Научно-практический журнал
Scientific and Practical Journal

ISSN 2307-9266
e-ISSN 2413-2241

# ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

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2019 Том / Volume VII

№ 6

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# PHARMACY & PHARMACOLOGY

Scientific and practical journal
**Volume VII, Issue 6, 2019**
The mass media registration certificate:
ПИ №ФС77–67428 от 13.10.2016
**ISSN 2307-9266 e-ISSN 2413-2241**

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*Union catalogue. Russian Press / Newspapers an journals. Code 94183*
A4 size, 1000 issues circulation. Price free

**Journal "Pharmacy & Pharmacology" is recommended International Comittee Of Medical Journal Editors and included in Higher Attestation Commission, Scopus, Web of Science (ESCI), Russian citation database, eLibrary, ARISTI (All-Russian Institute of Scientific and Technical Information), RSL (Russian State Library), CyberLeninka, Socionet, EMBASE, Chemical Abstracts (CAS), Directory of Open Access Journals (DOAJ), EBSCO Discovery Service, RNMJ, University of CAMBRIDGE, Ulrich'sWeb, Google Scholar, Biefeld Academic Search Engine (BASE), Directory of Open Access Scholarly Resources (ROAD), Research Bible, Open Archives Initiative, Academic Keys, JournalTOCs, WorldCat, OpenAIRE, University of Oxford, The British Library, Universitait Gent, Université de Montréal, University of Saskatchewan.**

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Научно-практический журнал

# ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

Периодичность 6 номеров в год

**Том 7, Выпуск 6, 2019**

Свидетельство регистрации СМИ:
ПИ №ФС77–67428 от 13.10.2016 г.

**ISSN 2307-9266 e-ISSN 2413-2241**

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ISSN 2307-9266 e-ISSN 2413-2241

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

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*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

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# 11-AMINO ACID PEPTIDE IMITATING THE STRUCTURE OF ERYTHROPOIETIN A-HELIX B IMPROVES ENDOTHELIAL FUNCTION, BUT STIMULATES THROMBOSIS IN RATS

**M.V. Korokin[1], V.O. Soldatov[1], A.A. Tietze[2], I.V. Golubev[1], A.E. Belykh[3], M.V. Kubekina[4], O.A. Puchenkova[1], T.A. Denisyuk[3], V.V. Gureyev[1], T.G. Pokrovskaya[1], O.S. Gudyrev[1], M.A. Zhuchenko[5], M.A. Zatolokina[3], M.V. Pokrovskiy[1]**

[1] Belgorod State National Research University
85, Pobeda St., Belgorod, Russia 308015
[2] University of Gothenburg, Department of Chemistry and Molecular Biology, Department of Organic and Medicinal Chemistry
PO Box 462; 9 C, Medicine Aregatan St., Göteborg, Sweden
[3] Kursk State Medical University
3, Karl Marx St., Kursk, Russia 305041
[4] Institute of Gene Biology of the Russian Academy of Sciences
34/5, Vavilov St., Moscow, Russia 119334
[5] Scientific Research Centre, Kurchatov Institute
1, Akademik Kurchatov Square, Moscow, Russia 123098

E-mail: pharmsoldatov@gmail.com

Received 6 December 2019 Review (1) 20 December 2019 Review (2) 26 December 2019 Accepted 27 December 2019

An 11-amino acid peptide imitating the natural structure of B erythropoietin α-helix (P-αB), has a specific affinity to the heterodimeric complex EPOR/CD131.

**The aim** of the study was to test whether P-αB can be positioned as a preventing and treating agent for cardiovascular diseases.

**Materials and methods.** The study was performed on sexually mature male Wistar rats. Endothelial dysfunction was modulated by a 7-days intraperitoneal administration of L-NAME at the dose of 2.5 mg/100 g. P-αB, or erythropoietin (EPO), was used for therapy at the dose of 2.5 μg/100 g × 3 times for 7 days, the total dose was 7.5 μg/100 g. The function of endothelium was estimated by an endothelium-dependent and endothelium-independent vasodilation. In addition, a histological assessment of the abdominal aortic wall state and the analysis of *eNos*, *Tnf* and *Il-1β* genes expression were performed. To estimate prothrombotic properties, P-αB and EPO were administered, at the doses of 2.5 and 5 μg/100 g (3 times a day for 7 days, the total doses were 7.5 μg/100 g and 15 μg/100 g, respectively) and on the 8th day, the time of ferric (III) chloride-induced carotid artery thrombosis was estimated.

**Results.** The results of the functional tests for endothelium-dependent and endothelium-independent vasodilatation, as well as the histological picture of the aorta have evidenced that P-αB and EPO do not affect L-NAME-induced hypertension but improve the endothelium function. At the same time, P-αB shows a significantly higher endothelial-protective activity, reducing the coefficient of endothelial dysfunction from 5.1±0.15 to 2.72±0.12. In addition, P-αB has significantly increased the expression of *eNos* and reduced the expression level of *Tnf* and *Il-1β* mRNA genes. Carrying out Ferric (III) chloride-induced carotid artery thrombosis has revealed that P-αB (5 μg/100 g × 3 times a day for 7 days, total dose was 15 μg/100 g) has a lower but statistically significant prothrombotic activity than EPO.

**Conclusion.** P-αB can be positioned as an atheroprotector because of its ability to prevent the death of endothelial cells, as well as to reduce remodeling and proinflammatory activation of the vascular wall. However, the prothrombotic properties of P-αB limit its use as a preventing and treating agent for atherosclerosis-associated diseases.

**Keywords:** atherosclerosis, erythropoietin, rats, P-αB, cibenitide, endothelium

**Abbreviations:** P-αB – α-helix of B erythropoietin; EPO – erythropoietin; L-NAME – N(ω)-nitro-L-arginine methyl ester; eNOS – endothelial nitric oxide synthase; ED – endothelial dysfunction; EDC – endothelial dysfunction coefficient; SBP – systolic blood pressure; DBP – diastolic blood pressure.

**For citation:** M.V. Korokin, V.O. Soldatov, A.A. Tietze, I.V. Golubev, A.E. Belykh, M.V. Kubekina, O.A. Puchenkova, T.A. Denisyuk, V.V. Gureyev, T.G. Pokrovskaya, O.S. Gudyrev, M.A. Zhuchenko, M.A. Zatolokina, M.V. Pokrovskiy. 11-amino acid peptide imitating the structure of erythropoietin α-helix b improves endothelial function, but stimulates thrombosis in rats. *Pharmacy & Pharmacology*. 2019;7(6): 312-320. **DOI:** 10.19163/2307-9266-2019-7-6-312-320

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**Для цитирования:** М.В. Корокин, В.О. Солдатов, А. Титце, И.В. Голубев, А.Е. Белых, М.В. Кубекина, О.А. Пученкова, Т.А. Денисюк, В.В. Гуреев, Т.Г. Покровская, О.С. Гудырев, М.А. Жученко, М.А. Затолокина, М.В. Покровский. 11-аминокислотный пептид, имитирующий структуру a-спирали b эритропоэтина, улучшает функцию эндотелия, но стимулирует тромбообразование у крыс. *Фармация и фармакология.* 2019;7(6): 312-320. **DOI:** 10.19163/2307-9266-2019-7-6-312-320

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2019-7-6-312-320*

# 11-АМИНОКИСЛОТНЫЙ ПЕПТИД, ИМИТИРУЮЩИЙ СТРУКТУРУ А-СПИРАЛИ В ЭРИТРОПОЭТИНА, УЛУЧШАЕТ ФУНКЦИЮ ЭНДОТЕЛИЯ, НО СТИМУЛИРУЕТ ТРОМБООБРАЗОВАНИЕ У КРЫС

**М.В. Корокин[1], В.О. Солдатов[1], А. Титце[2], И.В. Голубев[1], А.Е. Белых[3], М.В. Кубекина[4], О.А. Пученкова[1], Т.А. Денисюк[3], В.В. Гуреев[1], Т.Г. Покровская[1], О.С. Гудырев[1], М.А. Жученко[5], М.А. Затолокина[3], М.В. Покровский[1]**

[1] ФГАОУ ВО «Белгородский государственный национальный исследовательский университет»
308015, Россия, г. Белгород, ул. Победы, 85
[2] Гётеборгский университет, факультет химии и молекулярной биологии,
отдел органической и медицинской химии
41271, Швеция. Гетеборг, ул. Медицины Арегатан, 9С
[3] ФГБОУ ВО «Курский государственный медицинский университет»
305041, Россия, г. Курск, ул. Карла Маркса, 3
[4] ФГБУН «Институт биологии гена РАН»
119334, Россия, г. Москва, ул. Вавилова, 34/5
[5] НИЦ «Курчатовский институт» – ГосНИИгенетика
123098, Россия, г. Москва, пл. Академика Курчатова, 1

E-mail: pharmsoldatov@gmail.com

Получено 06.12.2019 Рецензия (1) 20.12.2019 Рецензия (2) 26.12.2019 Принята к печати 27.12.2019

**Цель.** 11-аминокислотный пептид, имитирующий природную структуру α-спирали В эритропоэтина (P-αB) обладает специфическим сродством к гетеродимерному комплексу EPOR/CD131. В нашем исследовании мы решили проверить может ли P-αB позиционироваться в качестве средства для профилактики и лечения сердечно-сосудистых заболеваний.
**Материалы и методы.** Исследование выполнено на половозрелых самцах крыс линии Wistar. Дисфункцию эндотелия моделировали путем 7-дневного внутрибрюшинного введения L-NAME в дозе 2,5 мг/100 г. В качестве терапии использовали P-αB или эритропоэтин (EPO) в дозе 2,5 мкг/100 г × 3 раза в течение 7 дней, суммарная доза 7,5 мкг/100 г. Функцию эндотелия оценивали путем проведения эндотелийзависимой и эндотелийнезависимой вазодилатации. В дополнение к этому проводили гистологическую оценку состояния стенки абдоминальной аорты и анализ экспрессии генов eNos, Tnf и Il-1β. Для оценки протромботических свойств P-αB и EPO вводили в дозах 2,5 и 5 мкг/100 г (3 раза в течение 7 дней, суммарная доза 7,5 мкг/100 г и 15 мкг/100 г, соответственно) и на 8-й день оценивали время железа (III) хлорид-индуцированного тромбоза сонной артерии.
**Результаты.** P-αB и EPO не влияют на L-NAME-индуцированную гипертензию, однако улучшают функцию эндотелия, о чем свидетельствуют результаты функциональных проб на эндотелийзависимую и эндотелийнезависимую вазодилатацию, а также гистологическая картина аорты. При этом P-αB демонстрирует значительно бóльшую эндотелиопротективную активность, снижая коэффициент эндотелиальной дисфункции с 5,1±0,15 до 2,72±0,12. Кроме того, P-αB значительно увеличил экспрессию eNos, и снизил уровень экспрессии мРНК генов Tnf и Il-1β. При проведении железа (III) хлорид-индуцированного тромбоза сонной артерии обнаружено, что P-αB (в дозе 5 мкг/100 г × 3 раза в течение 7 дней, суммарная доза 15 мкг/100 г) обладает меньшей, чем EPO, но статистически значимой протромботической активностью.
**Заключение.** P-αB может позиционироваться в качестве атеропротектора ввиду способности предотвращать гибель эндотелиоцитов, а также снижать ремоделирование и провоспалительную активацию сосудистой стенки. Тем не менее протромботические свойства P-αB ограничивают его применение в качестве средства для профилактики и лечения атеросклероз-ассоциированных заболеваний.
**Ключевые слова:** атеросклероз, эритропоэтин, крысы, P-αB, цибенитид, эндотелий
**Список сокращений:** P-αB – α-спираль В эритропоэтина; EPO – эритропоэтин; L-NAME – N-нитро-L-аргинин метиловый эфир; eNOS – эндотелиальная синтаза оксида азота; ЭД – эндотелиальная дисфункция; КЭД – коэффициент эндотелиальной дисфункции; САД – систолическое артериальное давление; ДАД – диастолическое артериальное давление.

## INTRODUCTION

The permanent growth of atherosclerosis-associated diseases in the overall structure of the death causes andthe disability rate in the developed countries, necessitatetheir in-depth study and the improvement of the correction methods [1, 2]. Hereby the experience accumulated since the first works by N.N. Anichkov [3] shows that the atherosclerotic damage of the vascular wall is a long-term multifactorial process [4]. According to the modern understanding of cardiovascular disease pathogenesis, endothelial dysfunction (ED) plays an integral role in the development of atherosclerosis and related complications [5–7]. The changes in the spectrum of molecules secreted and expressed by endothelium, and disruption of its barrier function, eventually lead to the vascular wall infiltration by atheromatous masses

and formation of atherosclerotic plaques [8]. The emerging pathogenetic cascade becomes an actual target for pharmacological influence [1, 2].

An effective way to prevent the endothelium injury is the use of molecules with a universal cytoprotective activity [9]. One of such molecules is an endogenous glycoprotein – erythropoietin (EPO) [10]. A number of our studies have demonstrated that EPO is capable of significantly improving the morphofunctional state of the vascular wall when simulating ED in rats [11–14]. Nevertheless, a long-term experience shows that promising results of EPO preclinical studies are poorly translated into clinical reality and its main niche is still treatment of anemia [15–17].

In 2004, Michael Brines et al. [17] proved that non-hematopoietic effects of EPO are realized via the heterodimeric complex of EPOR/CD131. The discovery of the fact that the erythropoiethic and tissue protective properties of EPO are realized by means of two different receptor systems, has led to the creation of prerequisites for a fundamentally new direction in the search for innovative molecules with a cytoprotective activity.

In 2008, the same authors [18] presented the generalized results of the study of the cytoprotective activity of an 11-amino acid peptide based on erythropoietin α-helix B. It imitates the spatial part of the molecule that interacts with the heterodimeric EPOR/CD131 receptor, but does not interact with the homodimeric EPOR/EPOR receptor.

This compound (P-αB, cibenitide, PubChem CID: 91810664) demonstrated the ability to significantly improve the morphofunctional state of tissues in diabetic macular edema, a renal ischemia/reperfusion injury, and significantly improve cognitive functions in the model of galantamine-induced amnesia in the absence of any effect on erythropoiesis.

**THE AIM** of the research is to evaluate the endothelial- and atheroprotective activity of P-αB and to evaluate the prothrombotic properties of this molecule in order to identify the obstacles in the clinical use of P-αB as a preventing and treating agent for cardiovascular accidents.

## MATERIALS AND METHODS

### Animals

The animals were obtained from the Charles River Laboratories Kennel (Massachusetts, USA). They were kept at the preclinical research center of the Research Institute of Pharmacology of Living Systems. After 14 days of quarantine, the rats were stratified by weight and placed by 9 individuals in separate conventional cages according to their experimental group. Before and during the study, the animals were kept in rooms with artificial lighting (12h/12h mode) at 21–23°C, the humidity of 38–50%, and had a free access to food and water. The number of the conclusion of the Independent Ethical Committee is 06-09/02-1 dated 16 May 2019.

The experiment was performed on 76 male rats (200-220 g) of the Wistar line. The requirements of the Law of the Russian Federation "On Protection of Animals from Cruelty" dated 24 June 1998, the rules of laboratory practice during preclinical studies in the Russian Federation (GOST 3 51000.3-96 and GOST R 53434-2009), Directives of the European Community 86/609EU 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 August 2010), were observed.

### Endothelial dysfunction modeling

Endothelial dysfunction was modeled by a 7-days' intraperitoneal administration of the blocker of endothelial nitric oxide synthase L-NAME (Sigma Aldrich, USA) at the dose of 2.5 mg/100 g. To estimate the endothelial-protective effect of P-αB in comparison with EPO, 4 groups of 9 animals each, were formed of 36 male Wistar rats (200–220 g):

1) ED + EPO (LLC "Pharmapark") (2.5 μg/100 g, 3 times a day for 7 days, the total dose was 7.5 μg/100 g);

2) ED + P-αB (Pharmapark LLC) (2.5 μg/100 g, 3 times a day for 7 days, the total dose was 7.5 μg/100 g);

3) ED + Solvent (0.9% sodium chloride solution 0.1 ml/100 g, 3 times a day for 7 days, the total dose was 0.3 ml/100 g);

4) Intact + Solvent (0.9% sodium chloride solution 0.1 ml/100 g, 3 times a day for 7 days, the total dose was 0.3 ml/100 g).

Exactly 24 hours after the last administration of L-NAME to each animal under anesthesia (Zolazepam (Virbac ( France)) 6 mg/100 g + Chloral hydrate (Panreac (Spain)) 15 mg/100 g), the left carotid artery was catheterized for intravascular blood pressure monitoring using Biopac MP150. Endothelium-dependent (Acetylcholine, 4 μg/100 g) and endothelium-independent (Sodium nitroprusside, 3 μg/100 g) kinds of vasodilation were stimulated against the background of continuous blood pressure monitoring. Vasoactive agents were injected at the intervals of 15 minutes through a catheter installed in the femoral vein. During all manipulations, the animal was assigned a unique code and the surgeon did not know the animal's belonging to the group. The ratio of the area above the pressure drop curve during the administation of sodium nitroprusside to the area above the pressure drop curve during the administation of acetylcholine,was taken as the endothelial dysfunction coefficient (EDC).After the functional vascular tests had been performed, the animals were euthanized by exsanguination and the abdominal part of the aorta was taken for histological studies, as well as for the evaluation of *eNos, Il-1b* and *Tnf* mRNA genes expression.

### Histology

The samples of the abdominal aorta were fixed in

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a 10% formaldehyde solution and then embedded into paraffin wax in a carousel-type machine "STP 120 (Microm International GmbH, Germany). Embedding of blocks with a standard orientation of pieces was carried out at the station for embedding biological material into paraffin wax EC 350 (Microm International GMBH, Germany). To ensure standardization, wax embedding was carried out in the form of multiblocks of 5–6 pieces each.

The sections 5 microns thick for histological examination, were made on a semi-automatic rotary microtome with the system of transportation and distribution of sections "HM 340 E" (Microm International GmbH, Germany).

Hematoxylin and eosin staining was carried out in a machine for staining histological sections and smears (Microm International GMbH, Germany).

Hematoxylin and eosin staining was carried out in a machine for staining histological sections and smears (Microm International GmbH , Germany). A descriptive study of histological preparations was performed under the microscope Axio Scope A1 (Carl Zeiss Microimaging GmbH, Germany).

### Determination of eNos, Tnf and Il-1β expression by quantitative polymerase chain reaction (qPCR)

The aortic tissue was taken out, homogenized and incubated in an "Extract RNA" solution for 10 minutes at 37°C. After the sample lysing in the reagent, it was chloroform cleaned, and the resulting RNA precipitate was washed with isopropyl alcohol and 70% ethyl alcohol. The concentration of the RNA was measured on an IMPLENNanoPhotometer®. The RNA yield was approximately 1000 ng/µl.A reverse transcription was performed using MMLVRTSK021 kit in accordance with the protocol of the manufacturer (Evrogen). The mixture was carefully mixed and heated for 2 minutes at 70°C to melt down the secondary RNA structures and the subsequent annealing of the OligoDT primer. Then the samples were transferred to ice. The entire reaction mixture was incubated for 60 min. at 40 °C in the T100™ThermalCycler (Bio-Rad). To stop the reaction, the mixture was heated at 70°C for 10 minutes. The obtained cDNA was diluted to the concentration of 1 ng/µl. The gene expression level was estimated relative to the Gapdh reference gene values. The calculation of the expression at a specific point was carried out by the formula: Gene expression = 2^[(Ct(Gapdh)-Ct(Gene of interest)] (Table 1).

### Prothrombotic Activity Study

40 rats, divided into five equal groups:

1) EPO (2.5 µg/100 g, 3 times a day for 7 days, the total dose was 7.5 µg/100 g);

2) EPO (5 µg/100 g, 3 times a day for 7 days, the total dose was 15 µg/100 g);

3) P-αB (2.5 µg/100 g, 3 times a day for 7 days, the total dose was 7.5 µg/100 g);

4) P-αB (5 µg/100 g, 3 times a day for 7 days, the total dose was 15 µg/100 g);

5) 0.9% sodium chloride solution (0.1 ml/100 g, 3 times a day for 7 days, the total dose was 0.3 ml/100 g).

24 hours after the last administration of the drug (or a solvent for the control group), the animals were anesthetized and fixed to the operating table. Then, a 10 mm incision was made to the left of the neck middle line, the common carotid artery was isolated and carefully separated from the surrounding tissues without damaging the vagus nerve. Using the ultrasound Minimax-Doppler (St. Petersburg, Russia), the best point for the signal was determined on the selected artery.

After that, a cotton wool moistened with a 50% ferric (III) chloride solution was applied, and the time was had been fixed until the initial signal was reduced to ≈10%. In all manipulations, the animal was assigned a unique code and the surgeon did not know the animal's belonging to the group.

### Statistical analysis

Statistical processing was performed using the R programming language. In the statistical sample, the data distribution type was determined using the Shapiro-Wilk test and the Spiegelhalter test ('normtest' package), the evaluation of the variance equality was determined using the Levene's test ('lawstat' package). Depending on the data distribution type and the variance equality, the significance of the obtained results was assessed using a parametric (ANOVA) or nonparametric (Kruskal–Wallis H test) one-way analysis of variance. The unpaired Student's t-test or Wilcoxon–Mann–Whitney test, respectively, were used as post-hoc analyses to identify the differences in the intergroup comparisons, with Benjamini–Hochberg procedure to decrease the false discovery rate. The results were considered statistically significant at $p \leq 0.05$.

## RESULTS

### Estimation of endothelial dysfunction

An 8-day administration of L-NAME (2.5 mg/100 g) resulted in a significant increase in blood pressure. At the same time, EPO and P-αB therapy had no statistically significant effect on systolic and diastolic blood pressure values (Table 2).

At the same time, the functional tests with acetylcholine and sodium nitroprusside revealed a significant effect of the therapy on the endothelium NO-producing function (Fig. 1A). The group treated with L-NAME and 0.9% sodium chloride solution, demonstrated almost 5-time increasing in EDC (5.1±0.15 c.u. in contrast to 1.21±0.09 in intact animals). In the group treated with EPO and P-αB, this indicator was 3.81±0.14 and 2.72±0.12, respectively.

**Table 1 – Primers for determination of mRNA expression of target and reference genes**

| Primer name | Nucleotide sequence 5'->3' | Melting point (°C) | PCR product size (nucleotide pairs) |
|---|---|---|---|
| Il-1b F | TCGTGCTGTCTGACCCATGT | 61.47 | 126 |
| Il-1b R | AGGCCACAGGGATTTTGTCG | 60.61 | |
| eNos F | GCCAACTCAAGGCAGGAGAC | 60.96 | 129 |
| eNos R | ATCCCCGGAAGGGTGCAATA | 60.99 | |
| Tnf-alpha F | TGAACTTCGGGGTGATCGGT | 61.19 | 152 |
| Tnf-alpha R | CGCTTGGTGGTTTGCTACGA | 61.2 | |
| Gapdh F | AGTGCCAGCCTCGTCTCATA | 60.68 | 141 |
| Gapdh R | TGAGGTCAATGAAGGGGTCGT | 61.11 | |

**Table 2 – Influence of EPO and 11-amino acid peptide P-αB on rat blood pressure in modeling L-NAME-induced endothelial dysfunction**

| Group | SBP (mm Hg) | DBP (mm Hg) |
|---|---|---|
| Intact | 121.5±3.4 | 97.5±3.1 |
| ED + NaCl (0.9%) | 189.9±4.8 | 133.2±4.1 |
| ED + EPO | 186.0±5.1 | 133.2±4.5 |
| ED + P-αB | 190.3±4.3 | 134.3±3.9 |

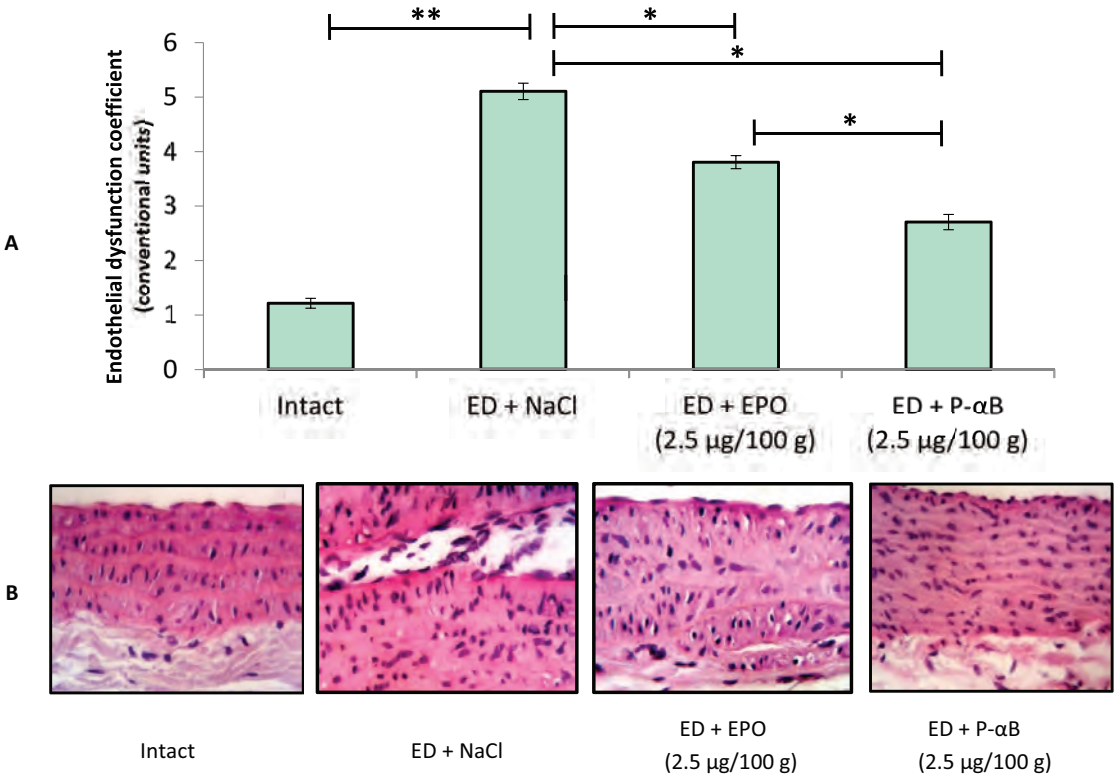


**Figure 1 – Morphofunctional state of the vascular wall**

Note: A) Influence of EPO and P-αB on the endothelial dysfunction coefficient calculated as the ratio of the area above the pressure drop curve during endothelium-independent vasodilation to the area above the pressure drop curve in endothelium-dependent vasodilation; B) Histological picture of the abdominal aorta wall. Intact – Endothelial lining is continuous, endothelial cells are flat. There are no signs of swelling or infiltration. Architectonics is not broken, the ratio of layers is preserved. ED + 0.9% sodium chloride solution – There is swelling of the outer shell, round cell infiltration of the middle shell and vacuolar dystrophy of smooth myocytes. The cell density is high. The ratio of layers is changed in comparison with that of intact animals, endotheliocytes are swollen, most of them are exfoliated from the surface of the basal membrane. ED + EPO – Against the background of disturbances of the architectonics of the middle shell and the initial signs of fibrosis, polymorphocytic infiltration of the outer shell of the vessel is observed; ED + P-αB – In the preparation, a complete safety of the architectonics of the vessel wall layers is visualized. The endothelial lining iskept safe, and the endothelial cells are located on the basal membrane in one layer. There are no signs of pericellular and perivascular edema (stained with hematoxylin and eosin. × 400). * – p≤0,05; ** – p≤0,01.

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### Histological picture of the aortic wall

In the histological study, a similar trend characterizing the endothelial-protective activity of EPO and P-αB, was determined (Fig. 1B).

– L-NAME + 0.9% sodium chloride solution. In the group of the animals with L-NAME-induced ED significant morphological changes in comparison with intact animals were revealed. They consisted in the presence of perivascular and pericellular edema signs, inflammation of all the membranes of the vessel wall (aorta). Single diapedemic haemorrhages were observed in the perivascular tissue, and there were mural microthrombi in the vascular lumen. In the area of endothelial lining, there was swelling of endothelial cells and their exfoliating from the surface of the basal membrane. The nuclei were located along the wall of the blood vessel in small areas with the preserved endothelium. In single endothelial cells, karyolysis, vacuolization of cytoplasm and wrinkling of the cells were observed up to their death. There was a round cell infiltration of the middle and outer shells. Vacuolar dystrophy was observed in the middle shell, in smooth myocytes. In the outer shell, there was deflaking and signs of swelling. The density of the cells in both middle and outer shells was high.

– L-NAME+EPO (2.5 μg/100 g). In this group, polymorphocytic infiltration of the outer shell of the vessel was observed against the background of minor disturbances in the architectonics of elastic membranes, the presence of functionally active fibroblasts (cells with dark-basophilic cytoplasm, large sized, visualized in the center of the middle shell), which may indicate the initial signs of fibrosis. A small number of reactively altered smooth myocytes located between the elastic fenestrated membranes, were visualized. At the same time, the majority of endotheliocytes had a flattened form and were located continuously, their nuclei were oriented parallel to the basal membrane.

– L-NAME+ P-αB (2.5 μg/100 g). In the study of histological slices in the group of the animals after the pharmacological correction by P-αB, almost a complete safety of the architectonics of the vessel wall layers was revealed. The ratio of the layer thicknesses did not differ visually from that in the intact group of the animals. The endothelial lining was kept safe, the endothelial cells were placed on the basal membrane in one layer, the cells were shaped flat, the cytoplasm was slightly oxyphilic. The stick-shaped nuclei were oriented along the blood vessel. No signs of pericellular or perivascular edema were visualized.

A slightly increased cell density per unit of the section area was revealed, but it was without any signs of destruction, observed in the animals without pharmacological therapy.

### Expression of eNos, Tnf and Il-1β according to quantitative PCR

When modeling L-NAME-induced ED in comparison with the intact animals, an increase in the relative expression of the *eNos* mRNA gene was revealed in all groups. At the same time, the *eNOS* expression grows in the following series: ED + 0.9% sodium chloride solution < ED + EPO < ED + P-αB. The level of proinflammatory cytokines mRNA genes *Tnf* and *Il-1*β is characterized by the highest increase in the group of ED without therapy, and the use of EPO and P-αB reduces the degree of their expression (Fig. 2).

### Estimation of prothrombotic activity

When estimating the time of the onset of ferric (III) chloride-induced thrombosis in the animals treated with EPO and P-αB for 8 days, a dose-dependent reduction of the thrombosis time was found out. A more significant prothrombotic effect was demonstrated by EPO, which reduced the thrombosis time to 16.7±1.2 min. (2.5 μg/100 g) and 14.2±1.3 min. (5 μg/100 g) compared to 19.5±0.9 min. in the group not treated with any preparations. P-αB at the dose of 1.25 μg/100 g did not significantly affect the thrombosis time, and at the dose of 2.5 μg/100 g, it accelerated the carotid artery thrombosis time up to 16.2±1.1 min. (Fig. 3).

## DISCUSSION

Our work has shown that the 11-aminoacid peptide P-αB, which has a selective affinity to the heterodimeric receptor EPOR/CD131, is able to reduce the endothelial damage in the L-NAME-induced nitrogen oxide deficiency. The eNOS blockade led to persistent hypertension, which provoked morphological changes in the vessel wall. It was manifested in its hypertrophy, necrosis and architectonics disorders.

With the use of a molecular-biological analysis, an increase in the mRNA expression of the eNos gene, which is probably a compensatory response to hypertension, has been found. At the same time, the level of the proinflammatory cytokine markers – TNF-α and IL-1β – has increased. The triple application of P-αB at the dose of 2.5 μg/100 g did not affect arterial hypertension associated with the introduction of L-NAME, but led to a more pronounced increase in the expression of the eNos mRNA gene. This may be explained by the fact that P-αB has increased the number of functioning cells capable of synthesizing eNOS due to its antiapoptotic properties.It is noteworthy that in the EPO group, despite the decrease in EDC, the eNOS mRNA level did not significantly increase with respect to the control. This is consistent with the data obtained by Sultan F. et al. showing that EPO is capable of suppressing the expression of eNOS [19]. Apparently, this property qualitatively distinguishes the vasotropic activity of P-αB from other erythropoietin preparations.

The results obtained also show that P-αB is able to prevent an inflammatory activation in the L-NAME-induced ED model. This phenomenon is most likely associated with both the antiapoptotic activity of the compound and the intrinsic anti-inflammatory activity of erythropoietin molecules. This property is very important for the potential atheroprotector, as a decrease in the cytokine activation is necessary to stabilize the atherosclerotic plaque and prevent its rupture.

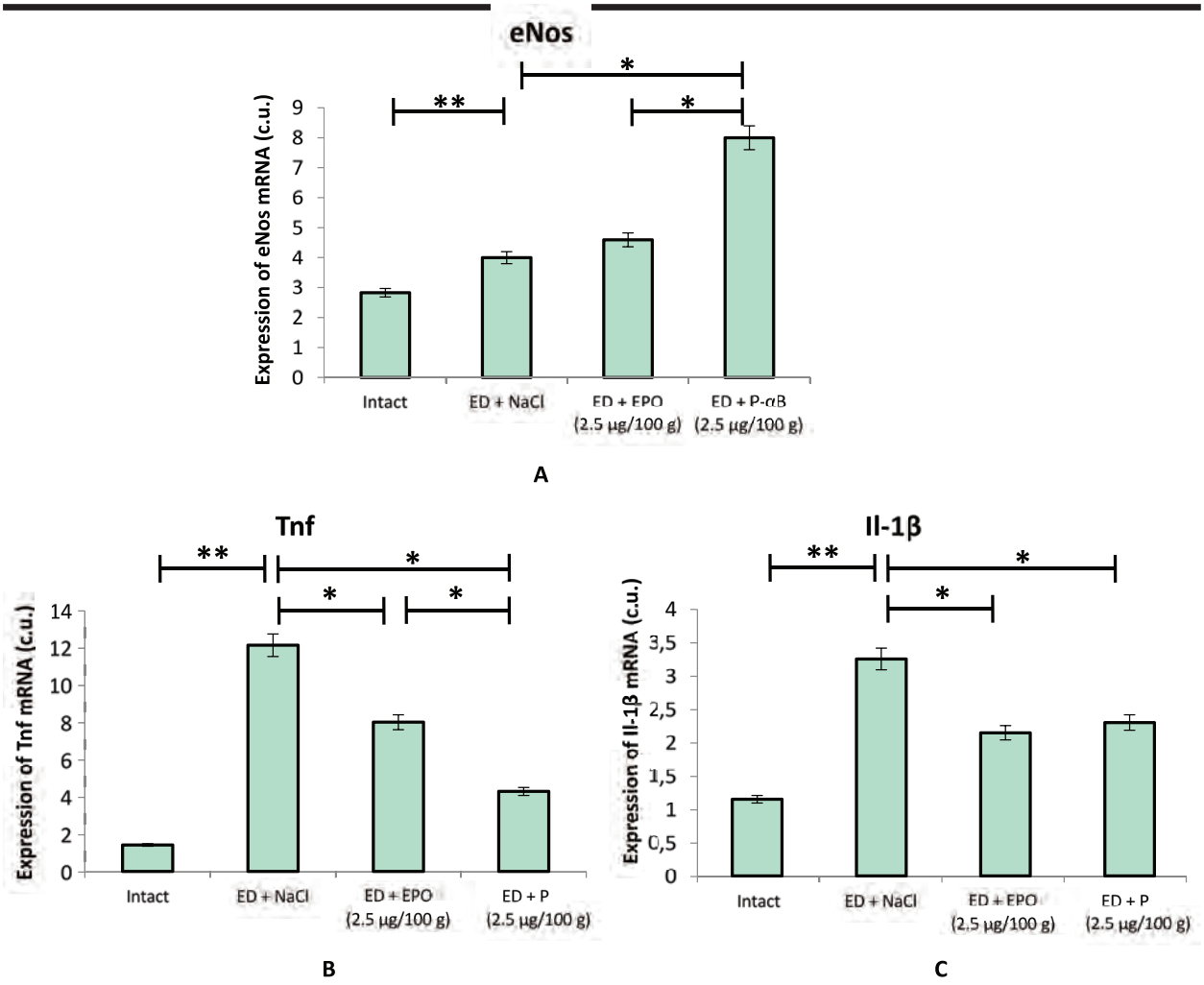


**Figure 2 – Influence of EPO and P-αB on the expression of eNos, Tnf, and Il-1β mRNA genes in the abdominal aorta against the background of L-NAME-induced ED modeling (based on quantitative PCR data)**
Note: * – p≤0,05; ** – p≤0,01.

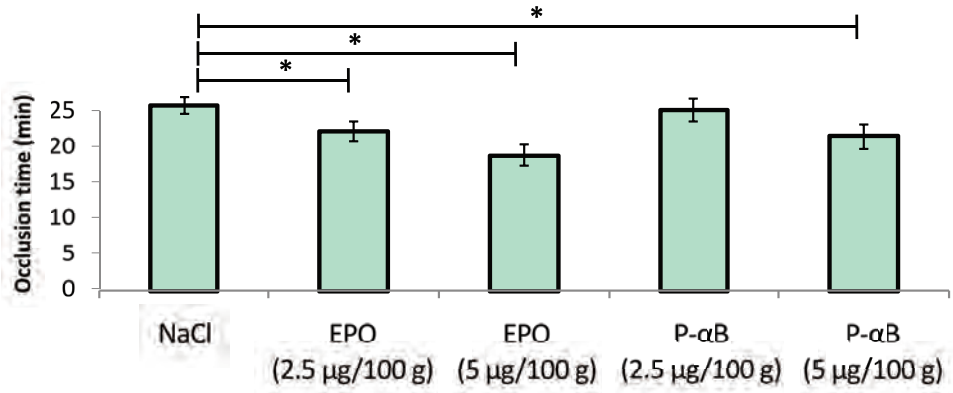


**Figure 3 – Influence of EPO and P-αB on the time of $FeCl_3$-induced thrombotic occlusion of the carotid artery (reduction of Doppler signal to the level of ≈10% from the initial one)**
Note: * – p≤0,05.

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Finally, the last stage of the study with the use of $FeCl_3$-induced carotid artery thrombosis in the rats has revealed that P-αB has prothrombotic properties. The prothrombotic activity of P-αB is less pronounced than that of EPO and has not appeared in the doses at which it demonstrates an endothelial-protective action (2.5 μg/100g × 3 for 7 days). However, this property is a significant limitation in the positioning of P-αB as a preventing and treating agent for cardiovascular diseases associated with atherosclerosis.

We see a perspective in the modification of P-αB by adding peptide motifs with an antiaggregant activity. To eliminate a prothrombotic activity, the amino acid sequences Arg-Gly-Asp and Lys-Gly-Asp, can be added to P-αB. Arg-Gly-Asp and Lys-Gly-Asp are known to have pronounced antiaggregant properties [20–22]. In a number of domestic studies, antithrombotic and antiplatelet properties of another auxiliary amino acid tripeptide, Pro-Gly-Pro, have also been revealed [23–25]. In addition, Pro-Gly-Pro stabilizes the molecule in the biological environment by inhibiting the activity of proteolytic enzymes [26] and has the ability to block angiotensin-converting enzyme [27], one of the most important proatherogenic factors that catalyzes the reaction of angiotensin II formation and promotes vascular wall remodeling [28].

The character (position, linkers, etc.) for the amino acid sequences in the base molecule still remains the key problem. The bioinformatics analysis will make it possible to determine the most optimal localizations for the tripeptide addition, allowing not to influence the interesting pharmacophores, preserving both endothelial-protective and antiplatelet kinds of activity.

## CONCLUSION

The 11-amino acid peptide imitating erythropoietin α-helix B, has a pronounced endothelial-protective and potentially atheroprotective effect due to its ability to prevent the death of endothelial cells, as well as to reduce remodeling and pro-inflammatory activation of the vascular wall. However, the prothrombotic activity of P-αB limits its use as a preventing and treating agent for atherosclerosis-associated diseases and necessitates further modifications of this molecule.

## FINANCIAL SUPPORT

The work is supported by the Russian Ministry of Education and Science. Subsidy Agreement No. 05.605.21.0109 (unique agreement identifier RFMEFI60519X0191).

## AUTHORS' CONTRIBUTION

**M.V. Korokin** – writing the article, the development of the research design, a sample preparation for the histological study, a morphological description of aortic wall sections; **V.O. Soldatov** – writing the article, developing a research design; **Alesia A. Tietze** – peptide synthesis, literature analysis; **I.V. Golubev** – the administration of drugs to animals, modeling a L-NAME-induced endothelial dysfunction; **A.E. Belykh** – mRNA isolation, reverse transcription reaction, analysis of mRNA expression of eNos, Tnf and Il-1*β* genes, translation of the article. **M.V. Kubekina** – mRNA isolation, reverse transcription reaction, analysis of mRNA expression of eNos, Tnf, and Il-1*β* genes, formalization of the literature list, work with graphic material; **O.A. Puchenkova** – work with the animals in all stages, separation of RNA, sampling for histological study. **T.A. Denisyuk –** participation in the evaluation of the endothelial dysfunction coefficient; **V.V. Gureyev** – estimation of the endothelial dysfunction coefficient; **T.G. Pokrovskaya** – consultation on planning, methodology and implementation of the experiment; **O.S. Gudyrev** –prothrombotic activity assessment, literature analysis; **M.A. Zhuchenko** – peptide synthesis, literature analysis; **M.A. Zatolokina** – preparation of samples for histological examination, morphological description of aortic wall sections; **M.V. Pokrovskiy** – the idea, research planning, consultation on the implementation of the individual phases of the pilot works.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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## AUTHORS

**Mikhail V. Korokin** – Doctor of Sciences (Medicine), Associate Professor, Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University. ORCID ID: 0000-0001-5402-0697. E-mail: mkorokin@mail.ru

**Vladislav O. Soldatov** – Assistant of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University.ORCID ID: 0000-0001-9706-0699. E-mail: pharmsoldatov@gmail.com

**Alesia A. Tietze** – Senior Lecturer (Assistant Professor) in Medicinal Chemistry with focus on total synthesis of bioactive peptides, Department of Chemistry and Molecular Biology, Division of Organic and Medicinal Chemistry, University of Gothenburg. ORCID ID: 0000-0002-9281-548X. *E*-mail: a.tietze@tietze-lab.com

**Ivan V. Golubev** – Applicant of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University. ORCID ID: 0000-0002-3754-0380. E-mail: golubevvano@yandex.ru

**Andrey E. Belykh** – Candidate of Sciences (Medicine), Associate Professor of Pathophysiology Department, Kursk State Medical University. ORCID ID: 0000-0001-9766-2104. E-mail: a.e.belykh@gmail.com

**Marina V. Kubekina** – Post-Graduate Student, Institute of Gene Biology of the Russian Academy of Sciences. ORCID ID: 0000-0002-8834-1111. E-mail: marykumy@gmail.com

**Olesya A. Puchenkova**– 5th – year student at the Medical Institute, Belgorod State National Research University. ORCID ID: 0000-0002-7657-0937. E-mail: lesya759@yandex.ru

**Tat'yana A. Denisyuk** – Doctor of Sciences (Medicine), Associate Professor of the Pharmacology Department, Kursk State Medical University. ORCID ID: 0000-0003-0974-4818. E-mail: denitatyana@yandex.ru

**Vladimir V. Gureyev** – Doctor of Sciences (Medicine), Associate Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University. ORCID ID: 0000-0003-1433-1225. E-mail: produmen@yandex.ru

**Tat'yana G. Pokrovskaya** – Doctor of Sciences (Medicine), Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University. ORCID ID: 0000-0001-6802-5368. E-mail: pokrovskaia-tg@mail.ru

**Oleg S. Gudyrev** – Candidate of Sciences (Medicine), Associate Professor, Associate Professor of the Department of Pharmacology and Clinical Pharmacology, Belgorod State National Research University. ORCID ID: 0000-0003-0097-000X. E-mail: gudyrev@mail.ru

**Maksim A. Zhuchenko** – Candidate of Sciences (Biology), the Section Chief, Kurchatov Institute. E-mail: maksim.zhuchenko@pharmapark.ru

**Mariya A. Zatolokina** – Doctor of Sciences (Medicine), Associate Professor, Professor of the Department of Histology, Cytology, Embryology, Kursk State Medical University. ORCID ID: 0000-0002-9553-1597. *E*-mail: marika1212@mail.ru

**Mikhail V. Pokrovskiy** – Doctor of Sciences (Medicine), Professor of the Department of Pharmacology and Clinical Pharmacology, the Head of the Research Institute of Pharmacology of Living Systems, Belgorod State National Research University. ORCID: 0000-0002-2761-6249. E-mail: mpokrovsky@yandex.ru

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2019-7-6-321-331*

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# STRUCTURAL TRANSFORMATIONS OF THERMAL BURN WOUNDS IN RATS UNDER THE INFLUENCE OF SEMAX AND SELANK NEUROPEPTIDES

**A.K. Azhikova[1], B.V. Feldman[1], L.A. Andreeva[2], D.D. Teply[3], M.A. Samotrueva[1], N. F. Myasoedov[2]**

[1] Astrakhan State Medical University
121, Bakinskaya Str., Astrakhan, Russia 414000
[2] Institute of Molecular Genetics, Russian Academy of Sciences
2, Acad. Kurchatov Square, Moscow, Russia 123182
[3] Astrakhan State University
1, Shahumyan Square, Astrakhan, Russia 414000

E-mail: alfia-imacheva@mail.ru

Received 8 August 2019 Review (1) 25 October 2019 Review (2) 18 November 2019 Accepted 11 December 2019

The morphological features of the skin of rats under the stress exposure, such as burn injury and against the background of the correction by the drugs of the peptide structure, have been studied.
**The aim of the study** was to investigate the regulatory effect of the neuropeptide drugs Semax (Met-Glu-His-Phe-Pro-Gly-Pro) and Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro) under the conditions of thermal burn exposure.
**Materials and methods.** The object of the study was integumentary tissues (skin, subcutaneous tissue) of laboratory non-linear male rats (n = 36) excised from the thermal injury zone. A thermal burn of the skin had been caused by the application of a copper object in the interscapular in the interscapular dorsal area. Starting from the first day after the injury, neuropeptide drugs Semax (Met-Glu-His-Phe-Pro-Gly-Pro) and Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro) had been administered intraperitoneally at the doses of 100 μg/kg daily during the entire period of the experiment. To assess the condition of the skin tissues, histological sections 5–6 μm thick were preparев, stained afterwards with hematoxylin and eosin. The consistency of the systemic effect of the neuropeptide drugs was estimated by a change in some indicators of the immune system.
**Results.** The thermal exposure led to the development of significant degenerative and dystrophic changes in the skin. The recovery of the burn wounds on rats' skins proceeded according to the type of the delayed partial reparative regeneration, accompanied by destructive phenomena and the formation of the scar tissue.
Under the influence of the drugs based on the regulatory peptides of Semax (Met-Glu-His-Phe-Pro-Gly-Pro) and Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro), the recovery of burn wounds in rats proceeded more intensively, compared with the animals from the group without any correction. This fact was confirmed by the earlier signs of the beginning of the reparative skin regeneration: the restriction of destructive processes within the epidermis and dermis, the absence of purulent-necrotic complications, the initial phases of granulation and epithelization, an early scab rejection and a partial closure of the defect.
**Conclusion**. The use of the neuropeptide drugs Semax and Selank as systemic remedies for the correction of wound skin defects in experimental animals proves their polypotent effectiveness, expands the therapeutic possibilities and opens up new prospects for their use.
**Keywords:** burn wound, structural transformations, neuropeptide regulation, Semax, Selank

# СТРУКТУРНЫЕ ПРЕОБРАЗОВАНИЯ ТЕРМИЧЕСКОЙ ОЖОГОВОЙ РАНЫ У КРЫС В УСЛОВИЯХ ВОЗДЕЙСТВИЯ НЕЙРОПЕПТИДОВ СЕМАКС И СЕЛАНК

**А.К. Ажикова[1], Б.В. Фельдман[1], Л.А. Андреева[2], Д.Д. Теплый[3], М.А. Самотруева[1], Н.Ф. Мясоедов[2]**

[1] Федеральное государственное бюджетное образовательное учреждение высшего образования «Астраханский государственный медицинский университет» Министерства здравоохранения Российской Федерации
414000, Россия, г. Астрахань, ул. Бакинская, д. 121
[2] Федеральное государственное бюджетное учреждение науки Институт молекулярной генетики Российской академии наук
123182, Россия, г. Москва, пл. акад. Курчатова, 2

**For citation:** A.K. Azhikova, B.V. Feldman, L.A. Andreeva, D.D. Teply, M.A. Samotrueva, N.F. Myasoedov. Structural transformations of thermal burn wounds in rats under the influence of Semax and Selank neuropeptides. *Pharmacy & Pharmacology*. 2019;7(6): 321-331. **DOI:** 10.19163/2307-9266-2019-7-6-321-331

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**Для цитирования:** А.К. Ажикова, Б.В. Фельдман, Л.А. Андреева, Д.Д. Теплый, М.А. Самотруева, Н.Ф. Мясоедов. Структурные преобразования термической ожоговой раны у крыс в условиях воздействия нейропептидов Семакс и Селанк. *Фармация и фармакология.* 2019;7(6): 321-331. **DOI:** 10.19163/2307-9266-2019-7-6-321-331

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

[3] Федеральное государственное бюджетное образовательное учреждение высшего образования
Астраханский государственный университет
414000, Россия, г. Астрахань, пл. Шаумяна, 1

E-mail: alfia-imacheva@mail.ru

Получено 08.08.2019 Рецензия (1) 25.10.2019 Рецензия (2) 18.11.2019 Принята к печати 11.12.2019

В работе изучены морфологические особенности кожи крыс при стрессорном воздействии – ожоговой травме и на фоне коррекции лекарственными средствами пептидной структуры.

**Цель исследования** – изучение регулирующего влияния нейропептидных лекарственных средств Семакса (Met-Glu-His-Phe-Pro-Gly-Pro) и Селанка (Thr-Lys-Pro-Arg-Pro-Gly-Pro) в условиях термического ожогового воздействия.

**Материалы и методы.** Объектом исследования служили покровные ткани (кожа, подкожная клетчатка) лабораторных животных-нелинейных крыс-самцов (n=36), иссеченные из зоны термической травмы. Термический ожог кожи вызывали наложением медного предмета в межлопаточной области спины крыс. Начиная с первых суток после травмы внутрибрюшинно вводили нейропептидные лекарственные средства Семакс (Met-Glu-His-Phe-Pro-Gly-Pro) и Селанк (Thr-Lys-Pro-Arg-Pro-Gly-Pro) в дозах 100 мкг/кг ежедневно в течение всего периода эксперимента. Для оценки состояния тканей кожи изготавливали гистологические срезы толщиной 5–6 мкм, которые окрашивали гематоксилином и эозином. Состоятельность системного влияния нейропептидных препаратов оценивали по изменению некоторых показателей иммунной системы.

**Результаты.** Термическое воздействие вело к развитию значительных дегенеративных и дистрофических изменений в коже. Восстановление ожоговой раны кожи крыс протекало по типу замедленной частичной репаративной регенерации, сопровождающейся деструктивными явлениями и формированием рубцовой ткани. Под влиянием лекарственных средств на основе регуляторных пептидов Семакса (Met-Glu-His-Phe-Pro-Gly-Pro) и Селанка (Thr-Lys-Pro-Arg-Pro-Gly-Pro) восстановление ожоговых ран у крыс протекало интенсивнее, по сравнению с животными из группы без коррекции, что подтверждалось более ранними признаками начала репаративной регенерации кожи: ограничением деструктивных процессов в пределах эпидермиса и дермы, отсутствием гнойно-некротических осложнений, начальными фазами грануляции и эпителизации, ранним отторжением струпа и частичным закрытием дефекта.

**Заключение.** Применение нейропептидных лекарственных средств Семакса и Селанка в качестве системных средств коррекции раневых дефектов кожи экспериментальных животных доказывает их полипотентную эффективность, расширяет терапевтические возможности и раскрывает новые перспективы их применения.

**Ключевые слова:** ожоговая рана, структурные преобразования, нейропептидная регуляция, Семакс, Селанк

## INTRODUCTION

In recent years, the study of pathogenesis of thermal skin injuries has been aimed at identifying the peculiarities of the response of immune, nervous and endocrine systems which provide mechanisms for activating regenerative processes. This study has also been aimed at searching for new means of correcting intersystem interactions in pathological changes [1–4]. Numerous studies consider complex relationships through which reactions of neurogenic and immunologically induced inflammation, are carried out under the conditions of skin damage [5–8].

Against the background of thermal stressful effects, alongside with destructive, dystrophic [9–13] and infectious manifestations in the skins, Imbalance, dysfunction of immunocompetent cells, insufficient local resistance [14–17], disorganization in the emotional zones of the brain [17–20], formation and triggering of stress-adaptive and / or stress-maladaptive reactions take place [22–26]. Currently, peptide drugs that act as regulators of the intersystem interaction, are actively used as effective remedies for the correction of stress-induced disorders of homeostasis in the clinical practice. In this regard, neuropeptide drugs with a nootropic, neurometabolic, immunomodulating effect, in particular, an adrenocorticotropic hormone analogue, Semax (Met-Glu-His-Phe-Pro-Gly-Pro) and a Tuftsin derivative, Selank (Thr-Lys- Pro-Arg-Pro-Gly-Pro), are of great interest [27, 28]. The use of synthesized peptide preparations increases the adaptive abilities of the body under stressful conditions, helps restore the structures of the central nervous system, and corrects immune and humoral disorders. In addition, under the stress exposure, these drugs have a corrective effect on somatic disorders, restoring adequate motivational activity and activating adaptive behavior [27–29]. Experimental and clinical data also confirm anxiolytic, antidepressant, psychoactivating, stress-protective, vegetotropic effects of these drugs [32–34]. However, the results of recent studies do not give a complete picture of the reparative potential of these drugs and the mechanisms of their stimulating dermatropic action.

Nowadays, the stimulation of the skin's regenerative potential is reduced to the use of the agents that comprehensively correct violations of neuroimmune endocrine interactions and the skin [35, 36]. One of the types of the systemic treatment is a neuropeptide correction. So, in the study on the immunotropic, antioxidant effects of a new thymogen analog H2N-L-Glu-L-Trp-D-Ala-COOH, modified with the D-alanine from the C-terminus of the peptide under the conditions of skin wounds, the reparative properties of the neuropeptide have also been established [37].

An experimental study of the peptide preparations Gly-His-Lys, dalargin and thymogen, revealed their participation in the reparative regeneration of the skin, ex-

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DOI: 10.19163/2307-9266-2019-7-6-321-331

pressed by the activation of reparative processes and stimulation of healing skin wounds [38]. A significant improvement in the course of psoriasis treatment has been proven: a decrease in the activity of the inflammatory process and a decrease in the area of psoriasis-affected skin in the patients taking Semax as a part of the complex therapy.

The results of the study describing a combined treatment of acne with the Semax neuropeptide drug and a supravenous laser blood irradiation, showed a decrease in skin manifestations and a change in personality self-esteem among alexithymic patients [39]. The use of Semax in the complex treatment of acne patients, contributed to an increase in the clinical efficacy of the therapy.

An important role of regulatory peptides in the correction of physiological stress-limiting mechanisms, is evidenced by the Semax correction as a part of the complex therapy of atopic dermatitis [40]. It has been found out that the use of heptapeptide has a beneficial effect on the reduction of itching, sleep quality and dynamics of the dermatological indices against the background of the absence of the side effects characteristic of a glucocorticoid hormone therapy.

The obtained results substantiate the position of a close structural and functional specific interaction of the neuroendocrine, immune systems and skin, both under physiological conditions and in the development of certain skin pathologies.

The research data on the detection of the healing neuropeptides effects, have determined the scientific interest and significance of studying the neuropeptide regulation of reparation under the conditions of burn exposure.

**THE AIM** of the study was to investigate the regulatory effect of the neuropeptide drugs Semax (Met-Glu-His-Phe-Pro-Gly-Pro) and Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro) under the conditions of thermal burn exposure.

## MATERIALS AND METHODS

### Laboratory research

The experiment was carried out on 36 non-linear white male rats, aged 7 months, weighing 200–230 g, provided by the Federal State Budget Educational Institution of Higher Education "Astrakhan State University" (Astrakhan). The animals were kept under standard vivarium conditions (natural light conditions, at the air temperature of 22–24°C and the humidity of 60±5%) at the Department of Pharmacognosy, Pharmaceutical Technology and Biotechnology (FSBEI HE Astrakhan State Medical University, Ministry of Health of Russia), in individual cages with a sawdust litter and a free access to water and food.

The animals were divided into 4 groups depending on the type of the exposure and correction:

Group I – the animals not exposed to burn effects – intact (n = 8);

Group II – the animals exposed to burn effects and not receiving correction remedies (control, n = 8);

Group III – the animals exposed to burn effects and receiving a daily intraperitoneal administration of 100 mg/kg Semax peptide dissolved in water (n = 10);

Group IV – the animals exposed to burn effects and receiving a daily intraperitoneal administration of Selank peptide dissolved in water at the dose of 100 μg/kg (n = 10).

During the experiment, the general condition of the laboratory animals was estimated. All manipulations with animals were carried out in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 1986), the Helsinki Declaration adopted by the General Assembly of the World Medical Association (1964–2013), Order No. 199n of the Ministry of Health of Russia dated April 1 2016 "On the approval of the rules of good laboratory practice".

### Burn injury model

The burn wounds on the rats' skins, were modeled in the depilated zone of the interscapular dorsal area with the help of a copper object with the diameter of 1.5 cm, heated in boiling water to the temperature of 100° C, under the conditions of weak ether anesthesia. The exposure duration was 5 seconds.

In the animals of the control group, the reparation of the skin defect took place naturally. Starting from the first day after the thermal exposure, the individuals of experimental groups III and IV were administrated intraperitoneally with Semax and Selank peptides dissolved in water, respectively (Peptogen Innovation Research and Production Center, Russian Federation, Institute of Molecular Genetics, Russian Academy of Sciences, Russia), at the dose of 100 mcg/kg a day for 10 days daily, throughout the whole period of the experiment.

During the experiment, the general condition of the animals was observed [41], the appearance and size of the burn wounds, the nature of the discharge and the timing of the scab discharge were evaluated, photographings surveys of the wound surfaces were carried out.

The complex effect of neuropeptide drugs was estimated by a change in some indicators of the immune system. The functional activity and adaptability of the immune system were estimated by the following tests – determining the total number of leukocytes, assessing the leukocyte formula and leukocyte coefficient, and determining the mass of immunocompetent organs.

The degree of reparation of the skin defect was estimated planimetrically. To do this, a transparent film coating was applied to the wound with the borders of the wound marked. The size and area of the wounds were measured immediately after the simulation of burn wounds and then daily till the end of the experiment. During the experiment, the wound surface was determined according to the method developed by V.Ya. Vasyukov, N.V. Protsenko (1993). The area of the wound surface corresponded to the area of the circle: $S = \pi \times R^2$, where R is the distance from the center of the wound to its periphery.

Based on the parameter of "the wound area", the percentage reduction of the wound area from the initial size was calculated, which served as a criterion for the rate of epithelization of the damaged skin. The degree of healing of a burn wound, or the rate of epithelialization (ΔS), was calculated as a percentage of a change in the area relative to the original group, according to L.N. Popova' formula (1942): $\Delta S = ((S - Sn)/S \times t) \times 100$, where: S is the size of the wound area in the first measurement ($cm^2$), Sn is the value of the area of the wound on the day of the subsequent measurement ($cm^2$), t is the number of the days between measurements.

### Sampling and preparation of biomaterial

Skin tissues were a biological material in the study. The material was sampled on the 10th day after the start of the experiment. The animals were taken out of the experiment under anesthesia by decapitation using sodium etaminal. For the morphological study of the skin, the tissues (epidermis, dermis, subcutaneous tissue) excised from the zone of the thermal damage in the interscapular dorsal area of the rats of all studied groups, were used.

The skin samples were fixed in 10% neutral buffered formalin (Biovitrum LLC, Russia). The material was washed, carried out on solutions of alcohols of various concentrations, solutions of xylene, then it was embedded in paraffin, and sections 5–6 μm thick were made. To assess the general morphological state of the skin layers, they were stained with hematoxylin and eosin.

Microscopic observations of histological preparations were carried out in transmitted light at 100x magnification (10x lens, WF-10x/18 eyepiece) of the Altami BIO 8 digital biological trinocular microscope (Altami LLC, Russia). Microphotography was carried out using a 3 megapixel camera and Altami Studio 3.4x64/Ink software.

### Methods of statistical processing of results

Statistical processing of the results was carried out using the Excel 2000 program by methods of variation statistics, including the calculation of average values (M), standard errors of average values (± m). To assess the reliability (significance) of differences between the two average values, Student t-test with Bonferroni correction was used. The differences were considered significant at $p \leq 0.05$.

## RESULTS

As a result of the experimental study it was established that a thermal burn on the skin of the rats caused not only local reactions, but also led to changes in the general condition of the body – there was "fading" and loss of hair, disorganized movements of the animals along the cage and a decrease in the dynamics of weight gain.

A microscopic analysis of histological skin tissue sections of the rats' burn wounds of all experimental groups, showed the presence of structural changes in the skin compared with the intact animals (group I – control) (Fig. 1). The epidermis of the interscapular area of the intact rats is represented by stratified squamous keratinizing epithelium. The stratum corneum is represented by a uniform layer. In the prickly layer, there are up to 2 rows of keratinocytes, in the granular layer there are 2–3 rows of nuclear-free cells. At the level of the germinal or malpighian layer, epithelial cells are visible. The cells of the basement membrane are arranged in a single row. On the level of the dermis, there are sebaceous glands and hair follicles, vessels of the microvasculature.

Along with the signs of the general behavioral disorganization, in the animals of the second group, pronounced destructive changes capturing the epidermis, dermis and hypodermis were observed. In this case, inflammation phenomena prevailed over reparative ones. In the area of the burn surface, a purulent inflammatory reaction was observed macroscopically, in some animals a hemorrhagic scab of a gray-brown color was observed. By the 10th day, there was a sucrose discharge in the animals, the wound cavity was covered with a necrotic mass.

It was microscopically revealed that the heat affected zone was devoid of stratified squamous epithelium, and in some places, necrotic elements in the form of folds were observed. The hyperemic dermis looked edematous due to the serous inflammation, a large amount of exudate and cellular elements; there was no pronounced differentiation into papillary and reticular layers. The papillary layer of the epidermis was disrupted. In the reticular layer, intercellular swelling and infiltration by macrophages and lymphocytes were observed. The basal membrane could not be clearly determined (Fig. 2).

Against the background of the thermal injury correction of the rats' skins with preparations of a neuropeptide nature, the animals of groups III and IV showed signs of improvement in the general condition, activity restoration and positive weight dynamics.

One of the stages of the work was the study of the rats' immune state in conditions of a "burn" stress and its correction.

As a result of the research of Semax and Selank effect on the total number of leukocytes and leukocyte counts under thermal burn conditions, it was established that these peptide preparations activated the leukocyte reaction: the total number of leukocytes increased; among them lymphocytes, eosinophils, monocytes, segmented neutrophils were restored to normal values.

The administration of neuropeptides led to a decrease in stab forms of neutrophils by more than 50% ($p<0.001$), to a normalization of the number of segmented neutrophils by 50% ($p<0.001$). (Tab. 1). An increase in the level of the Garkavi index under the conditions of thermal burns, can be interpreted as the degree of the response to the stress state, the so-called "reactivation" of the body in the conditions of an active inflammatory process. The use of Semax and Selank contributed to the restoration of the index to normal values, and the activation of adaptation reactions.

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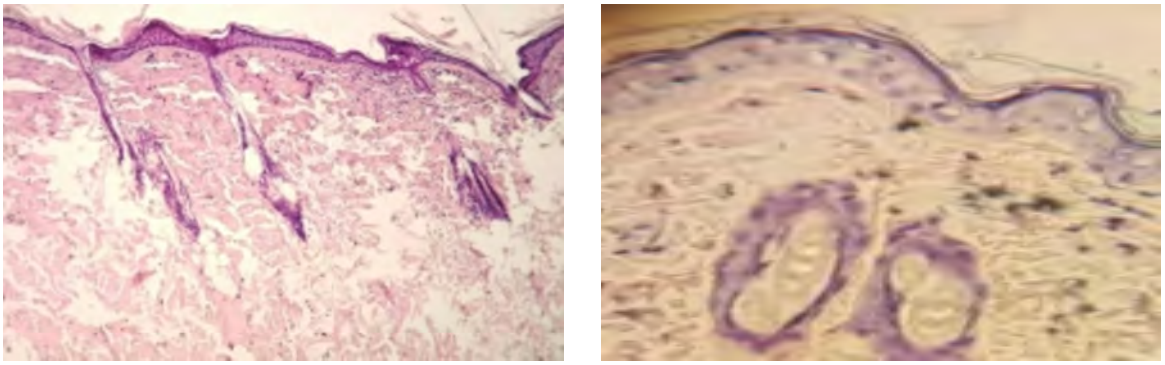
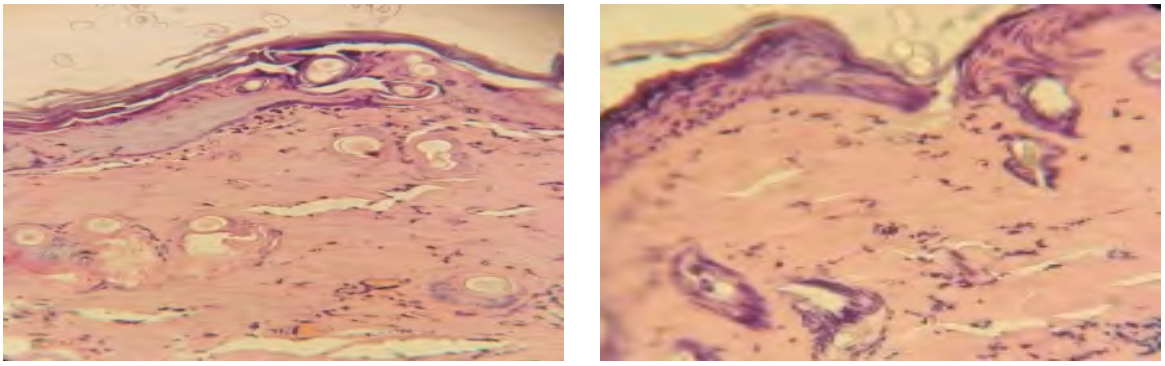
DOI: 10.19163/2307-9266-2019-7-6-321-331



A B

**Figure 1 – Micrograph of a skin flap excised in intact rats on day 10**

Note: stained with hematoxylin and eosin (UV. X100, vol. 10x (A). UV. X 400, vol. 40x (B), approx. WF-10x / 18)



A B

**Figure 2 – Micrograph of a skin flap excised in the wound 10 days after the burn exposure in the rats not treated with correction remedies**

Note: stained with hematoxylin and eosin (UV. X100, vol. 10x (A). UV. X 400, vol. 40x (B), approx. WF-10x / 18)

A – The edge of the burn wound. At the level of the epidermis and dermis, there is a pronounced coagulation necrosis and inflammatory infiltration; in the underlying fatty tissue there is lymphoid infiltration (hyperplasia of lymphoid follicles).

B – Necrotic changes in violation of the structure of tissues

**Table 1 – The effect of Semax and Selank neuropeptides on the functional activity of the immune system**

| Experimental groups (n = 10) / Indices (M ± m) | Group I Intact Group | Group II Control Group | Group III (animals treated with Semax) | Group IV (animals treated with Selank) |
|---|---|---|---|---|
| Total number of leukocytes, x109/l | 27.7±0.4 | 84.5±4.5* | 103.3±0.2### | 99.3±0.6## |
| Eosinophils, % | 5.7±0.2 | 4.3±0.2*** | 6.7±0.2### | 6.3±0.2### |
| Stab neutrophils, % | 12.0±0.4 | 26.5±0.6* | 14.2±0.2### | 14.9±0.4### |
| Segmentonuclear neutrophils, % | 19.3±0.2 | 13.9±0.4*** | 20.8±0.2### | 19.7±0.4### |
| Lymphocytes, % | 61±0.6 | 53.7±1.1* | 56.3±0.4## | 57.3±0.6# |
| Monocytes, % | 2.0±0.2 | 1.6±0.2* | 2.0±0.1## | 1.8±0.2## |
| Leukocytal coefficient | 3.1±0.4 | 3.9±0.4* | 2.7±0.2## | 2.9±0.4## |
| Thymus mass, mg | 51±0.4 | 31±5.4** | 73±6.0## | 68±0.4## |
| Spleen mass, mg | 352.3±0.6 | 456±10.3** | 572±39.6## | 583±31.4## |

Note: * – p <0.05; ** – p <0.01; *** – p <0.001 – relative to the intact group; # – p <0.05; ## –p <0.01; ### – p <0.001 - relative to the control group; Student t-test with Bonferroni correction for multiple comparisons.

The study has also established that thermal burns provoked involution of immunocompetent organs: the spleen – by more than 60% ($p < 0.01$), the thymus – by more than 40% ($p < 0.01$). The studied drugs contributed to an increase in the mass of the spleen by an average of 40% ($p < 0.01$), as well as the mass of the thymus by more than 50% ($p < 0.01$), compared with the control group.

In the course of macroscopic observations it was found out, that the use of Semax and Selank neuropeptides at the dose of 100 μg/kg a day under the conditions of thermal burn exposure leveled the development of the inflammatory process, helped to tighten the edges of the wound and, as a result, reduce its size on the 4th day. As the data in Tables 2 and 3 show, a decrease in the area of the burn wound by more than 60% ($p < 0.01$) in the experimental groups on the 10th day of the post-burn process was observed. It corresponded to the acceleration of wound recovery by 30% ($p < 0.05$), compared with the control group (Tab. 4, 5).

In Group III of the animals exposed to burn effects and treated with Semax, the wound surface was weakly edematous in the center, and along its edges there were growing hair shafts. There were inflammatory manifestations of the surrounding tissues in the form of a mild swelling. The boundaries of the wound narrowed significantly. Under a partially detached scab, an increase in granulation tissue was manifested. From the edges of the wound, there was also an epithelium growth.

A microscopic analysis of the skin fragments excised on the 10$^{th}$ day of the experiment in the area of the thermal injury, showed slightly destroyed epidermal layers and a partial necrosis of the stratified squamous epithelium. The epidermis was represented by a dark plate, sometimes exfoliated from the papillary dermis. Under the scab, the regenerate zone was determined in the form of a wide layer of newly formed granulation tissue (Fig. 3).

The papillary layer looked edematous, without contours of connective tissue fibers, but dark nuclei of connective tissue cells were identified. The papillary layer retained uneven edges. The reticular layer appeared to be broad, mildly swolen; dark nuclei of connective tissue cells were observed. The lower part of the mesh layer was infiltrated by lymphocytes. Hair follicles made of necrotically altered collagen fibers, were found out; single cells with light nuclei were found in hair sheaths. Mild neoangiogenesis was observed.

A macroscopic analysis of burn animal the IV groups wounds showed that on the 10$^{th}$ day after the burn exposure and Semax correction, a slightly swollen and hyperemic wound with uneven outlines, without purulent exudate and necrotic elements, was observed on the rats' skin. The inflammation of the tissue surrounding the wound, was moderate. The process of the peripheral wound epithelization was visualized. There was an increase in granulation tissue from the bottom of the wound. Under a partially detached scab, skin appendages appeared.

A morphological picture in the area of the burn wound of the skin excised on the 10th day after the burn exposure and Selank correction (Group IV) (Fig. 4), is comparable with the visualization of Semax activity. During this time period, activation of vascular neoplasm, hypertrophy of the border zone of the epidermis were established, and proliferating cells were noticeable. The upper layers of the epidermis were exfoliated. In their place, dark layers were found out. They had exfoliated from the basement membrane. The basal membrane itself was represented by a wide dark-colored plate on the papillary layer. The papillary layer was characterized by a small width and a wavy shape. In the upper part of it, there was a partial necrosis. The reticular layer was wide, its upper part was saturated with the nuclei of connective tissue cells. Necrotic changes were observed in the lower part of the reticular layer, although lymphocyte infiltration occurred in most fields of view. Hair follicles consisting of necrotic altered collagen fibers, were established. In the lower parts of the hair sheaths, separate epithelial light cells with rounded nuclei were determined. Around the hair follicles, lymphocyte infiltration was notified.

## DISCUSSION

The problem of post-burn skin regeneration is of great medical and social importance due to the significant prevalence of burn injuries, the complexity of the pathogenesis of burn injuries, disability, features of social disadaptation, and a high mortality rate [42]. The causes of mortality are microvascular and membrane lesions, burn sepsis, metabolic and hormonal changes, profound systemic disorders and the development of multiple organ failure syndrome [33]. Despite the experience gained in the treatment of burn diseases, the matter of morphogenetic changes during the thermal injury to the skin, remains poorly studied and determines the relevance of the problem.

During the experimental study it was established that under the conditions of the thermal injury to the skin during the first three days, not only local skin inflammatory reactions but also a change in the general condition of the body are observed: "fading" and hair loss, disorganized movement of animals along the cage, lethargy, lack of dynamism, a decrease in the dynamics of weight gain. Based on the results of a morphological study of fixed skin tissues, significant degenerative changes in the damaged skin and inhibition of reparative processes have been revealed. Recovery of a burn wound of the skin in the animals of the control group proceeded physiologically, accompanied by destructive phenomena and the formation of scar tissue.

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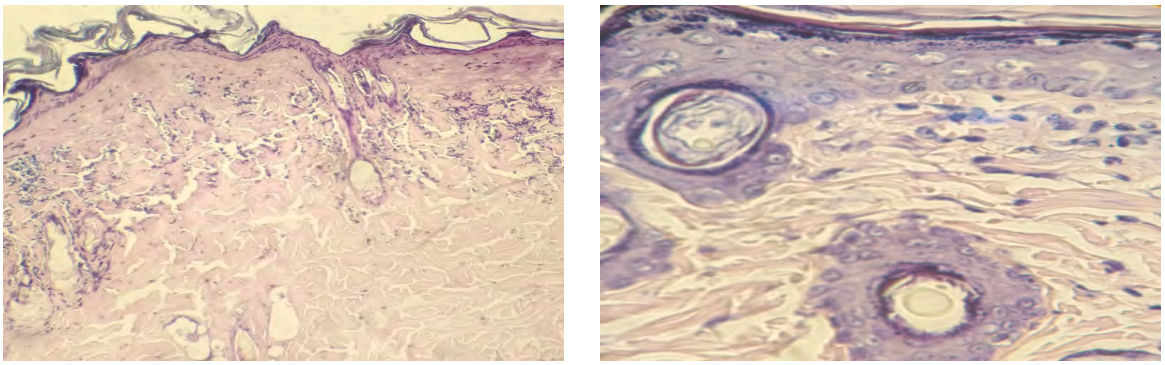
**Table 2 – Dynamics of changes in the areas of burn wounds in rats under conditions of thermal injury to the skin and against the background of exposure to Semax and Selank**

| Group | № | Observation time (days)/Wound square area, cm2 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 3 | 5 | 7 | 10 |
| Control group | 1 | 1.76 | 1.76 | 1.63 | 1.50 | 1.42 |
| | 2 | 1.76 | 1.76 | 1.63 | 1.51 | 1.41 |
| | 3 | 1.76 | 1.76 | 1.62 | 1.54 | 1.39 |
| | 4 | 1.76 | 1.75 | 1.62 | 1.52 | 1.42 |
| | 5 | 1.76 | 1.75 | 1.62 | 1.57 | 1.39 |
| | 6 | 1.76 | 1.76 | 1.63 | 1.52 | 1.41 |
| | 7 | 1.76 | 1.76 | 1.63 | 1.54 | 1.42 |
| | 8 | 1.76 | 1.75 | 1.61 | 1.53 | 1.34 |
| Group III (animals treated with Semax) | 1 | 1.76 | 1.54 | 1.34 | 1.14 | 0.78 |
| | 2 | 1.76 | 1.58 | 1.37 | 1.19 | 0.74 |
| | 3 | 1.76 | 1.55 | 1.35 | 1.18 | 0.77 |
| | 4 | 1.76 | 1.53 | 1.32 | 1.19 | 0.79 |
| | 5 | 1.76 | 1.57 | 1.34 | 1.13 | 0.76 |
| | 6 | 1.76 | 1.58 | 1.35 | 1.18 | 0.69 |
| | 7 | 1.76 | 1.58 | 1.39 | 1.19 | 0.78 |
| | 8 | 1.76 | 1.59 | 1.35 | 1.17 | 0.77 |
| | 9 | 1.76 | 1.59 | 1.36 | 1.18 | 0.78 |
| | 10 | 1.76 | 1.55 | 1.31 | 1.16 | 0.77 |
| Group IV (animals treated with Selank) | 1 | 1.76 | 1.59 | 1.33 | 1.19 | 0.74 |
| | 2 | 1.76 | 1.58 | 1.32 | 1.22 | 0.80 |
| | 3 | 1.76 | 1.59 | 1.31 | 1.24 | 0.77 |
| | 4 | 1.76 | 1.53 | 1.34 | 1.28 | 0.79 |
| | 5 | 1.76 | 1.58 | 1.33 | 1.24 | 0.77 |
| | 6 | 1.76 | 1.58 | 1.33 | 1.29 | 0.78 |
| | 7 | 1.76 | 1.58 | 1.32 | 1.25 | 0.79 |
| | 8 | 1.76 | 1.59 | 1.35 | 1.27 | 0.81 |
| | 9 | 1.76 | 1.57 | 1.28 | 1.24 | 0.79 |
| | 10 | 1.76 | 1.57 | 1.33 | 1.29 | 0.81 |

**Table 3 – Planimetric indicators of the wound surface in rats under conditions of thermal injury to the skin and against the background of exposure to Semax and Selank**

| Groups of animals | Wound square area, $cm^2$ | | | | |
|---|---|---|---|---|---|
| | Day | Day 3 | Day 5 | Day 7 | Day 10 |
| Group II (Control) | 1.76 | 1.76±0.01 | 1.62±0.01 | 1.53±0.01 | 1.4±0.01 |
| Group III (animals treated with Semax) | 1.76 | 1.56±0.01*** | 1.35±0.01*** | 1.17±0.01*** | 0.76±0.01* |
| Group IV (animals treated with Selank) | 1.76 | 1.58±0.01*** | 1.32±0.01*** | 1.25±0.01*** | 0.79±0.01* |

Note: * – p <0.05; ** – p <0.01; *** – p <0.001 – relative to the control group (Student t-test with Bonferroni correction for multiple comparisons).



**A** **B**

**Figure 3 – Micrograph of a skin flap excised in the wound area on day 10 after the burn exposure in rats treated with Semax**

Note: stained with hematoxylin and eosin (UV. X100, vol. 10x (A). UV. X 400, vol. 40x (B), approx. WF-10x / 18); A – Mild swelling of the tissues. Moderate leukocyte infiltration in the underlying fibrous and adipose tissue. B – Full-blood vessels and moderate leukocyte infiltration (individual sections of leukocyte diapedesis through the capillary wall into the tissue)

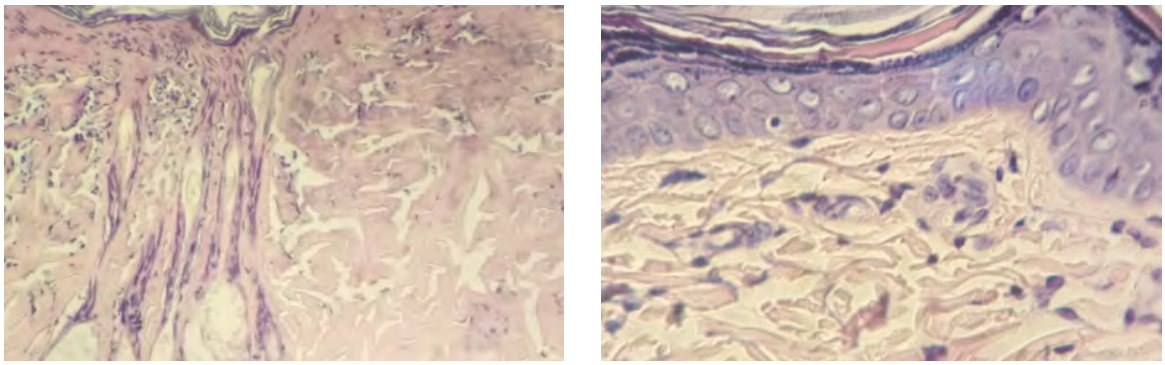
**Table 4 – Dynamics of burn wounds recovery in rats under conditions of thermal injury to the skin and against the background of exposure to Semax and Selank**

| Group | № | Observation time (days)/ Recovery rate (%) | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 3 | 5 | 7 | 10 |
| Control group | 1 | 0.00 | 0.00 | 7.39 | 14.77 | 19.32 |
| | 2 | 0.00 | 0.00 | 7.38 | 14.20 | 19.89 |
| | 3 | 0.00 | 0.00 | 7.95 | 12.50 | 21.02 |
| | 4 | 0.00 | 0.56 | 7.95 | 13.64 | 19.32 |
| | 5 | 0.00 | 0.56 | 7.95 | 10.79 | 21.02 |
| | 6 | 0.00 | 0.00 | 7.39 | 13.64 | 19.89 |
| | 7 | 0.00 | 0.00 | 7.39 | 12.50 | 19.32 |
| | 8 | 0.00 | 0.56 | 8.52 | 13.07 | 23.86 |
| Group III (animals treated with Semax) | 1 | 0.00 | 12.5 | 23.9 | 35.2 | 55.7 |
| | 2 | 0.00 | 10.2 | 22.2 | 32.4 | 57.9 |
| | 3 | 0.00 | 11.9 | 23.3 | 32.9 | 56.3 |
| | 4 | 0.00 | 13.1 | 25.0 | 32.4 | 55.1 |
| | 5 | 0.00 | 10.8 | 23.9 | 35.8 | 56.8 |
| | 6 | 0.00 | 10.2 | 23.3 | 32.9 | 60.8 |
| | 7 | 0.00 | 10.2 | 21.0 | 32.4 | 55.7 |
| | 8 | 0.00 | 9.7 | 23.3 | 33.5 | 56.3 |
| | 9 | 0.00 | 9.7 | 22.7 | 32.9 | 55.7 |
| | 10 | 0.00 | 11.9 | 25.6 | 34.1 | 56.3 |
| Group IV (animals treated with Selank) | 1 | 0.00 | 9.7 | 24.4 | 32.4 | 57.9 |
| | 2 | 0.00 | 10.2 | 25.0 | 30.7 | 54.5 |
| | 3 | 0.00 | 09.7 | 25.6 | 29.5 | 56.3 |
| | 4 | 0.00 | 13.1 | 23.9 | 27.3 | 55.1 |
| | 5 | 0.00 | 10.2 | 24.4 | 29.5 | 56.3 |
| | 6 | 0.00 | 10.2 | 24.4 | 26.7 | 55.7 |
| | 7 | 0.00 | 10.2 | 25.0 | 28.9 | 55.1 |
| | 8 | 0.00 | 9.7 | 23.3 | 27.8 | 54.1 |
| | 9 | 0.00 | 10.8 | 27.3 | 29.5 | 55.1 |
| | 10 | 0.00 | 10.8 | 24.4 | 26.7 | 54.1 |

**Table 5 – Epithelization rate of burn wounds in rats under conditions of thermal injury to the skin and against the background of exposure to Semax and Selank (M ± m)**

| Groups of animals | Epithelization rate (%) | | | | |
|---|---|---|---|---|---|
| | 1 Day | Day 3 | Day 5 | Day 7 | Day 10 |
| Group II (Control) | 0.00 | 0.21±0.24 | 7.74±0.35 | 13.14±1.04 | 20.46±1.31 |
| Group III (animals. treated with Semax) | 0.00 | 11.02±0.41*** | 23.42±0.55* | 33.45±0.40** | 56.66±0.68* |
| Group IV (animals treated with Selank) | 0.00 | 10.46±0.44*** | 24.77±0.48* | 28.9±0.68** | 55.42±0.45* |

Note: * – p <0.05; ** – p <0.01; *** – p <0.001 – relative to the control group (Student's t test with Bonferroni correction for multiple comparisons).



A B

**Figure 4 – Micrograph of a skin flap excised in the wound area on day 10 after burn exposure in rats treated with Selank**

Note: stained with hematoxylin-eosin (UV. X100, vol. 10x (A). UV. X 400, vol. 40x (B), approx. WF-10x / 18)
A – A fragment of the skin with underlying tissues. In the underlying tissues, there is a moderate diffuse leukocyte infiltration.
B – A fragment of skin with underlying tissues. At the level of the dermis, there is swelling; in the underlying fibrous tissue, there are foci of a moderate leukocyte infiltration reaching the dermis

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At present, the search and development of effective means of correcting skin lesions, as well as determining the role of pathogenetic links of complex systemic homeostasis disorders of the body, are of great interest in reparating skin wounds of various origins, stimulating tissue reparation. Taking into account a close relationship between the skin, immune, nervous and endocrine systems, in the study of the pathogenesis of skin wounds and its regenerative potential, special attention is paid to studying the regulation of these processes by the neuroimmunoendocrine interactions and their disorders.

Today, regulatory peptides are increasingly being used as effective remedies for correcting homeostasis disorders under the conditions of stress, in particular, the adrenocorticotropic hormone analogue Semax (Met-Glu-His-Phe-Pro-Gly-Pro) and a tuftsin derivative Selank (Thr-Lys-Pro-Arg-Pro-Gly-Pro). The use of synthesized peptide preparations increases the adaptive capabilities of the body under stressful conditions, helps restore the structures of the central nervous system, and corrects immune and humoral disorders.

However, the results of recent studies do not give a complete picture of the reparative potential of these drugs and the mechanisms of their stimulating dermatropic action.

The carried out study on the assessment of morphological characteristics of the rats' skins under the conditions of thermal injury to the skin in the animals treated with Semax and Selank, established mild degenerative and dystrophic changes in the burn wounds by stimulating start adaptive reparative processes in the skin and a systemic regulation of reparations.

A microscopic analysis of the structural features of the skin layers in the rats treated with neuropeptide preparations Semax and Selank intraperitoneally, confirmed the earlier onset of skin regenerative regeneration processes. That phenomenon manifested itself in the decrease of the destructive phenomena in the wound and leveling of inflammatory reactions, as well as the signs of early scab exfoliation, the formation of granulation tissue and accelerated epithelization of burn wounds. In the course of studying the systemic effect of Semax and Selank under the conditions of thermal exposure, it has been found out that these neuropeptide preparations have regulatory, stress-adaptive and immunocorrection properties. They reparate the leukocyte formula, leukocyte coefficient and weight of immunocompetent organs (the thymus and spleen). Such positive results prove the viability of systemic coordination in stimulating the regenerative potential of damaged skin and other pathological processes in the body.

## CONCLUSION

Up-to-date understanding of the molecular mechanisms of the complex regulation of the skin pathophysiological processes, Is at the heart of the systemic approach in. It proves their polypotent effectiveness, expands the therapeutic possibilities and opens up new prospects for their application.

The results of the experimental study make it possible to suggest the following qualities of neuropeptide drugs Semax and Selank: they contributes to the regulation and stimulation of reparative processes, which gives grounds to consider them as correction remedies for skin wounds with local and systemic effects, realized through neuroimmune endocrine mechanisms. The above determines the importance and necessity of the next stages of research in this direction.

The above analysis determines the importance and necessity of the next stages of research in this direction.

## FINANCIAL SUPPORT AND SPONSORSHIP

This study did not have any financial support from outside organizations.

## AUTHOR'S CONTRIBUTION

All authors equally contributed to the research work.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHORS

**Alfiya K. Azhikova** – Candidate of Sciences (Biology), Assistant Professor of the Department of Biology and Botany, Federal State Budgetary Educational Institution of Higher Education "Astrakhan State Medical University", the Ministry of Health of the Russian Federation. ORCID: 0000-0001-9758-1638. E-mail: alfia-imacheva@mail.ru

**Bronislav V. Feldman** – Doctor of Sciences (Biology), Associate Professor, the Head of the Department of Biology and Botany, Federal State Budgetary Educational Institution of Higher Education "Astrakhan State Medical University", the Ministry of Health of the Russian Federation. ORCID: 0000-0001-7205-986X. E-mail: agmafarm@gmail.com

**Lyudmila A. Andreeva** – the head of the regulatory peptides sector of the Department of Chemistry of Physiologically Active Substances, Federal State Budgetary Institution of Science Institute of Molecular Genetics of the Russian Academy of Sciences. ORCID: 0000-0002-3927-8590. E-mail: landr@img.ras.ru

**Dmitry D. Teply** – Candidate of Sciences (Biology), Senior Researcher at the Joint Laboratory for the Study of the Role of Apoptosis in the Formation of the Neuroendocrine System, Federal State Budgetary Educational Institution of Higher Education "Astrakhan State Medical University", the Ministry of Health of the Russian Federation. ORCID: 0000-0002-3484-9710. E-mail: dima.tepliy@yandex.ru

**Marina A. Samotrueva** – Doctor of Sciences (Medicine), the Head of the Department of Pharmacognosy, Pharmaceutical Technology and Biotechnology, Federal State Budgetary Educational Institution of Higher Education "Astrakhan State Medical University", the Ministry of Health of the Russian Federation. ORCID: 0000-0001-5336-4455. E-mail: ms1506@mail.ru

**Nikolay F. Myasoedov** – Doctor of Sciences (Chemistry), Professor, Academician of the Russian Academy of Sciences, the Head of the Department of Chemistry of Physiologically Active Substances, Federal State Budgetary Institution of Science "Institute of Molecular Genetics of the Russian Academy of Sciences". ORCID: 0000-0003-1294-102X. E-mail: nfm@img.ras.ru

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

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# CEREBROPROTECTIVE EFFECT OF SOME PHENOLIC ACIDS UNDER CONDITIONS OF EXPERIMENTAL BRAIN ISCHEMIA

**A.V. Voronkov, S.A. Nigaryan, D.I. Pozdnyakov**

Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University
11, Kalinin ave., Pyatigorsk, Russia 357532

E-mail: pozdniackow.dmitry@yandex.ru

Received 15 September 2019 Review (1) 14 October 2019 Review (2) 11 November 2019 Accepted: 16 December 2019

**The aim** of the study was to evaluate the cerebroprotective effect of some phenolic acids under the conditions of experimental cerebral ischemia in rats.

**Materials and methods**. The experiment was conducted on male Wistar rats weighing 220–240 g. Focal cerebral ischemia was modeled by irreversible right-sided thermocoagulation of the middle cerebral artery under chloral hydrate anesthesia (350 mg/kg, intraperitoneally). The experimental compounds (4-hydroxy-3.5-di-tert-butyl cinnamic acid, caffeic acid and gallic acid 100 mg/kg each compound) and a reference drug (Mexicor – 100 mg/kg) were administered intragastrically next day after the surgery and then for three daysrunning. The effect of the test-compounds on the cognitive functions of the rats was evaluated by CRPA and TEA tests. The influence of the compounds on the changes in the concentration of lactate, pyruvate, homocysteine, as well as the degree of cerebral edema formation and necrosis of the brain tissue, were studied.

**Results**. In the study, it has been established that against the background of the focal cerebral ischemia, the administration of caffeic, 4-hydroxy-3,5-di-tert-butylcinnamic and gallic acid, contributed to the preservation of a memorable trace in rats, as well as a decrease in lactate concentration (by 40.37% ($p<0.05$), 151.26% ($p<0.05$), 48.02% ($p<0.05$)) and pyruvate (by 96.6,% ($p<0.05$), 38, 78% ($p<0.05$), 33.3% ($p<0.05$)), homocysteine (by 59.6% ($p<0.05$), 102.18% ($p<0.05$), 28.8% ($p<0.05$)), anecrosis zone (by 122.79% ($p<0.05$), 165.11% ($p<0.05$), 12.38% ($p<0,05$)) and cerebral edema (by 10.47% ($p<0.05$), 11.08% ($p<0.05$), 9.92% ($p<0.05$)) relative to the NC group of rats.

**Conclusion.** The obtained data indicate the possibility of further detailed investigation of the cerebroprotective effect of 4-hydroxy-3,5-di-tert-butylcinnamic, caffeic and gallic acids.

**Key words**: cerebral ischemia, an insult to the brain, lactate, pyruvate, caffeic acid, gallic acid, 4-hydroxy-3,5-di-tert-butylcinnamic acid, ethylmethylhydroxypyridine succinate, choline alfoscerate.

**Abbreviations:** SO – sham-operated animals; NC – negative control**;** M – Mexicor; CA – caffeic acid**;** GA – gallic acid**;** HDTBCA – 4-hydroxy-3,5-di-tert-butylcinnamic acid**;** CRPA – Conditioned Reflex of Passive Avoidance; TEA – extrapolation avoidance test; ATP – adenosine triphosphate; AMP – adenosine monophosphate; ROS – reactive oxygen species.

# ЦЕРЕБРОПРОТЕКТИВНОЕ ДЕЙСТВИЕ НЕКОТОРЫХ ФЕНОЛОКИСЛОТ В УСЛОВИЯХ ЭКСПЕРИМЕНТАЛЬНОЙ ИШЕМИИ ГОЛОВНОГО МОЗГА

**А.В. Воронков, Д.И. Поздняков, С.А. Нигарян**

Пятигорский медико-фармацевтический институт – филиал ФГБОУ ВО
«Волгоградский государственный медицинский университет» Минздрава России
357532, Россия, г. Пятигорск, пр. Калинина, 11

E-mail: pozdniackow.dmitry@yandex.ru

Получено 15.09.2019 Рецензия (1) 14.10.2019 Рецензия (2) 11.11.2019 Принята к печати 16.12.2019

**For citation:** A.V. Voronkov, S.A. Nigaryan, D.I. Pozdnyakov. Cerebroprotective effect of some phenolic acids under conditions of experimental brain ischemia. *Pharmacy & Pharmacology*. 2019;7(6): 332-338. **DOI:** 10.19163/2307-9266-2019-7-6-332-338

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**Для цитирования:** А.В. Воронков, Д.И. Поздняков, С.А. Нигарян. Церебропротективное действие некоторых фенолокислот в условиях экспериментальной ишемии головного мозга. *Фармация и фармакология.* 2019;7(6):332-338. **DOI:** 10.19163/2307-9266-2019-7-6-332-338

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-6-332-338

**Цель исследования** – изучить церебропротективную активность некоторых фенолокислот в условиях экспериментальной ишемии головного мозга у крыс.
**Материалы и методы**. Эксперимент был проведен на крысах-самцах линии Wistar, массой 220–240 г. Фокальную ишемию головного мозга моделировали путем необратимой правосторонней термокоагуляции средней мозговой артерии под хлоралгидратным наркозом (350 мг/кг, интарперитонеально). Экспериментальные соединения (4-гидрокси-3,5-ди-трет-бутилкоричная кислота, кофейная кислота и галловая кислота 100 мг/кг каждое соединение) и референтные препараты (Мексикор – 100 мг/кг, Холина альфосцерат – 150 мг/кг) вводили на следующие сутки после операции интрагастрально и далее в течении трех дней. Влияние соединений на когнитивные функции крыс оценивали в тестах «условная реакция пассивного избегания» (УРПИ) и тесте экстраполяционного избавления (ТЭИ). Изучалось влияние данных соединений на изменение концентрации лактата, пирувата, гомоцистеина, а также степень формирования отека и некроза мозговой ткани.
**Результаты.** Установлено, что на фоне фокальной ишемии головного мозга применение кофейной, 4-гидрокси-3,5-ди-трет-бутилкоричной и галловой кислот способствовало сохранению памятного следа у крыс, а также снижению концентрации лактата (на 40,37% ($p<0,05$), 151,26% ($p<0,05$), 48,02% ($p<0,05$)) и пирувата (на 96,6,% ($p<0,05$), 38,78% ($p<0,05$), 33,3% ($p<0,05$)), гомоцистеина (на 59,6% ($p<0,05$), 102,18% ($p<0,05$), 28,8% ($p<0,05$)), зоны некроза (на 122,79% ($p<0,05$), 165,11% ($p<0,05$), 12,38% ($p<0,05$)) и отека (на 10,47% ($p<0,05$), 11,08% ($p<0,05$), 9,92% ($p<0,05$)) относительно группы крыс негативного контроля (НК).
**Заключение.** Результаты экспериментальных данных свидетельствуют о возможности дальнейшего углубленного изучения 4-гидрокси-3,5-ди-трет-бутилкоричной, кофейной, галловой кислот на предмет церебропротективной активности.
**Ключевые слова:** ишемия головного мозга, инсульт, лактат, пируват, кофейная кислота, галловая кислота, 4-гидрокси-3,5-ди-трет-бутилкоричная кислота, этилметилгидроксипиридина сукцинат, холина альфосцерат.
**Список сокращений:** Л/О – ложнооперированные животные; НК – негативный контроль; М – Мексикор; КК – кофейная кислота; ГК – галловая кислота; ГДТБКК – 4-гидрокси-3,5-ди-трет-бутилкоричная кислота; УРПИ – условная реакция пассивного избегания; ТЭИ – тест экстраполяционного избавления; АТФ – аденозинтрифосфат; АМФ – аденозинмонофосфат; АФК – активные формы кислорода.

## INTRODUCTION

Due to ischemic events, brain damage remains the leading cause for death and primary disability in the whole world [1, 2]. Social and economic consequences of an insult to the brain require the development of effective pharmacotherapeutic approaches, in connection with which the targeted search for new compounds used for the treatment and prevention of this pathology, becomes relevant. For the world scientific and medical community improving the pharmacological correction and rehabilitation of people who have suffered acute cerebrovascular accident, this is still an acute problem [3].

However, to date, the problem of pharmacological correction of an insult to the brain, remains an elusive goal, despite various major studies on the pathogenesis of cerebrovascular diseases and new compounds that can affect it [4, 5].

Based on the results of new domestic and foreign studies, it can be assumed that among the compounds that can influence patogenetic elements of an ischemic stroke, a special role is played by plant-origin bioactive substances, which can become an effective treatment of cerebral ischemia. Such assumptions are mainly associated with the positive effects possessed by plant-origin-substances. These include the breadth of the pharmacological activity spectrum, the ability to use these compounds in complex therapy, as well as the minimal risk of undesirable adverse effects [6, 7].

Cinnamic acid is knownto have antioxidant, anti-inflammatory, anti-apoptotic properties [8]. To date, many scientists have also found out a positive effect of various phenolic compounds on the course of neurodegenerative diseases, in particular, Alzheimer's disease [9]. Caffeic acid (3.4-dihydroxycinnamic acid) is a phenolic compound that is widely found in medicinal plants, as well as in fruits, vegetables, wine, coffee and olive oil [10]. Caffeic acid has various types of apharmacological activity: antioxidant [11], antihypertensive [12], antiviral [13], anti-inflammatory [14] and antidiabetic ones [15]. Recently, a cerebroprotective effect of caffeic acid has also been evaluated [16–18]. Gallic acid is a secondary metabolite present in most plants. This metabolite is known to have a number of pharmacological properties, including antioxidant, antimicrobial, anti-inflammatory and antitumor ones [19].

**THE AIM** of this study is to evaluate the cerebroprotective effect of caffeic, gallic and 4-hydroxy-3.5-di-tert-butylcinnamic acid under the conditions of experimental cerebral ischemia.

## MATERIALS AND METHODS

### Biological model

The experiment was performed on 42 Wistar male rats weighing 220–240 g, obtained from the «Rappolovo» laboratory animal nursery. All manipulations with animals, as well as their contents, met the requirements of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 1986). This study was approved by the Independent Ethics Committee of Pyatigorsk Medical and Pharmaceutical Institute. The rats were kept in macrolon cages, where a granular wood fraction was used as litter material at the relative humidity of 60 ± 5% and the air temperature of 22±2°C. The animals received food and water ad libitum. Previously, the rats had been randomized by behavioral activity byCRPA and TEA tests. During the study, seven experimental groups wereformed. The first group of rats wassham-operated (SO) animals (n = 6). The second group of animals (n = 6)

was a negative control group (NC); they received a 0.9% solution of sodium chloride in the equivalent volume. The second and subsequent groups of rats (n = 6in each group) were subjected to the ischemic damage to the brain by irreversible occlusion of the right middle cerebral artery, while the animalsof groups 3–7 received the test-compounds and referebce drugs (Fig. 1).

### Reference drug and test compounds

Ethylmethylhydroxypyridine succinate («Mexicor», 100 mg/kg, EcoFarmInvest, Russia) [20, 21] (Fig. 1) was chosen as a reference drug. The reference medicines and test compounds were administered per os next day after the simulation of cerebral ischemia and then for 3 days running. The test compounds-4-hydroxy-3,5-di-tert-butylcinnamic, caffeic, and gallic acids – were administered at the dose of 100 mg/kg (Fig. 1).

### Focal cerebral ischemia model

Focal cerebral ischemia was modeled by irreversible right-sided thermocoagulation of the middle cerebral artery under chloral hydrated anesthesia (350 mg/kg). The area below and to the right of the eye was depilated, an incision was made and soft tissues were spread apart, exposing a process us of the zygomatic bone, which was removed. Then, a trepanation hole was madewith a drill and the middle cerebral artery was burned by a thermocoagulator under the place of its intersection with the olfactory tract. After that, if possible, the topography of the soft tissues was restored. The seam was treated with a 5% iodine alcohol solution. The biomaterial was taken outon the 4th day after the reproduction of focal ischemia.

### Cognitive tests

Before modeling ischemia, the animals of all groups were skill-trained in the Conditioned Reflex of Passive Avoidance (CRPA) and extrapolation avoidance test (TEA) tests. The essence of the CRPA test is to form a memorial trace in animals, after which the latent entry time into the dark compartment, where electricity is supplied, is lengthened, or the rat does not enter it at all.

The TEA test, also makes it possible to evaluate the cognitive functions of the animals, based on the time of the rat dives from the cylinder. Subsequent reproductions of CRPA and TEA tests, as well as the assessment of the animal's behavioral activity and emotional status, were performed on the fourth day in theCRPA (latent time of the rat entry into the dark compartment was recorded) and TEA tests (the rat diving time was recorded) [22, 23].

### Studied laboratory parameters

As biomaterial, the animals' brain and blood were used in the work. The parameters under study were: concentrations of lactic and pyruvic acids in the blood serum, the size of the necrosis zone and the cerebral edema rate. The contents of lactic and pyruvic acids in the blood serum, were determined by an enzymatic colorimetric method using a standard reagents kits, manufactured by SMA«Arbis +» (St. Petersburg, Russia). The necrosis zone was evaluated using the triphenyltetrazolium method, which is based on a change in the absorbance of the formazan chloroform extract between the necrotic and intact parts of the brain [24]. The degree of brain hydration was estimated by the drying method, for which the animal'sbrain was removed and incubated for 24 hours at 60°C. The value of cerebral edema was determined by the difference in the brain masses before and after incubation [23].

### Statistical Methods

The experimental data were processed by the variation statistics method using the STATISTICA 6.0 software. (StatSoft, Inc., USA for the Windows). The data obtained, checked the normality of the distribution using the Shapiro-Wilk test. In case the data had been normally distributed, ANOVA with a posteriori Newman-Case criterion was used for statistical average calculation. In case the data of the experiment had been abnormally distributed further statistical processing of the data was carried out using the Wilcoxon test.

## RESULTS

In the CRPA test, after the reproduction of ischemia in rats of the NC group, a decrease in the latent time of rat entry into the dark compartment by 10% ($p<0.05$) was registered, the diving time of the animals in the TEA test was increased by 69.6% ($p<0.05$) relative to the SO group. The data of CRPA and TEA tests indicate that all the experimental compounds positively affect the cognitive functions of rats under the conditions of cerebral ischemia.

In the CRPA test, against the background of the administration of all the test compounds, a statistically significant increase in the latent time of entry into the dark compartment was observed. In comparison with the NC group of animals, the maximum effect was observed after the administration of caffeic acid: the latent time of entry into the dark compartment was increased by 239.39% ($p <0.05$), after the administration of gallic acid –by 129.09% ($p<0.05$), after the administration of 4-hydroxy-3,5-di-tert-butylcinnamic acid – by 90.15% ($p<0.05$). The administration of Mexicor to the animals, caused an increase in the time of entry into the dark compartment by 71.2% ($p <0.05$). It is worth noting that all phenolic compounds exceeded the reference drug in thepharmacological effect rate: caffeic acid – by 103.74% ($p <0.05$), gallic acid – by 33.8% ($p<0.05$), 4-hydroxy-3,5-di-tert-butylcinnamic acid –by 11.06 ($p<0.05$) (Fig. 2).

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DOI: 10.19163/2307-9266-2019-7-6-332-338

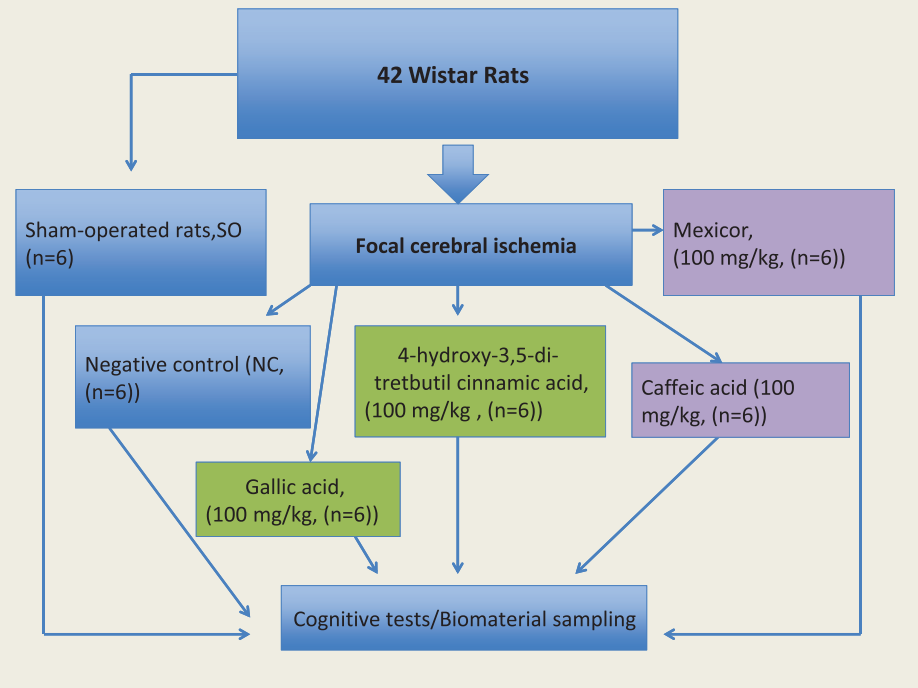


**Figure 1 – Model of the study**

**Table 1 – Study of the effect of experimental compounds on the concentration of lactate, pyruvate and homocysteine**

| Investigated parameter | SO | NC | Mexicor | Caffeic acid | Gallic acid | 4-hydroxy-3,5-di-tert-butyl cinnamic acid |
|---|---|---|---|---|---|---|
| Lactate, mmol/l | 1.08±0.1 | 2.99±0.024# | 2.57±0.16* | 2.13±0.040* | 2.02±0.079* | 1.19±0.043* |
| Piruvate, mmol/l | 100.38±1.1 | 200.68±15.664# | 116.89±5.82* | 102.07±3.48* | 150.51±3.787* | 144.60 ±7.357* |
| Homocysteine, ng/ml | 10.27±0.675 | 46.44±1.054# | 26.75±1.617* | 29.07±1.303* | 36.0±0.86* | 22.95±1.342* |

Note: * – statistically significant relative to the NC group of rats (Newman-Keulse test; p<0.05);
# – statistically significant relative to the SO group of rats (Newman-Keulse test; p<0.05)

In the TEA test, the latent diving time was evaluated. In this test, in comparison with the NC group of animals, a decrease in diving time by 1042.2% (p <0.05) was notedin the rats treated with 4-hydroxy-3,5-di-tert-butylcinnamic acid, whereas, when caffeic and gallic acids were used, this parameter decreased by 358.9% (p <0.05) and 229.48% (p <0.05), respectively. Under the same conditions, the administration of Mexicor reduced the diving time relative to the group of the NC rats by 96.69% (p <0.05). Therefore, in this test,4-hydroxy-3,5-di-tert-butylcinnamic, caffeic and gallic acids exceeded the reference drug effect by 477.7% (p <0.05), 132.14% (p <0.05) and 66.6% (p <0.05), respectively (Fig. 2).

The reproduction of focal cerebral ischemia in rats, caused the development of edema and necrosis of the brain tissue (52.38±3.03), which corresponds with the published data [25]. There was a significant increase in lactate (176.8% (p<0.05)), pyruvate (99.9% (p<0.05)), as well as homocysteine (352.2% (p<0.05)) in the NC group of animals (Tab. 1).

The administration of the reference drug contributed to a decrease in the concentration of lactate, pyruvate and homocysteine. Thus, in the group of the animals treated with Mexicor, a decrease in lactate by 16.3% (p<0.05), in pyruvate – by 71.68% (p<0.05), and in homocysteine content – by 73.45% (p<0.05) relative to the NC group of rats, was noted. (Tab.1).

When the animals were treated with 4-hydroxy-3,5-di-tert-butylcinnamic acid, a decrease in the serum lactate concentration by 151.26% (p<0.05), in pyruvate – by 38.78% (p<0.05) and in homocysteine – by 102.18% (p<0.05) relative to the NC group of the animals, was observed. It is worth emphasizing that against the background of the use of 4-hydroxy-3,5-di-tert-butyl cinnamic acid, a greater decrease in the formation of lactate – by 115.96% (p<0.05) and homocysteine – by16.5% (p<0,05) compared with the group of the rats treated with the reference drug, was observed (Tab.1).

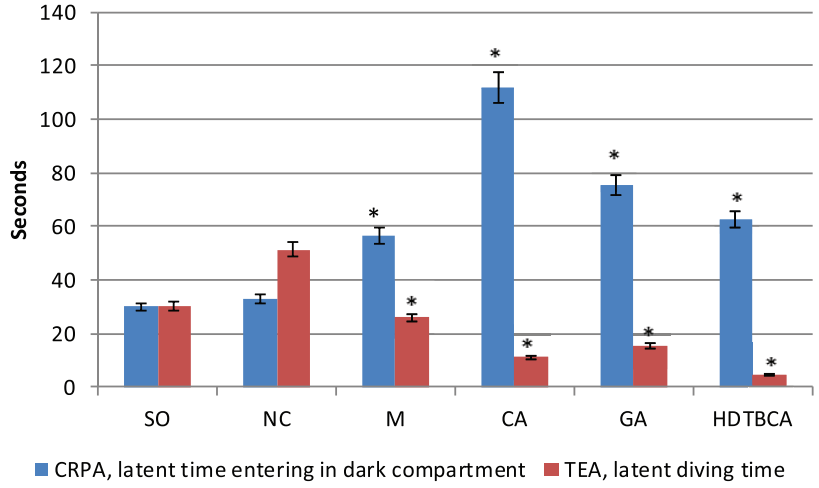


**Figure 2 - The influence of the test-compounds on the execution time of tasks in CRPA and TEA tests**

Note: * – statistically significant relative to the NC group of rats (Newman-Keulse test; p <0.05); SO – sham- operated animals; NC – negative control; M – Mexicor; CA – caffeic acid; GA – gallic acid; HDTBCA – 4-hydroxy-3,5-di-tert-butylcinnamic acid.

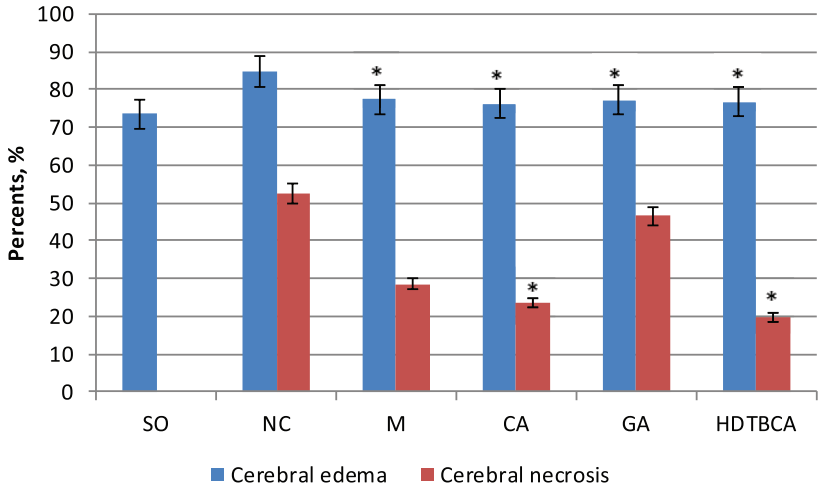


**Figure 3 – Influence of caffeic, gallic and 4-hydroxy-3,5-di-tert-butylcinnamic acids on the formation of cerebral edema and necrosis of brain tissue against the background of the experimental focal ischemia**

Note: * – statistically significant relative to the NC group of rats (Newman-Keulse test; p<0.05); SO – sham-operated animals; NC – negative control; M – Mexicor; CA – caffeic acid; GA – gallic acid; HDTBCA – 4-hydroxy-3,5-di-tert-butylcinnamic acid

In the group of the rats treated with gallic acid, a decrease in the lactate, pyruvate and a homocysteine content by 48.02% (p<0.05), 33.3% (p<0.05), 28.8% (p<0.05), respectively, relative to the NC group of rats, was noted. Compared with the Mexicor-treated group, gallic acid administration reduced the level of lactate formation by 27.2% (p<0.05) (Tab. 1).

The administration of caffeic acid also caused a decrease in the formation of lactate – by 40.37% (p<0.05), pyruvate – by 96.6% (p<0.05) and homocysteine – by 59.6% (p<0.05) relative to the NC group. When compared with the group of the animals treated with Mexicor, it was noted that the concentration of lactate and pyruvate in the blood serum was 20.65% (p<0.05) and 14.52% (p<0.05) higher than in this group of the animals treated with caffeic acid (Tab. 1).

In the rats treated with Mexicor, a decrease in cerebral edema relative to the NC group of rats by 9.36% (p <0.05) was noted. The administration of caffeic, 4-hydroxy-3,5-di-tert-butylcinnamic and gallic acids to the rats, caused a decrease in the hydration of the brain tissue, relative to the NC group, by 11.08% (p<0.05), 10.47% (p<0 05) and 9.92% (p<0.05), respectively (Fig. 3).

According to the effect on the degree of the brain necrosis formation, 4-hydroxy-3,5-di-tert-butylcinnamic was the most effective of the three experimental compounds and the reference drug. So, relative to the NC group of rats, the administration of 4-hydroxy-3,5-di-tert-butylcinnamic acid reduced the degree of cerebral necrosis by 165.11% (p<0.05), caffeic acid – by 122.79% (p<0.05), Mexicor – by

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DOI: 10.19163/2307-9266-2019-7-6-332-338

83.4% (p <0.05). In the rats treated with gallic acid, a decrease in brain tissue necrosis by 12.38% (p<0.05) was observed. It should be noted that 4-hydroxy-3,5-di-tert-butylcinnamic acid exceeded Mexicor by 44.8% (p<0.05) (Fig. 3).

## DISCUSSION

Focal cerebral ischemia leads to the depletion of brain energy reserves. In biochemical tests, this is manifested by a decrease in the content of macroergs (ATP, creatine phosphate), an increase in the number of semi-oxidized products (ADP, AMP, lactate, pyruvate, homocysteine), a decrease in the energy charge of the system, and the development of lactic acidosis [26]. Lactate, pyruvate, homocysteine are biomarkers of various neurodegenerative diseases, including an insult to the brain, Alzheimer's disease, as well as pathologies caused by a decrease in the mitochondrial function [27]. The conducted study has shown that against the background of the cerebral ischemia in rats, phenolic acids have a positive effect on theenergy metabolism in the brain decreasing the concentration of lactate; pyruvate and homocysteine in blood serum. Separately, it is worth noting a decrease in the degree of necrotization of the brain tissue when the experimental compounds were administered to the animals. The potential cerebroprotective effect of phenolic compounds may be associated with the chemical structure of these substances and their antiradical effect [28]. The cerebroprotective effect of 4-hydroxy-3,5-di-tert-butylcinnamic, caffeic, and gallic acids, can be also associated with their antioxidant, anti-apoptotic and anti-inflammatory properties [8–19]. It is possible that phenolic compounds improve the functional activity of mitochondria, which are most sensitive to ischemic conditions [29]. Mitochondria are organelles, which are the main producers of reactive oxygen species (ROS). Since ROS play one of the key role in the induction of mitochondrial pores, preventing the development of oxidative stress, is an effective method of stopping the cell death, and antioxidants, in particular phenolic acids, can be used as drugs of pharmacological correction of a mitochondrial dysfunction against the background of the focal cerebral ischemia.

## CONCLUSION

Against the background of rats' cerebral ischemia, 4-hydroxy-3,5-di-tert-butylcinnamic, caffeic and gallic acids improved metabolic processes and brain energy exchange, had a positive effect on cognitive functions. Experimental compounds may have a potential cerebroprotective activity against the background of the rat's cerebral ischemia, as evidenced by the experimental data.

This creates prerequisites for a further in-depth study of phenolic compounds, in particular, derivatives of cinnamic acid, in order to confirm cerebroprotective properties, as well as to continue the search for compounds of plant origin that can exert a cerebroprotective effect.

## FINANCIAL SUPPORT

This study did not have any financial support from outside organizations.

## AUTHORS' CONTRIBUTION

All authors equally contributed to the research work.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHORS

**Andrey V. Voronkov** – Doctor of Science (Medicine), professor, the Head of the department of pharmacology with a course of clinical pharmacology. Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID:0000-0001-6638-6223. E-mail: prohor77@mail.ru.

**Dmitry I. Pozdnyakov** – Candidate of Sciences (Pharmacy), Senior Lecturer of the Department of Pharmacology with a course of clinical pharmacology. Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID:0000-0003-0889-7855. E-mail: pozdniackow.dmitry@yandex.ru

**Siranush A. Nigaryan** – postgraduatestudentof the department of pharmacology with a course of clinical pharmacology. Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID ID: 0000-0001-9898-0518. E-mail: 79682650210@yandex.ru

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

*ОРИГИНАЛЬНАЯ СТАТЬЯ*

*DOI: 10.19163/2307-9266-2019-7-6-339-345*

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# ANTIULCER ACTIVITY OF 2-PHENYL-9-DIETHYLAMINO-ETHYLIMIDAZO[1,2-A]BENZIMIDAZOLE DINITRATE IN ETHANOL-PREDNISOLONE DAMAGE TO GASTRIC MUCOSA

**M.V. Chernikov, M.A. Oganova, S.A. Kalashnikova, L.V. Polyakova, N.A. Khromova**

Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University
11, Kalinin ave., Pyatigorsk, Russia 357532

E-mail: pharmax@list.ru

Received 12 August 2019 Review (1) 12 September 2019 Review (2) 10 October 2019 Accepted: 15 October 2019

Nowadays, the search for new effective and safe medicines for the treatment of acid-dependent gastrointestinal diseases remains an urgent problem of modern pharmacology.

**The aim** of this study was an experimentally study of the anti-ulcer activity of 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole dinitrate on the model of damage to the gastric mucosa caused by the administration of 80% ethanol and prednisolone combination (20 mg/kg).

**Materials and methods.** To simulate the damage to the gastric mucosa, the experimental animals (white male Wistar rats) were administrated with prednisone at the rate of 20 mg/kg and 80% ethyl alcohol at the dose of 0.6 ml/100 g of the animal body weight. Prednisolone was dissolved in 80% alcohol. Antisecretory antiulcer agents actively used in clinical practice, were selected as reference drugs: ranitidine (30 mg/kg, 10 mg/kg and 3 mg/kg) and omeprazole (3 mg/kg, 1 mg/kg and 0.3 mg/kg). The studied compound was used at the doses of 30 mg/kg, 10 mg/kg and 3 mg/kg. All the substances under study were administered intragastrically with the use of an atraumatic probe.

**Results.** It has been established that the benzimidazole derivative in the studied doses contributes to a dose-dependent reliable reduction in the area and depth of ulcerative lesions of the gastric mucosae relative to the control and reference drugs (ranitidine and omeprazole). In addition, in the maximum studied dose (30 mg/kg), the proportion of the animals with ulcerative lesions significantly decreases by more than 2 times. The calculated $ED_{50}$ values for the benzimidazole derivative and ranitidine were 5.09 mg/kg and 38.23 mg/kg, respectively.

**Conclusion.** The obtained experimental data indicate that the benzimidazole derivative has a pronounced dose-dependent antiulcer effect on the model of ethanol-prednisolone erosive-ulcerous defects of the rats' gastric mucosae, which is superior to the effects of the reference preparations. It makes its further study promising.

**Keywords:** 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole dinitrate, antiulcer action, ethanol-prednisolone damages / injuries, preclinical studies

# ПРОТИВОЯЗВЕННАЯ АКТИВНОСТЬ ДИНИТРАТА 2-ФЕНИЛ-9-ДИЭТИЛАМИНОЭТИЛИМИДАЗО[1,2-А] БЕНЗИМИДАЗОЛА ПРИ ЭТАНОЛ-ПРЕДНИЗОЛОНОВОМ ПОВРЕЖДЕНИИ СЛИЗИСТОЙ ОБОЛОЧКИ ЖЕЛУДКА

**М.В. Черников, М.А. Оганова, С.А. Калашникова, Л.В. Полякова, Н.А. Хромова**

Пятигорский медико-фармацевтический институт – филиал федерального государственного бюджетного образовательного учреждения высшего образования «Волгоградский государственный медицинский университет» Министерства здравоохранения Российской Федерации
357532, Россия, Ставропольский край, г. Пятигорск, пр. Калинина, 11

E-mail: pharmax@list.ru

Получено 12.08.2019 Рецензия (1) 12.09.2019 Рецензия (2) 10.10.2019 Принята к печати 15.10.2019

**For citation:** M.V. Chernikov, M.A. Oganova, S.A. Kalashnikova, L.V. Polyakova, N.A. Khromova. Antiulcer activity of 2-phenyl-9-diethylamino-ethylimidazo[1,2-a]benzimidazole dinitrate in ethanol-prednisolone damage to gastric mucosa. *Pharmacy & Pharmacology*. 2019;7(6): 339-345. **DOI:** 10.19163/2307-9266-2019-7-6-339-345

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**Для цитирования:** М.В. Черников, М.А. Оганова, С.А. Калашникова, Л.В. Полякова, Н.А. Хромова. Противоязвенная активность динитрата 2-фенил-9-диэтиламиноэтилимидазо[1,2-а]бензимидазола при этанол-преднизолоновом повреждении слизистой оболочки желудка. *Фармация и фармакология.* 2019;7(6):339-345. **DOI:** 10.19163/2307-9266-2019-7-6-339-345

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

Поиск новых эффективных и безопасных лекарственных средств для лечения кислотозависимых заболеваний желудочно-кишечного тракта до настоящего времени остаётся актуальным вопросом фармакологии.

**Цель** данного исследования – экспериментальное изучение противоязвенной активности субстанции динитрата 2-фенил-9-диэтиламиноэтилимидазо[1,2-а]бензимидазола на модели повреждения слизистой оболочки желудка, вызванной введением комбинации 80% этанола и преднизолона (20 мг/кг).

**Материалы и методы**. Для моделирования повреждения слизистой оболочки желудка экспериментальным животным (белые крысы-самцы линии Wistar) вводили преднизолон из расчета 20 мг/кг и 80% этиловый спирт в дозе 0,6 мл на 100 г массы тела животных. Преднизолон растворяли в 80% спирте. В качестве препаратов сравнения были выбраны антисекреторные противоязвенные средства, активно применяемые в клинической практике: ранитидин (30 мг/кг, 10 мг/кг и 3 мг/кг) и омепразол (3 мг/кг, 1 мг/кг и 0,3 мг/кг). Изучаемое соединение использовалось в дозах 30 мг/кг, 10 мг/кг и 3 мг/кг. Все исследуемые вещества вводились внутрижелудочно с помощью атравматичного зонда.

**Результаты и обсуждение**. Установлено, что производное бензимидазола в исследуемых дозах способствует дозозависимому достоверному относительно контроля и препаратов сравнения (ранитидина и омепразола) снижению площади и глубины язвенных поражений слизистой оболочки желудка. Кроме того, в максимальной исследованной дозе (30 мг/кг) достоверно снижается более чем в 2 раза доля животных с язвенными поражениями. Расчетные значения $ЕД_{50}$ для производного бензимидазола и ранитидина составили 5,09 мг/кг и 38,23 мг/кг, соответственно.

**Заключение**. Полученные экспериментальные данные свидетельствуют о том, что производное бензимидазола оказывает выраженный дозозависимый противоязвенный эффект на модели этанол-преднизолоновых эрозивно-язвенных дефектов слизистой оболочки желудка крыс, превосходящий эффекты препаратов сравнения, что делает перспективным его дальнейшее изучение.

**Ключевые слова:** динитрат 2-фенил-9-диэтиламиноэтилимидазо[1,2-а]бензимидазола, противоязвенное действие, этанол-преднизолоновые повреждения, доклинические исследования

**Список сокращений:** СОЖ – слизистая оболочка желудка, СИ – степень изъязвления, ИИ – индекс изъязвления

## INTRODUCTION

Nowadays, the search for new effective and safe medicines for the treatment of acid-dependent gastrointestinal diseases, remains an urgent problem of modern pharmacology [1, 2]. Chemical compounds from the group of benzimidazole derivatives, seem promising in terms of the development of new drugs, including those associated with an increase in acid production in the stomach [3, 4].

Nowadays, preclinical studies of the benzimidazole derivative, i.e., 2-phenyl-9-diethylaminoethylimidazo[1,2-a] benzimidazole dinitrate, which demonstrates antisecretory, antiulcer and gastroprotective effects in the experimental animal models of the laboratory pathologies associated with various pathogenetic mechanisms of ulcerogenesis in the gastric mucosae, are currently underway [5–7].

It is known that one of the mechanisms of the gastric ulcer formation is dissociation of the hypothalamic-pituitary-adrenal system of functions regulation caused by a chronic stress. In particular, an increased level of glucocorticoids, which cause an increase in catabolic processes that inhibit regeneration, disrupt microcirculation and stimulate the secretion of hydrochloric acid, provokes ulcerogenesis against the background of ischemia of the stomach wall [8, 9].

Moreover, under experimental conditions, the ulcer model caused by the action of the pure prednisolone glucocorticoid in its pure form, is characterized by low reproducibility. Therefore, a model modification had been used. It proposed a combination of prednisolone with 80% alcohol [10].

Ethanol contributes to the dissolution of the protective mucosal barrier, making the stomach wall vulnerable to damage by proteolytic and acidic factors. In addition, ethanol reduces the blood flow, damages the vascular endothelium, unbalances the cellular antioxidant defense, stimulating the formation of superoxidanion and hydroperoxide radicals [11].

Thus, the combination of ethanol and prednisone makes it possible to recreate erosive-ulcerous defects of the gastric mucosa with a high (100%) statistical probability.

**The aim** of this study was an experimentally study of the anti-ulcer activity of 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole dinitrate on the model of damage to the gastric mucosa caused by the administration of 80% ethanol and prednisolone combination (20 mg/kg).

## MATERIALS AND METHODS

### Animals

A study of the pharmacological activity was performed on outbred Wistar male rats (aged 10–12 weeks) weighing 180–250 g, obtained from the Rappolovo laboratory animal nursery (Rappolovo village, Leningrad region). At the time of the study, the rats were kept under standard vivarium conditions at the air temperature of 22 ± 20°C, the relative humidity of 60 ± 5% and a natural change in the daily cycle. Extruded food and tapwater were received by the rats ad libitum. The dispersion in the initial animals' mass in the group did not exceed 10% [12].

The conditions of keeping the animals met the requirements of the Decree of the Chief State Sanitary Doctor of the Russian Federation No.51 dated 29.08.2014 "On approval of SP 2.2.1.3218-14 "Sanitary and epidemiological requirements for the device, equipment and maintenance of experimental biological clinics (vivaria)".

Manipulations with the experimental animals were performed in accordance with the generally accepted ethical standards adopted by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (1986) and taking into account the International recommendations of the

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DOI: 10.19163/2307-9266-2019-7-6-339-345

European Convention for the protection of vertebrate animals used in experimental studies (1997) [13, 14].

### Study design

The substance of 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole dinitrate was synthesized at the Scientific Research Institute of Physical & Organic Chemistry (FSAEI HE "Southern Federal University", Rostov-on-Don).

The evaluation of the antiulcer effect of the substances was carried out at the doses of 3 mg/kg, 10 mg/kg and 30 mg/kg. The omeprazole substance of 0.3 mg/kg, 1 mg/kg and 3 mg/kg (Sigma Aldrich, USA) and the ranitidine substance of 3 mg/kg, 10 mg/kg and 30 mg/kg (Sigma Aldrich, USA), were used as reference drugs.

The maximum volume of the drugs for the intragastric administration to the rats did not exceed 3 ml for the animals weighing up to 200 g, 5 ml for the animals from 200 to 240 g and 6 ml for the animals weighing more than 240 g.

Peptic ulcers were caused by the administration of prednisone at the rate of 20 mg/kg and 80% ethyl alcohol at the dose of 0.6 ml per 100 g of the animal body weight. Prednisolone was dissolved in 80% alcohol.

24 hours before the simulation of the pathology, the animals were subjected to a food deprivation with a free access to water.

In the experimental study, the animals had been divided into 10 groups of 10 individuals in each. The individuals of the control group were given a combination of ethanol and prednisone intragastrically as a single dose. In the experimental groups, the studied benzimidazole derivative and comparison preparations were administered 1 hour before the ethanol and prednisolone combination. The animals were euthanized 12 hours after the administration of the ethanol and prednisone combination.

After opening the abdominal cavities, the stomachs were removed and bathed with physiological saline. Then a macroscopic assessment of the mucous membrane was carried out, the tissues were photographed (macro photography). For histological studies, the stomachs were fixed in 10% neutral formalin and embedded in paraffin. Then, with the use of a sled microtome, the sections were prepared. They were stained with hematoxylin-eosin and probed microscopically. The process was followed by a description of the histological picture. Then the assessment of the depth of damage to the wall of the stomach was carried out.

Microphotography was performed using a LeicaDM 1000 microscope and the Leica Application Suite software package (Leica, Germany). The morphometric study was carried out using Leica Application Suite software tools, where the depth of the damage to the gastric mucosa was determined.

### Defined indicators

To assess the severity of the damage in the study of the antiulcer action in the simulation of all pathological conditions, a point system was used [15].

In each group, the total score was calculated. The arithmetic mean value characterizing the average degree of ulceration in the group, was derived from that. In addition, the ulceration index was calculated in the group. The ulceration index reflects both the percentage of the frequency of animals with ulcers and the degree of degenerative disorders in the stomach. The depth of the damage was assessed by a histological examination.

According to the results of the study, the $ED_{50}$ values were calculated for the benzimidazole derivative and for ranitidine, since they had been used in the same doses. The $ED_{50}$ for omeprazole was not determined, since this substance has a different order of the doses used, and therefore, the comparison of the $ED_{50}$ values as a criterion for evaluating the effectiveness, is incorrect.

### Statistical processing

The obtained experimental data have been analyzed based on the method of variation statistics. The summary tables show the group averages (M) and the standard error of the mean (m). Intergroup differences have been analyzed based on a nonparametric criterion - the Mann-Whitney U test. The differences were determined at the 0.05 significance level. For statistical processing of the results, the StatPlus 2009 software package was used.

## RESULTS

Macroscopically, this model reproduces the ulcerous defects affecting a significant area of the gastric mucosa, while both erosive and ulcerative lesions up to the necrotic ones, are clearly expressed.

The obtained experimental data showed that the benzimidazole derivative at the doses of 3, 10 and 30 mg/kg, contributes to the dose-dependent reliable control and reference drugs (ranitidine and omeprazole) reducing the ulcer lesions, the degree of perforation (DP) and the index of perforation (IP) by 35%, 74% and 91%, respectively. In these conditions, the proportion of the animals with ulcerative lesions of the gastric mucosa**e** reduced by 60% at the dose of 30 mg/kg (Table 1).

Ranitidine at the dose of 10 and 30 mg/kg also caused a significant decrease in the area of peptic ulcer lesions relative to the control. However, taking into account the degree of perforation points and the frequency of manifestations of peptic ulcers, the decrease in the index of perforation did not exceed 38% and 44%, respectively.

The administration of omeprazole at the doses of 1 and 3 mg/kg contributed significantly to the control decrease in the area of ulcerous defects, the degree of perforation and the index of perforation by 35% and 46%, respectively, while the proportion of the animals with ulcerative lesions of the gastric mucosae did not differ significantly from the control ones with the administration of both ranitidine and omeprazole (Table 1).

*ISSN 2307-9266 e-ISSN 2413-2241*

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**Table 1 – Macroscopic analysis of the effect of the benzimidazole derivative substance and reference preparations on the gastric mucosae in response to the administration of the ethanol and prednisolone combination (male rats), n = 10, M ± m**

| Substance | Dose, mg/kg | Type of injury | Area of injury ($mm^2$/animal) | Proportion of animals with gastric mucosae (%) | Degree of perforation | Index of perforation | Index of perforation decline (%) |
|---|---|---|---|---|---|---|---|
| Control Ethanol + Prednisolone | – | Ulcers | 88.9±25.7 | 100% | 4.0 ±0.0 | 4.00 | – |
| | | | 36.7±15.9 | 90% | | | – |
| Benzimidazole derivative | 3 | Ulcers | 23.7±15.1* | 100% | 2.6±0.4* | 2,60 | –35 |
| | | Erosions | 16.3±6.5 | 90% | | | |
| | 10 | Ulcers | 3.2±0.9*#& | 70% | 1.5±0.4*# | 1.05 | –74 |
| | | Erosions | 7.9±5.8 | 80% | | | |
| | 30 | Ulcers | 4,4±3,4*#& | 40% | 0.9±0.4*# | 0.36 | –91 |
| | | Erosions | 16.3±7.1 | 80% | | | |
| Ranitidine | 3 | Ulcers | 38.6±7.8 | 90% | 3.3±0.4 | 2.97 | –26 |
| | | Erosions | 19,2±4,2 | 90% | | | |
| | 10 | Ulcers | 28,5±6,2* | 80% | 3.1±0.5 | 2.48 | –38 |
| | | Erosions | 15.7±5.7 | 70% | | | |
| | 30 | Ulcers | 18.1±5.6* | 80% | 2.8±0.6* | 2.24 | –44 |
| | | Erosions | 27.1±9.3 | 70% | | | |
| Omeprazole | 0,3 | Ulcers | 47.0±7.6 | 100% | 3.9±0.1 | 3.90 | –3 |
| | | Erosions | 29.7±8.2 | 80% | | | |
| | 1 | Ulcers | 42.9±10.8* | 90% | 2.9±0.5* | 2.61 | –35 |
| | | Erosions | 28.2±8.8 | 80% | | | |
| | 3 | Ulcers | 21.8±5.7* | 80% | 2.7±0.5* | 2.16 | –46 |
| | | Erosions | 17.6±6.7 | 70% | | | |

Note: * – significantly relative to control, P <0.05; # – significantly relative to the group receiving ranitidine, P <0.05; & – significantly relative to the group receiving omeprazole, P <0.05.

**Table 2 – Results of morphometric assessment of the benzimidazole derivative and reference drugs' effect on the development of ulcerative lesions of the gastric mucosae in response to the administration of the ethanol (80%) and prednisolone 20 mg/kg (n = 50) combination**

| Group | Dose, mg/kg | Depth of injury, mkm | Average reduction in injury depth, % |
|---|---|---|---|
| Control (ethanol 80% + prednisolone 20 mg / kg) | – | 311.22±9.83 | – |
| Benzimidazole derivative | 3.0 | 194.91±9.03*& | –38 |
| | 10.0 | 179.79±10.17*#& | –42 |
| | 30.0 | 89.76±10.27*#& | –71 |
| Ranitidine | 3.0 | 251.28±15.94* | –20 |
| | 10.0 | 283.03±21.53 | –9 |
| | 30.0 | 282.33±22.11 | –9 |
| Omeprazole | 0.3 | 225.76±12.48* | –28 |
| | 1.0 | 246.35±14.14* | –21 |
| | 3.0 | 189.55±16.95* | –39 |

Note: * – significantly relative to control, P <0.05; & – significantly relative to the group receiving ranitidine at the equivalent dose, P <0.05; # – significantly relative to the group receiving omeprazole at the equivalent dose, P <0.05

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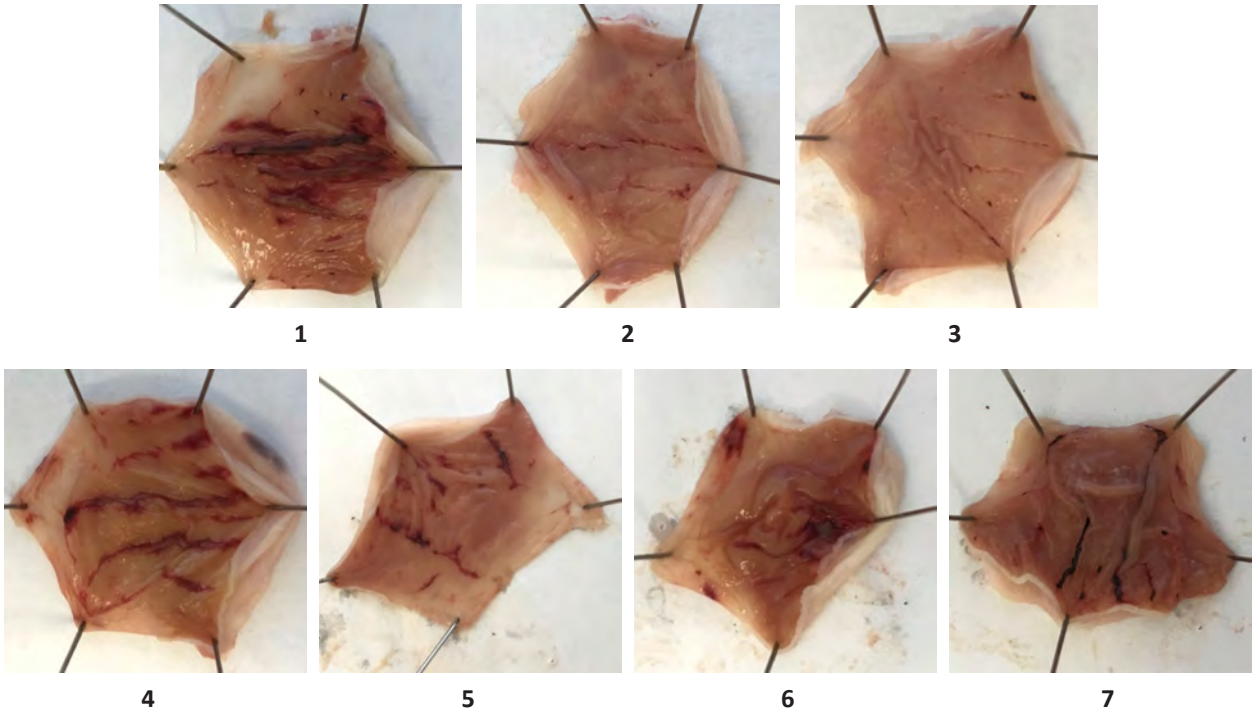


**Figure 1 – Macrographs of the rats' stomachs in modeling erosions caused by the administration of the ethanol and prednisolone combination (natural scale):**

Note: 1 – Control (ethanol + prednisolone), 2 – Benzimidazole derivative 10 mg/kg, 3 – Benzimidazole derivative 30 mg/kg, 4 – Ranitidine 10 mg/kg, 5 – Ranitidine 30 mg/kg, 6 – Omeprazole 1 mg/kg 7 – Omeprazole 3 mg/kg

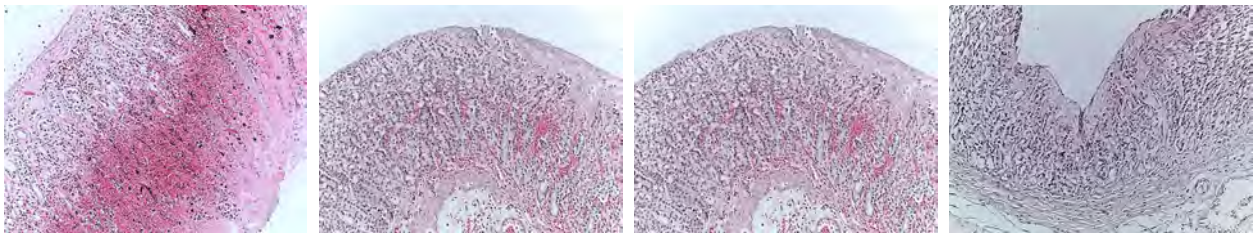


**Figure 2 – Micrograph of the mucous membrane of the rats' stomachs in modeling erosions caused by the administration of the 80% ethanol and prednisolone (20 mg/kg) combination against the background of the benzimidazole derivate administration**

Note: 1 – Control (ethanol + prednisone), 2 – Benzimidazole derivative 3 mg/kg, 3 – Benzimidazole derivative 10 mg/kg, 4 – Benzimidazole derivative 30 mg/kg

Thus, on the model of ethanol-prednisolone ulcers, the benzimidazole derivative had a pronounced antiulcer effect that exceeded the results in the ranitidine and omeprazole groups by an average of 2 times.

The calculated $ED_{50}$ value for the benzimidazole derivative was 5.09 mg/kg, and the $ED_{50}$ value for ranitidine was 38.23 mg/kg.

Fig. 1 shows the most typical macroscopic picture of the gastric mucosa against the background of the action of the studied substance of the benzimidazole derivative in comparison with ranitidine and omeprazole in modeling ethanol-prednisolone ulcerative lesions.

Histological studies showed that the combination of ethanol with prednisolone causes the development of extensive superficial defects of gastric mucosae with impressive zones of eosin masses affecting the submucosal base, profound leukocyte infiltration, hemorrhage and tissue necrosis (Fig. 2).

The benzimidazole derivative contributed to the dose-dependent decrease in the degree of mucosal injury, reduction of edema, hemorrhage, necrosis and destruction of blood vessels (Fig. 2).

In this model of injury, ranitidine and omeprazole showed a significantly less pronounced protective effect. The frequencies of the injuries reaching the submucosa, necrosis, destruction of glands and hemorrhages in the mucous membrane were comparable with the control (Fig. 3).

The results of morphometric studies indicate that the substance of the benzimidazole derivative at the doses of 3, 10 and 30 mg/kg contributed significantly to the control and reference drugs to reduce the injury depth by 38%, 42% and 71%, respectively (Table 2), significantly exceeding ranitidine and omeprazole effects.

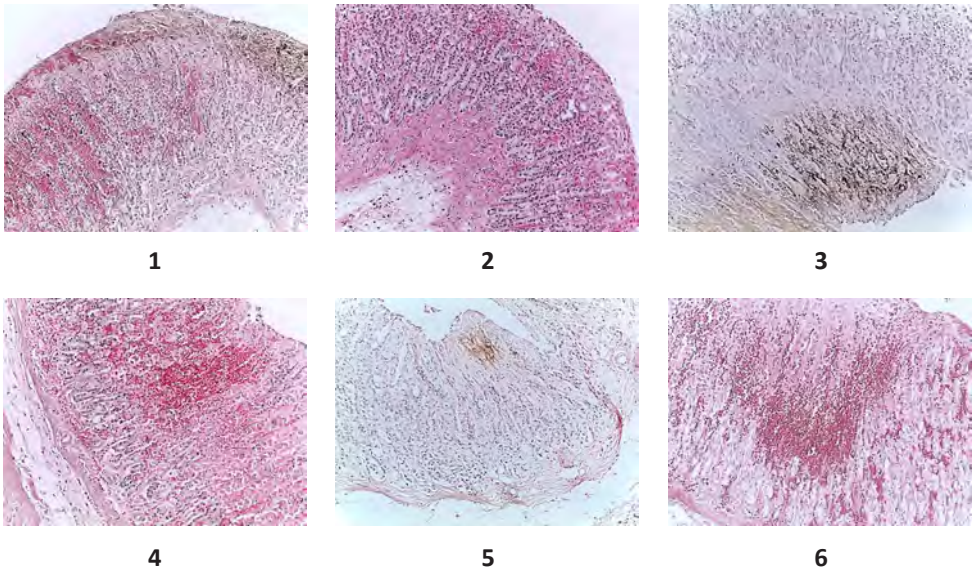


**Figure 3 – Microphotographs of the mucous membrane of the rats' stomachs in modeling erosions caused by the administration of the 80% ethanol and prednisolone (20 mg/kg) combination against the background of the reference drugs administration**

Note: 1 – Ranitidine 3 mg/kg, 2 – Omeprazole 0.3 mg/kg, 3 – Ranitidine 10 mg/kg, 4 – Omeprazole 1 mg/kg, 5 – Ranitidine 30 mg/kg, 6 – Omeprazole 3 mg/kg

## DISCUSSION

The mechanism of the damaging effect of glucocorticoids on the gastric mucosa is in reducing the resistance of the protective barrier by inhibiting the synthesis of prostaglandins, which have a gastroprotective effect. It is also in inhibiting the activity of peroxidase, which leads to an increase in the level of $H_2O_2$ and, accordingly, the level of hydroxyl radicals, which, in their turns, enhances the gastric mucosa injury with hydrochloric acid secreted by the parietal cells of the stomach [16]. Glucocorticoids also slow down the repair and regeneration of the gastric mucosa by inhibiting angiogenesis [17], suppressing EGF-stimulated proliferation of the gastric mucosa epithelial cells, and under these circumstances one of the ways is to inhibit the activation of ERK1/ERK2, followed by suppression of COX-2 and a decrease in Cyclin D1 expression and DNA synthesis [18]. Ethanol, in its turn, helps to dissolve the protective mucous barrier, reduces the blood flow, damages the vascular endothelium and stimulates the formation of superoxide anion and hydroperoxide radicals [11]. The studied pharmaceutical substance of 2-phenyl-9-diethylaminoethylimidazo[1,2-a]benzimidazole dinitrate has shown a pronounced dose-dependent antiulcer effect in modeling a gastric mucosa injury caused by a combination of 80% ethanol and prednisolone. It has been recorded at both – macro- and micro-morphological levels, reducing the area and degree of the damage to the gastric mucosa, reducing edema, the intensity of hemorrhages, necrosis and destruction of blood vessels. Under these circumstances, the data obtained are consistent with the results of several previous studies, indicating the presence of antiulcer and gastroprotective effects under the conditions of reproduction of helicobacter-like damage in rats modeled by intragastric administration of ammonia in combination with acute dosed blood loss [5] and immobilization stress [6].

The antiulcer action of the benzimidazole derivative can be associated with several mechanisms. First, it is due to the pronounced antisecretory effect, manifested by suppression of HCl secretion, both basal and under the conditions of stimulation with histamine [7]. It leads to a decrease in the acidity of the gastric contents and, accordingly, a decrease in its ulcerogenic potential. Second, it is connected with the probable antioxidant activity, which is shown in a number of studies on the biological activity of benzimidazole derivatives [19–21], which, however, requires additional studying.

## CONCLUSION

The obtained experimental data indicate that the benzimidazole derivative has a pronounced dose-dependent antiulcer effect on the model of ethanol-prednisolone erosive-ulcerous defects of the gastric mucosa, on average 2 times greater than the effects of the reference drugs.

The research results are consistent with the previously obtained data on the antisecretory and antiulcer activity of 2-phenyl-9-diethylaminoethylimidazo[1,2-a] benzimidazole dinitrate. A further study of the pharmacological effects of this compound in order to create a new antisecretory antiulcer drug, can be suggested as promising if we take into consideration the level of anti-ulcerogenic effect exceeding that of the reference drugs widely used in clinical practice, $H_2$-histamine blocker ranitidine and an inhibitor of hydrogen-potassium ATPase omeprazole.

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DOI: 10.19163/2307-9266-2019-7-6-339-345

## FINANCIAL SUPPORT

This study has been carried out as a part of the federal target program “Development of the pharmaceutical and medical industry of the Russian Federation for the period up to 2020 and the future perspective” is the State contract dated November 14, 2017 No. 14.N08.11.1042.

## AUTHOR’S CONTRIBUTION

All the authors have equally contributed to the research work.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHORS

**Maxim V. Chernikov** – Doctor of Sciences (Medicine), Associate Professor, Head of the Department of Biology, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. Research interests: pharmacology, clinical pharmacology. ORCID 000-0001-8340-1296. E-mail: pharmax@list.ru

**Marina A. Oganova** – Candidate of Sciences (Pharmacy), Associate Professor of the Department of Biology, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. Research interests: pharmacology, clinical pharmacology. E-mail: marina-oganova81@mail.ru

**Svetlana A. Kalashnikova** – Doctor of Sciences (Medicine), Professor, Head of the Department of Morphology, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID 0000-0002-7688-9366. E-mail: kalashnikova-sa@yandex.ru

**Lyudmila V. Polyakova** – Candidate of Sciences (Medicine), Associate Professor, Department of Morphology, Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. ORCID 0000-0002-7688-9366. E-mail: lypolyakova7@gmail.ru

**Natalya A. Khromova –** Postgraduate Student, Department of Biology and Physiology**,** Pyatigorsk Medical and Pharmaceutical Institute – branch of Volgograd State Medical University. E-mail: khromovanatasha1994@gmail.com

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

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# PHYTOCHEMICAL STUDY OF *TRIBULUS TERRESTRIS* L.

**A. Affaf, Yu.N. Karpenko, D.K. Gulyaev, V.D. Belonogova, E.I. Molokhova, O.L. Blinova, A.A. Gileva**

Perm State Pharmaceutical Academy
2, Polevaya Str., Perm, Russia 614990

E-mail: dkg2014@mail.ru

Received 09 September 2019 Review (1) 04 November 2019 Review (2) 26 December 2019 Accepted: 28 December 2019

*Tríbulus terrestris* L., an annual herb belonging to the *Zygophyllaceae* family and growing in moderate and tropical climates, has a rich chemical composition of biologically active substances and chemical elements.

**The aim** of the work is a phytochemical study of *Tribulus terrestris* L. growing in different geographical zones.

**Materials and methods.** The objects of study were herb specimens of *Tribulus terrestris* L. collected in different habitats. The samples of the raw materials were shade-dried. The determination of saponins in the raw materials, was carried out by high performance liquid chromatography with a mass spectrometric detection (HPLC-MS / MS). The study of the qualitative and quantitative composition of the elements was carried out on an X-ray fluorescence spectrometer.

**Results.** The saponins had been studied by HPLC-MS/MS, according to which in all the studied samples, dioscin and protodioscin were found. Their retention times coincided with the retention times of dioscin and protodioscin standards. It has been established that among the macroelements of *Tríbulus terrestris* L., potassium and calcium are mostly accumulated. They account for about 90% of the total content of the elements in the plant. It has been revealed that the distribution of macro- and microelements in the plant, varies significantly depending on their place and growing conditions.

**Conclusion.** The maximum dioscin content was observed in the samples harvested in Moldova, and the minimum – in the samples from the nursery garden of the All-Russian Scientific Research Institute of medicinal and aromatic plants. The largest amount of protodioscin was found out in the samples from the Crimea, and the minimum – in the samples from Moldova. The carried out study of the elements content of *Tríbulus terrestris* L. showed that the habitats (geographical zones) in which the studied samples of raw materials had been were collected, affect the accumulation of the elements by the plant. Based on the data obtained, biological absorption series have been compiled for the samples from each habitat.

**Keywords:** *Tríbulus terrestris* L., high performance liquid chromatography with a mass spectrometric detection, HPLC-MS/MS, elements content, phytochemistry

# ФИТОХИМИЧЕСКОЕ ИССЛЕДОВАНИЕ ТРАВЫ ЯКОРЦЕВ СТЕЛЮЩИХСЯ

**А. Аффуф, Ю.Н. Карпенко, Д.К. Гуляев, В.Д. Белоногова, Е.И. Молохова, О.Л. Блинова, А.А. Гилева**

ФГБОУ ВО «Пермская государственная фармацевтическая академия» Министерства здравоохранения Российской Федерации; 614990, Россия, г. Пермь, ул. Полевая, д. 2

E-mail: dkg2014@mail.ru

Получено 09.09.2019 Рецензия (1) 04.11.2019 Рецензия (2) 26.12.2019 Принята к печати 28.12.2019

Якорцы стелющиеся, или якорцы наземные – *Tríbulus terrestris* L., семейство парнолистниковые – *Zygophyllaceae*, однолетнее травянистое растение, произрастающее в умеренном и тропическом климате, имеет богатый химический состав биологически активных веществ и химических элементов.

**Цель работы –** фитохимическое исследование якорцев стелющихся, произрастающих в разных географических зонах.

**Материалы и методы.** Объектами исследования являлись образцы травы якорцев стелющихся, собранные в различных условиях

**For citation:** A. Affaf, Yu.N. Karpenko, D.K. Gulyaev, V.D. Belonogova, E.I. Molokhova, O.L. Blinova, A.A. Gileva. Phytochemical study of Tribulus Terrestris L. *Pharmacy & Pharmacology*. 2019;7(6):346-355. **DOI:** 10.19163/2307-9266-2019-7-6-346-355

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**Для цитирования:** А. Аффуф, Ю.Н. Карпенко, Д.К. Гуляев, В.Д. Белоногова, Е.И. Молохова, О.Л. Блинова, А.А. Гилева. Фитохимическое исследование травы якорцев стелющихся. *Фармация и фармакология.* 2019;7(6):346-355. **DOI:** 10.19163/2307-9266-2019-7-6-346-355

ОРИГИНАЛЬНАЯ СТАТЬЯ
DOI: 10.19163/2307-9266-2019-7-6-346-355

обитания. Образцы сырья были высушены воздушно-теневым способом. Определение сапонинов в сырье проводили методом высокоэффективной жидкостной хроматографии с масс-спектрометрическим детектированием (ВЭЖХ-МС/МС). Изучение качественного и количественного состава элементов проводили на рентгенофлуоресцентном спектрометре.
**Результаты.** Проведено исследование сапонинов методом ВЭЖХ-МС/МС, по результатам которого, во всех исследуемых образцах обнаружены диосцин и протодиосцин, времена удерживания которых совпадают с временами удерживания стандартов диосцина и протодиосцина. Установлено, что в образцах якорцев стелющихся травы среди макроэлементов в наибольшем количестве накапливаются калий и кальций, на их долю приходится около 90% от общего содержания элементов в растении. Выявлено, что распределение макро и микроэлементов в растении существенно различается в зависимости от места и условий произрастания.
**Заключение**. Максимальное содержание диосцина наблюдали в образцах, заготовленных в Молдове, а минимальное – в образцах из питомника ФГБНУ ВИЛАР. Наибольшее количество протодиосцина обнаружено в образцах из Крыма, а минимальное – в образцах из Молдовы. Проведенное исследование элементного состава якорцев стелющихся травы показало, что места обитания (географические зоны), в которых осуществлялся сбор исследованных образцов сырья, влияют на накопление элементов растением. С учетом полученных данных составлены ряды биологического поглощения для образцов из каждого места произрастания.
**Ключевые слова:** якорцы стелющиеся, *Tríbulus terrestris*, ВЭЖХ-МС/МС, элементный состав, фитохимия

## INTRODUCTION

Currently, a lot of attention is focused on complex preparations containing vitamins, amino acids and microelements. It has been established that manganese (Mn) and molybdenum (Mo) potentiate the effect of cardiac glycosides, Mn potentiates the effect of ascorbic acid and carotenoids in medicinal plants. In addition, the microelements found in plants, are better absorbed by the human body since they are found in plant materials in “biological” concentrations [1, 2].

*Tríbulus terrestris* L. is an annual herb belonging to the *Zygophyllaceae family*.

The plant has a tap-root system with a framework of fibrous lateral roots. Its leaves are pinnately-paired compound. The flowers with a diameter of 1–1.2 cm, are located in the leaf angles. The calyx consists of 5 long-pointed, externally pressed hairy calyx lobes 4–5 mm long, 1–1.3 mm wide [3–5].

It grows in moderate and tropical climates in southern Europe, South Asia, Africa, Northern Australia.

In the Russian Federation, *Tríbulus terrestris* L. grows in the European part, in the North Caucasus, in Western and Eastern Siberia and in the Crimea. It grows in dry, sandy and rocky steppes on clay-silty, saltierra, sandy loamy soils, moist meadows, along river valleys, like weeds in crops, along roads [6]. Raw materials base consist of wild plants.

One of the major groups of *Tríbulus terrestris* L. biologically active substances are steroidal saponins, represented by Dioscin, Protodioscin, Tribestin, Prototribestin, Methylprototribestin, methyl protodioscin, pseudo-protodioscin, Tribulosin and other compounds [7–10]. In the raw materials there are flavonoids, mainly derivatives of quercetin, astragalin, 3-rutinoside, 3-genciobioside kaempferol, 3-genciobioside isorhamnetin, tribuloside, rutin, kaempferol, quercetin, 3-O-rhamnoside quercetin [7, 11–13]. In the roots, aerial parts, fruits, flowers there is hecogenin, in the roots, aerial parts there is neo-tigogenin, in roots and flowers there are such substances as β-sitosterol, stigmasterol, campesterol; in the aerial part and flowers there is ruskogenin.

Carotenoids: in leaves there is α carotene; in fruits there are phenolcarboxylic acids – ferulic and p-hydroxybenzoic; alkaloids: tribulusamide C, tribulusterine, tribulusin, harmine. Harmine; other components include organic acids, amino acids; as for organic acids, there are benzoic, vanilla, ferulic and succinic acids. The main amino acids are alanine and threonine, in addition, there are the following substances there: coumarin, emodin and fission [6, 14–17].

Extracts from *Tribulus terrestris* L., have hypocholesteremic, anti-inflammatory, hepatoprotective, antihypertensive, immunomodulating and antioxidant effects, they enhance intestinal motility, stimulate the function of the sex glands, and have the properties of an aphrodisiac [18].

*Tríbulus terrestris* L. is widely used in folk medicine in many countries [19]. In folk medicine, it is used to treat impotence [19]. In folk medicine of Iran – as a diuretic, laxative, for poultices, in the treatment of syphilis [20]. In Nepal it is used for urogenital infections [21], in the Indian medicine – for radiculitis, inflammation of the pelvic and sacral organs, dry cough and respiratory disorders [22]. In the traditional Chinese medicine, it is used for the treatment of eyes, swelling, abdominal bloating, pathological pains and sexual dysfunctions. In the Shern-Nong Pharmacopoeia, *Tríbulus terrestris* L. is presented as a very valuable drug [19].

In the study conducted in Egypt in 2015 in an elder age group of men suffering from age-related androgen deficiency, the use of a *Tríbulus terrestris* L. extract showed a statistically significant difference in the increase in testosterone levels [23].

The study of 2016 carried out in Iran on human spermatozoids, showed improvements in various parameters of male sperm [24]. Another study on 65 males in Brazil, 2016, showed a significant increase in its quality [25]. A randomized, double-blind, placebo-controlled study confirmed a high effectiveness *Tríbulus terrestris* L. in the treatment of female sexual dysfunction [26].

In 2016, scientists from Iran showed that ethanol extract of *Tríbulus terrestris* L. effectively reduced glucose levels compared with placebo in women with type 2 diabetes [27]. The mechanism of hypoglycemic action, most likely, is associated with inhibition of the activity of α-glucosidase in the small intestine [28, 29]. A mild α-amylase inhibitory effect was also established [28].

Steroid saponins of *Tríbulus terrestris* L. have an antifungal effect against fluconazole-resistant fungi of the *Candida* genus [30]. Other *in vitro* studies have also shown both the antifungal and antibacterial properties of this plant [31].

In vitro studies of heart cells and whole animals' hearts showed that *Tríbulus terrestris* L. substances have a protective effect on the heart tissue [32], as well as the neuroprotective effect of steroidal saponins [33]. On the basis of *Furostanol-Type* Steroidal Saponins, Bulgarian scientists have created Tribestan, the drug which has hypolipidemic and hypocholesteremic effects [34].

A neogalenical drug Tribusponin (the sum of steroid saponins), has been obtained by Georgian scientists on the basis *Tribulus terrestris* L.; it reduces a blood cholesterol level and increases a lecithin/ cholesteric coefficient [35].

The drug "Tribusponin" has been registered in the Russian Federation in the dosage form of tablets (0.1 g) with *Tríbulus terrestris* L. extract as an antisclerotic agent; the extract is a part of the complex preparations "Fitovit", "Speman", "Speman forte". ZAO "Evalar" produces dietary supplements "Effex tribulus" for the treatment of erectile dysfunction and to increase potency.

Because of a widespread currency and use of the plant in scientific and folk medicine, it is of great interest in studying the chemical composition of the raw materials of *Tribulus terrestris* L. collected in different habitats.

**THE AIM** of the work is a study of *Tríbulus terrestris* L. harvested in different geographical zones.

## MATERIALS AND METHODS

### Object of the study

The object of the study is *Tríbulus terrestris* L. collected in different geographical zones: Syrian Arab Republic, the vicinity of Damascus; Republic of Moldova, the vicinity of Chisinau (Kishinev); the Botanical Garden of VILAR; the peninsula of the Crimea, the peak of the Mount Kush-Kaya (Table 1). The samples were collected during flowering and fruiting periods and represented the entire aerial part of the plant with the root. The primary processing of the raw materials was to remove the root, brown parts of the plant. After the shade drying, the raw materials met the requirements of All-Russian Federal Assembly No. 42-827-79 "Herba tribuli terrestris" in terms of "authenticity" and "good quality".

### Methods of determining of saponins

Quantitative determination of saponins in *Tribulus terrestris* L. was performed by HPLC-MS/MS using a liquid triple quadrupole chromatomass spectrometer LCMS-8050 (Shimadzu) under the following conditions:

– chromatographic column: Luna 5u C18(2) (2.1×150 mm 3.5-Micron);
– column thermostat temperature: 40°C;
– eluent: 0.1% formic acid solution – acetonitrile;
– elution mode: gradient;
– mobile phase flow rate: 0.4 ml/min.
–The conditions of mass spectrometric detection are presented in Table. 2.

### Sample preparation

About 0.5 g of the crushed raw materials (accurately weighed quantity) was placed in a conical flask with a capacity of 100 ml, 25 ml of methanol (c.p.) was added. Next, the contents of the flask were extracted in an ultrasonic bath for 30 minutes at the temperature of 30±1°C. 1 ml of the resulting extraction was transferred to a centrifuge tube and centrifuged for 5 minutes at 10.000 rpm.

50 µl of the supernatant was placed in a 2 ml vial, 950 µl of methanol was added and mixed up. The volume of the analyzed sample was 5 µl.

The identification and quantitative determination of saponins in the extracts from the plant raw materials was carried out using standard samples of dioscin and protodioscin (Sigma-Aldrich, Germany). Accurately weighed quantities (about 1 mg) were dissolved in 1 ml of methanol. The resulting solutions were used to prepare methanol solutions with the concentration of 1000 ng/ml of each standard.

The detection of the generated ions was carried out in the MRM mode (monitoring of multiple reactions). Ionic transitions for dioscin were 869.50 → 415.00, for protodioscin 1031.5 → 415.0 [36].

The determination was carried out in three replications.

### Methods of determining elements content

The elements content was studied using an X-ray fluorescence method recommended for the study of the elemental composition of medicinal raw materials (State Pharmacopoeia XIV) [37].

### Sample preparation

About 10 g of dry *Tribulus terrestris* L. was ground to a powdery state, placed in a crucible and burned on a stove in an exhaust fume hood until smoking stopped. The crucible was placed in a muffle furnace at a temperature of 500±1° C, kept there for about 2 hours, until it was completely salted and there was no black coal mass. After complete cooling in the fume hood, 50% nitric acid was added to the crucible and evaporated on a tile with a shut down spiral, avoiding splashing. Then it was placed in a muffle furnace at the temperature of 500±1 ° C for 2 hours [38].

After cooling the crucible, the qualitative and quantitative composition of the elements was determined in the ash residue on a Thermo Scientific QUANT'X X-ray fluorescence spectrometer [39]. The determination was carried out in in three replications.

### Statistical processing of results

Processing of chromatographic information was carried out using the software "LabSolutions" (Shimadzu).

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DOI: 10.19163/2307-9266-2019-7-6-346-355

**Table 1 – Zones for harvesting the samples of *Tribulus terrestris* L. raw materials**

| Habitat | Date of collecting |
|---|---|
| Syrian Arab Republic, vicinity of Damascus | June 2018 |
| The peak of the Mount Kush-Kaya, Laspi Bay, Republic of the Crimea, the Russian Federation | July 2017 |
| The vicinity of Chisinau (Kishinev), Republic of Moldova | June 2017 |
| The Botanical Garden of "All-Russian Scientific Research Institute of medicinal and aromatic plants" (VILAR) | July 2016 |

**Table 2 – Conditions of mass spectrometric analysis**

| Interface | DUIS (electrospray + chemical ionization) |
|---|---|
| Spray Gas Flow | 3 L/min |
| Heating gas flow | 10 L/min |
| Drying gas flow | 10 L/min |
| Interface temperature | 300°C |
| DL temperature | 200°C |
| Heat Block Temperature | 400°C |
| Drying gas Consumption | 10 L/min |
| Capillary voltage | 4000 V |
| Ionization source polarity | Positive |
| Impact Cell Energy | 30 eV |

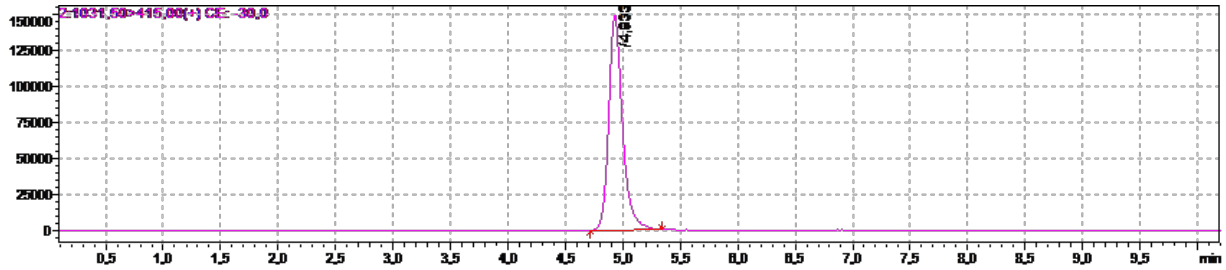

**Figure 1 – Chromatogram of a protodioscin standard sample solution (concentration 1333 ng/ml)**

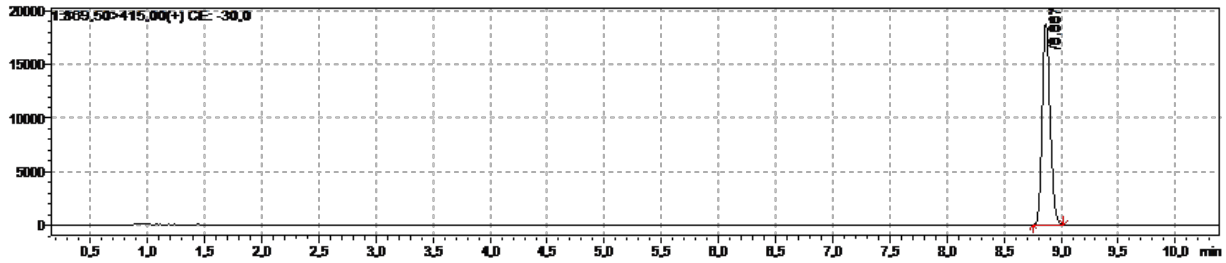

**Figure 2 – Chromatogram of a dioscin standard sample solution (concentration 900 ng/ml)**

For the statistical analysis of the results obtained, the statistical programming language R CRAN was used. The results were processed using Microsoft Excel. To compare the analyses of the results, Student's test was used with an assessment of the reliability of differences ($p<0.05$).

The results were visualized using non-metric multidimensional scaling. To make up the scaling, the Bray distance (also known as the *Sørensen* test) was applied [40, 41]. To minimize the stress function, centering and Procrustean rotation were performed

## RESULTS

Fig. 1–2 show chromatograms of solutions of dioscin and protodioscin standard samples in methanol.

The retention times of protodioscin and dioscin were 4.93 ± 0.03 and 8.86 ± 0.03 min, respectively.

Chromatograms of the extracts from the studied samples of plant materials are presented in Fig. 3 (A–D).

The identification of dioscin and protodioscin on the chromatograms was carried out by retention times in comparison with standard samples. Additional peaks observed on the chromatograms of the extracts corre-

sponding to the coextractive compounds, are well separated and do not interfere with the determination of the target analytes. To quantify saponins in plant materials, the external standard method was used.

The results of the studies showed that dioscin and protodioscin are found in all the studied samples. The maximum dioscin content is observed in the samples of the raw materials collected in the Crimea – 1.90 ± 0.02 ($p<0.05$), and the minimum – in the samples from the nursery garden of the nursery garden of the Federal State Budgetary Scientific Institution "All-Russian Scientific Research Institute of medicinal and aromatic plants" (VILAR). No statistically significant differences were found out between the dioscin content in the samples collected in the Crimea and Syria. The largest amount of proto was also found in the samples from the Crimea – 15.59 ± 0.28 ($p <0.05$), and the minimum – in the samples from Moldova (tab. 3).

The next stage of the work was the determination of the key elements in the elements content of *Tribulus terrestris* L. collected in different habitats, because these key elements are necessary for plant life and affect the processes occurring in the human body (tab. 4).

The data of statistical processing of the analyses results are presented in table. 5.

Fig. 7 shows a mutual disposition of the samples in the reduced attribute space and the contribution of each of the elements studied to the main coordinates.

Fig. 7 shows that the samples characterizing each geographical zone, are localized densely, especially the samples from the VILAR nursery garden. The least similar are the samples from Syria and Russian VILAR, whereas the samples from the Crimea and Moldova, by contrast, are relatively close.

As a result of the elements analysis of *Tribulus terrestris* L. samples, it has been found out that among the macroelements in all the studied samples, potassium and calcium are accumulated most of all, they account for about 90% of the total contents of elements in the plant. Among others, *Tribulus terrestris* L. contains essential elements, such as iron, copper, zinc, manganese, chromium and molybdenum.

The distribution of macro- and microelements in the plants differs significantly depending on the place and conditions of their growth. So, for example, the content of titanium in the samples of the raw materials collected in the VILAR nursery garden, is 5–6 times higher than that in other samples (tab.4). A high content of titanium in these samples is associated with the growing conditions of the plant (cultivated in the VILAR nursery garden). The minimal accumulation of titanium is found out in the samples from Syria. There was more Zinc in the samples collected on the Crimean Peninsula, and its minimum content was in the samples from the VILAR nursery garden. There was most of all of Manganese in the samples from the VILAR nursery garden; and in the samples from Syria, its minimum content was established.

Manganese is involved in metabolism, it improves physiological processes in the body. It takes part in oxidative processes, in the regenerative process of nitrates during photosynthesis, as well as in the antagonism between manganese and other chemical elements [42]. This leads to the conclusion that the intensity of metabolic processes is higher in the plants cultivated in the VILAR nursery garden. A high intensity of metabolic processes indicates favorable conditions for the plant.

The data provided by other researchers [42], showed a certain relationship between the contents of iron and manganese. With a decrease in the manganese content in the plant, an excess amount of active nitrous iron is accumulated [42]. A similar interdependence is found out in some of the studied samples.

The organic compounds containing ferrum, are necessary for plants to ensure the biochemical processes that occur during respiration and photosynthesis. Compounds with ferrum in the structure, act as electron carriers in biochemical processes, since they are components of the enzymes dihydrogenases and cytochromes. Ferrum takes part in the process of chlorophyll biosynthesis, so with a limited supply of it, severe plant diseases, in particular chlorosis, may occur, [42, 43].

The following pattern of ferrum accumulation is presented in the studied samples. Its content was higher in the samples collected in Moldova. There is less iron in the samples collected in Syria.

The maximum amount of silicon was found out in the samples from the VILAR nursery garden, while its minimum amount was found in the samples from Syria.

In the VILAR samples there was twice as much Aluminium as in the other samples. In the human body, aluminum is involved in the construction of epithelial and connective tissues, bone regeneration, mineral metabolism, etc. [1, 44]. It is also worth noting that a high content of aluminum can have a negative effect on the human body, since excessive intake may have an impact on the central nervous system, the progression of Alzheimer's disease and depression [1].

According to GPM 1.5.3.0009.15. "Determination of the content of heavy metals and arsenic in medicinal plant materials and herbal medicines", the maximum permissible concentration (MPC) of lead is 6.0 mg/kg. No lead has been detected in the samples from Syria, the Crimea and Moldova. The lead content in the samples collected in the VILAR nursery garden, did not exceed the maximum permissible concentration.

Based on the results obtained, a series of biological uptake of chemical elements has been compiled for *Tribulus terrestris* L.

The samples harvested in Syria: K>Ca>Na>S>P>Al>Mg>Si>Fe>Zn>Ti>Mn>Cu>Ni.

The samples harvested in the Crimean Peninsula: K>Ca>Na>S>Fe>P>Si>Al>Mg>Ti>Zn>Mn>Cu.

The samples harvested in Moldova: K>Ca>Na>S>Fe>Si>P>Al>Mg>Ti>Zn>Mn>Cu>Cr.

The samples harvested in the VILAR nursery garden: Ca>K>Na>Al>S>Si>Ti>Fe>P>Mg>Mn>Zn>Cu>Cr>Pb>Cu.

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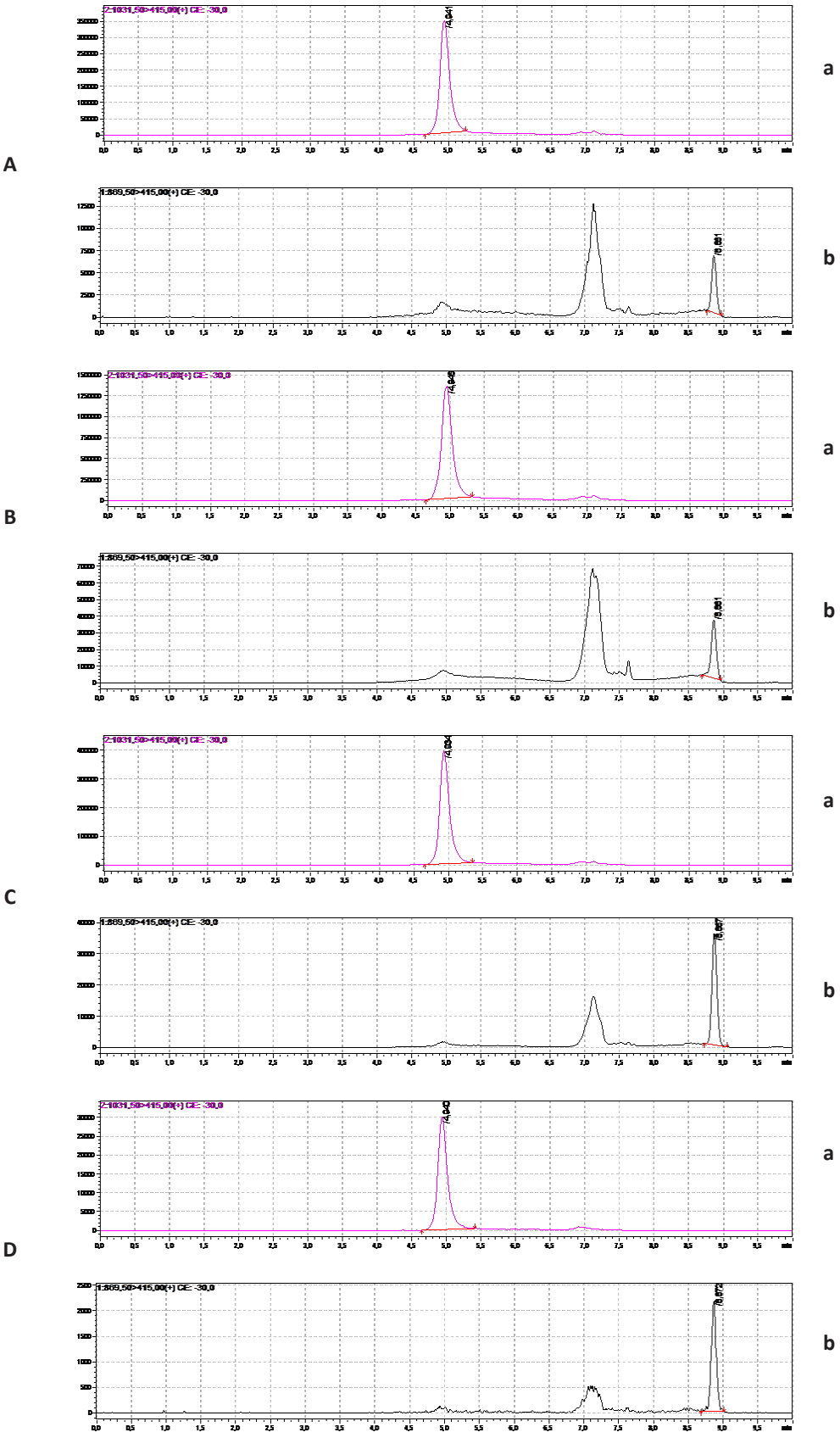


**Figure 3 – Chromatograms of extracts from the samples of raw materials collected in Moldova (A), Crimea (B), Syria (C) and VILAR nursery (D)**

Note: a – detection of protodioscin at ionic transitions (1031.5 → 415.0), b – dioscine (869.50 → 415.00)

**Table 3 – The results of dioscin and protodioscin quantitative determination in the samples of plant materials by method of HPLC-MS/MS**

| Samples | Content of dioscin, mg (in 1 g of raw materials) X cf ± SD | Content of protodioscin, mg (in 1 g of raw materials) X cf ± SD |
|---|---|---|
| Moldova (2017) | 0.265±0.011* | 3.17±0.13* |
| Crimea (2017) | 1.90±0.02 | 15.59±0.28* |
| Syria (2018) | 1.67±0.04* | 3.80±0.11* |
| VILAR (2016) | 0.097±0.02* | 1.435±0.38* |

Note: * – significance of differences p <0.05 significance of differences

**Table 4 – Elements content of *Tribulus terrestris* L. samples**

| Sl.No. | Element | % of the total content of elements in the samples, in terms of dried raw materials | | | |
|---|---|---|---|---|---|
| | | Syria (2018) | Crimea (2017) | Moldova (2017) | VILAR (2016) |
| | | Macroelements | | | |
| 1 | Na | 7.70±0.15 | 3.20±0.26 | 3.5±0.6 | 4.30±0.211 |
| 2 | K | 50.71±0.56 | 64.65±0.39 | 63.97±0.30 | 36.27±0.42 |
| 3 | Mg | 0.47±0.09 | 0.498±0.056 | 0.58±0.042 | 0.31±0.01 |
| 4 | P | 0.69±0.10 | 0.878±0.098 | 0.88±0.08 | 0.35±0.04 |
| 5 | S | 1.43±0.13 | 1.099±0.124 | 1.08±0.20 | 1.02±0.11 |
| 6 | Ca | 37.83±0.54 | 27.22±0.23 | 26.98±0.21 | 46.59±0.35 |
| | | Microelements | | | |
| 7 | Cu | 0.0323±0.001 | 0.0163±0.004 | 0.018±0.001 | 0.018±0.002 |
| 8 | Zn | 0.0685±0.008 | 0.0959±0.007 | 0.074±0.003 | 0.054±0.011 |
| 9 | Al | 0.600±0.079 | 0.628±0.048 | 0.736±0.045 | 1.555±0.124 |
| 10 | Si | 0.458±0.058 | 0.656±0.072 | 0.957±0.043 | 0.908±0.062 |
| 11 | Ti | 0.056±0.001 | 0.134±0.007 | 0.143±0.008 | 0.630±0.075 |
| 12 | Cr | 0 | 0 | 0.008±0.002 | 0.016±0.001 |
| 13 | Mn | 0.046±0.010 | 0.065±0.004 | 0.067±0.008 | 0.215±0.008 |
| 14 | Fe | 0.356±0.088 | 0.880±0.07 | 0.962±0.054 | 0.474±0.045 |
| 15 | Co | 0 | 0 | 0 | 0 |
| 16 | Ni | 0.004±0.002 | 0 | 0 | 0.011±0.001 |
| 17 | Pb | 0 | 0 | 0 | 0.011±0.003 |
| 18 | Mo | 0 | 0 | 0 | 0 |
| 19 | Sn | 0 | 0 | 0 | 0 |
| 20 | Ba | 0 | 0 | 0 | 0 |

Table 5 – Statistical processing of the results of the elements content analysis

| | Df | Sums of Sqs | Mean Sqs | FM model | $R^2$ | Pr (>F) |
|---|---|---|---|---|---|---|
| Group | 3 | 0.158631 | 0.052877 | 951.25 | 0.9972 | 0.001 |
| Residuals | 8 | 0.000445 | 0.000056 | 0.0028 | | |
| Total | 11 | 0.159075 | 1 | | | |

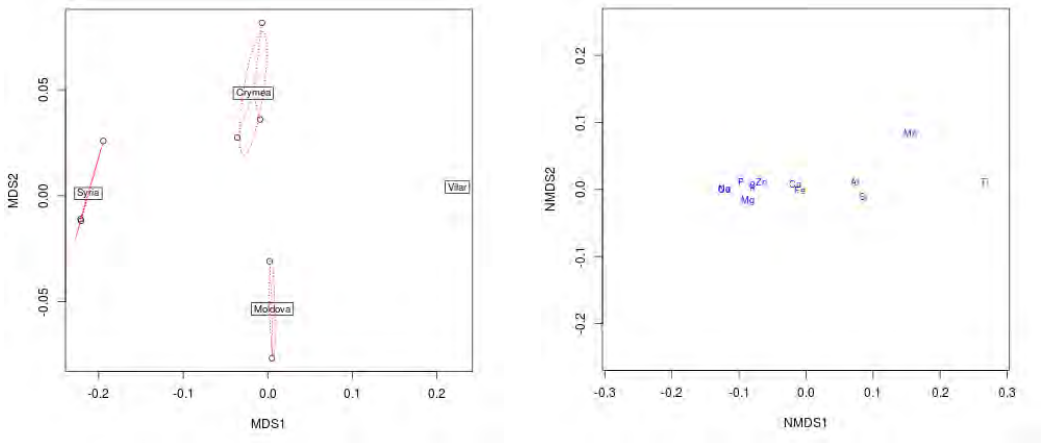


**Figure 7 – A mutual disposition of the samples and the contribution of each of the elements studied to the main coordinates**

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DOI: 10.19163/2307-9266-2019-7-6-346-355

## DISCUSSION

The plants of *Tribulus terrestris* L. from different geographical regions, are known to contain saponins different in composition [45, 46]. The accessible literature contains information about the saponins isolated from *Tribulus terrestris* L. growing in China, India, New Zealand, South Africa, Bulgaria, Georgia and Moldova [46].

According to Szakiel et al. [47], the structure of saponins and their quantitative contents in this plant depend on several factors: climate, light exposure, moisture and soil composition, as well as some other local regional characteristics. In addition, in the majority of previously performed studies, the stage (phase) of plant growth or when and how the raw material was harvested, has not been reported.

It is also clearly stated that the method of steroidal saponins extraction, obviously, affects the results of their determination in the *Tribulus terrestris* L. raw materials [45]. Therefore, the data on the absence, for example, of dioscin in some samples analyzed by other research groups [48] suggest that the phase of *Tribulus terrestris* L. development and the specific methods of the extraction of steroid saponins have a significant effect on the result.

The studies performed by our group, allow us to conclude that in the flowering and fruiting phases, all the samples, regardless of the sample preparation zone, contain dioscin and protodioscin. Moreover, in some samples, the content of protodioscin is several times higher than the content of dioscin.

The scientific literature devoted to the study of *Tribulus terrestris* L., provides information on various methods used to assess the contents of steroidal saponins, including dioscin and protodioscin, in this medicinal plant material [46, 48].

According to A.N. Stavrianidi et al. [45], "the best detection method (by informative value, selectivity and sensitivity) for determining saponins is considered to be electrospray ionization in combination with tandem mass spectrometry".

Our methods we based on using HPLC-MS/MS, allows us to reliably assess the quality of *Tribulus terrestris* L. raw materials by the content of dioscin and protodioscin in it using the external standard method.

According to some researchers, a characteristic set of macro- and microelements in this plant is considered an important component of its chemical composition [49–51].

R. Selvaraju et al. register *Tribulus terrestris* L. as a good source of Na, K, Ca, Mg, and Fe. However, a significant concentration of some heavy metals can accumulate in the flowers [49]. They also showed that the contents of minerals in *Tribulus terrestris* L. varies depending on the composition of the soil on which the plants grow. Our results also allow us to conclude that Na, K, and Ca are the predominant elements in *Tribulus terrestris* L.

A study by A. Ghani et al. has established that Tribulus accumulates $Cu^{2+}$ up to 0.044% (dry weight), $Ni^{3+}$ – 0.239%, $Zn^{2+}$ – 0.434%, $Co^{2+}$ – 0.161%, $Cr^{3+}$ – 0.241%, $Cd^{2+}$ – 0.384%, $Fe^{2+}$ – 0.349%, $Mn^{3+}$ – 0.527%, $Pb^{2+}$ – 0.494%, $Mg^{2+}$ – 0.541% [50]. The ability of this plant to accumulate cadmium and lead, depending on the composition of the soil, allows us to consider that control over the content of heavy metals in the *Tribulus terrestris* L. raw materials, is necessary.

Daur et al. [51] have found out that the accumulation of mineral elements in *Tribulus terrestris* L. is associated both with stressful conditions in the places where the plants grow and with their content and availability in the soil.

The series of biological uptake of macro- and microelements, which can be compiled on the basis of the results obtained for *Tribulus terrestris* L. samples collected by these researchers in Saudi Arabia and Pakistan – K> N> Mg> Ca> P> Na; Mn> Fe> Zn> Ni – allow, in comparison with the data obtained by us, to speak about the influence of the place (zone) of growth on the mineral composition of the raw materials of this plant.

## CONCLUSION

As a result of the studies by HPLC-MS/MS methods, dioscin and protodioscin have been found in all the studied samples. The maximum dioscin content was established in the samples of *Tribulus terrestris* L. raw materials harvested in Moldova, and the minimum was in the samples from the VILAR nursery garden. The largest amount of protodioscin was established in the samples of *Tribulus terrestris* L. raw materials collected in the Crimea, and the minimum – in the samples from Moldova. The obtained data and the methodology for assessing the quality of raw materials have been used in the preparation of the draft pharmacopeia article "*Tribulus terrestris* L.", sent to the "Scientific center for expert evaluation of medical products of the Ministry of Health of the Russian Federation".

The carried out study of the *Tribulus terrestris* L. elements content showed that the habitats (geographical zones) in which the studied samples of raw materials were collected, affect the accumulation of elements by the plant. Based on the obtained data, biological uptake series have been compiled for the samples from each habitat. All the tested samples of raw materials met the requirements of GPM.1.5.3.0009.15 SP on the content of heavy metals.

## FINANCIAL SUPPORT

This study did not have any financial support from outside organizations.

## AUTHORS' CONTRIBUTION

All authors equally contributed to the research work.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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## AUTHORS

**Abdulkarim Affuf** – post-graduate student of the Department of Pharmacognosy with a botany course at "Perm State Pharmaceutical Academy". E-mail: aboud.bashar89@gmail.com

**Julia N. Karpenko** – Candidate of Sciences (Pharmacy), Associate Professor of the Department of Toxicological Chemistry, Perm State Pharmaceutical Academy. E-mail: Karpenko@pfa.ru

**Dmitry K. Gulyaev** – Candidate of Sciences (Pharmacy), Senior Lecturer in the Department of Pharmacognosy with a course in Botany, Perm State Pharmaceutical Academy. ORCID: orcid.org / 0000-0001-9464-1869. E-mail: dkg2014@mail.ru

**Elena I. Molokhova** – Doctor of Sciences (Pharmacy), Professor of the Department of Industrial Technology with a biotechnology course, Perm State Pharmaceutical Academy. E-mail: molohova@pfa.ru

**Olga L. Blinova** – Candidate of Sciences (Pharmacy), Associate Professor of the Department of Pharmacognosy with a botany course, Perm State Pharmaceutical Academy. E-mail: oblinova@mail.ru

**Angelina A. Gileva** – Candidate of Sciences (Pharmacy), Associate Professor of the Department of Pharmacognosy with a botany course, Perm State Pharmaceutical Academy. E-mail: angelinaustinova@mail.ru

**Valentina D. Belonogova** – Doctor of Sciences (Pharmacy), Professor, Head of the Department of Pharmacognosy with a Botany Course, Perm State Pharmaceutical Academy. E-mail: belonogova@pfa.ru.

*RESEARCH ARTICLE*

*ISSN 2307-9266 e-ISSN 2413-2241*

Scientific and Practical Journal
PHARMACY & PHARMACOLOGY

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# DYNAMICS OF THE ACUTE VIRAL HEPATITISES MORBIDITY AND RANGE OF MEDICINAL PREPARATIONS FOR TREATMENT IN HOSPITAL ENVIRONMENT IN THE VOLGOGRAD REGION (THE RUSSIAN FEDERATION), 2016–2018

**L.M. Ganicheva[1], E.A. Ioannidi[1], O.A. Panfilova[1], I.N. Tyurenkov[1], Yu.S. Knyazeva[1], N.Sh. Kaysheva[2]**

[1] Volgograd State Medical University,
1, Pavshikh Boytsov Sq., Volgograd, Russia, 400131
[2] Pyatigorsk Medical and Pharmaceutical Institute, branch of Volgograd State Medical University
11, Kalinin ave, Pyatigorsk, Russia 357532

E-mail: olesya.panfilova.95@mail.ru

Received 18 July 2019 Review (1) 15 October 2019 Review (2) 20 October 2019 Accepted: 28 December 2019

**The aim** of the study was to investigate the prevalence of acute viral hepatitises A, B and C in the Volgograd region; the range and identification of the most frequently prescribed pharmacological groups to be used in hospital environment; and analyzing the price dynamics of medicines. These factors will make it possible to assess the state of the medicine provision for the patients with this disease in hospital environment.
**Materials and methods.** In the study, the following methods have been used: comparison, a method of grouping indicators and a structural-logical method. The materials were the hepatological department patients' treatment sheets (Volgograd Regional Clinical Infectious Diseases Hospital N1).
**Results.** Acute hepatitis A is the most common (46.9%), acute hepatitis B occupies the second position (27.4%), and acute hepatitis C – the third one (25.7%), where in 46.2%, 23.5% and 14.7%, respectively, are accounted for by icteric viral hepatitis. Hepatoprotectors, symptomatic medications and rehydration and detoxification medicinal preparations are prescribed for all forms and degrees of the disease severity.
**Conclusion.** The study has revealed a decreased morbidity of acute viral hepatitises A, B and an increased morbidity of acute viral hepatitis C in the Volgograd region. Among all the types of hepatitises, the prevailing one is the icteric form of moderate severity. The range of medicinal preparations prescribed for the treatment of acute viral hepatitises has been studied. Most often, doctors prescribe hepatoprotectors, rehydration and detoxification medicinal preparations. The study of price dynamics, showed a predominant increase in hepatoprotectors and a decrease in medicines for rehydration and detoxification. The results obtained indicate a tendency towards the improvement of drug provision in the Volgograd Region, for the patients with acute viral hepatitises. Besides, the results of the study give an opportunity to consider the ways of its further optimization.
**Keywords:** acute viral hepatitis, the form and severity of the disease, range of medicinal preparations
**Abbreviations:** ATC – Anatomical Therapeutic Chemical Classification System; HAV – viral hepatitis A; HBV – hepatitis B virus; HCV – viral hepatitis C; SRMP – state register of medicinal preparations; VEDL – Vital and Essential Drugs List; AVH – acute viral hepatitis.

# ДИНАМИКА ЗАБОЛЕВАЕМОСТИ ОСТРЫМИ ВИРУСНЫМИ ГЕПАТИТАМИ В ВОЛГОГРАДСКОМ РЕГИОНЕ И АССОРТИМЕНТ ЛЕКАРСТВЕННЫХ ПРЕПАРАТОВ ДЛЯ СТАЦИОНАРНОГО ЛЕЧЕНИЯ, 2016–2018 ГГ.

**Л.М. Ганичева[1], Е. А. Иоанниди[1], О.А. Панфилова[1], И.Н. Тюренков[1], Ю.С. Князева[1], Н.Ш. Кайшева[2]**

[1] ФГБОУ ВО «Волгоградский государственный медицинский университет» Минздрава России
400131, Россия, г. Волгоград, площадь Павших Борцов, д. 1
[2] Пятигорский медико-фармацевтический институт –
филиал ФГБОУ ВО «Волгоградский государственный медицинский университет» Минздрава России
357532, Россия, Ставропольский край, г. Пятигорск, пр. Калинина 11

E-mail: olesya.panfilova.95@mail.ru

Получено 18.07.2019 Рецензия (1) 15.10.2019 Рецензия (2) 20.10.2019 Принята к печати 28.12.2019

**For citation:** L.M. Ganicheva, E.A. Ioannidi, O.A. Panfilova, I.N. Tyurenkov, Yu.S. Knyazeva, N.Sh. Kaysheva. Dynamics of the acute viral hepatitises morbidity and range of medicinal preparations for treatment in hospital environment in the Volgograd region (the Russian Federation), 2016–2018. *Pharmacy & Pharmacology*. 2019;7(6):356-362. **DOI:** 10.19163/2307-9266-2019-7-6-356-362

*© Л.М. Ганичева, Е.А. Иоанниди, О.А. Панфилова, И.Н. Тюренков, Ю.С. Князева, Н.Ш. Кайшева, 2019*

**Для цитирования:** Л.М. Ганичева, Е.А. Иоанниди, О.А. Панфилова, И.Н. Тюренков, Ю.С. Князева, Н.Ш. Кайшева. Динамика заболеваемости острыми вирусными гепатитами в Волгоградском регионе и ассортимент лекарственных препаратов для стационарного лечения, 2016–2018 гг. *Фармация и фармакология.* 2019;7(6):356-362. **DOI:** 10.19163/2307-9266-2019-7-6-356-362

Научно-практический журнал
ФАРМАЦИЯ И ФАРМАКОЛОГИЯ

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DOI: 10.19163/2307-9266-2019-7-6-356-362

**Цель.** Исследование распространенности острых вирусных гепатитов А, В и С в Волгоградском регионе, изучение ассортимента, выявление наиболее часто назначаемых фармакологических групп для стационарного лечения и анализ динамики цен на лекарственные препараты, что позволит оценить состояние лекарственного обеспечения пациентов с данным заболеванием в условиях стационара.
**Материалы и методы**. Использовались методы сравнения, группировки показателей и структурно-логический. Материалами послужили листы назначений пациентов гепатологического отделения Волгоградской областной клинической инфекционной больницы №1.
**Результаты.** Наибольшее распространение имеет острый гепатит А – 46,9%, острый гепатит В – 27,4%, острый гепатит С – 25,7%, из которых соответственно 46,2%, 23,5% и 14,7% приходятся на желтушную форму средней степени тяжести. При всех формах и степенях тяжести заболевания назначают гепатопротекторы, симптоматические препараты и препараты для регидратации, дезинтоксикации.
**Заключение.** За исследуемый период в Волгоградском регионе выявлено снижение заболеваемости острыми вирусными гепатитами А и В, но, в свою очередь, и рост заболеваемости острым вирусным гепатитом С. Преобладающей среди всех видов гепатита является желтушная форма средней степени тяжести. Изучен ассортимент лекарственных препаратов, применяемых при лечении острых вирусных гепатитов в Волгоградской областной клинической инфекционной больнице № 1. Наиболее часто врачи назначают гепатопротекторы, препараты для регидратации, дезинтоксикации. Изучение динамики цен показало преимущественное повышение на гепатопротекторы и понижение на препараты для регидратации и дезинтоксикации. Полученные результаты свидетельствуют о тенденции к улучшению лекарственного обеспечения в Волгоградском регионе пациентов с острыми вирусными гепатитами и позволяют рассмотреть пути его дальнейшей оптимизации.
**Ключевые слова:** острый вирусный гепатит, форма и степень тяжести заболевания, ассортимент лекарственных препаратов
**Список сокращений:** АТХ – анатомо-терапевтическо-химическая классификация; ВГА – вирусный гепатит А; ВГВ – вирусный гепатит В; ВГС – вирусный гепатит С; ГРЛС – государственный реестр лекарственных препаратов; ЖНВЛП – перечень жизненно необходимых и важнейших лекарственных препаратов; ОВГ – острый вирусный гепатит.

## INTRODUCTION

Viral hepatitis is a widespread infectious disease that destroys a human liver. According to the World Health Organization, hundreds of millions of people in different countries are infected with viral hepatitises, which exceed the spread of HIV infections. Hepatitis is a medical and social problem both for the Russian Federation and for the whole world. A decrease in the incidence of acute hepatitises, is associated with increased preventive measures and the introduction of vaccination in accordance with the national vaccination calendar[1]. Vaccination against viral hepatitises A (HAV) and B (HBV) has been carried out in Russia for the past 20 years, but a vaccine against viral hepatitis C (HCV) has not been developed due to the severe variability of the virus genome [1, 2]. The main reason for the carrier state of this hepatitis virus is the epidemiological problems of countries over the previous 20–25 years. About 650 thousand deaths per year in the world are associated with hepatitis B virus. 2–3% of the Russia population, are infected with hepatitis C. The main routes of HBV and HCV transmission are parenteral, contact (through a skin microtrauma, sexual promiscuity), and the vertical routes (from a mother to a child) [3–9].

The mechanism of HCV transmission is the fecal-oral route. As the practical data have shown, in the Volgograd Region, the main route of hepatitis B infection is the parenteral route (administration of narcotic substances with non-sterile needles in drug addiction).

Viral hepatitis A is the most common and, although this disease is typical of the third world countries, rare cases or outbreaks of hepatitis A are also observed in developed countries. In the Russian Federation, HAV is the most common (more than 50% of cases). In its turn, mortality rate due to HAV is low and accounts for a fraction of a percent worldwide. About 1.5 million cases of HAV have been registered in the world annually, and in the Russian Federation in 2010, the mortality rate was 6.3 cases per 100 thousand people. High incidence of the disease is associated with the active release of HAV (hepatitis A virus) from the patient's body and its high resistance to the environment. As a rule, chronic hepatitis A does not develop when it comes to a single-agent infection. [10, 11].-Statistics of the Volgograd region on acute hepatitis A, are not available in the public information sources.

In the Russian Federation in 2012, acute hepatitis B amounted to 1.43 cases per 100 thousand people. Thanks to the vaccination, the morbidity decreased by 30 times in 2000–2012. 90–95% of adults with HBV recover and only 10% have the chronic form. The mortality rate due to acute hepatitis B is less than 1%. In the Volgograd region in 2015, the morbidity of acute hepatitis B was 1.7 times lower than its morbidity rate in the Russian Federation, in 2015 it amounted to 0.78 cases per 100 thousand people, in 2016 it was 0.54 [12, 13].

In the Russian Federation in 2012, acute hepatitis C amounted to 1.8 cases per 100 thousand people. 50–80% of the infected people have a chronic form. In the Volgograd region in 2015, the morbidity of acute hepatitis B amounted to 0.66 per 100 thousand people, which is 2.1 times lower than the average rate of this disease in the Russian Federation, and in 2016 it amounted to 0.16 [14].

**THE AIM** of the study was to investigate the state of affairs in acute viral hepatitises A, B and C in the Volgograd region during the period of 2016–2018: its prevalence, the forms and severity of the disease, the range of medicinal preparations used in hospitals for the acute hepatitises treatment, the dynamics of prices for medicines of the most widely used pharmacological groups based on the state register of medicinal preparations.

Early disease and treatment intelligence plays an important role in preventing health complications and saving budget funds, of both the state and the population.

## MATERIALS AND METHODS

In the study, the following methods have been used: comparison, a method of grouping indicators and a structural-logical method. The materials were 175 hepatological department patients' treatment sheets (Volgograd Regional Clinical Infectious Diseases Hospital No.1) and the data of the state register of medicinal preparations of the Russian Federation.

The research design is represented by 4 interconnected stages:

1 – the analysis of the morbidity dynamics of acute viral hepatitises A, B, C;

2 – the analysis of the data on the duration of treatment in hospital environment;

[1] On approval of the national calendar of preventive vaccinations and the calendar of preventive vaccinations according to epidemic indications: order of the Ministry of Health of the Russian Federation dated March 21, 2014 (as amended and added by the order of the Ministry of Health of the Russian Federation, dated 16 June 2016). Available at system "Consultant Plus"

3 – the detection of the most significant range groups of medicinal preparations for the treatment of acute viral hepatitises A, B, C.

4 – the analysis of the dynamics of prices for the medicinal preparations prescribed for the treatment of acute viral hepatitises.

## RESULTS AND DISCUSION

Viral hepatitises are classified according to the form and severity of the disease [15,16]. In the infectious hospital during 2016–2018, the following forms were registered:

– the icteric form (moderate and heavy severity level);

– the anicteric form (mild and moderate severity level).

At the first stage of the study, the analysis of the morbidity dynamics of the acute viral hepatitises A, B, C (according to the form and severity in the Volgograd region) was carried out (Tab. 1). Methods of comparison and grouping were used. From the data of Tab.1 it follows that acute hepatitis A was the most common – 82 (46.9%) cases; acute hepatitis B – 48 (27.4%) cases, it occupies the second place; and acute hepatitis C – 45 (25.7%) cases it occupies the third place.

In 2016–2018, the dynamics of the acute hepatitis A prevalence was characterized by a significant decrease in the morbidity rate from 59.8 to 12.2%, i.e. by 5 times; the morbidity of icteric moderate forms was decreased from 58.8 to 12.3%, i.e. by 4.5 times, although it should be noted that a single case of an icteric form of heavy severity was detected in 2016.

The dynamics of the acute hepatitis B prevalence in 2016–2018 was characterized by a tendency of a decrease in the morbidity rate from 39.5 to 29.2% (by 1.5 times); a decrease in the icteric form of moderate severity from 31.3 to 25% and in the anicteric form of moderate severity from 2.1 to 4.2% was also found out. A single case of acute hepatic encephalopathy has been detected for the whole period.

The dynamics of the acute hepatitis C prevalence in 2016-2018 was characterized by a tendency of an increase from 20 to 42.2% (by 2 times); there was a growth of the icteric form of moderate severity from 11.1 to 20%, of the anicteric form of mild severity – from 2.2 to 11.1%; and of an anicteric form of moderate severity from 4.4 to 11.1%. Two cases of icteric severity were also detected in 2016 and 2017 per annum.

A decrease in the acute hepatitises A and B morbidity is associated with an active use of the hepatitis vaccines [17]. Vaccination of newborns is carried out from the first days of their lives in accordance with the national calendar of vaccinations. Vaccination has also been prescribed for the adult population[1].

At the 2nd stage of the study, the data of the duration of treatment in hospital environment were reviewed and summarized. The therapy of acute viral hepatitis takes a different time depending on the type of hepatitis, the form and severity of the disease, and goes with clinical guidelines, standards of specialized medical care for patients with acute viral hepatitises [18]. The icteric form of hepatitis is severer than the anicteric form and, therefore, requires a longer treatment. Using the structural-logical method, we can conclude that among icteric forms of hepatitises, it is more difficult to treat acute severe hepatitis B (the treatment duration is 25±5 days).

The icteric form of acute severe hepatitis, occupies the 2nd position as its treatment duration is 19±0 days. Acute hepatitises A and B of the icteric form of moderate severity are in the third position; their treatment duration is 17±7 days. A period from 8 to 16 days is required for the treatment of the anicteric form of mild severity of acute hepatitis C; of moderate severity of acute hepatitises C and B; for the icteric form of acute hepatitis C of moderate severity; and for the icteric form of acute severe hepatitis A (Tab. 2). The cases of the anicteric form of moderate severity of acute hepatitises A and a mild degree of acute hepatitises A and B in hospital environment were not registered.

At the next stage of the study, the range groups of medicinal preparations and the frequency of their prescriptions were cleared up. In the treatment of acute viral hepatitises, pathogenetic therapy (hepatoprotectors, enzymes, vitamins, hormonal medicines, medicines for rehydration and detoxification for parenteral use) was used; besides, etiotropic (interferons and their inducers) and symptomatic kinds of therapy were used. These kinds of therapy also go with the clinical recommendations and standards of specialized medical care [19–23]. The use of groups of medicinal preparations in the corresponding types, forms and degrees of severity of acute hepatitises are presented in Tab. 3–5.

As it follows from Tab. 3, etiotropic therapy was not used for the icteric form of moderate severity of acute hepatitis A, and only interferons in the function of etiotropic therapy, are added to the main treatment of the icteric form of heavy severity of acute hepatitis A. It should be also noted that vitamins were not used at all in this hospital for the treatment of acute hepatitis A.

As Tab. 4 shows, vitamins were not used in the treatment of acute viral hepatitis B either. In its turn, interferon therapy was not used for the icteric form of heavy severity and the anicteric form of moderate severity of acute hepatitis B. Enzymatic and hormonal medicines were not prescribed for the treatment of acute hepatitis B anicteric forms of moderate severity either.

As it follows from Tab. 5, etiotropic therapy was not used in the icteric form of heavy severity and in the anicteric form of mild severity of acute hepatitis C. In the treatment of the anicteric form of moderate severity, only interferons were used in etiotropic therapy. In the treatment of the anicteric form of mild severity, enzyme medicines, hormonal, rehydration and detoxification medicines were not used. Hormonal medicines were not prescribed for the treatment of the acute hepatitis C anicteric forms of moderate severity either.

Thus, the data presented in Tab. 3–5, illustrate the specific treatment of acute viral hepatitises depending on their type, form and severity [24–27].

Tab.6 contains a range of medicinal preparations used in the treatment of acute viral hepatitis in Volgograd Regional Clinical Infectious Diseases Hospital N 1.

The medicinal preparations referring to pathogenetic and etiotropic therapy, have been examined in detail because they directly affect the outcome of the disease. In Tab.6, the groups of medicinal preparations are indicated in accordance with the anatomical-therapeutic-chemical classification [28]. All the presented medicinal preparations belong to the Vital and Essential Drugs List (dated 2016–2018).

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**Table 1 – Dynamics of the morbidity of acute viral hepatitises A, B, C during 2016-2018**

| Year | Type of hepatitis | Total | Icteric form | | Anicteric form | |
|---|---|---|---|---|---|---|
| | | | Moderate severity | Heavy severity | Moderate severity | Mild severity |
| 2016 | Acute hepatitis A | 49 (59.8) | 48 (27.4%) | 1 (0.7%) | – | – |
| 2017 | Acute hepatitis A | 23 (28%) | 23 (13.1%) | – | – | – |
| 2018 | Acute hepatitis A | 10 (12.2%) | 10 (5.7%) | – | – | – |
| | | 82(46.9%) | | | | |
| 2016 | Acute hepatitis B | 19 (39.5%) | 15 (8.6%) | 3 (1.7%) | 1 (0.5%) | – |
| 2017 | Acute hepatitis B | 15 (31.3%) | 14 (8%) | – | 1 (0.5%) | – |
| 2018 | Acute hepatitis B | 14 (29.2) | 12 (6.9%) | – | 2 (1.2%) | – |
| | | 48(27.4%) | | | | |
| 2016 | Acute hepatitis C | 9 (20%) | 5 (2.8%) | 1 (0.6%) | 2 (1.1%) | 1 (0.6%) |
| 2017 | Acute hepatitis C | 17 (37.8%) | 12 (6.8%) | 1 (0.6%) | 3 (1.7%) | 1 (0.6%) |
| 2018 | Acute hepatitis C | 19 (42.2%) | 9 (5.1%) | 5 (2.8%) | 5 (2.8%) | – |
| | | 5(25.7%) | | | | |

**Table 2 – Treatment duration of acute viral hepatitises in hospital environment of Clinical Infectious Diseases Hospital N1 (average statistics)**

| Type of hepatitis / form and severity | Icteric form | | Anicteric form | |
|---|---|---|---|---|
| | Moderate severity | Heavy severity | Moderate severity | Mild severity |
| Acute hepatitis A | 17±7 days | 14±0 days | – | – |
| Acute hepatitis B | 17±7 days | 25±5 days | 16±6 days | – |
| Acute hepatitis C | 15±5 days | 19±0 days | 9±4 days | 8±4 days |

**Table 3 – The use of medicinal preparations groups in the treatment of acute viral hepatitis A**

| Group of medicines / form and severity | Icteric form | |
|---|---|---|
| | Moderate severity | Heavy severity |
| Symptomatic medicines | + | + |
| Vitamin medicines | – | – |
| Hepatoprotecti.ve medicines | + | + |
| Enzyme medicines | + | + |
| Hormonal medicines | + | + |
| Rehydration and detoxification preparations for parenteral use | + | + |
| Interferons | – | – |
| Interferon inductors | – | + |

**Table 4 – The use of medicinal preparations groups in the treatment of acute viral hepatitis B**

| Group of medicinal preparations / form and severity | Icteric form | | Anicteric form |
|---|---|---|---|
| | Moderate severity | Heavy severity | Moderate severity |
| Symptomatic medicines | + | + | + |
| Vitamin medicines | – | – | – |
| Hepatoprotective medicines | + | + | + |
| Enzyme medicines | + | + | – |
| Hormonal medicines | + | + | – |
| Rehydration and detoxification preparations for parenteral use | + | + | + |
| Interferons | + | – | – |
| Interferon Inductors | + | + | + |

**Table 5 – The use of medicinal preparations groups in the treatment of acute viral hepatitis C**

| Group of medicines/form and severity | Icteric form | | Anicteric form | |
|---|---|---|---|---|
| | Moderate severity | Heavy severity | Moderate severity | Mild severity |
| Symptomatic medicines | + | + | + | + |
| Vitamin medicines | – | – | – | – |
| Hepatoprotective medicines | + | + | + | + |
| Enzyme medicines | + | – | + | – |
| Hormonal medicines | + | + | – | – |
| Rehydration and detoxification preparations for parenteral use | + | + | + | – |
| Interferons | + | – | + | – |
| Interferon Inductors | + | – | – | – |

**Table 6 – The range of medicinal preparations and the frequency of their prescriptions during the study period of 2016–2018**

| Medicinal preparations group | Trade name, prescribed dosage, dosage form, route of administration | Number of prescriptions * | | |
|---|---|---|---|---|
| | | 2016 | 2017 | 2018 |
| Hepatoprotective medicines | Heptor® 400 mg (pills, p.o.) | 1 (1.2%) | – | – |
| | Heptral® 400 mg (lyophilisate, i.v) | 2 (3.1%) | 7 (8.5%) | 4 (3.9%) |
| | Phosphogliv® 65 + 35 mg (capsules, p.o.) | 42 (60%) | 55 (66.3%) | 75 (70%) |
| | Phosphogliv® 2.5 g (lyophilisate, i.v) | 15 (21.4%) | 3 (3.6%) | 7 (6.7%) |
| | Ursodez® 250 mg (capsules, p.o.) | 3 (4,3%) | 15 (18%) | 20 (19.4%) |
| | Ursodez® 500 mg (capsule, p.o.) | 7 (10%) | 3 (3.6%) | – |
| | Total | **70 (100%)** | **83 (100%)** | **106 (100%)** |
| Enzyme medicines | Pancreatin 25 units (pills, p.o.) | – | – | 9 (16%) |
| | Pancreatin 0.5 (pills, p.o.) | – | – | 1 (2%) |
| | Pancreatin 30 units (pills, p.o.) | 1 (100%) | 1 (100%) | 46 (82%) |
| | Total | **1(100%)** | **1 (100%)** | **56 (100%)** |
| Hormonal medicines | Dexamethasone 8 mg (injection solution, i.v) | 1(14.3%) | 1 (4.4%) | 1 (4.5%) |
| | Prednisolone 5 mg (pills, p.o.) | – | 1 (4.1%) | 1 (4.6%) |
| | Prednisolone 10 mg (pills, oral) | – | 1 (4.1%) | – |
| | Prednisolone 15 mg (pills, p.o.) | – | 1 (4.1%) | – |
| | Prednisolone 30 mg (injection solution, i.v) | – | 2 (8.3%) | 1 (4.6%) |
| | Prednisolone 60 mg (injection solution, i.v) | | 6 (25%) | 3 (13.6%) |
| | Prednisolone 90 mg (injection solution, i.v) | 4(57,4%) | 9 (37.5%) | 11 (50%) |
| | Prednisolone 120 mg (injection solution, i.v) | – | 3 (12.5%) | 5 (22.7%) |
| | Total | **7 (100 %)** | **24 (100%)** | **22 (100%)** |
| Rehydration and detoxification preparations for parenteral use | Acesol 400 ml (infusion solution, i.v) | 6 (7.7%) | 23(16.5%) | 46(27.5%) |
| | Acesol 450 ml (infusion solution, i.v) | – | 2 (1.4%) | – |
| | Acesol 800 ml (infusion solution, i.v) | 1 (1.3%) | 1 (0.7%) | 2 (1.2%) |
| | Glucose 5% – 400 ml (infusion solution, i.v) | 32 (41%) | 44(31.7%) | 60 (36%) |
| | Glucose 10% – 400 ml (infusion solution, i.v) | 6 (7.7%) | 19(13.7%) | 8 (4.8%) |
| | Ringer's solution 400 ml (infusion solution, i.v) | 9(11.5%) | 11 (8%) | 4 (2.4%) |
| | Ringer's solution 500 ml (infusion solution, i.v) | 5 (6.4%) | 7 (5%) | 3 (1.8%) |
| | Reamberin® 200 ml (infusion solution, i.v) | - | 11 (8%) | – |
| | Reamberin® 250 ml (infusion solution, i.v) | 1 (1.3%) | – | – |
| | Reamberin® 400 ml (infusion solution, i.v) | 2 (2.6%) | – | – |
| | Remaxol® 200 ml (infusion solution, i.v) | – | – | 1 (0.6%) |
| | Remaxol® 400 ml (infusion solution, i.v) | – | – | 4 (2.4%) |
| | Remaxol® 500 ml (infusion solution, i.v) | – | – | 3 (1.8%) |
| | Potassium chloride 4% –10 ml (injection solution, i.v) | 7 (9.0%) | 7 (5%) | 3 (1.8%) |
| | Sodium chloride 0.9% -400 ml (infusion solution, i.v) | 9(11.5%) | 14 (10%) | 33 (19.7%) |
| | Total | **78 (100%)** | **139 (100%)** | **167 (100%)** |
| Interferons | Altevir® 3 mln UNITS (injection solution, IM) | 1 (12.5%) | 1 (16.6%) | 16 (100%) |
| | Alpharona® 1.5 million units (lyophilisate, IM) | – | 1 (16.7%) | – |
| | Alfarona® 3 million units (lyophilisate, IM) | 6 (75%) | 4 (66.7%) | – |
| | Layfferon® 3 mln UNITS (injection solution, lyophilisate, IM) | 1 (12.5%) | – | – |
| | Total | **8(100%)** | **6 (100%)** | **16 (100%)** |
| Interferon Inductors | Cycloferon® 12.5% -2ml (injection solution, IM) | 8 (100%) | 2 (100%) | 22 (100%) |
| | Total | **8 (100%)** | **2 (100%)** | **22 (100%)** |

Note: 1 prescription = 1 person

**Table 7 – Dynamics of prices for rehydration and detoxification medicinal preparations used in the treatment of acute viral hepatitises during the period of 2016–2018 (according to the data of the state register of medicinal preparations)**

| Group of medicinal preparations | Trade name, prescribed dosage, medicine form, route of administration, package | Limit price, rubles without VAT (per 1 unit) | | | Price change, % |
|---|---|---|---|---|---|
| | | 2016 | 2017 | 2018 | 2018 to 2016 |
| Rehydration and detoxification preparations for parenteral use | Acesol 400 ml (infusion solution, i.v), medicine bottle | 27.95 | 27.95 | 27.95 | 0 |
| | Glucose 5% – 400 ml (infusion solution, i.v), medicine bottle | 44.06 | 28.72 | 29.88 | 32.2 |
| | Glucose 10% – 400 ml (infusion solution, i.v), medicine bottle | 39.27 | 28.08 | 30.96 | 22.0 |
| | Ringer's solution 400 ml (infusion solution, i.v), medicine bottle | 45.93 | 35.78 | 24.89 | 46.0 |
| | Ringer's solution 500 ml (infusion solution, i.v), medicine bottle | 35.93 | 28.74 | 26.62 | 26.0 |
| | Reamberin ® 200 ml (infusion solution, i.v) | 128.31 | 132.99 | 138.00 | +7,5 |
| | Reamberin ® 250 ml (infusionsolution, i.v) | 113.83 | 113.83 | 113.83 | 0 |
| | Reamberin ® 400 ml (infusion solution, i.v) | 157.05 | 162.65 | 168.85 | + 7.5 |
| | Remaxol ® 400 ml (infusion solution, i.v) | – | – | 315.00 | – |
| | Potassium chloride 4% – 10 ml (injection solution, i.v) ampula | 2.53 | 4.43 | 4.60 | + 81.8 |
| | Potassium chloride 0,9% – 400 ml (injection solution, i.v) | 33.64 | 23.94 | 33.26 | – 1.2 |

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**Table 8 – Dynamics of prices for medicines of the group of hepatoprotectors used in the treatment of acute viral hepatitises in the period of 2016 – 2018 (according to the data of thestate register of medicinal preparations)**

| Group of medicinal preparations | Trade name, dosage, medicine form, route of administration | Limit price, rubles without VAT (per 1 unit) | | | Price change,% |
|---|---|---|---|---|---|
| | | 2016 | 2017 | 2018 | 2018–2016 |
| Hepatoprotective medicines | Heptor® 400 mg (pills, p.o.) | 44.80 | 44.80 | 44.80 | 0 |
| | Heptral® 400 mg (lyophilisate, i.v.) | 65.31 | 65.31 | 280.71 | + 329.8 |
| | Phosphogliv®.v 65 + 35 mg (capsules, p.o.) | 8.00 | 8.81 | 9.15 | + 14.4 |
| | Phosphogliv® 2.5 g (lyophilisate, i.v) | 264.45 | 264.45 | 270.12 | + 2.1 |
| | Ursodez® 250 mg (capsules, p.o.) | 9.03 | 9.03 | 12.05 | + 33.4 |
| | Ursodez® 500 mg (capsule, p.o.) | 24.11 | 24.11 | 24.11 | 0 |

The range of medicinal preparations expanded in 2017–2018 in comparison with 2016. Among hepatoprotectors, the most widely used was Phosphogliv® 35+65 mg, among enzyme preparations it was pancreatin 30 UAs, among hormonal preparations – prednisolone 90 mg, among rehydration and detoxification preparations – acesol 400 ml, among interferons – 3 mln UAs of Altevir® and among interferon inducers – Cycloferon® 12.5% – 2 ml.

It should be noted that the prescription of the same medicine in different dosages is associated with the type, form and severity of acute hepatitis. The following names and dosages are registered in both – the list of medicinal preparations presented in Tab. 6 and in the state register of medicinal preparations: Phosphogliv® 65+35 mg, Phosphogliv® 2.5 mg, Heptor® 400 mg, Heptral® 400 mg, Ursodez® 250 mg and 500 mg; pancreatin 30 UAs and 25 UAs; dexamethasone 4 mg, prednisone 30 mg and 5 mg; glucose 5%, 10% – 400 ml, potassium chloride 4% – 10 ml, sodium chloride 0.9% – 400 ml, Remaxol® 400 ml, Ringer's solution 400 ml and 500 ml, Reamberin® 20, 250, 400 ml, acesol 400 ml; Alpharona® 3 mln UAs, Liffeferon® 3 mln UAs, Altevir® 3 million UAs; Cycloferon® 12.5% – 2 ml.

According to Tab. 6, during the period of 2016–2018, rehydration and detoxification medicinal preparations were prescribed most often (47% of the entire range), as well as the group of hepatoprotectors (31.7%). In 2018, unlike the previous years, a group of enzyme medicinal preparations began to be prescribed quite widely.

The final stage of the investigation was devoted to the study of price dynamics for the most commonly prescribed medicines of two pharmacotherapeutic groups: medicines for rehydration and detoxification and hepatoprotectors. Since the presented medicines are included in the list of Vital and Essential Medicines, their prices and wholesale surcharges are fixed and controlled by the state. Tables 7, 8 show the dynamics of prices for rehydration and detoxification medicinal preparations, as well as the group of hepatoprotectors in the period of 2016–2018.

According to Tab. 6, in the period of 2016–2018, the most often prescribed were rehydration and detoxification medicines (47% of the entire range), as well as the group of hepatoprotectors (31.7%). In 2018, unlike the previous years, a group of enzyme medicines began to be prescribed quite widely. Tab. 7, 8 show the dynamics of prices for rehydration and detoxification medicines, as well as the group of hepatoprotectors.

Since the presented medicines are included in the list of Vital and Essential Medicines, their prices and wholesale surcharges are fixed and controlled by the state. From the data of Tab. 7 it follows, that in the study period in the rehydration and detoxification group (11 items taken as 100%) the prices increased for 3 medicines (27%), Reamberin® 200 ml and 400 ml, potassium chloride 4% – 10 ml; 2 medicines remained in the same price category: acesol 400 ml and Reamberin® 250 ml (18%); the prices decreased for 5 medicines: glucose 5% – 400 ml, glucose 10% – 400ml, Ringer's solution 400ml, Ringer's solution 500ml, sodium chloride 0.9% – 400 ml (45%). Remaxol® 400 ml (1%) belongs to new medicines of 2018, therefore its price dynamics is not possible to trace. A significant decrease in the price of 4medicines (–22–46%), a significant increase in the price of 1 medicine (+81.8%) and a slight increase (+7.5%) for 2 medicines were revealed in comparison with 2016.

Consequently, a larger percentage consists of the medicinal preparations the costs of which have decreased (by 45%). This fact may be associated with an increase in the affordability of medical care, and, accordingly, an increase in medical prescriptions for 2016–2018.

The data of Tab. 8 shows that in the group of 6 hepatoprotectors, the price of 2 medicines did not change (Heptor® 400 mg and Ursodez® 500 mg), the price of other medicines went up from 2.1 to 329.8%. However, this fact did not affect the frequency of prescriptions of this group of medicines, which may be due to an increase in the allocation for the purchase of these items for use in hospital environment. Attention should be also paid to the fact thataccording to Tab. 6 and 8, the prices for Heptor® 400 mg and Ursodez® 500 mg remained the same in 2016–2018, but their prescription was not registered in 2017 and 2018, which may be due to the lack of their procurement.

## CONCLUSION

The studies have shown the following. In the period of 2016–2018, there has been a steady tendency of decreasing in the morbidity of acute hepatitises A and B, but in its turn, there has been an increase in the incidence of acute hepatitis C (it was nicknamed "a gentle killer" because of the hidden course of the disease). The main groups of medicines used in hospital environment for the treatment of acute hepatitises A, B and C depending on the form and severity of the disease, have been identified. 3. A study of the range of medicines, the frequency of medical prescriptions and dosages showed that rehydration and detoxification medicines for parenteral use, and hepatoprotectors are the most commonly prescribed. 4. The dynamics of the prices for the groups of medicines has been studied. It has demonstrated a decrease in prices for rehydration and detoxification medicines (45% of all the numbers of the items) and an increase in prices for medicines of the hepatoprotectors group (66.7% of all the numbers of the items).

The results gained indicate a tendency towards the improvement of medicine provision of patients with acute viral hepatitises, and give an opportunity to consider the ways of their further optimization.

## ACKNOWLEDGMENT

The authors are grateful to the head physician of Volgograd Regional Clinical Infectious Diseases Hospital No. 1 Elena V. Dyomina for her assistance in conducting the study.

## FINANCIAL SUPPORT

This study did not have any financial support from outside organizations.

## AUTHORS' CONTRIBUTION

All authors equally contributed to the research work.

## CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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## AUTHORS

**Lyudmila M. Ganicheva** – Doctor of Sciences (Pharmacy), Associate Professor Head of the Department of Management and Economics of Pharmacy, Medical and Pharmaceutical Commodity Science, Volgograd State Medical University. ORCID ID 0000-0002-5647-0568. E-mail: Ganicheva@volgmed.ru

**Elena A. Ioannidi** – Doctor of Sciences (Medicine), Head of the Department of Infectious diseases with Epidemiology and Tropical Medicine, Volgograd State Medical University

**Olesya A. Panfilova** – Postgraduate Student, Department of Management and Economics of Pharmacy, Medical and Pharmaceutical Commodity Science, Volgograd State Medical University. ORCID ID: 0000-0003-3148-8371. E-mail: Olesya.Panfilova.95@mail.ru,

**Ivan N. Tyurenkov** – Doctor of Sciences (Medicine), professor, Correspondent Member of the Russian Academy of Sciences, Head of the Department of Pharmacology and Biopharmaceutics, Faculty of Advanced Medical Studies, Volgograd State Medical University; Head of laboratory of cardiovascular medications, Scientific Center of Drug Research, Volgograd State Medical University. ORCID ID: http://orcid.org/0000-0001-7574-3923. E-mail: fibfuv@mail.ru

**Julia S. Knyazeva** – Assistant, Department of Pharmacology and Biopharmacy, Volgograd State Medical University

**Nelly Sh. Kaisheva** – Doctor of Sciences (Pharmacy), Professor of the Department of Pharmaceutical and Toxicological Chemistry, Pyatigorsk Medical Institute – a branch of Volgograd State Medical University. E-mail: Caisheva2010@yandex.ru


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