaltrials.gov. Поиск проводился по следующим словам/словосочетаниям: индолокарбазолы (indolocarbazoles), производные индолокарбазолов (indolocarbazole derivatives), стауроспорин (staurosporine), ребеккамицин (rebeccamycin), производные стауроспорина (staurosporine derivatives), производные ребеккамицина (rebeccamycin derivatives). Поиск проводился с 11 января по 1 марта 2021 года; учитывались соединения с биологической активностью, проходящие или прошедшие доклинические и клинические испытания. Учитывались все материалы с 1977 года по 1 января 2021.

Результаты. Полученные материалы свидетельствуют о том, что производные индолокарбазола являются перспективными соединениями для создания противоопухолевых лекарственных препаратов благодаря их свойствам и особенностям механизма действия. Данные препараты обладают избирательностью действия, что обусловлено направленным взаимодействием с конкретными молекулярными мишенями: киназы (особенно протеинкиназа С и её изоферменты), ДНК и ДНК-топоизомеразы. К настоящему времени синтезировано и исследовано множество соединений из класса индолокарбазолов, показавших высокую противоопухолевую активность при терапии системных и солидных опухолей. Однако несмотря на это, только один лекарственный препарат на основе производного стауроспорина, зарегистрированный под ТН Rydapt® (в США и странах Евросоюза) и Митикайд® (в Российской Федерации), разрешен для применения в клинике.

Заключение. Таким образом проведено обобщение основных данных из научных публикаций, посвященным перспективным противоопухолевым препаратам на основе соединений из класса индолокарбазолов. В частности, приведены сведения об их молекулярном строении, происхождении, классификации, основных представителях класса, находящихся на различных стадиях исследований и разрешенных к применению в клинической практике.

Ключевые слова: индолокарбазолы; производные индолокарбазолов; противоопухолевые агенты; производные стауроспорина; производные ребеккамицина

Список сокращений: ЛС – лекарственное средство; ЛП – лекарственный препарат; ЛФ – лекарственная форма; РКС – протеинкиназа С; ДНК – дезоксирибонуклеиновая кислота; ТН – торговое наименование

INTRODUCTION

Cancer is often referred to as “the pathology of the century” in the context of an endemic disease spreading throughout the world. Cancer has also been identified as “a true disease of modernity” (Roy Porter) or even “an important product of modernity” (Siddhartha Mukherjee). These two definitions are generally accepted and justified by a sharp increase in morbidity and mortality, which has been observed since the end of the 18th century [1]. In 2020, cancer continued to be one of the leading causes of death and an important obstacle to increasing life expectancy in all countries of the world. In 2019, The World Health Organization estimates cancer as the first or second leading cause of people’s deaths under 70 in 112 out of 183 countries, and is ranked as the third or fourth in 23 more countries [2].

Over the past two decades, cancer treatment with the use of pharmacological approaches has changed dramatically. Long years of fundamental and clinical research have led to the transition from classical anticancer therapy, characterized by a low selectivity of a drug action and accompanied by severe intoxication of the body, to more targeted antitumor “snipers” that effectively destroy populations of tumor cells with fewer side effects [3].

Among a wide range of anticancer drugs, compounds from the group of indolocarbazole derivatives are of particular interest. Indolocarbazoles are a unique class of indole alkaloids of a natural or synthetic kinds of origin, which have a number of therapeutic properties – antitumor, antibacterial, antiparasitic, antiviral, and an immunomodulatory activity [4–7].

The most significant biological profile of compounds from the group of indolocarbazole derivatives is their potential antitumor effect [8]. A distinctive feature of the action mechanism of these drugs is their ability to interact with several targets and induce various pathways of tumor cell death [9]. For them, such targets are DNA, topoisomerase and protein kinase C enzymes, which are responsible for regulating the main aspects of cell metabolism, including the progression of the cell cycle [10, 11].

Protein kinases C are a family of protein kinases, enzymes that phosphorylate proteins and thus participate in cell signaling cascades. The term “protein kinase C” refers to all described isoenzymes [12]. PKC inhibitors can reduce the expression of P-glycoprotein in tumor cells and thereby increase their sensitivity to chemotherapy [13]. PKC activation is also required for tumor angiogenesis [14].

Topoisomerases affect the topology of DNA and are able to relax their supercoiled molecules by introducing single- or double-stranded breaks followed by their DSB repair, as well as negative supercoils, or catenans. Inhibitors of these enzymes are widely used to suppress the activity of type I and / or type II tumor topoisomerases, blocking cells in the G2 phase and delaying their entry into mitosis [15].

Inhibitors of topoisomerases are ones of the most effective inducers of apoptosis, i. e., a programmed death of tumor cells [10]. In addition, a number of indolocarbazole derivatives with antiangiogenic activity have been

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synthesized. They are able to block vasculogenic mimicry in a tumor and restore the sensitivity of resistant cells to chemotherapeutic drugs [16, 17]. These features of the action mechanism determine a wide range of cytotoxic and antitumor activities of indolocarbazole derivatives.

THE AIM of the work is to generalize the literature data on indolocarbazole derivatives with an antitumor activity.

MATERIALS AND METHODS

The objects of the study were the preparations based on indolocarbazole derivatives with the antitumor activity. To search for materials on the problem under study, the following search and information as well as library databases were used: Ebibrary, PubMed, CyberLeninka, ResearchGate, the State Register of Medicines, clinical trials registries clinline.ru and clinicaltrials.gov. The search for the following words / phrases was performed: indolocarbazoles, indolocarbazole derivatives, staurosporine, rebeccamycin, staurosporine derivatives. The search was conducted from January 11 until March 1, 2021. The compounds with a biological activity that were undergoing or had undergone preclinical and clinical trials were taken into account. All the materials from 1977 until January 1, 2021, were taken into account.

The article is a review of the publications devoted to indolocarbazole derivatives, i.e., to the information on their structure, origin, classification, the main representatives of the class, which are at various stages of research and are approved for use in the clinic.

RESULTS AND DISCUSSION

General characteristics of indolocarbazole group compounds

The first indolocarbazoles were found out in streptomycetes and subsequently isolated from numerous representatives of flora and fauna. To date, this class of compounds has also been supplemented by a wide variety of synthetic compounds [18][1].

Indolocarbazoles are a class of heterocyclic compounds that include a planar ring consisting of indole and carbazole elements (Fig. 1, 2) [18]. Indole, carbazole and their derivatives are colorless solid crystalline substances that do not dissolve in water. Under standard conditions, the melting point of indole is 52°C, of carbazole, it is 247–248°C, and their boiling points are 253°C and 354–355°C, respectively[2,3]. The carbazole fragment serves as a ligand for many receptors and has the property of reverse coupling to enzymes, in particular, to DNA topoisomerase I [19], and the indole element is responsible for the interaction with DNA [20, 21].

The class of indolocarbazoles includes 5 subclasses of compounds differing in the structure of a planar aromatic ring. In this case, 5 isomers of the polycyclic system – indolo[2,3-a]carbazole (1), indolo[2,3-b]carbazole (2), indolo[2,3-c]carbazole (3), indolo[3,2-a]carbazole (4) and indolo[3,2-b]carbazole (5) (Fig. 3) [18] – are meant.

The most extensive, biologically significant and studied in detail is the subclass of 11,12-dihydroindolo[2,3-a] carbazole derivatives, including mainly compounds having indolo[2,3-a]pyrrolo[3,4-c]carbazole ring, in which 2 indole fragments are linked through a benzene ring with an amide or imide group. The indole moieties are linked through 1 or 2 bonds to the carbohydrate moiety. At the same time, this subclass also includes a small group of compounds that do not include an additional pyrrole ring in their composition [18].

Based on the number of glycosidic bonds that bind the carbohydrate moiety to the isoindole backbone, indolocarbazole derivatives can be divided into 2 subclasses – compounds of the staurosporin group (a) and rebeccamycin (c). In staurosporin and its derivatives, for example, K252a (b), glycoside is bound to 2 indole groups through nitrogen atoms, in contrast to the representatives of the rebeccamycin group, for example, cholyrin A (d), in which the carbohydrate residue is attached to only one indole. The staurosporin heterocycle is connected to the lactone ring, the rebeccamycin heterocycle – to the imide ring (Fig. 4) [25]. Both monosaccharides [23, 24] and disaccharides [25–28] can be included in the structure of indolocarbazoles as carbohydrate residues.

However, the literature also describes indolocarbazole derivatives with a different structure, for example, Go 6976 (Fig. 5) and AEB071 (sotrastaurin) (Fig. 6) – selective inhibitors of protein kinase C α, β1, δ, υ and ζ isozymes. Unlike staurosporine, Go 6976 is methyl- and cyanoalkyl-substituted non-glycoside indolocarbazole [29]; sotrastaurin contains a piperazine ring, in which the nitrogen atom in the ring carries an aryl group [30].

Various modifications of natural and synthetic derivatives of indolocarbazoles lead to changes in their physicochemical properties and biological activity. These factors are important for the development of potential antitumor agents. It is obvious that the antitumor effect of the target compound can be influenced by both substituents in the aglycone and the nature of the glycosidic residue, changing the pharmacodynamic and pharmacokinetic properties. [31].

Representatives of indolocarbazoles class

Staurosporin, the first of the discovered compounds of indolocarbazole derivatives, was isolated in 1977 at the Kitasato Institute (Japan) from the cultures of *Strep-*

[1] A few examples of indolocarbazoles found in the ClassyFire database. The Metabolomics Innovation Centre (TMIC) [Electronic resource]. Available from: http://classyfire.wishartlab.com/tax_nodes/C0001866

[2] PubChem Compound Summary for CID 798, Indole. PubChem. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information [Electronic resource]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/6854

[3] PubChem Compound Summary for CID 6854, Carbazole. PubChem. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information [Electronic resource]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/6854

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tomyces staurosporeus and *Streptomyces actuosus.* Staurosporin has antifungal and hypotensive effects, inhibits platelet aggregation, and is a potent inhibitor of various protein kinases. These factors lead to its use as an antitumor drug [32, 33].

In 1983, the parent of the second group of indolocarbazole derivatives, rebeccamycin (NSC 655649) (Bristol-Myers Co., USA) [34], which is structurally similar to staurosporin, but has a weaker inhibitory activity against protein kinases, was isolated from the strain of actinobacteria C-38383 [35]. The mechanism of the antitumor action of rebeccamycin is associated with the inhibition of topoisomerase I, which is due to its ability to interact with DNA [36].

In order to increase the solubility and the biological activity, the hydrophobic indolocarbazoles staurosporin and rebeccamycin underwent various modifications: a) the addition of substituents to the upper heterocycle, replacement of atoms in the upper heterocycle or removal of a heterocycle, b) modification of flat chromophore, c) modification of replacement or removal of the carbohydrate moiety [37–39].

Staurosporine derivatives

Midostaurine (CGP 41251, PKC 412, NVP-PKC412) (Fig. 7) is an N-benzoyl[4] derivative of staurosporin; it is a synthetic inhibitor of many kinases, including FLT3 and KIT, with antiangiogenic and antitumor activities [40]. It is approved by the FDA[5] and EMA[6] by the TN of Rydapt® (Novartis Pharmaceuticals, Switzerland)[7], in Russia this drug is registered by the TN of Miticaid® (LP-005927)[8]. This MP is a liquid capsule for the oral administration, each capsule contains 25 mg of midostaurin[9].

Enzastaurine (LY-317615, LY317615) (Eli Lilly and Company, USA) (Fig. 8) is an acyclic bisindolylmaleimide derived from staurosporin that selectively inhibits protein kinase-β. The mechanism of the antitumor action of enzastaurin is due to several effects. First, the drug has anti-angiogenic properties associated with a decrease in the level of vascular endothelial growth factor. Second, enzastaurine directly induces the death of tumor cells by reducing the phosphorylation of protein kinase [41]. Numerous studies have been carried out in mono- and combined therapy of oncological diseases of various nosologies, for example, tumors of the nervous system [42–44], colon [45], lymphoma [46–49], Waldenstrom's myeloma and macroglobulinemia [50], non-small-cell lung cancer [51], prostate [52], ovaries [53], etc.

Sotrastaurine (AEB071) (Novartis Pharmaceuticals, Switzerland) is a selective inhibitor of PKC β [57], which prevents the activation of T cells, has a piperazine ring; therefore, this compound can be attributed to the class of organic compounds known as n-arylpiperazines (Fig. 6) [55]. The use of sotrastaurine in the treatment of diffuse large B-cell lymphoma, stomach cancer [56], ulveal melanoma [57], psoriasis [58], as well as in kidney transplantation, has been investigated [59, 60].

Lestaurtinib (A-154475, A-154475.0, CEP-701, KT-555, KT-5555, KT5555, SP-924, SP924, SPM-924) (Cephalon, Inc., USA) (Fig. 9)[10] has been studied in the treatment of infections of the central nervous system caused by free living amoebae [61], myeloid leukemia [62–64], polycythemia and essential thrombocythemia [65], myelofibrosis [66], prostate cancer [67, 68], neuroblastoma [69, 70] psoriasis [71].

Among the staurosporin derivatives, the antibiotic K-252a (Kyowa Hakko Kogyo Co., Ltd., Japan) (Fig. 10)[11], isolated from the culture of Nocardiopsis sp. K-252a, is a unique in its structure indolocarbazole glycoside, and exhibits a powerful neuroprotective antitumor activity. K-252a consists of K-252c and an extraordinary dihydrostreptose fragment linked together by two C-N bonds [72]. Its semi-synthetic derivative KT5720 inhibits cAMP-dependent protein kinase. The activity of KT5720 has been confirmed on granulosa cells of animal ovaries [73, 74].

A promising semi-synthetic staurosporine derivative is stauprimide (The Scripps Research Institute, USA), which inhibits the transcription of the MYC NME2 oncogene and also increases the efficiency of directed differentiation of embryonic stem cells [75, 76].

CEP-11981 (Cephalon, Inc., USA) (Fig. 11) is a targeted drug for oral administration, exhibiting a high inhibitory activity against several targets – receptors for vascular endothelial growth factor 1 and 2, tyrosine kinase 2, and a fibroblast growth factor-1, protooncogene c-SRC, and Aurora A. The studies of the pharmacological activity in animal and human tumor models have shown sustained dose-dependent antiangiogenic and antitumor effects. In addition, CEP-11981 has shown an excellent bioavailability, a metabolic stability, and other pharmacokinetic properties. Phase I clinical trials to evaluate the pharmacokinetics and pharmacodynamics of CEP-11981 in patients with advanced, recurrent / refractory solid tumors, have been completed [77, 78].

[4] PubChem. Compound Summary for CID 9829523, Midostaurin. PubChem. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information [Electronic resource]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Midostaurin.

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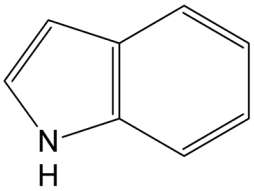


Figure 1 – Structural formula of indole

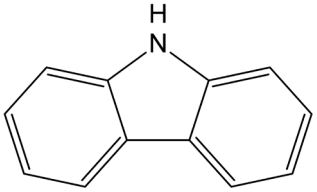


Figure 2 – Structural formula of carbazole

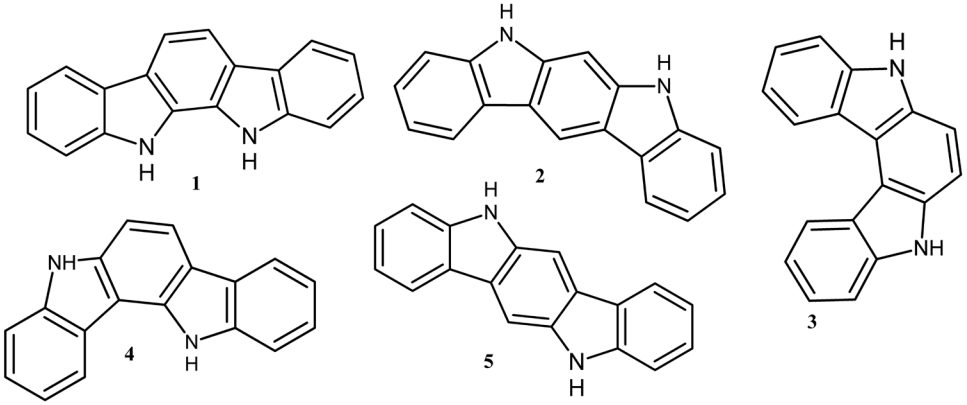
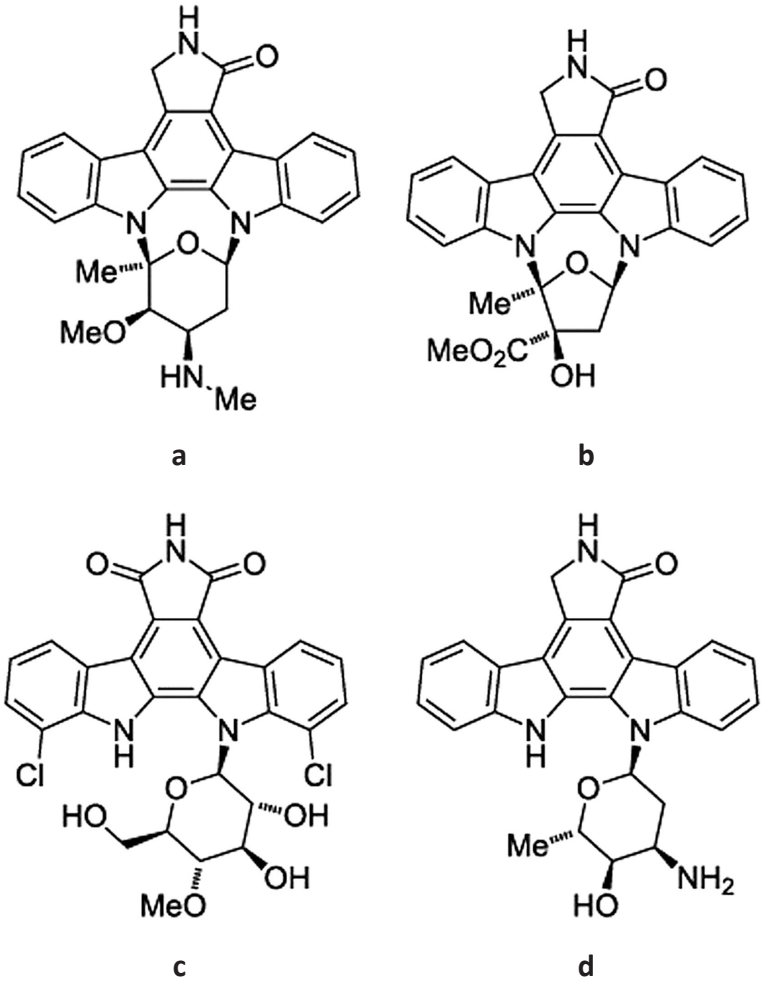


Figure 3 – Structural formulas of indolocarbazole isomers



a **b** **c** **d**

Figure 4 – Structural formulas of indolocarbazole derivatives

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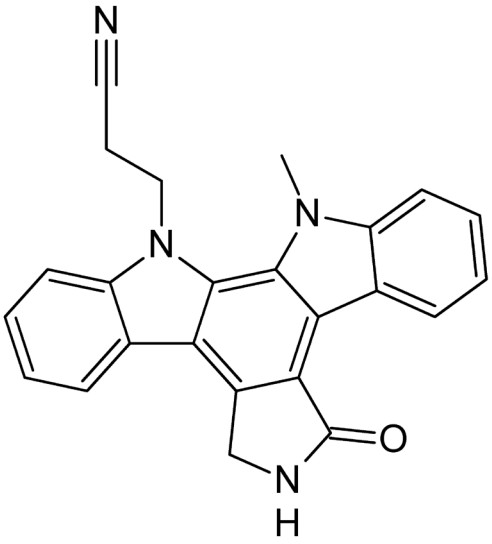

Figure 5 – Structural formula of Go 6976

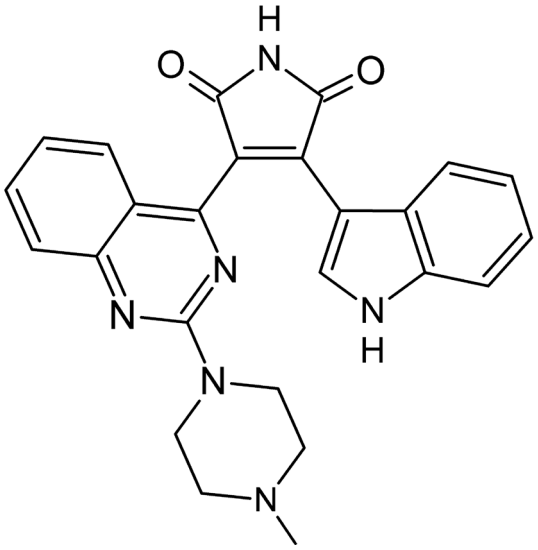

Figure 6 – Structural formula of sotrastaurine

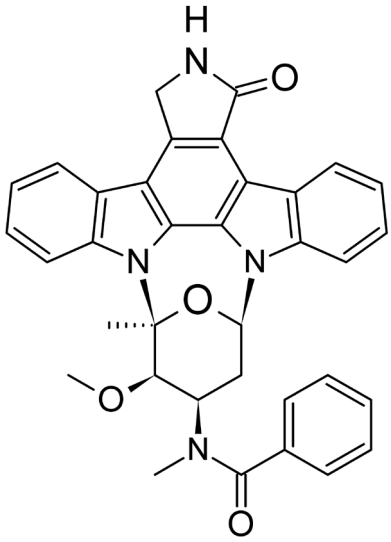

Figure 7 – Structural formula of midostaurine

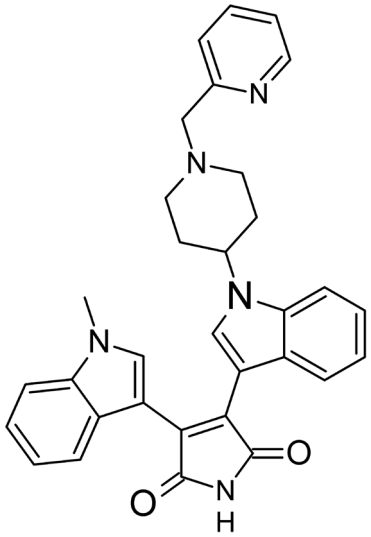

Figure 8 – Structural formula of enzastaurin

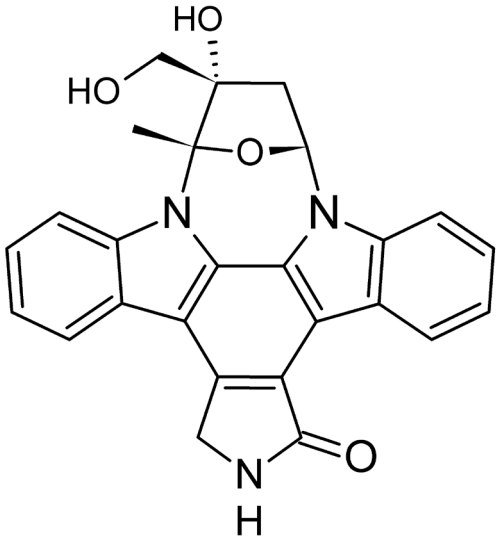

Figure 9 – Structural formula of Lestaurtinib

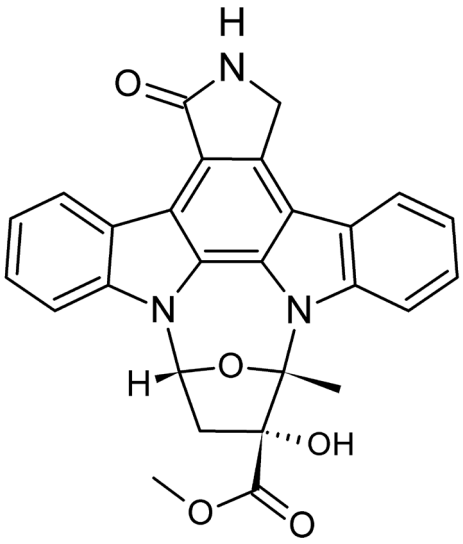

Figure 10 – Structural formula of K-252a

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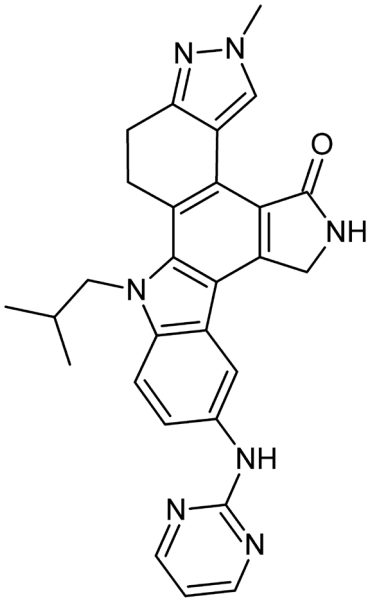

Figure 11 – Structural formula of CEP-11981

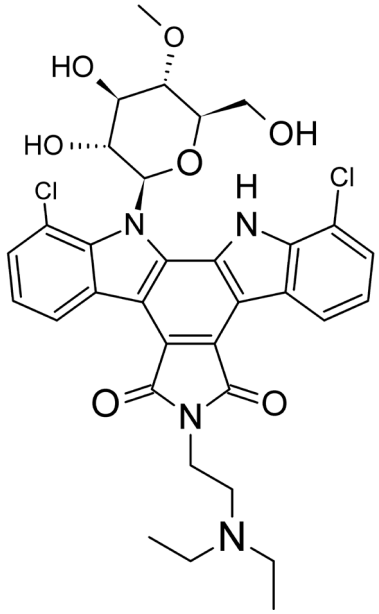

Figure 12 – Structural formula of becatecarin

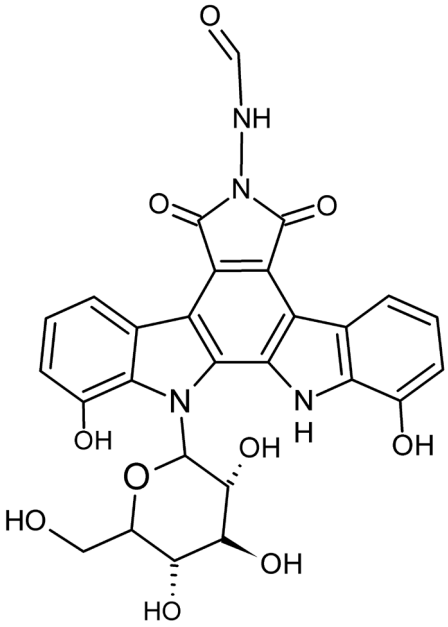

Figure 13 – Structural formula of NB-506

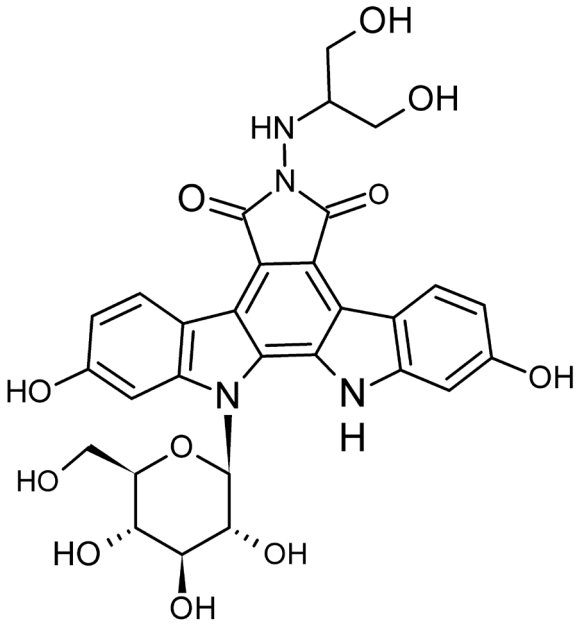

Figure 14 – Structural formula of edothecarin

Go 6976 (Godecke AG, Germany) (Fig. 5) is an indolocarbazole derivative containing a propane nitrile radical instead of a glycosidic residue[12]. Go 6976 is a selective inhibitor of PKC α and β, it moderately inhibits the activation of protein kinase regulated by extracellular signals [79]. In addition, this indolocarbazole is a potential anticancer drug due to its ability to stimulate the formation of cellular compounds (the formation of an increased number of desmosomes and adhesions), to suppress migration and invasion of tumor cells [80].

In the literature, there are also data on many other compounds of staurosporin derivatives: ZHD-0501 [81]; BMY-41950 (RK 1409) [82]; UCN-01 and UCN-02 [83]; CEP-7055 and CEP-5214 [84]; CEP-701; CEP-2563 and CEP-751 (KT-6587) [85]; KT5926 [86]; Ro 318220 and GF 109203X [87]; CEP-1347[13], and others.

Among the domestic compounds of staurosporine derivatives, the most famous are the N-glycosides of

[12] PubChem Compound Summary for CID 3501, Go-6976. PubChem. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information [Electronic resource]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Go-6976

[13] CEP-1347. DrugBank [Electronic resource]. Available from: https://go.drugbank.com/drugs/DB05403

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indolo [2,3-a] pyrrolo [3,4-c] carbazole-5,7-diones of indolocarbazoles: LHS-976, LHS-983, LHS-985, LHS-999, LHS-1006, LHS-1007, LHS-1040, LHS-1054, LHS-1098, LHS-1208, LHS-1269, etc. [88-93]. Today, compounds LHS-1208 and LHS-1269 are the most studied among them as antitumor agents.

An indolocarbazole derivative **LHS-1208** exhibits a strong inhibitory activity against kinases[14] – cyclin-dependent kinase, protein kinase C and tyrosine kinase; the second target is DNA and the DNA topoisomerase complex. To date, preclinical trials of an injectable dosage form LHS-1208 containing dimethyl sulfoxide and a solubilizer Kollidon 17PF as a co-solvent of the hydrophobic active substance, have been completed [94]. For this compound, a liposome-based DF was also developed in the form of a lyophilisate for the preparation of an injection emulsion [95].

LHS-1269 is an indolocarbazole derivative with a carbohydrate residue xylose, which has cytotoxic and antiangiogenic effects and has shown a high antitumor activity against a number of transplanted ascites and solid tumor models [96, 97]. To date, a composition and technology for producing an injectable liposomal dosage form have been developed for LHS-1269 [98].

Rebeccamycin derivatives

On the basis of rebeccamycin, a glycosyl-dichloroindolocarbazole analogue with the improved water solubility denoted as becatecarin (BMS-181176, BMY-27557, NSC-655649, XL 119, XL-119, XL119) (National Cancer Institute, USA) (Fig. 12)[15] was obtained [99]. Becatecarin is an antitumor antibiotic with an inhibitory activity against topoisomerase I and topoisomerase II, as well as the ability to intercalate DNA [100, 104]. It has been studied in the treatment of lung cancer [101, 104], blood cancer [102], tumors of the nervous system [99] and solid tumors [103].

NB-506 (Banyu Co., Japan) (Fig. 13) is a glycoside derivative of rebeccamycin, the antitumor activity of which is due to its ability to interact with DNA and inhibit topoisomerase I. The glucose residue attached to the planar chromophore of indolocarbazole, plays a significant role in the interaction of drugs with nucleic acids; it promotes the stabilization of covalent complexes of topoisomerase I – DNA [105]. It has been reported that NB-506 is in clinical trials [106].

Edothecarin (J-107088, J-107088, PF-804950, PHA-782615) (Banyu Co., Japan) (Fig. 14) is a NB-506 derivative with a broad spectrum of antitumor activity, it is a topoisomerase I inhibitor that induces cleavage of single-stranded DNA more effectively than original indolocarbazole or camptothecin. In contrast to other inhibitors of topoisomerase I, the antitumor activity is less dependent on the cell cycle. Despite the fact that J-107088 has a structure similar to staurosporin, this drug does not possess the properties of a protein kinase inhibitor [107]. It has been actively studied in mono- and combined therapy of oncological diseases [108–115].

It was also found out that when grown in a specific medium containing 0.05% potassium bromide, *Saccharothrix aerocolonigenes* ATCC 39243 produces a rebeccamycin analog which has been indicated as brombeccamycin. It has the same structure as rebeccamycin, except the replacement of two chlorine atoms with bromine atoms in the molecule. The authors of the study suggest that the compound has an activity against mouse P-388 leukemia [116].

Rebeccamycin-based compounds have also been obtained. They are: BMS-250749, BMS-210287, BMS-251873, SA315F, AT2433-A1, AT2433-A2, AT2433-B1, AT2433-B2, etc. [117].

CONCLUSION

An important issue in medical science is the creation of new MPs for the treatment of cancer. Indolocarbazole derivatives are a promising class of anticancer drugs characterized by a directed mechanism of the action on targets such as kinases (especially PKC and its isozymes), DNA and DNA topoisomerases I and II. These compounds, along with the antitumor effect, have a wide spectrum of a biological activity, which also makes it possible to use them in the therapy of other nosologies, including transplantology.

To date, a fairly large number of compounds that are at various stages of preclinical and clinical studies, have been synthesized. They belong to two subclasses – derivatives of staurosporin and rebeccamycin.

However, for clinical practice, only one drug based on a staurosporine derivative, midostaurine, registered abroad by the TN of Rydapt® (in the Russian Federation its TN is Miticaid) has been approved for use. Therefore, to expand the arsenal of targeted anticancer drugs, it is necessary to study the known synthesized indolocarbazole derivatives, as well as to search for new compounds with improved characteristics, further.

[14] Isoenmers are not specified.

[15] PubChem Compound Summary for CID 101524, Becatecarin. PubChem. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information [Электронный ресурс] Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Becatecarin

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Alexander P. Kolpaksidi – searching for materials, writing, planning and editing the review;
Maria V. Dmitrieva – searching for materials, planning and editing the review;
Ilya V. Yarosh – searching for materials; Ivan I. Krasnyuk – planning and searching for materials.

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DEVELOPMENT AND VALIDATION OF METHODS FOR QUANTITATIVE DETERMINATION OF ACTIVE PHARMACEUTICAL SUBSTANCES IN NASAL SPRAY

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Intranasal administration of H_1-histamine receptor blockers may be a promising approach to the treatment of allergic rhinitis. Earlier, an original composition of a nasal spray containing fexofenadine hydrochloride and ammonium glycyrrhizinate and demonstrating a high level of therapeutic efficacy, was developed.

The aim of the study was to develop and validate a method of the quantitative determination of active pharmaceutical ingredients fexofenadine hydrochloride and ammonium glycyrrhizinate in a spray for intranasal administration.

Materials and methods. During the development and validation of the method of the fexofenadine hydrochloride and ammonium glycyrrhizinate quantitative determination in a nasal spray, the method of high performance liquid chromatography was used: a Dionex Ultimate 3000 UV chromatograph with a Luna C18 column (2) containing octadecylsilicagel with a 5 μm grain size as a sorbent. The analysis and validation procedures were performed in accordance with the requirements of the State Pharmacopoeia of the Russian Federation, the XIVth edition.

Results. The study showed that for the simultaneous quantitative determination of fexofenadine hydrochloride and ammonium glycyrrhizinate, the optimal elution regime is a gradient mode with a mobile phase containing 50 mmol/L potassium dihydrogen phosphate solution with methanol (45:55), which ensured the separation of the components in the 20 minutes interval. The validation procedures showed that the developed methodology correspond to all the criteria of validity in terms of the following indicators: correctness, precision, specificity and linearity in the analytical area.

Conclusion. The obtained results indicate the possibility of using the method of high-performance liquid chromatography in a gradient elution mode with a mobile phase of the composition of a 50 mmol/L solution of potassium dihydrogen phosphate with methanol (45:55) for the simultaneous quantitative determination of active pharmaceutical ingredients – fexofenadine hydrochloride and ammonium glycyrrhizinate as parts of a promising nasal spray for the allergic rhinitis treatment.

Keywords: allergic rhinitis; quantification; high performance liquid chromatography; fexofenadine hydrochloride; ammonium glycyrrhizinate

Abbreviations: AR – allergic rhinitis; API – active pharmaceutical ingredient; UV – ultraviolet; HPLC – high performance liquid chromatography; SPRF – State Pharmacopoeia of the Russian Federation

РАЗРАБОТКА И ВАЛИДАЦИЯ МЕТОДИКИ КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ АКТИВНЫХ ФАРМАЦЕВТИЧЕСКИХ СУБСТАНЦИЙ В СПРЕЕ НАЗАЛЬНОМ

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ОРИГИНАЛЬНАЯ СТАТЬЯ
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Перспективным подходом к лечению аллергического ринита может стать интраназальное введение блокаторов H_1-гистаминовых рецепторов. Ранее был разработан оригинальный состав спрея назального, содержащего фексофенадина гидрохлорид и аммония глицирризинат, демонстрирующий высокий уровень терапевтической эффективности.
Цель состояла в разработке и валидации методики количественного определения активных фармацевтических субстанций фексофенадина гидрохлорида и аммония глицирризината в спрее для интраназального введения.
Материалы и методы. В ходе разработки и валидации методики количественного определения фексофенадина гидрохлорида и аммония глицирризината в спрее назальном применялся метод высокоэффективной жидкостной хроматографии: хроматограф с УФ детектором DionexUltimate 3000 с колонкой Luna C18 (2), содержащей в качестве сорбента октадецилсиликагель с зернением 5 мкм. Анализ и валидационные процедуры выполнялись в соответствии с требованиями Государственной Фармакопеи Российской Федерации XIV издания.
Результаты. Исследование показало, что для количественного определения при совместном присутствии фексофенадина гидрохлорида и аммония глицирризината оптимальным является градиентный режим элюирования с составом подвижной фазы 50 ммоль/л раствор калия дигидрофосфата и метанолом (45:55), который обеспечивал разделение компонентов смеси в интервале 20 минут. Валидационная оценка показала, что разработанная методика отвечает всем критериям валидности по показателям: правильность, прецизионность, специфичность и линейность в аналитической области.
Заключение. Полученные результаты свидетельствуют о возможности использования метода высокоэффективной жидкостной хроматографии в градиентном режиме элюирования с составом подвижной фазы 50 ммоль/л раствор калия дигидрофосфата с метанолом (45:55) для количественного определения активных фармацевтических субстанций – фексофенадина гидрохлорида и аммония глицирризината в составе перспективного спрея назального для лечения аллергического ринита.
Ключевые слова: аллергический ринит; количественное определение; высокоэффективная жидкостная хроматография; фексофенадина гидрохлорид; аммония глицирризинат
Список сокращений: АР – аллергический ринит; АФС – активная фармацевтическая субстанция; УФ – ультрафиолетовый; ВЭЖХ – высокоэффективная жидкостная хроматография; ГФ РФ – Государственная фармакопея Российской Федерации; х. ч. – химически чистый

INTRODUCTION

Allergic rhinitis (AR) is the most common disease resulting from the organism's hypersensitivity to various types of antigens. AR ranks the sixth place among the most common atopic diseases in the world, leading to a decrease of the quality of life and a deterioration of labor productivity, which negatively effects on the economic component of a human activity and requires significant financial investments, both personal and from the health care system [1, 2]. At the same time, the number of people diagnosed with allergic rhinitis is annually steadily increasing, which makes the development of effective and safe medicines for the treatment and prevention of AR relevant both for the patients themselves and for the state as a whole [3].

Currently available pharmacological approaches to AR therapy involve the elimination of the main symptoms of the disease. For this purpose, both intranasal and systemic medicines are used. Medicines administered intranasally are represented by glucocorticosteroids, which are first-line drugs. It is also possible to inject decongestants and anticholinergics into the nasal cavity. H_1-histaminolytics, mast cell membrane stabilizers and leukotriene receptor antagonists are used systemically in the AR treatment [4]. Despite the sufficient level of effectiveness, in some cases, the use of intranasal glucocorticosteroids does not provide the necessary pharmacological safety requirements, which limits their daily use [5]. In this regard, repeated attempts to overcome the existing disadvantages of glucocorticosteroids, including the ones by creating rational combinations of drugs or their complete replacement by the medicines of an alternative pharmacotherapeutic group, were made. In the latter case, the intranasal use of H_1 – histamine receptor blockers is relevant, while in order to increase the therapeutic effect, it is possible to develop synergistic combinations based on non-sedative histamine blockers and other antiallergic agents [6]. A promising direction in the correction of allergic rhinitis can be considered the use of a combination of the H_1-histamine blocker of the latest generation – fexofenadine hydrochloride and an antiallergic herbal agent – ammonium glycyrrhizinate – as parts of the nasal spray being developed [7].

Fexofenadine is an active metabolite of terfenadine, an anti-allergic agent with an antihistamine action. Fexofenadine belongs to the latest generation of long-acting H_1-histamine receptor blockers, devoid of a pronounced sedative effect. Fexofenadine has a favorable medicine with the safety profile that is superior to that of the first generation antihistamines. The absence of the sedative effect makes it possible to use this medicine by various groups of the population and including the working hours, since the attention concentration, motor and cognitive functions are not impaired [8–12].

Ammonium glycyrrhizinate is one of the effective medicines obtained from the licorice extract. The ammonium salt of glycyrrhizic acid has proven its anti-inflammatory, antinocciptive, antiallergic, antiviral, antioxidant, immunostimulating and hepatoprotective activity [13–17].

In the previous study devoted to the experimental evaluation of the pharmacological efficacy of the combination of the active substances fexofenadine hydrochloride + ammonium glycyrrhizinate (the adjuvants were benzalkonium chloride, polyethylene oxide – 400, propylene glycol), the following factors were established. In the animals with AR, the intranasal administration of the test composition in terms of the severity of its action

was comparable to glucocorticosteroids; that implies the relevance of the further investigation of this combination from the perspective of the pharmaceutical analysis [18].

THE AIM of the study was to develop and validate a method of the quantitative determination of active pharmaceutical ingredients fexofenadine hydrochloride and ammonium glycyrrhizinate in a spray for intranasal administration.

MATERIALS AND METHODS

Test objects and materials for analysis

Based on the current trends in the field of qualitative and quantitative analysis of pharmacologically active compounds, as well as on the features of the pharmaceutical analysis of the compounds similar in structure (terfenadine and glycyrrhizic acid, respectively) to the target ones (fexofenadine hydrochloride and ammonium glycyrrhizinate), the method of high performance liquid chromatography had been chosen in this study [19, 20]. The following objects were used in the work: the substance of ammonium glycyrrhizinate (the content was 99.4%, fexofenadine CJSC «VIFITECH», Russia); the substance of hydrochloride (the content was 101.6%, Ind – Swift Laboratories Limited, India); the standard samples of fexofenadine hydrochloride (Sigma – Aldrich, USA); the standard samples of ammonium glycyrrhizinate (Sigma – Aldrich, USA); acetonitrile; water for chromatography; methanol (UHPLC Grade; Panreac, Spain), potassium dihydrogen phosphate. The methods was developed using the following equipment: a Dionex Ultimate 3000 chromatograph (Thermo Scientific, USA) equipped with a UV detector UVD-3000, a Luna C18 column (2) with a size of 150 x 4.6 mm (octadecylsilicagel 5 µm granulation) (Phenomenex, USA); OPN centrifuge – 3.02 (Russia); analytical balance Sartogosm, LV 210-a (Russia).

The temperature of the test samples was 20°C, the temperature of the chromatographic column was 30°C, and it was maintained by a thermostat. The sample volume was 20 µL injected by an autosampler. The detection was carried out spectrophotometrically at the wavelength of 234 nm.

Methods for quantitative analysis of active pharmaceutical ingredients (APIs)

A quantitative analysis of the APIs of the developed spray for intranasal administration was carried out in accordance with the requirements of the State Pharmacopoeia of the Russian Federation (the XIVth edition of the GPA.1.2.1.2.0001.15 «Chromatography», GPA.1.2.1.2.0005.15 «High performance liquid chromatography» and GPA.1.1.0012.15 «Validation of analytical methods»[1].

[1] State Pharmacopoeia of the Russian Federation / Ministry of Health of the Russian Federation. XIV ed. T. I–IV. M., 2018. [Electronic resource]. Access mode: http: // http://femb.ru/femb/ pharmacopea.php

Preparation of standard sample solutions

The preparation of standard samples solutions was carried out as follows. An accurate weighed amount of a standard sample (30 mg for fexofenadine hydrochloride and 10 mg for ammonium glycyrrhizinate) was placed in a 10 ml volumetric flask. Then 5 ml of the mixture of methanol and 50 mmol/l potassium dihydrogen phosphate solution (55:45) was added, dissolved with stirring, and the volume of the flask was brought to the mark. 2 ml of the resulting solution was transferred into a 25 ml volumetric flask, and then the volume was brought to the mark with the same solvent. Before the injection, the solutions were filtered through a nylon filter with a pore size of 0.45 µm (Phenomenex, USA), discarding the first portions of the filtrate.

Preparation of test solution

Preparation of the test solution has undergone the following process. 2 ml of the medicines was transferred into a 25 ml volumetric flask, a mixture of methanol and 50 mmol/L potassium dihydrogen phosphate solution (55:45) was added to the mark, stirred and filtered through a nylon filter with a pore size of 0.45 µm (Phenomenex, USA), discarding the first portions of the filtrate (the test solution).

The calculation of the fexofenadine hydrochloride and ammonium glycyrrhizinate content in the spay in mg/ml was carried out according to the formula:

$$C_x = \frac{S_x \times a_{st} \times P \times W_x \times V_{st}}{S_{st} \times V_x \times W_{st} \times W_{st2} \times 100}, \quad (1)$$

where X is the content of the determined component, mg/ml; S_x is the area of the peak of the determined component of the test solution, mAU min, on the chromatogram; S_{st} is the area of the peak of the determined component of the standard solution, mAU min, on the chromatogram; V_x is the volume of the medicine aliquot, ml; a_{st} is the amount of the standard sample, g; P is a substance content in the standard sample solution, mg/ml; W_x is the volume of a volumetric flask taken to dilute the medicine, ml; W_{st}, W_{st2} are the volumes of volumetric flasks taken for dilution of the standard sample, ml.

The calculation of the content of the medicinal product components relative to the declared one was carried out according to the formula:

$$X = \frac{C_x \times 100}{L}, \quad (2)$$

where X is the content of the analyte relative to the declared one, %; C_x is the content of the analyte, mg/ml; L is the declared content of the substance in the nasal spray, mg/ml.

Preparation of solutions for validation assessment «linearity»

0.0300 g of a standard sample of fexofenadine hydrochloride was placed in a 10 ml volumetric flask, dissolved with stirring in 6 ml of the mixture of methanol

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and 50 mmol/L potassium dihydrogen phosphate solution (55:45), after which the volume of the flask was brought to the mark with the same composition.

The following amounts of the solution (1.2; 1.6; 2.0; 2.4; 2.8 ml, respectively) were transferred into volumetric flasks with a capacity of 25 ml and the volumes were brought to the mark with the same solvent.

Preparation of solutions for validation assessment according to the indicators «correctness» and «analytical area»

The model mixture was prepared with a content of fexofenadine hydrochloride and ammonium glycyrrhizinate in 60% relative to the declared spray (0.18 g of fexofenadine hydrochloride and 0.06 g of ammonium glycyrrhizinate per 100 ml of the medicine). Next, the obtained medicinal product was analyzed in accordance with the proposed methods.

At the same time, 2 ml of the solution of the obtained model mixture was placed into 25 ml volumetric flasks, then 0.4; 0.8 and 1.2 ml of a 0.3% solution of the standard sample of fexofenadine hydrochloride and a 0.1% solution of the standard sample of ammonium glycyrrhizinate to concentration levels of 80%, 100% and 120%, relative to the nominal, were added. After that, the volume of the flask was brought to the mark with the mobile phase in the ratio which was at the beginning of the analysis. Each dilution was repeated three times.

Statistical analysis

The results were processed by static methods using the Microsoft Excel v 13.0 software package with advanced statistical data analysis capabilities.

RESULTS AND DISCUSSION

Development of methods for simultaneous quantitative determination of fexofenadine hydrochloride and ammonium glycyrrhizinate in the nasal spray

The task of the present study was to develop HPLC – the methods of quantitative determination in the nasal spray. In the literature data, there is no HPLC methods for the simultaneous quantitative determination of fexofenadine hydrochloride and ammonium and glycyrrhizinate, however, methods for their individual determination have been described [21–24].

Preliminary studies on the development and optimization of the methods for the quantitative determination of the APIs of the nasal antiallergic spray, made it possible to establish the optimal aqueous component of the mobile phase, which additionally suppresses ionization. It is a solution of potassium dihydrogen phosphate at the concentration of 0.05–0.1 mol/l with a pH of 4.5–4.8. There was no pronounced difference when a 0.05 M – 0.1 M potassium dihydrogen phosphate solution was used. In order to minimize the potentially negative factors associated with the use of saline buffer solutions, a 50 mmol/L potassium dihydrogen phosphate solution was used.

An experimental study of the chromatography of fexofenadine hydrochloride and ammonium glycyrrhizinate in the mixtures of a 50 mmol/l potassium dihydrogen phosphate solution with methanol, made it possible to conclude that methanol and its mixtures with water easily dissolved the components of the medicine[2].

The baseline drift inherent in the gradient elution mode did not influenced the calculation of separation results. To shorten the analysis time, improve separation and decrease the viscosity of the mobile phase, the temperature of the chromatographic column was increased to 30°C. The optimized conditions for the chromatographic determination are presented in Table 1.

A typical nasal spray chromatogram is shown in Fig. 1.

At the same time, chromatography of the standard samples solutions of fexofenadine hydrochloride, ammonium glycyrrhizinate, as well as the solutions of the adjuvants in the composition of a nasal spray (benzalkonium chloride, polyethylene oxide – 400, propylene glycol) and the mobile phase, was carried out. Under the proposed conditions of the chromatographic determination, a reliable separation of the components is carried out with a certain baseline drift characteristic of the gradient elution mode, within 20 minutes.

To assess the suitability of the chromatographic system, the solutions of the nasal spray and standard samples of fexofenadine hydrochloride and ammonium glycyrrhizinate were sequentially analyzed in six replicates to determine retention times, asymmetry factors, resolution coefficients, and the efficiency of the chromatographic system.

The main characteristics of the fexofenadine hydrochloride and ammonium glycyrrhizinate peaks on the chromatograms of the nasal spray solution and standard sample solutions, are presented in Tables 2 and 3.

The presented results indicate that the retention times of the two main peaks on the chromatogram of the nasal spray solution match with the retention times of the peaks on the chromatograms of the standard samples solutions of fexofenadine hydrochloride and ammonium glycyrrhizinate.

The areas of the peaks obtained in the analysis of six consecutive injections of the standard samples solutions were used to calculate the value of the relative standard deviation (RSD). The results are shown in Table 4.

As follows from the presented results, the relative standard deviation of the peak areas of fexofenadine hydrochloride and ammonium glycyrrhizinate obtained by repeated administrations of the same standard solutions, does not exceed 2%. That matches the suitability requirements of the chromatographic system.

[2] State Pharmacopoeia of the Russian Federation / Ministry of Health of the Russian Federation. XIV ed. T. I – IV. M., 2018. [Electronic resource]. Access mode: http: // http://femb.ru/femb/ pharmacopea.php

Table 1 – Conditions for chromatographic determination

Time, min	Elution mode	
	Methanol, %	50 mmol/l potassium dihydrogen phosphate solution, %
0	55	45
10	55	45
30	95	5

Table 2 – Characteristics of APIs peaks on nasal spray chromatograms

Component of spray	Characteristics of chromatograms			
	t_R, min	A_s	R_s	N
Fexofenadine hydrochloride	6.2±0.2	Not more than 1.2	Not less than 1.5	2800
Ammonium glycyrrhizinate	16.4±0.2	Not more than 1.4	Not less than 1.5	30000

Table 3 – Characteristics of peaks of fexofenadine hydrochloride and ammonium glycyrrhizinate on chromatograms of standard sample solution

Component of spray	Characteristics of chromatograms			
	tR, min	A_s	R_s	N
Fexofenadine hydrochloride	6.2±0.2	Not more than 1.4	Not less than 1.5	2900
Ammonium glycyrrhizinate	16.4±0.2	Not more than 1.3	Not less than 1.5	31000

Table 4 – Evaluation of reproducibility of-peak areas on chromatograms of standard solutions of fexofenadine hydrochloride and ammonium glycyrrhizinate

Analyte	Injection replication, n	Peak area, mAU×min	RSD,%
Fexofenadine hydrochloride	1	33.12	Xm = 33.16 S^2 = 0.005667 SD = 0.07528 RSD = 1.25%
	2	33.26	
	3	33.08	
	4	33.24	
	5	33.18	
	6	33.10	
Ammonium glycyrrhizinate	1	8.13	Xm = 8.12 S^2 = 0.002417 SD = 0.04916 RSD = 0.82%
	2	8.04	
	3	8.12	
	4	8.18	
	5	8.16	
	6	8.10	

Table 5 – Initial data for evaluating of linearity methods in relation to fexofenadine hydrochloride

№	Concentration of fexofenadine hydrochloride standard solution, %	Peak area, mAU×min (3 sequential injections mean)
1	0.0144	19.90
2	0.0192	28.10
3	0.0240	33.16
4	0.0288	39.98
5	0.0336	48.26

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Table 6 – Initial data for evaluating of linearity methods in relation to ammonium glycyrrhizinate

№	Concentration of ammonium glycyrrhizinate standard solution, %	Peak area, mAU×min (3 sequential injections mean)
1	0,0048	5,01
2	0,0064	6,18
3	0,0080	8,12
4	0,0096	9,04
5	0,0112	10,45

Table 7 – Results of precision assessment of methods for quantitative determination of fexofenadine hydrochloride and ammonium glycyrrhizinate (replication level)

Component	Peak area, mAU × min	Found, mg/ml	Metrological characteristics
Fexofenadine hydrochloride	32.91	2.98	$\bar{x}$ = 2.92 S^2 = 0.00188 SD = 0.04336 RSD = 0.72% $\Delta\bar{x}$ = ±0.05 $\bar{\varepsilon}$ = ±1.56% $\bar{x} \pm \Delta\bar{x}$ = 2.92 ± 0.05 mg/ml
	31.86	2.88	
	32.13	2.91	
	31.76	2.87	
	32.27	2.92	
	32.76	2.96	
Ammonium glycyrrhizinate	8.36	1.03	$\bar{x}$ = 1.00 S^2 = 0.002657 SD = 0.05154 RSD = 0.86% $\Delta\bar{x}$ = ±0.05 $\bar{\varepsilon}$ = ±5.40% $\bar{x} \pm \Delta\bar{x}$ = 1.00 ± 0.05 mg/ml
	8.02	0.99	
	7.44	0.92	
	8.52	1.05	
	7.86	0.97	
	8.56	1.05	

Note: The peak area of the solution of the fexofenadine hydrochloride standard sample = 33.16 mAU×sec; the peak area of the solution of the ammonium glycyrrhizinate standard sample = 8.12 mAU×sec

Table 8 – Results of intra-laboratory precision assessment of methods for quantitative determination of fexofenadine hydrochloride and ammonium glycyrrhizinate

Fexofenadine hydrochloride					
Analyst 1		Analyst 2		Metrological characteristics	
Peak area, mAU × min	Found, mg/ml	Peak area, mAU × min	Found, mg/ml	Analyst 1	Analyst 2
32.91	2.98	32.64	2.96	$\bar{x}$ = 2.92 S^2 = 0.00188 SD = 0.04336 RSD = 1.48% $\bar{x} \pm \Delta\bar{x}$ =2.92 ± 0.05 mg/ml	$\bar{x}$ = 2.98 S^2 = 0.001987 SD = 0.04457 RSD = 1.49% $\bar{x} \pm \Delta\bar{x}$ =2.98 ± 0.05 mg/ml
31.86	2.88	33.42	3.03		
32.13	2.91	33.04	3.00		
31.76	2.87	32.08	2.91		
32.27	2.92	33.12	3.01		
32.76	2.96	32.54	2.95		
T_{calc} = 2,04 < t (95%; 10); F_{calc} = 1,06 < F (95%; 5; 5) – the differences between the results obtained are random					
Ammonium glycyrrhizinate					
Analyst 1		Analyst 2		Analyst 1	Analyst 2
8.36	1.03	8.84	1.08	$\bar{x}$ = 1.00 S^2 = 0.002657 SD = 0.05154 RSD = 5.15% $\bar{x} \pm \Delta\bar{x}$ = 1.00 ± 0.05 mg/ml	$\bar{x}$ = 1.02 S^2 = 0.003107 SD = 0.055737 RSD = 5.45% $\bar{x} \pm \Delta\bar{x}$ = 1.02 ± 0.06 mg/ml
8.02	0.99	8.56	1.04		
7.44	0.92	8.12	0.99		
8.52	1.05	8.92	1.09		
7.86	0.97	7.49	0.91		
8.56	1.05	7.84	0.95		
$t_{calc.}$ = 0,64 < t (95%; 10); $F_{calc.}$ = 1,17 < F (95%; 5; 5) the differences between the results obtained are random					

Note: Analyst 1: the peak area of the solution of the standard sample of fexofenadine hydrochloride = 33.16 mAU×sec; the peak area of the solution of the standard sample of ammonium glycyrrhizinate = 8.12 mAU×sec; Analyst 2: the peak area of the solution of the standard sample of fexofenadine hydrochloride = 33.04 mAU×sec; the peak area of the solution of the standard sample of ammonium glycyrrhizinate = 8.21 mAU×sec.

Table 9 – Scheme of preparation of model mixture solutions with adjuvants of API standard samples solutions

Analyte	Added as a model mixture, mg	Added as a standard sample amount, mg	The total calculated content of the component after dilution mg/ml	Concentration level relative to nominal,%
Fexofenadine hydrochloride	3,6	1,2	0,192	80
	3,6	2,4	0,240	100
	3,6	3,6	0,288	120
Ammonium glycyrrhizinate	1,2	0,4	0,064	80
	1,2	0,8	0,080	100
	1,2	1,2	0,096	120

Table 10 – The results of assessing the correctness of the method for the quantitative determination of fexofenadine hydrochloride and ammonium glycyrrhizinate

Additive added, mg	Additive found, mg	Detection rate, %	Characteristics calculated on the basis of the detection rate value
		Fexofenadine hydrochloride	
1.20	1.26	104.84	$\bar{x}$ = 100.52% SD = 3.76 RSD = 3.74%
1.20	1.15	95.74	
1.20	1.18	98.07	
2.40	2.29	95.39	
2.40	2.44	101.69	
2.40	2.52	104.95	
3.60	3.69	102.54	
3.60	3.53	98.07	
3.60	3.72	103.36	
		Ammonium glycyrrhizinate	
0.4	0.36	90.00	$\bar{x}$ = 95,79% SD = 7.51 RSD = 7.84%
0.4	0.34	85.00	
0.4	0.36	90.00	
0.8	0.82	102.50	
0,8	0.85	106.25	
0,8	0.78	97.50	
1,2	1.14	95.00	
1,2	1.09	90.83	
1,2	1.26	105.00	

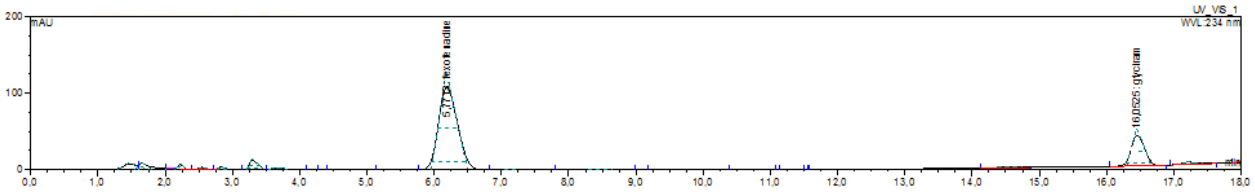

Figure 1 – Test solution chromatogram

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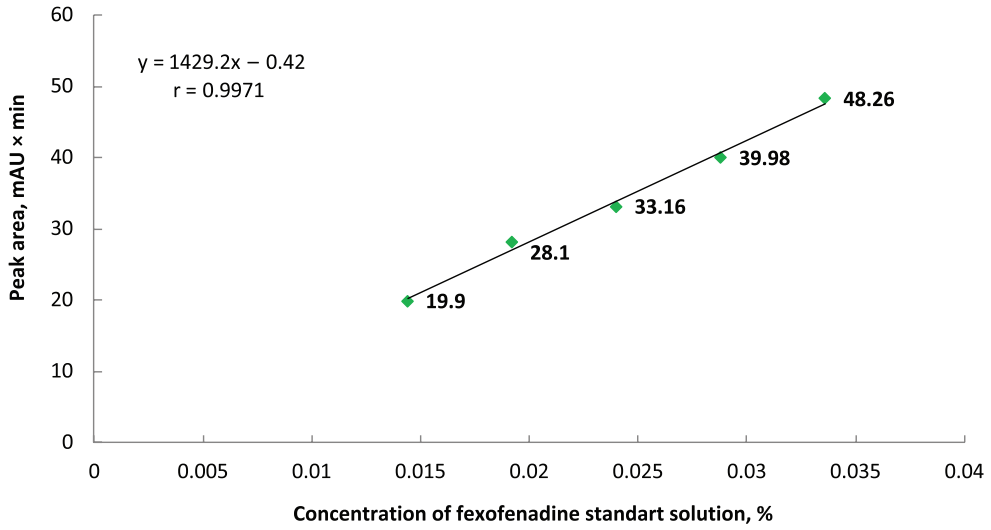

Figure 2 – Dependence graph of fexofenadine hydrochloride peak area on its concentration

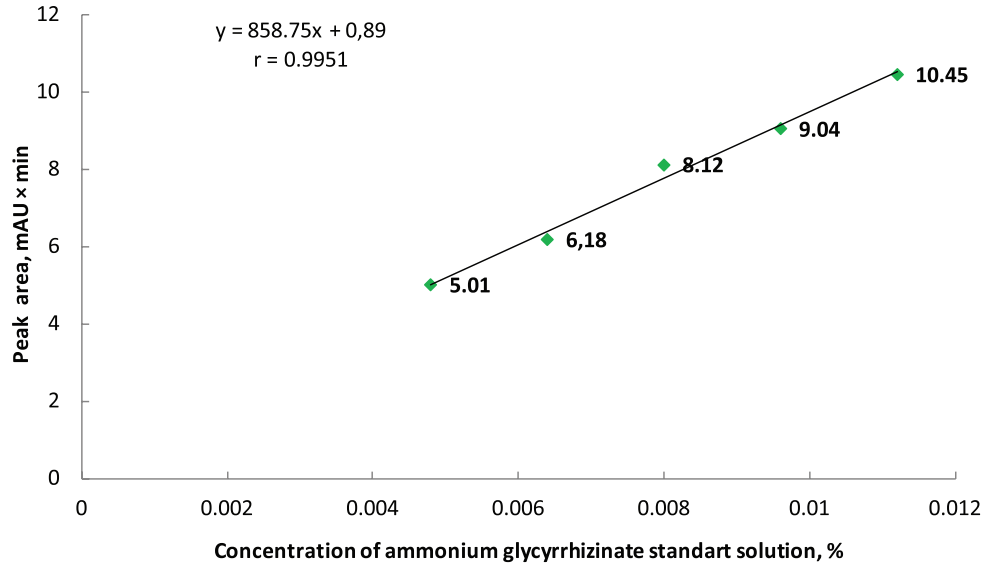

Figure 3 – Dependence graph of ammonium glycyrrhizinate peak area on its concentration

Thus, the characteristics of the peaks on the chromatograms of both the nasal spray solution and the standard sample solutions match the suitability parameters of the chromatographic system. They are: the efficiency of the chromatographic column, calculated from the peaks of the analytes, is not less than 2000 theoretical plates, the asymmetry factors of the peaks are in the range of 0.8 up to 1.5; the relative standard deviation of the peaks areas of the determined substances does not exceed 2%.

Validation of developed methods

The criterion for evaluating the analytical methods is its validation. The validation of the HPLC analysis method for the quantitative determination of the components of the nasal spray was carried out in accordance with State Pharmacopoeia of the Russian Federation of the XIVth edition; to solve practical issues of implementing validation procedures, ~~we~~ the authors of the article were guided by the literature data[3].

The specificity of the methods for determining fexofenadine hydrochloride and ammonium glycyrrhizinate in the nasal spray was confirmed by the correspondence of the retention times of the main peaks on the test solution chromatogram and the peaks of the standard samples solutions of fexofenadine hydrochloride and ammonium glycyrrhizinate. The analysis of the model mixture consisting of nasal spray excipients confirmed the absence of irrelevant chromatographic peaks in the domain of peaks output of fexofenadine hydrochloride and ammonium glycyrrhizinate[4].

[3] Validacija analiticheskih metodik dlja proizvoditelej lekarstv [Validation of analytical methods for drug manufacturers]. Edited by V.V. Beregovykh. M. Litterra, 2008:132.

[4] State Pharmacopoeia of the Russian Federation / Ministry of Health of the Russian Federation. XIV ed. T. I–IV. M., 2018. [Electronic resource]. Access mode: http: // http://femb.ru/femb/ pharmacopea.php

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The linearity relative to fexofenadine hydrochloride and ammonium glycyrrhizinate, was established using standard solutions. The concentration range of solutions included the proposed analytical area of the methods – from 80 to 120% of each component[5].

The solutions were chromatographed three times under the above-listed conditions; the results are shown in Table 5.

The obtained data were used to plot the dependence graph of the peak area on the concentration of fexofenadine hydrochloride (Figure 2).

A linear regression analysis of the results obtained by the least squares method made it possible to establish the dependence of the fexofenadine hydrochloride peak area on its concentration. It is linear and is described by the equation y = 1429 (± 264.2) x – 0.42; the correlation coefficient is 0.9971, and the free term of the equation is statistically insignificant, which is important for confirming the correctness of the method.

The linearity of the methods with respect to ammonium glycyrrhizinate was carried out in a similar manner. The solutions for chromatography were obtained by diluting the initial 0.1% solution of the ammonium glycyrrhizinate standard sample. The results of the determination are presented in Table 6.

The calibration graph based on the obtained data is shown in Fig. 3.

The analysis of the obtained dependence showed that it is described by a linear equation in the form of y = b × x + a, where b = 858.75 ± 204.59. The free term of the equation is 0.89, but its statistical significance is missing. The correlation coefficient is 0.9951, which meets the requirements (≥0.98)[6].-

Thus, the obtained results indicate a satisfactory linearity of the methods for determining fexofenadine hydrochloride and ammonium glycyrrhizinate.

The precision of the methods was evaluated by analyzing a sample of the nasal spray in a six-fold repetition (the replication level). To assess the intra-laboratory precision, the analysis of the test sample was carried out by another analyst on other days using the same equipment. The results of the precision assessment are presented in Tables 7 and 8.

The obtained results indicate the satisfactory precision of the proposed methods for the quantitative determination of the components of the developed nasal spray at the levels of replication and intra-laboratory precision.

It was found out that the average content of fexofenadine hydrochloride in the test sample of the nasal spray is 2.92 ± 0.05 mg/ml (97% of the declared; relative determination error ± 1.56%); ammonium glycyrrhizinate is 1.00 ± 0.05 mg/ml (100% of the declared value; relative determination error ± 5.4%).

Given that the nasal spray is multicomponent, the correctness of the methods was checked using the standard addition method.[7]

The scheme for obtaining solutions with adjuvants is shown in Table 9.

The results of determining the correctness of the methods are presented in table 10.

As follows from the obtained results, the detection rate of the added additives of fexofenadine hydrochloride was in the range from 95 to 105%, of ammonium glycyrrhizinate – from 85 to 106.25% with an RSD value of no more than ±3.74% and ±7.84%, respectively, which meets the requirements[8]. Thus, the proposed methods is characterized by the satisfactory correctness.

The analytical range of the method relative to the nominal concentration of the analytes in the nasal spray was from 80% to 120%.

The method of high performance liquid chromatography, along with other methods, is known to be increasingly used in both qualitative and quantitative analyses of active pharmaceutical ingredients. This method acquires particular relevance in the course of the analysis of pharmacologically active compounds combinations presented in one dosage form.

According to *Ibrahim F. A., et al., 2019* and using high performance liquid chromatography with an UV detection, it is possible to successfully identify and quantify the active components in combinations of moxifloxacin (a synthetic antibacterial agent of the fluoroquinolone group) with glucocorticosteroids intended for the systemic use – dexamethasone and prednisolone. Moreover, in this study, the authors used an original approach from the area of «green chemistry» without the use of toxic organic solvents: as an eluent in the isocratic determination mode, a mixture of ethanol:water in the ratio of 90:10 was used [25].

Another study conducted by the authors' team of *Al-Sanea M. M. et al, 2021,* showed that the method of high performance liquid chromatography makes it possible to quantitatively determine active substances in widespread combinations of antihypertensive medicines: hydrochlorothiazide + olmesartan medoxomil and hydrochlorothiazide + fosinopril-sodium. It should be noted that in this work, isocratic determination mode with a mobile phase based on potassium dihydrogen phosphate + orthophosphoric acid (pH = 3) with the addition of acetonitrile and methanol, made it possible not only to qualitatively and quantitatively determine the target compounds, but also to identify a number of specific impurities, for example, chlorothiazide, which is

[5] State Pharmacopoeia of the Russian Federation / Ministry of Health of the Russian Federation. XIV ed. T. I–IV. M., 2018. [Electronic resource]. Access mode: http: // http://femb.ru/femb/ pharmacopea.php

[6] State Pharmacopoeia of the Russian Federation / Ministry of Health of the Russian Federation. XIV ed. T. I–IV. M., 2018. [Electronic resource]. Access mode: http: // http://femb.ru/femb/ pharmacopea.php

[7] State Pharmacopoeia of the Russian Federation / Ministry of Health of the Russian Federation. XIV ed. T. I–IV. M., 2018. [Electronic resource]. Access mode: http: // http://femb.ru/femb/ pharmacopea.php

[8] State Pharmacopoeia of the Russian Federation / Ministry of Health of the Russian Federation. XIV ed. T. I–IV. M., 2018. [Electronic resource]. Access mode: http: // http://femb.ru/femb/ pharmacopea.php

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a product of the dehydrogenation reaction of hydrochlorothiazide [26].

Considering a widespread use of the HPLC method, as well as its high analytical characteristics, it is not surprising that HPLC is widespread in the pharmaceutical analysis of anti-allergic medicines and their combinations. So, *Shamshad N., et al., 2021* showed that it is possible to successfully identify and quantify cetirizine in the presence of chloroquine and pyrimethamine, using an isocratic elution mode with a mixture of methanol: water (70:30) and UV detection [27]. Furthermore, *Shamshad & Mirza, 2021* demonstrated the possibility of determining cetirizine in the presence of diclofenac sodium [28]. Loratadine can be successfully identified in combination with pseudoephedrine using a methanol: water (90:10) mixture as an eluent in an isocratic mode [29].

In addition, high performance liquid chromatography makes it possible to separate the substances with antiallergic properties from their metabolites (including those exhibiting a pharmacological activity), which was shown by *Sebaiy & Ziedan, 2019*. In this work, the authors identified and quantified loratadine and its active metabolite desloratadine (which is also an antiallergic drug that blocks histamine H_1 receptors) when eluted with a mixture of methanol + phosphoric acid (85:15) in an isocratic mode and detection with a UV spectrophotometric unit [30].

The methods for the qualitative and quantitative determination of fexofenadine hydrochloride in one dosage form with montelukast-sodium and ambroxol hydrochloride in isocratic elution mode with a mixture of methanol:water (70:30) and UV detection, are known [31].

Thus, based on the literature data, in this study for the qualitative and quantitative determination of the active substances of fexofenadine hydrochloride and ammonium glycyrrhizinate, the method of high performance liquid chromatography with UV detection was used. In the course of the work it was shown that due to the different solubility of the target substances, the isocratic elution mode does not allow achieving optimal separation of the components with an analysis duration of less than 30 minutes, which determined the use of a gradient mode. It should be noted that similar conditions of changing the analysis mode have been described in the literature. So, by *Leistner & Holzgrabe, 2021*, when analyzing impurities to the baclofen substance, a gradient mode was used, since the existing impurities for this substance are represented by sparingly soluble zwitter-ions [32] 11 impurities of the ivabradine substance [33] and a combination of pharmacologically active compounds of paracetamol and methionine [34] were investigated by the same approach.

The further course of the study showed that the developed analysis method is reproducible and matches all validity requirements, which is especially important in the analysis of medicines combinations. As *Narula & Pal, 2021* indicate the validation assessment of the analytical methods is a necessary step in the creation of rational methods for the medicines analysis and occupies one of the leading places in the course of their development [35]. There are cases when the optimal analytical methods (diazepam, metformin) did not meet the requirements of validity and, accordingly, could not be used in practical application [36]. In this regard, the developed method of the simultaneous determination of fexofenadine hydrochloride and ammonium glycyrrhizinate in an antiallergic nasal spray is a suitable analytical tool for a pharmaceutical analysis.

CONCLUSION

For the determination of fexofenadine hydrochloride and ammonium glycyrrhizinate using HPLC in a gradient elution mode, the selection of optimal conditions has been carried out and the methods has been developed. The results of the validation assessment showed that the developed methods matches the suitability parameters: it is correct, precise, specific and linear in the analytical field, which confirms its applicability for confirming the quantitative determination of fexofenadine hydrochloride and ammonium glycyrrhizinate in a medicine. It has been experimentally found out that during HPLC analysis, the average content of fexofenadine hydrochloride in the developed nasal spray with antiallergic action is 2.92±0.05 mg/ml (the relative determination error ±1.56%), ammonium glycyrrhizinate is 1.00±0.05 mg/ml (the relative determination error ±5.40%).

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHORS CONTRIBUTION

Mikhail V. Larskiy – carrying out the experimental part of the work, preparation a preliminary version of the manuscript; Anastasia E. Pozdnyakova – review of literary sources on the topic of research, conducting the experimental part of the work, preparation a preliminary version of the manuscript; Zara D. Khadzhieva – development of the research concept, approval of the final version of the manuscript; Dmitry I. Pozdnyakov – statistical processing of the obtained results, preparation of the preliminary version of the manuscript.

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RESEARCH ARTICLE

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EFFECT OF CRYOPRESERVED PLACENTA EXTRACT ON SOME BIOCHEMICAL INDICES OF THERAPEUTIC EFFICIENCY AND TOXICITY OF DICLOFENAC SODIUM IN ADJUVANT-INDUCED EXPERIMENTAL ARTHRITIS

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Relevance. Non-steroidal anti-inflammatory drugs are among the top requested ones in the clinic of internal medicine. However, these drugs are associated with a wide range of adverse reactions involving a number of organs and systems, in particular the gastrointestinal tract, cardiovascular system and kidneys.
The aim of the study is to characterize the effect of the combined use of cryopreserved placenta extract and diclofenac sodium on the prooxidant-oxidative system, the activity of inflammatory, destructive and cytolytic processes, as well as protein and lipid metabolism in rats with experimental rheumatoid arthritis.
Results. The administration of diclofenac sodium and cryopreserved placenta extract to rats with adjuvant arthritis normalized the level of active products of thiobarbituric acid and hence was indicative of the neutralization of an arthritis-induced oxidative stress. A statistically significant (p=0.01) increase ~~of~~ in a superoxide dismutase activity (by 30.6% relative as compared with rats of the control group) has also been established. An increase in the anti-inflammatory properties of diclofenac sodium in the combined use of diclofenac sodium with a cryopreserved placenta extract has been found out. The level of C-reactive protein decreased (p<0.001) by 61.1% as compared with the untreated rats, and the level of seromucoid has been significantly (p<0.01) decreased by 17.1% as compared with the rats of the monotherapy group treated with the studied NSAIDs. It was shown that alanine aminotransferase and aspartate levels were significantly lower (by 38.9%, p<0.01 and by 37.9%, p<0.01, respectively) as compared with those of the animals that had been administrated with diclofenac sodium. Their indices were by 16.7% (p=0.02) and 17.2% (p<0.001) lower than the indices of the control group rats with untreated adjuvant arthritis. The established changes of aminotransferases levels indicate the ability of a cryopreserved placenta extract to level not only an arthritis-induced cytolytic syndrome, but also a diclofenac-induced one. The combined use of cryopreserved placenta extract and diclofenac sodium was accompanied by the normalization of the total lipids level and phospholipids in the blood serum of rats against the background of experimental rheumatoid arthritis. Thus, the content of phospholipids in the lipid pool statistically significantly (p=0.02) increased by 22.6% as compared with the indices of the animals with adjuvant arthritis without treatment.
Conclusion. The study showed that the combined use of diclofenac sodium and cryopreserved placenta extract leads to the restoration of the balance of the prooxidant-antioxidant system that is more pronounced than monotherapy with diclofenac sodium. A decrease in the activity of inflammatory, destructive and cytolytic processes, as well as the restoration of lipid metabolism in the rats with experimental rheumatoid arthritis, has also been observed.
Keywords: cryopreserved placenta extract; adjuvant arthritis; anti-inflammatory activity; nonsteroidal anti-inflammatory drugs; diclofenac sodium
Abbreviations: AlAT – alanine-aminotransferase; AsAT – aspartate aminotransferase; AA – adjuvant arthritis; AOS – antioxidant system; COX – cyclooxygenase; CPE – cryopreserved placenta extract; C-RP – C- reactive protein; DS – diclofenac sodium; GGTP – gamma glutamyl traspeptidase; GI tract – gastrointestinal tract; i/g – intragastrically; i/m – intramuscularly; IPC & C NAS of Ukraine – Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine; LP – lipid peroxidation; LP-AOS – lipid peroxidation-antioxidant system; NSAIDs – non-steroidal anti-inflammatory drugs; OS – oxidative stress; RA – rheumatoid arthritis; SOD – superoxide dismutase; TBA-AP – active products of thiobarbituric acid; 95% CI – 95% Confidence interval

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ВЛИЯНИЕ КРИОКОНСЕРВИРОВАННОГО ЭКСТРАКТА ПЛАЦЕНТЫ НА ОТДЕЛЬНЫЕ БИОХИМИЧЕСКИЕ ПОКАЗАТЕЛИ ЛЕЧЕБНОЙ ЭФФЕКТИВНОСТИ И ТОКСИЧНОСТИ ДИКЛОФЕНАКА НАТРИЯ ПРИ АДЪЮВАНТ-ИНДУЦИРОВАННОМ АРТРИТЕ В ЭКСПЕРИМЕНТЕ

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Актуальность. Нестероидные противовоспалительные препараты являются одними из наиболее востребованных классов лекарственных средств в клинике внутренней медицины. Однако применение указанных препаратов ассоциируется с широким спектром нежелательных реакций с вовлечением ряда органов и систем, в частности желудочно-кишечного тракта, сердечно-сосудистой системы и почек.

Цель. Охарактеризовать влияние криоконсервированного экстракта плаценты и диклофенака натрия при их комбинированном применении на состояние прооксидантно-оксидантной системы, активность воспалительных, деструктивных и цитолитических процессов, а также состояние белкового и липидного обмена по данным биохимических исследований у крыс с экспериментальным ревматоидным артритом.

Результаты. Введение диклофенака натрия и криоконсервированного экстракта плаценты крысам с адъювантным артритом привело к нормализации уровня активных продуктов тиобарбитуровой кислоты, что указывает на нивелирование признаков артрит-индуцированного оксидативного стресса. Также выявлено статистически достоверное ($p=0,01$) повышение активности супероксиддисмутазы на 30,6% относительно значений у крыс контрольной группы. Установлено усиление противовоспалительных свойств диклофенака натрия на фоне комбинированного применения диклофенака натрия с криоконсервированным экстрактом плаценты, так как уровень С-реактивного белка снизился ($p<0,001$) на 61,1% относительно нелеченых крыс, а уровень серомукоида статистически достоверно ($p<0,01$) снизился на 17,1% относительно показателей крыс группы монотерапии исследуемым нестероидным противовоспалительным препаратом. Показано, что уровень аланин-аминотрансферазы статистически достоверно ($p<0,01$) был ниже на 38,9%, а аспартат-аминотрансферазы – ниже на 37,9% ($p<0,01$) относительно показателей животных, которым вводили диклофенак натрия, что соответственно на 16,7% ($p=0,02$) и 17,2% ($p<0,001$) было ниже показателей крыс контрольной группы с нелеченым адъювантным артритом. Установленные изменения со стороны аминотрансфераз указывают на способность криоконсервированного экстракта плаценты нивелировать не только артрит-индуцированный цитолитический синдром, но и диклофенак-индуцированный. Комбинированное применение криконсервованого экстракта плаценты и диклофенака натрия сопровождалось нормализацией уровня общих липидов и фосфолипидов в сыворотке крови крыс на фоне экспериментального ревматоидного артрита. Так содержание фосфолипидов в пуле липидов статистически достоверно ($p=0,02$) выросло на 22,6% относительно показателей животных с адъювантным артритом без лечения.

Заключение. Исследование показало, что комбинированное применение диклофенака натрия и криконсервированного экстракта плаценты приводит к восстановлению равновесия прооксидантно-антиоксидантной системы, более выраженному, чем при монотерапии диклофенаком натрия, снижению активности воспалительных, деструктивных и цитолитических процессов, а также восстановлению липидного обмена у крыс с экспериментальным ревматоидным артритом.

Ключевые слова: криоконсервированый экстракт плаценты; адъювантный артрит; противовоспалительная активность; нестероидные противовоспалительные препараты; диклофенак натрия

Список сокращений: АА – адъювантный артрит; АлАТ – аланин-аминотрансфераза; АОС – антиоксидантная система; АсАТ – аспартат-аминотрансфераза, в/ж – внутрижелудочно, в/м – внутримышечно; ГГТП – гамма-глутамилтраспептидаза; ДН – диклофенак натрия; ЖКТ – желудочно-кишечный тракт; ИПКиК НАН Украины – Института проблем криобиологии и криомедицины Национальной академии наук Украины; КЭП – криоконсервированный экстракт плаценты; НПВП – нестероидные противовоспалительные препараты; ОС – оксидативный стресс; ПОЛ – перикисное окисление липидов; ПОЛ-АОС – перекисное окисление липидов-антиоксидантная система; СОД – супероксиддисмутаза; С-РБ – С-реактивный белок; ТБК-РП – активные продукты тиобарбитуровой кислоты; ЦОГ – циклооксигеназа; 95% ДИ – 95% доверительный интервал

INTRODUCTION

Among the great number of drugs that clinicians have in their arsenal, non-steroidal anti-inflammatory drugs (NSAIDs) occupy a special place. This is due to the presence of nonspecific anti-inflammatory and analgesic properties in them, that justifies the annual increase in their sales against the background of the population aging, since, the frequency of their use is known to increase with age. NSAIDs play a special role in symptomatic therapy in patients with rheumatic diseases – rheumatoid arthritis (RA), ankylosing spondylitis, gout, etc. [1–5].

However, despite the generally recognized effectiveness of NSAIDs, their use in clinical practice is significantly limited by the risk of developing unwanted side effects on various organs and systems, in particular – the gastrointestinal tract (GI tract), cardiovascular system, liver, kidneys [2–6]. In addition, in 25.0–35.0% of cases, patients use NSAIDs irrationally and do not take into account the presence of risk factors for the development of undesirable effects.

It is known that the maximum risk of cardiovascular complications occurs when taking drugs with high selectivity to cyclooxygenase (COX) type 2 (coxibs – celecoxib, etoricoxib, etc.), while the risk of complications from GI tract occurs when taking non-selective NSAIDs (diclofenac, indomethacin, ibuprofen, etc.) [3, 4]. Thus, in the first 7 days of taking celecoxib, the increased risk of myocardial infarction is 92.0–99.0% [3, 7]. Adverse GI tract effects are the most common pathology caused by the inhibition of COX-1 and, as a consequence, a decrease in the protective potential of the mucous membrane of the digestive tract [8, 9]. Nonselective COX inhibitors increase the risk of GI bleeding by an average of 4 times, and selective inhibitors – by 3 times [4, 5].

The problem of GI-tolerance of widely used NSAIDs substantiates the relevance of the search for effective cytoprotective drugs that can increase the resistance of the gastrointestinal mucosa [10, 11]. The treatment of NSAID-induced esophagogastroenterocolonopathy is classically directed at acid suppressive therapy (proton pump inhibitors, H2-histamine blockers, etc.), the use of gastrocytoprotectors (de-nol, sucralfate, etc.) and the drugs that restore the content of prostaglandins in the mucous membrane, etc.) [5, 12–14]. However, none of these groups of drugs sufficiently satisfies clinicians, since they are mainly aimed at treating NSAID gastropathy, while NSAIDs are able of causing lesions, including the distal parts of the GI tract, and have their own side effects [13–19].

One of the promising areas of therapy for GI tract pathologies is the use of drugs of the biological origin, the advantages of which are their complex pharmacological action and high tolerability. [20–22].

As a means of correcting the ulcerogenic effect of NSAIDs, the authors' attention was drawn to a cryopreserved human placenta extract (CPE), which has a multivector spectrum of its biological activity [23–26].

For the first time, cryopreserved preparations of human placental tissue were obtained by scientists from the Institute for Problems of Cryobiology and Cryomedicine (IPC & C) of the National Academy of Sciences of Ukraine (IPC & C NAS of Ukraine), who developed and put into practice a unique technique for its long-term storage in a low-temperature environment. This ensured the primacy of Ukraine among European countries in the experience of cryopreservation of biological tissues [26–31].

In previous studies, it has been shown that CPE is able to neutralize the ulcerogenic effect of NSAIDs, in particular, of diclofenac sodium, meloxicam, ibuprofen, etc. [32–34]. At the same time, according to the literature data, CPE has its own anti-inflammatory properties [35], the mechanism of which is apparently associated with the action of hormones in it – progesterone, estradiol, prolactin, gonadotropin, etc.

In addition, CPE has pronounced antioxidant properties, and its anti-inflammatory effect is possibly associated with the elimination of the pro-inflammatory modulation of the blood system reactions by reactive oxygen species and products of lipid peroxidation (LP) as a result of stimulation of the physiological antioxidant system [35].

All of the forgoing facts justify the need for an in-depth study of the mechanisms of the therapeutic, in particular, the anti-inflammatory effect of CPE when used in combination with NSAIDs.

THE AIM of the study was to characterize the effect of cryopreserved placenta extract and diclofenac sodium in their combined use on the state of the prooxidant-antioxidant system, the activity of inflammatory, destructive and cytolytic processes, as well as the state of protein and lipid metabolism according to biochemical studies in rats with experimental rheumatoid arthritis.

MATERIALS AND METHODS

The study was conducted at the Department of Experimental Cryomedicine on the basis of IPC & C NAS of Ukraine. The work was performed in the frame of the planned research work "Destructive and regenerative processes in tissues in vivo after exposure to low temperatures and biologically active substances" (code 2.2.6.113, state registration number 0117U001049).

The study was carried out on 28 sexually mature nonlinear male rats obtained from the IPC & C NAS of Ukraine vivarium, weighing 200–220 g, divided into 4 groups of 7 animals each: I – intact rats (n=7); II (control) – rats (n = 7) with experimental RA without treatment; III – rats (n=7) with experimental RA administrated with DS (8 mg/kg [36], intragastrically (i/g)); IV (n=7) – rats with experimental RA administrated with DS (8 mg/kg, i/g) and CPE (0.16 mg/kg [37], intramuscularly (i/m)).

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Dose schedule of the investigational agents

AA treatment was carried out from 14 to 28 days. DS (Pr. JSC Chimfarmzavod Krasnaya Zvezda, ZAO "Zdorovye" Pharmaceutical Company, Ukraine) was administered i/g at the dose equal to ED_{50} for the anti-inflammatory activity in the carrageenin-induced edema model – 8 mg/kg in the form of an emulsion on polysorbate "Twin-80" [35, 37]. The indicated dose of DS corresponds to a single human dose of 88 mg (1.25 mg/kg), which is consistent with the clinical recommendations for the use of DS in patients at the dose of 75–100 mg/day in the long-term use and 1.7 times lower than its maximum daily dose of 150 mg [8].

According to the instructions, the CPE drug "Cryocell cryo-extract of the placenta" (State Enterprise "Interdepartmental Scientific Center of Cryobiology and Cryomedicine of the National Academy of Sciences, National Academy of Medical Sciences and the Ministry of Health of Ukraine", Ukraine), is used in patients parenterally in a single dose of 1.8 ml. Accordingly, a single dose for rats was (1.8 ml/70 kg) × 6.35 = 0.16 ml/kg of body weight [37]. Before using the "Cryocell cryo-extract of the placenta" preparation, a single dose (0.16 ml/kg) was extemporally diluted in a 0.9% NaCl solution at the rate of 0.1 ml of 0.9% NaCl solution/100 g of body weight. At the rate of at 0.16 ml / kg of body weight, CPE was injected i/m with an interval of 2 days (5 injections in total), on days 14, 17, 20, 23 and 26, respectively. That corresponded to the instructions for its clinical use – i/m by 1,8 ml with an interval of 2–3 days, a course of 1–5 injections.

Chronic immune inflammation model

To reproduce the conditions of NSAIDs administration to rats corresponding to their clinical application, a model of experimental rheumatoid arthritis (RA) – adjuvant arthritis (AA) in rats was chosen. It had all the morphofunctional signs of RA and was accompanied by a typical reaction, the main link of which is T-cell immunity [38–40]. AA was modeled by a subplantar injection of complete Freund's adjuvant (PAF, Thermo Fisher Scientific, USA) into the hind right limb at the rate of 0.1 ml per rat. The day of the administration of the adjuvant was taken as the "0" day of the experiment [40–42]. The maximum manifestation of the local inflammatory reaction, which was accompanied by a significant increase in the volume of the limb, was determined on the 12^{th}–14^{th} days, and then the activity of the inflammatory process gradually decreased.[1]

On the 28^{th} day of the experiment, the animals were withdrawn from the experiment, and after decapitation of the animals, blood samples were taken into tubes with a pre-introduced anticoagulant (2-substituted potassium salt of ethylenediaminetetraacetic acid). Plasma was separated by centrifugation for 15 min at 3000 revol/min. To obtain serum, blood was collected in glass tubes without anticoagulant and kept at room temperature (20–26°C) until complete separation of serum, which was taken into a test tube and centrifuged for 15–20 min at 3000 revol/min.

Biochemical methods of blood tests[2]

1. Content of active products of thiobarbituric acid (TBA-AP) in blood serum was determined spectrophotometrically by the method of Asakawa T. et al. [43] according to the reaction with TBA and was calculated from the optical density indices determined from the light absorption at the wavelength of λ=535 nm. The molar extinction coefficient of the red-colored complex, which is 1.56×105 mol-1/cm-1 and expressed in μmol/L, was taken into account.
2. The activity of superoxide dismutase (SOD) in blood serum was determined spectrophotometrically by the method of Kostyuk V.A. et al. [44], based on the suppression of the oxidation reaction of quercetin, according to the optical density, determined by the light absorption at the wavelength of λ=406 nm. The SOD activity was expressed as a percentage inhibition of quercetin oxidation.
3. The content of seromucoid in blood serum was determined spectrophotometrically by the method of Weimer H.E. and Moshin R.J., which consists in the precipitation of blood serum proteins with a 1.8 M solution of perchloric acid (HClO4), the isolation of seromucoid from the filtrate using phosphoric tungstic acid, and a further quantitative determination by the difference in light absorption at the wavelength of λ=260 nm and λ=280 nm. The content of seromucoid was determined in units of optical density (optical density units) and expressed in mmol/L (conversion factor = 1; 1 optical density unit = 1 mmol/L).
4. The content of C-reactive protein (C-RP) in blood serum was determined by the degree of agglutination and turbidity according to the instructions for using the latex diagnostic test for detecting C-reactive protein in blood serum "C-RP latex test" (NPL Granum LLC, Ukraine), and expressed in mg/L.
5. The activity of alanine aminotransferase (AlAT) in blood serum was determined spectrophotometrically by the method of Reitman S. and Frankel S. and expressed in μmol/(ml×h). The studies were carried out using kits for biochemical studies "ALT (Reitman-Frenkel with a calibrator)" (ZAO SPE "Filisit-Diagnostics", Ukraine).
6. The activity of aspartate aminotransferase (AsAT) in blood serum was determined spectrophotometrically by the method of Reitman S. and Frankel S. and expressed in μmol / (ml × h). The studies were carried out using kits for biochemical studies "AsAT (Reitman-Frenkel with a calibrator)" (ZAO SPE "Filisit-Diagnostika", Ukraine).

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7. The activity of gamma-glutamyl traspeptidase (GGTP) in blood serum was determined spectrophotometrically and expressed in U/L. The studies were carried out using kits for biochemical studies "GTP" (ZAO SPE "Filisit-Diagnostics", Ukraine).
8. The activity of alkaline phosphatase in blood serum was determined spectrophotometrically and expressed in μmol/h×l. The studies were carried out using kits for biochemical studies "Alkaline phosphatase" (ZAO SPE "Filisit-Diagnostics", Ukraine).
9. The content of total protein in blood serum was determined spectrophotometrically by biuret reaction and was expressed in g/l. The studies were carried out using kits for biochemical studies "General protein" (ZAO SPE "Filisit-Diagnostics", Ukraine).
10. The content of total lipids in blood serum was determined spectrophotometrically by the color reaction with the sulfophosphovaniline reagent, which is based on the fact that the decomposition products of unsaturated fatty acids formed after acid hydrolysis of lipids, interact with the phosphoricaniline reagent to form colored complexes with a maximum absorption at the wavelength of λ=530 nm. Lipid extracts were obtained according to the method of Bligh E.G. and Dyer W.I. [45]. Phospholipids were fractionated by the method of Svetashev V.I. and Vaskovsky V.E., by two-dimensional micro-ball chromatography [46]. Phospholipids were identified by the method [47] and their content was expressed by the level of inorganic phosphorus in g/L.

Bioethical aspects of research

The animals were kept in the conditions of the IPC&C NAS of a Ukraine vivarium. For 14 days, the rats were in quarantine (in accordance with the sanitary standards of "The structure and content of experimental biological clinics" (Order No. 755 of 08/12/1997)) on a water-food ration with free access to food and water (according to the Appendix dated 12/04/1977 to Order No. 163 of 03/10/1996 "About daily feeding rates for laboratory animals and producers")). All experimental studies on laboratory animals were carried out in accordance with the requirements of good laboratory practice and in compliance with the main provisions of the Council of Europe Convention on the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes dated 18 March 1986; Directive of the European Parliament and of the Council of the European Union 2010 / 63 / EC dated 22 September 2010 on the protection of animals used for scientific purposes; Order of the Ministry of Health of Ukraine dated December 14, 2009 No. 944 "On approval of the procedure for conducting preclinical study of drugs and examination of materials for preclinical study of drugs" and the Law of Ukraine dated February 21, 2006 No. 3447-IV "On the protection of animals from cruelty".

The comprehensive research program was reviewed and approved by the Bioethics Committee at IPC&C NAS of Ukraine (extract from Protocol No. 2 dated March 11, 2020).

Statistical processing

Statistical processing of the obtained results was carried out using the application program for working with electronic tables "Microsoft Office Excel 2003; 2013" (Microsoft Corporation, USA) using the "Real Statistics" extension (http://www.real-statistics.com/). The character of the values distribution in each group of the sample was assessed using the Shapiro-Wilk test. The homogeneity of the dispersions was determined according to the Levene's test. To assess the significance of the revealed differences in the studied indicators under different experimental conditions, a statistical analysis was carried out using parametric and nonparametric criteria. With a normal distribution of independent values, the differences between the groups were determined in pairs by Student's t-test. In the case of an abnormal distribution of at least one of the groups of independent values, the differences between them were determined in pairs using the Mann-Whitney nonparametric U-rank test. The obtained values were compared with the critical ones at the confidence level above 95.0% ($p<0.05$), above 99.0% ($p<0.01$), above 99.5% ($p<0.005$) and above 99.9% ($p<0.001$); and a conclusion about the error probability was made. The numerical data in the case of a normal distribution of values are given as "M±m" (M±SE), where M is the arithmetic mean, m(SE) is the standard error of the arithmetic mean, or M (95%CI:5%–95%), and 95% CI is a 95% confidence interval. In case of an abnormal distribution of the obtained values, the data are presented in the form of Me [LQ; UQ], where Me is the median, [LQ; UQ] – the upper border of the lower quartile (LQ) and the lower border of the upper quartile (UQ).

RESULTS AND DISCUSSION

Biochemical studies of the peripheral blood of rats with AA (control group) showed a statistically significant ($p<0.001$) increase in the TBA-AP content by 53.7% relative to the indices of intact animals (Fig. 1). That indicates an LP activation, which is probably due to the development of a systemic inflammatory process [47, 48].

LP products are known to promote platelet aggregation, decrease the synthesis of prostaglandins with an anticoagulant effect, form cytolysis syndrome, release blood coagulation factors, and suppress cell division and regeneration. TBA-AP acts as an endogenous aldehyde, which is a clinical and laboratory marker of oxidative stress (OS) and is widely used to control the effectiveness of treatment of a number of diseases – RA, osteoarthritis, coronary heart disease, etc. [49–51].

Monotherapy of AA rats with DS, led to a statistically significant ($p<0.01$) decrease in the level of TBA-AP by

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19.0% relative to the indicators of animals in the control group in the same period of the study. The combined use of DS and CPE was accompanied by a more pronounced decrease in the intensity of LP processes, which was indicated by a decrease in the content of TBA-AP in rats with AA by 31.7% relative to the indices of rats with AA without treatment. That was 1.7 times higher than similarly directed changes against the background of using only DS.

It should be noted that in the animals with AA, which had been injected with DS and CPE, on the 28-th day of the experiment, the level of TBA-AP was practically compared with the indices of the intact rats and it was 4.3 [3.6; 4.5] μmol/L (Fig. 1). That indicates the signs leveling of AA-induced OS and can be regarded as one of the mechanisms of the therapeutic action of the studied drugs.

The established LP activation in the rats against the background of AA development was consistent with the parallel depletion of the antioxidant system (AOS), which indicated a statistically significant ($p<0.01$) decrease in the SOD level by 27.9% relative to the indices of intact animals (Fig. 2).

Together with other AOS enzymes (catalase, ceruloplasmin, glutathione peroxidase, glutathione reductase, etc.), SOD is known to be an integral component of LP-AOS. The assessment of the functional state of AOS, determines the level of the compensatory and adaptive response of the organism in conditions of the possible development of OS [51, 52].

Monotherapy of AA rats with DS, led to a statistically significant ($p=0.03$) increase in the SOD activity by 20.4% relative to the indices of untreated animals (group II), but this value (59.0% inhibition of quercetin oxidation) was still 13.2% lower than the indices of the animals without AA (68.0 [64.0; 76.5]%).

Against the background of the combined use of DS and CPE, a statistically significant ($p=0.01$) increase in the SOD activity by 30.6% relative to the values in rats of the control group was noted, which was only 5.9% lower than the indices of the intact rats, and indicated the most pronounced restoration of the AOS activity.

To assess the state of connective tissue structures in rats against the background of AA development, the level of seromucoids was studied, since it is this group of glycoproteins that enters the blood plasma upon destruction, degradation or damage of connective tissue. In addition, seromucoids are usually considered acute phase markers of inflammation, which can serve indicators of the severity of the inflammatory process, since their life span is about 5 days [53–56].

It was found out that on the 28th day of the experiment, the rats with AA showed a statistically significant ($p<0.001$) increase in the seromucoid level by 2.2 times relative to the indices of intact rats (Fig. 3).

Monotherapy of AA rats with DS, led to a statistically significant ($p<0.01$) decrease in the level of the studied parameter by 31.4% relative to the level of the untreated animals. The combined use of DS and CPE, led to a statistically significant ($p<0.01$) even greater decrease in the seromucoid content by 17.1% relative to the indicators of rats in the DS monotherapy group, which amounted to 0.18 [0.17; 0.18] mmol/L.

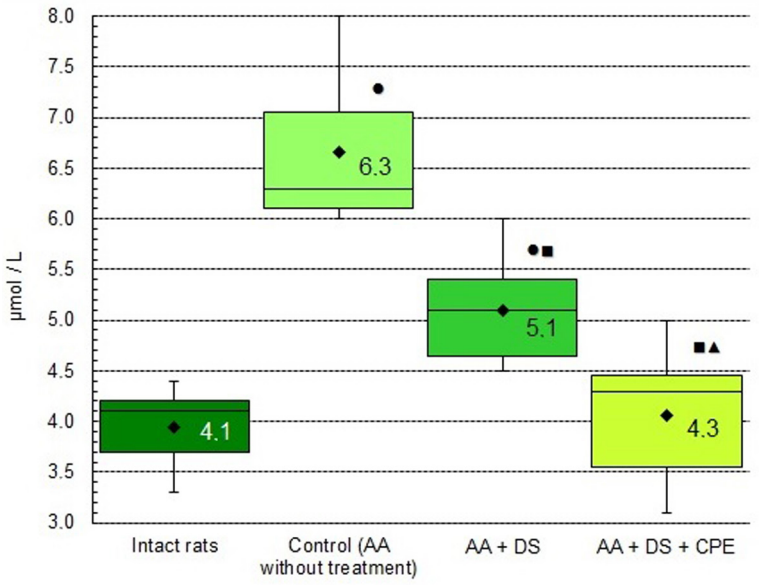

Figure 1 – Effect of DS and its combination with CPE on the level of TBA-AP in peripheral blood in rats with AA on the 28th day of the experiment

Note: the distribution of values is abnormal. Boxes include results from the 25th to 75th percentile, vertical lines outside the boxes are the minimum and maximum values. The horizontal line inside the box is the median (► – the median is 25 (75) percentile), ♦ – the average value; ● – $p<0.05$ relative to the indices of intact rats, ■ – $p<0.05$ relative to the indices of rats with AA without treatment; ▲ – $p<0.05$ relative to the indices of AA rats treated with DS

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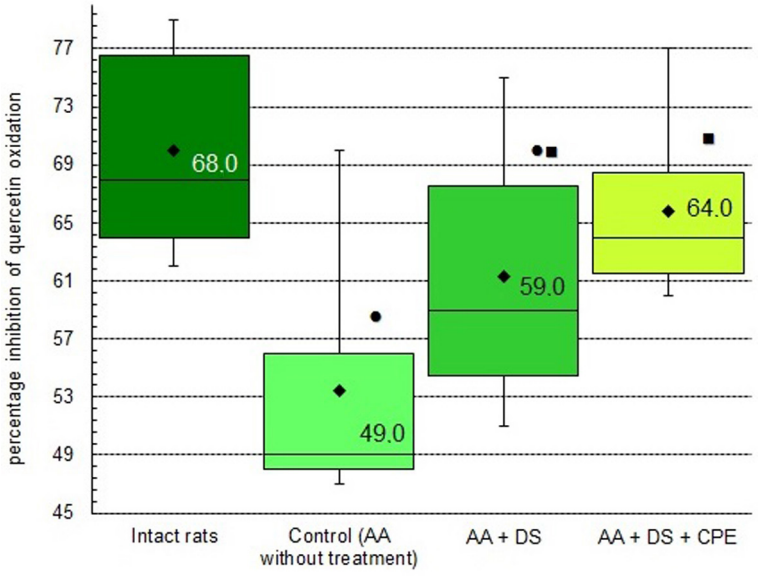

Figure 2 – Effect of DS and its combination with CPE on the level of SOD in peripheral blood in rats with AA on the 28th day of the experiment

Note: the distribution of values is abnormal. Boxes include results from the 25th to 75th percentile, vertical lines outside the boxes are the minimum and maximum values. The horizontal line inside the box is the median (► – the median is 25 (75) percentile), ♦ – the average value; ● – p<0.05 relative to the indices of intact rats, ■ – p<0.05 relative to the indices of rats with AA without treatment; ▲ – p<0.05 relative to the indices of AA rats treated with DS

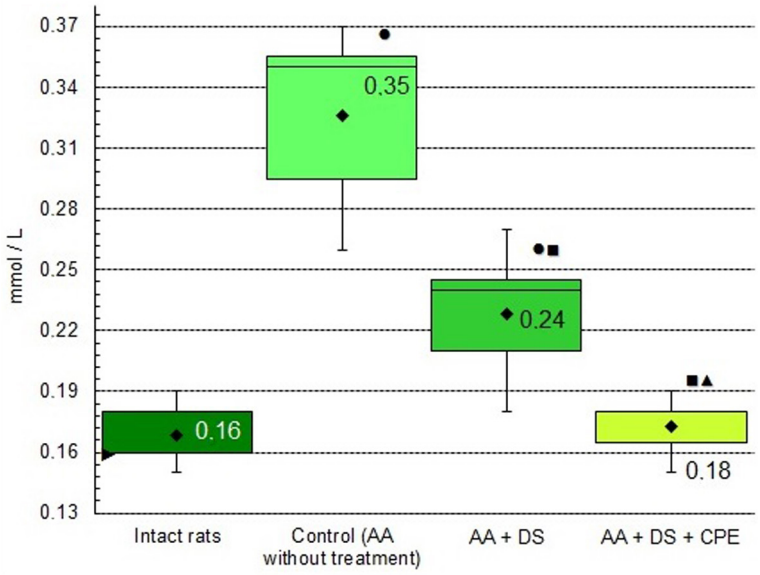

Figure 3 – Effect of DS and its combination with CPE on the level of seromucoid in peripheral blood in rats with AA on the 28th day of the experiment

Note: the distribution of values is abnormal. Boxes include results from the 25th to 75th percentile, vertical lines outside the boxes are the minimum and maximum values. The horizontal line inside the box is the median (► – the median is 25 (75) percentile), ♦ – the average value; ● – p<0.05 relative to the indices of intact rats, ■ – p<0.05 relative to the indices of rats with AA without treatment; ▲ – p<0.05 relative to the indices of AA rats treated with DS

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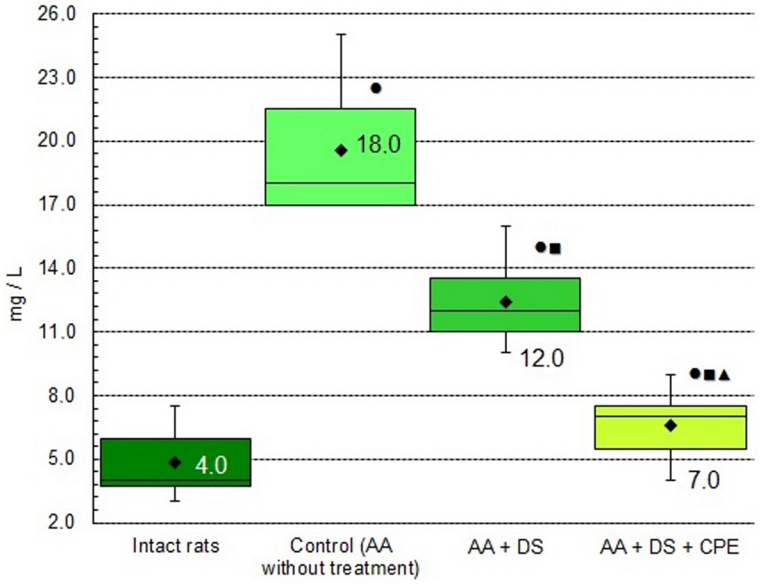

Figure 4 – Effect of DS and its combination with CPE on the level of C-RP in peripheral blood in rats with AA on the 28th day of the experiment

Note: the distribution of values is abnormal. Boxes include results from the 25th to 75th percentile, vertical lines outside the boxes are the minimum and maximum values. The horizontal line inside the box is the median (► – the median is 25 (75) percentile), ♦ – the average value; ● – p<0.05 relative to the indices of intact rats, ■ – p<0.05 relative to the indices of rats with AA without treatment; ▲ – p<0.05 relative to the indices of AA rats treated with DS

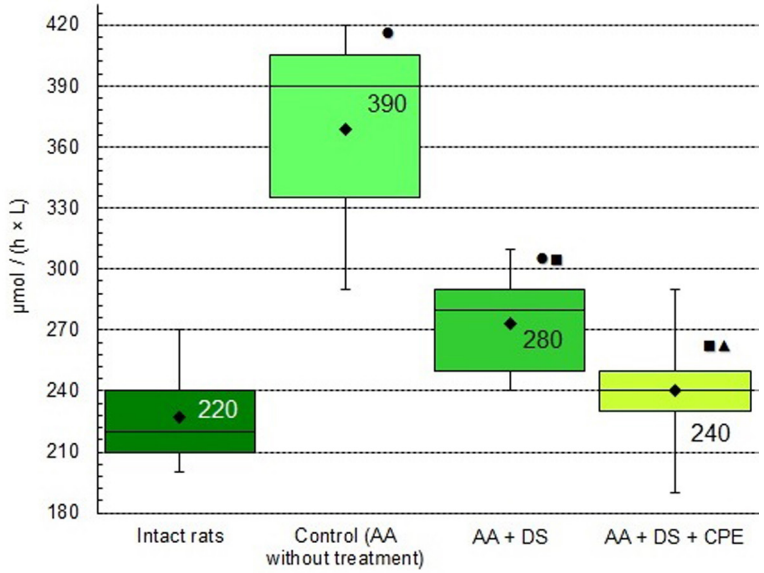

Figure 5 – Effect of DS and its combination with CPE on the level of alkaline phosphatase in peripheral blood in rats with AA on the 28th day of the experiment

Note: the distribution of values is abnormal. Boxes include results from the 25th to 75th percentile, vertical lines outside the boxes are the minimum and maximum values. The horizontal line inside the box is the median (► – the median is 25 (75) percentile), ♦ – the average value; ● – p<0.05 relative to the indices of intact rats, ■ – p<0.05 relative to the indices of rats with AA without treatment; ▲ – p<0.05 relative to the indices of AA rats treated with DS

Table 1 – Effect of DS and its combination with CPE on markers of cytolysis in the peripheral blood of rats with AA on the 28th day of the experiment (M ± m (95% CI) or Me [LQ; UQ]; n = 28)

Investigated indicator, units of measurement	Experimental conditions			
	Group I	Group II	Group III	Group IV
	Intact rats	Control (AA without treatment)	AA + DS	AA +DS + CPE
n	7	7	7	7
Alanine aminotransferase (AlAT), µmol/ml/h	1.4 [1.3; 1.6]	1.8 [1.6; 1.9] p_{1-2}=0.01	2.2 [2.1; 2.3] p_{1-3}<0.01 p_{2-3}=0.01	1,5 [1.5; 1.6] p_{1-4}=0.17 p_{2-4}= 0.02 p_{3-4}<0.01
Aspartate aminotrans-fe-times (AsAT), µmol/ml/h	2.3 [2.1; 2.4]	2.9 [2.9; 3.5] p_{1-2} < 0.001	3.5 [3.3; 2.4] p_{1-3} < 0.01 p_{2-3}= 0.2	2.4 [2.3; 2.5] p_{1-4}= 0.14 p_{2-4}<0.001 p_{3-4}< 0.01
Gammaglutamyl transpepti-dase (GGTP), U/L	22.3±1.81 (95% CI: 18.7–25.8)	36.1±0.80 (95% CI: 34.6–37.7) p_{1-2}<0,001	27.9±1.30 (95% CI: 25.3–30.4) p_{1-3}= 0.03 p_{2-3}<0.001	23.9±1.10 (95% CI: 21.7–26.0) p_{1-4}=0.5 p_{2-4}<0.001 p_{3-4}=0.04

Table 2 – Effect of DS and its combination with CPE on the indices of protein homeostasis in the peripheral blood of rats with AA on the 28th day of the experiment (M ± m (95% CI) or Me [LQ; UQ]; n = 28)

Investigated indicator, units of measurement	Experimental conditions			
	Group I	Group II	Group III	Group IV
	Intact rats	Control (AA without treatment)	AA + DS	AA +DS + CPE
n	7	7	7	7
Total protein, g/l	78.9±1.45 (95% CI: 76.0–81.7)	74.6±1.36 (95% CI: 71.9–77.2) p_{1-2}=0.05	74.7±0.92 (95% CI: 72.9–76.5) p_{1-3}=0.03 p_{2-3}=0.9	81.0±1.50 (95% CI: 78.1–83.9) p_{1-4}=0.3 p_{2-4}=0.01 p_{3-4}<0.01
Albumin, g/l	35.3±1.69 (95% CI: 32.0–38.6)	25.1±1.03 (95% CI: 23.1–27.2) p_{1-2}<0.001	27.9±1.30 (95% CI: 25.3–30.4) p_{1-3}<0.01 p_{2-3}= 0.1	37.4±1.90 (95% CI: 33.7–41.2) p_{1-4}=0.01 p_{2-4}<0.001 p_{3-4}<0.01
Globulins, g/l	45.7±1.54 (95% CI: 42.7–48.7)	49.6±1.29 (95% CI: 47.0–52.1) p_{1-2}=0.08	46.7±1.19 (95% CI: 44.4–49.0) p_{1-3}=0.6 p_{2-3}=0.1	40.4±0.65 (95% CI: 39.2–41.7) p_{1-4}<0.01 p_{2-4}<0.001 p_{3-4}<0.001
Albumin / globulin ratio	0.78±0.039 (95% CI: 0.70–0.85)	0.51±0.028 (95% CI: 0.46–0.57) p_{1-2}<0.001	0.60±0.035 (95% CI: 0.53–0.67) p_{1-3}<0.01 p_{2-3}=0.07	0.93±0.045 (95% CI: 0.84–1.02) p_{1-4}= 0.03 p_{2-4}<0.001 p_{3-4}<0.001

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Table 3 – Effect of DS and its combination with CPE on lipid homeostasis indices in the peripheral blood of rats with AA on the 28th day of the experiment (M ± m (95% CI) or Me [LQ; UQ]; n = 28)

Investigated indicator, units of measurement	Experimental conditions			
	Group I	Group II	Group III	Group IV
	Intact rats	Control (AA without treatment)	AA + DS	AA +DS + CPE
n	7	7	7	7
Total lipids, g/l	3.0 [2.7–3.3]	5.1 [4.5–5.2] p_{1-2}<0.001	4.4 [4.2–4.5] p_{1-3}<0.001 p_{2-3}=0.07	3,3 [3.2–3.4] p_{1-4}= 0.1 p_{2-4}<0.001 p_{3-4}<0.001
Phospholipids, g/l	2.1±0.05 (95% CI: 2.0–2.2)	2.5±0.09 (95% CI: 2.3–2.7) p_{1-2}<0.001	2.0±0.05 (95% CI: 1.9–2.1) p_{1-3}= 0.6 p_{2-3}<0.001	2.1±0.05 (95% CI: 2.0–2.2) p_{1-4}= 0.5 p_{2-4}<0.01 p_{3-4}=0.2
Phospholipids relative to total lipids,%	70.9±5.35 (95% CI: 60.4–81.4)	53.2±3.60 (95% CI: 46.2–60.3) p_{1-2}=0.02	46.9±1.80 (95% CI: 43.3–50.4) p_{1-3}<0.01 p_{2-3}=0.1	65.2±2.47 (95% CI: 60.3–70.0) p_{1-4}= 0.4 p_{2-4}=0.02 p_{3-4}<0.001

For a good reason, C-RP is considered the "golden" marker of inflammation in almost all pathological processes, since it tends to increase significantly in tissue damage, infections of a bacterial or viral nature, inflammation and malignant neoplasms; in tissue necrosis, it can even increase up to 300 mg/l during the day [47, 57–61].

It has been found out that the development of the experimental RA in rats led to a statistically significant (p<0.001) increase in the C-RP level by 4.5 times relative to the indices of the intact animals (Fig. 4).

On the photo of monotherapy with DS, this indicator decreased (p<0.001) by 33.3%, and against the background of the combined administration of DS and CPE, it decreased (p<0.001) by 61.1% relative to the values in the rats with AA without treatment. The data obtained indicate pronounced anti-inflammatory properties of DS, which are potentiated when combined with CPE, but may also be associated with the intrinsic anti-inflammatory activity of CPE.

The established biochemical signs of an active inflammatory process and the resulting imbalance in the LP-AOS system substantiate the advisability of a detailed analysis of the activity of cytolysis markers against the background of treatment of rats with AA – AlAT, AsAT and GGTP [62, 63].

The study showed that the development of AA in rats was accompanied by an increase in the content of AlAT by 28.6% (p=0.01), AsAT – by 26.1% (p<0.001) and GGTP – by 62.2% (p<0.001) relative to the indicators of the intact rats (Table 1).

Aminotransferases are complex enzyme proteins that catalyze transamination processes and ensure the synthesis of individual amino acids in the body. It has been proven that under the influence of damaging factors, the energy metabolism of hepatocytes is disrupted, which leads to an increase in the permeability of cell membranes and the appearance of cytoplasmic components in the blood serum.

Since the marker of the cellular damage, GGTP, is found to the greatest extent in the kidneys, liver and small intestine, it can be assumed that there is an inflammatory process in these organs. That is compared with the literature data on the systemic character of the inflammatory process in RA, which is analogous to AA in animals [50].

The use of DS led to an increase in cytolytic processes, which was indicated by an even greater increase in the studied markers in the peripheral blood – so, AlAT was higher by 22.2% (p<0.01), and AsAT – by 20.7% (p=0, 2) relative to the indices of rats with AA without treatment. That was 57.1% (p<0.01) and 52.2% (p<0.01), respectively, higher than the indices of the intact animals (Table 1). No mortality in the group treated with a combined use of DS and CPE, had been noted. These changes in the activity of aminotransferases are consistent with the data on the hepatotoxic effect of DS [3, 8, 64–66].

It was also found out that against the background of the use of DS, GGTP statistically significantly (p<0.001) decreased by 22.9% relative to the indices of the animals in the control group (Table 1).

Against the background of the combined use of DS and CPE, the indices of cytolytic processes in the rats with AA are of particular interest (Table 1). Therefore,

it was found out that AlAT was statistically significantly ($p<0.01$) lower by 38.9%, and AsAT was lower ($p<0.01$) by 37.9% relative to the indices of the animals injected with DS, which, respectively, were by 16.7% ($p=0.02$) and 17.2% ($p<0.001$) lower than the indices of the rats in the control group with untreated AA. The changes established on the part of aminotransferases, indicate the ability of CPE to level not only AA-induced cytolytic cider, but also concomitant cytolysis, enhanced by DS. That indicates the universality of its cytoprotective activity, probably due to its ability to stabilize cell membranes, as well as its antioxidant properties.

In addition, it was found out that the combined use of DS and CPE, was also accompanied by a statistically significant ($p<0.001$) decrease in the GGTP level by 28.6% relative to the untreated rats with AA, which was 11.4% lower ($p=0.01$) than in the rats treated with DS alone (Table 1).

The assessment of the level of alkaline phosphatase in the peripheral blood in the rats with AA showed that the development of a systemic autoimmune inflammation was accompanied by a statistically significant ($p<0.001$) increase in the indicated metalloprotein by 77.3% relative to the indices of the intact rats, and amounted to 390 [335; 405] µmol/h×L (Fig. 5).

Alkaline phosphatase is a heterogeneous enzyme consisting of separate isoenzymes, each of which is concentrated in a specific organ: liver, bone tissue, intestines, placenta. Localized on the cell membrane, the enzyme is connected to the transport of biologically important compounds, primarily phosphorus. Alkaline phosphatase is actively involved in the processes of phosphorus-calcium metabolism in the body. An increase in the activity of alkaline phosphatase indicates the activation of bone remodeling processes, since its bone fraction is synthesized by osteoblasts and their precursors, and is involved in the mineralization of the bone matrix [50, 67–69]. An increase in the activity of this enzyme may indicate an increase in an osteoblastic activity and an increase in destructive changes in the liver or the occurrence of cholestasis in the rats with AA [3].

DS monotherapy led to a statistically significant ($p<0.01$) decrease in the activity of alkaline phosphatase by 28.2%, and the combined use of DS and CPE was accompanied by a more pronounced decrease in the activity of this enzyme by 38.5% ($p<0.01$) relative to the indicators of the untreated rats with AA (Fig. 5).

The study of the protein spectrum of the blood of rats with AA showed that the level of total protein practically did not differ in the intact rats and the rats with AA (Table 2).

The content study of the individual protein fractions showed that the development of AA in the rats was accompanied by a statistically significant ($p<0.001$) decrease in the albumin content by 28.7% relative to the indices of the intact rats. Albumin is the most homogeneous fraction of proteins, the main function of which is to maintain colloidal osmotic pressure, as well as to transport carbohydrates, lipids, hormones and pigments. A patient's body with active RA, consumes albumin at the site of inflammation, resulting in hypoalbuminemia more actively.

In addition, it was noted that in the rats with AA, the content of globulins slightly increased by 8.4% ($p=0.04$) relative to the indices of the intact animals. As it is known, in a long subacute progressive process, hypergamaglobulinemia is primarily observed, which is associated with an increase in the level of autoantibodies [50, 51, 70, 71].

These changes led to a statistically significant ($p<0.001$) decrease in the albumin / globulin ratio of peripheral blood by 34.2% relative to the indices of intact animals (Table 3). The change in the ratio of protein fractions, as a rule, corresponds to the severity of RA and reflects the dynamics of the inflammatory process [50, 51, 70, 72].

DS monotherapy led to a statistically significant ($p=0.04$) decrease in the albumin / globulin ratio by 17.5% relative to the AA rats without treatment. The combined use of DS and CPE was accompanied by a complete increase ($p=0.01$) of the studied ratio by 19.5% higher than in the intact animals. That is due to an increase in the albumin level almost up to the level of the intact animals, and at the same time it is by 11.6% lower ($p<0.01$) than the values in the intact rats, the content of globulins (Table 3).

Lipids, which act as structural components of biomembranes, the energy substrate of the cell, participating in the reactions of signal transduction, exo- and endocytosis and the like, play an important role in the life of the cell. In addition, they participate in the fixation of proteins of the phospholipid bilayer and ensure their appropriate orientation in the cell membrane, are a non-polar medium for fat-soluble substrates and enzyme cofactors, cause their folding, and also act as regulators and modulators of an enzymatic activity [69, 73].

The study showed that against the background of the AA development, in the rats, there was a statistically significant ($p=0.02$) decrease in the proportion of phospholipids in the total lipid pool by 25.0%. At the same time, there was an increase in the total lipid level ($p<0.001$) by 1.7 times and a disproportionate increase in the content of phospholipids ($p<0.001$) by 1.2 times, which is due to the activation of the cyclooxygenase pathway of membrane phospholipid metabolism (Table 3).

Against the background of DS monotherapy, a decrease in the level of total lipids ($p=0.07$) was noted by 1.3 times and a decrease in the level of phospholipids ($p <0.001$) was also noted by 1.3 times relative to the indicators of the animals with AA without treatment. That corresponded to the development of even greater imbalance in the content of phospholipids in the pool of total lipids - their content decreased by another 6.3%,

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however, these changes did not reach the level of statistical significance (p=0.1).

Against the background of the combined use of DS and CPE in the rats with AA, a statistically significant (p=0.02) harmonization of the lipid balance was noted – the content of phospholipids increased by 22.6% relative to the indicators of the animals with AA without treatment and amounted to 2.1±0.05, respectively (95% CI: 2.0–2.2) g/l. These changes were observed against the background of a decrease in the level of total lipids in the blood serum (p<0.001) by 35.3% relative to the indices of the untreated rats, and amounted to 3.3 [3.2–3.4] g/l, respectively, which was compared with the indices of the intact animals 3.0 [2.7–3.3] g/l.

CONCLUSION

The administration of diclofenac sodium and cryopreserved placenta extract to rats with adjuvant arthritis normalized the level of active products of thiobarbituric acid, which indicates leveling of signs of the arthritis-induced oxidative stress. A statistically significant (p=0.01) increase in the level of superoxide dismutase (by 30.6%) as compared with the values in the rats of the control group, was also revealed.

An increase in the anti-inflammatory properties of diclofenac sodium was found out in the combined use of diclofenac sodium with cryopreserved placenta extract. The level of C-reactive protein decreased (p<0.001) by 61.1% as compared with the untreated rats, and the level of seromucoid statistically significantly (p<0.01) decreased by 17.1% relative to the indices of the rats in the monotherapy group with the studied NSAIDs.

The level of alanine aminotransferases was significantly (p<0.01) lower by 38.9%, and aspartate aminotransferase was lower by 37.9% (p<0.01) relative to the parameters of the animals that had been injected with diclofenac sodium., It was, respectively, 16.7% (p=0.02) and 17.2% (p<0.001) lower than the indices of the rats in the control group with untreated adjuvant arthritis. The established changes on the part of aminotransferases indicate the ability of a cryopreserved placenta extract to level not only an arthritis-induced cytolytic cider, but also a diclofenac-induced one.

A combined use of cryopreserved placenta extract and diclofenac sodium was accompanied by the normalization of the level of total lipids and phospholipids in the blood serum of rats against the background of experimental rheumatoid arthritis. Thus, the content of phospholipids in the phospholipid pool statistically significantly (p=0.02) increased by 22.6% relative to the indicators of the animals with adjuvant arthritis without treatment.

Prospects for further research. For a correct assessment of the activity of the inflammatory process, it is advisable to determine the level of matrix metalloproteinases and homocysteine.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Fedor V. Hladkykh – idea, concept and design of the study, coordination and participation in experimental work, collection of materials, statistical processing and analysis of the data obtained, writing the main text of the article and formulating conclusions; Mykola O. Chyzh – research concept, participation in experimental work, collection of materials, editing the text of the article; Anna O. Manchenko – participation in experimental work, collection of materials; Iryna V. Belochkina – participation in experimental work, collection of materials, search and analytical work; Iryna P. Mikhailova – participation in experimental work, collection of materials. All authors have read and approved the final version prior to publication.

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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RESEARCH ARTICLE

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STUDY OF THE PHARMACOLOGICAL ACTIVITY OF NOVEL EPOR/CD131 HETERORECEPTOR AGONISTS IN MICE WITH ENDOTHELIAL-SPECIFIC EXPRESSION OF MUTANT POLG GENE

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The aim of the research was to study antiatherosclerotic and endothelial kinds of a protective activity of peptides mimicking an erythropoietin a-helix B tertiary structure with laboratory codes EP-11-1 (UEHLERALNSS), EP-11-2. (UEQLERALNCS), EP-11-3 (UEQLERALNTS).

Materials and methods. The study was conducted on 96 C57Bl/6J male double transgenic $Polg^{mut/mut}$/Cdh5-CRE mice. Atherosclerosis was induced by a balloon injury accompanied by Western diet. Then, for 27 days, the drugs under study were administered once per 3 days at the dose of 20 μg/kg. On the 28th day, the animals were euthanized and the area of atherosclerotic plaques was collected for an assessment. The expression of genes associated with the processes of inflammation, apoptosis, and angiogenesis was determined in the tissues of the aorta. In addition, the endothelial protective effect of peptides in isolated segments of the thoracic aorta of wild and transgenic ransgenic $Polg^{mut/mut}$ mice was studied.

Results. The assessment of the plaque size in the animals with the $Polg^{mut/mut}$/Cdh5-CRE genotype against the background of the peptides under study did not reveal statistically significant differences in comparison to control. However, a quantitative PCR showed a statistically significant decreased expression of pro-apoptotic factors p-53 and Bax, and also increase the expression of anti-apoptotic factor Bcl-2 against the background of the peptides EP-11-1 and EP-11-2 administration. The administration of EP-11-1 and the original peptide pHBSP resulted in a statistically significant decrease in the Bax/Bcl-2 ratio. Compounds EP-11-1, EP-11-2, and EP-11-3 were more effective than the original peptide pHBSP, in reducing the increased expression of genes for inflammatory markers iNos, intercellular adhesion molecules Icam-1, Vcam-1 and E-selectin. The use of EP-11-1 led to a more efficient, in comparison with pHBSP, restoration of endothelial-dependent vasodilation of the aortic segments in mice with endothelial-specific overexpression of the mutant Polg gene.

Conclusion. The study carried out on a murine model of the endothelial-specific expression of mutant gamma polymerase has shown that derivatives of the pHBSP peptide with laboratory codes EP-11-1, EP-11-2, EP-11-3, obtained by BLAST-searching for groups of pHBSP related peptides, have atheroprotective and endothelial protective kinds of a protective activity, which is more pronounced in comparison with the original peptide pHBSP.

Keywords: atherosclerosis; erythropoietin derivatives; pHBSP derivatives; atheroprotective effect; endothelial protective effect

Abbreviations: PCR – polymerase chain reaction; pHBSP – pyroglutamate helix B surface peptide; HBSP – helix B surface peptide; iNOS – inducible NO synthase; Polg – polymerase gamma; ICAM-1 – Inter-Cellular Adhesion Molecule 1; VCAM-1 – Vascular cell adhesion molecule-1; EPO – erythropoietin; EpoR – erythropoietin receptor; mRNA – matrix ribonucleic acid; HUVEC – Human Umbilical Vein Endothelial Cells; AKT1 – RAC-alpha serine/threonine-protein kinase ; eNOS / NOS3 – endothelial nitric oxide synthase; NO – nitrogen oxide; Ach – acetylcholine; PI3K – phosphoinositide 3-kinase; NP – sodium nitroprusside

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ОРИГИНАЛЬНАЯ СТАТЬЯ

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ИЗУЧЕНИЕ ФАРМАКОЛОГИЧЕСКОЙ АКТИВНОСТИ НОВЫХ АГОНИСТОВ ГЕТЕРОРЕЦЕПТОРА EPOR/CD131 У ЖИВОТНЫХ С ЭНДОТЕЛИОСПЕЦИФИЧНОЙ ЭКСПРЕССИЕЙ МУТАНТНОГО ГЕНА POLG

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Цель. Изучение антиатеросклеротической и эндотелиопротективной активности пептидных производных, имитирующих а-спираль В эритропоэтина под лабораторными шифрами EP-11-1 (UEHLERALNSS), EP-11-2, (UEQLERALNCS), EP-11-3 (UEQLERALNTS).

Материалы и методы. Исследование проведено на 96 самцах мышей с генотипом $Polg^{mut/mut}$/Cdh5-CRE на фоне C57Bl/6J. Атеросклероз моделировали путем баллонного повреждения сосудистой стенки у животных, находящихся на западной диете. Затем в течение 27 дней вводили изучаемые соединения 1 раз в 3 дня в дозе 20 мкг/кг. На 28-й день животных эвтаназировали и оценивали площадь атеросклеротических бляшек. Также в тканях аорты определяли экспрессию генов, связанных с процессами воспаления, апоптоза и ангиогенеза. Кроме того, было изучено эндотелиопротективное действие пептидов на изолированных кольцах грудной аорты диких и трансгенных мышей $Polg^{mut/mut}$.

Результаты. При оценке размера бляшки у животных с генотипом $Polg^{mut/mut}$/Cdh5-CRE на фоне применения изучаемых пептидов мы не обнаружили статистически значимого изменения размера бляшки. При проведении количественной полимеразной цепной реакции показано, что пептиды EP-11-1 и EP-11-2 статистически значимо в сравнении с контрольной группой животных, снижают экспрессию проапоптических факторов p53 и Bax, а также увеличивают экспрессию антиапоптического фактора Bcl-2. Введение соединения EP-11-1 и исходного пептида pHBSP привело к статистически значимому снижению соотношения Bax/Bcl-2). Соединения EP-11-1, EP-11-2 и EP-11-3 более эффективно, чем исходный пептид, pHBSP, снизили повышенную на фоне баллонной травмы экспрессию генов воспалительных маркеров iNOS, молекул межклеточной адгезии ICAM-1, VCAM-1 и E-селектина. Применение EP-11-1 привело к более эффективному, в сравнении с pHBSP, восстановлению эндотелийзависимой вазодилатации кольца аорты мышей с эндотелий специфической гиперэкспрессией мутантного гена Polg.

Заключение. В исследовании, проведенном на мышиной модели эндотелиоспецифичной экспресии мутантной полимеразы гамма, нами показано, что производные пептида HBSP с лабораторными шифрами EP-11-1, EP-11-2, EP-11-3, полученные путем поиска групп родственных пептидов к молекуле pHBSP с помощью программы BLAST, обладают атеропротективной и эндотелиопротективной активностью, более выраженной в сравнении с исходным пептидом pHBSP.

Ключевые слова: атеросклероз; производные эритропоэтина; производные pHBSP, атеропротективное действие; эндотелиопротективное действие

Список сокращений: ПЦР – полимеразная цепная реакция; pHBSP – pyroglutamate helix B surface peptide / пироглутаматный поверхностный пептид спирали В; HBSP – helix B surface peptide / поверхностный пептид спирали В; iNOS – индубельная NO-синтаза; Polg – polymerase gamma / полимераза гамма; ICAM-1 – Inter-Cellular Adhesion Molecule 1 / межклеточная молекула адгезии – 1; VCAM-1 – Vascular cell adhesion molecule 1 / молекула адгезии клеточного эндотелия 1; ЭПО – эритропоэтин; EpoR – рецептор эритропоэтина; мРНК – ма́тричная рибонуклеи́новая кислота; HUVEC – Isolation of Human Umbilical Vein Endothelial Cells / культура эндотелиальных клеток из пупочной вены человека; AKT1 – RAC-alpha serine/threonine-protein kinase / RAC-альфа-серин/треонин-протеинкиназа; eNOS/NOS3 – эндотелиальная синтаза оксида азота; NO – оксид азота; АХ – ацетилхолин; PI3K – phosphoinositide 3-kinase / Фосфоинозитид-3-киназы; НП – нитропруссид натрия

INTRODUCTION

Erythropoietin (EPO) is one of the hormones produced by the kidneys. It was originally identified as a critical regulator of the hematopoiesis. Recombinant erythropoietin is widely used in the treatment of anemia associated with a chronic kidney disease, heart failure, and cancer [1]. In the human body, EPO stimulates the production of about 200 billion red blood cells daily. After being produced in the kidneys, EPO is secreted into the bloodstream and targets erythroid progenitor cells in the bone marrow [2, 3]. EPO acts by binding to its specific receptor on the surface of erythrocyte progenitor cells. The knockout of Epo (Epo-/-) or the EPO receptor (EpoR-/-) in mice leads to the embryo death caused by the development of severe anemia [4, 5].

Over the past decade, many non-hematopoietic effects of erythropoietin including its antiatherosclerotic action, have been identified [6, 7]. When non-hematopoietic effects are realized, locally produced hypoglycosylated erythropoietin acts in a paracrine-autocrine pathway and transmits signals mediated by the interaction with the tissue protective heterodimeric erythropoietin receptor EPOR/CD131 [8, 9]. The availability of recombinant EPO influenced the appearance of works devoted to the study of the non-hematopoietic activity of EPO, including its protective effect on endotheliocytes and neurons. Reports on the presence of EPO receptors (EpoR), expression of EpoR mRNA and/or EpoR protein besides the erythropoietic organs suggest the possibility of a non-hematopoietic receptor effect of EPO [3]. As reported, human umbilical vein endothelial cells became the first non-hematopoietic cells to express EpoR, bind erythropoietin and show a proliferative response to the EPO administration *in vitro* [8, 10]. It was found out that EPO protects rat brain microvascular endothelial cell cultures from the anoxia-induced damage by activating AKT1, maintaining mitochondrial membrane potential, and preventing oxidative stress-induced apoptosis [12]. An important function of endothelial cells is the expression of endothelial nitric oxide synthase (eNOS/NOS3), catalyzing the synthesis of nitric oxide (NO), the main regulator of vascular homeostasis. Using a cell culture of endotheliocytes, it was found out that a combination of a reduced oxygen content and EPO pretreatment in the cell culture increases the expression of mRNA and EpoR protein, increases the expression of eNOS, and thereby stimulates the production of NO [13]. In experimental models in transgenic mice with high hematocrit, it was shown that arterial hypertension does not develop due to a significant increased level of eNOS and NO in the vascular tissue and in the bloodstream [14].

Currently, it is clear that the thrombosis-related side effects of recombinant EPO prevent its clinical use in non-anemic patients [15]. To prevent thrombotic complications associated with the EPO therapy, EPO derivatives lacking a hematopoietic activity but having a tissue-protective effect, have been obtained. The 11-amino acid peptide imitating the tertiary structure of the erythropoietin B chain (HBSP) is one of EPO derivatives that exhibits a non-hematopoietic activity comparable to recombinant erythropoietin [16–19].

To search for new compounds with atheroprotective and endothelial protective effects, the HBSP amino acid sequence was changed by searching for groups of related peptides of the original compound, using the BLAST program. As a result, 3 compounds that mimic the a-helix B of erythropoietin, were obtained: EP-11-1 (UEHLERALNSS), EP-11-2, (UEQLERALNCS), EP-11-3 (UEQLERALNTS).

THE AIM of this research was to study the antiatherosclerotic and endothelial kinds of a protective activity of peptides EP-11-1, EP-11-2, EP-11-3.

MATERIALS AND METHODS

Animals and diet

The study comprised 96 C57Bl/6J double transgenic Polg$^{mut/mut}$/Cdh5-CRE male mice obtained from the Institute of Gene Biology, Russian Academy of Sciences. The requirements of the Law of the Russian Federation "On the Protection of Animals from Cruelty" dated June 24, 1998, the rules of laboratory practice during preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), European Community directives (86/609 EU), the rules of the International Recommendations of the European Convention for the Protection of Vertebrate Animals used in experimental research (1997) and the Rules of laboratory practice adopted in the Russian Federation (order of the Ministry of Health of the Russian Federation No.708 dated 29.08.2010) were followed. The experiments were approved by the local ethics committee of Belgorod State National Research University, Belgorod, protocol No.19/23. The Polg$^{mut/mut}$/Cdh5-CRE genotype is associated with the endothelial-specific expression of the mutant Polg gene encoding the polymerase gamma enzyme with the D257A mutation, leading to the absence of 3'exonuclease activity and the accumulation of mutations during the mitochondrial genome replication. The expression of the mutant protein leads to the development of a mitochondrial dysfunction with the formation of disturbances in the processes occurring in the vascular endothelium. 2 weeks before the start of the experiment, the animals were placed on a Western 2% cholesterol diet [20].

Modeling of balloon damage to vascular wall

The surgical procedure was performed on a heated platform under a preparative microscope. Under anesthesia (zolazepam 2.5 mg/100 g (Virbac, France) + xylazine 2 mg/100 g (Biogel, Russia) intraperitoneally), a common femoral artery was isolated through an incision in the medial femoral region, a balloon injury of endothelium was applied as described before. To alleviate the postoperative pain syndrome, within 3 days after the operation, the animals received metamizole sodium with drinking water *ad libitum* at the concentration of 50 mg

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metamizole sodium (Pharmstandard-Ufavita, Russia) per 100 ml of water [20–22].

Study design and drugs administration

The list of the studied peptides, their laboratory codes and amino acid sequences are presented in Table 1.

Animals with genotype Polg$^{mut/mut}$/Cdh5-CRE were divided into 6 equal groups:

1) Intact;

2) Control – the animals with modeling of balloon damage on a western diet;

3) pHBSP – the animals with modeling of pathology, administrated with pHBSP peptide (subcutaneously, s/c) from the 1st day at the dose of 20 μg/kg once per 3 days for 28 days (the total dose – 180 μg/kg);

4) EP-11-1 – the animals with modeling of pathology, administrated with EP-11-1 peptide (subcutaneously, s/c) from the 1st day at the dose of 20 μg/kg once per 3 days for 28 days (the total dose – 180 μg/kg);

5) EP-11-2 – the animals with modeling of pathology, administrated with EP-11-2 peptide (subcutaneously, s/c) from the 1st day at the dose of 20 μg/kg once per 3 days for 28 days (the total dose – 180 μg/kg);

6) EP-11-3 – the animals with modeling of pathology, administrated with EP-11-3 peptide (subcutaneously, s/c) from the 1st day at the dose of 20 μg/kg once per 3 days for 28 days (the total dose – 180 μg/kg).

The dose and route of administration of the studied peptides were selected according to previous experimental data obtained in the study of the pharmacological activity of several compounds based on pHBSP, with added tripeptide motifs RGD, KGD, and PGP to the original peptide [20].

Measurement of atherosclerotic plaque area

A macroscopic examination of atherosclerotic aortic plaques was performed using the material from 4 animals from each group. For this purpose, on the 28th day after modeling the balloon injury, the animals were euthanized by an overdose of anesthesia (zoletil 10 mg/100 g intraperitoneally) and the abdominal aorta was thoroughly removed from the bifurcation to the area at the level of the diaphragm.

Then the preparations were cut longitudinally, straightened on a foam pad, washed with a 50% ethanol solution, and immersed in the Oil Red O solution for 15 minutes. After that, the preparations were washed with distilled water and digital photographs were taken. Using the imageJ program, the ratio of the atherosclerotic plaque area (stained in red) to intact tissue was calculated [20].

Quantitative PCR

After euthanasia, the aortic tissue in the area of balloon injury was sampled from the rest of the animals, homogenized, and incubated for 10 minutes at 37°C in the Extract RNA solution. After lysis of the sample in the reagent, it was subjected to chloroform purification; the supernatant sample was collected and washed with isopropyl alcohol and 70% ethanol. The concentration of the obtained RNA, was measured on an IMPLENNano-Photometer® spectrophotometer and adjusted to the concentration of 300 ng/μl. A reverse transcription was performed using the MMLVRTSK021 kit in accordance with the manufacturer's protocol (Evrogen, Russia). The study was carried out in accordance with the previously described methodology [20]. The list of the primers used in quantitative PCR, is presented in Table 2.

Study of effect of aortic ring preparations on vascular endothelium

For the experiment, the following experimental groups were formed (n=8 animals per group):

1) Wildtype mice;

2) Polg$^{mut/mut}$/Cdh5-CRE mice;

3) Polg$^{mut/mut}$/Cdh5-CRE mice treated with pHBSP 20 μg/kg;

4) Polg$^{mut/mut}$/Cdh5-CRE mice treated with EP-11-1 20 μg/kg;

5) Polg$^{mut/mut}$/Cdh5-CRE mice treated with EP-11-2 20 μg/kg;

6) Polg$^{mut/mut}$/Cdh5-CRE mice treated with EP-11-3 20 μg/kg.

The compounds under study – innovative peptides with laboratory codes EP-11-1, EP-11-2, EP-11-3 – were administered intraperitoneally at the indicated doses for 7 days. On the 8th day from the beginning of the experiment, the experimental animals were anesthetized using the intraperitoneal injection of chloral hydrate at the dose of 300 mg/kg. Further on, in anesthetized mice, the thorax was opened to remove the thoracic aorta. The thoracic aorta was placed in a modified ice-cold Krebs Hanseleit solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM Na_2PO_4, 0.5 mM $MgCl_2$, 1.12 mM $CaCl_2$, 25 mM $NaHCO_3$ 0.03 mM EDTA, 11 mM glucose) with pH 7.4. The aorta was carefully removed from the surrounding adipose and connective tissue and cut into short 2 mm transverse segments. The aortic rings were suspended in an organ bath (Biopac Bas System Station, Biopac systems, USA) containing 10 ml of a K-H solution maintained at 37° C, and 95% O_2 and 5% CO_2 were bubbled between two parallel stainless steel hooks. The isometric tension during the experiments was measured and recorded using the Biopac Systems USA software and hardware complex. The data acquisition and processing were performed using the Biopac Icq 4.2 software. Each aortic segment had been gradually stretched to an initial tension of 0.8 g and allowed to equilibrate in a standard 10 ml organ bath for 60 minutes prior to the experiment. After the scales equilibration, the segments were first contracted with 60 mM KCl to induce their contractile response and achieve a reproducible maximum contractile response, then they were washed with Krebs-Hanseleit solution three times to restore the tension to the basal level. The response of the aortic segments contraction to the submaximal concentration of phenylephrine (1 μmol/L) was induced 30 min after the restoration of the basal

level. On the plateau of the epinephrine-induced contraction, the tests for endothelium-dependent and endothelium-independent vasodilation were performed. Acetylcholine (10^{-9}-10^{5} M) was cumulatively added to the aortic bath as an agent causing endothelium-dependent vasodilation, and sodium nitroprusside (10^{-9}–10^{-5} M) was added as an agent causing endothelium-independent vasodilation. Sensitivity was defined as a relaxation percentage of the baseline value obtained at the epinephrine administration plateau.

Statistical processing

Statistical processing was performed using the Statistics 10.0 software. The obtained data were checked for the normality of distribution using the Shapiro-Wilk test and the Spiegelhalter test (the normtest library), the assessment of the equality of variances – using the Leuven test (the lawstat library). Depending on the type of the feature distribution and the equality of variances, the significance of the results obtained was assessed using a parametric (ANOVA) or nonparametric (the Kruskal-Wallis test) one-way analysis of variance. The unpaired Student's t-test was used as a post-hoc analysis to identify differences in intergroup comparisons, or the Mann-Whitney test, respectively, with the Benjamini-Hochberg correction for a multiple hypothesis testing. The results were considered significant at $p \leq 0.05$.

RESULTS

Macroscopic evaluation of plaque size

In accordance with the experiment design, a macroscopic assessment of the balloon injury-induced plaque in wildtype (intact group) and $Polg^{mut/mut}$/Cdh5-CRE animals (the control group) was carried out. It was found out that in the control group of $Polg^{mut/mut}$/Cdh5-CRE animals, lipid deposits serving a marker of atherosclerosis, were visualized in all preparations stained with Oil Red O. That resulted in an increase in the size of the plaque in the control group by more than 11 times. Against the background of the test peptides, when processing the data obtained in the assessment of the plaque size in the animals with the $Polg^{mut}/^{mut}$/Cdh5-CRE genotype, no statistically significant change in the plaque size was found out (Fig. 1).

Quantitative PCR

In addition to the macroscopic plaque assessment, a molecular biological analysis of atherosclerotic plaque tissue after the balloon-induced injury was performed in all experimental groups. Fig. 2 shows that against the background of balloon injury modeling, in the animals with the $Polg^{mut/mut}$/Cdh5-CRE genotype, the expression of the markers of programmed cell death p53 and Bax is to a significant degree increased and the expression of the antiapoptic marker Bcl-2 is decreased. As the heat map presented in Figure 2A shows, peptides EP-11-1 and EP-11-2 statistically significantly compared with the control group of animals ($p<0.05$), reduce the expression of pro-apoptotic factors p-53 and Bax, as well as increase the expression of anti-apoptotic factor Bcl-2 ($p <0.05$). The most effective in terms of changing the expression of apoptosis factors was EP-11-1 – the expression values of the p53, Bax and Bcl-2 genes did not differ from those in the control group (Fig. 2A).

Fig. 2B shows the Bax/Bcl-2 ratio characterizing the integral pro-apoptotic orientation of the cell; the higher it is the more pronounced the activation of programmed cell death cascades. The figure shows that in the animals with the genotype $Polg^{mut/mut}$/Cdh5-CRE, the Bax/Bcl-2 ratio is significantly increased, and the introduction of a compound with the laboratory code EP-11-1 and the initial peptide pHBSP statistically significantly reduce the Bax/Bcl-2 ratio (Fig. 2B).

Table 1 – Amino acid sequence of test compounds

Laboratory code	Amino acid sequence
pHBSP	QEQLERALNSS
EP-11-1	UEHLERALNSS
EP-11-2	UEQLERALNCS
EP-11-3	UEQLERALNTS

Table 2 – Primers used for quantitative PCR

Gene	F-primer	R-primer	Product lenght	GenBank
Trp53 (p53)	CGACTACAGTTAGGGGGCAC	CCATGGCAGTCATCCAGTCT	95	NM_001127233.1
Bcl2	TCACCCCTGGTGGACAACAT	TTCCACAAAGGCATCCCAGC	102	NM_009741.5
Bax	CCCGAGCTGATCAGAACCAT	GAGGCCTTCCCAGCCAC	96	NM_007527.3
Vegfa (VEGF-A)	GGGCCTCCGAAACCATGAA	TGCAGCCTGGGACCACTTG	95	NM_001025250.3
Flt-1 (VEGFR-1)	CCCATCGGCAGACCAATACA	CGGTGCAGTTGAGGACAAGA	96	NM_001363135.1
Hif-1a	AGAACAACTTGAGCTGGCGT	TGGAGGTGAACTAGGCTCTGT	103	NM_001092957.1
Nos2 (iNOS)	GCTCTAGTGAAGCAAAGCCCA	GGGATTCTGGAACATTCTGTGC	103	NM_001313921.1
Icam-1	CTCCGGACTTTCGATCTTCCA	CCTTCCAGGGAGCAAAACAAC	98	NM_010493.3
Vcam-1	TACTGTTTGCAGTCTCTCAAGC	CGTAGTGCTGCAAGTGAGGG	101	NM_011693.3
Sele (E-selectin)	GGGAAGAAGACTGTCCTAGCC	AGGGGAGCTGGCTTCCTAAG	96	XM_006496715.3
Gapdh	GGGTCCCAGCTTAGGTTCATC	CCCAATACGGCCAAATCCGT	100	NM_001289726.1

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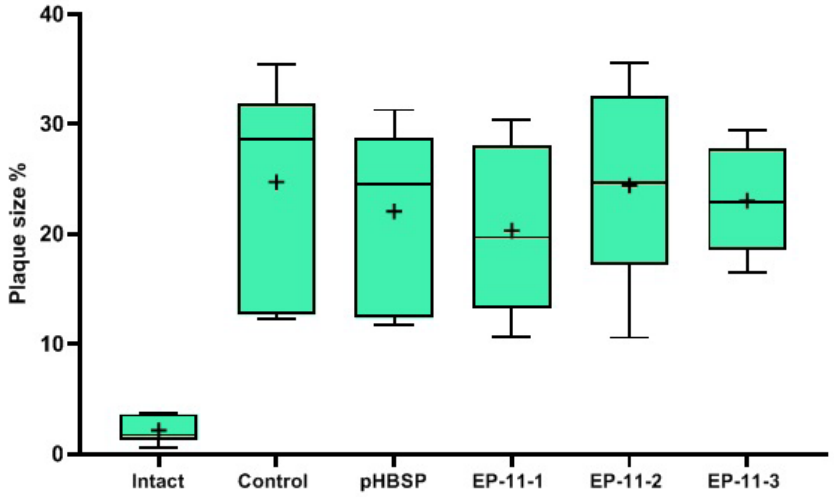

Figure 1 – Atherosclerotic plaque size in groups of wildtype and Polg^{mut}/^{mut}/Cdh5-CRE animals against the background of studied peptides use

Note: the "+" sign on the graph shows the arithmetic mean for each experimental group

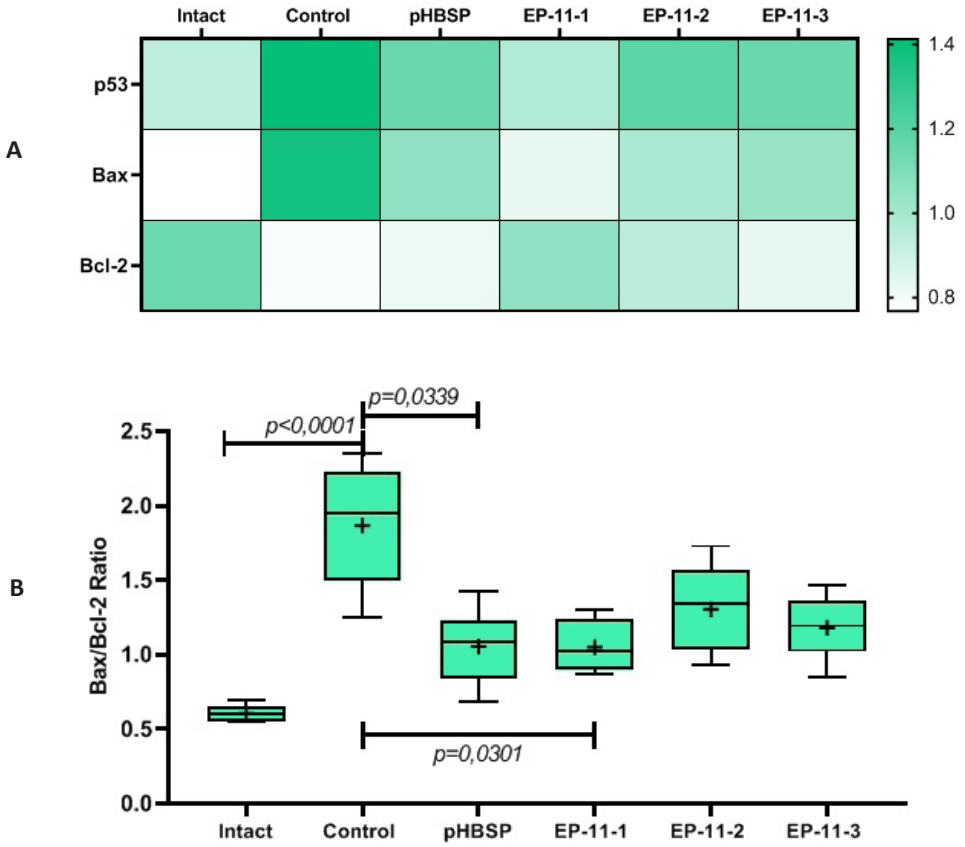

Figure 2 – Influence of test drugs on relative expression of apoptosis markers (2A) and-Bax/Bcl-2 ratio (2B)

Note: the + sign on the graph shows the arithmetic mean for each experimental group

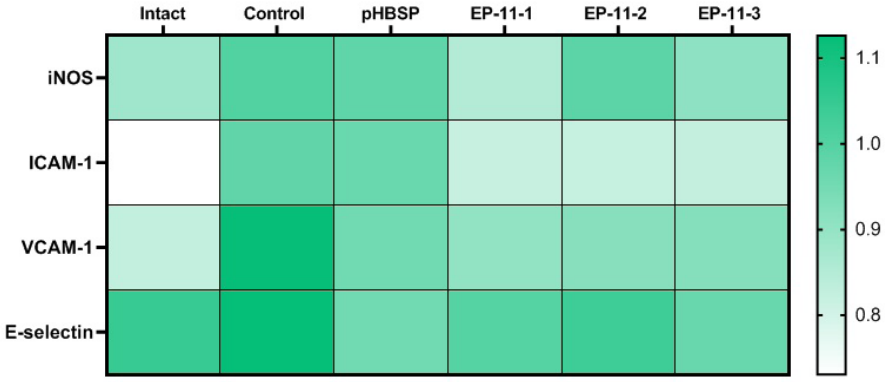

Figure 3 – Influence of test-compounds on inflammatory markers relative expression

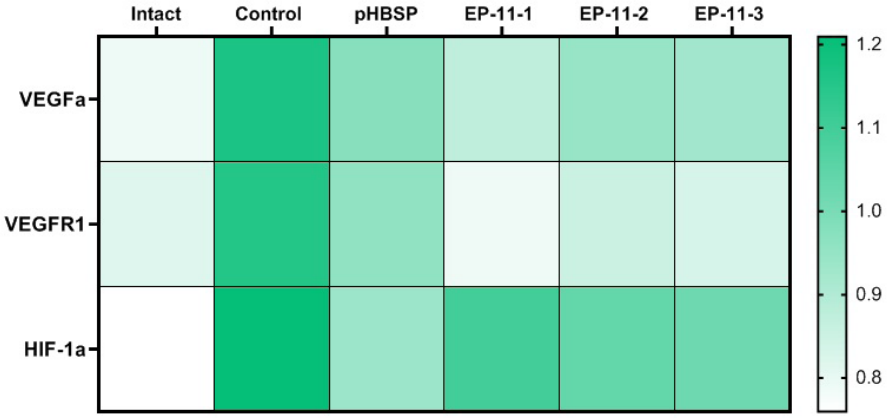

Figure 4 – Influence of test compounds on relative expression of related to angiogenesis factors

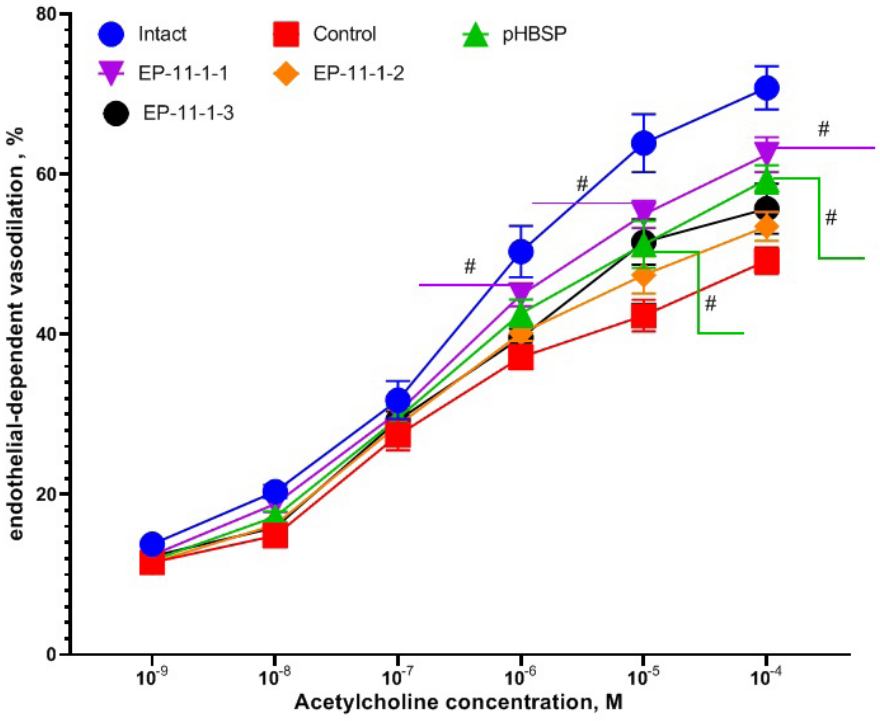

Figure 5 – Results of a test with endothelial-dependent vasodilation in response to ACh on the isolated segments of thoracic aortas of the Polg$^{mut/mut}$/Cdh5-CRE mice

Note: # – at p <0.05 in comparison with the untreated group

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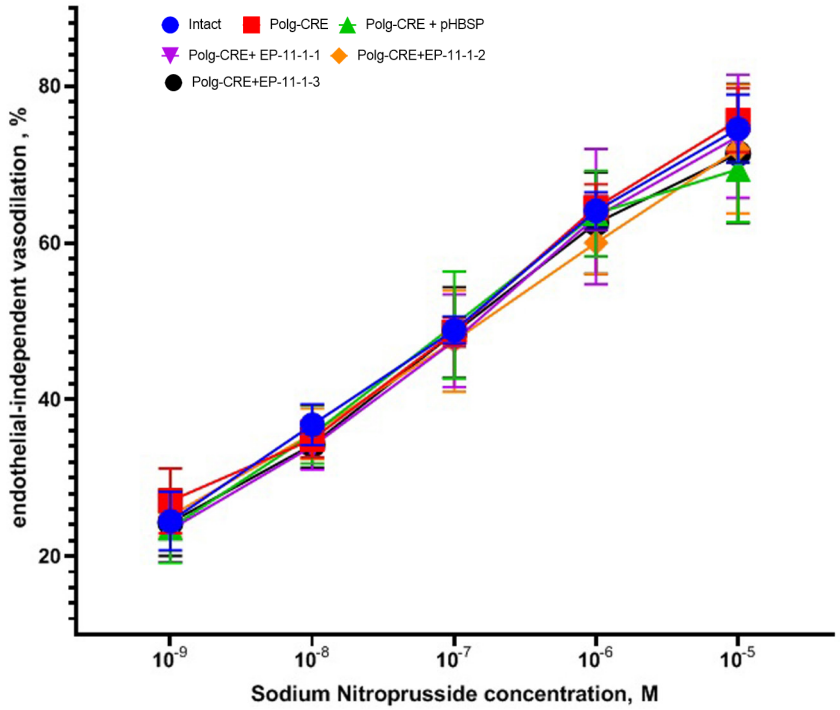

Figure 6 – Results of endothelial-independent vasodilation test in response to SN on isolated segments of thoracic aortas of Polg$^{mut/mut}$/Cdh5-CRE mice

The study of the expression of the inflammatory markers group showed a significantly increased expression of iNOS, Icam-1, Vcam-1, and E-selektin in the control group of the animals with the Polg$^{mut/mut}$/Cdh5-CRE genotype (Fig. 3). The test compounds with laboratory codes EP-11-1, EP-11-2, and EP-11-3, reduced the increased expression of the inflammatory genes iNos, Icam-1, Vcam-1 and E-selectin more effectively than the initial peptide pHBSP. The most pronounced effect was obtained in the group with the use of EP-11-1 (Fig. 3).

In addition, the test compounds with laboratory codes EP-11-1, EP-11-2, EP-11-3 were more effective in reducing the expression of the factors associated with angiogenesis than the original peptide pHBSP (Fig. 4). As in the previous series, EP-11-1 was the most effective in this test (Fig. 4).

Assessment of vascular endothelium functioning on preparations of isolated aortic rings

The effect of the compounds on the vascular endothelium functioning, was carried out on isolated segments of the thoracic aorta of Polg$^{mut/mut}$/Cdh5-CRE mice, kept on western diet. The endothelial function was assessed by performing endothelial-dependent vasodilation in response to ACh. As Fig. 5 shows, acetylcholine-induced endothelial-dependent vasodilation was significantly reduced in Polg$^{mut/mut}$/Cdh5-CRE mice. Notably, the impairment of endothelial-dependent vasodilation caused by ACh in Polg$^{mut/mut}$/Cdh5-CRE mice, was restored after the peptide EP-11-1 administration. The degree of the aortic segments relaxation upon administration of ACh at the concentrations of 10^{-6}M, 10^{-5}M and 10^{-4}M in this group, was significantly higher in comparison to the control group (Fig. 5). The administration of the original peptide pHBSP also led to an increase in vascular relaxation in response to the administration of ACh at the concentrations of 10^{-5}M and 10^{-4}M. The administration of peptides EP-11-2 and EP-11-3 did not significantly affect the response degree of the vascular segment to ACh (Fig. 5).

At the same time, the assessment of endothelium-independent vasodilation in response to SN did not reveal statistically significant differences between the experimental groups (Fig. 6).

DISCUSSION

The biological effects of recombinant EPO are wide and multifaceted, and much attention of researchers was attracted by its non-hematopoietic effects. In particular, the authors were interested in atheroprotective and endothelial protective effects. In vivo studies have shown that EPO reduces the manifestations of an ischemia/reperfusion damage to cardiomyocytes, which is partially explained by an increase in NO production and an acute response to an increase in hematocrit. The same study reported that the EPO-mediated eNOS activation is associated with PI3K signaling, while the EPO-associated reduction in cardiomyocyte ischemia

is not observed in the mice with the eNos -/- genotype [23]. It is assumed that the erythropoietin-induced NO production by endotheliocytes is mediated primarily by the induction and activation of eNOS, especially at a reduced oxygen content [24, 25]. In addition, the mice with ectopic transgenic EPO expression showed an increased eNOS activity and increased plasma NO levels, which prevent cardiovascular diseases such as hypertension and thrombosis, while the inhibition of NO synthase leads to cardiovascular diseases and deaths [3].

In terms of the known limitation of the recombinant EPO use in erythropoietic doses, the problem of finding new derivatives of tissue protective EPO with lack of hematopoietic properties, is relevant in modern medicine and pharmacology. The peptides that are agonists of the EPOR/CD131 heteroreceptor, trigger EPO-associated cytoprotective cascades, but do not have an erythropoietic effect. The previous studies have demonstrated that a peptide imitating the spatial structure of the erythropoietin B chain pHBSP, has a pronounced endothelial protective effect in modeling L-NAME-induced endothelial dysfunction in rats [26, 27]. However, in this study, a prothrombotic effect of pHBSP has also been shown. In view of the above, the need for further modifications of this molecule is obvious. In our opinion, the pHBSP modification to improve its pharmacokinetic and pharmacodynamic parameters may become a further promising development of pharmacotherapy for cardiovascular diseases based on short-chain peptides [28].

The search for such compounds can be solved in several ways, including the attachment of amino acid motifs with anticoagulant properties to the amino acid sequence or by searching for groups of related peptides of the original compound using the BLAST program. At the first stage of the study, an attempt to enrich the original pHBSP molecule by adding tripeptide motifs RGD, KGD, and PGP, having antiaggregant effect, was made. As a result, fundamentally new compounds that combine cytoprotective [29] and antiplatelet effects, were obtained [30]. It has also been shown that the EPO-based peptides are able to improve the functional state of the vascular wall against the background of atherosclerotic lesions and can ameliorate the pathobiological processes associated with a mitochondrial dysfunction. In addition, the studied peptides have a pronounced endothelial protective effect against the background of *in vitro* modeling of oxidative stress [20].

In this research, the pharmacological activity of 3 peptides that mimic the spatial structure of the EPO a-helix (EP-11-1 (UEHLERALNSS), EP-11-2. (UEQLERALNCS), EP-11-3 (UEQLERALNTS)), obtained by searching for groups of related peptides to the pHBSP molecule using the BLAST program, has been studied.

For the study, a line of animals with an endothelial-specific expression of the mutant Polg gene, was selected. Polymerase gamma is an enzyme that plays a key role in mitochondrial DNA replication. The pathology of this enzyme leads to the inclusion of "wrong" nucleotides without a subsequent correction, which causes a mitochondrial dysfunction with a subsequent increase in the production of active radicals and a cell damage. Homozygous animals with the systemic Polg mutation do not survive; therefore, in this work, the endothelial specific expression of an inducible transgene was used [20].

In the presented model, atherosclerosis is associated with a traumatic effect on the vessel against the background of damage to endothelial cells due to the mitochondrial dysfunction. The study of an atheroprotective activity showed that the studied peptides, as well as the original peptide pHBSP, did not significantly ameliorate the histological structure and size of the atherosclerotic plaque in the pathology model. The maximum reduction in the size of the atherosclerotic plaque, was established in the group of the animals treated with EP-11-1, which, however, was not statistically significant. Perhaps, in the further studies the effect of drugs on the histological structure and size of atherosclerotic plaques should be assessed in a model of atherogenesis that is not associated with a physical damage to the endothelium.

Using a molecular biological analysis of plaque samples, it was found out that the studied peptides EP-11-1 and EP-11-2 significantly reduced the expression of the pro-apoptotic factors p-53 and Bax, and also increased the expression of the anti-apoptotic factor Bcl-2. When calculating the ratio of Bax to Bcl-2 expression, it was found out that in the animals with the $Polg^{mut/mut}$/Cdh5-CRE genotype, the Bax/Bcl-2 ratio statistically significantly increased by more than 3 times. In addition, the introduction of a compound with a laboratory code EP-11-1 and the original peptide pHBSP statistically significantly reduced the Bax/Bcl-2 ratio by 57.2 and 56.4%, respectively. These findings are consistent with other studies showing that the administration of EPO for 10 weeks considerably decreases the Bax/Bcl-2 protein ratio in the aortic tissue of apolipoprotein E deficient mice fed a high-fat diet [31]. Along with the anti-apoptotic effect, the studied compounds with laboratory codes EP-11-1, EP-11-2, and EP-11-3 were more effective than the original peptide, pHBSP, in terms of the decrease of the iNos, Icam-1, Vcam-1 and E-selectin expression. At the same time, the maximum efficiency was found in the group of the animals that received the peptide with the laboratory code EP-11-1. The anti-inflammatory effect of EPO and its derivatives is widely known and has been studied [32], and this study confirmed the retention of this type of activity in derivatives that mimic the erythropoietin B chain.

The study of the peptides pharmacological effect ~~of~~ on isolated segments of the pulmonary aorta in $Polg^{mut/mut}$/Cdh5-CRE mice showed that the endothelium-dependent vasodilation induced by Ach, was significantly reduced (70.78% at the acetycholine concentration 10^{-4} M) compared to intact wild-type mice (49.2% at the

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acetycholine concentration 10^{-4} M). The use of peptide EP-11-1 led to the restoration of endothelium-dependent vasodilation induced by ACh at concentrations 10^{-6}M, 10^{-5}M и 10^{-4}M in mice with endothelial specific overexpression of the mutant gene Polg. The administration of the original pHBSP peptide also led to an increase in the vascular relaxation in response to the administration of ACh at concentrations 10^{-5}M и 10^{-4}M. The introduction of peptides with laboratory codes of another leader compound EP-11-2 and EP-11-3, did not statistically significantly affect the degree of response of the vascular segment to ACh. Noteworthy, no changes in the response of endothelium to independent vasodilation in any of the experimental groups were found. This fact confirms that in this study, the tissue protective effect of the peptides is in the normalization of the function of the vascular endothelium which determines the pronounced endothelial protective activity of these compounds.

CONCLUSION

At the first stage of the search for new EPO derivatives with tissue protective properties without manifesting a hematopoietic activity, the original pHBSP peptide was enriched by adding tripeptide motifs RGD, KGD, and PGP. The resulting compounds combined cytoprotective and antiplatelet effects, had an endothelial protective activity, and were able to attenuate atherosclerotic lesions. In the present study, the second pool of compounds – derivatives of the pHBSP peptide with laboratory codes EP-11-1 (UEHLERALNSS), EP-11-2. (UEQLERALNCS), EP-11-3 (UEQLERALNTS), obtained by BLAST-searching for groups of related to pHBSP peptides, was tested. In the study carried out on a mouse model of the endotheliospecific expression of the mutant Polg gene, it has been shown that the most active compound with laboratory code EP-11-1 has a more pronounced atheroprotective and endothelial protective activity than the original peptide pHBSP.

The results of this study, in combination with the previously obtained data characterizing the pharmacological activity of pHBSP derivatives containing RGD, KGD, and PGP, prove the effectiveness of this approach and reveal the prospects for further search for new EPO-derived nonhematopoietic peptides with tissue protective properties

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Mikhail V. Korokin – main idea, study planning, study conducting, statistical proceeding, article writing; Marina V. Kubekina – preparation of experimental animals, extraction of RNA, reverse transcription, analysis of the expression of targeted genes; Alexey V. Deykin – RNA extraction, reverse transcription, analysis of the expression of targeted genes; Oleg V. Antsiferov – observation, care and handling of animals, drugs administration, research of pharmacological activity; Vladimir M. Pokrovskii – observation, care and handling of animals, drugs administration, research of pharmacological activity; Liliya V. Korokina – statistical processing, development of research design; Pobeda A.S. – statistical processing, article writing, references formalization; Valeria A. Soldatova – observation, care and handling of animals, drugs administration, research of pharmacological activity; Natalia L. Kartashkina – reverse transcription, analysis of the expression of targeted genes; Elena V. Kuzubova – observation, care and handling of animals, drugs administration, research of pharmacological activity; Alexandra I. Radchenko – observation, care and handling of animals, drugs administration, research of pharmacological activity; Mikhail V. Pokrovskii – research planning, management of experimental work, quality assurance.

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RESEARCH ARTICLE

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INFLUENCE OF CERTAIN D-METALS ON FORMATION OF ADVANCED GLYCATION END PRODUCTS, AGGREGATION AND AMYLOID TRANSFORMATION OF ALBUMIN IN GLYCATION REACTION

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The aim of the research is to investigate the influence of the factor of the glycation behavior of bovine serum albumin (BSA) by glucose, and the factor of d-metal cations (nickel (II), cobalt (II), iron (II), iron (III), copper (II) or zinc (II)) presence, on the process of aggregation and the amyloid transformation of BSA and, therefore, to establish the effect of these cations on the rate of the formation of advanced glycation end products (AGEs), and the intensity of fluorescence of the amino acids tyrosine and tryptophan.

Materials and methods. Reagents in the glycation are: glucose (at the final concentration of 0.36 M), BSA (at the final concentration of 1 mg/ml), deionized water, one of the d-metal cations, i. e. nickel (II), cobalt (II), iron (II), iron (III), copper (II) or zinc (II) (in the form of chloride, sulfate or nitrate salts, at the final concentration of 40 μM). The conditions for the glycation reaction are the incubation for 24 hours at the temperature of 60°C. The influence of two factors (the factor of the glycation reaction and the factor of a d-metal ion presence in the reaction medium) on the concentration of glycation end products (AGEs) formed during the glycation reaction, on the fluorescence intensity of the amino acids tryptophan and tyrosine, on the aggregation of BSA, and on the ability of BSA to the amyloid transformation under the described conditions, have been studied.

Results. It was found out that the studied factors have a statistically significant effect on the considered parameters. The highest activity was found for the copper ion (II), which intensifies the formation of the AGEs in the samples where glycation occurs, reduces the fluorescence intensity of the amino acids' tryptophan and tyrosine (independently and increasing the effect against the background of glycation). Besides, it independently causes the aggregation of BSA hereby intensifying the effect against the background of glycation, it independently causes the amyloid transformation of BSA enhancing the effect against the background of glycation. The above-listed effects were the least pronounced in the reaction media with the addition of nickel (II) or cobalt (II). These cations reduce the rate of the AGEs formation, do not cause the formation of protein aggregates. In the presence of glucose, nickel (II) weakly suppresses the fluorescence intensity of tryptophan and tyrosine, and slightly enhances the amyloid transformation of BSA. Cobalt (II) slightly inhibits the amyloid transformation of BSA. In terms of the severity and nature of the effects, the iron (II), iron (III) and zinc (II) cations occupy an intermediate position between copper (II), on the one hand, and nickel (II) and cobalt (II), on the other hand, combining the influence on the AGEs formation, the intensity of fluorescence of tryptophan and tyrosine, the aggregation and amyloid transformation of BSA. In the absence of glucose, the ability of zinc (II) to induce the formation of protein aggregates turned out to be the highest, and its ability to stimulate the amyloid transformation of BSA corresponded to that of copper (II).

Conclusion. The presence of d-metal cations affects the rate of the AGEs formation in the glycation reaction, affects the rate of the BSA amyloid transformation and the protein aggregates formation. Among such ions as nickel (II), cobalt (II), iron (II), iron (III), copper (II) and zinc (II), copper (II) ions turned out to be the most active in their ability to accelerate the AGEs formation, suppress the fluorescence of tryptophan and tyrosine, enhance the aggregation and amyloid transformation of BSA in the glycation reaction. The least manifestation of these properties is observed for nickel (II) and cobalt (II) ions.

Keywords: advanced glycation end products; glycation; protein aggregation; amyloid transformation, d-metal

Abbreviations: AGEs – advanced glycation end products; BSA – bovine serum albumin; RAGEs – Receptor for advanced glycation end products; ThT – thioflavine T

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ВЛИЯНИЕ НЕКОТОРЫХ D-МЕТАЛЛОВ НА ОБРАЗОВАНИЕ КОНЕЧНЫХ ПРОДУКТОВ ГЛИКИРОВАНИЯ, АГРЕГАЦИЮ И АМИЛОИДНУЮ ТРАНСФОРМАЦИЮ АЛЬБУМИНА В РЕАКЦИИ ГЛИКИРОВАНИЯ

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Цель. Исследование влияния фактора протекания реакции гликирования бычьего сывороточного альбумина (БСА) глюкозой и фактора присутствия в среде реакции гликирования катионов d-металлов (никель (II), кобальт (II), железо (II), железо (III), медь (II) или цинк (II)) на процесс агрегации и амилоидной трансформации БСА. Установление влияния указанных катионов на интенсивность образования конечных продуктов реакции гликирования (КПГ) и интенсивность флуоресценции аминокислот тирозин и триптофан.

Материалы и методы. Реагенты в реакции гликирования: глюкоза (в конечной концентрации 0,36 М), БСА (в конечной концентрации 1 мг/мл), деионизированная вода, один из катионов d-металлов, а именно никель (II), кобальт (II), железо (II), железо (III), медь (II) или цинк (II) (в виде соли хлорида, сульфата или нитрата, в конечной концентрации 40 мкМ). Условия протекания реакции гликирования: инкубация 24 ч при температуре 60°С. Исследовано влияние двух факторов (фактор протекания гликирования и фактор присутствия иона d-металла в реакционной среде) на концентрацию КПГ, образуемых в ходе реакции гликирования, на интенсивность флуоресценции аминокислот триптофан и тирозин, на агрегацию БСА и на способность БСА к амилоидной трансформации в описанных условиях.

Результаты. Установлено, что исследуемые факторы статистически значимо влияют на рассматриваемые параметры. Наивысшая активность установлена для иона меди (II), который интенсифицирует образование КПГ в пробах, где протекает гликирование, снижает интенсивность флуоресценции аминокислот триптофан и тирозин (самостоятельно и усиливая эффект на фоне гликирования), вызывает агрегацию БСА (самостоятельно и усиливая эффект на фоне гликирования), вызывает амилоидную трансформацию БСА (самостоятельно и усиливая эффект на фоне гликирования). Наименее выражены перечисленные эффекты были в реакционных средах с добавлением никеля (II) или кобальта (II). Данные катионы снижают интенсивность образования КПГ, не вызывают образования белковых агрегатов. В присутствии глюкозы никель (II) слабо подавляет интенсивность флуоресценции триптофана и тирозина, незначительно усиливает амилоидную трансформацию БСА. Кобальт (II) незначительно подавляет амилоидную трансформацию БСА. Катионы железа (II), железа (III) и цинка (II) по выраженности и характеру эффектов занимают промежуточное положение между медью (II) с одной стороны, и никелем (II) и кобальтом (II) с другой стороны, в разной степени сочетая влияние на образование КПГ, интенсивность флуоресценции триптофана и тирозина, агрегацию и амилоидную трансформацию БСА. В отсутствии глюкозы способность цинка (II) вызывать образование белковых агрегатов оказалась наивысшей, а его способность стимулировать амилоидную трансформацию БСА соответствовала таковой у меди (II).

Заключение. Присутствие катионов d-металлов влияет на интенсивность образования КПГ в реакции гликирования, влияет на интенсивность амилоидной трансформации БСА и на образование агрегатов белка. В ряду таких ионов, как никель (II), кобальт (II), железо (II), железо (III), медь (II) и цинк (II), ионы меди (II) оказались наиболее активными по способности ускорять образование КПГ, подавлять флуоресценцию триптофана и тирозина, усиливать агрегацию и амилоидную трансформацию БСА в реакции гликирования. Наименьшая выраженность указанных свойств отмечается для ионов никеля (II) и кобальта (II).

Ключевые слова: конечные продукты гликирования; гликирование; агрегация белка; амилоидная трансформация; d-металл

Список сокращений: КПГ – конечные продукты гликирования; БСА – бычий сывороточный альбумин; РКПГ – рецептор к конечным продуктам гликирования; ThT – тиофлавин Т

INTRODUCTION

Non-enzymatic glycation is the source of toxic advanced glycation end products (AGEs). AGEs are a group of more than 20 molecules [1, 2], differing in their properties, including the ability to autofluorescence and the crosslinks formation. AGEs are important pathogenetic factors in the development of cognitive impairments (diabetic encephalopathy, conformational brain diseases) [3–6]. The structures of some AGEs are shown in Fig. 1.

AGEs are involved in the pathogenesis of cognitive impairment through various mechanisms. In the culture of hippocampal neurons, AGEs induced apoptosis, increased the production of pro-apoptotic Bax protein and acetylcholinesterase, decreased the level of antiapoptotic protein Bcl-2, glutathione peroxidase, superoxide dismutase and

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choline acetyltransferase, increased the concentration of malondialdehyde, etc. [7] AGEs are able of activating the AGEs receptor (RAGEs). It is assumed that the RAGEs links the pathogenesis of diabetes mellitus and Alzheimer's disease [8]. The RAGEs can be activated by Aβ protein [9] and is involved in its intraneuronal transport from the blood [10]. The RAGEs activation can lead to the development of the neuronal oxidative stress [11]. Under these conditions, if amyloid peptides are glycated, their RAGEs-mediated action can be enhanced [12]. For these reasons, it is advisable to consider the amyloid transformation and protein glycation as pathogenetically related processes.

In addition to the receptor-mediated relationship, AGEs are able to directly influence the amyloid transformation of proteins. By modifying the lateral chains of amino acids and the N-terminal residue of some proteins, AGEs can cause a change in the surface charge of the protein, a change in its hydrophobic properties and, as a consequence, lead to the amyloid transformation [13]. It is assumed that glycation contributes to the stabilization of protofibrillar structures, and the ability of AGEs to cross-link proteins to make the formation of larger conglomerates from amyloid aggregates possible [13, 14]. Under certain conditions, glycation slows down the formation of mature amyloid fibrils; however, this is associated with an extension of the life span of cytotoxic oligomeric forms [15, 16]. Amyloid oligomeric forms are able of destroying cell membranes, leading to a calcium imbalance, causing a mitochondrial dysfunction, and directly interacting with membrane proteins, leading to a change in their native state [14]. Thus, against the background of glycation, both acceleration and deceleration of the amyloid transformation have negative consequences, which makes the task of studying the effect of glycation on this process urgent.

The glycation reaction depends on many factors, in particular, on the presence of d-metal ions in trace concentrations (including transition metals and those close to them in properties), the presence of reactive oxygen intermediates, etc. [17, 18]. Transition metals include chemical elements the atoms of which have a partially filled d-sublevel or are able of forming cations with an incompletely filled d-sublevel (IUPAC[1]). The zinc subgroup and zinc itself belong to the metals close in properties to the transitional ones (some of them are called post-transition at times). Many properties of zinc are identical to those of transition metals, but its d-orbitals are filled[2]. To a great extent, a biological role of transition metals is due to the presence of an incomplete *d*-sublevel and how this sublevel is filled with electrons, since this, in turn, determines the tendency of a particular transition metal to form certain chemical bonds and their stability. In addition, the electronic configuration of the d-sublevel determines the presence of specific stable oxidation states and, as a consequence, the redox properties of the metal itself and its ions [19]. It was found out that the ability to change the oxidation state is an important property due to which some transition metals are able to stimulate glycation [20, 21]. Thus, in glycation reactions, copper (II) is reduced to copper (I), and oxygen is converted into superoxide anion with the participation of hydrogen peroxide, the source of which is some stages of glycation. After that, copper (I) ions are oxidized to copper (II), catalyzing the decomposition of hydrogen peroxide to a hydroxyl radical [20]. It should be noted that d-metals (zinc, iron, copper, etc.) are involved in the pathogenesis of cerebral conformational diseases by the mechanisms independent of glycation, associated with both the direct action of the ion on the protein (cross-linking of tyrosine residues, etc.) and indirectly, through the influence on the activity of enzymes (secretase, etc.) [22–25, 10]. Thus, the glycation reaction, the amyloid transformation of proteins, and the activity of d-metal ions, which can influence both of these processes, are pathogenetically related.

The amyloid protein transformation can be modeled using bovine serum albumin (BSA) [26, 27]. BSA is prone to the enhanced formation of β-sheets and the amyloid transformation under the physicochemical action (eg, heating), which causes its frequent use as a model protein in the study of amyloid transformation processes [28]. At the temperature that is borderline for the initiation of BSA amyloid aggregation, it becomes possible to assess the ability of the studied factor to accelerate or slow down the course of aggregation and amyloid transformation.

A comparative study of the d-metal ions ability to influence the rate of the AGEs formation in the glycation reaction, as well as their ability to stimulate or suppress the formation of amyloid and non-amyloid protein aggregates against the background of the glycation reaction and independently of it, are of interest.

THE AIM of the research is to evaluate the combined and independent influence of the factor of the BSA glycation reaction and the factor of the presence of d-metal cations (nickel (II), cobalt (II), iron (II), iron (III), copper (II) or zinc (II)) in the reaction medium, on the process of the BSA transformation into aggregates of the amyloid and non-amyloid nature. Besides, the influence of these factors on the fluorescence intensity of the amino acids' tryptophan and tyrosine and the ability of the indicated cations of d-metals to influence the intensity of the AGEs formation in the glycation reaction, are to be assessed.

MATERIALS AND METHODS

Modeling of glycation reaction

Glycation substrate is BSA (fraction V, 1 mg/ml, Himmed, Russia); glycating agent – glucose (0.36 M, Vekton, Russia); the reaction medium was deionized water (pH 6.2, deionizer Milli-Q, Germany) with the addition of one of the transition metal cations, i.e. nickel (II), cobalt (II), iron (II), iron (III), copper (II) or zinc (II) at the final concentration of 40 μM in the form of salts $NiSO_4 \cdot 7H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $FeSO_4$, $FeCl_3 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$ or Zn-

[1] IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A.D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). Online version (2019-) created by S. J. Chalk. ISBN 0-9678550-9-8. DOI: 10.1351/goldbook.

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SO_4•7H_2O, respectively (or without them). The studied concentration of metal salts was selected on the basis of the previously adjusted model of BSA glyoxidation by glucose in the presence of $CuSO_4$•5H_2O, with modifications [29, 30]. The interest in a comparative study of the activities of d-metals, determines the need to research them in equal concentrations. In the body, the concentrations of the studied metals are variable. Thus, the physiological concentrations of copper and zinc in the serum of healthy people are ~1 mg/L (~15.7 μM and ~15.3 μM, respectively) [31, 32]. At the same time, in Alzheimer's disease, a significantly higher concentration of copper in amyloid plaques (~400 μM) has been reported [10, 25]. During the neuronal activity, zinc is released into the synaptic cleft and can reach the concentration of 300 μM [25]. For these reasons, the selected concentration (40 μM) is a compromise with respect to a wide range of d-metal concentrations in the body under normal and pathological conditions, and is more related to the previously established activity of the copper (II) cation.

The reaction conditions are: the temperature is 60°C, the incubation duration is 24 hours. These conditions were selected on the basis of the literature data [33], indicating that the effect of the selected temperature on BSA (the author used a Tris buffer containing NaCl) is favorable for the transformation of protein α-helices into β-sheets (which may not occur at lower temperatures), while the transformation process is intensified at higher temperatures. In another study it was shown that the BSA aggregation does not occur when exposed to the given temperature (for BSA in a Na-phosphate buffer solution) [34].

Determination of glycation end products and fluorescence intensity of amino acids tryptophan and tyrosine

After the incubation, the aliquots of the samples (200 μL) were added to a 96-well flat-bottom black plate, the fluorescence intensity samples was determined at the excitation/emission wavelengths specific for the following AGEs: pentosidine (335/385 nm), vesperlysine C (345/405 nm), vesperlysines A and B (366/442 nm), crossline (379/463 nm) [35]. The fluorescence intensity at the excitation wavelengths exceeding 400 nm (440/520 nm), was also measured [36]. There is evidence that the product (or products) fluorescent at the excitation/emission wavelengths of 440/520 nm, belongs to the AGEs and is/are able of forming protein cross-links [37]. In addition to AGEs, the fluorescence intensity of the amino acids tryptophan and tyrosine was determined at the excitation/emission wavelengths of 295/335 nm. These wavelenghts are more specific for tryptophan, and 270/330 nm are characteristic of both amino acids (Infinite M200 Pro spectrofluorimeter, TECAN, Austria) [38–40]. The evaluation of the glycation effect and the d-metal ions action on the fluorescence of these amino acids is informative in view of the sensitivity of their fluorescence intensity to changes in the protein conformation and glycation of their amino acid environment [41, 42]. In addition, copper (II) cations promote the formation of dityrosine crosslinks, which is important for the pathogenesis of Alzheimer's disease [43], and, as a result, the fluorescent characteristics of tyrosine change. The possibility of a change in fluorescence upon oxidation of tryptophan and/or tyrosine, as well as an activation of tyrosine and its direct interaction with the reaction products upon glycation of its environment, cannot be ruled out [44, 42]. This makes the determination of the fluorescence of these amino acids relevant for the present study, and makes it possible to indirectly note changes in the course of both glycation and protein aggregation.

Investigation of aggregation and amyloid transformation of proteins

In the study using spectrophotometry, the intensity of the protein aggregates formation was assessed, and the spectrofluorimetric determination of their amyloid affiliation in the reaction with thioflavin T (ThT) was carried out. A spectrophotometric detection of BSA aggregates was carried out by the increase in the optical density at the wavelength of 405 nm [45] in aliquots of 200 μL in a 96-well flat-bottom transparent plate (Infinite M200 Pro spectrofluorimeter, TECAN, Austria). The study [45] showed that the optical density at the given wavelength is directly proportional to the aggregation degree. The confirmation of the amyloid belonging of the aggregates, was carried out in the reaction with ThT (Sigma Aldrich, USA), at the final ThT concentration of 20 μM [46], by determining the fluorescence intensity of the samples at the excitation/emission wavelength's of 450/482 nm (Infinite M200 Pro spectrofluorimeter, TECAN, Austria).

Statistical data analysis

In order to determine the contribution of each factor (both the factor of the glycation reaction and the factor of a metal ion presence), statistical data processing was carried out using a two-way analysis of variance, followed by a multiple comparison of data groups "all with all" according to Tukey test, at the significance level of $p \leq 0,05$ (GraphPad Prism 9). The correlation analysis was carried out using the Spearman rank correlation method (GraphPad Prism 9). The results are presented as mean with a standard error (M ± SEM).

RESULTS

Analysis of fluorescence intensity of tryptophan and tyrosine

As a result of the influence analysis of the investigated factors on the intensity of tryptophan and tyrosine fluorescence, it was found out that metal cations make a more significant contribution to the change in this parameter. For the cations of copper (II), iron (II), iron (III) and zinc (II), the ability to exert an effect was noted regardless of the glucose presence (a decrease in the fluorescence intensity of the amino acids tryptophan and tyrosine was noted not only in glycated, but also in glucose-free samples) (Table 1). At the same time,

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no differences were revealed when comparing the fluorescence intensity of tryptophan and tyrosine in the glycated samples that do not contain metals with the fluorescence intensity of the corresponding non-glycated samples (in which only BSA is present). Despite the absence of an intrinsic effect of the glycation reaction on the fluorescence intensity of amino acids, for some metals it was found out that they enhance their ability to suppress the fluorescence of amino acids in the presence of glucose, which indicates the importance of the glycation factor. Thus, a statistically significant decrease in the fluorescence intensity of tryptophan and tyrosine in glucose-containing samples (in comparison with the corresponding samples without glucose) was observed for the cases of glycation in the presence of copper (II) cations (at the excitation/emission wavelengths of 270/330 nm and 295/335 nm), iron (II) (at the excitation/emission wavelengths of 270/330 nm) and zinc (II) (at the excitation/emission wavelengths of 270/330 nm). Thus, these cations were able to enhance their action in the presence of glucose. With respect to cobalt (II), the effect of reducing the fluorescence of tyrosine and tryptophan in the presence of glucose was not observed when compared with the glucose-free samples. In the presence of nickel (II), this effect was weak (there were differences in the fluorescence intensity of the glycated samples containing nickel (II) and the glycated samples without metals, but there were no statistically significant differences in signals from the glycated and glucose-free samples containing nickel (II)).

Influence of d-metals on the rate of AGEs formation in glycation reactions

The intensity of fluorescence at the wavelength's characteristic of various AGEs, reflects the intensity of the glycation reaction. As expected, in all cases, glucose-containing samples showed an increase in the intensity of AGEs fluorescence in comparison with the corresponding glucose-free samples (Table 1). However, the attention is drawn to the differences in the nature and degree of d-metal cations influence on the glycation reaction. When comparing the AGEs fluorescence intensities of the metal-containing samples in which glycation occurred, with the indices of the corresponding samples without metals, the following was found out. Only copper (II) cations (at all the excitation/emission wavelengths except 335/385 nm) and zinc (II) (the excitation/emission wavelengths of 440/520 nm) had the ability to enhance the formation of AGEs. The rest of the metals, on the contrary, or with statistical significance, prevented an increase in fluorescence at the wavelength's characteristic of AGEs, or did not change the values of the indicator. At the wavelengths characteristic of pentosidine (335/385 nm), no metal was able to intensify the formation of AGEs in comparison with the samples without metals. At the same time, nickel (II), cobalt (II), iron (II), and iron (III) were able to reduce the intensity of the signal detected at these wavelengths. The established activity makes it possible to isolate copper (II) and zinc (II) into the category of d-metals capable of accelerating the glycation under the described conditions. The cumulative results are shown in Table 1.

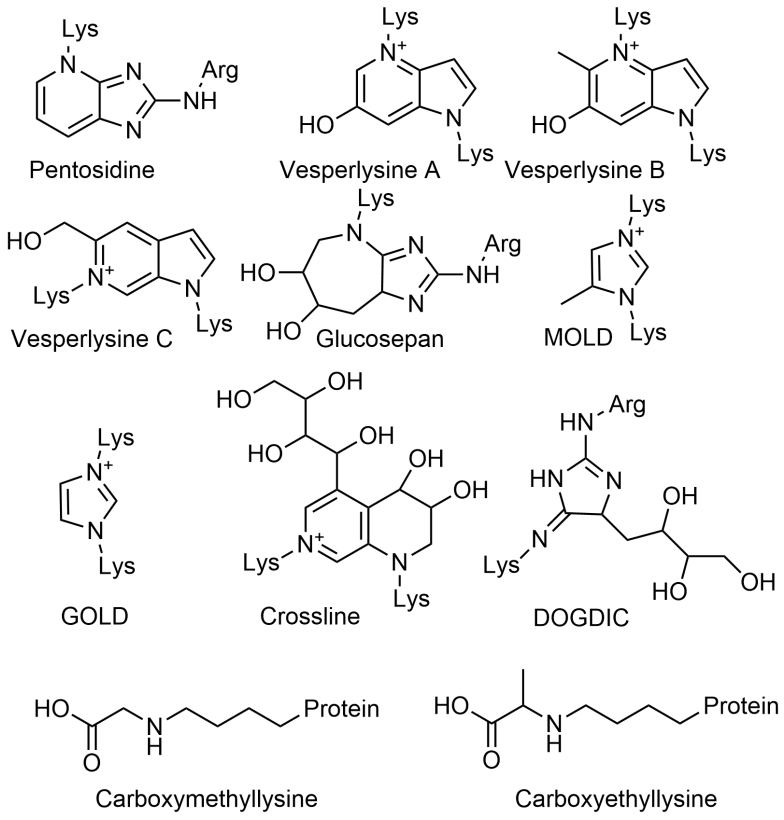

Figure 1 – Structures of various advanced glycation end products

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Table 1 – Influence of investigated factors on the intensity of fluorescence emission characteristic of various AGEs, as well as amino acids tryptophan and tyrosine; absolute values of fluorescence (M ± SEM)

Glucose factor	Fluorescence parameters (λexc/λtest), nm and corresponding product		Metal cation factor						
			None	Nickel (II)	Cobalt (II)	Iron (II)	Copper (II)	Iron (III)	Zinc (II)
Glucose	270/330	Tryptophan and tyrosine	83147.2±1561.7	75475.8±1415.6 **	78131.2±1441.9	68838.8±1290.9 ****##	30211.8±1680.2 ****####	63999.6±1399.6 ****	70210.8±1417.6 *****#
Glucose-free			83063.0±1000.5	80763.0±664.4	78733.8±926.2	76121.0±279.1 *	56691.4±1055.4 ****	62589.2±1609.8 ****	76616.8±837.1 *
Glucose	295/335	Tryptophan (predominantly)	41994.3±1290.6	37012.8±1205.5 *	39814.8±1459.9	34861.6±1056.5 ***	14637.2±587.9 ****####	31014.8±845.1 ****	33909.6±707.4 ****
Glucose-free			43126.5±303.6	40918.8±757.4	38680.0±881.2 *	36587.8±813.1 **	27971.5±785.8 ****	30535.0±1180.7 ****	37617.0±456.1 **
Glucose	335/385	Pentosidine	752.8±13.4 ####	538.6±15.8 ****####	580.6±17.2 ****####	490.2±15.0 ****####	757.0±29.5 ####	395.0±11.9 ****####	748.8±17.7 ####
Glucose-free			284.5±6.9	278.0±4.0	268.5±3.1	244.3±4.2	250.5±6.5	202.8±6.3 **	302.5±12.7
Glucose	345/405	Argpyrimidine	812.4±14.6 ####	578.8±15.3 ****####	609.4±14.5 ****####	594.0±10.2 ****####	1058.2±19.1 ****####	452.4±7.3 ****####	861.8±8.6 ####
Glucose-free			313.0±14.1	291.3±6.8	313.0±12.1	273.3±4.5	302.3±11.1	229.0±11.1 **	391.3±30.2 **
Glucose	366/442	Vesperlysines A and B	921.0±22.8 ####	744.0±17.0 **####	728.2±13.6 **#	874.0±61.5 ####	2009.8±37.0 ****####	666.0±13.2 ****####	977.0±5.6 ####
Glucose-free			491.0±11.0	477.2±9.2	561.2±33.9	504.8±49.4	594.8±21.5	390.0±27.6	661.4±40.8 *
Glucose	379/463	Crossline	826.6±21.7 ####	675.4±18.7 **####	644.2±10.9 ****##	786.6±47.2 ####	1917.8±34.9 ****####	618.8±12.1 ****####	828.6±7.6 ####
Glucose-free			477.8±11.2	471.6±9.8	500.0±23.9	439.3±5.9	594.4±22.9 *	365.2±20.7 *	603.6±32.9 *
Glucose	440/520	Cross-links	451.6±8.1 ####	355.4±9.2 ****####	348.8±9.3 ****####	416.2±9.9 ####	727.4±9.3 ****####	372.4±8.2 ****####	496.0±8.0 *####
Glucose-free			323.4±4.9	282.6±6.3	300.0±4.7	291.2±6.3	310.0±4.9	233.8±6.7 ****	375.0±15.0 **

Note: significance level p when compared with the corresponding metal-free samples (**** corresponds to p<0.0001; *** corresponds to p<0.001; ** corresponds to p<0.01; * corresponds to p<0.05). Significance level p when compared with corresponding glucose-free samples (#### corresponds to p<0.0001; ### corresponds to p<0.001; ## corresponds to p<0.01; # corresponds to p<0.05 (two-way ANOVA, Tukey's post-test).

Table 2 – Influence of investigated factors on formation of BSA aggregates, optical density of samples at wavelength of 405 nm; absolute values (M ± SEM)

Glucose factor	Metal cation factor						
	None	Nickel (II)	Cobalt (II)	Iron (II)	Copper (II)	Iron (III)	Zinc (II)
Glucose	0.06±0.0004	0.06±0.0006	0.06±0.0020	0.19±0.0021 ****####	1.08±0.0122 ****####	0.37±0.0188 ****	0.65±0.0236 ****####
Glucose-free	0.05±0.0004	0.06±0.0007	0.05±0.0007	0.06±0.0007	0.18±0.0036 ****	0.4±0.0090 ****	1.02±0.0180 ****

Note: significance level p when compared with corresponding metal-free samples (**** corresponds to p<0.0001; *** corresponds to p<0.001; ** corresponds to p<0.01; * corresponds to p<0.05). Significance level p when compared with corresponding glucose-free samples: #### corresponds to p<0.0001; ### matches p<0.001; ## matches p<0.01; # corresponds to p<0.05 (two-way ANOVA, Tukey's post-test

Table 3 – Influence of the studied factors on amyloid BSA transformation, intensity of fluorescent ThT emission at excitation/emission wavelengths of 450/482 nm; absolute values (M ± SEM)

Glucose factor	Metal cation factor						
	None	Nickel (II)	Cobalt (II)	Iron (II)	Copper (II)	Iron (III)	Zinc (II)
Glucose	6051.0±320.2 ####	7954.2±391.5 *####	5740.8±283.3 ####	6083.4±241.7 ####	18277.3±792.1 ****#	2704.8±190.4 ****####	13415.2±238.7 ****####
Glucose-free	11693.8±280.2	13841.6±109.3 **	9695.4±132.0 **	10887.6±157.8	16304.8±335.2 ****	7266.0±257.3 ****	17900.0±672.5 ****

Note: significance level p when compared with the corresponding metal-free samples (**** corresponds to p<0.0001; *** corresponds to p<0.001; ** corresponds to p<0.01; * corresponds to p<0.05). Significance level p when compared with corresponding glucose-free samples (#### corresponds to p<0.0001; ### corresponds to p<0.001; ## corresponds to p<0.01; # corresponds to p<0.05 (two-way ANOVA, Tukey's post-test)

In the absence of glucose, a slight increase in fluorescence was observed under the action of copper (II) cations (at all the excitation/emission wavelengths of 379/463 nm) and zinc (II) (at all the excitation/emission wavelengths of 345/405 nm, 366/442 nm, 379/463 nm and 440/520 nm, respectively). A partially similar effect, as well as the ability of iron (III) to reduce the fluorescence intensity at the excitation/emission wavelength's characteristic of AGEs, can be associated with the effect on residual amounts of glucose, the presence of which is probably explained by the technology of albumin production. At the same time, it was shown that, in the identical test system, the presence of zinc cations also leads to the appearance of fluorescence at the wavelengths of 320/438 nm, due to the coordination (or complexation) of zinc cations with amino acid residues at different sites of albumin [47]. In this case it can suggest the formation of coordination structures similar to those previously described by Wu F.-Y. et al. [47]. In the case of copper, the possibility of its interaction with the lysine residue resulting in the formation of a product, fluorescent at the wavelengths of 370/440 nm, was considered in the work by Zhang M. et al. [48]. The above-mentioned information does not exclude the possibility of the formation of other products associated with the specific effect of the metal ion on the protein, and significant for the pathogenesis analized in the article.

Effect of d-metals on BSA aggregation and amyloid transformation

It is known from the literature data that cations of transition metals (copper (II), iron (III)) are able to stimulate the amyloid transformation of proteins [10, 49]. The research of the glycation reaction influence on this metal's property is of great interest. Studying the optical density of the reaction medium at the wavelength of 405 nm is used to assess the kinetics of the protein aggregates formation in the process of the amyloid transformation [45]. The result obtained in the course of this study (during the glycation reaction and/or independently), confirms the ability of some of the studied d-metals to stimulate the BSA aggregation. The results are shown in Table 2.

Considering the intrinsic (independent of glycation) ability of the studied d-metals to cause the BSA aggregation, it should be noted that the activity of metals can be arranged in the ascending order: nickel (II) (inactive) = cobalt (II) (inactive) = iron (II) (inactive) < copper (II) < iron (III) < zinc (II) (Table 2).

The glycation reaction in a metals-free medium did not lead to any statistically significant aggregation of BSA. In this case, glycation affected the ability of some cations to induce the BSA aggregation. Nickel (II) and cobalt (II) cations did not cause any BSA aggregation either independently or in the presence of glucose.

The ability to enhance the BSA aggregation only in the presence of glucose and not without it, has been established for iron (II) cations. For copper (II), iron (III), and zinc (II), the BSA aggregation was observed both in the absence of glucose (due to its own activity) and under the action of cations against the background of the glycation reaction. In case of copper (II), the intensity of the aggregation was higher in the samples with glucose, for iron (III), the result is the same for glucose-containing and glucose-free samples, and in case of zinc (II), the in-

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tensity of the aggregation was higher in the glucose-free samples.

For the studied metal cations, there is a mismatch between the ability to influence the formation of AGEs in the glycation reaction and the ability to stimulate the BSA aggregation. Thus, in the presence of glucose, iron (II) and iron (III), causing the aggregation (Table 2), do not stimulate the AGEs formation (Table 1). The ability of these cations to aggregate BSA does not depend on the AGEs formation, and in case of iron (II), it does not depend on the AGEs formation, but is obviously associated with the presence of glucose. At the same time, when conducting a correlation analysis according to Spearman, a statistically significant inverse correlation between the ability of metal ions to aggregate BSA in the glycated samples and the fluorescence intensity of the amino acids tyrosine and tryptophan at the wavelengths of 270/330 nm specific for both amino acids ($r = –0, 85$, $p = 0.03$), and 295/335 nm, more specific for tryptophan ($r = –0.93$, $p = 0.01$), has been established.

Alongside with the study of the BSA aggregation, the samples were examined for the amyloid belonging of the aggregates in the reaction with thioflavin T (ThT), an amyloid-specific agent. A more pronounced amyloid BSA transformation in the glucose-free samples compared with the glucose-containing samples in all cases, except for the reaction in the presence of copper (II), is noteworthy.

In the presence of glucose, copper (II) stimulates the amyloid BSA transformation more intensively than without it (Table 3). Regarding the other metals and the reaction medium without the ones, the equally increased intensity of ThT fluorescence in the glucose-free samples in comparison with the corresponding glucose-containing samples, indicates that under the described experimental conditions, glycation prevents the amyloid BSA transformation. At the same time, in the samples glycated in the presence of copper (II) cations, a statistically significant higher intensity of ThT fluorescence indicates a mutual reinforcing effect of glucose and copper factors on the intensity of the amyloid transformation (Table 3). It follows from the result that copper showed both the ability to stimulate the amyloid BSA transformation, independent of glycation, and leveled the ability of the ongoing glycation reaction to slow it down.

When assessing the influence of the factor of each cation presence on the amyloid BSA transformation, it was found out that in both glucose and glucose-free samples, this process is enhanced by the cations of copper (II) and zinc (II) (to the maximum extent) as well as nickel (II) (least of all). Cobalt (II) cations slightly suppress the reaction (statistically significant only in the absence of glucose), while iron (II) cations were found to be inactive. Iron (III) was found out to be able to reduce ThT fluorescence in both glucose-containing and glucose-free samples. The ability of copper and zinc to transform proteins into the amyloid form has been previously described more than once. The authors' unexpected results regarding a weak activity of nickel, are consistent with the recently established role of nickel in the formation of human β-amyloid [24].

DISCUSSION

As a result of the study, it was found out that in the absence of metals, the glycation reaction does not affect the fluorescence intensity of tyrosine and tryptophan (the fluorescence intensity of glycated and non-glycated samples is the same). However, glycation can enhance the ability of metals to suppress the fluorescence of these amino acids. In the case of tyrosine, a possible mechanism for decreasing its fluorescence has been described for the reaction of collagen with ribose-5-phosphate [41]. It may be associated with such events as glycation of amino acids located in the spatial proximity to tyrosine (which leads to quenching of its fluorescence); a change in the spatial organization of the protein (which leads to quenching of the fluorescence of tyrosine), the reaction of tyrosine itself. Herewith tyrosine is not expected to directly interact with the glycating agent, but the participation of nearby lysine or arginine residues in the reaction can lead to the activation of tyrosine and its subsequent glycation, or oxidation.

At the same time, as for tryptophan, its residue can be oxidized during glycation [44], and this process can be enhanced in the presence of copper (II) [42]. Thus, a decrease in the intensity of tryptophan fluorescence during the glycation reaction in the presence of copper (II) is due to both the oxidation of the amino acid residue and a change in the protein conformation [42]. Summing up what has been said, it can be assumed that d-metals, which are able of suppressing the fluorescence of tyrosine and/or tryptophan during glycation more intensely, are active, influencing these mechanisms.

It is known that the ability of some d-metals to accelerate the AGEs formation in the glycation reaction is associated with their stimulation of oxidative reactions. This property is characteristic of metals such as copper, and the reaction that occurs with their participation is called the glyoxidation reaction [20]. Thus, the role of copper (II) in the course of glyoxidation is presumably in the catalysis of the electron transfer from enediols formed from reduced monosaccharides or during the fragmentation of Schiff bases and Amadori products. That leads to the formation of reactive oxygen species and dicarbonyl compounds. In these reactions, copper (II) is probably reduced to copper (I), and oxygen is converted to superoxide anion with the participation of hydrogen peroxide. Its source of some glycation stages. Then copper (I) ions are oxidized to copper (II), catalyzing the decomposition of hydrogen peroxide to a hydroxyl radical. Considering this, it can be concluded that the intensification of the glycation reaction in the presence of some transition metals under study can occur according to the described mechanism of glyoxidation.

The analysis of the zinc activity as a non-transition but d-metal, is of particular interest. According to the concept of the glyoxidation mechanism with the participation of copper (II), for the reaction to proceed, it is necessary for the metal ion to be able of changing the oxidation degree. A zinc ion has a constant oxidation degree, but it was able to slightly increase the fluorescence intensity at some excitation/emission wavelengths characteristic of AGEs. According to the literature data [50], zinc can exhibit antiglycating, antioxidant, and antiapoptotic properties, and its deficiency can contribute to the accelerated AGEs formation. However, according to the studies conducted by Zhuang X. et al. [51], in the *in vitro* glycation reaction, zinc (II) is able to enhance the formation of fluorescent AGEs, which is consistent with the results of the research. According to the same study [51], the transition metal manganese exhibits the ability to suppress the AGEs formation in the manner similar to that described for nickel, cobalt and iron, which are also transition metals. All these data indicate that belonging of the metal to transitional ones and the ability of its ion to change the oxidation degree is probably not a necessary and sufficient condition for accelerating a glycation reaction. This also indicates the uniqueness of the zinc properties among the studied metals.

Summarizing the investigation results of the d-metals effect on the fluorescence of the amino acids tyrosine and tryptophan, and the effect of the d-metals on the glycation reaction, it can be assumed that the mechanisms of the metals influence on both processes differ (at least, there are activity components associated with the effect on only one process). The results make it possible to assume the following: there is an additional mechanism of damage to tyrosine and/or tryptophan caused by the glycation factor (not limited by the intrinsic action of the metal ion), which manifests itself during the course of the reaction in the presence of copper (II), iron (II), zinc (II) cations, and, possibly nickel (II). Herewith, an increase in the fluorescence intensity at the wavelengths characteristic of AGEs, regarded as an intensification of the glycation reaction, was noted only when glycation proceeded in the presence of copper (II) cations (to a greater extent) and zinc (II) (to a lesser extent).

As for the results of albumin aggregation studies, it should be noted that, according to the literature data [43], one of the factors of the pathogenetic action of copper (II) cations in Alzheimer's disease, is its participation in the formation of amyloid dimers linked by dityrosine cross-links. This is consistent with the intense suppression of amino acid fluorescence observed in this study in the presence of copper, as well as with the presence of a statistically significant inverse correlation between the fluorescence intensity of these amino acids and the degree of BSA aggregation for the studied cations. Thus, a decrease in the fluorescence intensity of the amino acids tryptophan and tyrosine in the presence of d-metal ions, obviously accompanies the BSA aggregation process. The most intense aggregation of BSA proceeded in the presence of ions that showed the ability to enhance the formation of AGEs (copper, zinc).

As mentioned above, the amyloid transformation of a protein during glycation, depends on many factors, and in particular on the type of protein. Thus, according to the literature data, an increase in the amyloid transformation is observed in the process of albumin glycation (bovine and human), Aβ-protein, β2 microglobulin, etc. In contrast, the ability of α-synuclein to form amyloid fibrils after the glycation by methylglyoxal was reduced, and the resulting aggregates had the character of a molten glo

bule [52]. According to the results obtained, glycation under the described experimental conditions prevents the formation of ThT-sensitive amyloid forms. A similar result is observed when glycation occurs in the presence of nickel (II), cobalt (II), iron (II), iron (III), and zinc (II), but not copper (II), which has shown the ability to enhance the amyloid transformation and the BSA aggregation. Banerjee S. describes a decrease in the intensity of the amyloid transformation of chicken lysozyme after the exposure to the protein with methylglyoxal. Based on this result, the author puts forward a controversial assumption that carbonyl compounds can be used for pharmacological purposes [53]. Despite the doubtfulness of the therapeutic use of carbonyl compounds, this study and the similar ones confirm the fact that glycation can not only potentiate the amyloid transformation [13], but also prevent it [13, 53]. At the same time, it is known that the retardation of the amyloid transformation under the glycation action can be associated with the prolongation of the amyloid residence time in the oligomeric form – the form with the highest cytotoxicity. The latter negates the potential utility of glycation-induced slowing down of the amyloid transformation. According to the literature data [54, 55], ThT, in contrast to other forms (monomers, fibrils), may not detect the formation of amyloid oligomeric forms. It can be suggested that a comparatively lower intensity of ThT fluorescence in the samples glycated in the presence of all d-metal ions with the exception of copper (II), is related to the possibility of slowing down the reaction at the stage of the oligomer formation. However, that will be the subject of a further research. This property has not been found for the glycation in the presence of copper (II).

When evaluating the results obtained, one should obviously take into account all the conditions of the experiment (not only the factors of glycation and the presence of metal, but also heating, the content and pH of the reaction medium, etc.). It can be assumed that unaccounted for factors, can also affect the ability of albumin to undergo the amyloid transformation during glycation, and under other conditions, the course of the reaction can lead to a different result. Thus, for a comprehensive assessment of the glycation ability to influence the amyloid transformation, one should take into account the ability of a protein to transform into an amyloid form

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under various experimental conditions (different pH values, temperatures, ionic strength of the buffer solution, etc.), including conducting studies under the conditions close to physiological ones.

CONCLUSION

As a result of the research, it was shown that the course of the glycation reaction affects the amyloid transformation of BSA, and in the presence of d-metal ions it affects the ability of some of them to cause aggregation and the amyloid transformation. Under the described experimental conditions, copper (II) cations were the only ones able of enhancing the formation of AGEs, reducing the fluorescence intensity of the amino acids tryptophan and tyrosine in the glucose-mediated and glucose-independent ways, causing aggregation and the amyloid transformation of BSA. Ions of other metals showed these effects only partially, in various combinations. This makes it possible to suggest that in the series of d-metal ions such as nickel (II), cobalt (II), iron (II), iron (III), copper (II) and zinc (II), only copper ions (II) are probably the most significant factors in enhancing the amyloid transformation and BSA aggregation, and are the most active catalysts for the formation of AGEs in the glycation reaction. Thus, we believe that this element is a promising target for the development of methods for a pharmacological control of pathological conditions associated with all the processes considered – glycation, aggregation, and amyloid transformation of proteins (long term complications of diabetes mellitus, including diabetic encephalopathy; conformational brain diseases, etc.).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Roman A. Litvinov – development of the research idea, planning, preparation and writing of the publication text, organization and control of the research at all its stages; Arina V. Gontareva – setting up and carrying out the glycation reaction, preparation of reagents, obtaining primary data; Lyudmila E. Usmiyanova – setting and carrying out the glycation reaction, preparation of reagents, obtaining primary data; Daria R. Klimenko – preparation of reagents, statistical processing of primary data, correction of the publication text at the stage of its preparation, work with literature sources.

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RESEARCH ARTICLE

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ANTIMICROBIAL ACTIVITY STUDY OF NEW QUINAZOLIN-4(3H)-ONES AGAINST *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS PNEUMONIAE*

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Quinazolin-4(3H)-one derivatives exhibiting a wide spectrum of a pharmacological activity, represent a promising class of substances used to obtain antibacterial agents, which is especially important in the context of the emergence of pathogenic microorganisms' resistance to drugs used in medicine. It has been proved that compounds having a naphthyl radical in the molecule, as well as an amide group bound to the benzene ring as quinazolinone substituents, are characterized by a pronounced antimicrobial activity against *Staphylococcus aureus* and *Streptococcus pneumoniae.*

The aim of the research is a primary microbiological screening of the *in vitro* antimicrobial activity of new quinazolin-4(3H)-one derivatives against *Staphylococcus aureus* and *Streptococcus pneumoniae*, as well as the assessment of the relationship between the pharmacological effect and the structural transformation of the substance molecule, lipophilicity and the possibility of forming resistance to them.

Materials and methods. The experimental studies have been carried out using well-known nosocomial pathogens of infectious and inflammatory diseases *Staphylococcus aureus* and *Streptococcus pneumoniae* by a serial dilution method.

Results. A compound containing a naphthyl radical in its structure, which contributes to an increase in the hydrophobicity of the substance and its solubility in the membrane of a bacterial cell, has a bacteriostatic effect against both *Staphylococcus aureus* and *Streptococcus pneumoniae.* A similar pharmacological effect is exhibited by a derivative with an amide group as a substituent of the quinazolinone nucleus linked to a phenyl radical, which probably contributes to an increase in the degree of binding to active sites of enzymes involved in the DNA replication, and protein synthesis. Obviously, the increased lipophilicity, which promotes better binding to the efflux protein, cannot serve as objective characteristics of the emergence possibility of the pathogen's resistance to this substance.

Conclusion. Among the synthesized compounds, the leading substances that exhibit an antimicrobial activity against *Staphylococcus aureus* and *Streptococcus pneumonia,* have been identified. The assessment of the chemical structure made it possible to substantiate their pharmacological action and draw conclusions about the possibility of developing resistance to it in microbial cells.

Keywords: quinazolinone derivatives; antimicrobial activity; lead-compound; electron-donating centers; enzyme active site; minimum inhibitory concentration; minimum suppressing concentration; bacteriostatic action; bactericidal activity; resistance; ATP-dependent efflux pump; plasmids; transposones; large mobile element

Abbreviations: PBP – penicillin-binding protein; MRSA – methicillin-resistant *Staphylococcus aureus*; PBP2a – penicillin-binding protein; ATP – adenosine triphosphate, MIC – minimum inhibitory concentration; DMSO – dimethyl sulfoxide; DMF – dimethylformamide; MIB – meat infusion broth; MIA – meat infusion agar; AC – atypical colonies; TC – typical colonies; NMR – nuclear magnetic resonance; TLC – thin layer chromatography; NA – nucleic acid; FnBPs – fibronectin-binding proteins

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ИЗУЧЕНИЕ АНТИМИКРОБНОЙ АКТИВНОСТИ НОВЫХ ХИНАЗОЛИН-4(3*H*)-ОНОВ ПО ОТНОШЕНИЮ К *STAPHYLOCOCCUS AUREUS* И *STREPTOCOCCUS PNEUMONIAE*

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Производные хиназолин-4(3*H*)-она, проявляющие широкий спектр фармакологической активности, представляют перспективный класс веществ, используемых для получения антибактериальных средств, что особенно актуально в условиях возникновения резистентности патогенных микроорганизмов к используемым в медицине лекарственным препаратам. Доказано, что соединения, имеющие в молекуле нафтильный радикал, а также амидную группу, связанную с бензольным кольцом, в качестве заместителей хиназолинона, характеризуются выраженной противомикробной активностью в отношении *Staphylococcus aureus* и *Streptococcus pneumoniae.*

Цель. Первичный микробиологический скрининг антимикробной активности *in vitro* новых производных хиназолин-4(3*H*)-она по отношению к *Staphylococcus aureus* и *Streptococcus pneumoniae*, а также оценка взаимосвязи между проявляемым фармакологическим действием и структурным преобразованием молекулы вещества, липофильностью и возможностью формирования устойчивости к ним.

Материалы и методы. Экспериментальные исследования были выполнены с использованием общеизвестных нозокомиальных возбудителей инфекционно-воспалительных заболеваний *Staphylococcus aureus* и *Streptococcus pneumoniae* методом серийных разведений.

Результаты. Соединение, содержащее в структуре нафтильный радикал, вносящий вклад в увеличение гидрофобности вещества и его растворимости в мембране бактериальной клетки, обладает бактериостатическим действием как в отношении *Staphylococcus aureus*, так и к *Streptococcus pneumoniae*. Сходный фармакологический эффект проявляет производное с амидной группой в качестве заместителя хиназолинонового ядра, связанной с фенильным радикалом, которая, вероятно, способствует увеличению степени связывания с активными сайтами ферментов, принимающих участие в процессах репликации ДНК и синтеза белков. Очевидно, повышенная липофильность, способствующая лучшему связыванию с белком оттока, не может служить объективной характеристикой возможности возникновения резистентности патогенов к данному веществу.

Заключение. Среди синтезированных соединений были выявлены вещества-лидеры, проявляющее антимикробную активность в отношении *Staphylococcus aureus* и *Streptococcus pneumoniae.* Оценка химического строения позволила обосновать их фармакологическое действие и сделать выводы о возможности развития устойчивости к нему у микробных клеток.

Ключевые слова: производные хиназолинона**;** антимикробная активность; соединение-лидер; электронодонорные центры; активный сайт фермента; минимальная подавляющая концентрация; минимальная ингибирующая концентрация; бактериостатическое действие; бактерицидная активность; резистентность; АТФ-зависимый эффлюксный насос; плазмиды; транспозоны; большой мобильный элемент

Список сокращений: PBP – пенициллин-связывающий белок; MRSA – метициллин – резистентный *Staphylococcus aureus*; PBP2a – пенициллин-связывающий белок 2а; АТФ – аденозинтрифосфат, МПК – минимальная подавляющая концентрация; ДМСО – диметилсульфоксид; ДМФА – диметилформамид; МПБ – мясопептонный бульон; МПА – мясопептонный агар; АК – атипичные колонии; ТК – типичные колонии; ЯМР – ядерный магнитный резонанс; ТСХ – тонкослойная хроматография; НК – нуклеиновая кислота; FnBPs – фибронектин-связывающие белки

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INTRODUCTION

Currently, multi-resistance of pathogenic bacteria to antimicrobial agents used in medical practice, is a serious public health problem [1-6]. As a rule, the formation of resistance occurs in the course of antibiotic therapy, especially in the departments with more intensive use of this group drugs. Clinical studies have established the dominance of antibiotic-resistant strains in the structure of nosocomial infections. Thus, there is a need to search for new antibacterial substances characterized by high efficacy, low toxicity and insensitive to the suppressing action of pathogens [7–9].

It has been proven that *Staphylococcus aureus* and *Streptococcus pneumonia* are the most common and express various virulence factors. They are pathogens of a wide range of diseases in humans and animals, have the greatest resistance to antibiotics among gram-positive microorganisms [2, 10–14].

The emergence of *Staphylococcus aureus* resistance to β-lactam antibiotics, as well as to other antimicrobial agents, limits its use in medicine due to the following factors: its mutation and selection, the acquisition of new genetic material from other resistant organisms during the processes of transformation, transduction and conjugation, implying a change in the adhesive properties of the cell surface. It is known that functioning of ATP-dependent efflux pumps, which are carrier proteins that push antimicrobial agents out of the cell, contributes to the resistance formation of *Staphylococcus aureus* and *Streptococcus pneumoniae* to fluoroquinolones and the drugs of the tetracycline group [15–18].

Quinazolin-4(3H)-one and its derivatives, which are condensed heterocyclic nitrogen-containing compounds, are known as a promising class of substances exhibiting antibacterial, antifungal, anti-tuberculosis, and antiviral kinds of activity [3]. Its dependence on the nature and number of quinazolinone nucleus substituents has been described. It was found out that the compounds of this group have a pharmacological effect against *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* [3, 5, 19].

It has been proven that quinoline derivatives, which are the basis of the quinazolinones structure, inhibit the DNA synthesis, promoting the cleavage of bacterial DNA gyrase and type IV topoisomerase, resulting in the death of a bacterial cell [20–24]. The ability of compounds of the quinazolinone series, similar to β-lactam antibiotics used to prevent pathogenic processes in the body caused by *Staphylococcus aureus* and *Streptococcus pneumoniae,* to participate in the irreversible serine acylation of the active center of transpeptidase – penicillin-binding protein (PBP), catalyzing the formation of peptidaregine, an essential component of the bacterial cell wall, has been described. As a result of the formation of a stable lactam-acyl-enzyme complex, transpeptidase and carboxypeptidase kinds of the enzyme activity are inhibited, leading to the death of the pathogen.

A unique ability of quinazolinones, realized in synergy with piperacillin and tazobactam, to form bonds with the allosteric site of penicillin-binding protein 2a (PBP2a) of methicillin-resistant *Staphylococcus aureus* (MRSA) and coagulase-negative staphylococci, which cannot be inhibited by β-lactams, has been established [15, 25–27]. The possibility of the joint use of quinazoline derivatives with chloramphenicol to increase its intracellular concentration in pathogenic strains applying efflux pumping systems to resist the action of antimicrobial drugs, has been proven [28, 29]. Probably, during their passage, quinazolinone, having a lower polarity, binds to an efflux pump to a greater extent, undergoes an outflow more easily and facilitates the penetration of an antibiotic into a microbial cell with a constant concentration [16–18, 20, 21, 30].

The uniqueness of the structure of quinazolin-4 (3H)-one new derivatives, the possibility of using it together with other antimicrobial agents in order to increase their pharmacological effect and prevent the resistance emergence to them, creates the need for a comprehensive study of their activity.

THE AIM of the research is to study the antimicrobial activity of quinazolin-4 (3H)-one derivatives against *Staphylococcus aureus* and *Streptococcus pneumoniae in vitro* as well as to assess the effect of their structural changes on the biological activity of the analyzed substances, the lipophilicity of their molecules to predict the ability of inducing their resistance by the mechanism of the active outflow.

MATERIALS AND METHODS

Research objects

The objects of the study were new derivatives of quinazolin-4 (3H)-one.

The chemical structure of new quinazolinone compounds can be described by the general formula shown in Fig. 1. The yield and physicochemical properties of the new substances are presented in Table 1.

Synthesis of new derivatives of quinzolin-4(3*H*)-one

The synthesis of new derivatives was carried out according to the classical scheme of the nucleic bases alkylation with alkyl halides in anhydrous dimethylformamide (DMF) in the presence of a potassium carbonate excess. NMR^{1}H spectra were recorded on a BrukerAvance 400 spectrometer (400 MHz) in DSMO-d6, tetramethylsilane as the internal standard. The spectra were interpreted using the ACD/HNMR PredictorPro 3.0 licensed program from Advanced Chemistry Development (Canada). The melting points were measured in glass capillaries on a Mel-Temp 3.0 instrument (Laboratory Devices Inc., USA). The purity and individuality of the compounds were monitored by the TLC method.

N-[4-(Dimethylamino)phenyl]-2-[4-oxo-3(4H)-quinazolinyl] acetamide (Laboratory code: VMA-10-10).

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A mixture of 2.0 g (13.7 mmol) of quinazolin-4(3H)-one, 4.0 g (28.9 mmol) of anhydrous potassium carbonate and 50 ml of DMF is stirred at the temperature of 100-105°C for 30 min., then 3.2 g (15.1 mmol) of 2-Chloro-N-[4-(dimethylamino)phenyl]acetamide is added and stirred at the same temperature for 1 hour. After that, the mixture is cooled down to room temperature and filtered.

The filtrate is kept at the temperature of 0–5°C within 24 hours. The separated precipitate is filtered off, washed with cold DMF, water, and dried in air. It is recrystallized from DMF to get 2.95 g of the VMA-10-10compound, the yield is 67%, the mp. is 261–264°C.

The NMR^{1}H spectrum, δ, ppm, is the following: 2.78 s (6H, CH_3). 4.76 s (2H, CH_2); 6.63 d (8 Hz, 2H, phenyl); 7.34 d (8 Hz, 2H, phenyl); 7.51 t (7 Hz, 1H, H^6); 7.66 d (8 Hz, 1H, H^8); 7.78 t (7 Hz, 1H, H^7); 8.09 d (8 Hz, 1H, H^5); 8.29 s (1H, H^2); 10.08 s (1H, NH).

The rest of the compounds are obtained in the same way.

N-(4-Methoxyphenyl)-2-[4-oxo-3(4H)-quinazolinyl] acetamide (Laboratory code: VMA-10-18). The NMR^{1}H spectrum, δ, ppm is the following: 3.72 s (3H, OCH_3); 4.85 s (2H, CH_2); 7.51 d (8 Hz, 2H, phenyl); 6.90 d (8 Hz, 2H, phenyl); 7.57 t (7 Hz, 1H, H^6); 7.73 d (8 Hz, 1H, H^8); 7.86 t (7 Hz, 1H, H^7); 8.16 d (8 Hz, 1H, H^5); 8.37 s (1H, H^2); 10.31 s (1H, NH).

3-[2-Oxo-2-(4-phenylpiperazin-1-yl)ethyl] quinazolin-4(3H)-one (Laboratory code: VMA-10-21). The NMR^{1}H spectrum, δ, ppm is as follows: 3.14-3.32 m (4H, piperazine); 3.62-3.78 m (4H, piperazine); 5.01 s (2H, CH_2); 6.96-7.01 m (2H, phenyl); 7.23-7.29 m (3H, phenyl); 7.55 t (7.5 Hz, 1H, H^6); 7.71 d (8 Hz, 1H, H^8); 7.86 t (7.5 Hz, 1H, H^7); 8.17 d (8 Hz, 1H, H^5); 8.26 s (1H, H^2).

N-(2-Naphthyl)-2-[4-oxo-3(4H)-quinazolinyl] acetamide (Laboratory code: VMA-13-05). The NMR^{1}H spectrum, δ, ppm is as follows: 5.81 s (2H, CH2); 7.55-8.89 m (11H, H^5, H^6, H^7, H^8, naphthyl); 8.42 s (1H, H^2).

N-Phenyl-2-[4-oxo-3 (4H)-quinazolinyl]acetamide (Laboratory code: VMA-17-01). The NMR^{1}H spectrum, δ, ppm is as follows: 5.67 s (2H, CH_2); 7.54-7.77 m (5H, H^6, H^8, phenyl); 7.87 t (1H, 8 Hz, H^7); 8.07–8.19 m (3H, H^5, phenyl); 8.39 s (1H, H^2).

N-Phenyl-2-[4-oxo-3(4H)-quinazolinyl]propanamide (Laboratory code: VMA-17-04). The NMR^{1}H spectrum, δ, ppm is as follows: 1.53 d (3H, 7 Hz, CH_3) 5.49 q (1H, 7 Hz, CH); 7.56–7.80 m (5H, H^6, H^8, phenyl); 7.85 t (1H, 8 Hz, H^7); 8.06–8.19 m (3H, H^5, phenyl); 8.40 s (1H, H^2).

N- [6-Bromoquinazolin-3 (4H) -yl] acetylguanidine (Laboratory code: VMA-13-17). The NMR^{1}H spectrum, δ, ppm is as follows: 4.37 s (2H, CH2); 7.47 br. s (4H, NH); 7.60 d (1H, 8 Hz, H^8); 7.90 d (1H, 8 Hz, H^7); 8.17 s (1H, H^2); 8.28 s (1H, H^5).

Test cultures

A primary microbiological screening of the antimicrobial activity of the synthesized compounds in order to identify the lead compound, was carried out using cultures of *Staphylococcus aureus* and *Streptococcus pneumoniae* isolated from sick patients provided by the clinical diagnostic laboratory, City Clinical Hospital No. 3 n. a. S.M. Kirov, Astrakhan. The studies were approved by the Ethics Committee of Astrakhan State Medical University of the Ministry of Health of Russia (protocol No. 6 dated November 27, 2018).

Research methods

The analysis of substances with the assigned codes – VMA-10-10, VMA-10-18, VMA-10-21, VMA-13-05, VMA-17-01, VMA-17-04, VMA-13-17 – was carried out *in vitro* by the serial dilutions method in accordance with the requirements of the international standard ISO 20776-1:2006[1] and the National Standard GOST R ISO 20776-1-2010[2], identical to the international one.

The determination of the microorganism's sensitivity to quinazolinone derivatives was carried out by the macro method (test tube) in the medium of meat infusion broth (MIB) prepared in accordance with GOST 20729-75.

Preparation process of working solutions

The working solution was prepared by dissolving a 4 mg sample of the test substance in 0.5 ml of dimethyl sulfoxide (DMSO), followed by adding 4.5 ml of a physiological solution to it. The choice of the solvent was carried out in accordance with the Methodological Recommendations "Sensitivity determination of microorganisms to antibacterial drugs"[3], as well as taking into account the solubility of the compounds under study, with a preliminary assessment of DMSO effect on the strains of the microorganisms used [33]. It was found out that the compounds under study are insoluble in water, slightly soluble in 40 and 90% ethyl alcohol, and freely soluble in DMSO. A series of solutions with an exponentially decreasing concentration was obtained from the resulting initial solution: 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 μg/ml. A solution of ceftriaxone (JSC Sintez, Kurgan, P N000750/01) with the concentration equivalent to the process solution was used as a reference drug. Process solutions were introduced into 1 ml test tubes.

[1] CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement. CLSI document M100-S25. Wayne, PA: Clinicaland Laboratory Standards Institute; 2015.

[2] National Standard GOST R ISO 20776-1-2010 Clinical laboratory research and in vitro diagnostic test systems. Investigation of the sensitivity of infectious agents and assessment of the functional characteristics of products for the study of sensitivity to antimicrobial agents. Part 1. Reference method for laboratory study of the activity of antimicrobial agents against fast-growing aerobic bacteria that cause infectious diseases. Russian

[3] Methodical instructions 4.2.1890-04. Determination of the sensitivity of microorganisms to antibacterial drugs: Guidelines. M.: Federal Center for State Sanitary and Epidemiological Supervision of the Ministry of Health of Russia, 2004: 91 p. Russian

Inoculum preparation

Inoculum preparation was carried out in accordance with the requirements for the method of direct suspension of morphologically similar colonies collected using a sterile bacteriological loop in a sterile isotonic solution.

Methodology

Suspensions of *Staphylococcus aureus* and *Streptococcus pneumoniae,* diluted in a liquid nutrient medium to 10^6 cfu/ml, were added 1 ml each into the test tubes with the solutions of the studied substances.

The inoculations in the tubes closed with sterile cotton-gauze stoppers, were incubated for 24 hours at the temperature of + 37°C. At the end of the incubation period, they were visually assessed in the transmitted light. In the control tubes, in which a native culture had been grown without adding a reference drug or test compounds, complete turbidity of the culture medium indicating an intensive growth of the culture, was noted.

The determination of the minimum inhibitory concentration (MIC) of the test substance assumed the establishment of the lowest substance concentration at which there had been no bacterial growth. That was evidenced by the absence of turbidity of the solution, which was recorded visually.

Assessment of microorganisms growth

The assessment of the bacteria viability was carried out according to the value of the lowest concentration of the test substance that prevents the visible growth of bacteria, or, in other words, the minimum inhibitory concentration. The inoculation of 0.05 ml of the precipitate obtained by centrifuging the contents of each tube of the series at 1500 rpm for 10 min and separating the supernatant, was carried out on the meat infusion agar (MIA) placed in Petri dishes. The nutrient medium was prepared by dissolving the dry agar followed by autoclaving. After the inoculation, the Petri dishes were placed in a thermostat. The analysis of the characteristic growth was performed 24 hours after the incubation of the inoculation at the temperature of +37°C [33].

The determination of the antimicrobial activity of the test substances hypothesized a sixfold reproduction of the selected analysis method [31-32]. The absence of the culture growth was taken for the bactericidal effect manifested by the substance, while the inhibition of the culture growth, its intermittent growth, and the formation of single colonies indicated a bacteriostatic effect.

Statistical processing of research results

Statistical processing of the research results was carried out using the following software packages: Microsoft Office Excel 2007 (Microsoft, USA), BIOSTAT 2008 Professional 5.1.3.1. ("Analyst-Soft" Inc., USA). When processing the results obtained, a parametric method with the determination of the Student's t-test with the Bonferroni correction was used. The differences in the comparison groups were assessed at the constantly chosen significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

The analysis of the antimicrobial activity of the substances with codes VMA-10-10, VMA-10-18, VMA-10-21, VMA-13-05, VMA-17-01, VMA-17-04, VMA-13-17 showed that their manifestation depends on the multiplicity of dilution and a type of a pathogenic microorganism.

The experimental data obtained are summarized in Tables 2–5.

The growth pattern analysis of *Staphylococcus aureus* and *Streptococcus pneumoniae* in the meat infusion broth and on the meat infusion agar with DMSO showed a moderate growth of microorganisms at the concentration of 128 and 64 μg/ml, as well as an intensive growth in the concentration range from 32 to 0.25 μg/ml.

During the visual control of *Staphylococcus aureus* cultures in the meat infusion broth, the signs of growth in the test tubes with ceftriaxone were observed at low drug concentrations – 2–0.5 μg/ml. A moderate growth of the culture was observed in the presence of the VMA-10-10 compound in the concentration range of 128-4 μg/ml and in the case of VMA-17-01 – in the concentration range of 16–8 μg/ml. The intensive development of cells, accompanied by strong turbidity of the nutrient medium, the formation of flakes and abundant sediment, were observed in test tubes at the concentrations of the compound VMA-10-21 in the range of 128–0.25 μg/ml.

A significant culture growth was also recorded in the test tubes with substances VMA-17-04 with a concentration of 4–0.25 μg/ml and VMA-13-17 with its content of 2–0.25 μg in 1 ml of the solution.

Table 3 shows the results of inoculating *Staphylococcus aureus* on a solid nutrient medium – meat infusion agar.

The Table 3 data indicate that in the presence of the control, ceftriaxone at the concentrations of 128–64 μg/ml, the growth of the culture is completely suppressed, while at its content of 32–4 μg in 1 ml of the solution, the growth of single colonies of the pathogen is observed.

An intensive growth is recorded on the *Staphylococcus aureus* meat infusion agar when using VMA-10-10 at the concentrations of 128–0.25 μg/ml and VMA-10-21 at 64–0.25 μg/ml. No growth of colonies was observed in the concentration ranges of 128–16 μg/ml of the substance VMA-17-04, 128–64 μg/ml – VMA 13-05, 128–32 μg/ml – VMA-17-01. The results indicate the ability of these compounds to inhibit the development of *Staphylococcus aureus* and, as a consequence, to exhibit a pronounced antimicrobial activity against the pathogen.

Table 4 shows the results of *Streptococcus pneumo-*

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niae inoculations on a liquid nutrient medium (meat infusion broth).

During the visual control of *Streptococcus pneumonia* inoculations on the meat infusion broth, the signs of growth in the test tubes with ceftriaxone were observed at the concentration of 4–0.25 μg/ml. A moderate growth of the culture was observed in the presence of the VMA-10-21 compound in the concentration range of 64–0.25 μg/ml, and of the VMA-10-18 substance – at its content of 8–0.25 μg in 1 ml. Lower values were set for the VMA-13-17, VMA-13-05 derivatives – 2–0.25 μg/ml and for VMA-10-10, VMA-17-01 derivatives – 1–0.25 μg/ml.

A complete transparency of the medium was observed in the tubes with quinazolinone derivative VMA-13-05 at the concentration of 128–32 μg/ml, of compounds VMA-17-01 and VMA-17-04 – in the content of the active ingredient of 128–64 μg in 1 ml. The results obtained indicate a pronounced antipneumococcal activity of the substances.

Table 5 shows that the culture of *Streptococcus pneumoniae* gives a heavy growth on the MIA in the presence of VMA-10-10, VMA-10-18 compounds at the concentrations of 4–0.25 μg/ml, in the presence of VMA-13-05 substances – at 8–0.25 μg/ml and in the presence of the VMA-17-04 derivative – at the concentrations of 2–0.25 mg/ml. The results obtained indicate the lack of sensitivity of the pathogen to these substances in the given dilution.

When the content of VMA-13-05 is at the concentration of 128–16 μg/ml, the growth of the pathogenic strain colonies is not observed. This is similar to the effects of VMA-17-04 and VMA-17-01 in the concentration range of 128–64 μg/ml. Consequently, in this content in the solution, the substances are characterized by a high antimicrobial activity against *Streptococcus pneumoniae.*

Table 6 shows the average results of assessing the antibacterial action of the most active substances against the strains of *Staphylococcus aureus* and *Streptococcus pneumoniae.*

The analysis of the average results of the antibacterial action of the most active substances against pathogenic microorganisms, makes it possible to conclude the following. The bactericidal activity of the compounds VMA-13-05, VMA-17-01 and VMA-17-04 is comparable to the action of ceftriaxone at the concentrations of 128 and 64 μg/ml; their bactericidal activity against *Staphylococcus aureus* manifests itself at the concentration of 32 μg/ml. When analyzing the antimicrobial action of the most active quinazoline compounds in subsequent concentrations, it was found out that the bactericidal activity of VMA-13-05, VMA-17-01 and VMA-17-04 statistically significantly decreases in proportion to the decrease of the substances concentration in relation to the reference drug – ceftriaxone.

The heterocyclic nature of quinazolinone compounds determines their ability to inhibit a PBP2a activity due to the formation of hydrogen bonds with the amino acids of the allosteric enzyme site: lysine, glutamine and asparagine. As a result of this interaction, an active site, where the carbonyl group and the nitrogen atom of another molecule of the antimicrobial agent are covalently bound to the carboxyl and amino groups of lysine and arginine, is opened. The enzyme is suppressed and, therefore, the biosynthesis of the bacterial cell wall is blocked [37-40]. The analysis of various substituents effect in the molecule of quinazolinone derivatives made it possible to identify the functional groups and structural fragments that take part in the formation of a chemical bond with the amino acid residues of the enzyme, due to which the pharmacological effect of the substances is probably realized. The studies of the relationship between the structure and activity of quinazolinone derivatives have shown that the presence of a substituted aromatic ring at position 3 and a methyl group is essential for the compound to exhibit the antimicrobial activity [34]. In this case, the quinazolinone compounds containing a phenyl radical are characterized by a higher binding affinity than the substances with a methyl group, which can be explained by an increase in the number of hydrophobic bonds with amino acids of the active site [35]. It has been shown that the substituent in the phenyl ring also has a significant effect on the antibacterial activity. Methoxy, methyl, hydroxy groups, as well as bromine and chlorine atoms, increase the antimicrobial effect [24]. It has been proven that the combination of two or more biologically active fragments in one molecule also contributes to an increase in the antibacterial effect due to a change in the degree of polarity of the drug molecule [1].

The mechanism of the substances interaction with DNA gyrase has been described. It also depends on the substituents nature determining the polarity of the molecule, its ability to form various chemical bonds with the enzyme. In this case, the death of a bacterial cell is known to be mediated by a violation of the DNA synthesis during the DNA gyrase inhibition involved in the reduction (negative supercoiling) of a nucleic acid (NA) molecule, with a quinazolinone derivative [37]. It has been established that its effect can be explained by the formation of an intermediate complex “DNA-topoisomerase-quinazolinone” due to the donor-acceptor interaction of the carbonyl group oxygen atom of the antimicrobial agent and the phosphate group of DNA, nitrogen with guanine and NA asparagine, and the substituents of the quinazolinone molecule with its non-polar groups. Binding to the active site of the enzyme occurs due to the hydrogen bonds of the quinazolinone derivative with the amino acid residues of serine and arginine [37].

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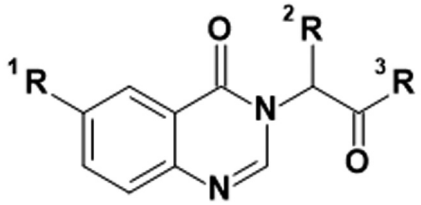

Figure 1 – General formula of quinazolin-4 (3H)-one derivatives

Table 1 – Chemical structure of new quinzolin-4 (3H)-one derivatives

Compound	R^1	R^2	R^3	Yield, %	Mp., °C
VMA-10-10	H	H	4-dimethylaminophenyl	67	261–264
VMA-10-18	H	H	4-methoxyphenyl	61	228–229
VMA-10-21	H	H	4-phenylpiperazin-1-yl	73	222–224
VMA-13-05	H	H	β-naphthyl	56	199–201
VMA-17-01	H	H	phenylamino	83	156–158
VMA-17-04	H	CH_3	phenylamino	72	222–224
VMA-13-17	Br	H	$NHC(NH)NH_2$	89	242–244

Table 2 – Indicators of visual assessment of compounds activity against growth of *Staphylococcus aureus* (MIB medium)

Series (compounds, drugs)	Concentration, μg/ml									
	128	64	32	16	8	4	2	1	0.5	0.25
DMSO	++	++	+++	+++	+++	+++	+++	+++	+++	+++
Ceftriaxone	–	–	–	–	–	+	++	+++	+++	+++
VMA-10-10	++	++	++	++	++	++	+++	+++	+++	+++
VMA-10-18	+	+	+	+	+++	+++	+++	+++	++++	++++
VMA-10-21	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
VMA-13-05	–	–	–	++	++	++	+++	+++	+++	+++
VMA-17-01	–	–	–	++	++	+++	+++	+++	+++	+++
VMA-17-04	–	–	–	–	++	++	++++	++++	++++	++++
VMA-13-17	+	+	++	++	++	++	+++	+++	+++	+++

Note: "–" – full medium transparency; "+ –" – incomplete medium transparency; "+" – weak growth; ++ – moderate growth; +++ – intensive growth

Table 3 – Indicators of visual assessment of compounds activity against growth of *Staphylococcus aureus* (MIA medium)

Series (compounds, drugs)	n	Concentration, μg/ml									
		128	64	32	16	8	4	2	1	0.5	0.25
DMSO	6	++	++	+++	+++	+++	+++	+++	+++	+++	+++
Ceftriaxone	6	–	–	+AC	+AC	+AC	+AC	+++AC	+++AC	+++AC	+++AC
VMA-10-10	6	+++AC	+++AC	+++AC	+++AC	+++AC	++++AC	++++AC	++++AC	++++AC	++++AC
VMA-10-18	6	+ AC	+AC	+ AC	+AC	+++AC	+++TC	+++TC	+++TC	++++TC	++++TC
VMA-10-21	6	++AC	+++TC	+++TC	+++TC	++++TC	++++TC	++++TC	++++TC	++++TC	++++TC
VMA-13-05	6	–	–	+AC	+++AC	++++AC	++++AC	++++AC	++++AC	++++AC	++++AC
VMA-17-01	6	–	–	–	++ AC	++ AC	+++ TC	+++ TC	+++ TC	+++ TC	+++ TC
VMA-17-04	6	–	–	–	–	++ AC	++AC	++++ AC	++++ AC	++++ AC	++++AC
VMA-13-17	6	+ AC	+ AC	++ AC	++ AC	++ AC	++AC	+++ TC	+++ TC	+++ TC	+++TC

Note: "–" – no colonies; "+" – single colonies; "++" – ≤ 50%, "+++" – ≤ 75%; "++++" – ≤ 100% of colonizating the Petri dish area; AC – atypical colonies; TC – typical colonies

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Table 4 – Indicators of visual assessment of compounds activity against growth of *Streptococcus pneumoniae* (MIB medium)

Series (compounds, drugs)	Concentration, μg/ml									
	128	64	32	16	8	4	2	1	0.5	0.25
DMSO	++	++	+++	+++	+++	+++	+++	+++	+++	+++
Ceftriaxone	–	–	–	–	–	+	+	+	+	+
VMA-10-10	+ –	+ –	+ –	+	+	++	++	+++	+++	+++
VMA-10-18	+	+	+	+	+++	+++	+++	+++	++++	
VMA-10-21	+	+++	+++	+++	+++	+++	+++	+++	+++	+++
VMA-13-05	–	–	–	+	+ –	++	+++	+++	+++	+++
VMA-17-01	–	–	+ –	+ –	+	++	++	+++	+++	+++
VMA-17-04	–	–	+	+	+	+	++	++	+++	+++
VMA-13-17	+	+	+	+	+	+	+++	+++	+++	+++

Note: "–" – full medium transparency; "+ –" – incomplete medium transparency; "+" – weak growth; ++ – moderate growth; +++ – intensive growth

Table 5 – Indicators of visual assessment of compounds activity against growth of *Streptococcus pneumoniae* (MIA medium)

Series (compounds, drugs)	n	Concentration, μg/ml									
		128	64	32	16	8	4	2	1	0.5	0.25
DMSO	6	++	++	+++	+++	+++	+++	+++	+++	+++	+++
Ceftriaxone	6	–	–	–	+AC	+AC	+AC	++AC	++AC	++AC	++AC
VMA-10-10	6	++AC	++AC	++AC	++AC	++AC	++++AC	++++AC	++++AC	++++AC	++++AC
VMA-10-18	6	++AC	++AC	++ AC	++ AC	+++AC	+++TC	+++TC	+++TC	++++TC	++++TC
VMA-10-21	6	+AC	+TC	++TC	++TC	+++TC	+++TC	+++TC	+++TC	++++TC	++++TC
VMA-13-05	6	–	–	–	–	++++AC	++++AC	++++AC	++++AC	++++AC	++++AC
VMA-13-17	6	+ AC	+ AC	++ AC	++ AC	++ AC	++ AC	+++ TC	+++ TC	+++ TC	+++ TC
VMA-17-04	6	–	–	+ AC	+AC	++ AC	++ AC	++++ TC	++++ TC	++++ TC	++++ TC
VMA-17-01	6	–	–	+AC	++ AC	++ AC	+++ TC	+++ TC	+++ TC	+++ TC	+++ TC

Note: "–" – no colonies; "+" – single colonies; "++" – ≤ 50%, "+++" – ≤ 75%; "++++" – ≤ 100% of colonizating the Petri dish area; AC – atypical colonies; TC – typical colonies

Table 6 – Average results of antibacterial action of the most active substances against *Staphylococcus aureus* and *Streptococcus pneumoniae* strains

Series (compounds, drugs)	Concentration, μg/ml									
	128	64	32	16	8	4	2	1	0.5	0.25
Ceftriaxone	0	0	0	0	0	18.1±2.3	18.8±2.2	19.3±2.2	22.5±3.6	22.7±3.2
Against *Staphylococcus aureus* strains										
VMA-13-05	0	0	0	29.5±2.4 ***	32.1±3.1 ***	38.4±3.8 **	59.4±4.7 ***	65.3±4.2 ***	65.8±5.6 ***	68.3±5.4 ***
VMA-17-01	0	0	0	28.3±2.1 ***	33.8±3.7 ***	39.9±4.2 **	64.4±4.3 ***	65.7±4.1 ***	65.6±6.6 ***	69.3±6.1 ***
VMA-17-04	0	0	0	0	27.3±3.1 ***	28.1±2.8 *	78.4±5.9 ***	81.3±7.1 ***	83.6±7.3 ***	85.2±6.5 ***
Against *Streptococcus pneumoniae* strains										
VMA-13-05	0	0	0	14.3±1.8 ***	16.4±2.1 ***	26.3±1.8 *	61.3±4.8 ***	63.8±5.6 ***	66.4±5.2 ***	71.6±6.9 ***
VMA-17-01	0	0	12.3±1.8 ***	14.9±2.0 ***	15.7±1.9 ***	25.9±1.8 *	27.3±2.0 *	56.4±4.6 ***	62.3±4.9 ***	68.3±6.0 ***
VMA-17-04	0	0	10.2±1.3 ***	12.7±1.8 ***	12.8±1.4 ***	13.2±1.9	26.2±1.9 *	28.6±2.2 **	53.8±5.2 ***	55.7±5.2 ***

Note: * – p <0.05; ** – p <0.01; *** – p <0.001 – by reference to the indicators of the antibacterial ceftriaxone action

The possibility of the quinazolinone derivatives interaction with peptidoglycan precursors cannot excluded. That leads to the inhibition of its polymerization (transglycosylation) and the subsequent stage of cross-linking (transpeptidation). The bactericidal effect of the drug is realized during the formation of an intermediate complex "quinazolinone – peptidoglycan derivative", as a result of which depolarization of the membrane occurs, its permeability increases, leakage of potassium ions and cytoplasmic ATP occurs resulting in the cell death [41, 42].

The idea of the efflux pumps functioning increases a number of requirements for the investigated antimicrobial substances, in the form of a combination of high efficacy with resistance to outflow. One of the options for achieving it can be the dissipation of the membrane potential [29, 34]. It has been proven that the presence of a keto group, a benzyl radical and nitrogen atom in the quinazolinone structure, contributes to a decrease in lipophilicity; covalently bound bromine in the quinazoline core; methoxyphenyl and methyl substituents, on the contrary, increase hydrophobicity [35, 36]. The saturation of the quinazolinone derivatives molecules by the centers that reduce hydrophobicity, suggests an insignificant degree of binding to efflux proteins and, as a consequence, a low probability of resistance to these substances from the point of view of the efflux theory [5, 7, 28].

The analysis of the results obtained shows that the compound VMA-17-04, and, to a lesser extent, VMA-13-05, are active against *Staphylococcus aureus* and have a bacteriostatic effect. The structure of the substance VMA-13-05 contains a naphthyl substituent, which makes the molecule more lipophilic and, as a result, increases its penetration into the cell membrane of the pathogenic culture. The polarity of VMA-17-04, due to the amide group associated with the quinazolinone moiety and the benzene ring, causes an increase in the interaction degree of the electron donor center in the form of a nitrogen atom with the active sites of enzymes that catalyze the DNA replication and protein synthesis.

The assessment of the test compounds antimicrobial activity against *Streptococcus pneumoniae* shows the manifestation of the bacteriostatic effect of the VMA-13-05 derivatives. The VMA-17-04 and VMA-17-01 compounds are characterized by a weakly expressed antimicrobial effect.

The VMA-10-10 substance has practically no effect on *Staphylococcus aureus* and *Streptococcus pneumoniae.*

Probably, the difference of the membrane components of gram-positive bacteria in the chemical composition can be the reason for the unequal manifestation of the pharmacological activity of the VMA-17-04 and VMA-13-05 substances in relation to the pathogens. The presence of the quinazolinone derivatives in the molecules differing from their substituents in the structure, determines the difference in the mechanism of their binding to the substances of the pathogens cell membrane acting as adhesives, which are one of the virulence factors of these microorganisms. It has been established that the main role in the adhesion process of *Streptococcus pneumonia,* is played by collagen-binding and fibronectin-binding proteins, lipoteichoic acid, as well as surface phosphoryl-choline, which is a part of teichoic acid with choline-binding proteins attached to it. The adhesive activity of Staphylococcus aureus is carried out due to fibrinogen-binding protein, the molecules of which are bound to the peptidoglycan of the cell wall, collagen adhesin, extracellular protein, fibronectin-binding proteins, teichoic acid, as well as staphylococcal haptoglobin receptor residues, consisting of 145 amino acid residues [43].

The nature of the substituents in the molecule determines the varying degrees of lipophilicity of the compounds, which, according to Gibbonson, is an important property of the substance that characterizes its solubility in the bacterial membrane, and the degree of binding to efflux proteins or pump substrates. The hydrophobicity of derivatives serves as a factor that reduces the recognition and transport of antimicrobial agents by a suction pump, which is especially important in the search for the inhibitors of their outflow [29]. Although the lipophilicity of the VMA-13-05 structure suggests better binding to the efflux pump protein, which can lead to the emergence of resistance in *Staphylococcus aureus* and *Streptococcus pneumoniae* due to a decrease in the concentration of the antimicrobial agent, it cannot serve as an objective characteristic of this process without additional data obtained by an alternative methods analysis.

CONCLUSION

Thus, among the synthesized derivatives of quinazolin-4(3H)-one, the substances that exhibit a pronounced antimicrobial activity against *Staphylococcus aureus* (VMA-17-04) and *Streptococcus pneumoniae* (VMA-13-05), have been identified. This is apparently due to the effect of the lipophilic site of their molecules on the manifestation of the antimicrobial action. The results obtained in the course of this study, determine the prospects for further research of the antimicrobial properties of new quinazoline-4(3H)-one compounds in order to increase their pharmacological effect and prevent the development of pathogenic microorganisms' resistance.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Marina A. Samotrueva – research concept and design, research planning, critical intellectual content review, final approval of manuscript for publication; Alexander A. Ozerov – scheme development of derivatives synthesis, obtaining data on physicochemical properties and spectral characteristics of substances, manuscript editing, its final approval for publication; Alla A. Starikova – data collection, text writing, chemical substantiation of ongoing processes based on structures of investigated substances, preparation of manuscript draft; Narmina Mutallimagakyzy Gabitova – carrying out microbiological research, assessment, substantiation and statistical processing of data obtained; Daria V. Merezhkina – implementation of quinazoline derivatives synthesis; Alexandra A. Tsibizova – data collection, assessment, substantiation and statistical processing of data obtained; Ivan N. Tyurenkov – research planning, research methodology, manuscript editing, assessment of results obtained by microbiological methods; final approval of manuscript for publication.

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Проводит обучение по следующим специальностям:

Код специальности	Наименование специальности	Квалификация	Срок обучения
СРЕДНЕЕ ПРОФЕССИОНАЛЬНОЕ ОБРАЗОВАНИЕ			
33.02.01	Фармация очно	ФАРМАЦЕВТ	2 года 10 месяцев
31.02.05	Стоматология ортопедическая очно	ЗУБНОЙ ТЕХНИК	2 года 10 месяцев
БАКАЛАВРИАТ			
38.03.02	Менеджмент очно-заочно, заочно	БАКАЛАВР	5 лет
СПЕЦИАЛИТЕТ			
31.05.03	Стоматология очно	ВРАЧ-СТОМАТОЛОГ ОБЩЕЙ ПРАКТИКИ	5 лет
33.05.01	Фармация	ПРОВИЗОР	5 лет
30.05.01	Медицинская биохимия очно	ВРАЧ-БИОХИМИК	6 лет
МАГИСТРАТУРА			
32.04.01	Общественное здравоохранение очно-заочно	МАГИСТР	2 года
АСПИРАНТУРА			
31.06.01	Клиническая медицина очно/заочно	ИССЛЕДОВАТЕЛЬ. ПРЕПОДАВАТЕЛЬ-ИССЛЕДОВАТЕЛЬ	3 года/4 года
33.06.01	Фармация очно/заочно	ИССЛЕДОВАТЕЛЬ. ПРЕПОДАВАТЕЛЬ-ИССЛЕДОВАТЕЛЬ	3 года/4 года
30.06.01	Фундаментальная медицина очно/заочно	ИССЛЕДОВАТЕЛЬ. ПРЕПОДАВАТЕЛЬ-ИССЛЕДОВАТЕЛЬ	3 года/4 года
ОРДИНАТУРА			
33.08.01	Фармацевтическая технология очно	ИССЛЕДОВАТЕЛЬ. ПРЕПОДАВАТЕЛЬ-ИССЛЕДОВАТЕЛЬ	2 года
33.08.02	Управление и экономика фармации очно	ИССЛЕДОВАТЕЛЬ. ПРЕПОДАВАТЕЛЬ-ИССЛЕДОВАТЕЛЬ	2 года
33.08.02	Фармацевтическая химия и фармакогнозия очно	ИССЛЕДОВАТЕЛЬ. ПРЕПОДАВАТЕЛЬ-ИССЛЕДОВАТЕЛЬ	2 года
31.08.73	Стоматология терапевтическая очно	ИССЛЕДОВАТЕЛЬ. ПРЕПОДАВАТЕЛЬ-ИССЛЕДОВАТЕЛЬ	2 года
31.08.75	Стоматология ортопедическая очно	ИССЛЕДОВАТЕЛЬ. ПРЕПОДАВАТЕЛЬ-ИССЛЕДОВАТЕЛЬ	2 года

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