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Contents
Содержание

CIRCULATING RNA IN BLOOD PLASMA AS DIAGNOSTIC TOOL FOR CLINICAL ONCOLOGY

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One of the key challenges facing today's oncology is the discovery of early predictors of malignant neoplasms in patients' biological samples. Liquid biopsy is a noninvasive diagnostic technique based on the detection and isolation of tumor cells, tumor-derived nucleic acid and exosomes circulating in the blood plasma of cancer patients. There is a plethora of research studies of circulating tumor DNA in patients with MN. The active proliferation of tumor cells occurs in the backdrop of altered gene expression. The presence of tissue-specific transcripts in the circulating RNA fraction suggests that levels of circulating RNA reflect the development of the primary tumor. We think that cell-free RNA circulating in the blood plasma is a promising molecular biomarker for early cancer detection.

Keywords: circulating nucleic acids, blood plasma, circulating tumor cells, circulating RNA, miRNA, biomarkers, oncology

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АНАЛИЗ ВНЕКЛЕТОЧНОЙ ФРАКЦИИ РНК ПЛАЗМЫ КАК ИНСТРУМЕНТ ДИАГНОСТИКИ В ОНКОЛОГИИ

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Одна из ключевых задач современной онкодиагностики — поиск ранних предикторов злокачественных новообразований (ЗНО) при анализе наиболее доступных видов биоматериала. Жидкостная биопсия представляет собой одну из неинвазивных методик и включает в себя обнаружение и выделение циркулирующих опухолевых клеток, циркулирующих опухолевых нуклеиновых кислот и экзосом из плазмы крови у пациентов со злокачественными заболеваниями. Множество работ посвящено исследованию внеклеточной фракции ДНК при ЗНО. Вместе с тем активную пролиферацию трансформированных клеток при развитии опухолей сопровождают значительные изменения экспрессии определенных генов. Обнаружение тканеспецифичных транскриптов в составе внеклеточной РНК плазмы крови (внРНК) позволяет предположить, что представленность циркулирующих в плазме РНК связана с развитием патологического процесса непосредственно в первичном очаге. На наш взгляд, внРНК плазмы крови представляют практическую ценность в качестве молекулярно-генетических маркеров ранней диагностики в онкологии.

Ключевые слова: циркулирующие нуклеиновые кислоты, плазма крови, циркулирующие опухолевые клетки, внРНК, микроРНК, биомаркеры, онкология

Финансирование: исследование выполнено в рамках работ по Государственному заданию №АААА-А18-118053190012-9 «Разработка тест-системы для ранней диагностики рака молочной железы и рака яичников на основе анализа свободно циркулирующих (внеклеточных) РНК периферической крови».

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Diagnostic tests known as liquid biopsies hold promise for the future of cancer screening. They are capable of detecting tumor-derived biomarkers in the blood serum of patients with malignant neoplasms (MN), including circulating tumor cells, circulating tumor DNA or RNA, and exosomes. Liquid biopsy samples can be analyzed using a few different types of analysis, such as quantification of individual analytes, including proteins, identification of nucleic acid sequences of the analyte, profiling DNA methylation, etc. [1]. The analysis of cell-free nucleic acids circulating in the blood plasma allows assessing the genetic heterogeneity of the tumor in response to anti-cancer therapy [2, 3].

It is known that apoptotic and necrotic cells release DNA or RNA fragments and exosomes (membrane-bound encapsulated subcellular structures containing proteins and nucleic acids derived from tumor cells) into the bloodstream [4]. From the early stages of carcinogenesis through the advanced stages of metastatic spread, tumor cells accumulate specific mutations and epigenetic modifications; these changes can be spotted by the analysis of cell-free nucleic acids.

Analysis of circulating DNA

The analysis of circulating DNA has been used in clinical oncology for over 20 years to aid the diagnosis and monitoring of the following cancers: lung [5, 6], head and neck [7], esophageal [8], breast [9], hepatic [10], colon [11], pancreatic [12], renal [13], and others. As a rule, the tests look for the presence of mutations in oncogenes, tumor suppressor genes and microsatellites [6, 9, 13]. Similarly, DNA methylation analysis has some diagnostic and prognostic value and can be employed for monitoring tumor growth [8, 14]. Quantitative aberrations of circulating DNA have been also reported in other pathologies besides MN, including preeclampsia [15], fetal chromosomal aneuploidy [16], and pernicious vomiting of pregnancy [17].

Analysis of circulating mRNA

The active proliferation of tumor cells and tumor evolution are accompanied by the pronounced changes in the abundance of various transcripts, some of which, like mRNA, can be quantified by RT-PCR [18]. RT-PCR was successfully used to measure the levels of circulating mRNA transcripts of housekeeping genes in the blood samples of healthy individuals and cancer patients [19]. Circulating RNA was also studied in patients with melanoma [20–22], follicular lymphoma [23], breast [22, 24–28], colon [23, 29], hepatic [30], esophageal [21], nasopharyngeal [31], thyroid [22], prostate [40, 41], lung [32] and other cancers. However, research into cell-free RNA is not limited to malignancies: its levels were investigated in patients with trauma [33, 34], diabetic myopathy [35], and pregnancy (fetal mRNA) [36].

A study demonstrated a statistically significant difference in hTERT mRNA levels between patients with early stages of *breast cancer* (BC) and healthy individuals. The presence of hTERT mRNA in the blood plasma of BC patients was affected by the surgical removal of the tumor [25]. However, it is unlikely that hTERT is a BC-specific marker because its concentrations also change in patients with melanoma and thyroid cancer [22]. The levels of hMAM mRNA expression in the blood plasma were correlated with unfavorable prognosis and poor survival in BC patients [26]. In another study, patients with BC were shown to have elevated Bmi-1 mRNA as compared to healthy donors [27]. According to a recent report, LincRNA-ROR (long intergenic non-protein coding RNA regulator of reprogramming) might be a potential biomarker of BC; considering that its plasma levels decline in the postoperative vs. preoperative period, this marker can be exploited to monitor a BC patient's condition [28].

It is reported that serum MMP-9 is elevated in the late stages of *ovarian cancer* and correlates with poor prognosis, which suggests the potential prognostic value of this biomarker [37]. The presence of circulating HMGA2 ctRNA may also be a promising tool for the diagnosis and monitoring of ovarian cancer [38].

Patients with advanced *prostate cancer* were shown to have higher levels of circulating cBMP6 mRNA than those with the localized lesion. At the same time, H3K27me3 is characterized by inverse distribution, and its levels are significantly lower in patients with metastatic prostate cancer than in those with early stages of the disease. Thus, post-treatment levels of circulating cBMP6 and H3K27 mRNAs are discriminators between metastatic and localized prostate cancer [39]. Levels of hTERT mRNA in the blood plasma might be another biomarker for distinguishing between localized and locally advanced prostate cancer [40].

Analysis of exosome composition

Ever more attention has been paid to the research into the extracellular vesicles (exosomes and microvesicles) secreted by the tumor that are thought to promote invasion and metastatic spread [41, 42].

Extracellular vesicles are specialized membrane organoids secreted by most cell types; they contain various molecules, including RNA, lipids, proteins, and metabolites [43, 44]. At present, extracellular vesicles are being increasingly recognized as mediators of cell-to-cell communication, transporting mRNA from cancer to normal cells across the extracellular matrix [45, 46].

Microvesicles contain microRNA, different types of long RNA, including mRNA, circular RNA and long non-coding RNA [47, 48]. RNA profiles of extracellular vesicles isolated from healthy individuals and patients with hepatocellular carcinoma are significantly different [48].

Analysis of circulating microRNA

MicroRNA comprises a group of non-coding regulatory RNA consisting of approximately 22 nucleotides and playing an essential role in the regulation of gene expression [49]. Relatively high stability makes microRNA a more advantageous biomarker than mRNA. MicroRNA is found both inside and outside exosomes [50, 51] and is highly stable due to its association with argonaute proteins [52] or lipoprotein complexes, like high density lipoproteins [53].

There were attempts to analyze circulating microRNA in patients with lymphoma [54] and in the plasma/serum samples of patients with prostate cancer [55]. Plasma levels of miR-26a can be indicative of ovarian epithelial cancer [56]. Patients with BC have significantly elevated concentrations of 4 different microRNAs (miR-148b, miR-376c, miR-409-3p, miR-801) [57]. Increased levels of miR-16, miR-21, and miR-451 and low miR-145 concentrations were observed in the plasma of patients with BC [58]. Used in combination, miR-145 and miR-451 were shown to be the best biomarkers of BC, helping to discriminate between BC patients and healthy individuals or patients with other cancers.

Challenges and limitations

Although the analysis of circulating RNA has impressive potential for the application in different fields of medicine, it is not free from drawbacks. Errors occurring during target amplification can affect the results of RNA quantification, especially when dealing with small numbers of analytes [59]. Some discrepancies might be due to the different efficacy of the applied reverse-transcriptase amplification techniques observed for different microRNA and mRNA sequences in different molecular environments. Therefore, PCR-free strategies for detecting circulating RNA seem to be most attractive [60, 61].

Today, most diagnostic approaches based on the analysis of circulating RNA have relatively low specificity and sensitivity [62]. Their improvement requires further large-scale prospective cohort studies.

CONCLUSIONS

The analysis of circulating RNA in cancer patients has a high diagnostic and prognostic value. The informative value of liquid biopsy can be considerably improved by separately analyzing the exosomal and cell-free circulating RNAs, including microRNA. Standardization of sample collection, circulating RNA extraction and the analysis of the obtained results will help to reduce the number of false-negative and false-positive results. Further large-scale prospective cohort studies are needed to select the most sensitive and specific circulating RNA panels.

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COLISTIN RESISTANCE OF CARBAPENEM-RESISTANT *KLEBSIELLA PNEUMONIAE* STRAINS: MOLECULAR MECHANISMS AND BACTERIAL FITNESS

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The increasing use of colistin in the clinic has led to the emergence and spread of colistin resistance. According to the literature, antibiotic resistance can have a metabolic cost, resulting in poor adaptation and survival, i.e. reduced bacterial fitness. The aim of this study was to investigate molecular mechanisms underlying resistance to colistin and their effect on the bacterial fitness of carbapenem-resistant (carba-R) strains of *K. pneumoniae* isolated from the patients of Moscow hospitals in 2012–2017. Of 159 analyzed carba-R isolates, 71 (45%) were resistant to colistin (minimum inhibitory concentration over 2 mg/L). By conducting Sanger sequencing, we were able to identify the mechanisms underlying colistin resistance in 26 (37%) isolates. Growth curves were constructed by measuring optical density at 600 nm wavelength for 15 hours. The competitive growth of colistin-resistant (col-R) *K. pneumoniae* isolates was assessed relative to the colistin-susceptible (col-S) isolate. Col-R and col-S cultures harvested in the exponential phase were combined at the ratio of 1:1, incubated in the Luria-Bertani medium and plated onto Luria-Bertani agar plates with 10 mg/L colistin and without it. The competition index was calculated as the ratio of grown col-R and col-S colonies. Resistance to colistin did not affect the growth kinetics of *K. pneumoniae*, but did reduce the competitive ability of the bacteria as compared to the col-S isolates. However, some col-R isolates were more competitive than the col-S strains of the same sequence type. Further research is needed to elucidate the effects of colistin resistance on bacterial fitness.

Keywords: *Klebsiella pneumoniae*, bacterial fitness, colistin resistance, *mgrB*, sequence type

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Author contribution: Shamina OV planned and conducted the study, analyzed the literature, analyzed and interpreted the obtained data, and wrote the manuscript; Kryzhanovskaya OA, Lazareva AV, Alyabieva NM planned and conducted the study; Mayanskiy NA planned, conducted and supervised the study, analyzed the literature, collected, analyzed and interpreted the obtained data, and wrote the manuscript.

Compliance with ethical standards: the study was carried out following the safety guidelines on the manipulations with pathogens of hazard groups III and IV.

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УСТОЙЧИВОСТЬ КАРБАПЕНЕМРЕЗИСТЕНТНЫХ ШТАММОВ *KLEBSIELLA PNEUMONIAE* К КОЛИСТИНУ: МОЛЕКУЛЯРНЫЕ МЕХАНИЗМЫ И БАКТЕРИАЛЬНЫЙ ФИТНЕС

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В последние годы широкое использование колистина в лечении инфекционных заболеваний привело к появлению и распространению колистинрезистентности. По данным литературы, формирование устойчивости может приводить к затратам внутренних биологических ресурсов и снижению уровня приспособленности и поддержания жизнедеятельности (бактериального фитнеса). Целью исследования было изучить молекулярные механизмы резистентности к колистину и их влияние на бактериальный фитнес карбапенемрезистентных (карба-Р) штаммов *K. pneumoniae*, выделенных у пациентов в г. Москве в 2012–2017 гг. Из 159 карба-Р-изолятов 71 изолят (45%) обладал резистентностью к колистину (минимальная подавляющая концентрация больше 2 мг/л); секвенирование по методу Сенгера позволило обнаружить механизмы устойчивости у 26 (37%) изолятов. Кривые роста были построены путем измерения оптической плотности при длине волны 600 нм в течение 15 ч. Конкурентный рост колистинрезистентных (кол-Р) изолятов *K. pneumoniae* оценивали относительно колистинчувствительного (кол-Ч) изолята. Кол-Р- и кол-Ч-изоляты в экспоненциальной фазе роста смешивали в пропорции 1 : 1, инкубировали в среде Луриа–Бертани и затем наносили на агар Луриа–Бертани, содержащий 10 мг/л колистина, и без него. Индекс конкуренции рассчитывали как отношение выросших кол-Р- и кол-Ч-колоний. Резистентность к колистину не влияла на кинетику роста *K. pneumoniae*, но снижала конкурентоспособность относительно кол-Ч-изолята. Тем не менее были обнаружены кол-Р-изоляты с высоким уровнем конкурентоспособности по сравнению с кол-Ч-изолятами такого же сиквенс-типа. Таким образом, необходимы дальнейшие исследования влияния резистентности к колистину на бактериальный фитнес.

Ключевые слова: *Klebsiella pneumoniae*, бактериальный фитнес, колистинрезистентность, *mgrB*, сиквенс-тип

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Соблюдение этических стандартов: исследование было проведено с соблюдением всех правил безопасности работы с микроорганизмами III–IV групп патогенности.

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Klebsiella pneumoniae is a common cause of infections that require medical attention [1]. The emergence and global spread of high-risk multidrug-resistant (MDR) *K. pneumoniae* sequence types is a worrying trend [2, 3]. Carbapenem-resistant (carba-R) *K. pneumoniae* are an especially serious concern because resistance to carbapenems often co-occurs with resistance to other antimicrobial drugs, which dramatically narrows the range of therapeutic options for *K. pneumoniae* infection. As revealed by multilocus sequence typing (MLST), the majority of carba-R isolates are represented by a small group of sequence types that universally dominate nosocomial populations [4, 5]. At present, the following sequence types are classed as globally disseminated: ST14/15, ST17/20, ST43, ST147, ST258, ST395 [5, 6], and ST307, which only recently has been recognized as clinically relevant [7].

The polycationic antibiotic colistin, also known as polymyxin E, retains activity against carba-R gram-negative microorganisms. However, its wide use in the clinic in the backdrop of rampant resistance to carbapenems has driven the emergence of colistin resistance [4, 8], which can significantly reduce the efficacy of antimicrobial therapy and result in increased mortality in patients infected with colistin-resistant (col-R) *K. pneumoniae* [9].

Resistance to colistin arises from the structural modification of bacterial lipopolysaccharides (LPS) that prevents the antibiotic from binding to the bacterial cell wall [10]. This modification is associated with alterations in the two-component PhoPQ/PmrAB system and its regulator MgrB caused by mutations in the *mgrB* gene, as well as with plasmid-borne *mcr* genes [8, 10].

Naturally, being able to thrive in the presence of an antibiotic, resistant strains have an advantage over susceptible strains; however, there is a biological cost to pay: resistant strains grow at a slower rate and are less competitive in the absence of selective pressure exerted by antibiotics, i.e. have lower bacterial fitness [11, 12] than their susceptible counterparts [13, 14]. Considering that resistance to colistin is linked to LPS modifications, which is the key component of the bacterial cell wall, colistin resistance might be associated with reduced bacterial fitness.

The aim of this study was to characterize the genotype of carba-R *K. pneumoniae* isolated from the inpatients of Surgery and Intensive Care Units of Moscow hospitals, describe molecular mechanisms underlying resistance to colistin and investigate the effect of colistin resistance on the growth kinetics and the competitive ability of this bacterial population.

METHODS

We analyzed 159 carba-R *K. pneumoniae* isolates (the minimum inhibitory concentrations (MIC) of meropenem and imipenem were > 8 mg/L and > 4 mg/L, respectively, as defined by EUCAST criteria) [15] with and without resistance to colistin that had been collected from the patients of Surgery and Intensive Care Units of Moscow hospitals in 2012 through 2017. Only one *K. pneumoniae* isolate per patient was included in the collection. Biological samples had been taken from normally sterile sites (blood, urine, cerebrospinal fluid), the respiratory tract (aspirates, sputum), the oropharyngeal cavity, stomas, wounds, and the anus.

MIC of meropenem, imipenem and tigecycline were determined by performing Etests (BioMerieux; France) on Mueller-Hinton agar plates (Bio Rad; France). Susceptibility to aminoglycosides (gentamicin, netilmicin, amikacin), ciprofloxacin, fosfomycin, cefotaxime, cefepime, and ceftazidime was evaluated using an automated VITEK 2 Compact instrument for bacterial identification and susceptibility testing (BioMerieux; France). Colistin MIC were measured by broth microdilution as recommended in the National Standards of the Russian Federation (GOST R ISO 20776-1-2010); colistin used in the experiments was a powder formulation. The ATCC 25922 strain of *Escherichia coli* served as a control. According to EUCAST, colistin susceptibility and resistance breakpoints for *K. pneumoniae* are ≤ 2 mg/L and > 2 mg/L, respectively [15].

Detection and/or Sanger sequencing of the *mcr-1*, *mgrB*, *pmrA*, *pmrB*, *phoP*, and *phoQ* genes and the analysis of amino acid sequences of the PmrA, PmrB, PhoP, and PhoQ proteins were carried out following the previously described protocols [16]. An *mcr-1*-positive strain of *E. coli* provided by the Research Institute of Antimicrobial Chemotherapy (Smolensk State Medical University, Russia) was used as a control for *mcr-1* detection. *K. pneumoniae* strains were genotyped by means of multilocus sequence typing (MLST) [17]. Insertions were identified using the ISfinder database [18].

Bacterial fitness was studied in a subset of colistin-susceptible (col-S) and colistin-resistant (col-R) *K. pneumoniae* isolates with disrupted and wild-type *mgrB*. The cultures were grown on Luria–Bertani agar (HiMedia Laboratories Pvt. Limited; India) for 24 h. Protocols for assessing bacterial fitness were previously described in [14]. One bacterial colony was suspended in LB and incubated in an orbital shaker incubator ES-20 (BioSan; Latvia) at 37 °C for 3 h at constant stirring at 250 rpm. Bacterial concentrations were measured using a Novocyte flow cytometer (ACEA Biosciences; USA).

To construct and compare growth curves for col-R and col-S strains, the obtained suspension was diluted to a concentration of 5×10^6 bacterial cells per 1 ml. The resulting suspension (250 µl) was plated on flat-bottom 96-well plates containing 0, 1, 4, 16, or 64 mg/L colistin and incubated in an Infinite 200 microplate reader (Tecan; Austria) at 37 °C for 15 h. Incubation was performed in 3 replicates for each strain. Every 30 min, the optical density of the incubated samples was measured at 600 nm (OD_{600}). Measurements were recorded in Magellan 6.6 software (Tecan; Austria). The area under the growth curve (AUGC) was an indicator of bacterial growth; it was calculated for the period between the beginning of exponential growth and the point when plateau was reached (Fig. 1). AUGC was expressed as OD_{600} per hour.

To evaluate the competitive ability of col-R and col-S *K. pneumoniae* isolates, the competition index (CI) was calculated. Briefly, the suspensions of col-R and col-S isolates were

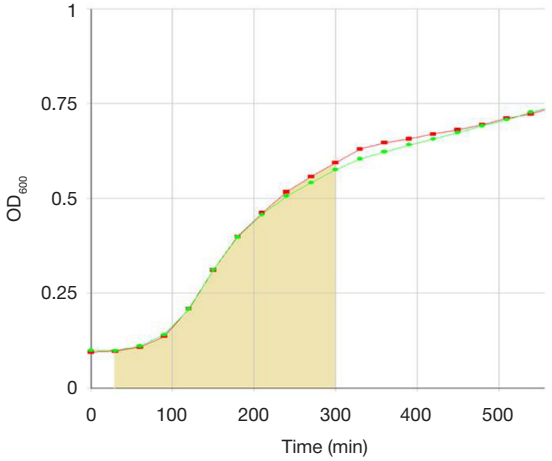

Fig. 1. Typical growth curves for *K. pneumoniae* isolates in the colistin-free culture medium. The shaded region on the graph represents an area under the growth curve (AUGC). The growth curve for col-S isolates is shown in red; the growth curve for col-R isolates is shown in green

Table 1. Genotypes, phenotypes and mechanisms underlying colistin resistance in carba-R *K. pneumoniae* isolates (n = 26)

Isolate ID	ST	Colistin MIC, mg/L	*mgrB* status [a]
69–77	23	128	IS*1A*, family IS-1 (+127/+128)
56–1790	307	64	IS*1R*, family IS–1 (+36/+37)
68–66–1	48	16	IS*Kpn14*, family IS–1 (+141/+142)
58–2876	48	128	IS*Kpn14*, family IS–1 (+141/+142)
58–3431	48	128	IS*Kpn14*, family IS–1 (+141/+142)
58–2966	48	512	IS*Kpn14*, family IS–1 (+141/+142)
56–1678	48	≥ 1024	IS*Kpn14*, family IS–1 (+141/+142)
56–1053	48	≥ 1024	IS*Kpn14*, family IS–1 (+141/+142)
71–1375	307	512	IS*Kpn14*, family IS–1 (+141/+142)
76–2089	377	512	IS*Kpn14*, family IS–1 (+141/+142)
64–574	307	256	IS*Kpn26*, family IS–5 (+74/+75)
4469	395	128	IS*Kpn26*, family IS–5 (+74/+75)
52–1659	395	256	IS*Kpn26*, family IS–5 (+74/+75)
58–1363	307	16	MITE*Kpn1*, family IS–5 (+74/+75)
55–148	307	64	MITE*Kpn1*, family IS–5 (+74/+75)
56–566	307	128	MITE*Kpn1*, family IS–5 (+74/+75)
58–1286	307	128	MITE*Kpn1*, family IS–5 (+74/+75)
56–613	307	512	MITE*Kpn1*, family IS–5 (+74/+75)
48–1594	307	≥ 1024	MITE*Kpn1*, family IS–5 (+74/+75)
78–296	37	16	Δ *mgrB* locus
37262	147	64	Δ *mgrB* locus
29423	70	128	Δ *mgrB* locus
36–2246	395	128	Δ *mgrB* locus
46–1574	307	128	Wild type [b]
48–2246	395	≥ 1024	Wild type [c]
56–410	48	128	Wild type [d]

Note: ST — sequence type; MIC — minimum inhibitory concentration; [a] — the position of the insertion sequence is specified in brackets; [b] — PmrB alteration (T157P); [c] — PmrA (A141T) and PmrB (L213M, G256R) alterations; [d] — PmrB alteration (deletion at 27–30 (QLIS)).

adjusted to 1.5 × 10^3 cells per 1 ml and combined at a 1 : 1 ratio (1.5 × 10^3 CFU per 1 ml for each strain). The mixture of col-R and col-S isolates and suspensions of unmixed col-S and col-R strains were grown in LB at 37 °C at 180 rpm for 16–18 h. Upon incubation, the suspensions were diluted 10^5-fold and plated on Petri dishes containing LB agar supplemented with 10 mg/L colistin or LB agar without colistin; plating was performed using an easySpiral automated spiral plater (Interscience; France). The cells were incubated at 37 °C for 16–18 h. CFU were counted using an automated Scan 500 colony counter (Interscience; France). CI was calculated as a ratio of col-R CFU in the LB dish with colistin to col-S CFU in the dish without colistin. CI < 1 was interpreted as reduced competitive ability of the resistant isolate, as compared to the susceptible isolate. The experiments were conducted in 3 replicates.

Statistical analysis was carried out in IBM SPSS Statistics 20.0 (IBM SPSS Inc; USA). Below, AUGC values and the number of colonies are presented as a median (P_{25}; P_{75}), CI is presented as a mean and a standard deviation. Differences in CI were evaluated using the Kruskal–Wallis test; pairwise comparisons were done using the Mann–Whitney U test. The differences were considered significant at $p < 0.05$.

RESULTS

Characterization of carba-R isolates of *K. pneumoniae*

All 159 carba-R *K. pneumoniae* isolates had an MDR-phenotype, i.e. were resistant to at least 3 classes of antimicrobial drugs. All studied strains were resistant to the third and fourth-generation cephalosporins and were highly resistant to ciprofloxacin (93%), fosfomycin (90%), netilmicin (82%), gentamicin (84%), amikacin (50%), and colistin (45%). The majority of carba-R *K. pneumoniae* isolates were susceptible to tigecycline; only 7% were resistant to this drug.

As revealed by MLST, the studied carba-R isolates were represented by 18 sequence types, of which only 5 dominated the collection, occurring in 86% of cases. Those included ST307 (n = 46, 29%), ST395 (n = 40, 25%), ST377 (n = 17, 10%), ST48 (n = 17, 10%), and ST23 (n = 16, 10%).

Mechanisms of colistin resistance

Resistance to colistin was observed in 71 (45%) carba-R *K. pneumoniae* isolates; for those isolates, colistin MIC varied from 4 to 1024 mg/L or was even higher. Investigation of

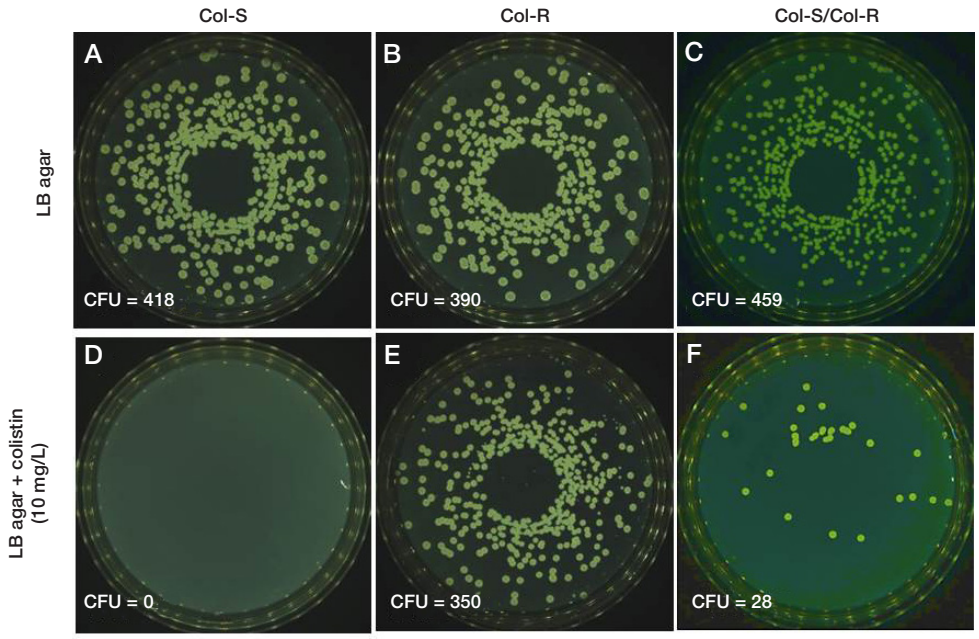

Fig. 2. Evaluation of the competitive ability of *K. pneumoniae* (a representative experiment). Col-S — colistin-susceptible isolates; col-R — colistin-resistant isolates; col-S /col-R — a mixture of susceptible and resistant strains; CFU — colony forming units. The photos of Petri dishes demonstrate the growth pattern for the col-S isolates (**A, D**), col-R isolates (**B, E**) and the mixture of col-R/col-S isolates (**C, F**) of *K. pneumoniae* on LB agar plates without colistin (**A–C**) and supplemented with 10 mg/L colistin (**D–F**). Numbers indicate the CFU count on each plate. The competition index (CI) is calculated as (the number of CFU on LB + colistin) divided by (the number of CFU on LB minus the number of CFU on LB + colistin), i.e. CFU F : (CFU C-CFU F)

molecular mechanisms underlying resistance to colistin was started with a search for the plasmid-borne gene *mcr-1*, which, according to the literature, is the most common cause of resistance [19]. We found that none of 71 col-R *K. pneumoniae* isolates carried the *mcr-1* gene.

Then, we went on to analyze the sequence integrity of the *mgrB* gene whose disruption might be associated with colistin resistance. Mutations in the *mgrB* gene were observed in 23 (32%) col-R isolates (Table 1). Deletion of the entire *mgrB* locus was detected in 4 (17%) isolates. In 13 (56%) isolates, there were insertions of 4 different types (IS1A, IS1R, IS*Kpn14*, and IS*Kpn26*), which occurred at different positions and represented the IS-1 and IS-5 families (Table 1). In 6 (26%) col-R isolates, the *mgrB* gene harbored a new mobile element (MITE*Kpn1*) described in our previous publication [16].

Summing up, the *mgrB* gene was wild-type in only 48 of 71 (68%) col-R *K. pneumoniae* isolates. Therefore, we had to continue looking for other mechanisms underlying colistin resistance. We analyzed the amino acid sequences of the proteins PmrA, PmrB, PhoP, and PhoQ in all 48 isolates. These proteins participate in LPS modification. Alterations in their sequences can cause resistance to colistin [10]. Significant alterations in PmrA and/or PmrB sequences were detected in 3 isolates from 3 different sequence types (ST307, ST395, ST48), with colistin MIC ranging from 128 to 1024 mg/L or being even higher (Table 1).

Effects of colistin resistance on bacterial fitness

In the absence of colistin, the growth kinetics of col-R and col-S *K. pneumoniae* did not differ significantly. Median AUGC values were 4.2 (3.9; 4.3) and 4.05 (3.9; 4.6) OD600 per 1 h, respectively (p = 0.842; Table 2). Addition of 1 mg/L colistin to the culture of col-S isolates caused AUGC to drop abruptly to 1.9 (0.95; 4.13) OD_{600} per 1 h (p = 0.065), whereas higher concentrations of colistin completely inhibited the growth of

Table 2. Effects of colistin resistance on the bacterial fitness (growth kinetics and competition index) of carba-R *K. pneumoniae* isolates

Isolates	Colistin MIC, mg/L; Me (P_{25}; P_{75})	AUC (OD_{600} per 1 H), Me (P_{25}; P_{75})					CI, mean (SD)
		Colistin concentration, mg/L					
		0	1	4	16	64	
Col-S (n = 6)	< 1 (< 1; < 1)	4.05 (3.9; 4.6)	1.9 (0.95; 4.13)	0 (0; 4.03)	0 (0; 0)	0 (0; 0)	n/a
Col-R (n = 32)	256 (128; 512)	4.2 (3.9; 4.3)[a]	4.1 (3.7; 4.2)[b]	3.9 (3.2; 4.15)[c]	3.3 (2.2; 3.45)[d]	0.9 (0; 3)[d]	0.15 (0.21)[f]
Of them:							
mgrB, disrupted (n = 15)	256 (128; 512)	4.1 (3.9; 4.2)	4 (3.9; 4.2)	3.9 (3.1; 4.1)	3.4 (0.9; 3.7)	1.1 (0; 3.3)	0.1 (0.1)[g]
mgrB, wild type (n = 17)	256 (96; 512)	4.3 (3.85; 4.4)[e]	4.2 (3.53; 4.25)[e]	3.8 (3.18; 4.25)[e]	3.25 (2.75; 3.43)[e]	0.9 (0; 3)[e]	0.19 (0.26)[h, i]

Note: MIC — minimum inhibitory concentration; AUGC — area under growth curve; Me — median; P_{25} and P_{75} — the 25th and 75th percentiles; CI — competition index; SD — standard deviation; col-S — colistin-susceptible isolates; col-R — colistin-resistant isolates; n/a — not applicable; [a] — p = 0.842 for comparison with col-S AUGC; [b] — p = 0.19 for comparison with col-R AUGC at 0 mg/L colistin; [c] — p = 0.016 for comparison with col-R AUGC at 0 mg/L colistin; [d] — p < 0.001 for comparison with col-R AUGC at 0 mg/L colistin; [e] — p > 0.05 for comparison with col-R AUGC for isolates with disrupted *mgrB*; [f] — n = 26; [g] — n = 11; [h] — n = 15; [i] — p = 0.283 for comparison with CI of the isolates with disrupted *mgrB*.

Table 3. The competition index of col-R and col-S isolates of carba-R *K. pneumoniae* representing the same sequence types

Col-R isolates		Mechanism of colistin resistance	CFU count (SD)		CI (SD)
ST	Isolate ID		Col-R (LB agar + colistin, 10 mg/L)	Col-S + col-R (LB agar)	
ST23	37261	Unknown	80	141	1.3
	69–77	Mutant *mgrB*	39	168	0.3
	37243	Unknown	25	112	0.29
	37224	Unknown	5	114	0.05
Total ST23:			**37 (32)**	**134 (26)**	**0.48 (0.56)**
ST395	52–1659	Mutant *mgrB*	88	135	1.87
	78–1127	Unknown	3	138	0.02
	59–397	Unknown	110	153	2.5
	4469	Mutant *mgrB*	17	141	0.14
Total ST395:			**55 (53)**	**142 (8)**	**1.1 (1.24)**
ST377	76–1648	Unknown	90	335	0.37
	76–2053	Unknown	38	282	0.16
	76–2089	Mutant *mgrB*	79	232	0.52
Total ST377:			**69 (27)**	**283 (52)**	**0.35 (0.18)**
ST307	64–574	Mutant *mgrB*	33	287	0.13
	56–566	Mutant *mgrB*	68	210	0.48
	71–1375	Mutant *mgrB*	63	196	0.47
Total ST307:			**55 (19)**	**231 (49)**	**0.36 (0.2)**
ST147	37–262	Mutant *mgrB*	3	201	0.02
ST48	58–2966	Mutant *mgrB*	8	152	0.06

Note: CI — competition index; SD — standard deviation.

susceptible isolates as anticipated (Table 2). Col-R isolates of *K. pneumoniae* demonstrated normal growth kinetics at 1 mg/L colistin but their growth slowed down at 4 and 16 mg/L colistin concentrations ($p = 0.016$ and $p < 0.001$, respectively). At 64 mg/L colistin, the growth of col-R isolates was almost completely inhibited at AUGC of 0.9 (0; 3.0) OD_{600} per 1 h (Table 2).

When comparing AUGC values between col-R isolates with disrupted and wild-type *mgrB* (Table 2), we found that the *mgrB* status only insignificantly affected the kinetics of bacterial growth regardless of colistin concentrations used.

Then, we calculated the CI for 26 col-R K. pneumonia isolates co-cultured with their carba-S/col-S counterparts in order to compare their competitive ability (Fig. 2; Table 2). The mean CI value was 0.15 (0.21); 25/26 (96%) of col-R isolates had IC < 1 ranging from 0.01 to 0.53; one isolate had CI of 1. Wild type isolates and those with disrupted *mgrB* had similar CI of 0.19 (0.26) and 0.1 (0.1), respectively ($p = 0.283$; Table 2). Thus, resistance to colistin was associated with a loss of competitive ability in the majority of analyzed col-R isolates, as compared to carba-S/col-S *K. pneumoniae* isolates, which did not depend on the *mgrB* status.

The effects of colistin resistance on bacterial fitness were additionally investigated in carba-R/col-S and carba-R/col-R pairs of *K. pneumoniae* of the same sequence types. We selected isolates of 5 most common ST (ST23, ST48, ST307, ST377 and ST395) and one rare ST (ST147); at least one isolate in this subset was colistin-sensitive (Table 3). The competitive ability of all col-R isolates belonging to types ST48, ST147, ST307 and ST377 was diminished compared to the col-S isolates of the same sequence types (CI < 1). However, the situation was different for the isolates represented by sequence types ST23 and ST395. One col-R isolate of type ST23 (CI = 1.3) and 2 col-R isolates of type ST395 (CI = 1.87 and CI = 2.5, respectively) were more fit than col-S isolates (Table 3).

DISCUSSION

The majority of carba-R isolates of *K. pneumoniae* in our collection were represented by 5 major sequence types; of them, types ST307 and ST395 made up 54% of the entire sample. A while ago, ST307 was not recognized as a dominant sequence type in Russia [20, 21], but at present, it is becoming one of the leading high-risk international sequence types [7], which is consistent with our findings.

Of all carba-R isolates analyzed in this paper, 45% were resistant to colistin. The multicenter study *MARAPHON* [2, 3] showed that the prevalence of col-R isolates in the large sample of nosocomial *K. pneumoniae* isolates was generally low, in spite of an increase from 4.5% in 2012 to 7.9% in 2014. Our data might reflect the global trend of growing antibiotic resistance, including resistance to colistin. According to a 15-year retrospective study conducted at a large hospital in Athens, the proportion of col-R *K. pneumoniae* isolates from blood cultures surged from 0% in 2002 to 26.9% in 2016 [22]. On the other hand, the high prevalence of col-R strains in our collection might be explained by the fact that our sample was dominated by nosocomial strains recovered from intensive care units, where, as reported by Feretzakis et al. [23], the proportion of col-R *K. pneumoniae* can be much higher than in other hospital departments (40 vs 13.8%). Besides, direct comparative analysis of colistin resistance studies that rely on different susceptibility testing techniques can be quite challenging. For example, false results are not rare in epsilometer tests in comparison with the reference method of microdilutions; therefore, such tests can fail in detecting the true rate of colistin resistance [9, 20].

Colistin resistance did not affect the kinetics of bacterial growth in the absence of this antibiotic and did not depend on the status of the *mgrB* gene, which is consistent with previously published data [24]. In contrast, in *Acinetobacter baumannii* and *Pseudomonas aeruginosa* resistance to colistin

undermines the dynamics of bacterial growth [13, 25], which might explain the relatively high prevalence of enterobacteria possessing chromosomal resistance to colistin in comparison with col-R *A. baumannii* and *P. aeruginosa*.

At the same time, the majority of col-R isolates were less competitive than col-S isolates of *K. pneumoniae*; this is also typically seen in other bacteria, such as *A. baumannii* [13] and *P. aeruginosa* [25]. There are reports of reduced bacterial fitness in col-R *K. pneumoniae* that carry the *mcr-1* gene [26].

Another interesting finding came from the experiments comparing bacterial fitness between col-R and col-S *K. pneumoniae* isolates of one sequence type, i.e. bacteria with very similar genotypes but different susceptibility to colistin. Six different sequence types were analyzed. The majority of col-R isolates had low CI. At the same time, 2 col-R isolates of type ST395 and 1 col-R isolate of type ST23 were more competitive than col-S isolates of the same sequence type. This finding can be explained by the presence of compensatory mutations in the bacterial genome, as was the case with resistance to fluoroquinolones and colistin in *Escherichia coli* [27] and *A. baumannii* [28]. Unlike mutations that confer resistance, compensatory mutations boost bacterial fitness and thus promote resistance even in the absence of selective pressure exerted by an antibiotic [27, 28].

We conclude that resistance to colistin is common in the population of carba-R *K. pneumoniae* isolated from Moscow patients. This alarming trend requires close monitoring.

CONCLUSION

Our collection of carba-R *K. pneumoniae* isolates was dominated by sequence types ST307 and ST395; disruption of the *mgrB* gene by a variety of insertion sequences was the leading mechanism of colistin resistance.

Resistance to colistin did not affect the kinetics of bacterial growth in col-R *K. pneumoniae* in the absence of the antibiotic and did not depend on the status of the *mgrB* gene; the overwhelming majority of col-R *K. pneumoniae* isolates were less competitive than col-S strains; but within one sequence-type, there could be col-R isolates with increased competitive ability. Further research into bacterial fitness might elucidate the causes underlying the spread of colistin resistance among enterobacteria.

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EFFICACY OF COMMERCIAL BACTERIOPHAGE PRODUCTS AGAINST ESKAPE PATHOGENS

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The ever-rising prevalence of multidrug-resistant bacteria necessitates the search for a therapeutic alternative to antibiotics. Using therapeutic products based on virulent bacteriophages might provide such an alternative. The aim of our study was to evaluate the efficacy of commercial phage products and natural bacteriophage monoisolates recovered from environmental sources against clinical strains of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. We compiled a collection of 147 strains that were subsequently genotypes using the MLST method. The efficacy of bacteriophages was evaluated in spot tests. The highest efficacy was demonstrated by "Staphylococcal bacteriophage" (86%, effective against *S. aureus*), "Purified polyvalent pyobacteriophage" (87.8%, effective against *K. pneumoniae*), and a group of phage products against *P. aeruginosa*, including "Pseudomonas aeruginosa bacteriophage" (87.5%), "Complex pyobacteriophage" (79.5–90%) and "Purified polyvalent pyobacteriophage" (90–92.5%). The efficacy of "Intesti bacteriophage", which targets *E. faecium*, was 4.2%. The efficacy of commercial phage products against *S. aureus* and *K. pneumoniae* was higher than the efficacy of individual phage monoisolates (60% for the *S. aureus* phage vB_SauP-436-3w and 5.9% for the *K. pneumoniae* phage vB_Kp_M_Seu621). Thus, all tested commercial phage products were highly effective against *P. aeruginosa*, *K. pneumoniae* and *S. aureus*. There are no commercial phage products on the market against other ESKAPE pathogens, including *Acinetobacter baumannii* and *Enterobacter cloacae*. Besides, there are no effective phage products against *E. faecium*. This dictates the need for new effective bacteriophages against these species.

Keywords: bacteriophages, phage therapy, microbiology, ESKAPE pathogens, bacteria

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Compliance with ethical standards: the study was carried out in strict compliance with the sanitary norms and epidemiological safety standards specified in the guidelines on the work with microorganisms belonging to hazard groups III–IV and causative agents of parasitic diseases (Guidelines 1.3.2322-08); supplementary guidelines № 1 to the guidelines on the work with microorganisms belonging to hazard groups III–IV and causative agents of parasitic diseases (Guidelines 1.3.2518-09), sanitary and epidemiologic requirements for the handling of medical waste (Sanitary norms and regulations 2.1.7.2790-10), and Federal clinical recommendations on the rational use of bacteriophages in clinical and epidemiological practice.

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ЭФФЕКТИВНОСТЬ ПРЕПАРАТОВ БАКТЕРИОФАГОВ ПРОТИВ ПАТОГЕНОВ ГРУППЫ ESKAPE

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Ежегодный рост числа случаев выявления бактерий с множественной лекарственной устойчивостью делает актуальной задачу поиска альтернативы применяемым антибиотикам. Такой альтернативой могут быть препараты на основе вирулентных бактериофагов. Целью работы было оценить эффективность коммерческих фаговых препаратов и моноизолятов бактериофагов, выделенных из природных источников, против клинических штаммов *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae* и *Pseudomonas aeruginosa*. Была собрана коллекция из 147 штаммов, типированных методом МЛСТ. Оценку эффективности бактериофагов проводили методом спот-тестирования. Наиболее эффективными оказались препараты против *S. aureus* («Бактериофаг стафилококковый», 86%), *K. pneumoniae* («Пиобактериофаг поливалентный очищенный», 87,8%) и *P. aeruginosa* («Бактериофаг псевдомонас аеругиноза», 87,5%; «Пиобактериофаг комплексный», 79,5–90%; «Пиобактериофаг поливалентный очищенный», 90–92,5%). Для *E. faecium* эффективность препарата «Интести-бактериофаг» составила лишь 4,2%. При этом эффективность терапевтических препаратов, активных против *S. aureus* и *K. pneumoniae*, была выше эффективности отдельных моноизолятов бактериофагов (фаг *S. aureus* vB_SauP-436-3w — 60%, фаг *K. pneumoniae* vB_Kp_M_Seu621 — 5,9%). Таким образом, исследуемые препараты обладают высокой активностью против штаммов *P. aeruginosa*, *K. pneumoniae* и *S. aureus*. В свою очередь препаратов, действующих против остальных членов группы ESKAPE-патогенов (*Acinetobacter baumannii* и *Enterobacter cloacae*), а также эффективных против *E. faecium*, не представлено на рынке, что подчеркивает необходимость поиска новых бактериофагов.

Ключевые слова: бактериофаги, бактериофаговая терапия, микробиология, ESKAPE-патогены, бактерии

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Every year, multidrug resistant (MDR) bacteria are becoming more prevalent. MDR strains are defined as having resistance to three or more antibacterial drugs [1]. Bacterial infections caused by MDR strains pose a critical threat to global healthcare. Most MDR strains are found among the so called ESKAPE pathogens (an acronym for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter spp*.). These bacteria cause life-threatening nosocomial infections and are especially dangerous for individuals with compromised immunity and chronic conditions [2–4].

According to the World Health Organization, pathogenic bacteria can be classified in terms of threat prioritization as having critical, high or medium priority [1]. Carbapenem-resistant *A. baumannii*, *P. aeruginosa*, *Enterobacteriaceae spp*., as well as *K. pneumoniae*, are critical priority pathogens. In some countries, the proportion of carbapenem-resistant isolates among *P. aeruginosa* and *K. pneumoniae* can be as high as 50 and 64%, respectively [5]. Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* belong to the high-priority group. In some countries, MRSA strains amount to 43% of all *S. aureus* isolates, whereas vancomycin-resistant *E. faecium* makes up 59.1% [5]. The number of antibiotic-resistant isolates is constantly increasing.

Infections caused by drug-resistant ESKAPE pathogens dictate the need for novel therapeutic approaches. One of them involves using virulent bacteriophages as a complement or an alternative to antibacterial therapy. The first attempts to exploit bacteriophages in clinical practice were made in the early 20th century. So far, phages have proved to be effective antibacterial agents [6, 7]. Using virulent bacteriophages as therapeutic agents has several advantages. Most importantly, their interaction with a bacterial cell does not depend on the resistance profile of the latter. Phages co-evolve with their bacterial hosts and thereby learn to overcome the host's defenses.

Phage products available on Russia's pharmaceutical market are cocktails composed of several virulent phages. Such cocktails allow targeting an array of different bacterial strains. In Russia, most commercial phage products are manufactured by two companies: Microgen Scientific and Production Association and Micromir Research and Production Center. The manufacturers claim that their phage cocktails are effective against ESKAPE pathogens, including *E. faecium*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*. At present, there are no commercial phage preparations on the Russian market exerting activity against *A. baumannii* and *Enterobacter spp*. This emphasizes the importance of their development.

The aim of this work was to evaluate the efficacy of commercial phage cocktails and monoisolates of bacteriophages from environmental sources against clinical strains of *E. faecium*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*.

METHODS

Bacterial isolates

Isolates of *E. faecium*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* (n = 147) were obtained from the inpatients of the Federal Research and Clinical Center of Physical-Chemical Medicine of the Federal Medical Biological Agency in 2018–2019. The cultures were grown on Columbia agar or soya broth (both by Oxoid; UK) at 37 °C for 18–24 h.

Bacterial species were identified by means of direct mass spectrometry profiling of bacterial lysates as described in [8]. A saturated solution of α-cyano-4-hydroxycinnamicacid (Bruker Daltonics; Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid was used as a matrix solution. Mass spectra were recorded on a Microflex MALDI TOF mass spectrometer (Bruker Daltonics; Germany). A bacterial test standard (Bruker Daltonics; Germany) was used for calibration. Mass spectra were recorded, processed and analyzed in flexControl 3.0 and flexAnalysis 3.0 (Bruker Daltonics; Germany). Species identification was aided by MALDI Biotyper 3.0 (Bruker Daltonics; Germany).

Determining bacterial sensitivity to antibiotics

Sensitivity of bacterial strains to antibiotics was evaluated by disk diffusion as recommended by the international Performance Standards for Antimicrobial Susceptibility Testing (Clinical and Laboratory Standards Institute) (CLSI) published in 2019 [9]. Gram-negative *K. pneumoniae* and *P. aeruginosa* were tested for sensitivity to ceftriaxone, gentamicin, ciprofloxacin, and meropenem. Gram-positive *S. aureus* and *E. faecium* were tested for sensitivity to erythromycin, ciprofloxacin and tetracycline. Additionally, *S. aureus* isolates were tested for resistance to oxacillin and gentamicin. Sensitivity of *E. faecium* to vancomycin was evaluated using a method of serial dilutions following CLSI recommendations [9].

Molecular genetic testing of bacterial strains

K. pneumoniae, *P. aeruginosa* and *E. faecium* strains were genotyped using multilocus sequence typing (MLST) following standard schemes [10–14]. For *S. aureus*, spa-typing was applied according to the standard protocol; this technique allows determining the sequence of the Staphylococcus protein A gene [15].

Bacterial DNA was isolated using a DNA-express kit (Lytech; Russia) following the manufacturer's protocol. DNA samples were stored at –20 °C. Genes targeted by genetic typing were amplified in a TETRAD DNA ENGINE thermocycler (MJ Research; USA). Amplification was carried out in 25 µl of the reaction mix containing 66 mM Tris-HCl (pH 9), 16.6 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 1 Taq DNA polymerase unit (Lytech; Russia), and 10 pmol of primers. Amplification products were separated in 2% agarose gel stained with ethidium bromide for DNA visualization.

Sanger sequencing was performed in a 3730 DNA Analyzer (Thermo Fisher Scientific; UK). Gene sequences were analyzed in the Ridom StaphType TM software (Ridom GmbH; Würzburg, Germany) and Vector NTI Suite 9 (Thermo Fisher Scientific; UK). Allelic profiles and MLST types were determined by comparing the obtained nucleotide sequences to the sequences stored in the international PubMLST database [11].

Commercial phage products

In this study, we evaluated the efficacy of 14 commercial products of virulent bacteriophages manufactured by Microgen (Table 1). All phage products were bought at Moscow pharmacies and are approved for clinical use.

Isolation of bacteriophages from environmental sources

Bacteriophages capable of infecting some *K. pneumoniae* and *S. aureus* strains were isolated from water samples collected in different water reservoirs; isolation was performed using the enrichment culture method. Briefly, a 50 ml water sample was

Table 1. Commercial bacteriophage products used in the study

Name	Activity spectrum	Batch number	Manufactured in
"Staphylococcal bacteriophage"	*Staphylococcus aureus* and some other coagulase-negative staphylococci	N33	Nizhny Novgorod
		P332	Perm
"Pseudomonas aeruginosa bacteriophage"	*Pseudomonas aeruginosa*	N7	Nizhny Novgorod
"Klebsiella pneumoniae purified bacteriophage"	*Klebsiella pneumoniae*	P252	Perm
		P251	
"Klebsiella pneumoniae purified polyvalent bacteriophage"	*Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Klebsiella rhinoscleromatis*	U27	Ufa
"Purified polyvalent pyobacteriophage"	*Staphylococcus spp, Streptococcus spp, Proteus spp, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli*	U1	Ufa
		U25	
"Complex pyobacteriophage"	*Staphylococcus spp, Enterococcus spp, Streptococcus spp,* enteropathogenic *Escherichia coli, Proteus vulgaris, Proteus mirahilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Klebsiella oxytoca*	N74	Nizhny Novgorod
		N45	
"Intesti bacteriophage"	*Shigella flexneri, Shigella sonnei, Salmonella typhimurium, Salmonella spp, Escherichia coli, Proteus spp, Enterococcus spp, Staphylococcus spp, Pseudomonas aeruginosa*	N101	Nizhny Novgorod
		N123	
		N86	
		N175	

filtered through a 0.45 μm Millipore filter (Merck Millipore; USA). A 2x lysogeny broth (LB) (Oxoid; UK) was combined with the water sample; 300 μL of the overnight bacterial culture were added to the mixture and incubated on a rocking shaker at 37 °C for 18 h. Then, bacterial cells were centrifuged at 3,500 g, and the supernatant was filtered through a 0.22 μm Millipore filter (Merck Millipore; USA). Monoisolates were obtained through a series of 3 sequential isolations from negative colonies. The obtained bacteriophages were grown in 50 ml of LB containing 300 μl of the overnight bacterial culture. Bacteriophage concentrations in the phage lysate were measured using a classic double layer agar method proposed by A. Gratia [16].

Evaluating the efficacy of commercial phage products and monobacteriophage lysates

The efficacy of lytic phages (titers of over 10^7) was evaluated in a spot test. Briefly, 0.1 ml of the overnight culture was combined with 0.6% semi-liquid LB agar. The resulting suspension was applied onto Petri dishes coated with 1.5% LB agar. After the top LB agar layer hardened, 5 μl of the studied phage was applied onto it and incubated at 37 °C for 18–24 h. In 24 h, either individual negative colonies or a transparent lysis zone were observed where the agar drop had been applied. If this was the case, the bacterial strain was considered sensitive to the tested phage. In the absence of a lysis zone, the

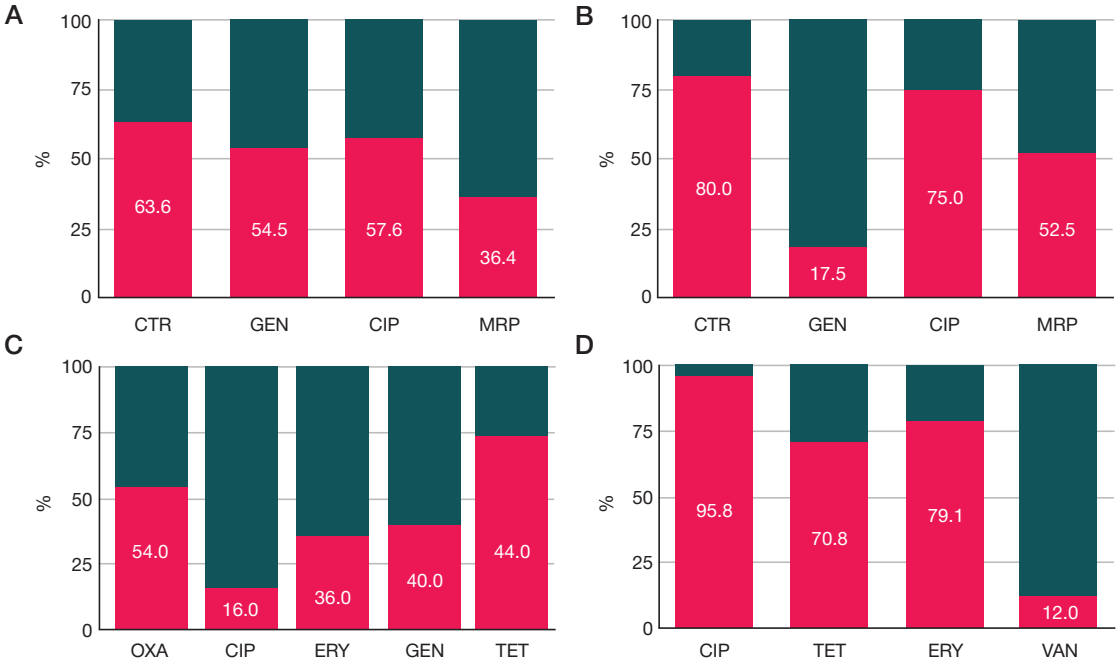

Fig. 1. Resistance to antibiotics among the strains of *K. pneumoniae* (**A**), *P. aeruginosa* (**B**), *S. aureus* (**C**), and *E. faecium* (**D**). The pink shows the proportion of resistant strains. CIP — ciprofloxacin, TET — tetracycline, ERY — erythromycin, MRP — meropenem, VAN — vancomycin, OXA — oxacillin, CTR — ceftriaxone, GEN — gentamicin

bacterial strain was considered resistant to the tested phage. The efficacy of a phage against a certain bacterial strain was determined as percentage of susceptible bacterial strains of a given species in the total pool of strains of this species included in our collection.

RESULTS

We compiled a collection of 147 bacterial strains, which included 33 strains of *K. pneumoniae* (22.5%), 40 strains of *P. aeruginosa* (27.2%), 50 strains of *S. aureus* (34%), and 24 strains of *E. faecium* (16.3%). Susceptibility profiles were obtained for all strains included in the collection (Fig. 1).

Of 33 *K. pneumoniae* strains, 9 (27.3%) were sensitive to all antibiotics they were tested against, 4 (12.1%) strains were resistant to only one antibacterial drug, and 17 (51.5%) strains exhibited multidrug resistance. Of 40 *P. aeruginosa* strains included in the collection, 7 (17.5%) were sensitive to all antibiotics they were tested against, 15 (37.5%) were resistant to one antibacterial drug, and 6 (15%) strains fell into the MDR category.

Of 50 *S. aureus* strains included in the collection, 19 (38%) were sensitive to all antibiotics they were tested against, 7 (14%) were resistant to one antibacterial drug, and 22 (44%) were classified as MDR. Twenty-seven (54%) *S. aureus* strains were resistant to oxacillin. There were no susceptible strains among *E. faecium* isolates; 3 (12.5%) of 24 *E. faecium* strains were resistant to one antibacterial drug, and 19 (19.2%) were multidrug-resistant. Vancomycin-resistant *E. faecium* strains amounted to 12%.

Using MLST, we identified 15 sequence types among *K. pneumoniae* strains (Fig. 2A). The most common of them were ST395 and ST23 represented by 14 (42.4%) and 5 (15.2%) strains, respectively. In addition, two unique sequence types were identified in this group of pathogens (2-1-1-1-9-4-1 and 2-1-1-1-9-4-18). According to MLST, *P. aeruginosa* strains fell into 26 different sequence types (Fig. 2B). ST12 was the most common sequence type among *P. aeruginosa* strains (5 out of total 40 strains; 12.5%). In addition, 3 unique sequence types were identified: type 15-5-11-8-4-4-1 represented by 2 strains, type 15-2-11-3-3-38-3 represented by 2 strains and type 17-5-12-3-14-4-7 represented by 1 strain. *E. faecium* strains belonged to 12 different sequence types, the most common being ST18 (4 out of 24 strains; 16.7%), ST17 (3 of 24 strains; 12.5%), ST78 (3 of 24 strains; 12.5%) and ST192 (3 of 24 strains; 12.5%) (Fig. 2C).

Spa-typing revealed the diversity of *S. aureus* strains (Fig. 2D) in our collection. This species was represented by 18 spa-types; the types t008 and t308 prevailed, accounting for 20 (40%) and 6 (12%) of the total 50 *S. aureus* strains.

The efficacy of 14 commercial phage products (see Table 1; Fig. 3) was tested on the compiled collection of characterized ESKAPE pathogens. The best effect against *K. pneumoniae* was observed for "Purified polyvalent pyobacteriophage", batch number U1, which killed 29 (87.9%) of 33 *K. pneumoniae* strains (Fig. 3A). The efficacy of the commercial phage products against *P. aeruginosa* varied from 76.9 to 92.5% (Fig. 3B). "Staphylococcal bacteriophage" was effective against 43 (86%) of 50 *S. aureus* strains (Fig. 3C). "Intesti bacteriophage", batch number P86, was the only

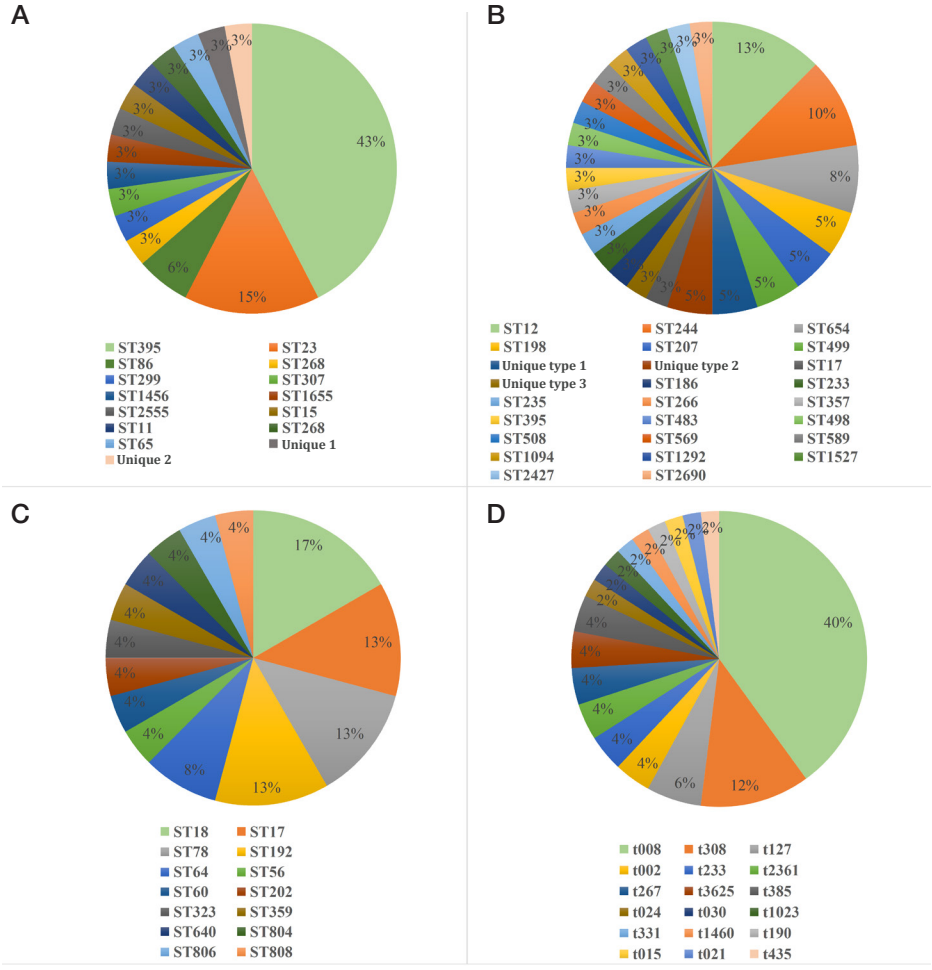

Fig. 2. Results of molecular genetic typing for *K. pneumoniae* (**A**) *P. aeruginosa* (**B**) *E. faecium* (**C**), and *S. aureus* (**D**)

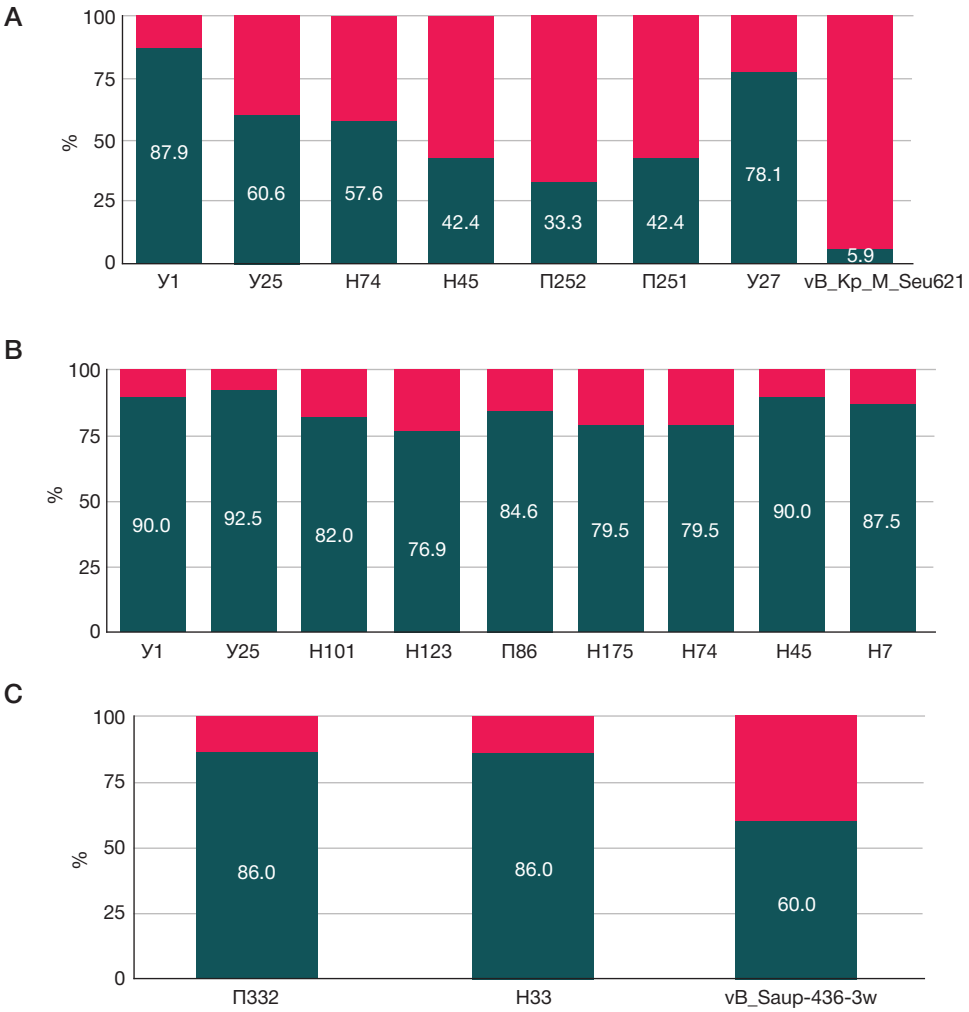

Fig. 3. The efficacy of commercial phage products against *K. pneumoniae* (**A**), *P. aeruginosa* (**B**) and *S. aureus* (**C**). The green shows the proportion of strains sensitive to the tested phage products. Batch numbers represent the tested products: "Purified polyvalent pyobacteriophage" (U1, U25); "Complex pyobacteriophage" (N74, N45); "Klebsiella pneumoniae purified bacteriophage" (P252, P251); "Klebsiella pneumoniae purified polyvalent bacteriophage" (U27); "Pseudomonas aeruginosa bacteriophage" (N7); "Staphylococcal bacteriophage" (P332, N33).

available bacteriophage against *E. faecium*; it successfully infected 24 (4.2%) *E. faecium* strains.

To compare the efficacy of commercial phage products with that of natural phages, bacteriophage monoisolates exhibiting activity against *K. pneumoniae* and *S. aureus* were recovered from natural reservoirs (vB_Kp_M_Seu621 and vB_SauP-436-3w, respectively). Their titers were 10^{12} PFU/ml (for vB_Kp_M_Seu621) and 10^{11} PFU/ml (for vB_SauP-436-3w), respectively. The efficacy of the vB_Kp_M_Seu621 and vB_SauP-436-3w monoisolates was 5. 9 and 60%, respectively (see Fig. 3A and 3C).

DISCUSSION

The efficacy of polyvalent phage products against *K. pneumoniae* varied from 42.4 to 87.9%; for monoisolates, this range was narrower: from 33.3 to 78.1% (see Fig. 3A). This suggests that the phage cocktails used in the study differed in their composition and should be updated and tested against currently circulating bacterial strains. The efficacy of the phage vB_Kp_M_Seu621 (5.9%) isolated from environmental sources was much lower than the efficacy of the tested commercial phage products which might be associated with the diversity of *K. pneumoniae* capsule types. The capsule can serve as a receptor for bacteriophages and determine the efficacy of interaction between the phage and its host [17].

It should be noted that almost all strains of *K. pneumoniae* included in the collection (32 of 33; 97.9%) were sensitive to at least one of the tested phage products. There was no significant difference in the efficacy of lysis between MDR and susceptible strains. The majority of MDR strains belonged to the sequence type ST395. Strains of this sequence type are very common among nosocomial pathogens and are associated with the spread of the *blaOXA-48* gene, which confers resistance to β-lactams [18]. MDR strains representing this sequence type were susceptible to "Purified polyvalent pyobacteriophage" (U1); the efficacy of this phage product against ST395 strains was 81.8% (9 of 11). It also caused lysis of other MDR strains of K. pneumoniae belonging to the types ST15, ST23, ST268.

The highest efficacy of virulent phages was observed for *P. aeruginosa* strains. The efficacy of polyvalent phage products against this pathogen was 76.2–90%, whereas the efficacy of monovalent phage products was 87.5% (see Fig. 3B). These findings correlate with previously published data. A Turkish study carried out on a small sample of 10 *P. aeruginosa* strains demonstrated that the efficacy of "Complex pyobacteriophage" and "Intesti bacteriophage" was 90 and 80%, respectively [19].

Similar to their effect on *K. pneumoniae*, the tested products caused lysis of almost all *P. aeruginosa* strains included in our collection (39 of 40; 97.5%). MDR strains represented by the types ST235, ST357 and ST654 were successfully lysed by the majority of the tested phage preparations.

Monovalent bacteriophage products demonstrated 86% efficacy against *S. aureus* ("Staphylococcal bacteriophage", Fig. 3C). High efficacy of the phage product was earlier reported by other researchers. For example, the efficacy of the phage vB_SauM-fRuSau02 isolated from this commercial product was previously evaluated against 135 staph strains, including 30 strains of coagulase-negative staphylococci [20]. Notably, *S. aureus* strains used in the study had different origins: 51 strains were isolated from humans, whereas 54 strains, from pigs. The efficacy of the phage vB_SauM-fRuSau02 was very high (96%) against *S. aureus* isolated from humans. In turn, the efficacy of this phage against coagulase-negative staphylococci species and *S. aureus* strains isolated from animals was lower (50 and 33%, respectively) [20]. Another study investigated the efficacy of the commercial phage product "Stafal phage" (Bohemia Pharmaceuticals; Czech Republic). The study revealed that bacteriophages isolated from this preparation effectively killed 83% of MRSA and 99% of MSSA (methicillin susceptible Staphylococcus aureus) [21].

In our study, all MRSA, as well as MDR strains, were sensitive to "Staphylococcal bacteriophage" (batch number N33). One more MRSA strain from the MDR group was sensitive to another batch of this commercial product (P332). This strain was represented by the spa-type t127.

The efficacy of the phage monoisolate vB_SauP-436-3w against the strains included in our collection was lower (30 of 50; 60%) than the efficacy of the commercial product "Staphylococcal bacteriophage" (43 of 50; 86%), but still significantly higher than the efficacy of the phage vB_Kp_M_Seu621, which effectively killed *K. pneumoniae*. This can be explained by the fact that receptors for staphylophages are represented by teichoic acids of bacterial cells [22], whose variability is much lower than that of gram-negative bacteria capsules.

The efficacy of all tested commercial phage products against *E. faecium* was poor (1 of 24; 4.2%). The only strain sensitive to the tested phages was represented by the type ST-17. Bacteriophages that exert activity against this species are listed as ingredients of commercial phage products, which are claimed to have a broad activity spectrum. Monovalent lytic phage products against *E. faecium* are not available on the Russian market.

A possible correlation between a bacterial strain's resistance to a phage and its resistance to antibacterial agents might have serious clinical implications. Another important finding would be a correlation between the resistance of a bacterial strain to a phage and the clonal complex the bacterium belonged to. In this study, we conducted a search for such correlations. We established that phage products induced lysis of both susceptible and sensitive (in terms of antibiotic resistance) bacteria. This is a crucial factor in deciding whether phages can be used as a clinical alternative to antibiotics. We established no correlations between the sensitivity of bacterial strains to the tested phages and their sensitivity to antibacterial agents ($p > 0.05$). We also found that bacterial strains representing one sequence type could be sensitive or resistant to a phage. This was true for all tested bacterial species. Thus, there was no clear correlation between the type of interaction between a phage and a bacterial cell, and a bacterial MLST sequence type ($p > 0.05$).

CONCLUSION

We found that strains included in our collection belonged to different genetic groups and have increased resistance to antimicrobial drugs, which makes them suitable for investigating the efficacy of commercial phage products. Commercial phage products available on the Russian market are highly effective against such ESKAPE pathogens as *P. aeruginosa* and *S. aureus*. However, not all tested phage products were equally effective against *K. pneumoniae*. Phage cocktails should be preferred to monovalent phages in the therapy of infections caused by gram-negative microorganisms, including *K. pneumoniae*.

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IMMUNITY TO COVID-19 AND ISSUES OF SCREENING FOR SARS-COV-2 ANTIBODIES

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This paper briefly presents the known data on the immune response to SARS-CoV-2, and also analyzes the possibilities and limitations of serological testing for antibodies that should be accounted for when planning population studies and interpreting their results.

Keywords: COVID-19, SARS-CoV-2, immunity, antibodies, screening

Author contribution: Mayanskiy NA — literature analysis, text authoring.

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ИММУНИТЕТ К COVID-19 И ВОПРОСЫ ПРОВЕДЕНИЯ СКРИНИНГОВЫХ ИССЛЕДОВАНИЙ АНТИТЕЛ К SARS-COV-2

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В работе кратко представлены известные данные об иммунном ответе на SARS-CoV-2, а также проанализированы возможности и ограничения серологического тестирования на антивирусные антитела, которые следует учитывать при планировании популяционных исследований и интерпретации их результатов.

Ключевые слова: COVID-19, SARS-CoV-2, иммунитет, антитела, скрининг

Вклад авторов: Н. А. Маянский — анализ литературы и написание текста.

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The evolving COVID-19 pandemic caused by the SARS-CoV-2 coronavirus is unprecedented in modern history. SARS-CoV-2 quickly spread throughout the world, affecting over 5 million and causing death of more than 300 thousand people (WHO data as of May 25, 2020) [1]. It dramatically changed the way of life in many countries, threatening further economic shocks. The reaction of scientific community to the pandemic is also distinguished with speed and scope. As soon as they receive papers from authors, world leading journals publish the latest information about the pathogen and its effect on the body, approaches to treatment and principles of curbing the spread of the virus. Such papers allowed developing the tools to detect SARS-CoV-2 RNA and test for its antibodies within the shortest time. This paper briefly overviews the known data on the immune response to SARS-CoV-2, and also analyzes the possibilities and limitations of serological testing for antibodies that should be accounted for when planning population studies and interpreting their results.

SARS-CoV-2 infection immune response

The information accumulated to date allows a degree of certainty to the statement that the immune response to SARS-CoV-2 infection develops following a typical scenario. In most SARS-CoV-2 patients specific antibodies of various classes appear 1–2 weeks after manifestation of the symptoms [2]. One study reports 40-55% of patients admitted with COVID-19 developing antibodies by days 5 to 7, with their number reaching 100% by days 17 to 19 [3]. In these patients, the antibody titer increased 2–4 times throughout the observation period (up to 27 days from the onset of symptoms) with seroconversion, i.e. appearance of specific antibodies, in the background. Other researchers report the average of 10–15 days as the term of seroconversion in symptomatic SARS-CoV-2 cases [4]. Another important factor is that most COVID-19 patients, including those that had it in a mild form, develop specific functional antibodies capable of neutralizing the virus that make them effectively immune. Two weeks after the onset of symptoms, such antibodies were found in 94% of COVID-19 patients [5].

Cellular immunity to SARS-CoV-2 is developing in parallel with antibodies thereto. Within 2–4 weeks after infection, the body generates a pool of virus-specific T-lymphocytes [7, 8]. The hypothesis is that CD4- and CD8- T-lymphocytes will reliably protect their carriers from SARS-CoV-2 reinfection if they had no antibodies detected.

At this stage of development of the pandemic, the data available disallow conclusions about the term of persistence of SARS-CoV-2 immunity. The earlier research into seasonal coronaviruses, MERS-CoV and SARS-CoV-1, which are related to SARS-CoV-2, suggests certain ideas. SARS-CoV-1 patients had the IgG concentration remaining high for 4–5 months, then it was gradually decreasing over the course of 2–3 years, although after 2.5 years up to 90% of those who had SARS-CoV-1 retained neutralizing antibodies. The antibody response followed the like path in MERS-CoV patients: those who recovered retained the antibodies for up to 34 months [4, 6].

The SARS-CoV-2 reinfection potential question remains open. So far, no confirmed SARS-CoV-2 reinfection cases have been described. A study on primates showed that having been infected with SARS-CoV-2, the body cannot be reinfected with this virus [6]. There are also no SARS-CoV-1 and MERS-CoV reinfection cases described. However, the cases of reinfection with seasonal coronaviruses are quite common, with the conditions brought by them typically being mild acute

respiratory diseases. Here, reinfection may be associated with a rapid decline of protective immunity or contracting a new serovariation of the virus [4, 6].

COVID-19 seroepidemiology

So far, most COVID-19 seroepidemiology studies have only considered cohorts of patients admitted to the hospitals and patients with symptoms of the infection. It is possible that the bodies of individuals that had the symptoms manifesting minimally or not manifesting at all generate antibodies at a different rate and their post-infection immunity has different properties [9]. Various media outlets report the frequency of asymptomatic (or subclinical) seroconversion in various populations sharing a territory or an occupation, such reports being of varying degrees of reliability. The scientific community has produced a very small amount of publications covering this topic. A serological examination of Los Angeles residents that aimed to assess the cumulative incidence of COVID-19 revealed IgG and/or IgM antibodies in 35 (4.65%) of 863 participants [10], with 10 (29%) of the 35 reporting no symptoms of acute respiratory viral infections in the last two months, which could point to seroconversion as a result of asymptomatic SARS-CoV-2 infection. The possibility of subclinical seroconversion was demonstrated in a small study showing 3 (23%) of 13 patients and 11 (44%) of 25 employees of an outpatient dialysis center in the USA generating anti-SARS-CoV-2 IgM and/or IgG 21 days after contact with a COVID-19 patient. In this study, the majority of participants with antibodies (2 of 3 patients and 9 of 11 employees) had no COVID-19-like symptoms [11].

The problems of anti-SARS-CoV-2 serological testing

There is no doubt that serological tests for antibodies to SARS-CoV-2 can be extremely useful for diagnosing infection, studying population immunity, evaluating the response to vaccination etc. At the same time, such tests have specific limitations, which should be acknowledged on par with their advantages. A number of materials published in the respected scientific journals [6, 9, 12–14] recommend caution in the use of anti-SARS-CoV-2 serological tests, most of which are flawed.

Like any serological test, the anti-SARS-CoV-2 test will inevitably produce a certain percentage of erroneous (false-positive and false-negative) results. Obviously, their number will depend on the analytical capabilities of the test system, such as sensitivity (ability to detect antibody carriers; proportion of true-positive results) and specificity (selectivity of the test; proportion of true-negative results).

The sensitivity and specificity of 90–95% are often misleading; the figures are interpreted as a guaranteeing a low probability of error, 5–10%, respectively, which can be simply neglected. However, it should be remembered that the number of false results will vary depending on the prevalence of antibodies in a given population. This fact is often ignored, which leads to an inaccurate interpretation of the results. Below are some cases exemplifying the concern outlined above. Suppose there is a test system with a declared sensitivity and specificity of 95%. It is applied to 1) examine a population of former COVID-19 patients (the expected prevalence of antibodies is 90%); 2) examine a population not infected with COVID-19 and showing no symptoms of acute respiratory viral infections in the last 2 months (the expected prevalence of antibodies is 5%). The following formula allows assessing reliability of test result with the help of the positive prognostic value (PPV), i.e. the probability that a positive test result is true-positive [14]:

$$PPV = \frac{Sensitivity \times Prevalence}{Sensitivity \times Prevalence + (1 — Specificity) \times (1 — Prevalence)}.$$

For the first population (former COVID-19 patients) the PPV is 0.99, i.e., the probability that with a positive test result the individual actually has antibodies is 99%. However, the situation looks radically different in the second hypothetical population, for which the PPV value (with the same sensitivity and specificity of the test system) is only 50%. Thus, selectivity of the test system with a specificity of 95% is insufficient for a population with a low level of occurrence of antibodies; such a system produces false-positive results in half (!) of the cases.

Therefore, the degree of sensitivity and specificity of a test system should be evaluated depending on the characteristics of the population subject to examination, and interpretation of results of the tests should factor in the possible error. Simple calculations show that in order to have PPV > 80% in a population 5% of which has antibodies, the test system's specificity must be over 99%.

Currently, the market offers dozens of SARS-CoV-2 antibody test systems from various manufacturers. They are actively promoted, the campaigns' messages declaring accuracy and reliability of testing. Not all manufacturers openly report analytical characteristics of their products; probably, some of them simply have no knowledge of such since they did not set up the studies needed to acquire such information [9]. The use of such unreliable tests during a pandemic can be dangerous both for a specific person and for the population as a whole. The decisions about admission of medical personnel to work, making restrictive measures milder/stricter, as well as stigmatization of "people without antibodies" and, conversely, granting complete indulgence to individuals with SARS-CoV-2 antibodies down to giving them the so-called "immunological passports" based on a single study with uncertain level of confidence, can lead to serious consequences [9, 13].

In addition, the scale of serological testing should be reasonable. Testing the entire population is impractical and not necessary. For example, there are detailed guidelines issued by Rospotrebnadzor describing the procedure for organizing and conducting serological monitoring of the status of herd immunity to vaccine-controlled infections [15]. These guidelines describe indicator representative groups that should be tested, the frequency of their examination, etc. The SARS-CoV-2 antibodies screening activities should be clearly planned so that the results obtained in relatively small samples could allow reliable conclusions about herd immunity in the population as a whole and in individual high-risk (medical workers in particular) or vulnerable groups (elderly people, patients with chronic diseases, etc.).

In the view of the stated considerations, the gigantic scale of anti-SARS-CoV-2 population screening launched in May 2020 in Moscow, the effort involving 3 to 6 million people and costing 0.5–1 billion rubles [16], is bewildering, to say the least. The test system used for this effort has a specificity of 95.38%, which is clearly not enough for use in a population with a low prevalence of antibodies, as mentioned above. With a high degree of certainty, it can be said that Moscow residents, who have been in isolation for almost two months, constitute just such a population. Even if we assume that the real proportion of seropositive individuals is 10%, a test with the indicated specificity will give over 30% of false-positive results.

CONCLUSIONS

Mass-scale testing of individuals that did not have COVID-19 (i.e., populations with a low incidence of SARS-CoV-2

antibodies) can generate a large number of false-positive results, significantly exceeding the number of true-positive results. The sample size for such studies should be reasonably sufficient, and the data interpreted with the systems' analytical characteristics factored in. These characteristics should be publicly available and verifiable. Otherwise, the widespread use of imperfect serological tests can be a source of serious errors in the medical and managerial decisions made.

[1] This value is indicated in the decision of the Moscow Department of Health COVID-19 Clinical Committee of 12.05.2020 (not published officially). Other sources of data on the analytical characteristics of this test system could not be found.

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IgM AND IgG ANTIBODIES AGAINST SARS-COV-2 IN NEONATES BORN TO MOTHERS WITH COVID-19

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Immunity against the novel coronavirus infection in neonates born to mothers with PCR-confirmed COVID-19 is an understudied field of research. The aim of this study was to analyze the levels of IgM and IgG antibodies against SARS-CoV-2. The study was carried out in 20 mothers aged 19 to 39 years and 21 neonates (including a pair of twins). Babies born to mothers with elevated IgM and IgG against SARS-CoV-2 also had elevated IgG. There is a hypothesis that anti-SARS-CoV-2 IgM are not passed on to the child across the placenta. In all cases studied in this work, neonates were PCR-negative for the virus, which suggests the absence of vertical COVID-19 transmission. Further research is needed.

Keywords: novel coronavirus, COVID-19, SARS-CoV-2, transmission routes, neonate, mother, neonatal immunity, immunoglobulin, IgM, IgG, placenta

Author contribution: Semeshkin AA — data acquisition, blood collection for antibody tests in neonates, nasopharyngeal swab collection for PCR tests in neonates, analysis of the obtained data, manuscript preparation; Vechorko VI, Averkov OV — study planning, analysis of the obtained data; Silaev BV — study planning, blood collection for antibody tests in mothers, nasopharyngeal swab collection for PCR tests in mothers, data analysis, literature analysis, manuscript preparation; Levchuk NN — data acquisition, laboratory tests for IgM and IgG, analysis of the obtained data; Polikarpova SV — data acquisition, PCR tests in mothers and neonates, literature analysis, analysis of the obtained data.

Compliance with ethical standards: the study was approved by the Ethics Committee of Filatov City Clinical Hospital No. 15 (Protocol No. 5 dated May12, 2020). Informed consent was obtained from all study participants or their legal representatives.

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АНТИТЕЛА IgM И IgG К ВИРУСУ SARS-COV-2 У НОВОРОЖДЕННЫХ ОТ МАТЕРЕЙ С COVID-19

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Исследование иммунитета у новорожденных, родившихся от матерей с доказанной новой коронавирусной инфекцией COVD-19, — малоизученный в настоящее время вопрос. Целью работы было проанализировать уровень иммуноглобулинов IgM и IgG к вирусу SARS-CoV-2. Исследование проводили у 20 матерей в возрасте 19–39 лет и 21 новорожденного (родилась одна двойня). В случае обнаружения повышенного уровня иммуноглобулинов IgM, IgG к вирусу SARS-CoV-2 у матери у новорожденного выявляли повышенный уровень IgG. Имеется предположение, что иммуноглобулины IgM к вирусу SARS-CoV-2 не проникают через плаценту от матери к ребенку. Во всех наблюдениях исследование с помощью ПЦР у новорожденных показало отрицательный результат, таким образом, вертикального пути передачи COVID-19, по всей видимости, нет. Необходимы дальнейшие исследования.

Ключевые слова: новая коронавирусная инфекция, COVID-19, SARS-CoV-2, пути передачи инфекции, новорожденные, матери, иммунитет новорожденных, иммуноглобулины, IgM, IgG, плацента

Вклад авторов: А. А. Семешкин — сбор материала, забор крови для исследования IgM и IgG у новорожденных, взятие мазков для ПЦР-диагностики у новорожденных, анализ полученных данных, написание статьи; В. И. Вечорко, О. В. Аверков — планирование исследования, анализ полученных данных; Б. В. Силаев — планирование исследования, забор крови для исследования IgM и IgG у матерей, взятие мазков для ПЦР-диагностики у матерей, анализ полученных данных, поиск литературных источников, написание статьи; Н. Н. Левчук — сбор материала, выполнение лабораторных исследований IgM и IgG, анализ полученных данных; С. В. Поликарпова — сбор материала, выполнение ПЦР-диагностики у матерей и новорожденных, поиск литературных источников, анализ полученных данных.

Соблюдение этических стандартов: исследование одобрено этическим комитетом Городской клинической больницы № 15 имени О. М. Филатова (протокол № 5 от 12 мая 2020 г.) все участники исследования и их законные представители подписали добровольное информированное согласие на участие в исследовании.

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The ongoing COVID-19 pandemic has raised keen interest in studying the immunity to the novel coronavirus infection in neonates born to mothers with laboratory confirmed COVID-19. So far, there is only a paucity of international literature on this matter, based on few observations that require further in-depth analysis. Currently, vertical (mother-to-child) transmission of the virus is deemed unlikely [1]. A few cases of possible vertical transmission reported in China [2, 3] and Peru [4] are controversial. According to the interim guidance by WHO, testing for COVID-19 should be performed using reverse-transcriptase polymerase chain reaction (RT-PCR), which is a highly accurate and reliable diagnostic tool [5]. RT-PCR can detect the smallest amounts of viral RNA in a human biological specimen. Testing for anti-SARS-CoV-2 IgM and IgG antibodies is widely used in patients with COVID-19 and might be an alluring technique for studying the immunity of infants born to SARS-CoV-2-infected mothers. The aim of this paper was to study the immunity of neonates born to mothers with PCR-confirmed COVID-19. The presence of IgG antibodies against SARS-CoV-2 in newborns suggests the innate immune response to the virus and might serve as a prognostic marker.

METHODS

The study was carried out in a group of pregnant females (n = 20) aged 19 to 39 years admitted to the maternity unit of Filatov City Clinical Hospital No. 15 between May 1, 2020 and May 20, 2020. On admission, all females underwent PCR-based tests for COVID-19. Additionally, all neonates (n = 21,

including a pair of twins) were also tested for COVID-19 on the first or second day after birth. The following inclusion criteria were applied: COVID-19 diagnosed on admission, with mild or moderate clinical symptoms. The severity of the disease was assessed following the temporary COVID-19 guidelines of the Russian Ministry of Healthcare [6]. Females with acute respiratory infection other than COVID-19 and a negative SARS-CoV-2 test were excluded from the study.

Laboratory (molecular) tests for the novel coronavirus infection were carried out following the temporary COVID-19 guidelines of the Russian Ministry of Healthcare [6].

Biological samples were collected for subsequent PCR as recommended in COVID-19 guidelines [7]. The etiologic diagnosis of COVID-19 is based on establishing the fact of infection with SARS-CoV-2 from the presence of SARS-CoV-2 RNA in the collected specimen by means of nucleic acid amplification. In our laboratory, we use AmpliTest SARS-CoV-2 kits designed at the Center of Strategic Planning and Management of Medical and Biological Health Risks (FMBA; Russia).

IgM and IgG antibodies against SARS-CoV-2 were measured in the serum of mothers and neonates on the first/second day after birth. Collection, shipment and storage of the samples were performed following the manufacturer's instructions.

The samples were analyzed on a CL 6000i Chemiluminescence Immunoassay Analyzer (Shenzhen Mindray Bio-Medical Electronics Co.; China).

Levels of IgG and IgM antibodies against SARS-CoV-2 (SARS-CoV-2-IgG, SARS-CoV-2-IgM) were measured using an immunochemiluminescent technique (the claimed sensitivity and specificity of the assay were 97.8 and 97.9%, respectively). The reference ranges for IgM and IgG titers were 0.00–0.999 OSE and 0.00–9.90 un/ml, respectively.

Sixteen deliveries were vaginal. Cesarian section by the conventional technique was performed in 4 cases. Considering the epidemiological situation, all patients were wearing face coverings and the medical personnel were wearing protective gowns and FFP2-3 face masks.

RESULTS

Newborns were isolated from their mothers immediately after birth. All babies scored 8–9 points on the Apgar scale at 1 minute after birth and 9–10 points at 5 minutes after birth. All neonatal nasal and nasopharyngeal swabs collected on day 1 or 2 after birth were PCR-negative for SARS-CoV-2. The collected specimens were also tested for the presence of anti-SARS-CoV-2 IgM and IgG antibodies.

Antibodies against SARS-CoV-2 were detected in all neonatal samples. Both IgM and IgG were elevated in one case. IgM and IgG were within the reference range in 4 neonates; similar results were observed in their mothers. Only IgG was elevated in 14 newborns; their mothers had high titers of both IgM and IgG outside the reference range (Table 1).

No RNA fragments of SARS-CoV-19 were detected in any of the neonates born to mothers with confirmed COVID-19. However, all neonates had virus-specific IgG antibodies in their serum.

IgG was elevated in 16 neonates but only in those cases when maternal IgG levels were also elevated (n = 16).

DISCUSSION

The obtained data allows us to hypothesize that IgG antibodies are passively carried to the fetus across the placenta by the maternal blood flow at the end of the third trimester and reach their peak level by the time of delivery. Some authors report that due to high molecular weight, IgM antibodies, which were detected in only one neonate in our study, are not passed on from the mother to the child [1–4]. It might be possible, though, that IgM is produced by the baby itself if the virus

Table 1. Levels of IgM and IgG antibodies against SARS-CoV-2 in mothers and their children

Case	IgM		IgG	
	Mother	Infant	Mother	Infant
1	2.51	0.15	0.40	0.49
2	8.84	0.30	34.76	11.24
3	9.29	0.43	136.02	109.84
4	0.96	0.26	0.53	0.97
5	1.19	0.24	17.54	15.01
6	0.48	0.24	0.32	0.70
7	2.01	0.24	24.81	9.69
8	1.19	0.18	92.40	42.12
9	6.70	0.26	116.04	24.41
10	6.80	0.14	66.32	89.12
11	0.68	0.25	1.54	1.20
12	0.31	0.21	0.32	0.58
13	14.43	0.15	38.86	23.9
14	2.85	1 infant 0.14 2 infants 0.16	47.77	1 infant 8.44 2 infants 6.83
15	1.26	0.62	39.30	12.67
16	2.52	0.95	6.52	2.11
17	0.41	0.12	103.02	38.64
18	1.94	0.41	19.86	12.54
19	2.48	0.94	6.34	1.15
20	10.08	8.54	24.12	16.87

has already crossed the placenta. Our findings are consistent with the findings of our Chinese colleagues who worked with a smaller sample size [8–10]. We did not perform any tests on the placenta; therefore, the proposition above is merely a hypothesis. Our study has a few limitations. Our sample size was small and we did not test the amniotic fluid and breast milk for the presence of the virus and the antibodies. Nevertheless, our findings might be helpful in better understanding the serologic features of neonates born to SARS-CoV-2-infected mothers.

CONCLUSION

1. Elevated IgG levels are detected on the first or second day after birth in the blood of neonates born to mothers with PCR-confirmed COVID-19. 2. Elevated virus-specific IgG might indicate the presence of innate immunity to the novel coronavirus. 3. Unelevated virus-specific IgM in neonates born to mothers with PCR-confirmed COVID-19 could be explained by the fact that due to high molecular weight, maternal IgM cannot be carried across the placenta.

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MICROCEPHALY-CAPILLARY MALFORMATION SYNDROME

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Microcephaly-capillary malformation syndrome (MICCAP: OMIM 614261) is a severe monogenic disorder inherited in an autosomal recessive manner caused by mutations in the *STAMBP* gene. There are less than 20 published cases of the syndrome to date. The paper reports three new cases of rare MICCAP syndrome. The cause of the disorder was confirmed in three affected individuals from two unrelated families by pedigree analysis, biochemical analysis, RFLP analysis and automated Sanger sequencing. The two brothers were homozygous for the potentially pathogenic *STAMBP* gene variant c.188A>G (p.Tyr63Cys). Clinical phenotype of the girl from the second family resulted from the combination of two genetic disorders: galactosemia caused by the compound heterozygosity for the pathogenic *GALT* gene variants (c.563A>G and c.855G>T), and MICCAP caused by the *STAMBP* gene variants (c.204-5C>G and c.668_669delCA), one of which originated *de novo*. The prevalence of microcephaly-capillary malformation syndrome in Russia is evaluated, it is one per 120,000 people (CI: 1/356 724–1/62 691). The carrier frequency is one per 173 people. The target *STAMBP* gene analysis makes the genetic confirmation of the MICCAP syndrome quicklier. When determining the tactics of diagnosis and therapy in each particular case, the possibility of combination of two rare genetic disorders in one patient should be considered.

Keywords: microcephaly, hemangioma, capillary malformation, STAMBP, galactosemia, GALT

Author contribution: Shchagina OA — study design, molecular genetic analysis, frequency estimation, statistical analysis; Semenova NA, Bessonova LA — clinical examination and genetic counseling of the patients' families; Larshina EA — biochemical assays, GALT gene analysis; Beskorovainiy NS — exome sequencing data processing; Zakharova EYu — biochemical analysis, prevalence calculation; Ryzhkova OP — pathogenicity analysis of genetic variants, exome sequencing; Poliakov AV — selection of primers for molecular genetic analysis.

Compliance with ethical standards: the study was approved by the Ethics Committee of the Research Centre for Medical Genetics (protocol № 5/8 dated November 12, 2018). The informed consent to molecular genetic testing, and anonymity-preserving clinical and molecular genetics data publishing (including photos and videos) was submitted by all participants or their legal representatives.

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СИНДРОМ МИКРОЦЕФАЛИИ В СОЧЕТАНИИ С КАПИЛЛЯРНЫМИ МАЛЬФОРМАЦИЯМИ

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Синдром микроцефалии в сочетании с капиллярными мальформациями (MICCAP) (OMIM #614261) — тяжелое моногенное заболевание с аутосомно-рецессивным типом наследования, причиной которого являются мутации гена *STAMBP*. На сегодняшний день в мировой литературе описано менее 20 случаев данного синдрома. В работе представлены три новых случая редкого синдрома MICCAP: на основании клинико-генеалогического анализа, методов биохимического исследования, анализа полиморфизма длин рестрикционных фрагментов, прямого автоматического секвенирования по Сенгеру установлена причина болезни у трех больных из двух неродственных семей. У двух братьев выявлен вероятно-патогенный вариант гена *STAMBP* c.188A>G (p.Tyr63Cys) в гомозиготном состоянии. У девочки из второй семьи причиной клинического фенотипа явилось сочетание двух наследственных заболеваний: галактоземии, обусловленной патогенными вариантами c.563A>G и c.855G>T гена *GALT* в компаунд-гетерозиготном состоянии и MICCAP, обусловленного вариантами c.204-5C>G и c.668_669delCA гена *STAMBP*, один из которых возник *de novo*. Оценены частота синдрома микроцефалии в сочетании с капиллярными мальформациями в России — один случай на 120 000 человек (ДИ: 1/356 724–1/62 691) и частота носительства данного синдрома — один случай на 173 человека. Исследование гена *STAMBP* позволяет быстро найти молекулярно-генетическую причину синдрома MICCAP. При выборе тактики диагностики и терапии в каждом конкретном случае необходимо учитывать возможность сочетания у одного больного двух редких наследственных патологий.

Ключевые слова: микроцефалия, гемангиома, капиллярная мальформация, STAMBP, галактоземия, GALT

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Соблюдение этических стандартов: исследование одобрено этическим комитетом Медико-генетического научного центра имени Н. П. Бочкова (протокол номер 5/8 от 12 ноября 2018 г.). Все пациенты или их законные представители подписали добровольное информированное согласие на проведение молекулярно-генетических исследований, публикацию клинических, молекулярно-генетических данных, включая фото- и видеоматериалы, с сохранением анонимности.

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Microcephaly-capillary malformation syndrome (MICCAP: OMIM 614261) is a severe monogenic disorder inherited in an autosomal recessive manner. The patients have progressive microcephaly, deep mental retardation, early-onset drug-resistant epilepsy and cutaneous capillary malformations due to abnormal capillary formation. Moreover, facial abnormalities, hypoplasia of distal phalanges and congenital heart defects are typical for a number of patients [1, 2].

The syndrome is associated with the *STAMBP* gene mutations The STAMBP protein is involved in the regulation

of the ubiquitinated proteins' endosomal sorting, as well as in the trafficking them from endosomes to lysosomes. Endosomal sorting is a highly dynamic process essential for protein homeostasis regulation via receptor-mediated signal transduction active regulation and autophagy [3]. Impaired endosomal sorting leads to intracellular accumulation of ubiquitinated proteins [4, 5]. Aggregated proteins cause the brain cells damage [6]. Meanwhile, the elevated level of GTP linked Ras protein (active form of Ras) has been revealed in the cell lines of patients affected with the *STAMBP* gene mutations, which indicates the involvement of the STAMBP protein in the Ras-MAPK signal transduction pathway [1]. It is known that capillary malformations are characteristic for various RASopathies since the mutations in the genes involved in the pathway are responsible for the congenital capillary formation abnormalities [7].

Currently there are less than 20 patients known to have MICCAP syndrome. Various types of the *STAMBP* gene mutations in the homozygous or compound heterozygous state have been identified in all patients [1, 8, 9]. Two patients with MICCAP syndrome have been reported in Russia: a girl with compound heterozygosity for the c.204-5C>G and c.273delA variants [10], and a boy with homozygosity for the c.188A>G (p.Tyr63Cys) variant of the *STAMBP* gene [11].

The study was aimed to present the results of clinical examination and molecular genetic testing of three patients with MICCAP syndrome belonging to two unrelated families.

Clinical cases

Samples of DNA of the patients from two unrelated families (ST1 and ST5) and their relatives who referred to the Research Centre for Medical Genetics for consultation were analysed.

The analysis of the *STAMBP* gene (NM_201647.3) was carried out by automated Sanger sequencing with primers flanking each of the exons of the gene. For the proband of the ST5 family, the galactose-1-phosphate uridyl transferase activity was evaluated. The search for frequent mutations c.563A>G (p.Gln188Arg), c.855G>T (p.Lys285Asn) and c.940A>G (p.N314D) polymorphic Duarte variant in the *GALT* gene (MIM 606999; RefSeq: NM_000155.3) was performed by RFLP analysis.

The results of sequencing 1036 exomes (2072 chromosomes) of the unrelated patiens living in Russia affected with various genetic disorders other than MICCAP were used as the control. The sequencing was performed with the IlluminaNextSeq 500 Sequencing System (Illumina; USA) using the IlluminaTruSeq® ExomeKit (Illumina; USA), and IDT xGen® (IDT; USA) for sample preparation.

Statistical analysis was carried out using the MS Excel 2016 application (Microsoft Corporation; USA).

Family ST1. The proband was an affected boy ST1.1, born from the first pregnancy complicated by the lingering ARVI during the 1st trimester and the threatened miscarriage since the beginning of the 2nd trimester. Intrauterine growth retardation was diagnosed at 20 weeks of gestation. The delivery occurred at 38–39 weeks of gestation. The birth weight was 2540 g, the birth length was 47 cm, the head circumference was 32 cm, and the Apgar score was 8/9. The boy developed normally until 3-months old. At the age of 3 months the parents noted the first convulsive seizure (myoclonus) followed by rapid psychomotor regression.

MRI at the age of 4 months revealed the diffuse cortical and subcortical atrophy of the cerebral hemispheres. The sleep video-EEG monitoring revealed generalized, high-amplitude, polymorphic slow-wave hypersynchronous activity with spike-and-wave complexes. The predominance of spike-wave activity in the parieto-occipital regions of brain hemispheres throughout the time of investigation together with the recurrent pattern of hypsarrhythmia amplitude decrease were registered.

On admission, at the age of 1 year 7 months the boy was completely immobile, with vision and hearing loss, and he was fed via gavage. The seizures' duration and frequency dramatically increased. The anticonvulsant therapy had completely no effect. The boy's height was 81 cm (50th percentile), the weight was 9.4 kg (below the 3rd percentile), and the head circumference was 43 cm (below the 3rd percentile). The patient had severe protein-energy undernutrition, microcephaly and mild positional skull deformity, multiple cutaneous hemangiomas over the buttocks, lumbar region, right thigh and back, moderate hypertrichosis over the forehead, bushy eyebrows and lashes (Fig. 1).

The child died at the age of 2.5 of the respiratory infection septic complication.

The proband's sibling was the affected boy ST1.4 born from the second pregnancy. Ultrasonography at 28–30 weeks of gestation revealed mismatch between the fetal head circumference and the gestational age. The boy was born in time, the birth weight was 3100 g, the birth length was 51 cm, and the head circumference was 32 cm. At the age of 2 months the parents noted the first convulsive seizure. After that, multiple seizures occurred daily. The paroxysmal events consisted of sudden freezing and the upward rolling of the eyes. Until 2 months old the patient's development was age-appropriate. The bottle-fed infant experienced swallowing difficulties with frequent choking on. The parents complained about frequent squeaks, agitation with head bouncing associated with the increase in blood pressure up to 130/70 mm Hg, myoclonus of limbs even when falling asleep and sometimes during sleep, frequent sudden freezing together with gaze freezing, occasional salaam seizures, lack of eye contact. The patient received valproic acid and vigabatrin as an anticonvulsant therapy, against which the moderate positive changes were observed. The infant was examined by neurogenetics specialist at the age of 5 months. Phenotype: height 63 cm (50–75th percentile), weight 6820 g (50th percentile), head circumference 37 cm (below the 3rd percentile), microcephaly, anterior fontanelle sized 2.5 × 2.5 cm, multiple cutaneous hemangiomas 0.5–2 cm in diameter. On admission, the infant failed to maintain gaze, fix eyes on objects and hold his head. The muscle tone was diffuse and moderately decreased, the reflexes were brisk. During the examination, paroxysmal events were repeatedly observed consisting of sudden freezing and upward rolling of the eyes. In addition, the infant's pronounced agitation, squeaks, myoclonus of limbs even during sleep and episodes of sudden freezing were noticeable. The continuous roving eye movements together with elements of horizontal and rotational nystagmus were noted. The tongue in the oral cavity was tense and raised up. The oral automatism reflexes were brisk. The tendon reflexes were brisk, with ankle clonus. The pronounced startle reflex was observed when touched. The patient demonstrated a developmental delay in his psycho-emotional sphere.

Based on the clinical picture characterized by specific phenotypic manifestations (combination of microcephaly and capillary abnormalities), and the existence of two affected siblings, the patient was diagnosed with rare autosomal recessive disorder, the MICCAP syndrome. Since in all described patients the disease was caused by the *STAMBP* gene mutations, a search for mutations in that gene was carried out by automated Sanger sequencing.

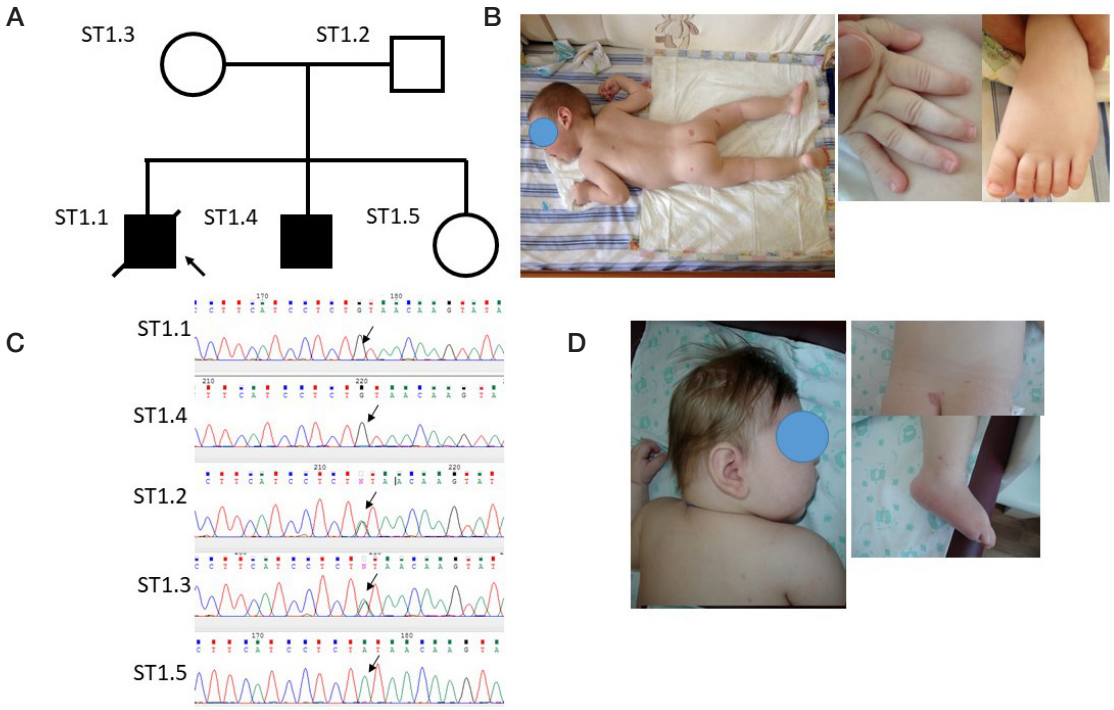

Fig. 1. Phenotype, pedigree and sequencing results for the ST1 family members. **A**. ST1 family pedigree. **B**. General appearance of patient ST1.1 at the age of 2. **C**. General appearance of patient ST1.4 at the age of 5. **D**. Sanger sequencing chromatograms for patient ST1.1, patient ST1.4, father (ST1.2), mother (ST1.3) and healthy sibling ST1.5 (proband is denoted by the *arrow*)

As a result, a *STAMBP* gene variant NM_201647.3: c.188A>G (p.Tyr63Cys) in the homozygous state previously described twice in patients with MICCAP syndrome was identified [1, 11]. Both parents turned out to be heterozygous carriers of the variant. According to the gnomAD database [12], the described gene allele frequency was 0.0000637. In the control group of exomes of Russian patients with various genetic disorders the described variant was detected in one chromosome of 2072, i. e. the frequency was 0.00048 (male patient born in 1973 diagnosed with hemophilia A having a pathogenic variant in the *F8* gene). In Russia, the variant in the homozygous state was also reported in patient with MICCAP syndrome [11]. Among all patients with that syndrome known in Russia, the variant c.188A>G was identified in four independent chromosomes of eight. Thus, the prevalence of the variant in affected individuals was significantly higher than in the control group. The odds ratio (OR) calculated during the case-control study was 2071 (95% CI: 187–22849).

The *STAMBP* gene has a low level of benign missense variants, and the missense mutations are a common cause of MICCAP syndrome. Thus, in accordance with the ACMG criteria, the discribed variant has been recognized as likely pathogenic (PS4, PM2, PP2, PP3, PP5) [13].

Two years after the birth of a second child the third pregnancy occurred in the family. The parents applied for prenatal diagnosis of the fetus at 11–12 weeks of gestation. No changes in the STAMBP gene nucleotide sequence were identified. The pregnancy ended in the birth of healthy girl.

Family ST5. The ST5 family applied to refine the daughter's diagnosis. The 3-week old girl was the fourth child born wihin exogamous marriage of healthy parents. The family included other three children (healthy boy and girl, and a son with cerebral palsy, a pre-term baby born at 31 weeks of gestation). The maternal half-sibling (brother) was healthy. The mother had medical abortion and ectopic pregnancy in her history. The pregnancy was complicated by anemia, rhinopharyngitis and influenza vaccination at 35 weeks of gestation. The delivery occurred in time. The infant's birth weight was 2910 g (10th percentile), the birth length was 51 cm (50th percentile), the head circumference was 30 cm (below the 3rd percentile), and the Apgar score was 8/8 (Fig. 2).

From birth, multiple flat roundish cutaneous hemangiomas not raised above the skin surface were visible on the trunk and limbs. After delivery, the infant was in moderately grave condition due to central nervous system depression syndrome. It was transferred to the intensive care unit for observation. There was no need in the respiratory support. On the 5th day, the hyperbilirubinemia increase was registered, as well as cholestasis and cytosis (transaminase concentration increased five times to normal; the peak level of aspartate aminotransferase (AST) was 229 U/l, the level of alanine aminotransferase (ALT) reached 143 U/l). On the 10th day of life the patient required a plasma transfusion due to coagulopathy. A complete blood count revealed normal hemoglobin level and normal platelets amount. Neurosonography on the 6th day of life revealed echo-signs of subependymal pseudocyst on the left side. The heart ultrasound revealed no heart defects, the atrial septal aneurysm together with functioning foramen ovale were noted. The neonatal screening center reported increased galactose level (the total galactose level remained unknown). The activity of galactose-1-phosphate uridylyltransferase (GALT) was analyzed. A significant decrease in enzyme activity to 0.63 U/gHb was detected (normal value 4.4–15 U/gHb), which made up 7% of normal value.

Phenotype: microcephalic skull, microgenia, long philtrum; generalized multiple roundish "port-wine" stained cutaneous hemangiomas on the belly, back, buttocks and limbs. Hemangiomas faded slightly when pressed.

The study of frequent mutations NM_000155.3 in the *GALT* gene was carried out using PCR-RFLP analysis. The frequent pathogenic variants c.563A>G (p.Gln188Arg) and c.855G>T (p.Lys285Asn) in heterozygous state were identified [14]. The examination of parents revealed the trans-position of the detected pathogenc variants (substitution c. 563A>G

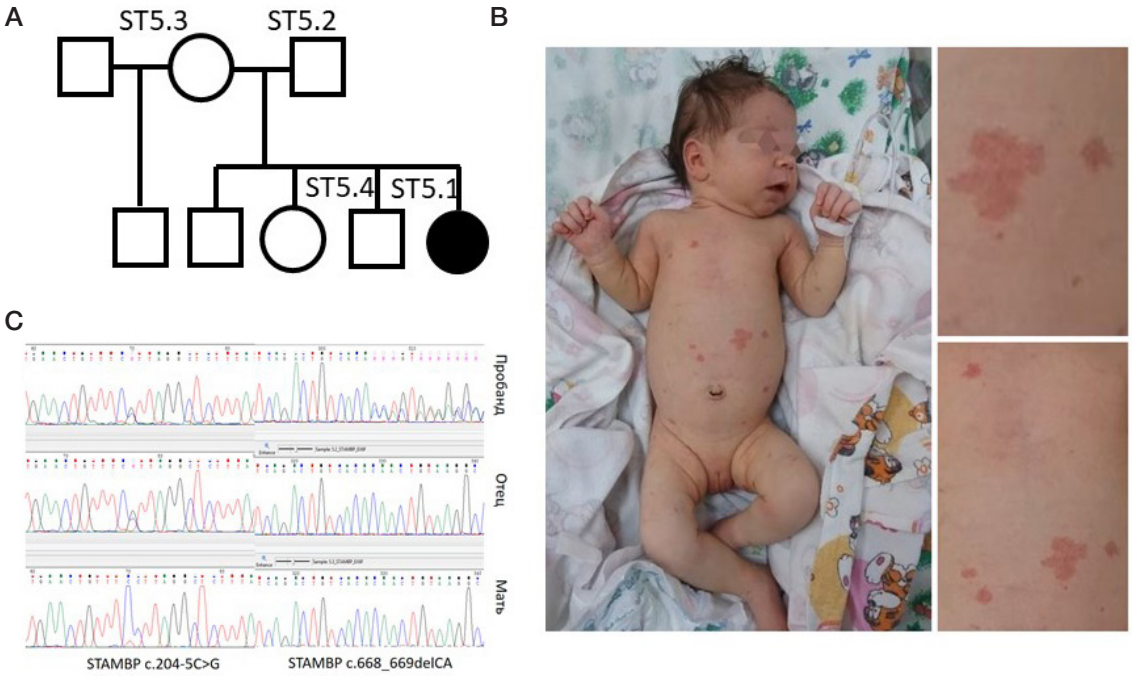

Fig. 2. Phenotype, pedigree and sequencing results for the ST5 family members. **A**. ST5 family pedigree. **B**. Patient's general appearance at the age of 3 weeks. **C**. Sanger sequencing chromatograms for proband, father and mother

(p.Gln188Arg) was inherited from mother, and c.855G>T (p.Lys285Asn) was inherited from father).

The study of *STAMBP* gene identified variants c.204-5C>G and c.668_669delCA in heterozygous state. The variant NM_201647.3:c.204-5C>G (rs746354315) was previously reported as likely pathogenic in Russian female patient with MICCAP syndrome, in compound heterozygous state with pathogenic variant c.273delA [10]. According to gene prediction programs, the described variant affected splicing. According to gnomAD database, the frequency of the variant was 0.00000399. In the control group of exomes of Russian patients with various genetic disorders the variant was identified in one chromosome of 2072, and the frequency of the variant was 0.00048 (in female patient born in 1980, being a mother of proband diagnosed with expressive language delay). Among all patients with MICCAP syndrome known in Russia, the variant c.204-5C>G was identified in two independent chromosomes of eight. Thus, the prevalence of the variant in affected individuals was significantly higher than in the control group. The odds ratio (OR) calculated during the case-control study was 690 (95% CI: 55–8672). According to the ACMG criteria, the variant c.204-5C>G was pathogenic (PS4, PM2, PM3, PP4, PP5) [13]. The variant NM_201647.3:c.668_669delCA was responsible for frameshift and formation of premature termination codon p.Thr223AsnfsTer6. The described deletion had not been reported previously in patients with MICCAP syndrome. The variant was not registered in the gnomAD database and in the control group of the Russian patients' exomes. The study of relatives revealed the variant c.204-5C>G in heterozygous state in patient's parents and sibling. The variant c.668_669delCA was not detected in proband's parents and sibling. Maternity and paternity were established using the microsatellite markers. According to the ACMG criteria, the variant was pathogenic (PVS1, PS2, PM2, PP4) [13].

The family decided not to pursue further the study in order to define the *cis-trans* position of variants at the RNA level due to the nontransportable infant's severe grave condition and lack of plans for further reproduction.

Heterozygous variants at exons and splicing sites of the *STAMBP* gene, the allele frequency of which was lower than 1% in the gnomAD database, were selected in 1036 exomes of Russian patients. The variants' pathogenicity was assessed in accordance with the ACMG criteria using the VarSome application [15] (Table 1).

The carrier frequency was estimated and the prevalence of MICCAP syndrome in the Russian Federation was calculated based on the frequencies of pathogenic (PAT) and likely pathogenic (LPAT) variants of the *STAMBP* gene (see Table 1, denoted with *bold*). In the exomes of residents of the Russian Federation the pathogenic and likely pathogenic *STAMBP* gene variants in heterozygous state were detected in 6 people of 1,036. According to data received, *STAMBP* gene mutations carrier frequency was one per 173 people (1/126–1/299). Thus, the prevalence of the disorder in the Russian Federation is likely to be one per 120,000 (CI: 1/356 724–1/62 691).

The phenotype of proband ST5.1 was caused by combination of two autosomal recessive genetic disorders: galactosemia and MICCAP syndrome. Severe grave condition during the neonatal period (cytosis, cholestasis, coagulopathy) was due to metabolic disorders associated with the *GALT* gene mutations. The *STAMBP* gene mutations were responsible for microcephaly, capillary malformations, and low birth weight. The galactosemia manifestations were adjusted in the first year of life. However, overlaying seizures and severe central nervous system damage due to MICCAP syndrome made the patient's prognosis unfavorable.

The main clinical data of patients are presented in Table 2. The clinical manifestations of the syndrome in all examined patients were quite typical: all patients had congenital microcephaly and multiple cutaneous capillary malformations which not shrinked but increased with the infants' growth. During infancy, epilepsy with polymorphic seizures debuted resistant to anticonvulsant therapy. All examined infants had profound psychomotor development delay and neurological deficit. One of the patients had hypoplastic phalanges of fingers and nails, and the siblings of the ST1 family had myoclonus of limbs and roving eye movements. Severe central nervous system damage is typical

Table 1. *STAMBP* gene variants found in the exomes of residents of the Russian Federation

Variant		Number of chromosomes with variants	Allele frequency (2072 chromosomes)	Pathogenicity
Position (GRCh37/hg19)	Effect			ACMG [13]
chr2-74058017-G-A	c.34G>A p.Glu12Lys	1	0.00048	VUS (PM1,PM2,PP2)
chr2-74058041-C-A	c.58C>A p.Gln20Lys	1	0.00048	VUS (PM1,PM2,PP2,BP4)
chr2-74058095-C-A	**c.112C>A p.Arg38Ser**	**1**	**0.00048**	**LP (PM1,PM2,PM5,PP2,PP3)**
chr2-74058143-G-T	**c.160G>T p.Gly54Cys**	**1**	**0.00048**	**LP (PM1,PM2,PP2,PP3)**
chr2-74058171-A-G	**c.188A>G, p.Tyr63Cys**	**1**	**0.00048**	**LP (PS4,PM2,PP2, PP3,PP5)**
chr2-74071935-C-G	**c.204-5C>G**	**1**	**0.00048**	**PAT (PS4,PM2,PM3,PP4,PP5)**
chr2-74072291-C-A	c.280-3C>A	1	0.00048	VUS (PM2,BP4)
hr2-74072359-C-A	c.345C>A p.Thr115Thr	1	0.00048	VUS (PM2,BP7)
chr2-74074592-C-T	**c.454C>T p.Gln152***	**2**	**0.00096**	**PAT (PVS1 PM2,PP3)**
chr2-74074722-C-T	c.584C>T p.Pro195Leu	1	0.00048	VUS (PM2,PP2,BP4)
chr2-74074796-G-A	c.658G>A p.Asp220Asn	2	0.00096	VUS (PM2,PP2,BP4)
chr2-74074815-G-A	c.677G>A p.Arg226Lys	1	0.00048	VUS (PM2,PP2,BP4)
chr2-74076511-G-A	c.764G>A p.Arg255His	5	0.0024	VUS (PP2,PP3,BP4)
chr2-74076532-G-T	c.785G>T p.Arg262Leu	1	0.00048	VUS (PM2,PP2,PP3)
chr2-74076576-C-A	c.829C>A p.Arg277Arg	1	0.00048	VUS (PM2,BP7)
chr2-74077551-G-A	c.916G>A p.Ala306Thr	1	0.00048	VUS (PM2,PP2,BP4)
chr2-74086389-C-A	c.1014C>A p.Pro338Pro	1	0.00048	VUS (PM2,BP7)

for MICCAP syndrome, the patients often die early due to septic complications (like the elder affected boy of the family ST1).

Discussion

The currently existing literature data make it possible to define the "core" of the syndrome: microcephaly, intractable neonatal seizures, profound psychomotor development delay and the unique manifestation, the randomly arranged multiple red spots which do not shrink with age. The examined patients had all the listed signs. According to literature data, the infants with MICCAP syndrome may have dysmorphic facial features: low anterior hairline, sloping forehead, epicanthus, ptosis, low-set ears, micrognathia, short nose. The examined patients also had some facial abnormalities: these were low anterior hairline in proband ST1.1, microgenia and long philtrum in patient ST5.1. Many authors report brachydactyly and nail hypoplasia. However, the digital features (shortening) of hands and feet were present only in the girl of the family ST5.1. On the contrary, myoclonus of limbs together with rowing eye movements were observed in patients of the famly ST1 and abscent in the family ST5.1 [7–11]. Thus, the presence and severity of phenotype features in various patients (except for microcephaly and spots on the skin) may be variable and probably depend on the genotype.

The combination of two genetic disorders in patient ST5.1 is a rare case. Such combination is particularly difficult in newborns, when there is a need for quick decision making in order to save the life and stabilize the patient. According to estimates, the prevalence of galactosemia in Russia in from 1 : 60,000 to 1 : 70,000 newborns, and the predicted MICCAP syndrome prevalence is even lower, 1 per 120,000 [16, 17]. The probability of two rare genetic disorders in one patient is extremely low. However, the reported case demonstrates that such combination may occur, and therefore should be considered when defining tactics of treatment, family planning and genetic counseling of families.

CONCLUSION

MICCAP syndrome is a rare genetic disorder. Currently less than 20 patients in the world have been reported to have MICCAP. Congenital microcephalies are the large heterogenous group of genetic disorders. However, the specific clinical symptom, the presence of multiple capillary malformations, makes it possible to suspect a particular disorder associated with mutations of the *STAMBP* gene. The study of the gene allows one to quickly find the molecular genetic cause of the disorder.

The reported case of galactosemia and MICCAP syndrome combination in one patient demonstrates that despite the low probability of two rare genetic disorders in one individual the described variant should be considered when defining the tactics of diagnosis and therapy for each particular family.

Table 2. Comparison of patients with MICCAP syndrome

Trait	ST1.1	ST1.4	ST5.1
STAMBP genotype	c.[188A>G]; [188A>G] (p.Tyr63Cys)	c.[188A>G]; [188A>G] (p.Tyr63Cys)	c.[204-5c>g(;) 668_669delCA]
Gender	м	м	f
Age of onset	3 months	2 months	6 months
Congenital microcephaly	+	+	+
Birth head circumference	< 4th percentile	< 4th percentile	< 3rd percentile
Head circumference upon admission	< 3rd percentile	< 3rd percentile	< 3rd percentile
Capillary malformations	+	+	+
Small for gestational age	+	-	+
Early-onset resistant seizures	+	+	+
Myoclonus	+	+	-
Hypoplastic phalanges	-	-	+
Profound developmental delay	+	+	+
Additional information	Died at the age of 2.5		Galactosemia *GALT* c.[563A>G]; [c.855G>T]

In one of the reported patients, the new nucleotide sequence variant was identified, which had not been described before in the literature and databases. That makes it possible to expand knowledge of the MICCAP syndrome allele heterogeneity. The provided information on genotypes and phenotypes of the affected individuals may be of interest to scientists studying clinical genetic correlations and STAMBP protein functions.

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DIAGNOSTIC SIGNIFICANCE OF *LACTOBACILLUS SPP*. IDENTIFICATION IN EJACULATE

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Popularization of the real-time polymerase chain reaction method (RT-PCR), which is a trend of the recent years, allowed to significantly expand of the range of microorganisms that can be detected in the genitourinary tract of men. Moreover, the available picture of the microbiome's bacterial component structure became more detailed. *Lactobacillus spp.* remains one of the least studied groups of microorganisms. Treating patients with reproductive disorders, the authors have accumulated clinical experience demonstrating the possible relationship between presence of *Lactobacillus spp.* in the ejaculate and changes in the level of sex hormones and the key values registered with a spermogram. This study aimed to compare the levels of luteinizing hormone, follicle-stimulating hormone, testosterone, estradiol, prolactin, progesterone, and sex hormone binding globulin (SHBG) in blood serum and changes in spermogram values in 210 men with and without *Lactobacillus spp.* detected in their ejaculate. The treatment group included 105 men whose ejaculate had *Lactobacillus spp.* in the amount of (Lg) ≥ 10^3, as detected by RT-PCR. The control group included 105 men whose ejaculate did not have *Lactobacillus spp.* detected; the microbiome's bacterial component structure of their ejaculate was normal. Compared to the control group, treatment group had hormonal disorders registered more often: abnormal levels of three or more hormones ($p = 0.04$), hyperestradiolemia ($p = 0.05$), increased level of SHBG ($p = 0.01$). It was established that the presence of *Lactobacillus spp.* in the ejaculate of treatment group participants is associated with oligoastenoteratozoospermia ($p < 0.01$), decreased concentration of spermatozoa ($p = 0.01$), their decreased motility ($p < 0.01$) morphology abnormalities ($p < 0.01$). Thus, the presence of *Lactobacillus spp.* in the ejaculate can be interpreted as an additional marker of hormonal imbalance and fertility dysfunction in men.

Keywords: *Lactobacillus spp.*, lactobacilli, ejaculate, male infertility, ejaculate microflora, Androflor, pathospermia, oligoastenoteratozoospermia, hyperestradiolemia, hyperprosterolemia

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Compliance with ethical standards: the study was approved by the Ivanovo State Medical Academy ethics committee and is a part of the earlier research (protocol № 5 of June 03, 2009). All patients signed a voluntary informed consent to participate in the study.

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ДИАГНОСТИЧЕСКАЯ ЗНАЧИМОСТЬ ВЫЯВЛЕНИЯ *LACTOBACILLUS SPP.* В ЭЯКУЛЯТЕ

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В последние годы благодаря внедрению метода полимеразной цепной реакции в реальном времени (ПЦР-РВ) значительно расширился спектр микроорганизмов, выявляемых в мочеполовом тракте мужчин, детализировано представление о структуре бактериальных компонентов микробиома. Одной из наименее изученных групп микроорганизмов остается *Lactobacillus spp*. При ведении пациентов с репродуктивными нарушениями авторами накоплен клинический опыт, демонстрирующий возможную взаимосвязь изменения уровня половых гормонов и основных показателей спермограммы с наличием *Lactobacillus spp*. в эякуляте. Целью работы было сравнить уровни лютеинизирующего гормона, фолликулостимулирующего гормона, тестостерона, эстрадиола, пролактина, прогестерона, а также глобулина, связывающего половые гормоны (ГСПГ), в сыворотке крови и изменения показателей спермограммы у 210 мужчин при выявлении в эякуляте *Lactobacillus spp*. и при их отсутствии. В основную группу были включены 105 мужчин, в эякуляте которых по данным ПЦР-РВ выявлены *Lactobacillus spp*. в количестве (Lg) ≥ 10^3. Контрольная группа включала 105 мужчин, у которых отсутствовали *Lactobacillus spp*. и структура бактериального компонента микробиома эякулята соответствовала норме. В основной группе по сравнению с контрольной у мужчин чаще встречались гормональные нарушения: отклонение от нормы уровней трех и более гормонов ($p = 0,04$), гиперэстрадиолемия ($p = 0,05$) и повышение уровня ГСПГ ($p = 0,01$). Установлено, что наличие *Lactobacillus spp*. в эякуляте мужчин основной группы ассоциировано с олигоастенотератозооспермией ($p < 0,01$), со снижением концентрации сперматозоидов ($p = 0,01$), с ухудшением их подвижности ($p < 0,01$) и нарушением морфологии ($p < 0,01$). Таким образом, присутствие в эякуляте *Lactobacillus spp*. может служить дополнительным маркером нарушения гормонального фона и фертильности у мужчин.

Ключевые слова: *Lactobacillus spp.,* лактобактерии, эякулят, мужское бесплодие, микрофлора эякулята, «Андрофлор», патоспермия, олигоастенотератозооспермия, гиперэстрадиолемия, гиперпрогестеронемия

Вклад авторов: Д. Г. Почерников — планирование исследования, анализ литературы, интерпретация и анализ данных, подготовка черновика рукописи, подготовка финального варианта статьи; Н. Т. Постовойтенко — анализ литературы, сбор, анализ и интерпретация данных, подготовка черновика рукописи, подготовка финального варианта статьи; В. В. Гетьман — сбор, анализ и интерпретация данных, подготовка черновика рукописи; И. С. Галкина — планирование исследования, интерпретация данных, подготовка черновика рукописи, подготовка финального варианта статьи.

Соблюдение этических стандартов: работа одобрена этическим комитетом Ивановской государственной медицинской академии и является фрагментом начатого ранее исследования (протокол № 5 от 03 июня 2009 г.). Все пациенты подписали добровольное информированное согласие на участие в исследовании.

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Currently, there is no consensus among the researchers as to what is the normal composition of men's urogenital tract microflora [1]. Clinical recommendations of the recent years suggest performing only the cultural analysis of the ejaculate of infertile men or preconception patients [2–4]. However, this method does not fully uncover the specifics of a man's urogenital microbiota: it does not allow identification of uncultivated microorganisms, e. g., obligate anaerobic and some facultative anaerobic ones, including lactobacilli [5, 7–10]. According to the leading European and Russian urologists, prostatitis diagnostics should include a two-glass test, ejaculate bacterial examination and additional RT PCR examination thereof [3, 5, 7–10]. In the recent years, popularization of the advanced diagnostic methods allowed to significantly expand the range of microorganisms detected in the urogenital tract of men and women [1, 8, 11–13]. In our opinion, one of the promising methods is RT-PCR performed with the Androflor testing kit for men; the method allows uncovering both the qualitative and the quantitative composition of the ejaculate microbiota, including lactobacilli [5, 7, 9, 10, 14–19].

Among the published papers, there are but a few publications addressing the occurrence of lactobacilli in various biotopes of the genitourinary tract of men. According to several researchers [16, 19, 20], *Lactobacillus spp*. is one of the most common genus of microorganisms found both in healthy men and those with urethritis or prostatitis; the bacteria are identified by the bacteriological method [21], 16S rRNA sequencing [6, 20] and RT-PCR [5, 8, 14, 16, 19]. Thus, *Lactobacillus spp.* bacteria were detected in 9–73.3% of samples of ejaculate of infertile men and those examined as part of a preconception course [5, 6, 8, 16]. One study reported registering a statistically significant correlation between the presence of lactobacilli in the urethra and hormonal disruptors in the seminal fluid of infertile men, which is especially interesting [19].

Some papers highlight the link between the presence of lactobacilli in the ejaculate and normal characteristics of the semen as registered by a spermogram. The research showed that normal sperm morphology can coexist with an increased relative content of *Lactobacillus spp*. in semen samples [22]. Moreover, there was detected a positive correlation between the presence of lactobacilli in semen and normal sperm characteristics [23].

The least studied to date are Lactobacillus spp. bacteria taken from men who observed the biomaterial donation rules, i.e. abstained sexually or used barrier contraception to reduce the risk of receiving lactobacilli from a vagina. According to a number of researchers, in most cases, *Lactobacillus spp*. are transient microflora of a man's genitourinary tract [7, 10, 14, 17]. Lactobacilli can play the part of a probable microbial agent that promotes emergence and persistence of a chronic inflammation of the prostate gland [24]. In the recent years, the role of hormonal changes, in particular, the effect of testosterone levels on bacterial contamination of the prostate gland secretion, has been discussed increasingly often [25, 26]. However, analyzing the literature available to us we failed to discover data pointing to the correlation between the key fertility hormones — estradiol, prolactin, progesterone, and SHBG, traditionally examined in men with reproductive disorders [2–4], — with infectious agents identified in prostate secretions or ejaculate.

Results of a pilot study translated into a patent for an invention we obtained [27]. The essence of the discovery is that the presence of *Lactobacillus spp*. in the ejaculate with the bacterial titer of (lg) ≥ 10^3 can be interpreted as an additional marker of hormonal disorders and thus call for further extended examination of the man. The subject of this research is extremely relevant since there is no data describing the effect high content of lactobacilli has on the men's sperm fertility. This study aimed to compare the levels of LH, FSH, testosterone, estradiol, prolactin, progesterone, SHBG in serum and the key sperm property indicators in men with *Lactobacillus spp*. detected and not.

METHODS

The comparative prospective study lasted from November 2016 to July 2019 and included 210 men that visited urological clinic of the Ivanovo State Medical Academy seeking treatment for infertility, preconception course and/or having a concomitant erectile dysfunction. The inclusion criteria were: male, reproductive age; infertility or preconception preparation course; no hormonal and antibacterial drugs, as well as any other medicines, taken within the last 4 weeks. The exclusion criteria were: hypogonadotropic and hypergonadotropic hypogonadism, diabetes mellitus, hypo- and hyperthyroidism; sexually transmitted infections and clinical manifestations of prostatitis, such as pain and dysuria; karyotype abnormalities, CFTR gene mutations, microdeletions in the AZF locus of Y chromosome.

All men had their ejaculate examined with the help of the Androflor testing kit (RT-PCR); their serum was examined to determine the levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), total testosterone, estradiol, prolactin, progesterone, and sex hormone binding globulins (SHBG), free androgen index (FAI), testosterone to estradiol ratio (TER), which are typically checked during examination of infertile men or men undergoing a preconception course [2, 3]. Blood samples for the hormone concentration study were taken in the morning, from 8 to 10 am; the patients had to abstain from eating any food before sample taking. They were also not

Table 1. Level of the hormones studied in the two compared groups

Hormone in serum hormone	Reference values	Group with *Lactobacillus spp.* (*n* = 105), mean ± STD	Group without *Lactobacillus spp* (*n* = 105), mean ± STD	*p*
LH, mIU/ml	1–12	5.1 ± 2.1	4.4 ± 2.0	0.09
FSH, mIU/ml	0.9–12	5.2 ± 2.5	4.5 ± 2.7	0.1
Prolactin, ng/ml	4–15	14.1 ± 7.8	12.8 ± 5.9	0.3
Progesterone, ng/ml	0.05–0.15	0.23 ± 0.14	0.25 ± 0.17	0.3
Estradiol, pg/ml	11–43	26.5 ± 13.5	23.4 ± 9.9	0.04
Testosterone ng/ml	3.5–9	5.3 ± 2.6	5.1 ± 2.5	0.2
SHBG, nmol/l	18–54	41.1 ± 23.2	35.8 ± 20.7	0.08
FAI, %	15–102	51.6 ± 23.3	51.3 ± 27.6	0.5
T/E_2 ratio	83 and above	232.8 ± 134.4	252 ± 157.1	0.2

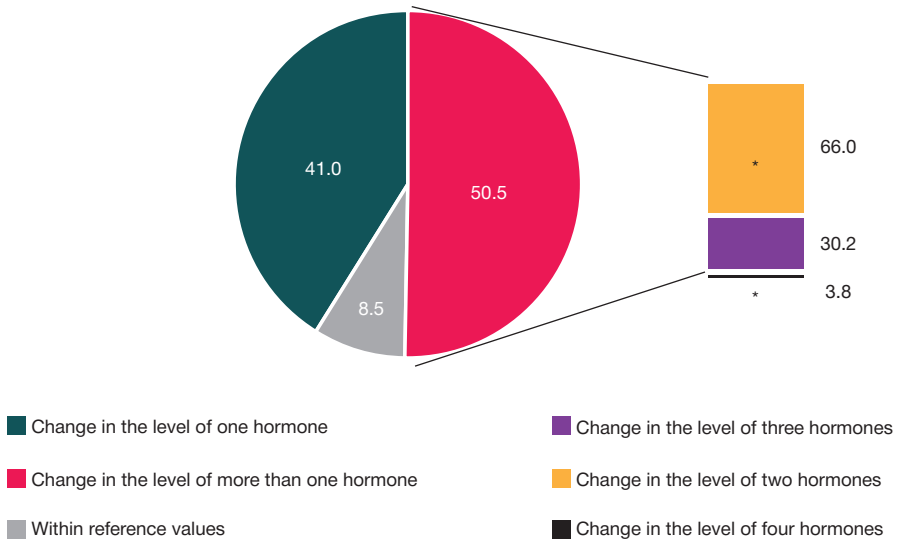

Fig. 1. Hormonal changes in the group with *Lactobacillus spp.*, *n* = 105

supposed to have sexual intercourses for 24 hours before the procedure.

All men were divided into two groups, randomized by age, body mass index, alcohol intake and smoking status, complaints, established diagnosis and serum testosterone level (in order to exclude its influence on the bacterial content of the ejaculate) [25, 26]. The treatment group included 105 men whose ejaculate had *Lactobacillus spp.*, titer of (Lg) ≥ 10^3, as detected by RT-PCR. The control group included 105 men whose ejaculate did not have Lactobacillus spp. detected; the microbiome's bacterial component structure of their ejaculate was normal, as registered with the Androflor testing kit. The average age of the treatment group patients was 35.5 ± 8.1 years, that of the control group patients 35.8 ± 8.3 ($p > 0.05$). Before biomaterial sample collection, all patients urinated, thoroughly cleaned their external genitalia (without antiseptics), and masturbated to deliver the ejaculate samples into sterile polymer containers. The containers were delivered to the laboratory within one hour from collection or less.

For RT-PCR examination, we used the DT-96 detection amplifier (DNK-Tekhnologiya; Russia) [28] and the Androflor testing kit (medical product registration certificate RZN 2016/4490 of 07.25.2016). For the hormone concentration study we took 5 ml of venous blood from each participant. The samples were collected from 8 am to 10 am under aseptic conditions, the blood put into 5 ml tubes. After coagulation, the liquid part was transferred to clean sterile tubes, centrifuged in a laboratory centrifuge for 10 min at 1500 rpm, then the supernatant was transferred to disposable plastic Eppendorf tubes. Hormone concentration was determined with the help of Roche Cobas e8000 602 analytical system (Roche Diagnostics; Sweden). Table 1 contains reference levels of the hormones studied. Ejaculate samples were studied with the help of the SQA-V analyzer (Medical Electronic System Ltd.; Israel). The amount of leukocytes in semen was determined by staining smears with Leukodif 200 dyes (Erba Lachema; Czech Republic); assessing the quantitative and qualitative indicators, we relied on the normal values approved by WHO (2010) [29]. Microsoft Excel 2013 and Statistica 12.0 (Stat Soft Inc.; USA) software packages enabled statistical analysis. Wilcoxon and Fisher tests were applied to determine reliability of the data obtained; the differences were considered significant at $p \leq 0.05$.

RESULTS

Nine (8.5%) patients of the treatment group (with *Lactobacillus spp.* detected) had the levels of all studied hormones corresponding to the reference values. In the control group, the

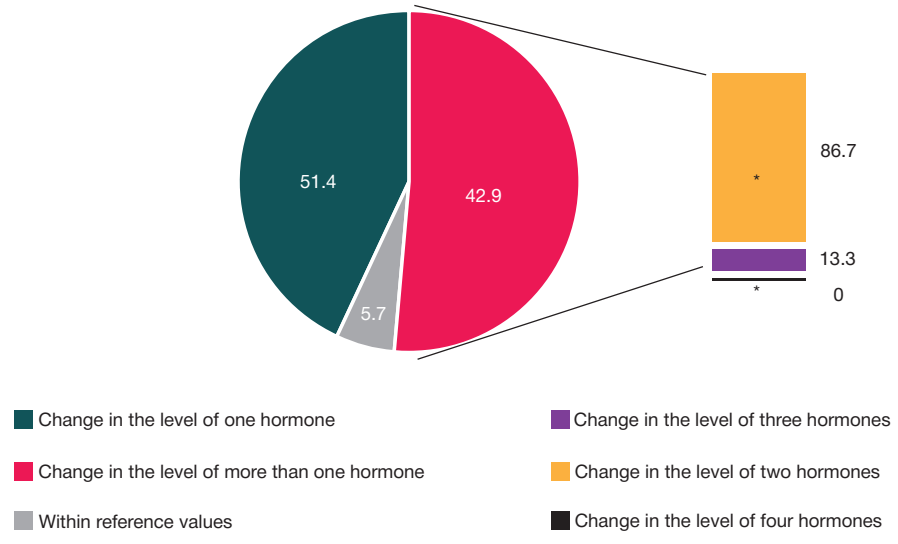

Fig. 2. Hormonal changes in the group without *Lactobacillus spp.*, *n* = 105

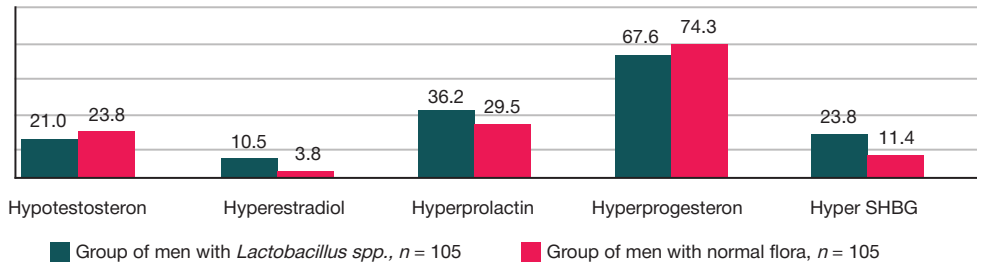

Fig. 3. Changes in the hormones studied, two compared groups

number of such patients was 6 (5.7%) ($p > 0.05$). Combined hormonal disorders were more common in the treatment group (50.5% versus 42.9%; $p > 0.05$) than in the control group (Fig. 1 and 2, respectively). Men with lactobacilli had more pronounced hormonal disturbances in the form of deviations from the normal levels of three or more hormones, the difference with the values registered in the control group significant ($p = 0.04$).

Table 1 contains the average hormone levels registered in the two groups. The group with *Lactobacillus spp*. had their average level of estradiol significantly different from that registered in the control group. Treatment group patients were also likely to have increased levels of prolactin and SHBG. Higher SHBG level and hyperestradiolemia were discovered in the treatment group more often (23.8% versus 11.4% at $p = 0.01$ and 10.5% versus 3.8% at $p = 0.05$, respectively), the difference with the corresponding values registered in the control group significant. As for the remaining hormones studied, there were no statistically significant differences revealed in their concentrations, however, the group with lactobacilli had a higher incidence of hyperprolactinemia (Fig. 3).

Compared to the control group, patients of the treatment group had their ejaculate samples more saturated with bacteria, the content measured as the total bacterial load ($10^{4.5 \pm 0.6}$ versus $10^{4.2 \pm 0.9}$, respectively, $p < 0.01$).

The results of RT-PCR examination (Fig. 4) show that the majority of identifiable microorganisms were registered in the treatment group more often than in the patients that had no lactobacilli detected. The difference in the incidence was significant ($p < 0.05$).

The results of the spermiological study (Fig. 5.) revealed that the more common types of disorder in the treatment group was oligoastenoteratozoospermia (30.0% versus 9.3%; $p < 0.01$) and asthenoteratozoospermia (28.8% versus 20.0%; $p = 0.1$). In the control group, the disorders diagnosed significantly more often were normozoospermia (42.7% versus 25.0%; $p = 0.01$) and isolated teratozoospermia (20.0% versus 7.5%; $p = 0.01$). Asymptomatic leukospermia was twice as common in the control group as in the treatment group (26.7% versus 13.8%; $p = 0.03$). The analysis of the key spermogram indicators (Table 2) showed that in the treatment group the motility, morphology of spermatozoids, as well as sperm concentration, were significantly worse compared to the control group.

DISCUSSION

There is an opinion that lactobacilli in men can only exist as transient flora. However, as registered in the clinical practice, some patients had Lactobacillus spp. in their ejaculate and reported over a month of sexual abstinence or strict use of barrier contraception, which minimizes the chance that such bacteria are transient in them. This research, in contrast with the pilot study [27], proved the hypothesis about the association between hyperestradiolemia and appearance of *Lactobacillus spp*. in the ejaculate. In the treatment group, leukospermia was a less common diagnosis than in the control group, which is probably related to the higher incidence of prostate acinus obstruction or fibrosis cases [30]. We found the ejaculate samples of the treatment group patients to be greatly contaminated with bacteria compared to those

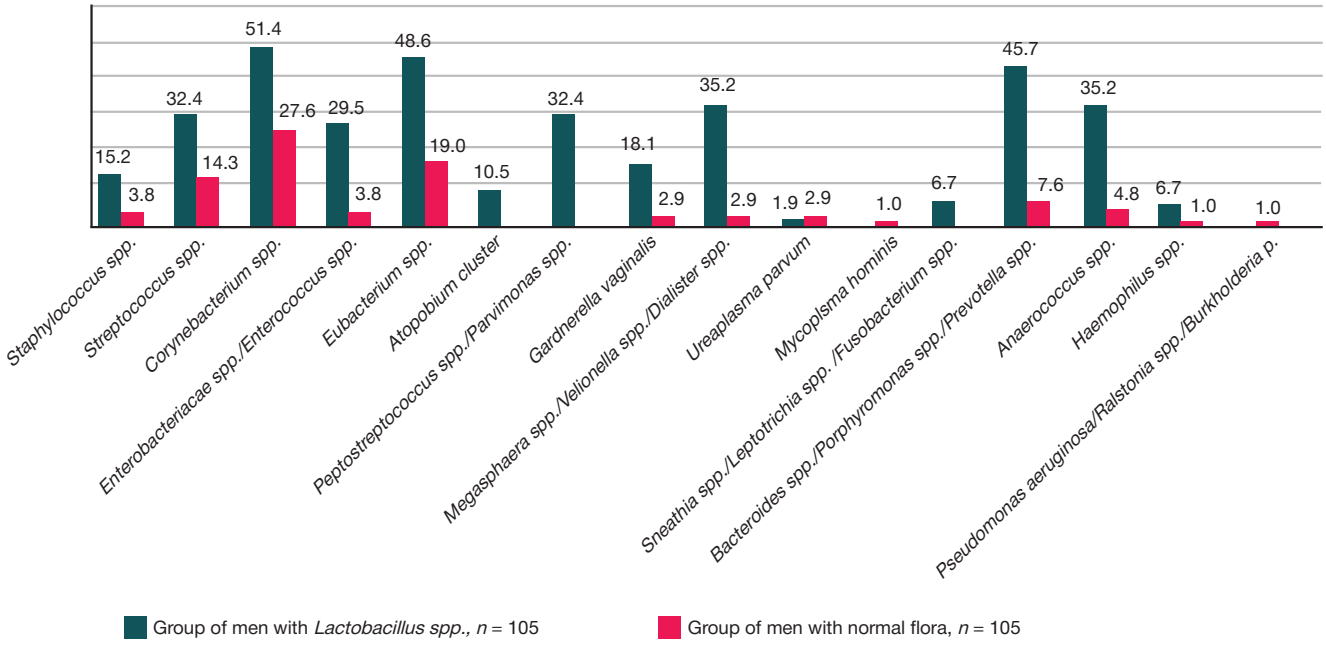

Fig. 4. The frequency of occurrence of microorganisms in the ejaculate as detected by RT-PCR, two compared groups

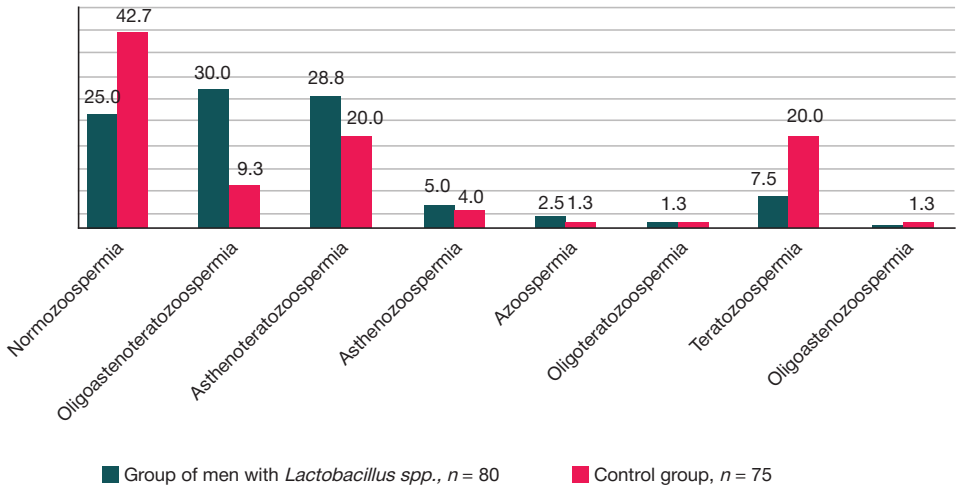

Fig. 5. Changes in spermogram values, two compared groups

of the control group ($p < 0.05$), which can be explained by hyperestradiolemia, increased levels of SHBG and prolactin, since the latter dampen biological activity of testosterone. One of the SHBG growth mechanisms is associated with an increased level of estradiol in blood; it often is a signal of "latent hyperestradiolemia", the latency here meaning the indicators remain within the reference range. The increased level of lactobacilli can be considered as a protective-compensatory mechanism triggered to maintain normal ejaculate microbiome and prevent genital tract invasion with opportunistic pathogenic microorganisms [6, 21, 23].

In our opinion, subject to strict adherence to the rules of preparation for RT-PCR ejaculate examination, including sexual abstinence or barrier contraception for at least three days, the detection of *Lactobacillus spp*. in the titer of Lg ≥ 10^3 can be regarded as an additional reason to investigate the concentrations of estradiol, prolactin, progesterone and SHBG.

The analysis of the data obtained does not allow deriving whether the lactobacilli play a negative or a positive role; this may be subject of further research that, employing RT-PCR, will establish the amount of Lactobacillus spp. and determine the type of this group of microorganisms.

CONCLUSIONS

Thus, *Lactobacillus spp*. bacteria are more likely to be found in the ejaculate of men with hyperestradiolemia and more severe combined abnormalities as detected by the spermogram. Detection of *Lactobacillus spp*. in semen can be an additional marker of hormonal imbalance in men, even with the spermogram values being normal.

Table 2. The studied spermogram values in the two compared groups

Spermogram value	Group with *Lactobacillus spp.* ($n = 80$), mean ± STD	Group without *Lactobacillus spp.* ($n = 75$), mean ± STD	*p*
Spermatozoa concentration, mln/ml	52.6 ± 44.8	70.1 ± 48.2	0.03
The total number of spermatozoa in the ejaculate, mln	167.1 ± 167.3	221.6 ± 169.4	0.01
Progressively motile spermatozoa (PR), %	25.4 ± 23.7	40.4 ± 23.7	< 0.01
Non-progressively motile spermatozoa, (NP), %	11.3 ± 9.4	12.3 ± 9.7	0.2
Immobile spermatozoa, (IM), %	61.2 ± 27.8	46.8 ± 23.5	< 0.01
Normal forms, %	2.4 ± 2.1	3.6 ± 2.7	< 0.01

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A CASE REPORT OF DOPA-RESPONSIVE DYSTONIA IN A YOUNG WOMAN

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Dopa-responsive dystonia (DRD) is a rare progressive genetically heterogenous disorder with pediatric onset. DRD is 3 times as prevalent in women than in men. This article reports a clinical case of DRD in a young female presenting with paraparesis, foot dystonia (more pronounced in the right foot) and pronounced walking impairment, who was admitted for emergency treatment to a Neurology Unit. Based on the additional tests, which included a levodopa trial and Sanger sequencing, the patient was diagnosed with DRD. Levodopa caused a considerable improvement of the symptoms. The article describes the clinical features of the disease, talks about its differential diagnosis, genetic predisposition and treatment strategy.

Keywords: DOPA-responsive dystonia, Segawa syndrome, hereditary dystonia

Author contribution: Belykh NA analyzed the literature, made the differential diagnosis, participated in establishing the definitive diagnoses followed the patient up for 3 years. Akhkyamova MA examined the patient, collected her medical history, participated in establishing the diagnosis, followed the patient up for 3 years, wrote the manuscript. Gusev VV followed the patient up; ordered diagnostic tests, prescribed treatment and monitored its course; provided the patient's medical history; helped with the application to the Ethics Committee. Lvova OA advised the patient on the genetic test and interpreted its results.

Compliance with ethical standards: the study was approved by the Ethics Committee of Ural State Medical University (Protocol № 1451/19 dated September 20, 2019). The patient gave informed consent to participate in the study.

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КЛИНИЧЕСКИЙ СЛУЧАЙ ДОФА-ЗАВИСИМОЙ ДИСТОНИИ У МОЛОДОЙ ЖЕНЩИНЫ

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ДОФА-зависимая дистония (ДЗД) — это редкое прогрессирующее генетически гетерогенное заболевание с манифестированием в детском возрасте, в три раза чаще встречающееся у женщин. В статье описан клинический случай синдрома Сегавы у молодой женщины с нижним парапарезом, дистонией стоп (больше справа), нарушением функции ходьбы, поступившей в неотложном порядке в неврологическое отделение с жалобами на выраженное ограничение ходьбы и самообслуживания. В результате дообследования, включающего в себя тест с Леводопой и прямое автоматическое секвенирование по Сенгеру, у пациентки была диагностирована ДЗД. Проведено лечение Леводопой, в ходе которого у пациентки регрессировали клинические симптомы дистонии. В статье представлены особенности течения заболевания и дифференциальной диагностики, а также генетическая детерминированность и тактика лечения.

Ключевые слова: ДОФА-зависимая дистония, синдром Сегавы, наследственная дистония

Вклад авторов: Н. А. Белых — сбор материала по заболеванию, дифференциальная диагностика, участие в постановке окончательного диагноза, наблюдение пациента в течение трех лет; М. А. Ахкямова — осмотр пациента, сбор анамнеза заболевания, участие в диагностике, наблюдение пациента в течение трех лет, редактирование рукописи; В. В. Гусев — наблюдение пациента в течение трех лет, назначение диагностических методов исследования, подбор и контроль лечения, предоставление истории болезни пациента для написания статьи, организация этического комитета; О. А. Львова — рекомендации по генетическому исследованию, интерпретация результатов, генетическое консультирование.

Соблюдение этических стандартов: исследование одобрено этическим комитетом Уральского государственного медицинского университета (протокол № 1451/19 от 20 сентября 2019 г.). Получено добровольное информированное согласие на участие пациента в научном исследовании.

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Dopa-responsive dystonia (DRD) is a form of torsion dystonia first described by the Japanese neurologist Segawa in 1971 as progressive hereditary dystonia with prominent diurnal fluctuations [1].

In most patients, the disease starts in one leg in the first decade of life and spreads to other limbs by the time the patient enters their late 20s [2]. Gait disturbance is typically the earliest manifestation of DRD often mistakenly interpreted as cerebral palsy [3]. In the early stages of the disease, the symptoms are absent in the morning but worsen towards evening; such aggravation depends more on the number of waking hours than on physical activity itself. As the disease progresses, the patient starts to experience morning symptoms as well [4].

There are 2 types of dystonia: classic postural dystonia, e.g. postural instability due to increased muscle tone, and phasic dystonia, which is a combination of postural instability and phasic dystonic movements. There are single reports of isolated hand tremor, permanent foot deformities (clubfoot) and dystonia of individual muscles [5].

A trial of levodopa is the main diagnostic test in patients with suspected DRD. A good, sustained response to this drug indicates a high probability of DRD.

The recommended starting dose of levodopa is 1 mg/kg per day. It should be gradually increased until sustained improvement is achieved or side effects are reported by the patient. The majority of patients improve at 4–5 mg/kg per day.

In the absence of a positive response to levodopa, the drug should discontinue no sooner than 3 months after the onset of therapy [6].

To differentiate between DRD and other conditions alleviated by levodopa, pterins are measured in the cerebrospinal fluid of the patient (CSF). The positive levodopa trial and elevated CSF pterins are strongly suggestive of DRD [7]. The diagnosis can be confirmed by means of molecular genetic testing for mutations in the *GCH1* gene [8].

Clinical case

A female patient underwent a series of medical examinations and received treatment for DRD at the Neurology Unit of the Central Clinical Hospital № 23 (Yekaterinburg) in 2015–2017. The diagnosis was based on the presence of the heterozygous nucleotide sequence variant c.248g>a (p.Gly83Asp) detected using PCR .

The patient was born in 1982. Her family members first became aware of her symptoms when she was 12 years old. The symptoms included weakness in the legs, gate disturbance, muscle fatigue in the legs exacerbated on walking, a feeling of knee rotation, toe curling while walking, and psychological tension. According to the patient, she also felt occasional weakness in her arms. The patient's parents sought medical advice with a neurologist. Provisional diagnoses included Strümpell–Lorrain disease, paraparesis and paraplegia. The patient was prescribed Baclofen, Neuromultivit, Sirdalud (tizanidine), paraffin wax therapy, massage, and exercise, to no avail. Her condition continued to deteriorate. The patient started feeling very weak, lost control over her legs, discontinued Baclofen and Sirdalud without consulting the neurologist and stopped attending PE classes at school after failing her normative assessment tests (especially, the jumping portion).

Family history. As a child, the patient was raised in a two-parent family and did not have any developmental delays. At the age of 3 years, the patient had chickenpox. After graduating from high school, she went on to earn a degree in economy but was out of work at the time of this study. The patient denied any unhealthy habits. In 2010 she delivered a child by Cesarian section. The patient had no past history of serious infections or allergies. Due to her condition, she was found disabled in 1995 and qualified for category 2 of disability in 2000.

In 2015, she was referred for botulinum toxin therapy, but never received it for reasons unknown. At about that time, her symptoms started to aggravate: she experienced pronounced difficulty walking and could no longer take care of herself. Eventually, she was admitted to the Neurology Unit for further tests.

On admission, her condition was moderately severe, the skin appeared pale and dry. The pulse was regular, 72 beats per min; BP was 130/75, and respiration rate was 16 breaths per min. On a neurological examination, the patient was fully conscious and oriented; speech was scaning. Her cranial nerves were unremarkable. Muscle strength was reduced in the distal legs (the patient scored 3.5 points); muscle tone was increased on the right side (extrapyramidal signs) (Fig. 1); tonic spasms were observed in the lower limbs. Upper limb reflexes were moderate and symmetric; in the lower limbs, reflexes were diminished. No pathologic reflexes were observed. In coordination tests the patient was uncertain; a positive Romberg was present. The patient showed no signs of meningeal irritation. The range of motion was limited in all spinal cord segments. The preliminary diagnosis based on the patient's complaints, medical history and clinical presentations was consistent with encephalo-polyradiculoneuropathy, paraparesis, feet dystonia (more pronounced on the right side) and walking impairment. Results of laboratory tests, including complete blood count, urinalysis and blood biochemistry test, were within the normal reference range. On ECG, the sinus rhythm was 73 beats per minute. Electromyoneurography of the lower and upper limbs revealed mild radiculopathy of L4–L5, S1, C7–C8, and Th1, moderate ulnar neuropathy at the elbow and mild axonal neuropathy of the right tibial and peroneal nerves. MRI of the brain revealed grade 1 cortical atrophy. Nothing was suggestive of a focal or diffuse cerebral pathology (Fig. 2) An earlier cervical spine MRI scan performed in 2007 had been suggestive of stage 1 degenerative disc disease at C3–C6 and a small disc osteophyte complex at C5–C6, with no pathologic foci in the spinal cord (Fig. 3). DNA testing for *GCHI* mutations was conducted on March 13, 2017: the coding sequence and the adjacent introns of the *GCHI* gene responsible for torsion dystonia (DYT5) were analyzed using Sanger sequencing. The heterozygous variant c.248g>a (p.Gly83Asp) was detected. Thus, torsion dystonia was confirmed by molecular genetic testing.

Based on the data from a follow-up MRI scan, the diagnosis was revised and corrected to G24.8 Dopa-responsive dystonia involving both feet, walking impairment; polyradiculoneuropathy in the presence of degenerative disc disease of the cervical and lumbosacral spine; pain syndrome; motor impairment.

The patient was prescribed regular daily exercise, a dopamine mimetic drug, a vasodilator, a hepatoprotective agent, vitamins, and a cholinesterase inhibitor. She was followed up by her local healthcare provider. The patient started

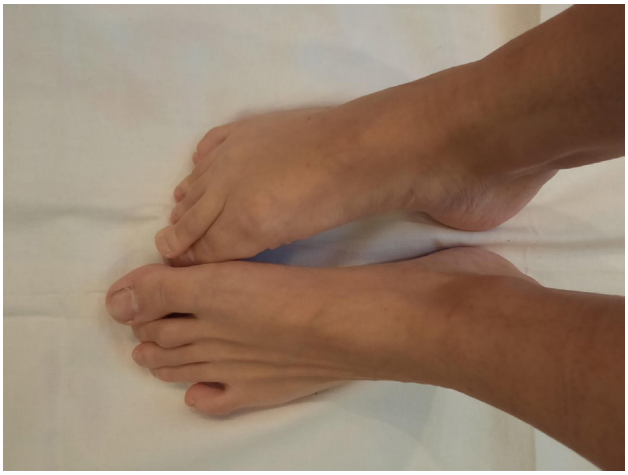

Fig. 1. Dystonia of the right foot before therapy with levodopa

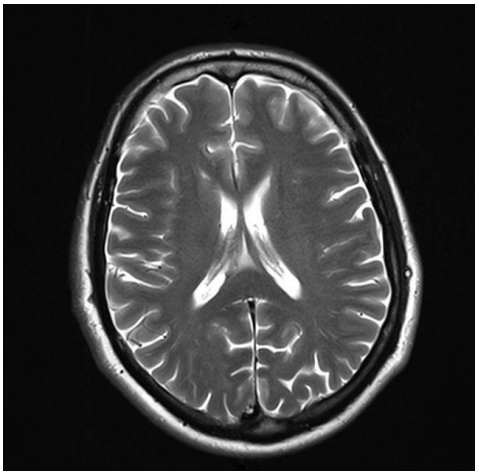

Fig. 2. Brain MRI: grade I cortical atrophy, no signs of focal or diffuse cerebral pathology

taking the dopamine mimetic drug after the definitive diagnosis was made and continued through pregnancy. When she got pregnant, she reduced the dosage by 50%, with no loss of effect.

Discussion

Patients with DRD can have 4 different mutations in the gene involved in the synthesis of guanosine triphosphate cyclohydrolase-I (GCH1) [9]. This enzyme participates in the synthesis of tetrahydrobiopterin (BH4), the tyrosine hydroxylase (TG) cofactor that converts L-tyrosine into L-DOPA, which leads to a reduction in dopamine concentrations in the striatum. Patients with the autosomal-dominant type of inheritance carry this mutant gene on chromosome 14 (14qll-q24.3). Patients with autosomal-recessive inheritance carry this gene on chromosome 11p15.5 of the TG gene [10].

The prevalence of the neurometabolic disease is 0.5-1 cases per 1 million population [11]; it is probably underreported because some patients present with very mild symptoms. Manifestation of symptoms at the age of 4–8 years is accompanied by parkinsonian features and muscle dystonia resulting in gait disturbance. Because of increased muscle tone in the legs and signs of damage to the pyramidal tracts, the condition can be mistakenly interpreted as cerebral palsy or inherited spastic paraplegia [12]. Over time, dystonia of the lower limbs progresses to generalized dystonia. The disease is characterized by diurnal fluctuations of motor symptoms, which improve in the morning after sleep and aggravate towards evening. Evaluation of the therapeutic effect of levodopa is a good diagnostic technique in patients whose dystonia is not associated with hypoxic-ischemic encephalopathy [13].

In the described clinical case, the patient had reduced muscle strength in the distal leg (3.5 points), increased muscle tone on the right side (extrapyramidal signs) gait disturbance with dystonic movements of the lower libs, diminished reflexes in the lower limbs and a positive Romberg.

Given that the patient's symptoms regressed after the combination therapy with levodopa and carbidopa, the definitive diagnosis was DRD. At present, the patient is free of dystonia symptoms.

CONCLUSION

Dopa-responsive dystonia is a rare genetic pediatric-onset disease. Due to diagnostic difficulties, the definitive diagnosis can be delayed. In the early stages of the disease, gait disturbance may be overlooked by the parents. An accurate medical history and a thorough neurological examination focused on detecting the presence of diurnal fluctuations and evaluating the patient's response to medication therapy are instrumental in establishing the accurate diagnosis. The clinical case described in this article might remind healthcare practitioners to stay vigilant about this pathology when examining a patient.

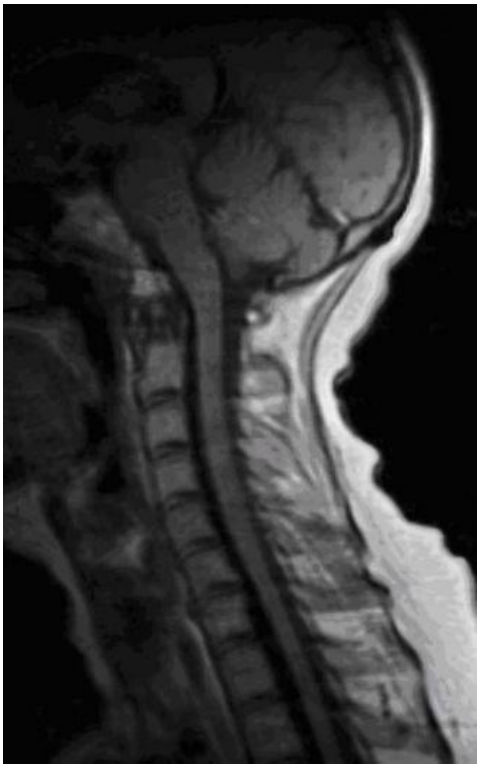

Fig. 3. Cervical spine MRI: stage 1 degenerative disc disease at C3–C6, small disc osteophyte complex at C5–C6. No pathological foci detected

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EFFECTS OF EMPAGLIFLOZIN AND L-ORNITHINE L-ASPARTATE ON BEHAVIOR, COGNITIVE FUNCTIONS, AND PHYSICAL PERFORMANCE IN MICE WITH EXPERIMENTALLY INDUCED STEATOHEPATITIS

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Non-alcoholic fatty liver disease (NAFLD) is a chronic condition characterized by disturbed carbohydrate and lipid metabolism and often complicated by psychoneurological symptoms, including anxiety, depression, memory deficit, and asthenia. Most studies of pharmacotherapy candidates for NAFLD focus on the ability of the tested drugs to restore the biochemical functions and morphology of the liver while their potential effects on the co-existing conditions remain overlooked. The aim of this paper was to investigate the effects of empagliflozin and L-ornithine L-aspartate (OA) on behavior, memory, and physical performance in C57BL/6 mice with experimentally induced NAFLD (6 months of a Western diet + weekly carbon tetrachloride injections). The disease affected animal behavior (locomotion speed decreased by 38% and 35%, $p < 0.01$; rearing increased by 432% and 279%, $p < 0.05$ etc.), induced long-term memory deficit (latency to find the target box increased by 108% in the Barnes maze, the number of errors increased by 439%, $p < 0.05$), and compromised physical performance (swimming time in the forced swim test dropped by 50%, $p < 0.05$ etc.). When administered during the high-calorie diet period, both drugs reduced anxiety (empagliflozin: the number of grooming bouts rose by 160%, $p < 0.05$ and 2173%, $p < 0.01$; time spent in the light compartment in the light/dark box test increased by 275%, $p < 0.05$, etc.; OA: time spent in the open arms of the maze increased by 267%, $p < 0.05$), and promoted memory retention in mice with NAFLD. OA improved physical performance (swimming time in the forced swimming test improved by 106%, $p < 0.05$, etc.). Thus, empagliflozin and OA can have a beneficial effect on cognitive functions, as well as behavior, and ameliorate asthenia in NAFLD.

Keywords: non-alcoholic fatty liver disease, steatohepatitis, cognitive disorders, physical performance, empagliflozin, L-ornithine L-aspartate

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Author contribution: Prikhodko VA analyzed the literature; conducted the experiments; participated in statistical analysis and interpretation of the obtained data; wrote the manuscript and prepared the figures. Sysoev Yul planned the study; analyzed the literature; conducted the experiments; participated in statistical analysis and data interpretation; wrote the manuscript and prepared the figures. Poveryaeva MA conducted the experiments; Bunyat AV planned the study; conducted the experiments; Karev VE analyzed and interpreted the obtained data; wrote the manuscript and prepared the figures; Ivkin DYu planned the study; analyzed the literature; Sukhanov DS planned the study; analyzed and interpreted the obtained data; wrote the manuscript; Shustov EB, Okovityi SV planned the study; analyzed the literature; analyzed and interpreted the obtained data; wrote the manuscript.

Compliance with ethical standards: the experiments were conducted in compliance with the Basel Declaration, the Order № 199 on the *Principles of Good Laboratory Practice* of the Ministry of Healthcare of the Russian Federation dated April 01, 2016, and the recommendations of the Bioethics Committee of Saint Petersburg State Chemical and Pharmaceutical University. The animals were housed in a vivarium under standard controlled laboratory conditions.

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ВЛИЯНИЕ ЭМПАГЛИФЛОЗИНА И L-ОРНИТИНА L-АСПАРТАТА НА ПОВЕДЕНЧЕСКО-КОГНИТИВНЫЕ ФУНКЦИИ И ФИЗИЧЕСКУЮ РАБОТОСПОСОБНОСТЬ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ СТЕАТОГЕПАТИТЕ

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Неалкогольная жировая болезнь печени (НАЖБП) — хроническое заболевание, характеризующееся не только изменениями углеводного и липидного обменов, но и рядом психоневрологических нарушений, включая тревожно-депрессивные расстройства, ухудшение памяти и астенический синдром. Большинство фармакологических исследований направлено на оценку способности препаратов восстанавливать биохимические функции и гистоморфологическую картину печени при НАЖБП; их влияние на течение сопутствующих нарушений изучают редко. Целью работы было оценить влияние эмпаглифлозина и L-орнитина L-аспартата (ОА) на поведение, память и физическую работоспособность мышей линии C57BL/6 при моделировании НАЖБП (6-месячная «западная диета» с еженедельным введением тетрахлорметана). Данная модель вызывает у животных изменение поведения (снижение скорости передвижения на 38 и 35%, $p < 0,01$; увеличение частоты стоек на 432 и 279%, $p < 0,05$ и др.), ухудшение долговременной памяти (время поиска в «Лабиринте Барнс» возрастало на 108%, число ошибок — на 439%, $p < 0,05$), а также снижение физической работоспособности (время вынужденного плавания сократилось на 50%, $p < 0,05$ и др.). Оба препарата при введении во время диеты снижали тревожность (эмпаглифлозин: число грумингов возросло на 160%, $p < 0,05$ и на 2173%, $p < 0,01$; время в белой зоне черно-белой камеры — на 275%, $p < 0,05$ и др.; ОА: время в открытых рукавах лабиринта увеличилось на 267%, $p < 0,05$) и способствовали сохранению памяти у мышей с НАЖБП. Особенностью ОА было повышение физической работоспособности животных (время вынужденного плавания увеличилось на 106%, $p < 0,05$ и др.). Таким образом, эмпаглифлозин и ОА могут положительно влиять на поведенческие и когнитивные функции, а также снижать выраженность астенического синдрома при НАЖБП.

Ключевые слова: неалкогольная жировая болезнь печени, стеатогепатит, когнитивные нарушения, физическая работоспособность, эмпаглифлозин, L-орнитина L-аспартат

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Вклад авторов: В. А. Приходько — анализ литературы, проведение экспериментов; анализ, статистическая обработка и интерпретация данных; подготовка рукописи и иллюстраций. Ю. И. Сысоев — планирование исследования, анализ литературы, проведение экспериментов; анализ, статистическая обработка и интерпретация данных; подготовка рукописи и иллюстраций. М. А. Поверяева — проведение экспериментов; А. В. Бунят — планирование исследования, проведение экспериментов; В. Е. Карев — анализ и интерпретация данных, подготовка рукописи и иллюстраций; Д. Ю. Ивкин — планирование исследования, анализ литературы; Д. С. Суханов — планирование исследования, анализ и интерпретация данных, подготовка рукописи; Е. Б. Шустов и С. В. Оковитый — планирование исследования; анализ литературы; анализ и интерпретация данных; подготовка рукописи.

Соблюдение этических стандартов: все эксперименты проводили в соответствии с принципами Базельской декларации, Приказом Минздрава РФ от 01.04.2016 № 199н «Об утверждении правил надлежащей лабораторной практики» и рекомендациям биоэтической комиссии ФГБОУ ВО СПХФУ Минздрава России. Животных содержали в стандартных условиях вивария с соблюдением нормативных требований к питанию и освещению.

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Non-alcoholic fatty liver disease (NAFLD) is a chronic condition caused by the pathologic deposition of fat in liver cells which does not result from alcohol intake or exposure to toxic substances. NAFLD is closely associated with carbohydrate and lipid metabolism disorders, obesity, and insulin resistance. It is estimated that up to 25% of the world's population today suffers from NAFLD, suggesting that it is the leading cause of chronic liver disease [1, 2].

Besides metabolic alterations, NAFLD is associated with a number of non-specific symptoms, including depression, anxiety, asthenia, and cognitive impairment [3–5]. A study revealed that 53% of patients with NAFLD had signs of subclinical depression whereas 14% were clinically depressed [6]. Mood swings and irritability observed in such patients often co-exist with weakness and chronic fatigue [3]. Some authors hold the opinion that almost all NAFLD patients experience cognitive complications; mild cognitive symptoms are observed in half of such patients, whereas the other half have moderate or severe symptoms [4].

The aims of treatment for NAFLD are to eliminate its causes, mitigate the symptoms and prevent the progression of the disease [2, 7]. Apart from diet and physical exercise, some patients with NAFLD should be advised to take hypolipidemic, hypoglycemic and hepatoprotective agents [2, 7]. The efficacy of these drugs against metabolic disorders has been studied extensively; however, few publications discuss their use in the therapy of NAFLD-associated psychoneurological deficits [8, 9]. This paper investigates the effects of empagliflozin and L-ornithine L-aspartate on behavior, cognitive functions, and physical performance in C57BL/6 mice with experimentally induced NAFLD.

METHODS

The study was carried out on 52 3-month-old male C57BL/6 mice with an average body weight of 23 g, purchased from the Rappolovo breeding farm (Leningrad region). The study was hosted by Saint Petersburg State Chemical and Pharmaceutical University. The animals were fed a complete feed ration (Laboratorkorm; Russia) and provided with drinking water (Russian GOST 2874-82), except for the period of high-calorie diet. Food and water were available *ad libitum*. C57BL/6 mice develop metabolic, behavioral and cognitive disorders when fed a high-fat diet, which determined the choice of this strain for the study [10, 11]. After a 14-day acclimation period, the mice were randomized into the following experimental groups: group 1 (intact animals; n = 10), group 2 (control animals with induced NAFLD that did not receive any treatment; n = 14), group 3 (EMPA; NAFLD+2 mg/kg empagliflozin marketed as Jardiance®; n = 14), and group 4 (OA; NAFLD + 1.5 g/kg L-ornithine L-aspartate marketed as Hepa-Merz®; n = 14). The drugs were administered by intragastric gavage once a day throughout the experiment; the control and intact groups received equivalent volumes of sodium chloride 0.9%.

NAFLD (steatohepatitis) was modeled by feeding the mice a Western diet and administering carbon tetrachloride (CCl_4) intraperitoneally (Fig. 1A). The control, EMPA and OA groups were fed a high-calorie diet (HCD) for 6 months. The diet was composed of 36.65% standard chow + 21.1% beef tallow + 41% D-fructose + 1.25% cholesterol. Additionally, the drinking water was replaced with a 42 g/L D-fructose solution. Throughout the HCD part of the experiment, the animals received intraperitoneal injections of CCl4 (0.32 μg/kg) once a week [12]. The intact animals received the standard feed ration and pure drinking water.

After the HCD part of the experiment was completed, animal behavior was assessed in the open field test (OF), the light/dark box test (LDB), and the elevated plus maze test (EPM) (all by Open Science; Russia). The mice were video recorded using a VideoMot2 system (TSE Systems; Germany).

The short-term and long-term spatial memory of the animals was evaluated in the Barnes maze (BM) (Open Science; Russia) [13]. After 4 days of training (4 trials per day), probe trials were conducted on days 5 and 12. In this test, we measured the time it took the animal to reach the target box (seconds) and counted the number of mistakes.

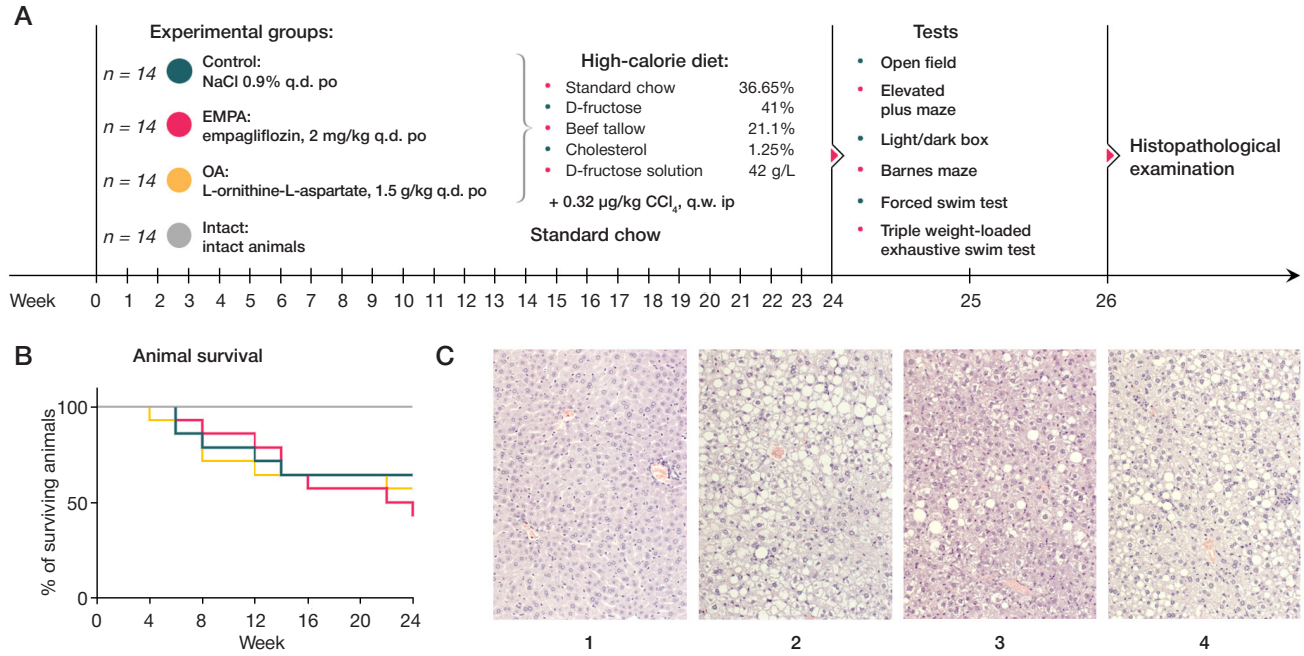

Fig. 1. A. Description of the NAFLD model, animal groups and the experimental design. Abbreviations: q.d. — once a day, q.w.— once a week, po — orally, ip — intraperitoneally. **B**. Survival dynamics. **C**. Histopathological slides of the liver. **1**. No structural abnormalities in liver tissue in the intact group; **2**. Morphological features of NAFLD in the control group, including marked macrovesicular steatosis and marked ballooning degeneration of hepatocytes, focal parenchymal infiltration by polymorphonuclear leukocytes; **3**. Morphological features of NAFLD in the EMPA group, including mild macrovesicular steatosis and mild ballooning degeneration of hepatocytes, no pathological infiltration of the liver parenchyma; **4**. Moderate macrovesicular steatosis and moderate ballooning of hepatocytes, moderate parenchymal infiltration by polymorphonuclear cells in the OA group. A, B, C, D — hematoxylin-eosin staining, ×200

Two days after the behavioral tests, the weight-loaded (7.5% of body weight) forced swim test (FS) and the triple weight-loaded exhaustive swim test (TES) [14, 15] were conducted to evaluate the physical performance of the animals. In the second test, swimming time was measured for each mouse at the beginning of the trial, 5 minutes later and 45 minutes later.

Morphological changes in the liver were evaluated by histology. Samples of liver tissue were fixed in 10% buffered formalin, dehydrated, cleared in isopropanol, and embedded in paraffin using a conventional technique. Four-μm sections prepared from paraffin blocks were mounted on slides, stained with hematoxylin-eosin and coverslipped. The slides were examined in incident light. Steatosis, ballooning degeneration of hepatocytes, and infiltration of the parenchyma were evaluated qualitatively [2, 16].

Statistical analysis was carried out in GraphPad Prism 8.0.2 (GraphPad Software; USA). Normality of distribution of quantitative variables was tested using the Shapiro-Wilk W-test. If the distribution was normal, the differences were assessed using ANOVA followed by Dunnett's post-hoc test. If the distribution was non-normal, the Kruskal-Wallis was applied followed by Dunn's multiple comparison. Below, numeric data appearing in the figures are presented as M ± SE. The principal component analysis was done in MS Excel XLStat 2016 (Addinsoft; France).

RESULTS

Animal survival and pathomorphological liver features in experimentally induced NAFLD

Forty percent of the animals from all the experimental and control groups died during the 6 months of the study. Neither empagliflozin nor OA had any effect on animal survival (Fig. 1B).

On histology, the liver specimens of mice with induced NAFLD were characterized by steatosis, ballooning degeneration of hepatocytes, and parenchymal infiltration by polymorphonuclear leukocytes. The changes in liver morphology were the most pronounced in the control group: steatosis affected at least 30% of hepatocytes; ballooning degeneration occurred in at least 30% of liver cells. Focal infiltration of the parenchyma by polymorphonuclear leukocytes was another remarkable histologic feature in the experimental groups. Empagliflozin and OA reduced the degree of these NAFLD-associated pathological changes (Fig. 1C).

Effects of experimentally induced NAFLD on measured parameters in the control group

Behavioral tests demonstrated that steatohepatitis affected animal behavior, cognitive functions and physical performance. In the OF test, mice with experimentally induced NAFLD moved more slowly (–38%; $p < 0.01$), had a longer total freezing time (–35%; $p < 0.01$) and a higher number of rearing episodes (+432%; $p < 0.05$) (Fig. 2A). In the control group, the number of rearing episodes and head dips in the EPM test was also higher (+279%, $p < 0.05$ and +553%, $p < 0.05$, respectively; Fig. 2B). In the LDB and OF tests, mice with NAFLD had a lower locomotion speed (–35%; $p < 0.01$) than healthy animals (Fig. 2C).

Although the overall learning performance in the BM test was similar between the groups (the animals made fewer errors over time and needed less time to locate the target box), the latency to locate the target box was increased in the control group (+108%) and the error rate was higher (+439%) on day 12, as compared to day 5 ($p < 0.05$ in both cases) (Fig. 3).

Active swimming time in the forced swim test (–50%; $p < 0.05$), as well as time spent swimming during the first trial of

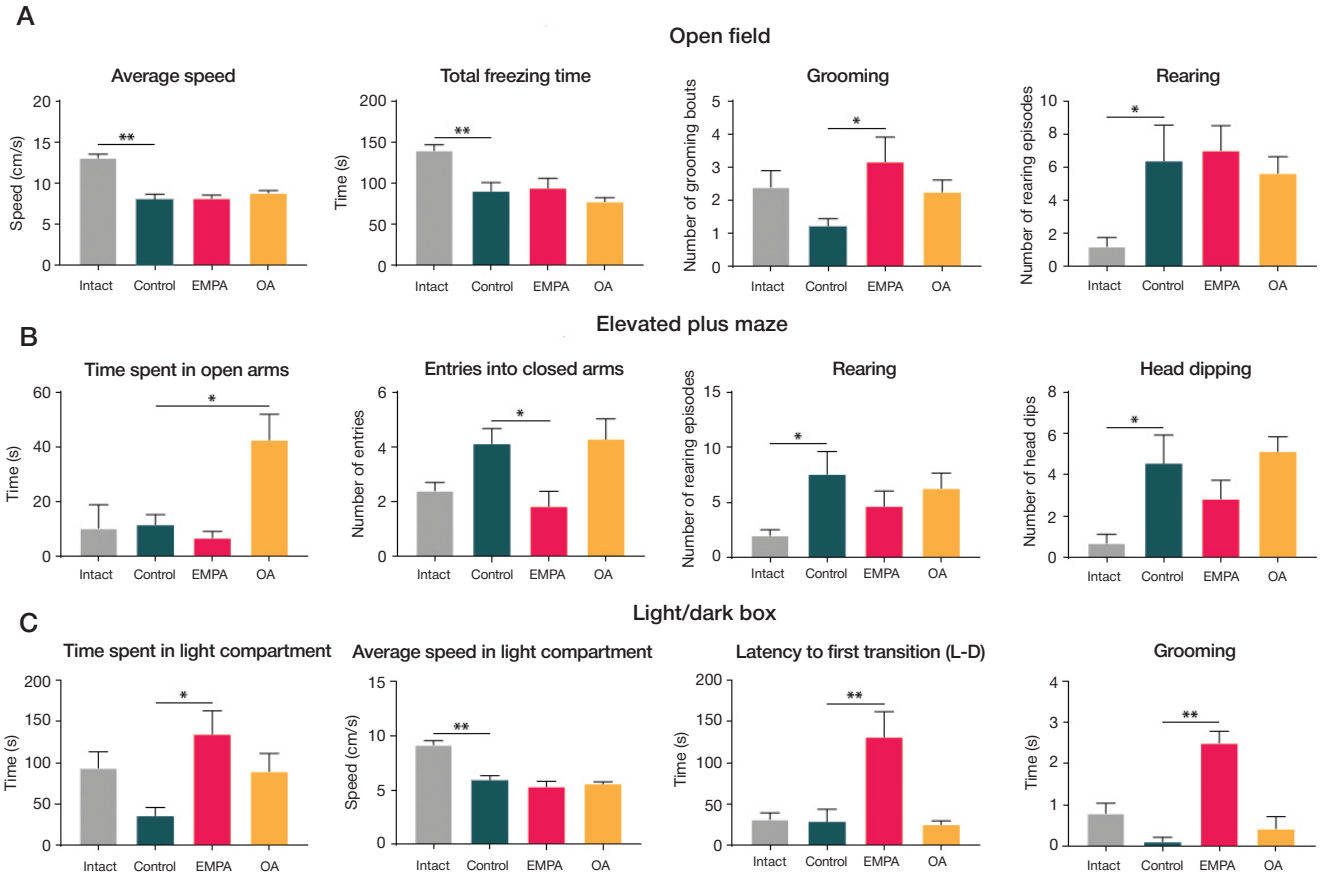

Fig. 2. Results of (**A**) the Open field test, (**B**) the Elevated plus maze test and (**C**) the Light/dark box test. * — $p < 0.05$; ** — $p < 0.01$

the triple weight-loaded exhaustive swim test (–69%; $p < 0.05$), was shorter in the untreated NAFLD group than in the intact group. Besides, during the triple weight-loaded exhaustive swim test the control group required more time to recover, and their results during the second trial (5 min after the 1st trial) were not as good as those of healthy animals (–63%; $p < 0.05$) (Fig. 4).

Effects of empagliflozin on measured parameters

The number of grooming bouts in the OF test was higher in the animals treated with empagliflozin (+160%; $p < 0.05$) (Fig. 2A), whereas the number of visits to the closed arms in the EPM test was lower in those animals than in the control group (–56%; $p < 0.05$) (Fig. 2B). In the LDB test, mice treated with empagliflozin spent more time in the illuminated compartment (+275%; $p < 0.05$), took more time to enter the dark compartment for the first time (+355%; $p < 0.01$), and engaged in significantly more grooming bouts in comparison with the control group (+2173%; $p < 0.01$) (Fig. 2C).

Empagliflozin did not have any significant effect on the short- and long-term memory and physical performance of mice with induced NAFLD in the BM test and both forced swim tests, respectively.

Effects of L-ornithine L-aspartate on measured parameters

No significant effect of OA on animal behavior and performance was noticed in the OF, LDB and BM tests. In the EPM test, time spent in the open arms of the maze was longer in the animals treated with OA than in the control group (+267%; $p < 0.05$) (Fig. 2B).

The animals treated with OA spent more time swimming actively than the control group (+106%; $p < 0.05$); their results were comparable with those of the healthy animals. Similarly, in the TFS test, the mice from the OA group were able to keep their heads above the water surface longer than the untreated NAFLD animals (+137%; $p < 0.01$) 45 minutes after the first trial (Fig. 4).

DISCUSSION

This study investigated potential effects of empagliflozin and OA on behavior, memory and physical performance in C57BL/6 mice with the steatohepatitis stage of NAFLD.

In the OF and EPM tests, the mice with experimentally induced NAFLD had more episodes of rearing behavior than the controls; they also performed more head dips in the open arms of the EPM. The increased rearing frequency could be interpreted as a sign of anxiety [17, 18]. Head dipping in the open arm areas is not only an exploratory, but also a risk assessment behavior [19]. Therefore, increased head dipping is an ambiguous behavioral marker. Considering the increased rearing frequency and the propensity to visit the closed arms, head dipping in our experiment might be interpreted as a sign of anxiety [20, 21].

Unlike the intact group, mice with NAFLD engaged in grooming less often in the LDB test and tended to spend more time in the brightly illuminated compartment. Preference of dark enclosed spaces based on the hole reflex indicates increased anxiety in rodents [22]. High anxiety levels in rodents with induced NAFLD were previously reported by other authors [23, 24]. Along with mood swings and apathy, anxiety-like behavior is a typical neurological symptom in human patients with NAFLD [3, 6].

On average, animals with experimentally induced NAFLD ambulated more slowly in the OF and LDB tests than intact animals (this parameter was not evaluated in the EPM test). The decrease in locomotion speed was not improved by empagliflozin or OA. Besides, total freezing time was shorter in all groups of animals with NAFLD. A reduction in average locomotion speed might be interpreted as a manifestation of anxiety. In the classic sense, shorter freezing time indicates an anxiolytic effect. On the other hand, it might suggest that mice

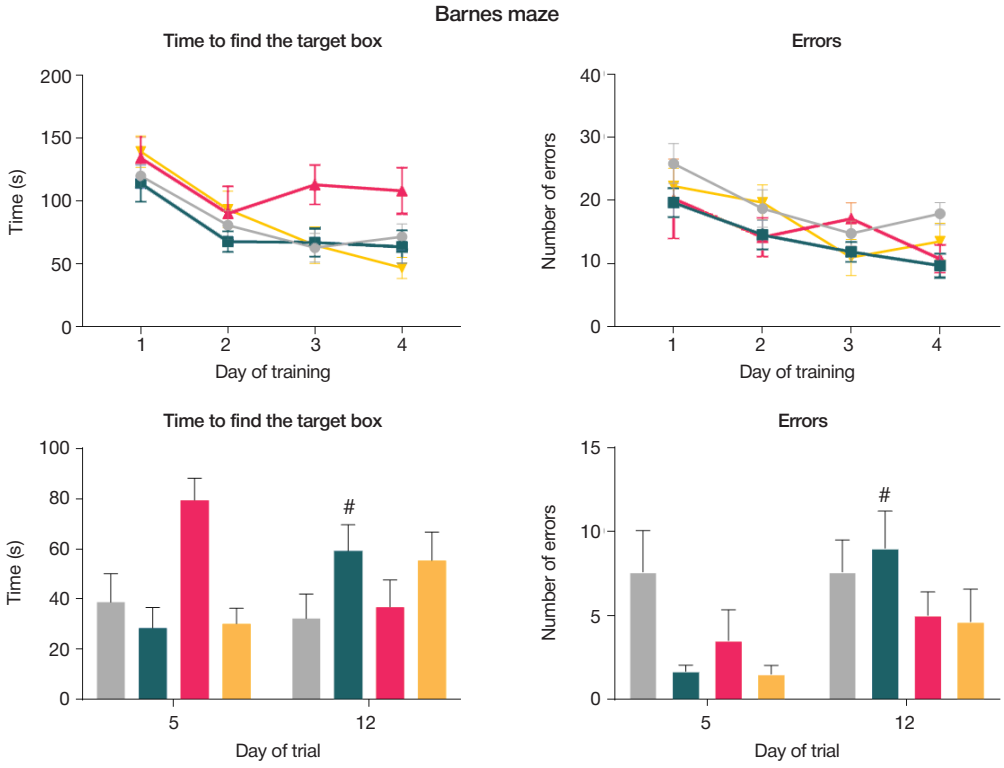

Fig. 3. Results of spatial memory assessment in the Barnes maze. ■ — Intact, ■ — Control, ■ — EMPA, ■ — OA; # — $p < 0.05$, day 12 vs day 5

in our experiment were actively seeking shelter because of high anxiety levels. We decided to give special emphasis to changes in these parameters since it can be difficult to interpret them unequivocally. It is possible that NAFLD causes more complex behavioral changes that cannot be interpreted only in terms of high/low anxiety levels.

Mice treated with empagliflozin spent more time in the brightly illuminated compartment in the LDB test than the control group. Apparently, the animals felt less discomfort and a less pronounced need to seek shelter when placed in the light compartment. As a result, latency to the first transition into the dark compartment increased. Besides, during their visits to the light compartment, mice treated with empagliflozin performed more grooming bouts than the control animals. A similar increase in the number of grooming bouts was observed in the OF test. There are thought to be two sides to this phenomenon. On the one hand, stressed animals show a propensity to engage in shorter yet more frequent grooming bouts, which, along with increased urination and defecation, might indicate high levels of anxiety. In this case, grooming replaces other behaviors and activities inhibited by acute stress [25]. On the other hand, rodents perform more grooming bouts when they feel safe, calm and free of anxiety [26]. Considering that animals in our experiment preferred open, brightly illuminated spaces, the increased grooming frequency might reflect the anxiolytic effect of the tested drug.

Behavioral changes described above might be directly linked to the beneficial effect of empagliflozin on the morphology of glial cells [27] and to elevated BDNF (brain-derived neurotrophic factor) levels observed in mice with obesity and insulin resistance [28]. This allowed us to hypothesize that empagliflozin might have additional central mechanisms mediating its anxiolytic action.

Mice treated with OA spent more time in the open arms of the EPM, which indicates an anxiolytic effect of this drug. The unchanged closed arm entry and head dipping frequencies may indicate retention of spontaneous motor and exploratory activities in this group of animals [29].

Following a period of training for the BM test, the animals in all experimental groups were able to find the target box faster and to make less errors. The control group was the only one whose performance on day 12 was much worse than on day 5. This might indicate a long-term memory deficit in mice with induced NAFLD. Previously, spatial memory deficits were reported in rats with NAFLD [30]. NAFLD-associated cognitive impairment was experimentally correlated to shrinkage of both gray and white brain matter and was accompanied by brain mass reduction [31]. Another study demonstrated that non-alcoholic steatohepatitis and liver fibrosis, especially in its late stages, caused multiple focal lesions in the white matter regardless of the presence of comorbidities [32]. In our experiment, long-term memory was compromised neither in the group of mice treated with the tested drugs nor in the intact animals.

On days 3, 4 and 5 of the experiment, mice treated with empagliflozin performed slightly worse, needing more time to find the target box in comparison with other animal groups. Those mice did not attempt to explore the maze and seek shelter but instead preferred to stay in the open space in a random hole. This is consistent with our previous proposition about the anxiolytic effect of empagliflozin, which in our case promoted resilience to stress caused by open spaces and bright light.

Earlier studies demonstrated that transfer from a normal feed ration to a high-calory diet used to model NAFLD resulted in a relatively fast (by the end of month 1) twofold drop in physical performance [33]. Starting from month 4 of the high-calorie diet, the mice were gradually restoring their initial level of performance. However, the metabolic burden of excess calories was more extreme in our experiment. This might explain why the mechanisms involved in energy production had not adapted to the new conditions by month 6 and physical performance of the animals in the forced swim test was significantly worse (45% relative to the results of the intact group that had fully restored the level of physical performance by month 6 of a less strict diet).

Importantly, even milder models of NAFLD can result in a significant decline in glycogen levels in skeletal muscles (by 33%) and liver (by 44%) [33]. A combination of a high-calorie diet and injections of CCl_4, which exerts hepatotoxic and prooxidant effects, leads to the development of pronounced disturbances in lipid metabolism with typical morphological changes to liver tissue visible on histology, can further aggravate cognitive and behavioral deficits and compromise physical performance [12]. Carbohydrate deficiency in skeletal muscles could be the underlying metabolic cause of poor physical performance in laboratory animals. In our study, repeated administration of empagliflozin did not have a positive effect on the physical performance of mice in the forced swim test. This was predictable because empagliflozin does not improve glucose transport to muscle cells and does not stimulate glycogen synthesis.

Breakdown of muscle proteins and participation of amino acids in the production of substrates for the Krebs cycle provide energy for muscle work. These processes are accompanied by vigorous production of ammonia, which is then utilized in

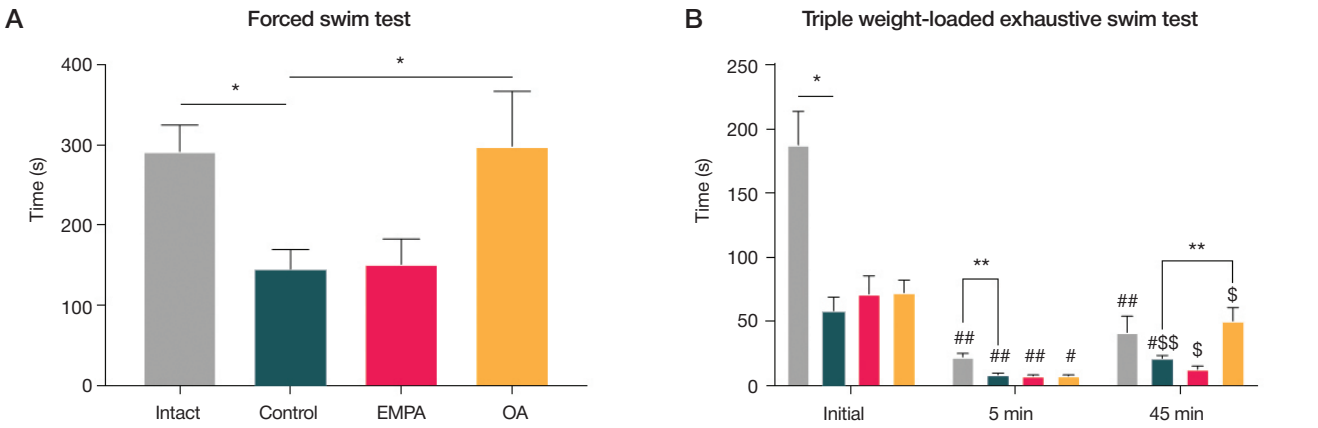

Fig. 4. Results of the Forced swim test (**A**) and the Triple weight-loaded exhaustive swim test (**B**). ■ — Intact, ■ — Control, ■ — EMPA, ■ — OA; * — $p < 0.05$; ** — $p < 0.01$; # — $p < 0.05$; ## — $p < 0.01$, comparison with the initial result; $ — $p < 0.05$, $$ — $p < 0.01$, comparison with the second trial (5 min after the first trial)

the ornithine cycle of urea synthesis. In humans, OA activates the urea cycle, enhances lipid metabolism, slightly elevates the level of ketone bodies and free fatty acids, lowers blood ammonia, and alleviates the subjective feeling of fatigue [34]. In our study, repeated administration of OA significantly mitigated the noxious effect of NAFLD on the physical performance of mice in the forced swim tests, demonstrating the special role of amino acid utilization in providing energy for muscle work in a situation of a high-calorie diet, when the organism's metabolism has to adjust to the new conditions.

To elucidate the mechanism of OA effects on the physical performance of mice with experimentally induced NAFLD, the TFS test was conducted. The initial-to-exhausting swimming time ratio (the total duration of the first and second trials) in the intact group revealed that the animals refused to continue swimming because of physical exhaustion; the contribution of the central nervous system component was minor [35]. In mice with experimentally induced NAFLD, the duration of active swimming was three times shorter in the first trial, suggesting diminished endurance with a central nervous system and a peripheral organ components. Repeated administration of empagliflozin and OA decreased the contribution of the central (but not the peripheral) component, which was indicated by a 40% decline in the physical performance compared to the intact group.

The main TFS parameter (exhaustion index, EI) characterizes the efficacy of recovery mechanisms in the first phase of recovery after exhaustive exercise. This parameter is calculated as a ratio of weight-loaded swimming duration after 45 min of the recovery period to the duration of exhaustive swimming. Immediate recovery occurs in the first 0.5–1.5 h of the resting phase and is essentially based on the excretion of anaerobic metabolism products accumulated during exercise and the repayment of oxygen debt. It should be noted that in the immediate phase of post-exercise recovery, oxidative phosphorylation does not play a key role in the resynthesis of adenosine triphosphate (ATP). More important here (especially in a situation of exhaustive exercise, fatigue or overtraining) is the alactic pathway, which uses adenosine diphosphate (ADP), the product of ATP degradation), for ATP resynthesis. This mechanism is launched when ADP concentrations are high in muscle cells, which occurs only when other pathways for ADP resynthesis have been depleted. To ensure the high rate of the myokinase reaction, adenosine monophosphate needs to be degraded further and its metabolite, inosine monophosphate, needs to be excreted from the muscles. These events are closely associated with ammonia synthesis by actively working skeletal muscles [36].

The EI analysis revealed that empagliflozin did not affect the processes unfolding in the first recovery phase (TI was 0.20 in the intact group, 0.19 in the EMPA group and 0.38 in the control group due to a steep reduction in endurance and shorter duration of the exhaustive exercise). At the same time, EI was 0.61 in mice treated with OA, suggesting a significant effect of the drug on post-exercise recovery. Perhaps, this could explain the restoration of physical performance in mice treated with OA. The effect of L-ornithine and its salts on post-exercise recovery, as well as the achievement of the desired training effect, in healthy humans and laboratory animals is a well-known phenomenon in sports medicine [37–39]. However, its impact on physical performance and the efficiency of the first phase of recovery after exhaustive exercise in a NAFLD model has never been demonstrated before.

The principal component analysis of all experimental data detected significant differences between intact and control animals. The effects of the studied drugs were not limited to mitigation of NAFLD symptoms: some of them were unrelated to the studied pathology (Fig. 5). Repeated administration of empagliflozin and OA can improve the histomorphological appearance of the liver and correct some symptoms often present in NAFLD, including anxiety, depression, cognitive deficit and asthenia. These effects might be clinically relevant due to the high prevalence of NAFLD in the economically active population.

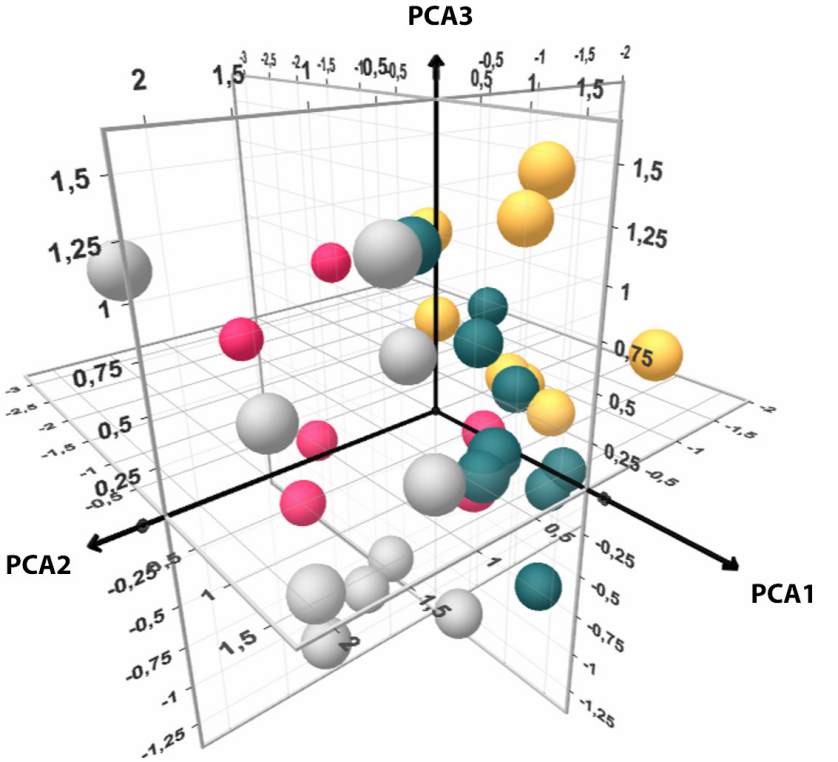

Fig. 5. Graphic representation of principal component analysis results. ■ — Intact, ■ — Control, ■ — EMPA, ■ — OA

CONCLUSIONS

1. Experimentally induced NAFLD causes pronounced behavioral changes in C57BL/6 mice. These changes include increased anxiety, long-term memory deficit, and poor physical performance. 2. Repeated administration of empagliflozin and OA can mitigate anxiety-like behavior and improve the affected cognitive functions to some extent. 3. OA can correct asthenia manifesting as poor physical performance in mice with NAFLD.

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ASPECTS OF FREE RADICAL OXIDATION IN THE LARGE BOWEL IN ULCERATIVE COLITIS AND CROHN'S DISEASE

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Research into the accumulation patterns of protein oxidative modification (POM) products and lipids in Crohn's disease (CD) and ulcerative colitis (UC) could have important implications for understanding the pathogenesis and improving the diagnosis and therapy for these diseases. The aim of this study was to investigate the aspects of free radical oxidation (FRO) in the large bowel and their possible correlations with clinical symptoms of UC and CD. In the Wistar rat model used in the experiment, CD was induced with 2,4,6-trinitrobenzenesulfonic acid, and UC was induced with oxazolone. Clinical status was assessed using the Disease activity index (DAI). Lipid peroxidation (LPO) products were measured in the heptane and isopropanol phases of the intestinal mucosa extract. POM products were measured following spontaneous and stimulated oxidation. The DAI (Me (Q_{25}–Q_{75})) was increased in both CD and UC on days 3 and 7 of the experiment: for CD, it was equally increased on days 3 and 7 (7 (3-7)) and was 11 (11–11) and 11 (9–11) for UC on days 3 and 7, respectively. The amount of primary, secondary and end LPO products in the heptane and isopropanol phases, as well as the total amount of POM products, was increased in the homogenized mucosa of the large bowel. In the CD group, the relative content of secondary basic POM products was increased on day 7 of the experiment. The following patterns of FRO were revealed: accumulation of LPO products in the UC group and accumulation of POM products in the CD group; UC is characterized by the accumulation of mostly LPO products in the heptane phase and secondary LPO products in the isopropanol phase; CD is characterized by the accumulation of secondary basic POM products. DAI scores were correlated with the amount of LPO products in the isopropanol phase and the amount of POM products in the spontaneous oxidation mode. The highest number of strong correlations was observed in the UC group. Our findings suggest a very serious contribution of FRO changes to the pathogenesis of UC and CD, meaning that LPO and POM products could be regarded as diagnostic markers and indicators of treatment efficacy.

Keywords: oxidative stress, lipid peroxidation, protein oxidative modification, large bowel, ulcerative colitis, Crohn's disease

Author contribution: Osikov MV conceived and designed the study, analyzed the experimental data and contributed to writing the manuscript; Davydova EV conceived and designed the study, analyzed the experimental data and contributed to writing the manuscript; Boyko MS, Bakeeva AE, Kaygorodtseva NV, Galeeva IR collected the samples, performed statistical analysis and data interpretation; Fedosov AA analyzed the experimental data and contributed to writing the manuscript; Ilyinyh MA, Vorgova LV analyzed the experimental data and contributed to writing the manuscript. All authors read and approved the final version of the manuscript for publication.

Compliance with ethical standards: the study was approved by the Ethics Committee of South Ural State Medical University, Chelyabinsk (Protocol No. 11 dated December 27, 2017; Protocol № 1 dated January 22, 2020). The experiment was carried out under standard vivarium conditions in strict compliance with the guidelines on the care and use of animals for scientific purposes provided in the European Convention (ETS № 123 dated March 18, 1986, Strasbourg), European Commission Recommendation 2007/526/EC dated June 18, 2007, and Directive 2010/63/EU of the European Parliament and European Council dated September 22, 2010.

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ОСОБЕННОСТИ СВОБОДНОРАДИКАЛЬНОГО ОКИСЛЕНИЯ В ТОЛСТОМ КИШЕЧНИКЕ ПРИ ЯЗВЕННОМ КОЛИТЕ И БОЛЕЗНИ КРОНА

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Изучение особенностей накопления продуктов окислительной модификации белков (ОМБ) и липидов в кишечнике при болезни Крона (БК) и язвенном колите (ЯК) может иметь значение в патогенезе, диагностике и терапии этих заболеваний. Целью работы было изучить особенности свободно-радикального окисления (СРО) в толстом кишечнике, связь с клиническими симптомами при БК и ЯК. Для моделирования БК крысам Wistar вводили тринитробензосульфат, для ЯК — оксазолон. Клинический статус оценивали по Disease activity index (DAI). В толстом кишечнике определяли продукты пероксидного окисления липидов (ПОЛ) в гептановой и изопропанольной фазах, продукты ОМБ в спонтанном и металл-зависимом режимах. При БК и ЯК на 3-и и 7-е сутки увеличивается DAI (Me (Q_{25}–Q_{75}): 7 (3–7) при БК на 3-и и 7-е сутки одинаково; 11 (11–11) и 11 (9–11) при ЯК на 3-и и 7-е сутки соответственно), в толстом кишечнике повышается количество первичных, вторичных и конечных продуктов ПОЛ в гептановой и изопропанольной фазах, суммарное количество продуктов ОМБ, при БК на 7-е сутки увеличивается доля вторичных продуктов ОМБ основного характера. Особенности СРО: при ЯК — накопление продуктов ПОЛ, при БК — продуктов ОМБ; при ЯК прежде всего накапливаются конечные продукты ПОЛ в гептановой фазе и вторичные продукты в изопропанольной; при БК — вторичные продукты ОМБ основного характера. При БК и ЯК установлена ассоциация DAI с содержанием продуктов ПОЛ преимущественно в изопропанольной фазе, продуктов ОМБ в спонтанном режиме; наибольшее количество сильных связей зафиксировано при ЯК. По результатам исследования, роль изменений СРО в патогенезе БК и ЯК гораздо больше, что является предпосылкой для обозначения продуктов ПОЛ и ОМБ как диагностических маркеров, показателей эффективности терапии.

Ключевые слова: окислительный стресс, пероксидное окисление липидов, окислительная модификация белков, кишечник, язвенный колит, болезнь Крона

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The incidence of gastrointestinal diseases doubles every decade, both in Russia and globally, posing a serious concern to public health and society [1]. Although there is great variability in the epidemiological data across studies, an increase in the incidence of ulcerative colitis (UC) and Crohn's disease (CD), especially in young, socially active and employable individuals, is being reported by many authors [2, 3]. Worldwide, there are 3 to 62 and 50 to 70 incident cases of UC and CD, respectively, per 100,000 population a year [4]. Currently, as many as 1.6 million people are suffering from UC and CD in the USA [5, 6]. UC and CD are also associated with a variety of intestinal and extraintestinal complications that add to hospitalization, surgery and outpatient care costs.

The pathogenesis of UC and CD is not fully clear: there are a multitude of etiological factors that can activate cell-mediated and humoral components of the immune system by eliciting Th1 and Th2 immune responses and cause an imbalance between Th17 and T_{reg} cells [7]. Just like cytokines, enzymes, immunoglobulins, and some other factors, reactive oxygen and nitrogen species (ROS, RNS) produced by activated neutrophils, monocytes/macrophages, endothelial and epithelial cells are involved in triggering and maintaining inflammation in the intestinal wall when there is deficit of antioxidant defense factors [8–10]. ROS and RNS, as well as products of their interaction with proteins and lipids, can act as markers of intestinal tissue damage, indicating the severity of the disease and the efficacy of treatment; they are also potential targets for novel personalized therapeutic and preventive approaches to the regulation of the local redox state in the large bowel affected by CD and UC [11–13]. Therefore, it would be interesting to study the aspects of free radical oxidation (FRO) and the relationship between the redox state of the intestinal lesion and the severity of clinical manifestations in UC and CD.

In our *in vivo* experiment described below, we aimed to investigate the aspects of FRO in the large intestine and the relationship between FRO and the clinical manifestations of UC and CD.

METHODS

The experiment was conducted in 35 male Wistar rats weighing 200–230 g. The animals were randomized into 3 groups: group 1 served as the intact control (n = 7); group 2 included animals with CD (n = 14); group 3 consisted of rats with UC (n = 14). The following procedure was applied to induce CD in the animals: 30 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma-Aldrich; USA) was dissolved in 150 ml of 50% ethanol; 0.2–0.3 ml of the solution (depending on the animal's weight) was injected through a 2 mm polyurethane catheter (Sintez; Russia) inserted rectally to a depth of 8 cm [14]. A two-step oxazolone-based scheme was used to induce UC. In the first step, the animals were sensitized with 150 µl of 3% alcohol solution of oxazolone (Sigma-Aldrich; USA) applied onto the skin in the interscapular region; in the second step, 150 µl of 3% alcohol solution of oxazolone was rectally injected to a depth of 7–8 cm [15]. Before the procedure, the animals were anesthetized with 20 mg/kg Zoletil 100 (tiletamine hydrochloride; Virbac Sante Animale; France). CD and UC were evaluated based on the clinical symptoms and the morphology of the intestinal lesion on days 3 and 7. Symptoms were evaluated on a daily basis using a modified disease activity index (DAI) adapted to rats [16, 17]. Parameters assessed by DAI and the corresponding scores are provided in Table 1. Liquid stools were defined as unformed fecal matter of mash-like or watery consistency. Diarrhea was defined as passage of unformed stools more than 3 times a day. Rectal bleeding was defined as the presence of fresh blood on the animal's fur around the anus and in the feces. The presence of occult blood in the feces was evaluated using the benzidine test.

To prepare a sample of 10% intestinal mucosa homogenate, the proximal colon was removed and placed in a cooled 0.1M phosphate buffer solution (pH 7.4); then 100 mg of the tissue was homogenized in a mechanical glass tissue grinder (1 : 10) for 3 min at ≤ 4 °C. These conditions were applied to obtain 1 ml of homogenized intestinal mucosa. Lipid peroxidation (LPO) products contained in the homogenate were extracted in a mixture of heptane and isopropanol and measured using an SF-56 spectrophotometer (LOMO-Spectr; Saint-Petersburg) following the technique proposed by Volchegorsky et al. [18, 19]. Absorbance of heptane and isopropanol extracts was measured at 220 nm (isolated double bonds), 232 nm (diene conjugates, DC), 278 nm (ketodienes, KD, and conjugated trienes, CT), and 400 nm (Schiff bases, SB). The relative content of LPO products was expressed in the units of oxidation indices (UOI): E232/E220 (DC), E278/E220 (KD and CT) and E400/E220 (SB). To quantify products of oxidative protein modification (POM) in the homogenized tissue, carbonyl protein derivatives were allowed to react with 2,4-dinitrophenylhydrazine (two

Table 1. Parameters assessed by DAI

Parameter	Score	Description
Body weight loss	0	No weight loss
	1	1–5%
	2	6–10%
	3	11–20%
	4	Over 20%
Stool consistency	0	Normal stool consistency
	2	Unformed stool
	4	Diarrhea
Rectal bleeding	0	No blood in feces
	1	Positive benzidine test
	2	Positive benzidine test and visible blood in feces
	4	Rectal bleeding, blood around anus

types of oxidation were tested: spontaneous and catalyzed by the Fenton reagent) and the resulting aldehyde/ketone DNPH derivatives were spectrophotometrically measured in the UV and visible regions of the spectrum [20, 21]. Measurement results were expressed in absorbance units per 1 mg of the protein (au/mg) and as percentage (%). Adaptive reserve potential (ARP, %) was calculated as a ratio of spontaneous oxidation products to induced oxidation products. The results were processed in IBM SPSS Statistics 19 (SPSS: An IBM Company; USA). The obtained values are presented below as a median (Me) and an interquartile range [Q_1–Q_3]. Significance of differences between the groups was assessed using the Kruskal–Wallis, Mann–Whitney and Wald–Wolfowitz tests. Correlations between the studied parameters were evaluated using Spearmen's rank correlation test. The differences were considered significant at $p < 0.05$.

RESULTS

On day 3 of the experiment, the animals with experimentally induced CD started exhibiting the following symptoms: frequent defecation, changes in stool consistency and blood in feces. On day 7, the symptoms deteriorated and were now accompanied by weight loss; those changes were reflected in the heightened DAI scores, as shown in Table 2. In addition, the animals became less active. On day 7, the DAI scores were significantly higher than on day 3 ($p < 0.05$). Changes were also observed in the amount of LPO products in the lipid extract from the homogenized intestinal mucosa (Table 3). On day 3, there was an increase in the amount of primary and secondary LPO products in the heptane phase and primary, secondary and end LPO products in the isopropanol phase. On day 7, there was an increase in the amount of primary, secondary and end LPO products in both heptane and isopropanol phases. Thus, the primary and end products of lipid peroxidation were building up between days 3 and 7 in the animals with CD, as seen from the isopropanol extracts ($p < 0.01$). In the next step, we quantified products of spontaneous and stimulated oxidative protein modification in the homogenates of large bowel tissue (Table 3).

We found that the total amount of carbonyl protein derivatives was elevated on days 3 and 7 of observation. Carbonyl protein derivatives are irreversible products of oxidative stress. They are formed when a number of amino acid residues undergo oxidation and also when lipid peroxidation products interact with reducing sugars. The total amount of POM products was higher on day 7 than on day 3 ($p < 0.01$). When analyzing their relative content in the animals with CD, we discovered that on day 7 the share of primary products (aldehydes) shrank while the proportion of secondary products (ketones) increased. As neutral carbonyl derivatives absorb in the UV spectrum, whereas basic carbonyl derivatives absorb in the visible light region, the analysis of cumulative absorption in the UV and visible regions of the spectrum can shed light on the nature of POM products and their accumulation dynamics. On day 3, the proportion of neutral and basic POM products did not differ between the animals with CD and the intact group. On day 7, there was a shift towards accumulation of basic POM products, signaled by a decline in their total relative content in the UV spectrum and an increase in their total relative content in the visible region.

The adaptive reserve potential was estimated by calculating the total amount of POM products generated by spontaneous and stimulated oxidation. Briefly, protein oxidation in the intestinal mucosa homogenate was catalyzed by adding Fe^{2+} and H_2O_2 to the reaction mix; the reaction yielded a highly reactive $OH^{\cdot}$ radical. We found that the total amount of POM products yielded by stimulated oxidation was elevated on days 3 and 7 of the experiment in the animals with experimentally induced CD. On day 3, the proportion of primary and secondary POM products (both basic and neutral) did not differ from that in the intact animals; on day 7, the proportion of neutral aldehydes decreased and the proportion of basic ketones went up. So, changes in POM characteristics were similar for both spontaneous and stimulated oxidation modes. The total adaptive reserve potential was significantly increased on day 3 of the experiment and did not differ from that in the intact group on day 7. This phenomenon can be explained by the enhancement of the adaptive reserve potential of aldehyde/ketone DNPH derivatives registered in the UV and visible light regions; however, ketone DNPH derivatives registered in the visible light region were the biggest contributor.

On day 3, the following symptoms were observed in the animals with UC: body weight loss, frequent defecation, loose stools, visible blood in feces, positive benzidine tests. On day 7, the symptoms deteriorated, as reflected in the heightened DAI scores (Table 2). The DAI scores were significantly higher on day 7 than on day 3 ($p < 0.05$). On day 3, primary, secondary and end products of lipid peroxidation were significantly elevated in the heptane and isopropanol phases in this group of animals (Table 3). Similarly, primary, secondary and end products of lipid peroxidation were significantly elevated in the heptane and isopropanol phases on day 7. Thus, primary and secondary LPO products were declining in the isopropanol phase between the days 3 and 7 in the animals with experimentally induced UC ($p < 0.01$).

In this group of animals, the total content of POM products yielded by stimulated oxidation was significantly increased on days 3 and 7 of the experiment (Table 3). On days 3 and 7, the share of aldehyde DNPH derivatives shrank, whereas the share of ketone DNPH derivatives went up. On day 7, the relative amount of basic carbonyl derivatives was increased, in contrast to the reduced share of neutral carbonyl derivatives (this was inferred from an increase in the total relative content of POM products in the visible light region and their low total relative content in the UV region). The total amount of POM products yielded by stimulated oxidation was elevated on days 3 and 7. The share of aldehyde DNPH derivatives decreased whereas the share of ketone DNPH derivatives increased on days 3 and 7. The total relative content of POM products registered in the visible light spectrum was significantly elevated on day 3, suggesting a shift towards formation of basic aldehyde and ketone DNPH derivatives. The total adaptive reserve potential was significantly reduced on days 3 and 7.

Table 2. Disease activity index in rats with UC and CD (Me (Q_{25}–Q_{75}))

Parameter	Group 1 Intact animals ($n = 7$)	Day 3		Day 7	
		Group 2 CD ($n = 7$)	Group 3 UC ($n = 7$)	Group 2 CD ($n = 7$)	Group 3 UC ($n = 7$)
DAI, a.u.	0	7 (3.00–7.00)*	7 (3.00–7.00)*	11 (11.00–11.00)*	11 (9.00–11.00)*

The correlation analysis established correlations between DAI scores (the integral indicator of the clinical status) and the amounts of LPO/POM products in the homogenized intestinal mucosa of the large bowel (Table 4). On day 3, there were moderate correlations in the CD group between DAI scores and the amount of secondary LPO products in the heptane and isopropanol phases, as well as the total amount of POM products yielded by spontaneous oxidation. In the UC group, DAI scores were strongly correlated with the amount of secondary LPO products in the heptane phase, primary and secondary LPO products in the isopropanol phase, and the total amount of POM products yielded by spontaneous and stimulated oxidation. On day 7, DAI scores correlated with the amount of primary LPO products in the heptane phase, primary, secondary and end LPO products in the isopropanol phase, and the total amount of POM products yielded by spontaneous and stimulated oxidation; for UC, DAI scores were strongly correlated with the amount of primary and end LPO products in the heptane phase, primary, secondary and end LPO products in the isopropanol phase, and the total amount of POM products yielded by spontaneous and stimulated oxidation. The largest number of strong correlations was observed for UC: 10 out of 12 correlations were strong; by contrast, only 1 of 9 correlations established in the CD group was strong.

DISCUSSION

In our experiment, clinical manifestations of experimentally induced CD and UC were consistent with the actual symptoms and signs of these diseases, suggesting that the proposed TNBS and oxazolone-based animal models can be used to study the pathogenesis of homeostatic changes characteristic

Table 3. Markers of free radical oxidation in the homogenate of large intestinal mucosa of rats with CD and UC (Me (Q_{25}–Q_{75}))

Parameter	Group 1	Day 3		Day 7	
	Intact animals	Group 2	Group 3	Group 2	Group 3
	(n = 7)	CD (n = 7)	UC (n = 7)	CD (n = 7)	UC (n = 7)
DC (h), UOI	0.63	0.79	0.79	0.75	0.76
	(0.55–0.65)	(0.79–0.81)*	(0.75–0.81)*	(0.74–0.81)*	(0.75–0.77)*
KD and CT (h), UOI	0.06	0.09	0.08	0.07	0.09
	(0.05–0.06)	(0.06–0.09)*	(0.07–0.08)*	(0.06–0.09)	(0.09–0.11)*#
SB (h), UOI	0.01	0.01	0.03	0.03	0.05
	(0.01–0.02)	(0.01–0.01)	(0.02–0.04)*#	(0.03–0.04)*	(0.04–0.06)*#
DC (i), UOI	0.34	0.38	0.38	0.43	0.43
	(0.32–0.36)	(0.38–0.45)*	(0.33–0.43)*	(0.41–0.45)*	(0.43–0.45)*
KD and CT (i) UOI	0.31	0.61	0.72	0.51	0.58
	(0.29–0.32)	(0.61–0.71)*	(0.56–0.91)*#	(0.51–0.55)*	(0.57–0.59)*#
SB (i), UOI	0.01	0.08	0.07	0.14	0.11
	(0.01–0.02)	(0.08–0.11)*	(0.07–0.09)*	(0.12–0.14)*	(0.11–0.14)*
S POM spont., a.u./mg	141.86	324.21	194.91	392.31	343.48
	(136.04–166.74)	(313.48–340.93)*	(182.07–201.07)*#	(272.17–497.71)*	(332.13–358.22)*#
Aldehyde DNPH derivatives, %	93.71	92.59	91.79	89.13	90.71
	(93.69–93.71)	(91.61–93.41)	(91.48–91.98)*	(88.99–90.02)*	(90.69–90.71)*
Ketone DNPH derivatives, %	6.29	7.41	8.51	11.02	10.58
	(6.09–6.31)	(6.59–8.38)	(8.21–9.44)*#	(10.93–11.12)*	(10.31–11.34)*
uv spont., %	96.57	95.57	95.32	92.88	94.58
	(96.41–96.58)	(95.27–96.01)	(95.29–95.74)	(92.72–93.59)*	(94.23–95.21)*#
vs spont., %	3.42	4.42	4.71	7.11	5.76
	(3.41–3.59)	(3.99–4.72)	(4.67–5.85)	(6.41–7.27)*	(5.41–6.77)*#
S POM stim., a.u./mg	266.76	380.93	321.71	662.05	544.66
	(256.21–280.81)	(373.56–427.51)*	(284.89–377.77)*#	(643.29–690.09)*	(479.92–600.42)*#
Aldehyde DNPH derivatives stim., %	86.94	89.34	83.13	81.15	83.07
	(85.98–88.02)	(89.03–90.07)	(82.89–85.89)*#	(81.01–83.48)*	(82.62–87.41)*
Ketone DNPH derivatives stim., %	13.05	10.65	16.86	18.84	16.92
	(11.97–14.01)	(9.92–10.96)	(14.11–17.11)*#	(16.51–18.99)*	(12.58–17.37)*
uv, stim., %	88.99	91.91	85.67	84.52	86.13
	(88.99–90.83)	(91.27–92.31)	(85.44–85.44)*#	(83.91–86.37)*	(85.01–89.91)
vs stim., %	11.01	8.09	14.32	15.47	13.89
	(9.16–11.01)	(7.69–8.72)	(12.19–14.55)*#	(13.62–16.08)*	(10.09–14.98)
ARP, %	54.71	80.25	49.51	57.15	42.31
	(51.53–56.71)	(74.89–87.87)*	(46.77–51.14)*#	(50.11–59.71)	(28.17–47.78)*#

Note: * — difference is significant (p < 0.01) relative to group 1, # — relative to group 2. The listed parameters reflect the content of LPO products in the heptane (g) and isopropanol (i) phases of the lipid extract from the mucosal homogenate of the large intestine

of these conditions. On average, the rats did not lose more than 10% of their body weight. Such weight loss is traditionally associated with diarrhea and systemic inflammatory response and more specifically with the anorexigenic effect of some proinflammatory cytokines [22]. It is thought that TNBS acts as a hapten and, if administered rectally, induces a Th1 immune response, which involves proinflammatory cytokines and some mediators, against hapten-modified autologous proteins/luminal antigens or against intestinal microbiota proteins, causing transmural infiltration by leukocytes and inflammation [23–25]. Oxazolone is also seen as a hapten that mediates a Th2 immune response typical to UC, and the majority of researchers prefer it as the most popular agent for modeling UC in rats [26]. The 50% ethyl alcohol solution used as a solvent for TNBS and oxazolone aggravates damage to the mucosa of the large bowel [27].

Experimental CD and UC are characterized not only by weight loss, frequent defecation and changes to stool consistency but also by the presence of blood in feces and the accumulation of LPO products in the large bowel mucosa. In our experiment, accumulation of LPO products was observed in the isopropanol phase, which primarily accumulates phospholipids of cell membranes, and in the heptane phase (in triglycerides). Besides, there was accumulation of POM products, mainly secondary basic ketone DNPH derivatives, following spontaneous and stimulated oxidation. This led us to hypothesize that combined effects of $OH^{\cdot}$ and $O_2^{\cdot}$ cause accumulation of late markers of oxidative protein destruction and protein fragmentation [20, 28, 29]. Protein fragments are highly resistant to proteolysis, very toxic and can trigger apoptosis or necrosis, expanding the area of secondary alterations [30]. We have identified a few specific aspects of FRO occurring in the large bowel affected by CD and UC that are related to oxidative destruction of lipids and proteins. Firstly, comparative analysis reveals that the amount of end LPO products in the heptane phase and secondary products in the isopropanol phase measured on day 3 of the experiment, as well as the amount of end and secondary LPO products in the heptane phase and secondary LPO products in the isopropanol phase measured on day 7, was significantly higher in rats with UC (Table 3). Secondly, comparison of POM products in the homogenized mucosa of the large bowel demonstrates that the adaptive reserve potential and the total content of POM products were higher in the spontaneous and stimulated oxidation modes on days 3 and 7. Besides, in the CD group, as opposed to the UC group, basic primary POM products yielded by spontaneous and stimulated oxidation prevailed on day 3 of observation; on day 7, basic POM products prevailed in the spontaneous oxidation mode.

We think that escalation of oxidative stress resulting from production of ROS by activated neutrophils, monocytes/macrophages and endothelial cells in the primary lesion in a situation when the enzymes responsible for antioxidant defense are insufficiently active/scarce increases damage, dysfunction and death of large bowel cells, expands the area of secondary alteration and causes exacerbation of symptoms in patients with CD and UC. This conclusion is supported by the discovered correlations between DAI scores and the amount of LPO/POM products in the homogenized large bowel mucosa of rats with induced UC and CD.

CONCLUSIONS

We found that on days 3 and 7 of TNBS-induced CD and oxazolone-induced UC, DAI scores, the amount of primary, secondary and end LPO products in the heptane and isopropanol phases of homogenized intestinal mucosa and the total amount of POM products were increased. In UC, the proportion of secondary basic POM products increased on day 7. In the setting of UC, FRO was characterized by the accumulation of mostly LPO products, in contrast to CD characterized by the accumulation of mostly POM products. In UC, end LPO products were accumulated in the heptane phase of the lipid extract, whereas secondary products, in the isopropanol phase. CD was characterized by the accumulation of mostly secondary basic POM products. In CD and UC, there was a correlation between DAI scores and the amount of LPO products in the isopropanol phase, as well as the amount of POM products generated by spontaneous oxidation. The highest number of strong correlations was observed for UC. These findings broaden our knowledge about the role of redox state changes in the pathogenesis of inflammatory bowel diseases and encourage further research into FRO affecting the large bowel of patients with CD and UC. LPO and POM products should be investigated in the clinical setting as potential diagnostic markers of the disease and indicators of the efficacy of treatment aimed at reversing exacerbations and prolonging remission.

Table 4. Correlations between DAI scores (c.u.) and FRO in rats with experimentally induced UC and CD

Parameter	Day 3		Day 7	
	Group 2	Group 3	Group 2	Group 3
	CD (n = 7)	UC (n = 7)	CD (n = 7)	UC (n = 7)
DC (h), UOI	R = 0.15	R = 0.58	**R = 0.43**	**R = 0.72**
KD and CT (h), UOI	**R = 0.51**	**R = 0.82**	R = 0.32	**R = 0.66**
SB (h), UOI.	R = 0.13	R = 0.17	R = 0.43	**R = 0.88**
DC (i), UOI	R = 0.27	**R = 0.75**	**R = 0.51**	**R = 0.92**
KD and CT (i), UOI	**R = 0.64**	**R = 0.76**	**R = 0.51**	**R = 0.88**
SB (l), UOI	R = 0.32	R = 0.31	**R = 0.67**	**R = 0.72**
S POM spont., a.u./mg	**R = 0.69**	R = 0.77	**R = 0.83**	**R = 0.89**
S POM stim., a.u./mg	R = 0.41	R = 0.85	**R = 0.63**	**R = 0.81**

Note: significant correlations ($p < 0.05$) are shown in bold.

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DIAGNOSIS AND TREATMENT OF ACUTE SURGICAL DISEASES IN PATIENTS WITH COVID-19

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Emergency surgery in the infectious diseases hospital is an urgent problem during the COVID-19 pandemic. Municipal Clinical Hospital No.15 named after O. M. Filatov has been providing emergency surgical care after conversion, from March 27, 2020 until now. The hospital's medical staff has built up extensive experience: 194 surgical procedures were carried out in April, and 289 surgical procedures were carried out in May 2020. The paper reports the experience of emergency surgery at the stage of conversion to an infectious diseases hospital. Among all hospitalized patients, 482 (5.29%) people had acute surgical pathology requiring emergency surgery. Among patients who underwent urgent surgery, 472 (98%) people had the caused by COVID-19 community-acquired pneumonia of various degrees of severity. The paper discusses some features of acute surgical pathology and complications identified in patients with COVID-19. The surgical care features in the hospital after conversion are proper epidemiological regime implementation, minimization of the number of staff in the operating room, possible minimization of the number and reduction of the duration of surgical procedures. The most important challenge during the COVID-19 pandemic is medical staff safety.

Keywords: acute surgical pathology, peritonitis, acute intestinal obstruction, bleeding, acute appendicitis, acute cholecystitis, hernias, acute pancreatitis, COVID-19, mesenteric thrombosis

Author contribution: all authors made an equal contribution to study planning, hospital management, data acquisition and summarizing, as well as to manuscript writing.

Compliance with ethical standards: all patients submitted the informed consent to personal data processing and surgical treatment. When it was not possible to obtain the patient's informed consent due to the severity of the disease, a consultation was issued in accordance with the Ethics Committee requirements and local regulations.

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ДИАГНОСТИКА И ЛЕЧЕНИЕ ОСТРЫХ ХИРУРГИЧЕСКИХ ЗАБОЛЕВАНИЙ У ПАЦИЕНТОВ С COVID-19

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Экстренная хирургическая помощь в условиях инфекционного стационара — актуальная проблема во время пандемии новой коронавирусной инфекции COVID-19. Городская клиническая больница № 15 имени О. М. Филатова оказывает экстренную хирургическую помощь в условиях перепрофилирования с 27 марта 2020 г. по настоящее время. Специалистами больницы накоплен большой практический опыт: в апреле 2020 г. выполнено 194, а в мае — 289 оперативных пособий. В статье представлен опыт работы экстренной хирургической службы на этапе перепрофилирования в инфекционный стационар. Среди всех госпитализированных пациентов 482 (5,29%) имели острую хирургическую патологию, потребовавшую экстренного оперативного вмешательства. У 472 (98%) экстренно прооперированных пациентов присутствовала вызванная COVID-19 внебольничная пневмония разной степени тяжести. В статье рассмотрены некоторые особенности острой хирургической патологии и осложнения, встречающиеся у пациентов с COVID-19. Особенности хирургической помощи в перепрофилированном стационаре — это строгое соблюдение эпидемиологического режима, минимизация численности персонала в операционной, возможная минимизация числа оперативных пособий и сокращение их длительности. Наиболее важной задачей в условиях пандемии COVID-19 является безопасность персонала.

Ключевые слова: острая хирургическая патология, перитонит, острая кишечная непроходимость, кровотечение, острый аппендицит, острый холецистит, грыжи, острый панкреатит, COVID-19, мезентериальный тромбоз

Вклад авторов: все авторы внесли равный вклад в планирование исследования, организацию работы стационара, сбор и обобщение материала, а также написание статьи.

Соблюдение этических стандартов: все пациенты подписали согласие на обработку персональных данных, а также согласие на оперативное лечение. В случае невозможности получения информированного согласия пациента ввиду тяжести его состояния, в соответствии с требованиями этического комитета и действующих нормативных актов был оформлен консилиум.

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The coronavirus infection outbreak in 2019 (COVID-19) posed a serious challenge to the global community [1]. Introduction of the self-isolation and social distance regime all over the world led to the incidence rate decrease. However, it is too early to talk about full control over the situation.

During the pandemic, the burden on surgical services has increased significantly [2–5]. The world surgical communities (Society of American Gastrointestinal and Endoscopic Surgeons (SAGES) [6, 7], American College of Surgeons [8], Royal College of Surgeons of England [9]), together with Russian communities of surgeons [10], urologists and obstetrician-gynecologists have published the joint guidelines on the surgical service management under the new conditions [10, 11]. The framework of the "Temporary Guidelines" aimed, firstly, at preventing the medical staff infection, and, secondly, at developing principles for the provision of medical care to the infected patients, which cannot be postponed until the pandemic ends up. The "Temporary Guidelines" are as follows:

– all planned surgical procedures, manipulations and studies should be postponed;

– when conducting emergency medical procedures, the possibility of medical staff infection should be considered, therefore, the amount of intervention should be minimized;

– minimum number of personnel should be involved in the

treatment process; the full advantage should be made of remote communication between the medical staff and the patient, as well as of the remote medical specialists' consultations;

– surgical service is quite likely to experience the shortage of personnel, as surgeons may be called upon to provide medical care to patients in the infectious diseases hospitals.

The study was aimed to report the results of diagnosis and treatment of acute surgical diseases during the COVID-19 pandemic.

METHODS

Since March 27, 2020, after conversion to the infectious diseases hospital which took about five days, the Municipal Clinical Hospital № 15 named after O. M. Filatov started to provide medical care to patients with COVID-19.

In a short time, a gateway was built with channels for entry, staff changing in protective clothing, and leaving the "red zone".

The surgical service staff number was reduced to the minimum, since most doctors of all specialties, including surgeons, were called upon to provide medical care to patients with COVID-19. The planned surgical procedures were cancelled. Along with patients with an infection, hospitalization of patients with acute surgical diseases, injuries, acute urological and gynecological pathology against the background of COVID-19 began.

Despite the problems, the high quality emergency surgical care had to be provided to all patients in a timely manner. For that purpose, compliance to the following was fundamental:

a) proper patient routing;

b) selection of the sufficient (usually minimum) volume of surgical procedure;

c) creation of the safe working environment for the operating room team.

To date, the experience gained by countries which have already faced a new infection has made it possible to develop principles for carrying out surgical procedures during COVID-19 pandemic. These principles have been implemented in the Municipal Clinical Hospital № 15:

– the operating suite ventilation mode should be changed: air extraction should take precedence over the air inflow, and the negative pressure should be created in the operating room;

– all operating room team members enter the operating suite wearing the personal protective equipment (PPE): protection suit, bouffant cap, safety glasses or plastic face shield, shoe covers, latex gloves and respirator;

– in addition to PPE, surgeons and operating room nurses wear disposable sterile operating clothing and sterile gloves;

– the patient is provided with disposable bouffant cap and disposable surgical mask;

– optimal temperature and humidity should be maintained in the operating room (given that operating room team members are wearing a double outfit);

– maximum use of disposable surgical instruments;

– only the operating room team members stay in the operating room; during the surgical procedure, the doors of the supportive units and operating rooms are closed tightly;

– the nurse on duty responsible for the delivery of the instruments stays in supportive unit;

– intercom is used for communication between operating room and supportive units.

All surgical procedures were carried out by the pre-trained and instructed personnel in accordance with the epidemiological regime and the rules of aseptics and antiseptics.

The total of 482 patients underwent surgery in the Municipal Clinical Hospital № 15 from April 1 to May 23, 2020, which made up 5.29% of the total number of all hospitalized patients. Among patients with surgical diseases, there were 226 men (46.8%) and 256 women (53.2%), the average age was 57.2 ± 6.9 (Table 1).

In 436 patients (90.4%), the coronavirus infection was confirmed by the laboratory test results (nasopharyngeal swab, PCR). In other 46 patients (9.6%), the coronavirus infection had no laboratory confirmation, however, all the patients demonstrated clinical manifestations of highly probable viral pneumonia.

Highly probable viral pneumonia of varying severity was identified in 472 patients (97.9%). The patient distribution according to pneumonia severity is presented in Table 2.

Inclusion criteria: patients of both genders, of any age, with new coronavirus infection COVID-19 confirmed by PCR, and acute surgical disease, requiring emergency surgery. Exclusion criteria: no COVID-19 confirmation in patients of both genders.

Statistical processing was performed by standard methods using the Microsoft Excel application (Microsoft; USA).

RESULTS

The categories and number of surgical procedures performed in the hospital from April 1 to May 23, 2020 are listed in Table 3.

Of all operated patients, only 32 (6.6%) were placed in the intensive care units. The others were transferred to the ward from the operating room immediately after waking up.

DISCUSSION

Based on the data obtained, it is necessary to discuss the diagnosis and treatment of some acute surgical diseases in the context of conversion to the infectious diseases hospital for patients with COVID-19.

Prior to conversion to the infectious diseases hospital, the diagnostically "obscure" patients with suspected *acute appendicitis* were prescribed multislice spiral computed tomography (MSCT), but in the context of overburdened

Table 1. Patient age distribution

Age	Number of patients	% of patients' total number
18–44 years	107	22.3
44–60 years	192	39.9
60–75 years	104	21.5
75–90 years	73	15.1
> 90 years	6	1.2
Total:	482	100

CT-rooms the exploratory laparoscopy started to be used more often. Thus, of 36 patients with suspected acute appendicitis, the diagnosis was confirmed by exploratory laparoscopy in 28 people.

Some papers suggest upfront surgery instead of laparoscopy since it is believed that laparoscopic procedures increase the risk of medical staff infection [7, 8]. Other specialists perform laparoscopic appendectomy even in patients with viral pneumonia [12]. We decided not to change the usual surgical tactics. The total of 27 laparoscopic appendectomies was performed after the conversion to the infectious diseases hospital. The average duration of the procedure was 44.7 min (for reference, the average duration of 25 laparoscopic appendectomies performed in January–February 2020 was 42.4 min). In one patient, we used laparotomy, and performed the open appendectomy. No complications were observed during the postoperative period.

In the literature, different approaches to the treatment of *acute cholecystitis* in patients with somatic comorbidities are discussed [6, 7, 13]. During the described period, 16 patients with acute calculous cholecystitis were treated in the hospital. Nine of them received the effective non-surgical treatment, in 6 patients laparoscopic cholecystectomy was performed. Six patients had acute cholecystitis contributed to by viral pneumonia with CT-3–4 score of lung parenchymal lesion. These patients underwent the US-guided biliary drainage. The postoperative period was uneventful, the cholecystostomy tube was removed in 9–18 days after surgery.

Twelve patients with *acute destructive pancreatitis* were treated. The diagnosis was confirmed by MSCT, the US-guided percutaneous drainage of fluid was carried out. One patient diagnosed with destructive pancreatitis and severe (CT-4) viral pneumonia died.

Nine patients underwent surgery due to *acute bowel obstruction* contributed to by viral pneumonia (lung parenchymal lesion score CT-3–4). In 5 patients, the obstruction was associated with adhesions, therefore, the dissection of adhesions was carried out (in 2 patients it was performed by laparoscopy). In 4 patients, the obstruction was associated with the sigmoid and rectum tumor. Two of these patients underwent the sigmoid resection with colostomy due to the signs of tumor perforation, the other 2 patients underwent the double-barrel colostomy. Two patients with severe viral pneumonia (CT-3–4) and comorbidities died during the early postoperative period.

Three patients underwent surgery due to *strangulated hernias*. The 1st patient had strangulated inguinal hernia (herniorrhaphy was performed successfully followed by recovery), the 2nd female patient had giant ventral hernia with multiple comorbidities (herniorrhaphy was performed followed by the patient's death due to the comorbidities' severity), the 3rd female patient had diaphragmatic hernia on the 3rd day after the cesarean section. The patient underwent laparotomy, reduction of the stomach into the abdominal cavity and diaphragm defect closure. Postoperative course was uneventful, the patient was discharged from the hospital.

After conversion to an infectious diseases hospital, 3 patients underwent surgery due to *perforated ulcer of stomach or duodenum*. In all patients, the open suture of ulcers was carried out. One 92-year old female patient had diffuse peritonitis, the ulcer suture failure occurred during the postoperative period. Two relaparotomies were performed, the patient died on the 8th day after hospitalization.

The total of 34 patients had ulcers of stomach or duodenum complicated with bleeding. Emergency fibrogastroduodenoscopy (FEGDS) in patients with COVID-19 has an inherent risk of the endoscopy team infection, therefore, some experts recommend performing it only on special indications within 24 hours from the patient's hospitalization [7, 14]. Our patients underwent emergency FEGDS during the first 3 hours after hospitalization. Primary hemostasis was achieved during the endoscopic examination, however, the recurrent bleeding occurred in 12 patients. The repeated endoscopic hemostasis was effective in 11 of them. One patient underwent emergency surgery due to relapse: laparotomy, duodenotomy, suture repair of bleeding ulcer making full recovery.

Almost simultaneous hospitalization of 2 patients with *spontaneous spleen rupture* of the may be considered the feature of the described period. Of the disease symptoms, the patients complained of pain in the left half of the chest when coughing. In both patients the diagnosis was verified by ultrasonography. The patients' subcapsular hematomas volume was 55 and 120 ml. We selected the conservative "wait-and-see" approach. On the 1st day, no negative dynamics were detected, and on the 2nd day the hematoma volume increase to 150 and 220 ml respectively was observed with a strip of free fluid in the abdominal cavity. Both patients underwent splenectomy followed by full recovery.

Two patients hospitalized with severe pneumonia (CT-3–4) were supported by mechanical ventilation. They had *pneumoperitoneum and pneumomediastinum*. The surgeon on duty suspected perforation of the hollow organ, but no abdominal lesions were found during surgery. Both patients died due to increasing acute respiratory distress and multiple organ failure. The described cases require further analysis and evaluation.

Patients with *mesenteric thrombosis* should be discussed specifically. Even in normal conditions, the mortality in this group exceeds 75% [15]. The discussed group included 8 patients with mesenteric thrombosis. Four patients were hospitalized with symptoms of peritonitis. The total necrosis of small bowel and right side of the colon was detected during the emergency surgical procedure. Four patients underwent superior mesenteric artery thrombectomy with resection of small bowel. One patient underwent endovascular thrombectomy. However, despite anticoagulation with therapeutic dose, all patients had retrombosis, necrosis of the remaining part of the small bowel during the early postoperative period, and died.

The data obtained indicated a significant increase in the number of emergency and urgent surgical interventions in May

Table 2. Patient distribution according to pneumonia severity

Pneumonia severity	Number of patients	% of patients' total number
COVID-19 with no pneumonia	10	2
Pneumonia CT-1	118	24.5
Pneumonia CT-2	228	47.4
Pneumonia CT-3	85	17.6
Pneumonia CT-4	41	8.5
Total:	482	100

Table 3. Surgical procedures carried out in the Municipal Clinical Hospital № 15 after conversion to infectious diseases hospital for patients with COVID-19

Surgical procedures	April 2020	May 2020	Procedures number increase, %
General surgery			
Appendectomy	9	19	
Amputation (toes, lower leg, thigh)	14	25	
Sigmoid colectomy in patients with perotonitis	3	1	
Laparotomy, superior mesenteric artery thromboembolectomy in patients with mesenteric arterial thrombosis	2	2	
Adhesiolysis in patients with acute bowel obstruction	3	2	
Splenectomy	2	1	
Laparoscopic cholecystectomy	0	6	
Microcholecystostomy	2	4	
Strangulated hernia surgery	0	2	
Perforated ulcer suturing	0	2	
Percutaneous transhepatic biliary drainage (PTCD)	1	0	
Endoscopic retrograde papillotomy	5	5	
Phlegmon lancing	14	24	
TOTAL:	55	93	69.09
Cardiovascular surgery			
Coronary angioplasty	13	21	
Lower extremity arterial thrombectomy	12	21	
Inferior Vena Cava (IVC) filter placement	1	3	
Arteriovenous fistula formation	1	5	
Aortic prosthetic reconstruction	1	0	
Pacemaker insertion	0	4	
Coronary artery bypass grafting (CABG)	0	1	
Valve replacement surgery	0	2	
Stenting in the leg arteries	0	2	
Vascular catheter insertion	6	4	
TOTAL:	34	63	85.29
Trauma surgery			
Osteosynthesis	8	15	
Transpedicular fixation of the spine	0	1	
Hip replacement in patients with fractures	0	3	
TOTAL:	8	19	137.5
Neurosurgery			
Cranial trepanation in patients with intracranial hematomas	0	6	
TOTAL:	0	6	
Urologic surgery			
Nephrostomy	5	10	
TOTAL:	5	10	100
Other procedures (exploratory laparoscopy, primary surgical treatment of wounds, tracheostomy, thoracostomy, etc.)	92	98	
TOTAL:	194	289	48.96

2020 compared to April 2020. In April 2020, mainly patients with viral pneumonia caused by COVID-19 were hospitalized to the clinic, and only a few of them had emergency surgical diseases. The situation changed in May 2020. Due to opening of a number of other large clinics in Moscow for treatment of patients with COVID-19, the Municipal Clinical Hospital № 15 named after O. M. Filatov started to admit patients with acute surgical diseases contributed to by COVID-19.

The built up experience allows us to state that there are no fundamental changes in the management of patients with acute surgical diseases. However, in patients diagnosed with acute surgical disease contributed to by COVID-19 and viral pneumonia of varying severity, it is necessary to consider the increased risk of both bacterial and thromboembolic complications.

Based on the incomplete two months of work, it is difficult to draw conclusions about the features of the acute surgical disease course contributed to by viral pneumonia. However, it is obvious that at the peak of the COVID-19 incidence the correct management of surgical care in the infectious diseases hospital for patients with the new coronavirus infection makes it possible to establish balance between reducing the volume

of surgical care to the emergency level on the one hand and reducing the risk of infection of medical staff on the other hand.

CONCLUSION

In patient with acute surgical disease contributed to by COVID-19 and viral pneumonia of varying severity, the increased risk of both bacterial and thromboembolic complications should be considered. The surgical care features in the infectious diseases hospital for patients with COVID-19 are proper epidemiological regime implementation, minimization of the number of staff in the operating room, possible minimization of the number and reduction of the duration of surgical procedures. The most important challenge is medical staff safety.

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STRUCTURE OF ANXIETY ASSOCIATED WITH COVID-19 PANDEMIC: THE ONLINE SURVEY RESULTS

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The COVID-19 pandemic imposed not only serious threats to the physical health of the population, but also provoked a wide range of psychological problems. The study was aimed to define the structure of anxiety in the population during the epidemic period, as well as to identify the most vulnerable social groups (including individuals with affective disorders) which were most in need of psychological and/or psychiatric help. The online survey of 1957 Russian-speaking respondents aged over 18 was carried out from March 30 to April 5, 2020. The anxiety distress level was verified using the Psychological Stress Measure (PSM-25), the stigmatization of individuals experiencing respiratory symptoms was assessed using the modified Perceived Devaluation-Discrimination Questionnaire (PDD; Cronbach's $\alpha = 0.707$). In 99.8% of respondents, the combination of various concerns associated with COVID-19 was observed, the mean psychological stress score was increased to moderate level (score 104.9 ± 34.4), and the stigmatization score exceeded the whole sample median value (19.5±3.4; Me = 17). About 35% of respondents had concerns associated with anxiety distress (Cohen's d = 0.16–0.39): these were the "risk of social isolation" and the "possible lack of medication for daily use". The following groups of respondents were the most susceptible to the stress: people with affective disorders, young people (aged ≤20), unemployed persons, single persons, people with no formal education, and women. Thus, the broad sectors of the population need correction of anxiety distress associated with the COVID-19 pandemic. Therefore, the measures' implementation should be targeted, and in terms of coverage and content oriented to the identified vulnerable social groups.

Keywords: coronavirus infection, pandemic, COVID-19, mental health, anxiety, affective disorders, associated stigma

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Compliance with ethical standards: the study was performed in accordance with the World Medical Association Declaration of Helsinki (2013). All participants submitted the consent to personal data processing.

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СТРУКТУРА ТРЕВОЖНЫХ ПЕРЕЖИВАНИЙ, АССОЦИИРОВАННЫХ С РАСПРОСТРАНЕНИЕМ COVID-19: ДАННЫЕ ОНЛАЙН-ОПРОСА

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Пандемия COVID-19 не только создала серьезные угрозы для физического здоровья населения, но и вызвала широкий спектр психологических проблем. Целью работы было выявить структуру тревожных переживаний населения в период эпидемии и определить наиболее уязвимые социальные группы (в том числе среди лиц с аффективными расстройствами), больше всего нуждающиеся в психологической и/или психиатрической помощи. Было проведено онлайн-анкетирование 1957 русскоговорящих респондентов старше 18 лет в период с 30 марта по 5 апреля 2020 г. Уровень тревожного дистресса верифицировали по шкале психологического стресса (PSM-25), стигматизацию лиц с респираторными симптомами — по модифицированному опроснику обесценивания/дискриминации (PDD; Cronbach's $\alpha = 0{,}707$). У 99,8% респондентов обнаружено сочетание нескольких типов тревожных переживаний о COVID-19, показатель стресса был повышен до уровня средней интенсивности (104,9 ± 34,4 балла), а показатель стигматизации превосходил медианное значение по выборке (19,5 ± 3,4; Me = 17). До 35% респондентов имели опасения, ассоциированные с тревожным дистрессом (Cohen's d = 0,16–0,39): «риск изоляции» и «возможное отсутствие лекарств для ежедневного приема». Особенно подверженными психологическому стрессу оказались страдающие аффективными расстройствами, лица молодого возраста (≤ 20 лет), безработные, холостые/незамужние, не имеющие высшего образования и женщины. Таким образом, широкие слои населения нуждаются в коррекции дистрессовых опасений на фоне пандемии COVID-19, поэтому их проведение должно быть адресным, ориентированным по степени охвата и содержанию на выявленные уязвимые социальные группы.

Ключевые слова: коронавирусная инфекция, пандемия, COVID-19, психическое здоровье, тревога, аффективные расстройства, ассоциированная стигма

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First cases of a novel coronavirus infection, caused by the SARS-CoV-2 coronavirus, (COVID-19 from COrona VIrus Disease 2019) were detected in November 2019 [1]. The infection spread quickly in Wuhan (the capital of the Chinese province of Hubei), then throughout whole China, and later spread to other countries including the Russian Federation leading to a global public health emergency [2]. As early as March 11, 2020, due to the high prevalence of COVID-19 cases, the World Health Organization (WHO) announced the current situation as a pandemic [3]. First patients with COVID-19 in the Russian Federation were identified on January 31, 2020. In early April, more than 5,000 Russians had confirmed diagnoses [4, 5].

The COVID-19 pandemic imposed serious threats to people's physical health and life. Moreover, the risk of coronavirus infection caused a wide range of psychological problems among the population of countries with a high spread of viral infection, such as panic, anxiety, and depression [6]. Since March 2020, many governments around the world have introduced specific quarantine measures to limit the spread of the virus and minimize the burden on healthcare services. People over 65, individuals with comorbidities and pregnant women were proposed to isolate themselves from direct contact with other people for at least 12 weeks, and the patients suspected of carrying coronavirus together with those living with them were instructed to stay at home and isolate themselves for at least 14 days [7].

Thus, the current situation involves a number of factors significantly affecting the mental health of the population:

1) unprecedented, potentially life-threatening situation of indefinite duration;

2) large-scale quarantine measures in all major cities, which force the residents to stay at home;

3) undefined viral infection incubation period and its possible transmission from asymptomatic patients;

4) reported lack of protective remedies for medical professionals;

5) unstable information background with excess controversial data;

6) uncertainty related to the possible COVID-19 coronavirus infection impact on the economic situation in the country.

According to Chinese researchers, the COVID-19 coronavirus infection pandemic provoked a parallel epidemic of anxiety and depressive reactions [8, 9]. Moreover, certain sectors of the population may be more vulnerable to psychological stress associated with the disease. This is especially true for individuals with affective disorders who are more susceptible to the COVID-19 pandemic related emotional responses, which could manifest in mental symptoms relapses or worsening. This is due to such patients' high sensitivity to stress compared to the general population, and also due to the scheduled psychiatric outpatient appointment limitations. Furthermore, in addition to stress level escalation among the population, stigmatization and discrimination against certain sectors of the population increase [10], even with no evidence of increased morbidity risks in the discriminated groups.

The study was aimed to reveal the structure of anxiety in the population during the epidemic period, as well as to identify the most vulnerable social groups (including individuals with affective disorders) which were most in need of psychological and/or psychiatric help.

METHODS

Data acquisition

The data was acquired using the online survey which was carried out from March 30 to April 5, 2020. The participants were proposed to complete the Questionnaire via the Google Forms online platform, which on average took about 15 minutes. The Questionnaire was distributed through social media, as well as via websites of public organizations and communities of interest (see Acknowlegements).

Inclusion criteria: skill of reading in Russian, submitted consent to personal data processing (completion of all proposed Questionnaire forms was considered a consent). Exclusion criteria (defined as freely as possible in order to represent as many social groups as possible among the respondents): 1) age <18; 2) blank sections in the Questionnaire.

The Questionnaire included social and demographic information about the respondents, as well as the information on the presence or absence of affective disorders (major depressive disorder, bipolar affective disorder, generalized anxiety disorder, cyclothymia, dysthymia) and somatic pathology.

The participants were proposed to mark any amount of 10 Questionnaire paragraphs describing various types of concerns associated with the COVID-19 pandemic, as well as any amount of 6 behavioral patterns of infection prevention (for the Questionnaire full version see Appendix). Furthermore, the respondents could determine how often they requested information about a pandemic during the last week in the range from "never' to "every hour" (according to the 8-point scale). Questions from the Psychological Stress Measure (PSM-25) were used for the anxiety distress assessment [11]. Based on the widely used Perceived Stigmatization Questionnaire (Devaluation-Discrimination section, PDD) [12], the statements

Table 1. Social and demographic features correlation with psychological and behavioral responses related to COVID-19

	Information about COVID-19 search frequency	Number of concern themes associated with COVID-19	Number of COVID-19 infection prevention measures	Stress (PSM-25)	Stigmatization
Age	0.06***	–0.23***	–	–0.38***	0.06**
Education	0.08***	–0.15***	–	–0.22***	–
Information about COVID-19 search frequency	1.000	–	–	–	–
Number of concern themes associated with COVID-19	0.22***	1.000	–	–	–
Number of COVID-19 infection prevention measures	0.17***	0.30***	1.000	–	–
Stress (PSM-25)	0.14***	0.28***	0.05*	1.000	–
Stigmatization	0.10***	0.12***	0.12***	–	1.000

Note: Spearman's rank correlation coefficients; $N = 1957$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq .001$.

were formulated describing the negative perception of people with the signs of cold (coughing, runny nose, sneezing). The levels of agreement with the Questionnaire statements were evaluated using the 4-point Likert scale. The higher total scores corresponded to more severe stigma intensity.

Statistical analysis

Statistical data processing was carried out using the SPSS-16 software package (SPSS Inc.; USA). The descriptive statistics were used. Distribution normality test was performed using the skewness and kurtosis calculation. Dispersion for nominal scales was analyzed using the Pearson's χ^2 test, and the data for ordinal scales were obtained using the Mann–Whitney U-test. Effect sizes obtained using the Cohen's d and Cramer's V measures were calculated for groups, the differences between which had the significance level $p \leq 0.05$. When comparing the nominal data with more than two gradations, the interpretation of the effect size was carried out adjusted for the number of degrees of freedom and indicator threshold values for a weak/moderate/strong effect. Spearman's rank correlation coefficients were calculated. The original stigmatization Questionnaire internal consistency assessment was performed using the Cronbach's α.

RESULTS

The final register included 2117 records obtained during the 1st week of the self-isolation regime recommended in Russia (from March 30 to April 5). The data of 160 respondents were excluded from analysis due to age. Thus, the statistical analysis of the 1957 respondents' data was carried out.

Demographics

Among the participants women prevailed (1649 people, 84.3%). The average age of the respondents was 31 (Me = 27; Q_{25} = 22, Q_{75} = 38). The sample included people living in the cities of federal importance (Saint-Petersburg, 21.1%, Moscow, 16.8%), all federal districts of Russian Federation (57.6%), and abroad (4.5%).

Social characteristics

About a half of all respondents had a university degree (55.3%). The 25.6% of participants reported on the incomplete higher education. The majority of respondents were employed in private (23.6%) and public (32.2%) organizations. The medical professionals made up 10.3% of the sample. The 22.2% of participants had no permanent employment. The 51.8% of the sample were single. The 26.9% of respondents were officially married, and the 12.4% lived in the *de facto* marriage.

Comorbidities

The 54.8% of respondents reported on the concominant somatic pathology. The 29.5% of participants confirmed they were diagnosed with affective disorders. Most often the participants mentioned major depressive and bipolar affective disorders (19.8%), and less frequently the anxiety disorders (6.0%), cyclothymia or dysthymia (3.7%).

Characteristics of the participants' psychological and behavioral responses

Correlation analysis of the whole data set demonstrated that adaptation that adaptation to new living conditions during the COVID-19 spread was a multi-level process with a complex structure of interrelated factors. The higher number of strategies used for coronavirus infection prevention (4 on average: Me = 4; Q_{25} = 3, Q_{75} = 4) and the more frequent search for the epidemic information (twice a day on average: Me = 6; Q_{25} = 5, Q_{75} = 7) correlated in a predictable way. These were associated with the respondents' psychological reactions to the pandemic intensification: the number of anxious concerns about COVID-19 increased, as well as the associated psychological stress level and the tendency to stigmatize people with respiratory symptoms. Almost all of the mentioned above characteristics were also sensitive to the social and demographic parameters of the sample (Table 1).

The 99.8% of the study participants reported at least two coronavirus related concern themes, and the most common themes number was 5 (Me = 5; Q_{25} = 4, Q_{75} = 6) (Table 2). The anxiety responses diversity was associated with the psychological stress measure (PSM-25) reaching the moderate level of 104.9 in the whole sample (Me = 106; Q_{25} = 80, Q_{75} = 130). Qualitative analysis of the relationship between the specific COVID-19 associated concern themes and the psychological stress and people with respiratory symptoms stigmatization/discrimination levels revealed the multidirectional effects of specific concerns.

The concern about the threat to the life and health of relatives was not associated with significant stress level or stigmatization increase. Possibly, it was due to the maximum

Table 2. Types of COVID-19 related concern themes and corresponding levels of anxiety distress and people with respiratory symptoms stigmatization

Concern type	Prevalence (people/%)	Indicators change (SE):	
		stress	stigma
Threat to the life and health of relatives and important people	1527 / 77.2	– *	+0.06
Possible financial difficulties	1128 / 57.0	+0.16	–0.04
Harsh social consequences	980 / 49.5	+0.14	–0.08
Lack of specific treatment for COVID-19	789 / 39.9	+0.1	+0.19
Disrupted normal routine	766 / 38.7	+0.17	–0.16
Virus transmissibility	708 / 35.8	+0.1	+0.27
Threat to the own life	619 / 31.3	+0.14	+0.36
Lack of commercially available protection remedies	544 / 27.5	+0.16	+0.23
Possible lack of medication for daily use	434 /21.9	+0.39	+0.19
Risk of social isolation	351 / 17.7	+0.43	–0.14

Note: effect size (SE) is considered weak when $0.2 \leq$ Cohen's $d \leq 0.49$; $p \leq 0.05$.

Table 3. Anxiety experience features depending on the respondents' health group

Concern themes associated with COVID-19		Healthy people $n = 643$	Disorders			Significance level
			Affective disorders $n = 242$	Somatic disorders $n = 737$	Comorbidities $n = 336$	
Risk of social isolation	+	11.0%	21.5%	17.9%	31.0%	$x^2 = 63.8$; $p = 0.000$ SE = 0.25
	–	89.0%	78.5%	82.1%	69.0%	
Lack of medication for daily use	+	22.2%	34.3%	14.4%	30.4%	$x^2 = 59.6$; $p = 0.000$ SE = 0.21
	–	77.8%	65.7%	85.6%	69.6%	

Note: effect size (SE) is considered medium when 0.17≤Cramers's $V \leq 0.29$.

experience prevalence in the vast majority of respondents. At the same time, the clinically significant psychological stress increase (weak in magnitude) was associated with the two (of 10) most rare concern themes: the "possible lack of medication for daily use" and the "risk of social isolation" (Table 2). In total, 688 study participants (35% of the sample) reported experiencing at least one of those concerns.

The average total score for the Questionnaire on the people with respiratory symptoms stigmatization was 19.5, with Me = 17 (Q_{25} = 15, Q_{75} = 19) and sufficient internal consistency of the instrument (Cronbach's α = 0.707). The "risk of social isolation" was associated with a significant decrease in the respondents' tendency to stigmatize people with respiratory symptoms. However, the stigmatization increase effects became practically significant only in people with the "virus transmissibility", "threat to the own life" and "lack of commercially available protection remedies" concerns.

Psychological reactions of specific sectors of the population

Among the groups of respondents, the specific concern themes had some features. Two themes most closely related with psychological stress were observed in participants who had reported being diagnosed with affective disorders (Table 3). Moreover, the "risk of social isolation" caused apprehension mostly in individuals with comorbid affective and somatic disorders. At the same time, the "lack of medication for daily use" concern theme was more frequently reported by participants with affective disorders and no comorbidities.

It is important to note, that among 688 participants reporting at least one of the two main psychological stress associated concern themes, the respondents without mental disorders were as common as those with affective disorders. Unexpectedly, the external validity of the online Questionnaire was confirmed by the prevalence of the specific for people with anxiety disorders fear for their security, which distinguished them from people with mood disorders (Table 4).

In addition to traditional sectors of the population considered most vulnerable to anxiety reactions (patients with affective and somatic disorders) many other cohorts demonstrated various prevailing concerns about COVID-19. Thus, women were worried about the lack of commercially available protection remedies and threat to their own life more often than men (Table 4). Single people, as well as the unemployed and public institutions employees were more likely to be aware of social isolation (Table 5).

In respondents with higher education and academic degrees, as well as in people over 31, the concerns about the risk of social isolation were significantly fewer. A group of participants over 60 tended to be the most wary of financial difficulties the possibly caused by pandemic (Table 6).

DISCUSSION

The data coming from the online survey made it possible to assess the structure of psychological experience characteristic for Russian-speaking respondents during the first week of the proposed self-isolation regime in Russia. The analysis demonstrated high prevalence of various COVID-19 pandemic associated anxiety trends among the study participants, which cumulatively increased the total psychological stress level in the surveyed sample.

Amidst the changing due to quarantine measures living conditions and routine, various COVID-19 pandemic related concerns predictably arose in the respondents. It is essential to note that concerns about the "threat to the life and health of relatives and important people" did not lead to the psychological stress level increase. Therefore, those could be considered adaptive personality and psychological reactions. At the same time, the concern themes number expansion led to the breakdown of adaptive mechanisms, provoking both the intensification of psychological (higher anxiety) and social stress. The social stress was consciously or unconsciously projected outside, causing the increased stigma. It is important

Table 4. Anxiety experience features depending on the affective disorder type and gender

Concern themes associated with COVID-19		Representation by groups		Significance level
		1	2	
Gender: 1 — men, 2 — women				
Lack of protective remedies	+	20.1%	29.2%	$x^2 = 10.7$; $p = 0.001$ SE = 0.14
	–	79.9%	70.8%	
Threat to the own life	+	23.1%	33.2%	$x^2 = 12.4$; $p = 0.000$ SE = 0.14
	–	76.9%	66.8%	
Affective disorder type: 1 — mood disorder, 2 — anxiety disorder				
Threat to the own life	+	26.7%	40.7%	$x^2 = 8.8$; $p = 0.003$ SE = 0.12
	–	73.3%	59.3%	

Note: effect size (SE) is considered weak when 0.1 ≤ Cramers's $V \leq 0.3$.

Table 5. Anxiety experience features depending on the occupation and marital status

Concern themes associated with COVID-19		Representation by groups:					Significance level
		1	2	3	4	5	
Occupation: 1 — student, *n* = 271; 2 — unemployed, *n* = 435; 3 — private sector employee, *n* = 462; 4 — public sector employee, *n* = 631; 5 — businessman, *n* = 158							
Risk of social isolation	+	19.6%	25.1%	8.2%	20.9%	12.0%	x^2 = 52.6; *p* = 0.000 SE = 0.19
	–	80.4%	74.9%	92.8%	79.1%	88.0%	
Marital status: 1 — widowers/widows, *n* = 30; 2 — divorced, *n* = 144; 3 — single, *n* = 1014; 4 — *de facto* marriage, *n* = 243; 5 — registered marriage, *n* = 526							
Risk of social isolation	+	3.3%	13.2%	22.5%	16.9%	11.8%	x^2 = 34.5; *p* = 0.000 SE = 0.16
	–	96.7%	86.8%	77.5%	83.1%	88.2%	

Note: effect size (SE) is considered medium when 0.15 ≤ Cramers's *V* ≤ 0.25.

that psychological stress escalated notably amid the "possible lack of medications for daily use" and the "risk of social isolation" concerns. The first could be due to the subjective perception deterioration, and the second due to quarantine measures provoking a wave of anxiety and anger itself. The stigmatization attitudes increase turned out to be related mostly with the following experiences: "threat to one's own life", "virus transmissibility", and "lack of commercially available protection remedies", which were, to a greater extent, caused by the feeling of loss of control over the situation.

Noteworthy was the data obtained from respondents who reported being diagnosed with affective disorders. For them, as well as for individuals who had not reported any mental disorders, the same types of categories most closely related with psychological stress were common: the "risk of social isolation" and "lack of medications for daily use". However, the participants with comorbid affective and somatic disorders were more wary of "social isolation". At the same time, the "possible lack of medication for daily use" often worried respondents with affective disorders and no somatic comorbidities. Moreover, in people with anxiety disorders compared to participants with affective disorders, the prevalence of a specific "threat to their own life" concern was observed, which emphasized the clinical diversity of their experience.

The obtained data on the respondents' anxious experience structure make it possible to distinguish the features of different sectors of population, which is important for the further design of differentiated psychological and social assistance programs. In particular, the "risk of social isolation" concerns are most common in young respondents (under 31), single people, individuals with no formal education and the unemployed, as well as in people with comorbid affective and somatic disorders. In the first three social groups this may be due to personal immaturity, unformed self-control and self-employment skills, as well as to temporary loss of the ability to communicate. In the unemployed people, the main reason is the financial support reduction. In the older age group the "financial difficulties" become a specific concern theme, which obviously calls for different informational and social interventions.

The WHO COVID-19 Strategic Preparedness and Response Plan includes no strategies addressed the emerging mental health needs [13]. Although, the need for such strategies is likely to increase both during the epidemic and after it.

There are no literature data on psychological reactions at the initial stages of the epidemiological situation deterioration and the quarantine announcement (the official epidemiological distress increase endorsement). In China, which was the first to deal with the medical care organization in order to limit the spread of coronavirus, the Principle for Supporting Mental Well-being was developed. The Principle included the following: 1) determining the current status of mental health in the population; 2) determining the group of people at high risk of suicide and aggression; 3) developing the structured assistance measures [14]. However, the psychological assistance effectiveness in the region was considered insufficient, which was due to the lack of experience in teaching the mental health maintenance principles [15].

Thus, the size and social heterogeneity of the risk group require the use of broader social interventions to overcome the pandemic social and psychological consequences. The interventions which may be implemented in accordance with the aid separation principle should include the following: psychosocial support stage, specialized psychological assistance, and clinical and psychological assistance involving psychiatrists. Based on the Chinese experience of psychological assistance arrangement, as well as our data, stigmatization/discrimination may be one of the barriers making it impossible to establish the effective population assistance service [16].

Table 6. Anxiety experience features depending on the education and age

Concern themes associated with COVID-19		Representation by groups						Significance level
		1	2	3	4	5	6	
Education: 1 — incomplete secondary, *n* = 31; 2 — secondary, *n* = 98; 3 — professional, *n* = 164; 4 — incomplete higher, *n* = 501; 5 — higher, *n* = 1082; 6 — academic degree, *n* = 81								
Risk of social isolation	+	25.8%	24.5%	22.6%	24.0%	14.5%	6.2%	x^2 = 35.1; *p* = 0.000 SE = 0.14
	–	74.2%	75.5%	77.4%	76.0%	82.5%	93.8%	
Age: 1 — 18–20 years, *n* = 310; 2 — 21–30 years, *n* = 859; 3 — 31–40 years, *n* = 363; 4 — 41–50 years, *n* = 231; 5 — 51–60 years, *n* = 136; 6 — 60–78 years, *n* = 58								
Risk of social isolation	+	28.7%	20.0%	14.3%	8.2%	8.8%	12.1%	x^2 = 54.0; *p* = 0.000 SE = 0.18
	–	71.3%	80.0%	85.7%	91.8%	91.2%	87.9%	
Financial difficulties	+	31.3%	32.2%	30.6%	29.9%	30.9%	39.7%	x^2 = 101.6; *p* = 0.000 SE = 0.13
	–	68.7%	67.8%	69.4%	70.1%	69.1%	60.3%	

Note: effect size (SE) is considered medium when 0.13 ≤ Cramers's *V* ≤ 0.22.

Study limitations. The study results were obtained using the respondents' reports. Although the reports' correlation with the objective experimental psychology test results is usually quite high, the additional profiles verification via expert assessment performed by the researcher may lead to corroboration increase. At the same time, the cross-cutting evaluation would significantly limit the sample size and participation of various sectors of the population (including through experiences of stigma of respondents with affective disorders), as well as scale the period of the first results acquisition. Furthermore, in the context of strongly recommended physical distancing the possibility of face-to-face counseling involving the visit to the clinic is extremely limited. Besides, the rates of epidemic process development and the population psychological response to the COVID-19 pandemic determine the importance of the research promptness and psychological and psychiatric assistance recommendations statement based on the research results.

It is worth noting, that the revealed strength of correlations between the population psychological reactions, applied infection prevention measures and the frequency of searching for information about the pandemic corresponded only to the weak or moderate level of signs association. The described above situation is quite typical for the human psychology studies. On the one hand, this illustrates the inadmissibility of interpreting the correlation as causal even in the context of the study (when the observed features are semantically closely related). On the other hand, the revealed relationships' strength emphasizes the behavior regulation multidimensional nature, when none of the parameters can be considered determinative.

CONCLUSION

Psychological crisis intervention should be considered an important part of a public health response to the COVID-19 outbreak. The greater involvement of competent specialists in infectious diseases, epidemiology and mental health by the mass media in order to inform the citizens about the effective infection and psychological stress increase prevention measures as opposed to flooding the Internet unprofessional judgments should become the first step. Moreover, the obtained results make it possible to recommend the broader but more precise correction of distress concerns. Additional attention should be paid not only to patients with affective disorders, but also to young people, women, people with no formal education, out-of-work and out-of-school people, and single individuals. The most urgent need for the COVID-19 pandemic anxiety correction was revealed in people aware of the possible lack of medication for daily use and the risk of social isolation, since these types of experience are related to the maximum anxiety distress escalation in the surveyed sample. The access to individual online psychological counseling establishment for general public at the state level, as well as the arrangement of psychological and psychiatric assistance for people in need providing the adequate epidemiological safety, are extremely important, since the psychological stability contributes to the population physical health maintenance.

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THE USE OF ELECTRONIC DEVICES BY STUDENTS, PARENTS AND TEACHERS BEFORE AND AFTER THE TRANSITION TO DISTANCE LEARNING

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Transition to distance education in spring 2020 led to the overuse of information and communication technologies by the participants of the educational process. The aim of this study was to characterize the patterns of using electronic devices in high school students, their parents, and teachers in the settings of traditional brick-and-mortar education and distance learning. We created online questionnaires that were used to survey 200 high school students, 389 teachers and 251 parents before the transition to distance learning and also 658 teachers and 500 parents after the transition. Statistical analysis was conducted using Student's t test, χ^2, and Pearson's contingency coefficient; relative risks were calculated using fourfold contingency tables. Differences were considered significant at $p \leq 0,05$. After the transition to distance learning, the number of electronic devices used by each student increased for 96.6% of the surveyed students; the average screen time also increased. About 80% of the surveyed parents reported that their children had more health complaints; of them, 60% reported symptoms typical of computer vision syndrome. We established a correlation between the readiness to cut down on screen time and the subjective assessment of vision as perfect or good by the respondents (Pearson's contingency coefficient 0.3; $p \leq 0.05$). Our study confirms the relative risk for subjectively assessing one's vision as satisfactory or poor in individuals who use ED on a daily basis; the risk is 1.13 for students, 1.41 for parents, and 1.27 for teachers ($p \leq 0.05$). The study proves that eliminating screen time from daily activities for at least one day per week is an effective measure for preventing vision disorders.

Keywords: students, teachers, parents, distance learning, health risk behavior, electronic devices, information and communication technologies

Author contribution: Milushkina OYu, Popov VI, Skoblina NA planned and supervised the study, analyzed the obtained data and wrote the manuscript; Markelova SV, Sokolova NV analyzed the literature, collected and processed the questionnaires. All authors participated in manuscript revision.

Compliance with ethical standards: the study was approved by the Ethics Committee of Pirogov Russian National Research Medical University (Protocol № 159 dated November 21, 2016). The survey did not encroach upon human rights, did not expose the respondents to any dangers and complied with the principles of biomedical ethics.

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ИСПОЛЬЗОВАНИЕ ЭЛЕКТРОННЫХ УСТРОЙСТВ УЧАСТНИКАМИ ОБРАЗОВАТЕЛЬНОГО ПРОЦЕССА ПРИ ТРАДИЦИОННОЙ И ДИСТАНЦИОННОЙ ФОРМАХ ОБУЧЕНИЯ

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Внедрение дистанционного обучения весной 2020 г. привело к увеличению интенсивности использования информационно-коммуникационных технологий участниками образовательного процесса. Цель работы — дать гигиеническую характеристику использования электронных устройств школьниками, их родителями и учителями организаций общего образования в условиях различных режимов обучения (традиционного и дистанционного). В исследовании посредством онлайн-опроса приняли участие 200 школьников, 389 учителей и 251 родитель в период традиционного обучения, 658 учителей и 500 родителей — в период дистанционного обучения. При статистической обработке результатов использовали *t*-критерий Стьюдента, критерий χ^2, коэффициент сопряженности Пирсона, относительный риск определяли с помощью четырехпольных таблиц сопряженности, $p \leq 0,05$. В период дистанционного обучения увеличились число используемых электронных устройств у 96,6% школьников и время работы с ними. Увеличение числа жалоб на самочувствие учащихся отметили до 80% родителей, из них более 60% указывали на симптомы, характерные для компьютерно-зрительного синдрома. Установлена связь между возможностью отказа от использования электронного устройства и субъективной оценкой респондентами своего зрения как «отличное» и «хорошее» (КС Пирсона 0,3; $p \leq 0,05$). Подтвержден риск субъективной оценки зрения как «удовлетворительное» и «плохое» при ежедневном использовании электронного устройства: для школьников — 1,13, родителей — 1,41, учителей — 1,27 ($p \leq 0,05$). Обоснован в качестве меры профилактики нарушения зрения отказ от использования электронного устройства минимум на один день в неделю.

Ключевые слова: школьники, учителя, родители, дистанционное обучение, поведенческие риски, электронные устройства, информационно-коммуникационные технологии

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The regulatory framework for e-learning and distance education technologies was first captured in Russian legislation back in 2012 [1]. However, at that time teachers were not ready for the new teaching modalities, lacked knowledge of computer workstation ergonomics and safety, and did not fully understand the importance of good practices for safe computer use [2].

Nevertheless, information and communication technologies (ICT) have been gradually integrated into the educational process, and it is now hard to imagine education without computers, interactive digital boards, the Internet, or audio and video resources. Smartphones, tablets, and computers have become a vital part of daily lives of children, adolescents, and young adults. Today, there are about 10 million active Internet users below 14 years of age in Russia [2–6].

Because e-learning is a multifaceted educational challenge, some of its aspects are yet to be addressed, including workstation safety and ergonomics. New requirements for safe computer use imposed on education providers cannot solve the complexity of problems encountered in e-learning today [7–11].

The coronavirus pandemic of spring 2020 and the implemented containment measures dictated the need for transitioning from traditional brick-and-mortar education (TE) to distance learning (DL). This resulted in the overuse of ICT by all groups of the population, including students. The foreign literature on the transition from TE to DL discusses its economic implications, such as staff redundancy or saving costs, and learning outcomes (by comparing the results of final tests before and after the transition to DL), but do not mention the barrage of health complaints and the ways of preventing computer-associated damage to students' health [12–13].

The mass spread of electronic devices (ED) driven by the occupational and social necessity puts ED users at risk for various health conditions in a situation when good computer use practices are not followed. It is reported that young ED users suffer from increased emotional strain, irritability, sleep problems, and addiction [14–19]. The lack of physical activity and awkward static posture negatively affect the musculoskeletal, respiratory and cardiovascular systems, cause attention deficit and poor memory retention [20–23]. Poor image quality, wrong viewing distance, excessive or low lighting, and continuous screen time are all risk factors for vision disorders [24–27].

The high incidence of vision disorders witnessed over the past 15 years in children and adolescents has coincided with the mass spread of ED [28–29].

Increased use of ICT (stationary computers and portable devices) has resulted in high information load on students. Unfortunately, the existing screen time requirements are ignored [28].

Awareness should be raised about the possible detrimental impact of ED on the health of ED users, especially children; students should be taught skills allowing them to minimize the risk of ED-associated health problems in a situation when the teacher cannot control the ergonomics of their workstations. This means more responsibility on parents, who are expected to take proper care of their child's health and create the best environment for healthy DL from home.

So far, there have been no studies of health-related aspects of mass transition to DL in Russia.

The aim of this study was to characterize the patterns of using electronic devices in high school students, their parents, and teachers in the settings of traditional brick-and-mortar education and distance learning.

METHODS

Teachers working at the Department of Hygiene (Faculty of Pediatrics, Pirogov Russian National Research Medical University) and certified in hygiene education, hygiene of children and adolescents, and general hygiene designed 5 questionnaires and uploaded them to Google Forms. The sensitivity of the online vs. offline questionnaires was slightly below 82% (CI: 80.5–83.5); their specificity was at least 90% (CI: 88.1–92.2). The questionnaires for health assessment in the TE period were designed to target students, their parents and teachers (Appendix 1); the questionnaires for health assessment in the DL period were developed for parents and teachers only (Appendix 2). The following inclusion criteria were applied: being a high school student, a parent of a high school student or a high school teacher; the correctly filled out questionnaire form. Exclusion criteria: not being a high school student, a student's parent or a high school teacher; inability to correctly fill out the questionnaire form. The questionnaires contained 4 sections: background information, health complaints, questions about the patterns of using stationary and portable ED, questions about the skills necessary to safely use ED.

Brick-and-mortar education, 2019

Before the transition to distance learning, 200 high school students (grades 9–11) were surveyed from 8 Russian regions, including cities with over 1,000,000 residents; 43% of them attended public schools with traditional curricula, the rest attended schools with advanced curricula, lyceums, etc.

Of 251 surveyed parents residing in 15 Russian regions, including cities with over 1,000,000 population, the majority (86%) were 30–49 years old. Over 85% of them were women.

Of 389 teachers residing in 25 Russian regions, including cities with over 1,000,000 population, 31% were 40–49 years of age. Over 90% of them were women.

Distance learning, 2020

In the DL period, 500 parents were surveyed from more than 15 Russian regions, including cities with over 1,000,000 residents. The majority of them (> 90%) were women. The parent sample was dominated by individuals aged 30 to 49 years (89%).

We also surveyed 658 teachers from over 30 Russian regions, including cities with a population of over 1,000,000.

Table 1. Subjective vision assessment and behavioral risk factors for ED overuse in high school students, their parents and teachers in the brick-and-mortar setting, %

Risk factor	High school students	Parents	Teachers
No screen breaks	64.5 ± 3.4	63.0 ± 3.1	53.1 ± 2.5*
Dim lighting	87.0 ± 2.4	87.3 ± 2.1	81.2 ± 2.0*
Wearing digital protecting lenses	5.5 ± 1.6	7.5 ± 1.7	11.0 ± 1.6
Subjective assessment of vision as satisfactory or poor	49.0 ± 3.5	51.4 ± 3.2	59.4 ± 2.5*

Note: * — differences are significant at $p \leq 0.05$.

Table 2. Subjective vision assessment by high school students, parents and teachers who were willing or unwilling to reduce the amount of screen time, %

Reducing screen time	High school students	Parents	Teachers
Not possible	24.0 ± 3.0	41.8 ± 3.1	38.4 ± 2.5
Can do without ED 1-3 days a week	38.5 ± 3.4	33.1 ± 3.0	47.8 ± 2.5*
Can do without ED more than 3 days a week	37.5 ± 3.4	25.1 ± 2.7	33.5 ± 2.4*
Subjective vision assessment as perfect or good	51.0 ± 3.5	48.6 ± 3.2	40.6 ± 2.5*

Note: * — differences are significant, $p \leq 0,05$.

The majority of them (> 85%) were women. The most prevalent age group (32%) was 40 to 49 years.

Statistical analysis was conducted in Statistica 13.0. (StatSoft Inc.; USA). The following statistics were used: Student's t test, χ^2, Pearson's contingency coefficient. Relative risk (RR, i.e. the probability of a certain outcome depending on the environmental factor) was determined using fourfold contingency tables. Differences were considered significant at $p < 0.05$.

RESULTS

In the TE period, the respondents were asked about their skills for safe ED use and measures they were possibly taking to prevent ICT-associated health damage.

Most high school students and their parents (64.5% and 63.0%, respectively) reported that they took screen breaks less often than once every hour; one in 6 teens (17.0%) and one in 6 parents (17.5%) did not take any breaks at all while using their ED (Table 1).

Of all the teachers surveyed in that period, over half of the respondents (53.1%) took breaks less often than once every hour; one in 7 teachers (15.2%) did not take any breaks at all (Table 1).

We established that only 1 in 8 students (13%) and parents (12.7%) preferred not to work on their ED if the lighting was dim, which is a standard ergonomics recommendation, whereas every third student (37%) and every fifth parent (20.7%) would continue using their device despite poor lighting conditions (Table 1).

One in 5 teachers (18.8%) wrote that they would stop using ED if the lighting was poor, whereas one in 8 teachers (12.9%) would continue using their ED (Table 1).

The majority of the surveyed teachers (86.6%) think it necessary to promote healthy lifestyle in their students from early age. At the same time, 26.5% do not organize small screen breaks during the lesson and about half of the teachers (44.4%) do not do eye exercises with their students, i.e. do not take measures aimed at maintaining their students' health, which is part of their professional duties. Interestingly, most teachers (67.5%) report health problems in their students.

Half of the students (51%) and half of the parents (48.6%) assessed their vision as perfect (24.5% and 19.5%, respectively) or good (26.5% and 29.1%, respectively). One in 6 students (18.0%) and one in 3 parents (31.5%) said their vision was satisfactory; poor vision was reported by one in 3 students (31.0%) and one in 5 parents (19.9%). Perfect vision was reported by 10.7% of the teachers; good, by 29.9%; satisfactory, by 46.0%; and poor, by 13.4% (Table 1).

Only half of the students (49.0%) said they did not need screen protection glasses; this percentage was consistent with the proportion of students who thought their vision was perfect or good. Only 5.5% of the students said that they wore computer glasses when working on their computers, whereas others either denied wearing computer glasses or never used glasses at all (Table 1).

No need for vision correction was reported by 47.4% of the parents and 40.6% of the teachers. These figures were comparable to the proportion of adult respondents who thought their vision was perfect or good. Only 7.5% of the parents and 11.0% of the teachers wore computer glasses; others did not wear glasses although their vision acuity was poor, or wore glasses unsuitable for working with ED (like distance glasses) (Table 1).

Contingency tables revealed that about 50% of the surveyed students, parents and teachers did not pay due attention to their deteriorating vision or wore unsuitable glasses ($p \leq 0.05$).

Based on the survey results, we distributed the respondents into several groups depending on their readiness to reduce the amount of screen time (Table 2).

There were significant differences in the subjective assessment of vision acuity and the readiness to reduce the amount of screen time (having 1 or more days a week free of ED) between the teachers and parents ($p \leq 0.05$) (Table 2).

Of all the students, parents and teachers who emphasized they could not do without ED, one-third (37.5%, 38.1% and 36.0%, respectively) subjectively assessed their vision as perfect or good; of those who could do without ED longer than 3 days, good or perfect vision was reported by a significantly higher number of respondents (64.0%, 58.75% and 48.0%, respectively; $p \leq 0.05$).

A correlation was established between the readiness to cut down on screen time and the subjective assessment of vision as perfect or good (Pearson's contingency coefficient equaled to 0.3; $p \leq 0.05$).

During the DL period, significantly more students were using ED in their learning process (Table 3).

According to the parents, during the DL period screen time increased by 2 h for 15.0% of students, by 3 h for 20.8%, by 4 h for 18.6%, by 5 h for 10.0%, and by 6 h for 25% of students.

Statistics generated by the Screen time app revealed that teachers used ED for 5.0 ± 0.3 h on average in the TE period and for 8.0 ± 0.3 h in the DL period ($p \leq 0.01$).

Table 3. The proportion of high school students who used ED for traditional and distance education, according to parents' reports, %

Number of ED used for learning	Traditional education	Distance learning
No ED used	36	3.4*
One ED	47.4	29.8*
Two ED	11.4	51.8*
Three of more ED	5.2	15.0*

Note: * — differences are significant, $p \leq 0,05$.

The majority of the surveyed parents (80%) reported that their children had more health complaints during the DL period. The complaints were grouped into a few categories based on their association with computer vision syndrome (eye fatigue 60.6%, pain in the eyes 27.0%, blurred vision 19.4%, seeing dark spots 11.4%, gritty eyes 16.2%), musculoskeletal disorders (back pain 37.6%, neck pain 31.8%), or psychoneurological disorders (headache 40.2%, heavy head 21.0%, muffled hearing or ringing in the ear 7.8%, fatigue 58.0%, psychoemotional strain 49.8%, disrupted daily schedule, difficulty falling asleep 30.4%). Only 17.2% of the students did not have any health complaints.

We calculated statistically significant relative risks for subjectively assessing one's vision as satisfactory or poor depending on the frequency of ED use (daily or with 1–3 day or longer breaks). In the respondents who used ED on a daily basis, the relative risk for subjectively assessing their vision as satisfactory or poor was 1.13 for students (CI: 1.07–1.47); 1.41 for parents (CI: 1.11–1.79), and 1.27 for teachers (CI: 1.07–1.52).

DISCUSSION

ICT have been developing rapidly in the past 20 years and are now an indispensable part of our daily lives and education. The pandemic of spring 2020 compelled general education providers to transition to DL.

It was demonstrated before the pandemic that only 0.5% of students did not have access to ED; the rest were using one to several ED on a daily basis. The amount of screen time was comparable between children and adults, varying from 7 to 10 h per day depending on age, sex, and season (school year/holidays) [19, 25]. It is reported that screen time is rising [30].

In the DL period, the number of ED used by each student increased from 1 to 3 or more for almost all students (96.6%) ($p \leq 0.05$) (Table 3). Screen time increased from 2 h (15.0% of students) to 6 h and more (25.0%). As a result, the majority of parents (80%) noticed that their children had more health complaints, most of which (60%) were consistent with the clinical manifestations of computer vision syndrome, musculoskeletal disorders or psychoneurological problems.

Statistics generated by the Screen time app revealed that screen time had increased by 3 h a day in teachers in the DL period ($p \leq 0.01$).

This is the result of ED overuse during quarantine and transition to DL, aggravated by the lack of digital technologies properly adapted to the educational process and the absence of specialized software. There are no clear criteria for the acceptable amount of screen time for different age groups in different seasons (holidays or school year); there are no transparent safety requirements for ED image quality and technical specifications. The situation is further complicated by low awareness of workstation ergonomics and safety.

All groups of respondents heavily depended on their ED. One in 4 students (24.0%), one in 2 parents (41.8%) and one in 3 teachers (38.4%) were not ready to cut down on their screen time (Table 2).

The surveys of teachers, students and parents conducted in the brick-and-mortar setting and after the transition to DL identified behavioral risk factors for ED overuse.

We found that the majority of the respondents did not follow the simple rules for minimizing computer-associated health damage: they did not take breaks every hour (64.5% of students, 63.0% of parents, 53.1% of teachers), worked under poor lighting conditions (87.0% of students, 87.3% of parents, 81.2%, of teachers), had uncorrected vision problems, or wore unsuitable glasses (45.5% of students, 44.8% of parents, 48.4% of teachers).

These risk factors for ED overuse were more frequent among parents than among teachers ($p \leq 0.05$); similarly, more parents than teachers characterized their vision as satisfactory or poor ($p \leq 0.05$) (Table 1).

Obviously, the family and the educational institution play a tremendous role in encouraging (or discouraging) students to develop a healthy and safe attitude to using ED. Our respondents demonstrated low awareness of this problem. This means that teachers, parents and students should be better educated in the health and safety issues relating to the use of ED. The expansion of distance learning, less control exerted by the teacher, and increased use of ICT by children outside the classroom demand that parents should have sufficient knowledge of good computer use practices.

The majority of teachers (67.5%) report health problems in their students and realize the need to encourage students to lead a healthy lifestyle, which also means a healthy attitude to working with ED (86.6%). At the same time, one-third of teachers (26.5%) do not use short breaks during the lesson and one in 2 teachers (44.4%) does not do eye exercises with their class. Perhaps, the teachers are not motivated to do so or simply do not have the time due to high curricular intensity.

Due to low awareness, 50% of the respondents were not concerned with prophylaxis of computer-associated health problems or took ineffective measures like wearing unsuitable glasses ($p \leq 0.05$).

Previously, we proved the risk of moderate or high myopia (RR 6.62) associated with| the frequency of using a laptop and a computer ($p \leq 0.05$) [25].

The established correlation between the readiness of the participants to cut down on screen time and the subjective assessment of their vision as perfect or good suggests that vision disorders can be effectively prevented by limiting screen time. Respondents who were ready to eliminate ED from their daily activities for at least once a week assessed their vision as perfect or good significantly more often ($p \leq 0.05$).

We discovered that there were significant relative risks for eye condition subjectively assessed as satisfactory or poor vision and associated with daily ED use; the RR values were 1.13 for children, 1.41 for parents, and 1.27 for teachers ($p \leq 0.05$).

Thus, we found that daily use of ED is a behavioral risk factor that can be modulated through implementing good computer use practices.

Based on our findings, we recommend that screen time should be strictly regulated or even eliminated from daily activities for at least one day per week. This will prevent the negative effects of ED in the traditional brick-and-mortar and distance learning settings. This approach will help to reduce the exposure to the negative factors and allow students to find time for physical activities or sports, thereby promoting a healthy lifestyle, improving the efficacy of prevention measures and eventually benefiting public health.

Once effective screen time guidelines are developed and the public becomes aware of how to use ED safely, one can expect an increase in the number of respondents with good skills for safe ED use.

CONCLUSIONS

1. We have identified behavioral risk factors for ICT overuse by students, teachers and parents after the transition to DL. Those include no screen breaks, poor lighting, uncorrected vision or

unsuitable glasses, daily use of ED, prolonged screen time, using more than 1 ED on a regular basis. 2. After the transition, the number of ED used by each student increased, screen time rose, and students had more health complaints. Teachers also used ED more, as compared to the TE period. 3. Due to the lack of skills for safe computer use, parents cannot be a role model for their children when it comes to a healthy attitude to ICT. 4. Eliminating screen time from daily activities for at least one day per week is an effective measure for preventing vision disorders. 5. The study provides rationale for expanding the scope of educational programs promoting healthy technology use in students, teachers and parents.

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ANALYSIS OF LEECH THERAPY EFFECTS IN PATIENTS WITH CHRONIC APICAL PERIODONTITIS

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Chronic apical periodontitis (CAP) is characterized by tissue inflammation around the tooth tip. Unstable outcomes of current treatments against CAP dictate the need for novel therapeutic techniques and medications. The aim of this study was to analyzed the effects of hirudotherapy on the treatment course in patients with CAP. Forty-one study participants aged 25 to 40 years were divided into the main group (20 patients) and the control group (21 patients). Pain level and the gingival index (GI) were measured in all study participants. During the first visit, pain scores did not differ significantly between the control (5.81 ± 0.65) and the main (5.75 ± 0.92) groups. During the second visit, pain was almost unnoticeable in the main group patients (1.05 ± 0.34), whereas pain scores were higher in the control group (4.10 ± 0.7). Our findings suggest a positive effect of hirudotherapy used in combination with standard treatment regimens.

Keywords: hirudotherapy, chronic apical periodontitis, inflammation, pain

Author contribution: Abdullaeva AI — literature and experimental data analysis, experimental data analysis; Prityko AG — experimental data analysis, experimental data analysis; Voronin PA — statistical analysis, manuscript revision; Mikhailova EG — statistical analysis, manuscript revision.

Compliance with ethical standards: the study was approved by the Ethics Committee of Pirogov Russian National Research Medical University (Protocol № 947 dated February 4, 2019). Informed consent was obtained from all study participants.

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АНАЛИЗ РЕЗУЛЬТАТА ПРИМЕНЕНИЯ МЕТОДА ГИРУДОТЕРАПИИ ПРИ ЛЕЧЕНИИ ХРОНИЧЕСКОГО АПИКАЛЬНОГО ПЕРИОДОНТИТА

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Для хронического апикального периодонтита (ХАП) характерно воспаление тканей, окружающих верхушку корня зуба. Отсутствие стабильности результатов стандартного лечения объясняет поиск новых лекарственных средств и методов лечения данного заболевания. Целью исследования было проанализировать влияние гирудотерапии в лечении ХАП при динамическом наблюдении с использованием клинических методов исследования. Пациенты в возрасте от 25 до 40 лет (всего 41 человек) были разделены на основную группу (20 человек) и группу сравнения (21 человек). У всех участников исследования определяли показатели выраженности постоянных болевых ощущений и десневого индекса GI. В первое посещение показатели выраженности болевых ощущений в группе сравнения (5,81 ± 0,65) и основной группе (5,75 ± 0,92) сильно не различались. К третьему посещению у пациентов основной группы боль почти отсутствовала (1,05 ± 0,34), а в группе сравнения показатели были выше (4,10 ± 0,7). Результаты клинических исследований указывают на положительное влияние гирудотерапии в составе комплексного лечения.

Ключевые слова: гирудотерапия, хронический апикальный периодонтит, воспаление, боль

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The prevalence of chronic apical periodontitis (CAP) in dentistry patients over 18 years of age is 95–100% [1]. Unstable outcomes of current treatments against CAP dictate the need for novel therapeutic techniques and medications. Hirudotherapy, i.e. application of medicinal leeches *Hirudo medicinalis*, dates back centuries [2]. It has proved to be effective in treating a number of conditions, including battle wounds [3]. Among absolute contraindications for hirudotherapy are malignancies, blood clotting disorders, thrombocytopenia, and intolerance of leech saliva components [4, 5]. At present, leeches are used to relieve pain and treat asthma, high blood pressure, migraine, phlebitis, varicose veins, and dental diseases [2, 6–7]. In most cases, leech therapy has positive outcomes; however, adverse events are possible. When the leech bites, it injects into the wound a complex mixture of bioactive ingredients, including hirudin [8]. Hirudin is a polypeptide that irreversibly binds to thrombin; the resulting complex blocks conversion of fibrinogen into fibrin [9–10]. This improves microcirculation in tissues, produces anti-inflammatory and analgesic effects, and stimulates tissue regeneration [11]. Leech therapy has been approved by the Russian Ministry of Healthcare; there is a pharmacopeial monograph (PM-42-702-97) containing a list of quality control guidelines for this treatment type. The literature on the efficacy of hirodutherapy in periodontitis is scarce, and the question remains whether it is feasible for treating CAP [12].

The aim of this study was to investigate the effect of hirudotherapy on the pace of clinical improvement in patients receiving a combination therapy for CAP.

METHODS

The study was carried out in 41 patients with exacerbated CAP undergoing treatment at the facilities of Voyno-Yasenetsky Research and Practical Center for Specialized Medical Care

Table 1. Demographics of the participants

	Main group (n = 20)	Control group (n = 21)
Sex, m/f	8/12	9/12
Mean age (years), M ± m	31.4 ± 0.4	30.2 ± 0.3

for Children in January through March 2020. Of all study participants, 17 were men and 24 were women aged 25 to 40 years. The mean age of the participants was 29.1 ± 1.7 years in the female group and 34.6 ± 1.4 year in the male group, respectively. The study included patients with CAP who required endodontic retreatment, had no dental fistulas and were 25 to 40 years old. The following exclusion criteria were applied: decompensated comorbidities, a flare-up of a chronic comorbidity, inflammatory periodontal disease, pregnancy, HIV, blood clotting disorders, cancer, refusal to participate. Chronic apical periodontitis (ICD code: K04.5) was diagnosed based on CT findings (GENORAY; South Korea).

The patients were divided into 2 groups. The main group consisted of 20 patients who received standard treatment for CAP complemented by hirudotherapy. The mean age in the main group was 30.0 ± 1.0 years for women and 33.5 ± 1.3 years for men. The control group included 21 patients who received only standard treatment. The mean age in this group was 31.5 ± 1.2 years for women and 28.4 ± 1.4 years for men (Table 1). The groups did not differ in terms of age and sex.

Endodontic treatment was carried out following the guidelines proposed by the European Society of Endodontology in 1994 (tooth preparation; rubber dam isolation; access cavity preparation; determination of the working length; cleaning, shaping and filling of the root canal system). Dental materials and medications were the same for all study participants. In the main group, endodontic procedures were followed by leech therapy. Briefly, a medicinal leech was applied to the alveolar mucosa over the apical aspect of the causative root using an aspiration technique; the leech was allowed to feed for 20–30 min until it detached. The leeches used in the study were purchased at the HirudoCenter (Russia; Certificate of Conformity ROSS RU.AD77.H00310, valid from January 29, 2018 through January 28, 2021). The main group received 3 courses of leech therapy: on days 1, 4 and 7 of treatment, as recommended by the hirudotherapy guidelines [13].

The efficacy of the applied treatment was assessed based on the level of pain and the gingival index (GI) developed by Löe и Silness (1963); patient dynamics were compared between the groups [8]. Pain was measured on a visual analog scale (VAS): 0 — no pain; 1–3 — mild pain; 4 — moderate pain; 5–6 — severe pain; 7–9 — very severe pain; 10 — unbearable pain. GI is traditionally used to evaluate the periodontium for the clinical signs of gingival inflammation, which include redness, edema and bleeding on probing (in our case, at the site of the examined tooth). GI scores were interpreted as follows: 0 — no signs of inflammation; 1 — a slight change in color, mild edema, no bleeding on probing (mild inflammation); 2 — redness, edema, bleeding on probing (moderate inflammation); 3 — marked redness, edema, ulceration, a tendency to spontaneous bleeding (severe inflammation) [8].

Statistical analysis was done in SPSS 21 (IBM SPSS Statistics; USA). Differences were considered significant at $p \leq 0.05$ (95%).

RESULTS

On day 1, pain scores did not differ significantly between the groups. In the main group, pain was less pronounced during the second visit and almost unnoticeable during the third visit; the main group patients reported the analgesic effect of hirudotherapy (Table 2). On day 4 of treatment (the second visit), pain scores in the control group were also lower than at the onset of treatment; during the third visit, however, pain scores in the control group were much higher than in the main group. An increase in pain scores observed in the control group on day 7 was linked to the normal body reaction to a medical intervention.

In both groups, GI scores were decreasing steadily over the entire course of treatment. However, on days 4 and 7 GI was lower in the main group than in the control group. Hirodutherapy reduced edema and redness and had an analgesic effect.

Thus, the analysis of clinical symptoms suggests a positive effect of leech therapy on the outcome of combination therapy in patients with CAP.

DISCUSSION

This study demonstrates a positive effect of hirudotherapy used in combination with standard treatment regimens in patients with CAP and confirms its analgesic and anti-inflammatory effects previously reported by Russian researchers. Another study consistent with our findings has shown that hirudotherapy reduces endogenous toxic effects in the affected dentoalveolar region in patients with periodontal tissue destruction [14]. Our study suggests that hirudotherapy is also effective in reducing periodontal inflammation. During the third visit, no hyperemia was observed in the main group, whereas in the control group its level remained almost unchanged throughout the entire course of treatment. A dissertation written in 2003 is also consistent with our findings and points to the fact that hirudotherapy used in combination with standard treatment regimens can relieve pain, reduce edema, inflammation and the duration of therapy [15]. In our experiment, additional parameters were used, including pain scores on the 10-point scale and the gingival index.

Table 2. Dynamics of the gingival index (GI) and pain scores in patients with CAP in the main and control groups

	Day 1		Day 4		Day 7	
Parameters	Main group	Control group	Main group	Control group	Main group	Control group
Pain, points (M ± m)	5.75 ± 0.92	5.81 ± 0.65	2.5 ± 0.54	3.29 ± 0.68	1.05 ± 0.34*	4.1 ± 0.75
Gingival index GI, points (M ± m)	1.88 ± 0.06	1.92 ± 0.05	1.13 ± 0.10	1.45 ± 0.13	0.38 ± 0.06*	1.13 ± 0.06

Note: * — $p < 0.05$; comparison with the control group

Based on the pain, edema and hyperemia scores on days 1, 4, and 7 of treatment, we conclude that these parameters were lower in the patients who received hirudotherapy. In the main group, leech secretions expediated the resolution of inflammation and had a pronounced decongestive and pain-relieving effect, resulting in clinical improvement. No adverse effects of hirudotherapy were observed.

CONCLUSIONS

Our findings suggest that hirudotherapy can be recommended as a complement to conventional treatment of CAP and used in the clinical setting for relieving pain and reducing inflammation. Further research is needed to study the long-term effects of leech therapy using modern diagnostic modalities, including radiography.

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