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МЕДИЦИНА ЭКСТРЕМАЛЬНЫХ СИТУАЦИЙ

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N PROTEIN BASED VACCINE AGAINST SARS-COV-2 PRODUCES A STRONG T CELL IMMUNE RESPONSE TO N PROTEIN OF NOVEL STRAINS

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The second generation COVID-19 vaccines should produce the long-term protective immune response to the existing and novel strains of SARS-CoV-2. The Convacell® vaccine was designed to produce such immune response by using N protein as an antigen. N-protein is not susceptible to fast accumulation of mutations and is highly homologous to nucleocapsid proteins of other β-coronaviruses. The study was aimed to perform *in vitro* assessment of the Convacell® vaccine ability to produce immune response to the Wuhan, Delta, and Omicron strains. Mononuclear cells of vaccinated volunteers and survivors were subjected to N protein stimulation. After that specific activation of the cells was assessed by flow cytometry. The results showed that a sibstantial percentage of CD4 and CD8 cells produced IFNγ and IL2 in response to stimulation. No significant reduction of the response to strains Delta and Omicron compared to the Wuhan strain was revealed. The findings support the direction of the N protein based vaccine design towards creation of the universal vaccine.

Keywords: SARS-CoV-2, vaccine, immunity, novel strains, N protein, nucleocapsid, Delta, Omicron, Wuhan, coronavirus

Author contribution: Rabdano SO — study design, data analysis, data interpretation, manuscript writing; Mukhin VE — bioinformatic analysis, experimental procedure, data acquisition, statistical analysis, manuscript writing; Makarov VV — study design, data interpretation, manuscript writing; Rudakov GO — data analysis, statistical analysis, data interpretation, graphics preparation, manuscript writing; Ruzanova EA, Arakelov SA, Khaitov MR, Yudin SM, Kryuchko DS, Berzin IA, Evtushenko AE — study design, manuscript editing; Truhin VP, Skvortsova VI — research idea, study concept, manuscript editing.

Compliance with ethical standards: the study was approved by the Ministry of Health of the Russian Federation (clinical trial approval № 388 of 19 July 2021), Ethics Committee of MH RF (protocol № 282 of 19 July 2021) and Independent Ethics Committee (IEC) of the research center (protocols № 163 of 15 July 2021 and № 164 of 20 July 2021); the study was conducted in accordance with the principles of the World Medical Association (WMA) Declaration of Helsinki (1964) and its latest update (2013), tripartite guideline for Good Clinical Practice approved by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (E6 (R2) of 09 November 2016) and current legislation of the EEU and RF. Two copies of the informed consent form (volunteer information sheet) were to be signed and dated by the subjects and the researcher by their own handwriting.

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ВАКЦИНА НА ОСНОВЕ N-БЕЛКА SARS-COV-2 ФОРМИРУЕТ ВЫРАЖЕННЫЙ Т-КЛЕТОЧНЫЙ ИММУНИТЕТ НА N-БЕЛОК НОВЫХ ШТАММОВ

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Вакцины второго поколения против COVID-19 должны создавать долговременный протективный иммунный ответ по отношению к существующим, а также новым штаммам SARS-CoV-2. Дизайн вакцины Конвасэл® направлен на формирование такого иммунного ответа путем использования N-белка в качестве антигена. Белок N не подвержен быстрому накоплению мутаций и имеет высокую гомологию с нуклеокапсидными белками других β-коронавирусов. Целью работы было провести *in vitro* исследование возможности Конвасэл® создавать иммунный ответ против штаммов Ухань, Дельта и Омикрон. Мононуклеарные клетки вакцинированных или переболевших добровольцев стимулировали N-белком, а затем методом проточной цитометрии анализировали их специфическую активацию. Результаты показали, что существенный процент $CD4^+$- и $CD8^+$-клеток продуцирует IFNγ и IL2 в ответ на стимуляцию. Статистически значимого снижения ответа для штаммов Дельта и Омикрон по сравнению с штаммом Ухань не выявлено. Полученные результаты поддерживают направление в дизайне вакцин на основе N-белка для создания универсальной вакцины.

Ключевые слова: SARS-CoV-2, вакцина, иммунитет, новые штаммы, N-белок, нуклеокапсид, Дельта, Омикрон, Ухань, коронавирус

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The COVID-19 pandemic has been a global challenge since 2020. High infectivity of SARS-CoV-2, high morbidity and mortality [1, 2], as well as rapid virus evolution [3] are the main features of the pandemic. More than 600.5 million cases of COVID-19 and more than 6.5 million deaths were officially reported by September 2022 [4]. Additionally, even non-fatal cases of the disease can have long-term effects on human health, especially on the cardiovascular system [5–7]. The development of universal COVID-19 vaccine capable of producing long-lasting immunity against common viral strains is therefore an urgent task for world pharmaceutical industry.

It is difficult to develop such a vaccine due to high antigenic variability of the virus [8], including the viral spike (S) protein [9]. Today, all of the certified and widely used vaccines are based on the S protein [10]. High specificity of antibodies and T-cell receptors, which manage the humoral and cell-mediated immune responses to the virus, combined with its fast evolution causes the efficiency of both virus neutralization and elimination of infected cells to decrease over time. Past studies present data on the reduction of neutralization of new viral strains by sera of individuals vaccinated with S protein-based vaccines [11, 12]. There are also many publications describing the reduced efficiency of viral epitope recognition by T-cells, and therefore T-cell activation by protein epitopes of new viral strains [11,13–16]. However, there are controversial studies describing the persistence of T-cell immune response to peptides containing mutations typical for new viral variants [17].

Overall, the effectiveness of existing S protein-based COVID-19 vaccines decreases over time due to evolution of the virus [18]. As such, the conserved nucleocapsid (N) protein, which is less susceptible to mutation, is a much better target for the formation of long-lasting immunity against the virus than the S protein [19–21]. The protective potential of vaccines based on viral nucleocapsid proteins was demonstrated for influenza [22–26], dengue [27–29] and coronaviruses [20, 30]. An immune response targeted against viral nucleocapsid proteins allows T-cells and antibody-mediated natural killer cells to rapidly destroy infected cells and ensure complete elimination of the virus from the body. Furthermore, the conserved nature of N protein entails its high similarity in different stains, which results in the capability of N-protein-based vaccines to provide protection against a wide range of SARS-CoV-2 variants.

The Saint Petersburg Scientific Research Institute of Vaccines and Serums has developed Convacell®, an N protein-based vaccine against SARS-CoV-2. The amino acid sequence of the antigen in the vaccine corresponds to the N protein of the Wuhan variant. The vaccine is currently undergoing combined phase I and II clinical trials. Due to its target selection, the Convacell® vaccine should produce a long-lasting immunity against all variants of SARS-CoV-2. This advantageously distinguishes it from the currently available S protein-based vaccines. This study was aimed to demonstrate the capability of Convacell® to evoke specific immune response to a range of currently existing SARS-CoV-2 strains.

METHODS

Laboratory studies were performed in the Department of Analysis and Forecasting of Medical and Biological Health Risks, Centre for Strategic Planning and Management of Biomedical Health Risks of FMBA of Russia. A total of 12 subjects were enrolled. Convalescents: six patients with the diagnosis of COVID-19 confirmed by laboratory tests, who had a mild disease in January 2022 (three men and three women aged 18–51). Vaccinated individuals: volunteers fully vaccinated with Convacell® (two doses with an interval of 21 days), the other data on vaccinated subjects have been blinded until the end of the clinical trial. The inclusion/exclusion criteria and other details of the studies are provided in the ClinicalTrials.gov database (NCT05156723).

The convalescents' blood was collected on the day of recovery, and blood of vaccinated individuals was collected on day 42 after administration of the first dose. Peripheral blood samples for laboratory testing were collected by venipuncture of a cubital vein in the morning in the fasting state (at least 8 hrs after the last meal). Blood was collected in sterile vacuum tubes containing K3-EDTA as anticoagulant.

Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood samples. The peripheral blood sample volume was at least 6 ml. All the procedures were performed not later than 8 hrs after the collection of biomaterial. The samples were stored and transported at room temperature (18–25 °C) while maintaining the cold chain.

PBMCs were isolated from peripheral blood by the Ficoll density gradient centrifugation (1.077 g/l). Whole blood was gently mixed several times by inverting the tube 5–6 times by 180°, diluted in Dulbecco's phosphate buffered saline (DPBS) to obtain a 1:1 dilution, and then layered onto a Ficoll-based medium with the density of 1.077 g/mL and centrifuged at 450 g for 30 min with disabled rotor brake at room temperature. After the end of centrifugation, the interphase ring located on the boundary between the Ficoll-based medium and blood plasma was harvested with a serological pipette and transferred to a clean 15 ml conical tube. Then the cells were twice washed with DPBS by centrifugation at 300 g for 10 min (Eppendorf; Germany). After the second washing, cells were enumerated, and cell viability was assessed by trypan blue staining with the Countess 3 Cell Counter (Thermo; USA). The cells were subsequently diluted with RPMI 1640 complete medium (10% fetal bovine serum, 1% penicillin-streptomycin) to a concentration of 10^6 cells/mL. Cell viability was at least 95%.

The quantity and phenotypes of antigen-specific cells were defined by multicolor flow cytometry based on the cell capability to produce cytokines in response to stimulation with peptides covering N protein regions of various SARS-CoV-2 variants. 10^6 PBMCs in 100 µl of culture medium were introduced to flow cytometry tubes, supplemented with 1 µg/ml of antigen (protein or pooled peptides) and incubated for 12 hrs at 37 °C in 5% CO_2. Two hours after adding antigen, 10 µg/ml of brefeldin A were added to the cells (Sigma-Aldrich; USA). After the end of incubation cells were washed with DPBS, then cell surface was stained with the anti-CD3 (UCHT1), anti-CD4 (13B8.2), anti-CD8 (B9.11), anti-CD45RA (2H4), anti-CD197 (G043H7) antibody conjugates (all antibodies manufactured by Beckman Coulter; USA). Zombie Aqua™ Fixable Viability Kit (Biolegend; USA) was used to assess cell viability. Then, cells were washed with DPBS and fixed, cell membranes were permeabilized using the IntraPrep Permeabilization Reagent Kit (Beckman Coulter; USA), and intracellular staining was performed using the anti-IL2 (IL2.39.1) and anti-IFNg (45.15) antibody conjugates (Beckman Coulter; USA).

The CytoFlex flow cytometer (Beckman Coulter; USA) was used for analysis. At least 100,000 T-cells per sample were recorded. The results were analyzed with the CytExpert Acquisition and Analysis Software, ver. 2.4 (Beckman Coulter; USA), plots were prepared using the GraphPad Prism 9 software. Analysis of variance (ANOVA) was used for statistical analysis in all cases.

```
REF          MSDNGPQNQRNAPRITFGGPSDSTGSNQNGERSGARSKQRRPQGLPNNTASWFTALTQHG 60
B.1.617.2    ............................................................ 60
BA.2         ............L.................---........................... 57
BA.4         ............L.................---........................... 57
BA.5         ............L.................---........................... 57

REF          KEDLKFPRGQGVPINTNSSPDDQIGYYRRATRRIRGGDGKMKDLSPRWYFYYLGTGPEAG 120
B.1.617.2    ..G......................................................... 120
BA.2         ............................................................ 117
BA.4         ............................................................ 117
BA.5         ............................................................ 117

REF          LPYGANKDGIIWVATEGALNTPKDHIGTRNPANNAAIVLQLPQGTTLPKGFYAEGSRGGS 180
B.1.617.2    ............................................................ 180
BA.2         ............................................................ 177
BA.4         ............................S............................... 177
BA.5         ............................................................ 177

REF          QASSRSSSRSRNSSRNSTPGSSRGTSPARMAGNGGDAALALLLLDRLNQLESKMSGKGQQ 240
B.1.617.2    ......................M...........C........................ 240
BA.2         ......................KR.................................. 237
BA.4         ......................KR.................................. 237
BA.5         ......................KR.................................. 237

REF          QQGQTVTKKSAAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQGNFGDQELIRQGTDYKH 300
B.1.617.2    ............................................................ 300
BA.2         ............................................................ 297
BA.4         ............................................................ 297
BA.5         ............................................................ 297

REF          WPQIAQFAPSASAFFGMSRIGMEVTPSGTWLTYTGAIKLDDKDPNFKDQVILLNKHIDAY 360
B.1.617.2    ............................................................ 360
BA.2         ............................................................ 357
BA.4         ............................................................ 357
BA.5         ............................................................ 357

REF          KTFPPTEPKKDKKKKADETQALPQRQKKQQTVTLLPAADLDDFSKQLQQSMSSADSTQA  419
B.1.617.2    ..............Y.....................................  419
BA.2         ..............................................R......  416
BA.4         ..............................................R......  416
BA.5         ..............................................R......  416
```

Fig. 1. Alignment of the SARS-CoV-2 N protein sequences carrying mutations typical for Delta (B.1.617.2) and Omicron (BA.2/4/5) strains with the NC_045512 reference sequence (REF). Alignment was performed using the MUSCLE tool (https://github.com/rcedgar/muscle). The matching amino acids are marked with dots, the differences are reported using the single-letter amino acid code, deletions are marked with the dash character

RESULTS

Cytometry of PBMCs stimulated by N proteins of the Wuhan, Delta and Omicron strains

Simulation of the contact between the immune cells obtained from vaccinated individuals and convalescents and the Wuhan, Delta and Omicron strains of SARS-CoV-2 was done by incubation of the cells after adding the representative antigens of each strain. The cells were incubated with the following three variants of the SARS-CoV-2 antigen: a) full-length N protein with the sequence corresponding to the that of the reference strain (Wuhan); b) Delta strain N protein peptide pool; c) Omicron strain N protein peptide pool.

The following analysis was performed to provide the Delta and Omicron strain peptide pools.

Mutations in the nucleocapsid protein of the virus of the clades we were interested in were selected in the Lineages/Mutations section of the Coronavirus 3D website [31].

The reference nucleotide sequence of N protein (Wuhan strain) and full-genome reference sequence for the Delta B.1.617.2 strain (EPI_ISL_1663516) were downloaded from the GISAID website [32]. Currently there are no reference full-genome sequences for the strains Omicron BA.2, BA.4, BA.5 available from the GISAID database.

N protein nucleotide sequences were translated into amino acid sequences with the VIGOR4 tool [33].

Mutations selected in paragraph 1 were incorporated into the reference N protein sequence using UGENE [34]. Four resulting FASTA files were integrated into one multi-FASTA file.

Multiple sequence alignment was performed using the MUSCLE tool [35].

The alignment of N protein sequences typical for various SARS-CoV-2 strains (Fig. 1) showed that the differences between the nucleocapsid proteins of the Delta and Omicron strains could be limited to the listed below mutations, compared to the Wuhan reference strain N protein. Omicron: P13L, 31-33del, P151S, R203K, G204R, S413R; Delta: D63G, R203M, G215C, D377Y.

Peptide pools with five mutations, three peptides per mutation and 11 overlapping amino acids were selected based on the analysis to represent each strain (Fig. 2). Despite the availability of data on the immunodominant epitopes in vaccinated individuals and COVID-19 convalescents [36],

Omicron peptide pool (BA.2/4/5):

1	NGGARSKQRRPQGLP
2	GSNQNGGARSKQRRP
3	SDSTGSNQNGGARSK
4	KEDLKFPRGQGVPIN
5	TQHGKEDLKFPRGQG
6	FTALTQHGKEDLKFP
7	SKRTSPARMAGNGGD
8	TPGSSKRTSPARMAG
9	SRNSTPGSSKRTSPA
10	KKADETQALPQRQKK
11	KDKKKKADETQALPQ
12	TEPKKDKKKKADETQ

Delta peptide pool (B.1.617.2):

1	NGERSGARSKQRRPQ
2	GSNQNGERSGARSKQ
3	SDSTGSNQNGERSGA
4	KEGLKFPRGQGVPIN
5	TQHGKEGLKFPRGQG
6	FTALTQHGKEGLKFP
7	SMGTSPARMAGNGCD
8	TPGSSMGTSPARMAG
9	SRNSTPGSSMGTSPA
10	KKAYETQALPQRQKK
11	KDKKKKAYETQALPQ
12	TEPKKDKKKKAYETQ

Fig. 2. Selected Omicron and Delta peptide pools. The differences between strains are highlighted in *yellow*

we chose epitopes containing mutations, since these were the most representative in terms of changes in the immune response due to virus evolution.

To assess and compare cell-mediated immune responses evoked by the Convacell® vaccine and SARS-CoV-2 infection, blood was collected from participants of the Convacell® vaccine clinical trials and volunteers who survived COVID-19. Then, PBMCs were isolated and frozen until the analysis was performed. After thawing, cells were divided into aliquots and subjected to specific stimulation with antigens of the Wuhan, Delta and Omicron strains. Furthermore, the corresponding negative (culture medium) and positive (phorbol myristate acetate/ionomycin) controls were introduced. The details of stimulation protocol are provided in the Methods section.

After stimulation, PBMCs were stained with fluorescent antibodies against markers corresponding to the major T cell phenotypes (CD4, CD8) and markers of their activation (IFNγ, IL2). Then, cytometry analysis of the stained PBMCs was performed.

The results of the analysis (Fig. 3) showed that for samples from vaccinated individuals the stimulation with N protein generally evoked similar immune responses in all the studied subpopulations of T-helper cells and killer T cells for all strains of SARS-CoV-2.

$CD4^+INF\gamma^+$ PBMC subpopulation: vaccination with Convacell® evoked the response similar to that produced due to infection; no significant differences in the number of activated cells between N proteins of various strains were found in both vaccinated individuals and convalescents.

$CD8^+INF\gamma^+$ PBMC subpopulation: a slightly stronger immune response was observed in convalescents compared to vaccinated individuals; stimulation with N proteins of the Wuhan and Delta strains also evoked stronger immune response than stimulation with Omicron N protein. However, no significant differences were revealed. N protein stimulation produced strong immune response in all cases.

$CD4^+IL2^+$ cells: strong immune response was produced in vaccinated individuals, and a significantly weaker response was observed in convalescents. No differences were revealed during stimulation with N proteins of various strains.

$CD8^+IL2^+$ cells: equally weak responses were observed in all subpopulations, regardless of the cell phenotype and protein strain used for stimulation.

Phenotyping of memory cells

Further analysis of the phenotype of specific memory cells (Fig. 4) that were stimulated in samples from individuals that were vaccinated with Convacell® and COVID-19 convalescents after stimulation with N proteins of various viral strains revealed no differences in the structure of immune response in all populations.

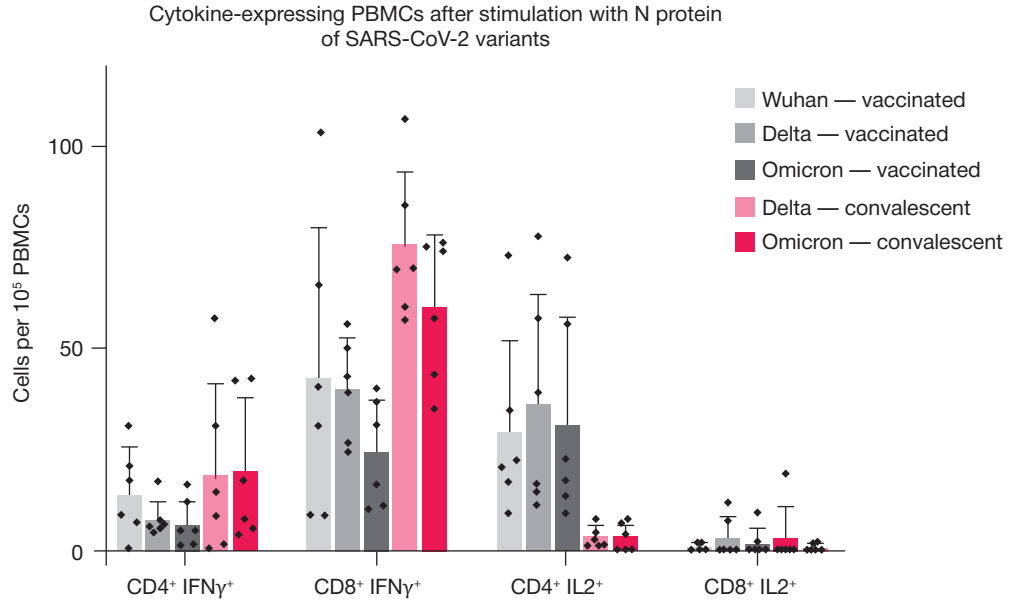

Fig. 3. Quantification of PBMCs expressing cytokines for individuals vaccinated with Convacell® and COVID-19 convalescents after stimulation with viral N protein or N protein-based peptide pools

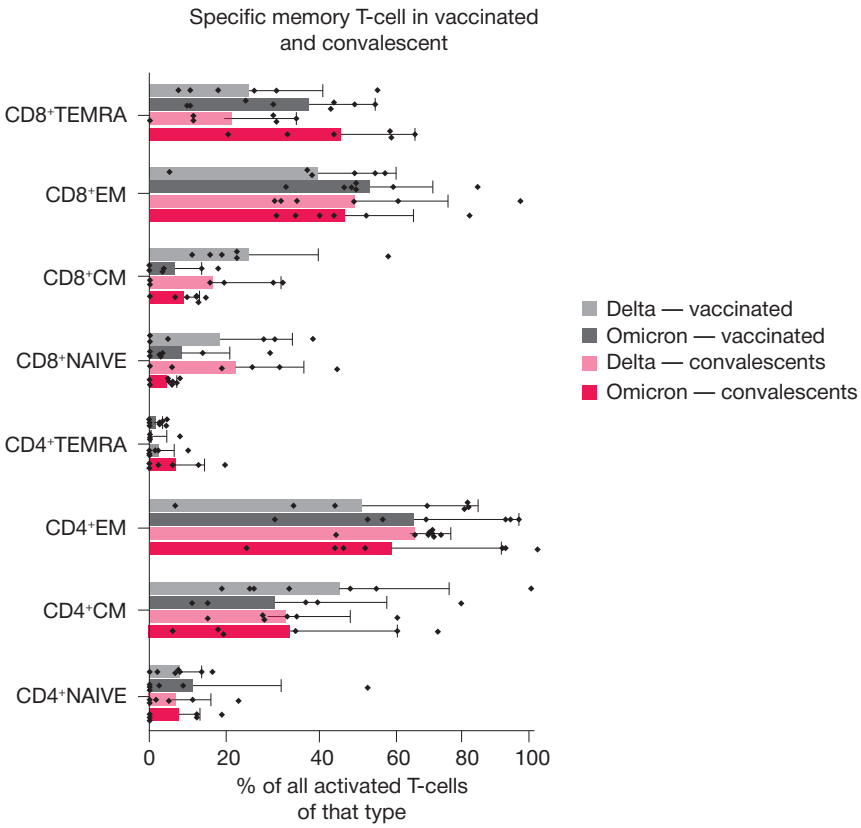

Fig. 4. Phenotyping of specific memory T cells of individuals vaccinated with Convacell® and COVID-19 convalescents after stimulation with N protein

Predominance of effector memory (EM) T cells at the level of about 60% of all activated cells and prevalence of central memory (CM) T cells at the level of about 30% of the total cell number are typical for T-helper cells. Naive T cells constitute about 10%, while TEMRA cells are rare. There are no significant differences between the convalescents and vaccinated individuals, as well as between the SARS-CoV-2 strains.

Cytotoxic T-cells are characterized by the presence of effector memory T-cells at 40–50% of total number and TEMRA cells at 20–40% of total number. Naive T cells and central memory T cells constitute 10–20% of total number. Stimulation with the N protein of Omicron strain results in higher proportion of TEMRA cells and lower proportion of naive T-cells and central memory T-cells compared to stimulation with the Delta strain protein, however, the differences are non-significant. There are no differences between the convalescents and vaccinated individuals.

DISCUSSION

Studies assessing the proportion of CD4 and CD8 cells expressing IFNγ in response to stimulation with antigens were also published earlier [17, 37]. In individuals vaccinated with mRNA vaccines and S protein from homologous variant, the immune response was stronger than in COVID-19 convalescents [17]. A stronger T-cell response, in particular $CD4^+IL2^+$ cells (see comparison of gray and red bars in Fig. 3), is observed for vaccinated with Convacell® compared to convalescent patients, however, unlike S-protein vaccines, this effect is spread and non-homologous strains. In blood samples obtained from convalescents, about 40 of 10^5 $CD4^+$ cells and 50 of 10^5 $CD8^+$ cells express IFNγ when stimulated with N protein peptides [17]. Our findings show that the number of cells responsive to stimulation in convalescents is slightly lower than the number reported in the paper [17]. It should be noted that the number of T cells responsive to stimulation with S protein peptides rapidly decreases in both convalescents and individuals vaccinated with mRNA vaccines [37, 38]. The number of cells decreases by a factor of two within 1–1.5 months on average.

The findings show that the Convacell® vaccine is capable of evoking the same or even stronger immune response as the disease: there are no significant differences in the number of activated cytotoxic T cells and T helper cells between the vaccinated subjects and convalescents, and the number of T helper cells producing IL2 is significantly higher among vaccinated individuals. The data obtained confirm Convacell®'s capability to produce highly potent cell-mediated immune response to the SARS-CoV-2 N protein.

There are also no significant differences in the number of activated cells in all the vaccinated populations when stimulated with N protein of any of the viral strains used. This demonstrates Convacell® capability to produce equally strong immunity against new strains.

Phenotyping of CD4 memory cells in vaccinated individuals and convalescents was also conducted earlier [37]. The $CD4^+$-CM and $CD4^+$-EM cell predominance (40–50% of all responsive cells) revealed in our study is consistent with the data provided by these studies.

The lack of differences in the structure of immune response in vaccinated individuals stimulated with proteins of various SARS-CoV-2 strains confirms Convacell® capability to produce equally strong cell-mediated immune response against all viral strains. Similarities in the structure of immune responses resulting from both vaccination and disease testify in favor of the efficiency of the immune response produced by the vaccine.

CONCLUSIONS

The Convacell® vaccine showed capability to produce equally strong cell-mediated immune response against the SARS-CoV-2

Wuhan, Delta and Omicron strains. This emphasizes the potential of the N-protein-based approach to the development of universal COVID-19 vaccine. Our study demonstrates Convacell®'s capability to produce highly potent cell-mediated immune response against the currently circulating SARS-CoV-2 strains. In view of rapid virus evolution, availability of universal vaccine would ensure protection of the population against new waves of COVID-19.

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PATHOLOGIES OF PETROUS APEX

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The review of papers, focused on studying various neoplasms, diagnosis, selection of surgical approach, complications, and recurrence rates of the petrous apex lesions, that have been published in 2008–2022, is provided. Effusion, mucocele, cholesterol granuloma, cholesteatoma are the most common benign lesions of the petrous apex. Such surgical approaches as translabyrinthine, transcochlear, endoscopic endonasal approach and the middle cranial fossa approach are most often used during treatment. The middle cranial fossa approach, infracochlear approach and endoscopic transnasal approach are recommended for patients with preserved hearing. In case of disseminated lesions, when the carotid artery and the jugular bulb should be additionally controlled, transcochlear and translabyrinthine surgical corridors could be used.

Keywords: petrous apex, neoplasm, middle cranial fossa, surgical approach, skull base, internal carotid artery, facial nerve, liquorrhea, complications, cholesteatoma, cholesterol granuloma, effusion, diagnosis

Author contributions: Diab KhM — research design, manuscript editing; Daikhes NA — manuscript editing; Pashinina OA, Shamkhalova AM — manuscript writing; Shamkhalova AM, Panina OS — literature review.

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ПАТОЛОГИЯ ВЕРХУШКИ ПИРАМИДЫ ВИСОЧНОЙ КОСТИ

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Представлен обзор публикаций, посвященных изучению различных новообразований, диагностики, выбора хирургического доступа, осложнений и частоты рецидивирования патологии верхушки пирамиды височной кости за период с 2008 по 2022 г. Наиболее распространенной доброкачественной патологией верхушки пирамиды височной кости являются выпот, мукоцеле, холестериновая гранулема, холестеатома. При лечении чаще всего используют такие хирургические доступы, как транслабиринтный, транскохлеарный, эндоскопический эндоназальный и доступ через среднюю черепную ямку. Для пациентов с сохранной слуховой функцией рекомендовано использование доступов через среднюю черепную ямку, инфракохлеарного, либо эндоскопического трансназального доступов. В случае распространенных патологических процессов, когда необходим дополнительный контроль сонной артерии и луковицы яремной вены, могут быть использованы транскохлеарный либо транслабиринтный хирургические коридоры.

Ключевые слова: верхушка пирамиды височной кости, новообразование, средняя черепная ямка, хирургический доступ, основание черепа, внутренняя сонная артерия, лицевой нерв, ликворея, осложнения, холестеатома, холестериновая гранулема, выпот, диагностика.

Вклад авторов: Х. М. А. Диаб — дизайн исследования; редактирование статьи; Н. А. Дайхес — редактирование статьи; О. А. Пащинина, А. М. Шамхалова — написание текста рукописи; А. М. Шамхалова, О. С. Панина — обзор литературы.

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The petrous apex, located in the petrous part of the temporal bone, is an anatomical area difficult for surgical treatment, since it lays adjacent to vital anatomical structures (second segment of the internal carotid artery (ICA), cavernous sinus, cranial nerves V and VI, temporal lobe of the brain, subarachnoid cistern).

Various pathologies are found at the site, among which space-occupying lesions, that require only surgical treatment, are the most common, and selection of optimal approach remains the main problem in such cases. Extradural and intradural lesions of the petrous apex are distinguished based on etiology. The most common extradural lesions are cysts (asymmetric effusion), which are more prevalent than cholesterol granulomas, cholesteatomas, chondromas, mucocele. Intradural lesions include meningioma and schwannoma [1, 2]

The petrous apex lesions are usually incidental findings, they do not produce any clear symptoms given the small mass size and petrous pyramid limited with bony walls. This makes them difficult to diagnose. Disruption of bony edges of the petrous apex and compression of important anatomical structures produce a vivid clinical picture (pain on one side of the face (trigeminal nerve (cranial nerve V)) or headache, diplopia (abducens nerve (cranial nerve VI)), weakness of facial muscles (facial nerve (cranial nerve VII)), hearing loss and vestibular disorders (vestibulocochlear nerve (cranial nerve VIII)), Gradenigo's syndrome) that motivates the patient to seek urgent medical care [3, 4]. The diversity of symptoms results from the petrous apex position on the boundary between the otorhinolaryngological and neurosurgical practices. Surgical treatment of patients with the pathologies of the petrous apex is difficult due to the petrous apex medial position at the base of the skull, location in the close proximity to vital anatomical structures, and therefore high risk of injury to these natomical structures during surgery. Surgical techniques are limited to the use of microscopic, endoscopic or combined methods involving creation of the anterior (from the upper surface of the pyramid or transnasal), lateral (translabyrinthine, transcochlear, infracochlear approach) or posterior (retrosigmoid approach in case of intradural lesion) surgical corridor.

Each of these approaches has certain advantages and disadvantages. To date, no standard surgical algorithm has been developed. Some patients do not receive adequate treatment due to the surgeons' (otosurgeons'/neurosurgeons') lack of experience with this zone; there are almost no publications on this topic in Russian journals. In case of suspected benign lesion the need to select a gentle treatment strategy involving maximum preservation of adjacent anatomical structures is noted.

The review describes the most common petrous apex lesion, surgical approaches and the petrous apex anatomy.

Epidemiology and pathophysiology

Asymmetric pneumatization and effusion in the cells of the petrous pyramid are the most common manifestations of the petrous apex lesions [5]. The estimated incidence of the petrous apex granuloma is 0.6 cases per 1 million population. Cholesterol granuloma is 10 times more prevalent than the petrous apex cholesteatoma and 40 times more prevalent than mucocele [5].

There are two theories about the cholesterol granuloma formation. The obstructive vacuum theory is based on the obstruction of air space resulting in gas absorption an resorption, vacuum, mucosal hemorrhage, inflammation, and degradation of red blood cells followed by formation of cholesterol crystals. During the cholesterol crystal formation, the inflammatory cascade is triggered by bone resorption and inflammatory body reaction [6]. The theory of the spongy bone involvement in based on the development of abnormal link between the air cell system mucosa during intense pneumatization of the petrous apex with subsequent gradual replacement with spongy bone accompanied with hemorrhage into the petrous apex air cells [6]. Red blood cells decay to the form cholesterol crystals, thus inducing the inflammatory cascade [6].

The hypothesis has been proposed that cholesterol granulomas are formed due to eustachian tube dysfunction, negative pressure not only in the tympanic cavity, but also in mastoid cells and cells of the petrous pyramid, which communicate with the tympanic cavity, most often indirectly [7]. The resulting negative pressure leads to the leakage of fluid (plasma) from the submucous blood vessels through the mucous membrane of the tympanic cavity, mastoid cells and cells of the petrous pyramid. In some cases, blood vessel rupture and hemorrhage between the mucous membrane and bony wall occur in certain areas of the mucous membrane [7]. In the conditions of oxygen deficit, hemolysis of red blood cells (hemoglobin) occurs that also results in the formation of cholesterol crystals from cell membranes, causing the giant cell reaction, which in turn leads to the cyst formation [7]. When the bone surface becomes deficient, with coaptation of richly vascular mucosal air cell lining, accumulation of blood leads to abnormal blood outflow pathways, and increased accumulation of blood enables progressive cyst expansion [7]. As the cyst expands, bone erosion increases the area of exposed bone surface along the cyst wall [7]. Based on the above theory, is was shown that six patients with cholesterol granuloma out of 13 definitely had a gap between the bony wall and the mucous membrane of the petrous apex air cells, while the control patients with pneumatized apices showed no signs of such gap [7]. This theory was confirmed by additional hystological assessment of the temporal bone.

Cholesteatoma (epidermoid cyst) consists of the epithelial torus structure, subepithelial fibrous tissue, and keratinized desquamated debris. It is believed that the remaining epidermis is formed in close vicinity to the foramen lacerum during embryonic development [6] or due to migration of ectoderm of the external auditory canal [6]. Expansion of cholesteatoma may result in bone erosion due to osteolytic enzymes of epithelium and subepithelial fibrous tissue. Epidermoid and dermoid cysts can be distinguished by the presence of skin appendages (for example, sweat glands). Mucocele, which is formed due to obstructed drainage from the excessively pneumatized petrous apex, can also cause the development of cystic mass [6].

Diagnosis

Primary lesions of the petrous apex are usually diagnosed at late stages since such lesions are rare and asymptomatic at early stages. Defects of the petrous apex and around it could be caused by cholesteatoma, meningioma, schwannoma, giant cell tumor, cholesterol granuloma, ICA aneurysm, metastases or mucocele. The clinical features result from the compression of cranial nerves IV, V and VI. Larger lesions may affect the cranial nerve complexes VII–VIII. Erosion in the dura mater may result in otoliquorrhea [8]. Masses in the petrous apex are revealed mostly by CT and MRI. These diagnostic methods are also used to define the postresection cavity size and exclude postoperative complications.

Surgical treatment

Surgical treatment of the petrous apex lesions is recommended to patients with such symptoms, as hearing loss and vestibular disorder, i.e. when the lesion spreads beyond the petrous apex and extends to the labyrinth.

The main factor that should be analyzed prior to surgery in case of the petrous apex lesion is the lesion localization: extradural (cholesterol granuloma, clival chordoma or chondrosarcoma) or intradural (meningioma, epidermoid and dermoid tumors). This is the only factor that provides a key to understanding of both etiopathogenesis and the question of which neural and vascular components might be involved in the process [9]. Furthermore, surgical treatment is selected based on the assessment of dysfunction of the facial and vestibulocochlear nerves [9, 10].

Several surgical approaches to the petrous apex have been described, which could be classified as transtemporal/transnasal or hearing-preserving/non-hearing-preserving [5]. Surgical treatment includes mass excision or marsupialization (minor surgical procedure to drain out cysts or granulomas). Excision (for example, of cholesterol granuloma) usually involves wide exposure of the lesion and often requires craniotomy with appropriate temporal lobe retraction. The *advantage* of excision lies in the chance to reduce the number of relapses, and *disadvantages* lie in the need for brain retraction (which may result in encephalomalacia and sometimes in seizures) and the increased risk of postoperative intracranial hemorrhage [5–7]. Liquorrhea also contributes to the risk of intracranial surgery, especially in patients with cholesterol granulomas, who usually demonstrate hyperpneumatization of the temporal bone [11].

A major shortcoming of marsupialization is the relapse of symptoms due to scarring that blocks the surgical drain [5].

A clinical case is reported of the 24-year-old patient with cholesterol granuloma complicated by Gradenigo's syndrome, who initially presented with nausea, fever, photophobia, left retro-orbital pain, and headache [11]. At first, the following diagnosis was established: bacterial meningitis based on the data of lumbar puncture due to detection of *H. influenzae* in cerebrospinal fluid (CSF); the patient received a course

of antibiotic therapy. After a few days, diplopia emerged in addition to the listed above symptoms.

Brain MRI revealed an expansile erosive cholesterol granuloma, which was removed during the combined operation performed by neurosurgeon and otorhinolaryngologist with endoscopic transsphenoidal drainage. Following evacuation of the cyst contents, the left petrous apex defect was marsupialized using the right middle turbinate mucosal graft, harvested earlier in the appropriate procedure. Histopathology revealed xanthogranulomatous inflammation consistent with the radiologically suspected cholesterol granuloma [11].

Such atypical manifestation of Gradenigo's syndrome with the rare complication of meningitis associated with granuloma of the petrous apex demonstrates the importance of early detection, mandatory use of radiological assessment methods (MRI), and consideration of the possibility of performing surgery in patients with pre-existing lesions of the petrous apex, who are potentially at higher risk of dangerous complications. Thus, surgical approach and operation procedure are selected based on the clinical manifestations, lesion, space-occupying lesion site, position of blood vessels, and surgeon's experience. Anterior, lateral (posterior) and anterolateral (transpetrosal) approaches to the petrous apex are distinguished. Anterior approaches include the following: medial transsphenoidal approach; medial transsphenoidal approach with lateralization of the ICA; sphenoidal approach in the lower petrous part of the temporal bone; endoscopic approach through the foramen lacerum; endoscopic anterior resection of the petrous apex. Lateral (posterior) approaches include: translabyrinthine, transcochlear, transcanal infracochlear, and the middle cranial fossa approaches. Anterolateral (transpetrosal) approaches are as follows: open anterior resection of the petrous apex.

Translabyrinthine approach

It is considered a standard otosurgical approach that provides the widest lateral corridor to the petrous apex with the best control over the the entire length of the facial nerve. However, this approach entails removal of semicircular canals, vestibule, and identification of internal auditory canal. The approach provides limited access to the petrous apex. The risks of this surgical procedure include the development of liquorrhea, facial nerve injury, vestibular disorders, and the loss of residual hearing.

Infracochlear or supracochlear approach

It is indicated for lesions located under the internal auditory canal between the jugular bulb, ICA and cochlea [8–10,12].

When using this approach, the bony wall of the lowest basal cochlea curl, jugular bulb and petrous segment of the carotid artery are the key targets that are exposed and identified within the medial wall of meso-hypotympanum. Sometimes, larger and more lateral cholesterol granulomas are found in the lateral part of the infralabyrinthine tract, which can easily expand into the middle ear. The medial cholesterol granulomas require more extensive resection that could be sometimes difficult due to the presence of jugular bulb or the petrous segment of the carotid artery located posterior at this site. The risk of liquorrhea increases when the resection angle points to the posterior cranial fossa (PCF) and cochlear aqueduct. Hearing loss and vertigo may develop in case of injury to the cochlear aqueduct. Injury to the carotid artery is rare, however, it requires emergency angiographic intervention [12].

Transotic approach

It provides access to entire petrous apex. With this approach, the same stages are used as in the translabyrinthine aproach, with the mandatory closure of the external auditory canal and removal of the posterior wall of the carotid canal and cochlea. The facial nerve is preserved in the canal, unlike the transcochlear approach that involves the backward shift of the entire intratemporal part of the nerve. Transotic approach has the same risks, as the translabyrinthine aproach. Injury to the carotid artery and iatrogenic cholesteatoma formation due to incomplete removal of skin from the external auditory canal are also possible. Transotic and transcochlear approaches usually require eustachian tube obliteration, since liquorrhea is often observed during cochleectomy. These approaches make it possible to perform marsupialization, and in some cases complete excision of the neoplasm.

Transcanal transpromontorial approach

It is an improved method for endoscopic transcanal transpromontorial approach developed for patients with hearing loss or no hearing [9,10,12] that is indicated for removal of lesions located in the internal auditory canal and extended to the petrous apex, more medial to the ICA [8, 9]. This is the access between the tympanic and mastoid segments of the facial nerve, jugular bulb, and the ICA through the cochlea, that is why complete hearing loss should be expected in the postoperative period.

A clinical case is reported of the 51-year-old female patient with trigeminal schwannoma, who presented complaining of pain in her face persisting for three months (along the branches of the right trigeminal nerve) [4]. MRI revealed a large homogenous contrasting mass in the base of the right middle fossa, extending to the most apical and medial areas of the posterior cranial fossa through the petrous apex. The patient underwent surgery via the middle cranial fossa (MCF) approach. The mass was completely resected during surgery; facial nerve paralysis with preserved lacrimal gland function, that was completely resolved six months after surgery, was observed during the postoperative period [4].

There is one more clinical case of the 33-year-old female patient with the gradually progressive facial nerve palsy (grade IV according to the House–Brackmann Facial Paralysis Scale), vertigo and nausea. Neuroimaging revealed the growing tumor that involved the geniculate ganglion and extended to the MCF, internal auditory canal (IAC) and the cerebellopontine angle (CPA) [13].

The patient undervent surgery via the MCF approach that involved the use of the facial nerve (FN) neurophysiological monitoring. The facial nerve involvement in the neoplastic process was revealed during surgery. Surgery allowed the surgeon to remove as much mass as possible and preserve the FN. The FN palsy improved gradually during the postoperative period; after 10 months of postoperative follow-up grade III of the FN function was achieved (House–Brackmann Facial Paralysis Scale), and there were no signs of tumor regrowth [13].

The middle cranial fossa (MCF) approach is used to ensure hearing preservation in patients with benign, non-tumor or cystic lesions of the petrous apex. It should be borne in mind that MCF approach to the petrous apex is used to remove, rather than just drain out the cyst; this approach is preferable when the cyst localization and abnormal hypotympanic pneumatization make it impossible to use infracochlear approach.

Middle cranial fossa (transpetrosal) approach

It proceeds from the top down towards the anterior (uppermost) surface of the pyramid and the petrous apex; the approach makes it possible to excise limited lesions. This hearing-preserving approach is unique, since it provides direct access to the petrous apex and identification of the facial nerve over a considerable length (intracranial/cisternal, meatal (mastoid), labyrinthine and tympanic segments) of the vestibulocochlear nerve and structures of the CPA [7]. Since hearing preservation is a hallmark of the MCF approach, the approach requires precision knowledge about microsurgical anatomy of the lateral skull base, given the frequent absence of the clearly defined landmarks, anatomical variability, and high risk of complications in case of surgeon's disorientation during the surgical procedure [7].

The main landmarks of the lateral skull base when using the MCF approach are as follows: middle meningeal artery, greater and lesser petrosal nerves (GSPN); trigeminal nerve (V3); projection of the IAC; arcuate eminence (EA); lacerum segment (C3 of the ICA), region of trigeminal impression.

When the lesions are large, and additional control over the carotid artery is required (when there are cysts that go down and surround the carotid artery), transcochlear access may be used, extended to the infratemporal fossa, if necessary.

Endoscopic endonasal approach

In some cases, endoscopic transnasal access can provide broad drainage pathways in patients with cholesterol granulomas of the petrous apex. The approach usually requires wide sphenoidotomy that involves creation of the large corridor to the lesion along the posterior-lateral wall of the sphenoid sinus, as well as extended maxillary antrostomy and complete ethmoidectomy in addition to removal of the posterior wall of the maxillary sinus to expose the pterygomaxillary fissure and infratemporal fossa. This approach has some advantages over transtemporal access. A significantly wider drainage tract, that is less likely to be occluded by the scar, could normally be created using transnasal approach. The disadvantages of the approach include nosebleed, liquorrhea, restenosis of the drainage tract, dry nose, complications in the form of injury to the structures of eye socket and the brain, injury to the carotid artery and facial nerve, chronic serous otitis media resulting in the need to install the tympanostomy tube; chronic sphenoiditis and transient abducens nerve palsy (cranial nerve VI), tension pneumocephalus [9,10,12].

The literature also describes the role of otoendoscopy in performing the middle cranial fossa approach. Surgical treatment of patients with cholesteatoma of the petrous apex has been assessed. The researchers have found that the use of endoscopic surgery, indicated for removal of the remaining cholesteatoma surrounding the ICA (especially in the medial part of the ICA), dura mater and facial nerve near the petrous apex, results in less invasive surgical intervention and lower number of relapses due to complete removal of the mass, especially from the so-called blind spots [14].

Meanwhile, one of the papers reports the importance of using endoscopic techniques for treatment of supralabyrinthine cholesteatoma. The techniques enabled imaging of the medial parts of the tumor surrounding the cochlea and internal carotid artery by performing transcranial approach via MCF. The 70 degree rigid endoscope was used for visualization of the internal carotid artery and the lower surface of the cochlea [3].

The case of using the combined microscopic and endoscopic approach to remove the giant petrous bone cholesteatoma is reported [15]. The authors have noted that the combined approach is mobile, it enables surgeon's manipulation in the surgical wound and provides access to the lesion from various angles.

CONCLUSION

According to literature, effusion, mucocele, cholesterol granuloma, cholesteatoma are the most common benign lesions of the petrous apex. The published sources describe various surgical approaches, among which translabyrinthine, transcochlear, endoscopic endonasal approaches and the middle cranial fossa approach are the most common.

The use of the middle cranial fossa approach, infracochlear or endoscopic transnasal approach is recommended for patients with preserved hearing. In case of disseminated lesions, when the carotid artery and the jugular bulb should be additionally controlled, transcochlear and translabyrinthine surgical corridors could be used.

Despite the wide use of endoscopic techniques in the middle ear surgery, surgery of the lateral skull base is still based on the use of microscopy, since the lateral skull base lesions are often large. It is more feasible to use lateral approaches for removal of such lesions, particularly middle cranial fossa approach, to reach the petrous apex lesion, ensure high-quality lesion removal and maximum preservation of vital vascular and neural structures, as well as the patient's hearing.

In this regard, despite all the advantages, it is better to combine endoscopic and microscopic approaches to ensure visualization of blind spots and provide additional assistance to the surgeon. The issue of the need to use endoscopic approach as a solo surgical technique remains controversial.

The middle cranial fossa approach is a hearing-preserving approach that ensures a good view of the entire petrous apex and controlled position of the facial nerve. In is necessary to have a precision knowledge of microsurgical anatomy of the bony floor of the middle cranial fossa and adjacent areas, good spatial sense, good surgical technique and sufficient experience in excisions to prevent complications when performing the middle cranial fossa approach to the petrous apex.

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STUDYING VISUAL GNOSIS THROUGH EEG MICROSTATE ANALYSIS

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Objective diagnostic assessment of the human thought processes is an important issue of modern neurophysiology. The study was aimed to develop a system to analyze visual gnostic processes as a model of higher nervous function. A total of 30 people aged 30–60 having no acute disorders, exacerbations of chronic disorders or significant vision problems were examined. Electroencephalography analysis included EEG artifact removal, clustering and distinguishing specific EEG microctates according to the selected model with subsequent localization of the main source of activity, that had generated the EEG microstate, through the algorithms for solving the inverse EEG problem implemented in the sLORETA software package. When running the visual gnosis test (looking at written symbols), activity was recorded within a larger number of Brodmann areas compared to the state of relaxed wakefulness. Activity was detected within Brodmann areas 18 and 19 (11 and 45%, respectively) responsible for visual perception of images, area 39 being a part of Wernicke's area (6%), and the structures of premotor and prefrontal areas (areas 6–11) (up to 11%) ($p < 0.001$; Pearson's chi-squared test). Microstates defined when a subject is in a state of relaxed wakefulness or under visual load are not identical. Rather these are gauge derivatives of clustering in the context of used mathematical model. Solving the inverse EEG problem at the final stage of the study makes it possible to define the average sequences of rhythmic activity associated with realization of visual gnostic function.

Keywords: EEG, inverse problem, microstates, model, gnosis, vision, function

Compliance with ethical standards: the study was approved by the Ethics Committee of the Federal Center for Brain and Neurotechnologies of FMBA (protocol № 148-1 dated June 15, 2021) and carried out in accordance with the principles of biomedical ethics set out in the Declaration of Helsinki (version issued in 1964 and the next updated versions). The informed consent was submitted by all study participants.

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ИССЛЕДОВАНИЕ ЗРИТЕЛЬНОГО ГНОЗИСА С ПОМОЩЬЮ АНАЛИЗА ЭЭГ-МИКРОСОСТОЯНИЙ

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Объективная диагностика мыслительных процессов человека представляет собой важную проблему современных нейрофизиологических исследований. Целью исследования было разработать систему анализа процессов зрительного гнозиса как модели высшей нервной функции. Обследовано 30 человек в возрасте 30–60 лет, не имеющих острых заболеваний или обострений хронических заболеваний, а также выраженных проблем со зрением. Анализ электроэнцефалограмм включал подавление артефактной ЭЭГ-активности, кластеризацию с выделением отдельных ЭЭГ-микросостояний согласно выбранной модели и последующим установлением локализации основного источника активности, формирующего ЭЭГ-микросостояние, посредством алгоритмов решения обратной задачи ЭЭГ пакета программ eLORETA. При тесте на зрительный гнозис с рассматриванием письменных знаков активность была зарегистрирована над большим числом полей Бродмана, чем в состоянии пассивного расслабленного бодрствования, и затрагивала поля Бродмана 18 и 19 (11 и 45% соответственно), ответственных за зрительное восприятие образов, 39-е поле — дополнительную часть области Вернике (6%), а также структуры премоторной и префронтальных областей (поля 6–11) (до 11%) при ($p < 0,001$; тест хи-квадрат Пирсона). Микросостояния, определяемые во время пребывания обследуемого в состоянии расслабленного бодрствования и при выполнении зрительной нагрузки, не представляют собой идентичные феномены, а являются градуированными производными кластерного анализа в рамках используемой математической модели. Решения обратной ЭЭГ-задачи на конечном этапе исследования позволяют определить усредненные последовательности ритмической активности, связанные с реализацией функции зрительного гнозиса.

Ключевые слова: ЭЭГ, обратная задача, микросостояния, модель, гнозис, зрение, функция

Соблюдение этических стандартов: исследование одобрено этическим комитетом ФГБУ «ФЦМН» ФМБА России (протокол № 148-1 от 15 июня 2021 г.), проведено в соответствии с принципами биомедицинской этики, сформулированными в Хельсинкской декларации 1964 г. и ее последующих обновлениях. Каждый участник подписал добровольное информированное согласие на участие в исследовании.

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Establishing a link between the objective research data and the thought process is the most important issue of modern neurophysiology.

Following the development of the method for human electroencephalography recording by H. Berger in 1929, the researchers proposed different approaches to the issue, from attempting to convert brainwaves into audio recording [1] to the use of top-down artificial intelligence system that involved using the data sets obtained when examining people with various disorders. However, despite the range of the used technologies, we are still a long way from resolving the problem.

Currently, researchers are less interested in neurophysiological techniques based on EEG/MEG recording of various modalities due to a number of objective reasons. Thus, conventional EEG tests involve recording of continious rhythmic activity of the brain with the pool of electrodes evenly distributed over the scalp surface. All electrodes are equal in design and function, that is why a composite signal resulting from the activity of brain structures together with multiple artifacts of physiological and physical origin are registered on each electrode [2]. There is a need for careful processing of the composite signal to distinguish distinct components by mathematical methods [3] that require

in-depth training of specialists. The other feature of the technique is that it is difficult to establish an objective link between the characteristics of the studied signals and the changes in environmental conditions, i.e. signal sensitivity. Discrete methods, such as evoked potential tests, involve recording the changes in biopotentials after stimulus presentation, thus allowing the researcher to set the zero reference point to assess the association of the changes observed with significant and non-significant stimuli. However, a wide range of different stimuli is needed to assess a complex cognitive stimulus, that is why different method implementation is required [4, 5].

Among EEG data processing methods designed for this purpose that make it possible to establish an objective link between the changing characteristics of the signal recorded and external exposure, clustering algorithms [6] and techniques for solving the inverse EEG problem [7–9] are the most interesting. These methods allow for determining the pattern and detection of changes in bioelectric activity of distinct brain structures.

Currently, clustering of continuous EEG signal proposed in the 1990s [10] enables real-time acquisition of data from the neuronal structures of the brain. The technique is based on monitoring the characteristics of distribution of the head surface biopotentials that show discrete changes in the total energy density of the total scalp potential. Thus, the first variant remains stable within a short time frame, then it quickly gives way to the other one that also remains stable for some time. In 1995 R. Pasqual-Marque introduced a mathematical method for active detection of EEG microstates by clustering continuous EEG data [11, 12]. These studies confirmed the perception that each EEG microstate reflected the function of certain neural network of the brain or functionally linked group, and microstate sequences could be considered a reflection of realization of certain brain function [13]. Such approach has made it possible to divide the continuous flow of EEG data acquired during initial assessment into components that currently allow us to detect as many as 39 distinct EEG microstates. However, only six first microstate classes can be fully representative, which is possibly due to the activity of large neural networks responsible for realization of basic and the most stable brain functions; impairment of these functions manifests in the form of severe mental disorders [14–16]. Therefore, the functional study performed using the method outlined above makes it possible to define interconnection system, and the technique for solving the inverse EEG problem allows one to map brain structures involved in implementation of the studied brain function. However, when using the technique for solving the inverse EEG problem, it is necessary to take into account that the terms “EEG activity” and “functional activity of nervous tissue” are not synonymous in the context of physiological research [17]. When performing physiological research, the power of scalp potential of the cortical areas that produce rhythmic activity is registered as the area of high signal strength in contrast to the areas of the nervous tissue excitation characterized by low amplitude and disorganized EEG patterns [18, 19].

That is why the use of combined analysis based on various functional imaging techniques (EEG-fMRI, MEG-fMRI, etc.) to confirm the physiological research data could be ineffective [20]. The study was aimed to develop a system for analysis and assessment of visual gnostic processes as a model of higher nervous function by solving the inverse EEG problem based on the cluster EEG microstate model.

METHODS

A total of 30 generally healthy people aged 30–60 (32.4 ± 9 years) were enrolled. Inclusion criteria: no significant visual problems. Exclusion criteria: history of head or eye injury; taking medications on a regular basis. The resting state EEG was continiously recorded in all subjects with their eyes closed, eyes open, and when continiously looking at symbols (letters, numbers) in the black and white contrast image on the computer screen. The subjects were allowed to rest for 3 minutes between tests to recuperate.

Subsequent analysis of encephalograms included removal of artifacts resulting from interference during EEG recording by filtering using digital filters with fixed bandwidth of 1–35 Hz in order to suppress the galvanic skin response and myographic artifacts. Later physiological artifacts were removed using the independent component analysis performed with the RUNICA utility of the EEGLAB Ver. 2022a software package (USA). Clustering of the continious EEG signal was performed by distinguishing distinct stable EEG microstates in accordance with the selected six-component model using the atomize and agglomerate hierarchical clustering (AAHC) that showed higher sensitivity compared to K-means clustering. Six components were selected based on the current two-streams hypothesis of functional brain network organization and the existence of functional links between certain microstates and information processing. In this case two additional microstates were considered the reserve quantities. Spatial localization of rhythmic activity for each EEG microstate was calculated at the final stage using the algorithms for solving the inverse EEG problem implemented in the sLORETA software package.

Histograms of bioelectric activity recorded within Brodmann areas were created based on the results of each test for each participant and each group (average).

Hardware and software used during the study

A 128 channel EGI-GES-300 bioamplifier combined with the original GSN-128 sensor net system (analogue for the 10-5 system) (MAGSTIM; USA). EEGLAB software package for MATLAB_ Runtime 2021b (Center of the Institute for Neural Computation, the University of California San Diego; USA). eLORETA v20210701 software package (University Hospital of Psychiatry, University of Zurich; Switzerland). Statistical software package GNU PSPP (International). Operating system Linux Mate 21.10 (Canonics; UK). Suite of office applications LibreOffice 7.2 (Document Foundation; USA). No commercial licenced software was used during the study.

Selection of functional study design

The study involved stimulation of the visual sensory system since its neuronal networks occupied the largest area of the cortex, and excitation in these networks caused the most significant changes in rhythmic activity of the brain thus allowing one to get more accurate results compared to analysis of activity of other neuronal systems. The following situations were chosen as loading tests: the state of relaxed wakefulness with eyes closed or open, and the state of visual gnosis realization through looking at various symbols and signs on the screen of LED monitor. Our aim was to define the characteristics of bioelectric activity with the sensory system in inactive (eyes closed) or active state, in the situation of no mental task performed (relaxed wakefulness with eyes open) or during realization of mental task in the form of test that involved looking at symbols.

Statistical analysis

A linear model that included one factor, the impact on the visual sensory system, was used in the study. Regarless of the direct

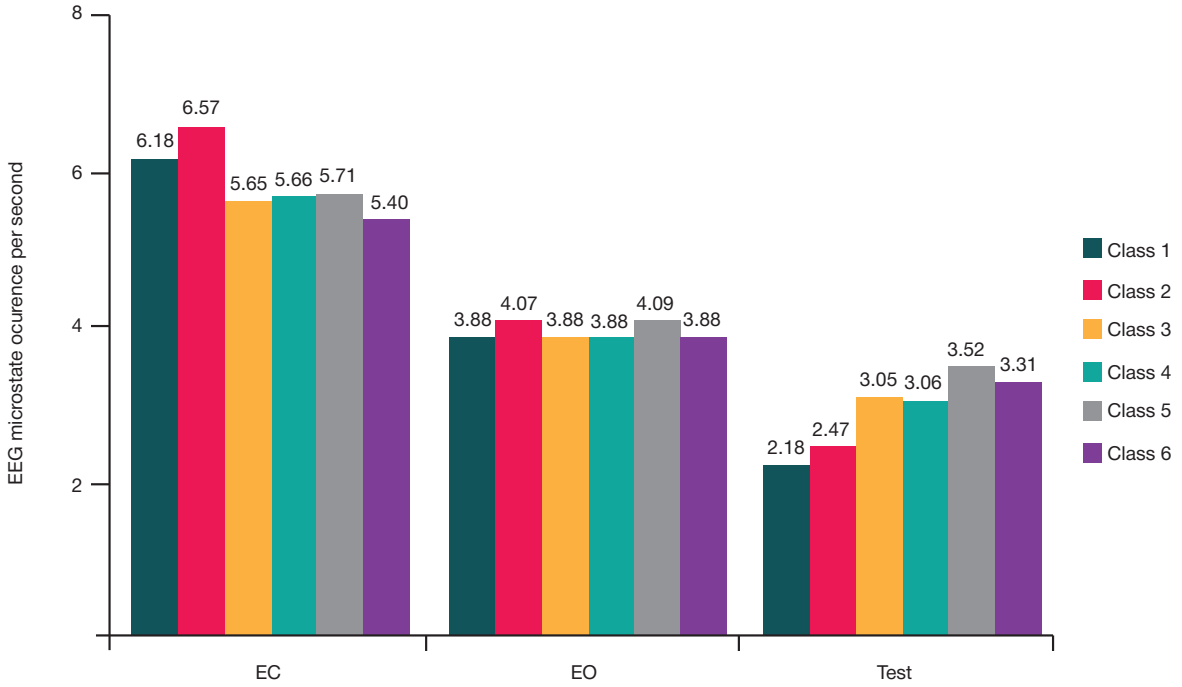

Fig. 1. Average values of EEG microstate occurrence per second for six microstate classes obtained when peforming loading tests

correlations between the experimental data, one-way analysis of variance (ANOVA) was chosen after testing for normality due to the differences between data obtained using the model for dividing the studied activity into classes. Significance of the results obtained by solving the inverse EEG problem was tested using the Pearson's chi-squared test, since the changes were of a qualitative nature. Assessment was performed in accordance with the guidelines [21].

RESULTS

Characteristics of EEG microstates obtained during the experiment

The main objective measures of EEG microstates used in the study were as follows: microstate occurrence per second, microstate duration in seconds, and microstate percentage in the structure of the total EEG potential (coverage). These indicators conditionally reflected the characteristics of certain neuronal network that had generated every single microstate, the frequency of its functional activation during realization of the studied function, and relative amount of functional elements contained in this network.

Studying the EEG microstate occurrence showed that the biggest changes took place when comparing the tests performed in the state of relaxed wakefulness with eyes open with the tests performed with eyes open. Thus, indicators of the first and second class significantly ($p < 0.05$; ANOVA) changed during all tests. However, in other classes of EEG microstates, significant differences ($p < 0.05$; ANOVA) were observed only with the state of eyes-closed relaxed wakefulness. In other cases no significant differences in occurrence were found when peforming tests with eyes open ($0.1 < p < 0.8$; ANOVA) (Fig. 1).

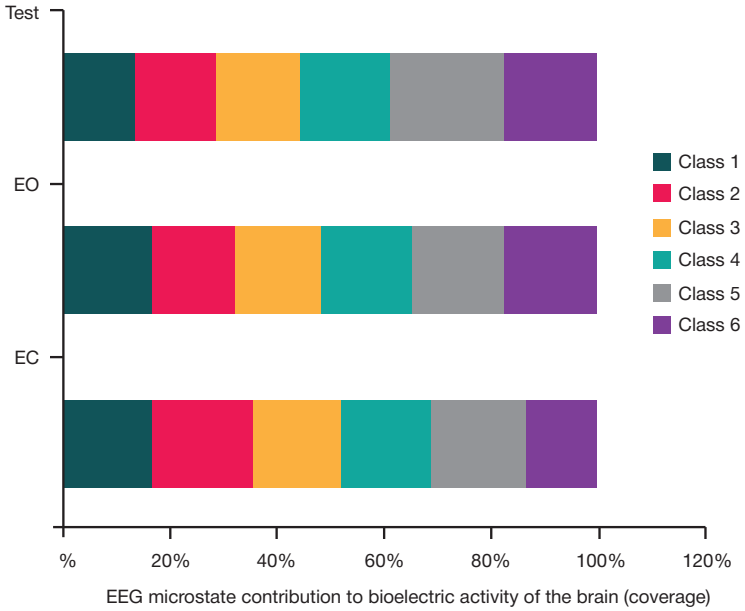

Fig. 2. Average values of changes in the EEG miscrostate contribution to the total energy of the scalp field (coverage) obtained when performing the main tests during the study

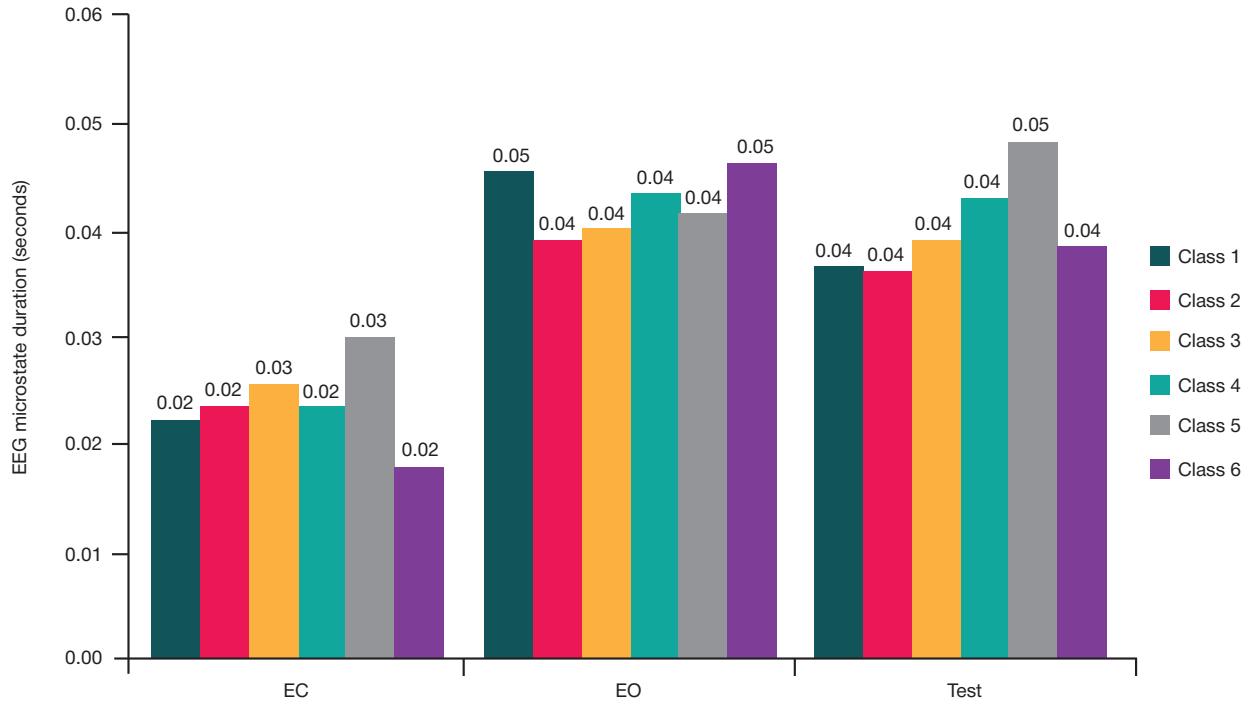

Fig. 3. Changes in EEG microstate duration (average values) depending on the load

Calculation of the EEG miscrostate contribution to the total energy of the scalp field (coverage) revealed no signficant differences between the characteristics obtained during three tests. The significance level exceeded 0.5 (Fig. 2).

Assessment of EEG microstate duration showed that this indicator significantly increased when the subjects opened their eyes or performed the loading test that involved looking at symbols ($0.01 < p < 0.05$; ANOVA). Comparison of the values obtained when the subjects had their eyes closed or were actively engaged in performing the visual test revealed almost no differences ($0.1 < p < 0.9$; ANOVA).

Solving the inverse EEG problem for the used model of the EEG microstate classes

The inverse EEG problem was solved using the e-LORETA software package. Source localization and verification against Brodmann's map was performed by clustering for each EEG microstate class.

In the state of relaxed wakefulness with eyes closed, activity was detected within the following Brodmann areas: 11 — areas of olfactory system, 18 and 19 — secondary zones of visual sensory system, 21 — vestibular area, 37–47 — areas responsible for perception of music. In the context of detecting rhythmic EEG activity these reflected the centers' readiness to perceive and analyze the tonal stimuli, or to respond in case of human reaction to stimulation in the form of sound (screaming, sounds having no morpho-phonetic structure).

Eye-opening changed the pattern recorded. Three main zones of activity were distinguished: area 18 — areas of visual sensory system responsible for the written text recognition, area 20 — cortical center of the vestibular system / complex pattern recognition, area 37 — acoustic-gnostic speech center.

When performing the test for visual gnosis that involved looking at written symbols, activity was detected within a larger number of Brodmann areas compared to the state of relaxed wakefulness (Fig. 4). Activity was found in Brodmann areas 18 and 19 responsible for visual perception of images, area 39 being a part of Wernicke's area, and the structures of premotor and prefrontal areas (areas 6–11).

DISCUSSION

The study showed that the technique for continuous EEG monitoring supplemented by methods for EEG microstate analysis and solving the inverse EEG problem could be used as a research tool for assessment of changes in functional brain activity during realization of higher nervous functions.

Analysis of the main EEG microstate characteristics has shown that activation of visual sensory system has an overall impact on the occcurence and duration of each EEG microstate class. However, no significant changes in the contribution of each class to the total energy of the scalp field (coverage) are observed. This fact correlates with the results of a number of studies [22, 23], since such stability results from intact structure of neuronal networks involved in generation of each microstate in the generally healthy experimental subjects.

Significant variability of the first and second class EEG microstate occurrence was also the expected result that had been previously described in literature. Such variability was related to rearrangement of large neuronal network during realization of functional response. As previously reported, the lack of similar dynamic changes in the characteristics of other four classes was possibly due to the fact that these were more difficult to distinguish when assessing the recording [24].

Observations suggest that microstates detected when the subject is in a state of relaxed wakefulness with his/her eyes closed or in a situation of visual load are not identical, since the activity of neuronal networks that generate these microstates shows up significant differences in both occurrence and duration of each of the distinguished EEG microstate classes. It may be assumed that the posiibility of EEG signal discretization involving division into separate groups of brain activity that correspond to functioning of distinct neuronal networks is the main effect of the used cluster model. However, calculation of basic EEG microstate characteristics makes it impossible to define their specificity.

The experiment showed that EEG microstates detected when the subject was in a state of relaxed wakefulness or in the situation of visual load were not identical. Rather these

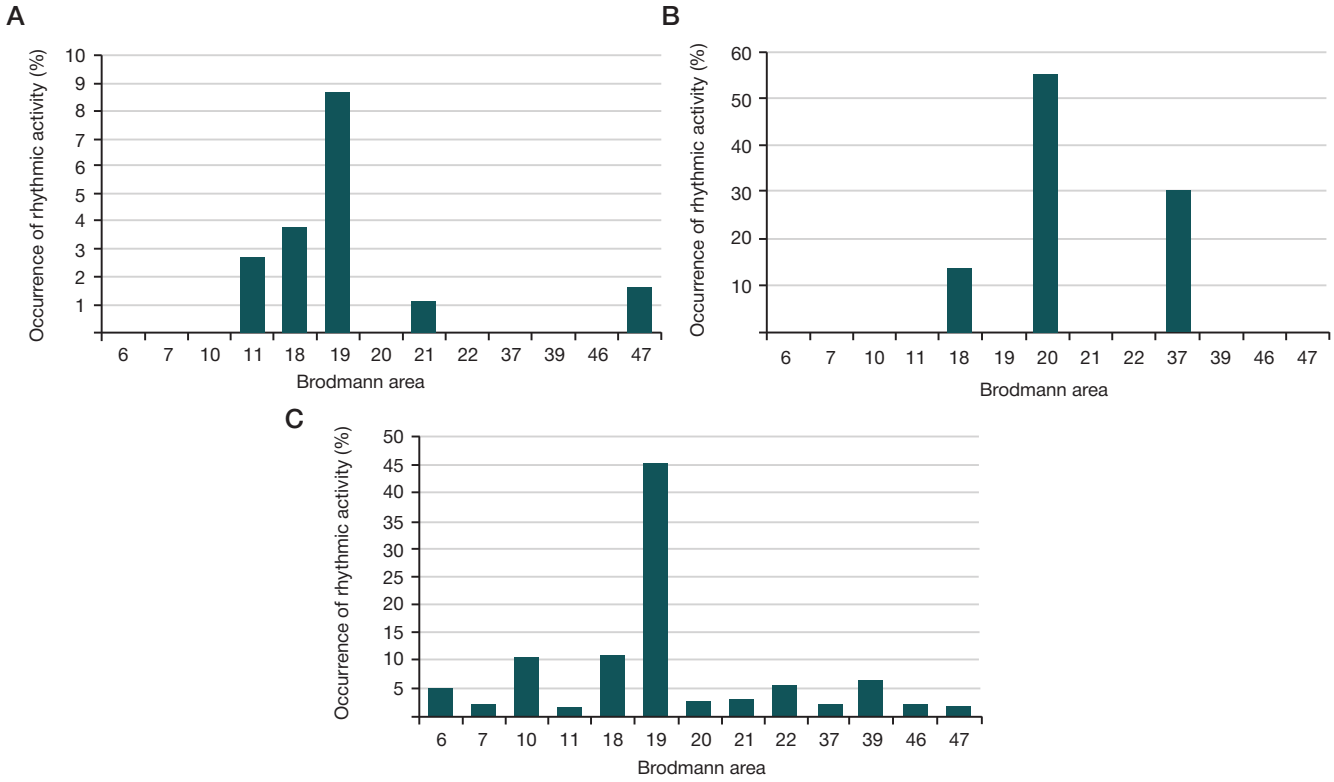

Fig. 4. Comparison of average (for the group) quantitative characteristics of the Brodmann area signal recorded in the situation of solving the inverse EEG problem when performing tests during the study based on the model of six EEG microstate classes ($p < 0.001$; Pearson's chi-squared test). a — eyes closed, b — eyes open; c — performing recognition test

were gauge derivatives of clustering in the context of used mathematical model, which was confirmed by the differences in their basic characteristics measured in a resting state, with eyes open, and when performing the loading test. However, no reports of similar findings were found in the reviewed literary sources.

Solving the inverse EEG problem at the final stage of the study made it possible to localize and define (based on Brodmann areas) three average sequences typical for the loads used during the study. Taking into account the features of the EEG signal recording and frequency analysis, these reflected the processes of preparing/selecting the appropriate neuronal networks for further functional activity. Such processes can be considered the idle state [25–27]. However, during realization of complex (gnostic) brain functions, excitation and idling processes change each other in a rhythmic manner, thus allowing us to characterize certain sections as active and consider these as related to realization of the studied function. Thus, in the state of relaxed wakefulness with eyes open almost no rhythmic activity was detected within the Brodmann area 19, since this neuronal network was in the excited state and showed minimum bandpower of the signal. At the same time bandpower of the areas responsible for complex pattern recognition (area 20) and gnostic centers of sound perception and analysis (area 37) indicated that these structures were ready for engagement, which actually corresponded to the state of increased attention while awaiting speech addressed to a person. No such components related to being more focused on a specific task were observed in a state of passive wakefullness with eyes closed. The changes in rhythmic activity affected visual areas (areas 18 and 19) and the center involved in processing of music (area 47), which corresponded more closely to awaiting perception of external signals, readiness for analysis of these signals and primary non-specific response. Under the test load in the form of looking at symbols the patterns of rhythmic activity were more complex than in the state of passive wakefulness, since these involved a larger number of Brodmann areas. This observation was considered as a consequence of the neural network cyclic transitions between the excited state and idle state during realization of visual gnostic function.

Thus, we believe that the method proposed in our study based on using EEG microstates makes it possible to reveal functional sequences associated with realization of distinct brain functions. In the future the method may be used in developing the new diagnostic equipment for objective assessment of thought processes in the brain.

CONCLUSIONS

The study has shown the potential of studying higher nervous functions by recording the changes in characteristics of the scalp surface biopotentials. However, implementation of the technique requires a radical revision of both EEG test organization and analysis of the results. It is necessary to develop the new diagnostic equipment that should be quite different from the currently used conventional EEG systems. The analytical unit should be completely revised in favor of systems for automated analysis of activity generated by structures in the brain. The prospects of such inventions are not only of great interest for modern neuroscience, but also have a significant potential for the development of human monitoring systems in harsh environmental conditions.

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DEVELOPMENT OF MICROBIAL PREPARATION FOR BIOREMEDIATION OF SOILS CONTAMINATED WITH ROCKET FUEL COMPONENTS

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Heptyl rocket fuel and aviation kerosene are widely used in the propulsion systems of the Proton and Soyuz spacecraft. The propellant components (RFC) enter the environment, causing strong toxic effects, when the separating first stages of rockets fall away or in case of emergencies. The study was aimed to isolate strains of microorganisms-destructors of RFC, as well as to assess their safety for bioremediation of contaminated soils. Microorganisms capable of decomposing heptyl, formalin, and aviation kerosene were isolated from natural soils. An association of two strains of bacterial destructors *Pseudomonas putida* 5G and *Rhodococcus erythropolis* 62M/3 was obtained, and a method of their use in recultivation of soil contaminated with RFC was developed. The results of laboratory and field tests showed high efficiency of the microbial destruction of pollutants, the decrease in integral toxicity and phytotoxicity of the cleaned soil to safe levels, and an increase in the soil biological activity. Thus, dehydrogenase activity increased by 2.4 times, hydrolase activity by 2.1 times, and cellulase activity by 5.1 times. Microbial association can be recommended for recultivation of soil contaminated with RFC.

Keywords: rocket fuel, heptyl, dimethylhydrazine, formaldehyde, aviation kerosene, rocket fuel components, degrading microorganisms, soil bioremediation

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Compliance with ethical standards: animals were treated in accordance with the principles of Good Laboratory Practice. Veterinary protocols № 669 and № 677 for strains 5G и 62M/3 were approved by the Bioethics Commission (protocol № 165/2019 of 19 February 2019, protocol № 169/2019 of 16 April 2019).

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РАЗРАБОТКА МИКРОБИОЛОГИЧЕСКОГО ПРЕПАРАТА ДЛЯ БИОРЕМЕДИАЦИИ ПОЧВ, ЗАГРЯЗНЕННЫХ КОМПОНЕНТАМИ РАКЕТНЫХ ТОПЛИВ

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Ракетное топливо, гептил и авиационный керосин, широко используют в двигательных установках космических кораблей «Протон» и «Союз». При падении отделяющихся первых ступеней ракет и в случае аварийных ситуаций компоненты ракетных топлив (КРТ) попадают в окружающую среду, вызывая сильные токсические эффекты. Целью исследования было выделить штаммы микроорганизмов-деструкторов КРТ и изучить их безопасность для биоремедиации загрязненных почв. Из природных почв выделены микроорганизмы, способные разлагать гептил, формалин и авиационный керосин. Получена ассоциация из двух штаммов бактерий *Pseudomonas putida* 5Г и *Rhodococcus erythropolis* 62М/3, отработана методика их применения для рекультивации загрязненной КРТ почвы. Результаты лабораторных и полевых испытаний показали высокую эффективность микробной деструкции загрязнителей, снижение интегральной токсичности и фитотоксичности очищаемой почвы до безопасных уровней, повышение ее биологической активности. Так, было отмечено повышение дегидрогеназной активности в 2,4 раза, гидролазной — в 2,1 раза, целлюлазной — в 5,1 раза. Ассоциацию микроорганизмов можно рекомендовать для рекультивации почв, загрязненных КРТ.

Ключевые слова: ракетное топливо, гептил, диметилгидразин, формальдегид, авиационный керосин, компоненты ракетных топлив, микроорганизмы-деструкторы, биоремедиация почв

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Соблюдение этических стандартов: работы с животными выполняли в соответствии с принципами надлежащей лабораторной практики. Ветеринарные протоколы № 669 и № 677 по штаммам 5Г и 62М/3 утверждены комиссией по биоэтике (протокол № 165/2019 от 19 февраля 2019 г., протокол № 169/2019 от 16 апреля 2019 г.).

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Heptyl (unsymmetrical dimethylhydrazine, UDMH) is used as a liquid rocket fuel to launch into orbit the Proton, Cyclon, Kosmos, Rokot, Strela spacecraft and unmanned satellites of the Earth. Extensive use of UDMH in the rocket and space technology is due to its unique operational properties. It can hardly be replaced by any other fuel in the near future [1]. Because of its physical and chemical properties (high volatility and solubility in aqueous solutions), dimethylhydrazine migrates in the natural environment, is decomposed into a number of highly toxic products, and persists for a long time in soil. UDMH is assigned hazard class 1, it shows carcinogenic, mutagenic, embryotoxic (yellow children), and teratogenic (abnormal development of an embryo) effects, and causes cancer in people engaged in working with UDMH of living in contaminated areas [1, 2]. Aviation kerosene, that is used in the manned spacecraft of the Soyuz type, also shows high toxicity [1].

To date, no effective, environment-friendly and cheap techniques for cleaning of soils contaminated with heptyl and aviation kerosene have been developed. All the existing techniques can be conditionally divided into three groups: thermal methods (burning); methods of the UDMH deep oxidation that involve the use of aqueous solutions containing active substances capable of interacting with UDMH – these substances form insoluble or poorly soluble complexes in one case, and promote decomposition into simpler compounds in another. Chemical agents (hydrogen peroxide, potassium permanganate solutions, quicklime) are mainly used, which is expensive, environmentally harmful and results in the reduced fertility of recultivated soil. Methods of the other type involve the use of aqueous solutions containing active substances, such as meta-nitrobenzoic acid that forms a complex with UDMH at certain pH values in the form of solid phase. Then, in accordance with the proposed method, solutions contaminated with UDMH undergo thermal deactivation in the specialized furnace. Fixation of UDMH in soil with the formilations containing humic acids, turf, schungite is also used. However, this method does not provide soil decontamination to the level of PEL (0.1 mg/kg) [3].

Calcium peroxide, decomposition of which results in the release of atomic oxygen that is involved in degradation of UDMH, is used for detoxication of soil and deactivation of UDMH on the surface of metal constructions, walls of shelters, etc. Poor decontamination of contaminated areas and long process of detoxication are the disadvantages of this method [3].

Biological techniques for recultivation of contaminated areas are the most preferable due to environmental safety, low cost and relatively high efficiency, which were demonstrated repeatedly when coping with various ecological issues. There are numerous known environmentally friendly biological preparations containing aerobic bacterial strains that are targeted on biochemical destruction and utilization of pollutants, mostly hydrocarbons (pertoleum and its industial derivatives) [4–7]. Biological methods based on the controlled bio-composting are known. Furthermore, biological preparations have been created that contain microoorganisms, for which hazardous waste is a source of nuitrition. This method of soil detoxication does not involve the use of toxic chemicals; microorganisms-destructors die after destruction of UDMH due to the lack of nuitrition, and the soil treated retains biological activity and fertility [8].

Microorganisms are increasingly being used for decontamination of soil contaminated with RFC and water in Russia and abroad. Thus, studies are conducted at the Baikonur Cosmodrome (Kazakhstan) that are focused on using indigenous soil microorganisms for degradation of dimethylhydrazine. Microorganisms are isolated from soil and grown in the fermentation devices to be introduced into the contaminated soil [9]. Currently, there are sporadic reports of the microbial species and associations of microorganisms capable of utilizing UDMH. That the method for biodestruction of heptyl has been developed that involves the use of the association of microorganisms *Acinetobacter sp*. H-1, *Rhodococcus sp*. H-2, *Arthrobacter sp*. H-3 [10]. The method has been proposed for biological decontamination of water and soil contaminated with petroleum and oil products using the Centrum-MMS ecobiopreparation [11], also capable of the UDMH biodestruction in the aqueous solutions. Ecobiopreparation contains microorganisms *Pseudomonas fluorescens* BKM B-6847 and *Rhodococcus erythropolis* AC-1769. However, according to the authors of this invention, Centrum-MMS ecobiopreparation is not capable of cleaning up soil contaminated with heptyl [11].

The development of effective techniques for bioremediation of soils contaminated with highly toxic RFC and their introduction into practice are extremely relevant. Currently, there are no ready-to-use microbial preparations and highly efficient industrial bacterial strains for bioremediation of soil contaminated with UDMH and aviation kerosene, which provides grounds for the study.

The study was aimed to isolate strains of microorganisms-destructors of RFC, as well as to assess their toxicological and environmental safety together with the possibility of application for bioremediation of contaminated soils.

METHODS

The culture collection of microorganisms, that degrade various toxic chemicals (petroleum products, polycyclic aromatic hydrocarbons, mineral oils, phenols, polychlorinated biphenyls, ethylene glycol, heptyl, aviation kerosene, pesticides, mustard gas, lewisite, organochlorines, and organophosphate compounds), was created at the Research Center for Toxicology and Hygienic Regulation of Biopreparations after many years of expeditionary works on the contaminated soil sampling and further laboratory testing. Soil samples collected from the territories contaminated with pesticides and petroleum products for a long time, as well as from the areas of heptyl spill emergencies and the site of the Proton-M launch vehicle crash (Site 81 at the Baikonur Cosmodrome, Kazakhstan), were used to isolate microbial strains capable of degrading heptyl and aviation kerosene.

The method of enrichment culture with subsequent inoculation of minimal medium, containing formaldehyde (primary product of heptyl degradation) or primary degradation product of heptyl as the only source of carbon, was used to isolate microorganisms-destructors of RFC [12].

Pure cultures of isolated microorganisms were identified by MALDI in the All-Russian Collection of Microorganisms (IBPM RAS, Pushchino; Russia).

The biomass of microorganisms-destructors of RFC for the laboratory and field tests was built up in the Certomats-BS1 incubation shaker (Sartorius; Sweden) at a temperature of 28 °C and speed of 180 rpm until the culture entered stationary phase (24–48 h depending on the strain).

Strain 19F of *Rhodococcus globerulus*, the UDMH (heptyl) biodestructor [13], was used as a reference strain.

The sod-podzol soil was used for both laboratory and field experiments.

Bacterial suspension was treated with polyurea microcapsules (BNT LLC; Russia).

Integral (overall) toxicity of water and soil samples was assessed in the laboratory culture of *Daphnia magna* grown

Table 1. Changes in microbial cell counts of microorganisms-destructors and saprophytic microflora during the microbial remediation of soil performed in laboratory environment, CFU/g of soil

Variant	Test duration, days				
	0	7	14	21	30
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	$(3.2 \pm 0.62) \times 10^4$ $< 10^3$ $(1.2 \pm 0.17) \times 10^5$	$(1.1 \pm 0.34) \times 10^5$ $< 10^3$ $(2.7 \pm 0.31) \times 10^5$	$(1.5 \pm 0.21) \times 10^4$ $< 10^3$ $(3.1 \pm 0.24) \times 10^5$	$(1.0 \pm 0.4) \times 10^4$ $< 10^3$ $(6.5 \pm 0.24) \times 10^5$	$(1.0 \pm 0.24) \times 10^4$ $< 10^3$ $(6.5 \pm 0.24) \times 10^5$
Soil + formalin + aviation kerosene + microencapsulated microorganisms	$(3.3 \pm 0.54) \times 10^4$ $< 10^3$ $(1.0 \pm 0.12) \times 10^5$	$(1.8 \pm 0.41) \times 10^5$ $< 10^3$ $(1.6 \pm 0.45) \times 10^5$	$(1.7 \pm 0.38) \times 10^4$ $< 10^3$ $(4.5 \pm 0.34) \times 10^5$	$(1.2 \pm 0.24) \times 10^4$ $< 10^3$ $(7.2 \pm 0.54) \times 10^5$	$(1.2 \pm 0.24) \times 10^4$ $< 10^3$ $(7.2 \pm 0.48) \times 10^5$
Soil + formalin + aviation kerosene + strain 19F (reference strain)	$< 10^3$ – $(1.6 \pm 0.48) \times 10^5$	$(1.5 \pm 0.44) \times 10^4$ – $(4.2 \pm 0.61) \times 10^5$	$(1.2 \pm 0.41) \times 10^5$ – $(1.4 \pm 0.37) \times 10^5$	$(2.4 \pm 0.45) \times 10^4$ – $(6.5 \pm 0.59) \times 10^5$	$(1.1 \pm 0.44) \times 10^4$ – $(8.5 \pm 0.24) \times 10^5$
Soil + formalin + aviation kerosene (control)	– – $(1.5 \pm 0.24) \times 10^5$	– – $(4.5 \pm 0.54) \times 10^5$	– – $(3.8 \pm 0.24) \cdot 10^5$	– – $(2.5 \pm 0.45) \times 10^6$	– – $(6.5 \pm 0.44) \times 10^6$
Clean soil (control)	– – $(2.7 \pm 1.2) \times 10^6$	– – $(3.2 \pm 0.26) \times 10^6$	– – $(4.3 \pm 0.18) \times 10^6$	– – $(5.4 \pm 0.24) \times 10^6$	– – $(3.4 \pm 0.24) \times 10^6$

Note: the columns show indicators for strain 5G, strain 62M/3, soil saprophytes.

in the climatostat at the Research Center for Toxicology and Hygienic Regulation of Biopreparations. Biotests were performed in accordance with the methods [14, 15].

Integral toxicity of soil was assessed by the bioluminescence method using the Ecolum bacterial test system (MSU; Russia) in the Biotox-10M test device (MSU; Russia) in accordance with the guidelines [16, 17].

The colorless 2,3,5-triphenyltetrazolium chloride was used as a substrate for assessment of the soil dehydrogenase activity. Total hydrolase activity of the soil was assessed using the fluorescein diacetate hydrolysis assay. The application method was used to assess the soil cellulose-destroying capacity [18].

Phytotoxicity of soil samples was tested using the oat seeds by the method introduced by O.A. Berestetsky [19].

The content of aviation kerosene was defined with the petroleum product analyzer, the KN-2 infrared spectrometer (Novolab; Russia) in accordance with the method [20].

Pathogenicity (safety) of microorganisms-destructors of RFC was evaluated in accordance with the guidelines of the Ministry of Health of the USSR № 2620-82, № 4263-87 taking into account the guidelines issued by the World Health Organization [21–23]. Evaluation included asessment of virulence, toxicity, toxigenicity, and dissemination in the internal organs of white mice and rats.

Statistical processing of the experimental data was performed with the Excel 7.0 (Microsoft; USA) and Statistica 10.0 (StatSoft; USA) software packages. Experimental data were represented as mean values and 95% confidence intervals.

RESULTS

Isolation of microorganisms-destructors of RFC from contaminated soil

Microorganisms-destructors of heptyl were isolated from the samples of soil contaminated with UDMH. A total of 100 bacterial isolates were obtained. Then microorganisms were subcultured in the minimal medium containing formaldehyde (concentration gradient 0–500 mg/L) and incubated for a long time (up to 10 days) to monitor bacterial growth. Strains 2G, 4G, 5G, G8/2, Y-21 were capable of growing at the formaldehyde level of 200 mg/L; the reference strain 19F and strain G-803 grew at a concentration of 100 mg/L; strains 19 S/1 и 37M/1 remained viable at a concentration of 80 mg/L.

Microorganisms-destructors of aviation kerosene were isolated from the samples of soil contaminated with petroleum products collected from airports. petroleum storage depots,

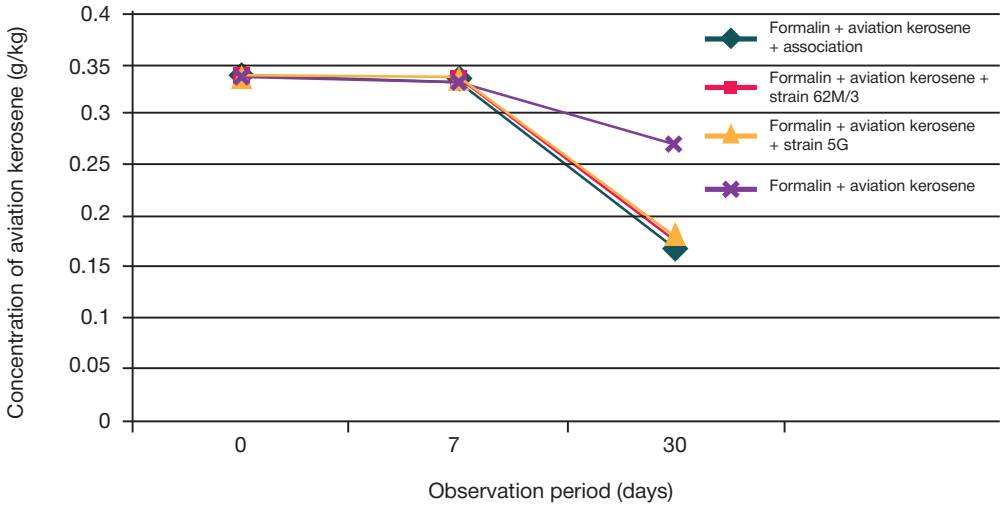

Fig. 1. The process of aviation kerosene degradation by microorganisms-destructors in laboratory environment, g/kg of soil

Table 2. Acute toxicity of the soil to Daphnia assessed during microbial degradation of formalin and aviation kerosene performed in laboratory environment

Experiment variant	Number of surviving Daphnia, *n*		Daphnia mortality rate, %	Acute toxic effects/no acute toxic effects
	control	experiment		
Soil (control)	30	30	0	No acute toxic effects
Soil + formalin + aviation kerosene	30	0	100	Acute toxic effects
After 7 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	13	57	Acute toxic effects
Soil + formalin + aviation kerosene + strain 19F (reference strain)	30	18	40	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	8	74	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects
After 14 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	20	33	No acute toxic effects
Soil + formalin + aviation kerosene + strain 19F (reference strain)	30	18	40	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	8	74	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects
After 21 day				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	18	40	No acute toxic effects
Soil + formalin + aviation kerosene + strain 19F (reference strain)	30	15	50	Acute toxic effects
Soil + formalin + aviation kerosene (control)	30	8	74	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects
After 30 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	30	0	No acute toxic effects
Soil + formalin + aviation kerosene + strain 19F (reference strain)	30	29	3	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	15	50	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects

and filling stations. Six most active bacterial strains capable of growing on the minimal medium containing 5% of diesel fuel were selected among 34 isolates obtained. Three most active strains-destructors of aviation kerosene, 12R, 37M/1 and 62M/3, were selected based on the laboratory test results.

The strains *Pseudomonas putida* 5G (UDMH) and *Rhodococcus erythropolis* 62M/3 (aviation kerosene) were selected for further experiments on the creation of the RFC destructor association based on the laboratory tests of contaminated soil and assessment of the parameters of bacterial growth in the culture medium. The association of microorganisms-destructors reproduced quickly in the soil contaminated with RFC, destroying heptyl and aviation kerosene. Integral toxicity of the soil contaminated with these toxicants gradually decreased after being treated with the association of microorganisms-destructors of RFC to reach a safe level, and the soil enzyme activity increased.

Toxicity testing of the isolated strains of microorganisms-destructors of RFC in animals

Evaluation of pathogenicity of microorganisms-destructors of heptyl (*Ps. putida* 5G) and aviation kerosene (*Rh. erythropolis* 62M/3) involved asessment of virulence, toxicity, toxigenicity, and dissemination in the internal organs of the outbred white mice and rats. The irritating effect of these bacteria on the ocular mucous membrane of rabbits was also defined.

Assessment of virulence involved single intragastric and intraperitoneal administration of bacteria to white mice and rats. All experimental animals were still alive by the end of the observation period. The clinical status of animals, as well as food and water consumption were normal. It was found that in case of intragastric administration of *Ps. putida* 5G and *Rh. erythropolis* 62M/3 to rats and mice, LD_{50} exceeded 109 bacterial cells, and in case of intraperitoneal administration LD_{50} exceeded 10^8 bacterial cells.

Toxicity testing involved intragastric administration of the suspensions of the studied strains 24-hour cultures to white mice. All experimental animals were still alive by the end of the observation period. The clinical status of animals, as well as food and water consumption were normal. It was found that the tested strains *Ps. putida* 5G and *Rh. erythropolis* 62M/3 showed no toxicity in warm-blooded animals.

Assessment of toxigenicity involved intraperitoneal and intragastric administration of filtrates of the 3-day and 7-day broth cultures of the studied strains to white mice. All experimental animals

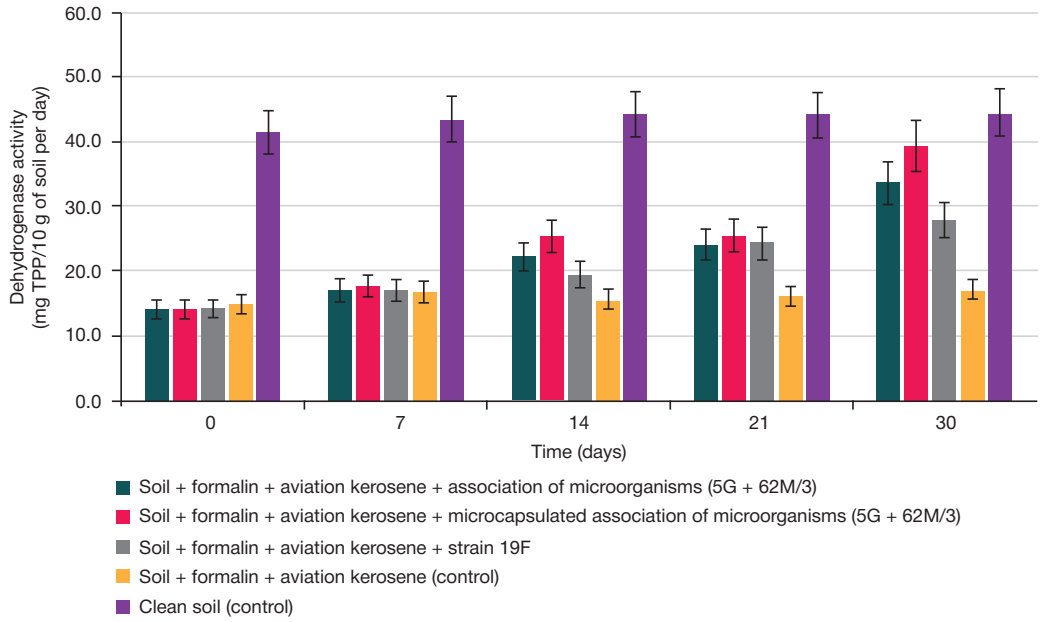

Fig. 2. Dehydrogenase activity in the soil contaminated with formalin (0.05%) and aviation kerosene (0.1%) during microbial remediation in laboratory experiments

were still alive by the end of the observation period. The clinical status of animals, as well as food and water consumption were normal, no toxic effects were observed. Thus, the tested strains of microorganisms-destructors *Ps. putida* 5G and *Rh. erythropolis* 62M/3 showed no toxigenicity in warm-blooded animals.

To assess dissemination in the internal organs, white mice and rats were infected with the isolated strains by intragastric and intraperitoneal injection. No animals died by the end of the observation period. Autopsy revealed no differences between the organs of experimental animals and controls. Normal positioning and macrostructure of organs in the thoracic and abdominal cavities were observed, no pathological changes were revealed at the macro level. No growth was observed in the bacterial cultures isolated from animal organs. Thus, based on the bacterial cultures isolated from the internal organs, the studied strains of microorganisms-destructors of RFC are not capable of dissemination and never cause bacterial contamination of organs in warm-blooded animals.

When studying the irritating effect on the ocular mucous membrane, no irritating effect was observed both 4 h after the injection of the bacterial suspension containing *Ps. putida* 5G or *Rh. erythropolis* 62M/3 into the rabbit's conjunctival sac and throughout the observation period. There were no differences between the eyes injected with bacteria and control eyes in all animals. Research has shown that bacteria *Ps. putida* 5G and *Rh. erythropolis* 62M/3 do not irritate the ocular mucous membrane of warm-blooded animals.

Thus, toxicity testing, that involved asessment of virulence, toxicity, and toxigenicity, showed that microorganisms-destructors of formaldehyde (heptyl) (*Ps. putida*, strain 5G) and aviation kerosene (*Rh. erythropolis*, strain 62M/3), were not pathogenic (harmless) to warm-blooded animals. Microorganisms are harmless and can be used for bioremediation of contaminated soils without limitation.

Defining the main parameters and conditions for cultivation of microorganisms-destructors in the fermentation device

The selected strains *Ps. putida* 5G and *Rh. erythropolis* 62M/3 belong to different genera. That is why we assessed the possibility of co-growth in the agar Petri dishes. Co-culture of the strains showed no growth suppression.

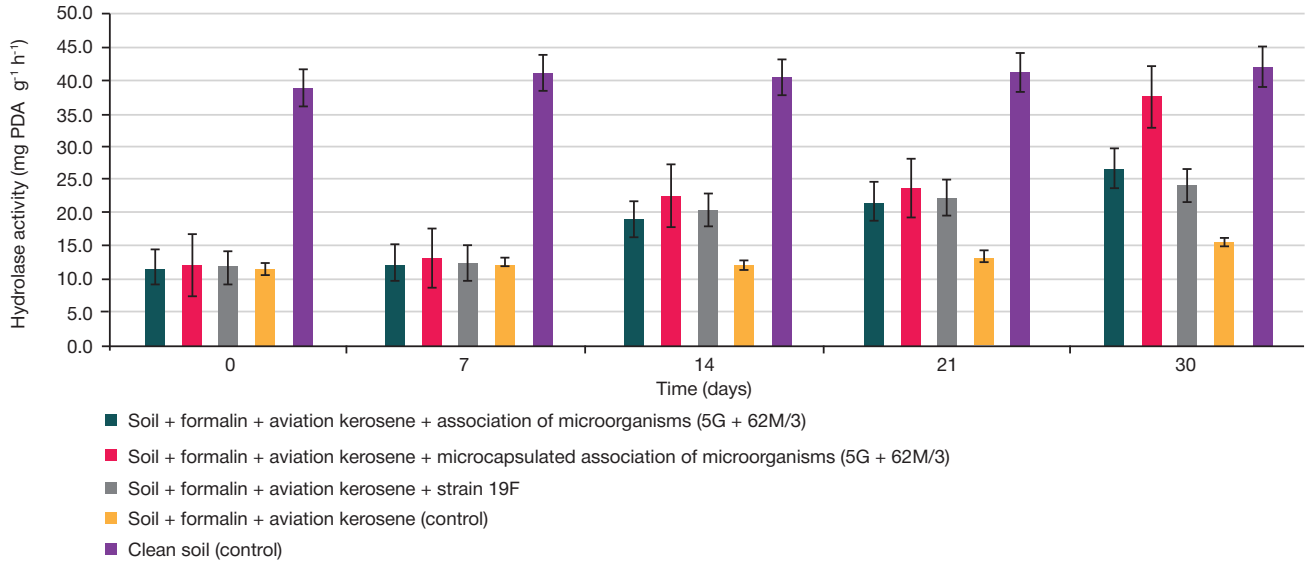

Fig. 3. Hydrolase activity in the soil contaminated with formalin and aviation kerosene during microbial remediation in laboratory experiments

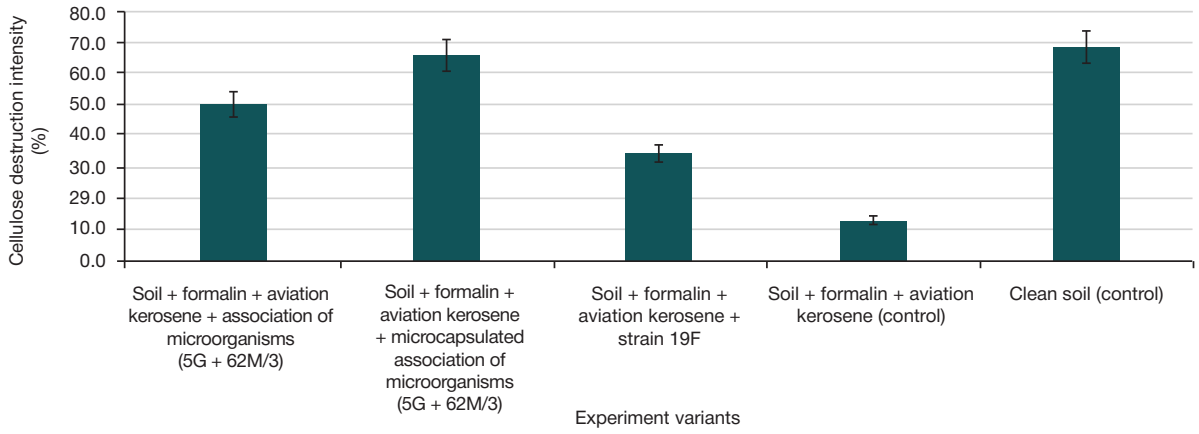

Fig. 4. Cellulase activity (%) in the soil contaminated with formalin and aviation kerosene after 30 days of microbial remediation in laboratory experiments

We defined the main parameters and conditions for cultivation of the RFC destructor strains *Ps. putida* 5G and *Rh. erythropolis* 62M/3 on various culture media. The optimum regime was cultivation at 28 °C for 24 h.

Efficiency of the 0.05% formalin and 0.1% aviation kerosene destruction in soil by the association of microorganisms-destructors of RFC in the laboratory environment

Microbial remediation of soil contaminated with RFC was performed in the laboratory settings in the 0.5 L plastic glasses. The working concentrations of pollutants in the soil were achieved by adding the following solutions: 0.05% (500 mg/кг) formalin (simulator of heptyl being the primary product of heptyl degradation) and 0.1% (1000 mg/kg) aviation kerosene.

Soil samples for chemical analysis and toxicity testing were collected before adding microorganisms-destructors, after 7, 14, 21 and 30 days (after the end of experiment).

The experimental procedure included a whole range of studies, such as determination of the aviation kerosene concentration, integral toxicity of soil to Daphnia, concentrations of microorganisms-destructors and soil microphlora, dehydrogenase, hydrolase, and cellulose-destroying activity, phytotoxicity to oat seeds.

Assessment of the soil microbial contamination showed that microbial cell counts of microorganisms-destructors of heptyl and aviation kerosene was stable throughout the period of bioremediation; these microorganisms were suppressed by saprophytic microflora (Table 1). Microbial cell counts of saprophytic soil bacteria remained almost the same throughout the experiment (Table 1).

The concentration of pollutant in the soil gradually decreased during microbial remediation (Fig. 1).

Integral toxicity of the contaminated soil before and after treatment with microorganisms-destructors was tested using Daphia bioassay. Before treatment, soil contaminated with aviation kerosene and formalin caused death in 100% of Daphnia. After 14 days of microbial remediation soil, toxicity decreased to reach a safe level (Table 2).

We defined the enzyme activity in the contaminated soil (dehydrogenase, hydrolase, and cellulase activity) before and after treatment with microorganisms-destructors. Soil contamination with aviation kerosene and formalin reduced the

Table 3. Phytotoxicity of the soil contaminated with formalin and aviation kerosene to oat seeds after the soil microbial remediation in laboratory experiments

Experiment variant	Measurement	Units	Mean value (M ± 6)	Number of seeds per iteration, *n*	Number of iterations
Soil + formalin + aviation kerosene (negative control)	Roots	mm	34.0 ± 23.9	25	3
	Sprouts	mm	46.5 ± 30.0		
	Weight (roots and sprouts)	g	3.5 ± 0.1		
	Number of ungerminated seeds	*n*	16		
Soil (control)	Roots	mm	62.0 ± 30.0	25	3
	Sprouts	mm	45.0 ± 24.6		
	Weight (roots and sprouts)	g	4.0 ± 0.4		
	Number of ungerminated seeds	*n*	7		
Soil + formalin + aviation kerosene + association 5G + 62M/3	Roots	mm	30.5 ± 22.8	25	3
	Sprouts	mm	58.0 ± 39.1		
	Weight (roots and sprouts)	g	3.3 ± 0.7		
	Number of ungerminated seeds	*n*	11		

Note: M — mean, 6 — standard deviation.

Table 4. The changes in microbial cell counts of microorganisms introduced into soil and saprophytic microbiota during the field experiment, CFU/g

Variant	Test duration, days					
	0	7	14	30	45	60
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	$(2.4 \pm 0.24) \times 10^5$ $(2.6 \pm 0.28) \times 10^5$ $(5.0 \pm 0.31) \times 10^4$	$(5.9 \pm 0.48) \times 10^6$ $(6.5 \pm 0.55) \times 10^6$ $(2.7 \pm 0.28) \times 10^5$	$(1.9 \pm 0.26) \times 10^6$ $(1.2 \pm 0.27) \times 10^6$ $(1.2 \pm 0.14) \times 10^6$	$(1.2 \pm 0.17) \times 10^6$ $(8.3 \pm 1.03) \times 10^5$ $(1.5 \pm 0.37) \times 10^6$	$(6.0 \pm 0.68) \times 10^5$ $(3.7 \pm 0.34) \times 10^5$ $(9.0 \pm 0.17) \times 10^6$	$(3.0 \pm 0.75) \times 10^5$ $(1.9 \pm 0.20) \times 10^5$ $(1.1 \pm 0.45) \times 10^6$
Soil + formalin + aviation kerosene (control)	– – $(2.3 \pm 0.41) \times 10^4$	– – $(3.2 \pm 0.24) \times 10^5$	– – $(2.1 \pm 0.21) \times 10^5$	– – $(2.0 \pm 0.31) \times 10^5$	– – $(7.6 \pm 0.27) \times 10^5$	– – $(6.54 \pm 0.48) \times 10^5$
Clean soil (control)	– – $(1.2 \pm 0.45) \times 10^6$	– – $(2.4 \pm 0.48) \times 10^6$	– – $(2.8 \pm 0.34) \times 10^6$	– – $(3.2 \pm 0.21) \times 10^7$	- - $(2.3 \pm 0.48) \times 10^6$	– – $(6.4 \pm 0.25) \times 10^6$

Note: the columns show indicators for strain 5G, strain 62M/3, soil saprophytes.

enzyme activity. After 30 days of microbial remediation enzyme activity gradually increased (Fig. 2–4).

Phytotoxicity of the soil significantly decreased during the microbial remediation. Based on such parameters, as the "sprouts length" and the "number of ungerminated seeds" it approached the level of the conditionally clean soil (Table 3).

Field testing of the microbial destruction of formalin and aviation kerosene in soil by the association of microorganisms-destructors of RFC

Microbial destruction of formalin and aviation kerosene in the soil was studied under field conditions on the experimental plots. The working concentrations of pollutants in the soil were achieved by adding 0.1% formalin and 0.1% aviation kerosene. Microbial association of the destructor strains *Ps. putida* 5G and *Rh. erythropolis* 62M/3 with a concentration of 1×10^7 CFU/mL was added at a rate of 1 L/m^2 of soil. Soil samples for further assessment were collected within 60 days. The extract obtained from soil samples was cultured in Petri dishes with the fish meal hydrolysate and formalin (100 mg/L) for strain 5G, and in Petri dishes with the minimal salt medium and 1% of diesel fuel for strain 62M/3. The concentration of saprophytic (indigenous) bacteria was assessed using the GRM medium.

The field experiment included a whole range of studies, such as determination of the aviation kerosene concentration

Table 5. Integral toxicity of soil to Daphnia in the field experiment

Experiment variant	Number of surviving Daphnia, *n*		Daphnia mortality rate, %	Acute toxic effects/no acute toxic effects
	control (water)	experiment		
Baseline				
Soil + formalin + aviation kerosene	30	10	67	Acute toxic effects
Soil (control)	30	30	0	No acute toxic effects
After 7 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	20	33	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	14	54	Acute toxic effects
After 14 days				
Soil + formalin + aviation kerosene + microbial association (5G+ 62M/3)	30	25	17	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	16	53	Acute toxic effects
After 30 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	27	10	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	15	50	Acute toxic effects
After 45 days				
Soil + formalin + aviation kerosene + microbial association (5G+ 62M/3)	30	27	10	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	14	46	No acute toxic effects
After 60 days				
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	30	30	0	No acute toxic effects
Soil + formalin + aviation kerosene (control)	30	20	33	No acute toxic effects
Soil (control)	30	30	0	No acute toxic effects

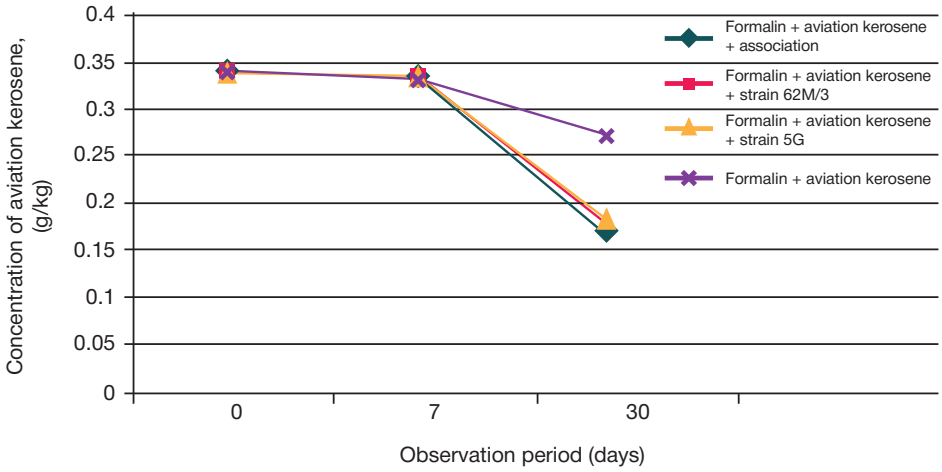

Fig. 5. Dynamic changes in the soil concentration of aviation kerosene during the field experiment, g/kg of soil

in the soil, integral toxicity of soil to Daphnia, bacteria counts for microorganisms-destructors and soil microbiota (Table 4), dehydrogenase, hydrolase, and cellulose-destroying activity, phytotoxicity.

The baseline microbial cell counts of both strains (after being added to the soil) were at the level of 10^5 CFU/g of soil, and the bacteria counts of soil saprophytes were at the level of 10^4 CFU/g of soil. The highest microbial cell counts were observed on day 7: $(5.9 \pm 0.48) \times 10^6$ CFU/g of soil for strain 5G, $(6.5 \pm 0.55) \times 10^6$ CFU/g of soil for strain 62M/3. The microbial cell counts of biodestructors slightly decreased starting from day 45 due to degradation of soil pollutants

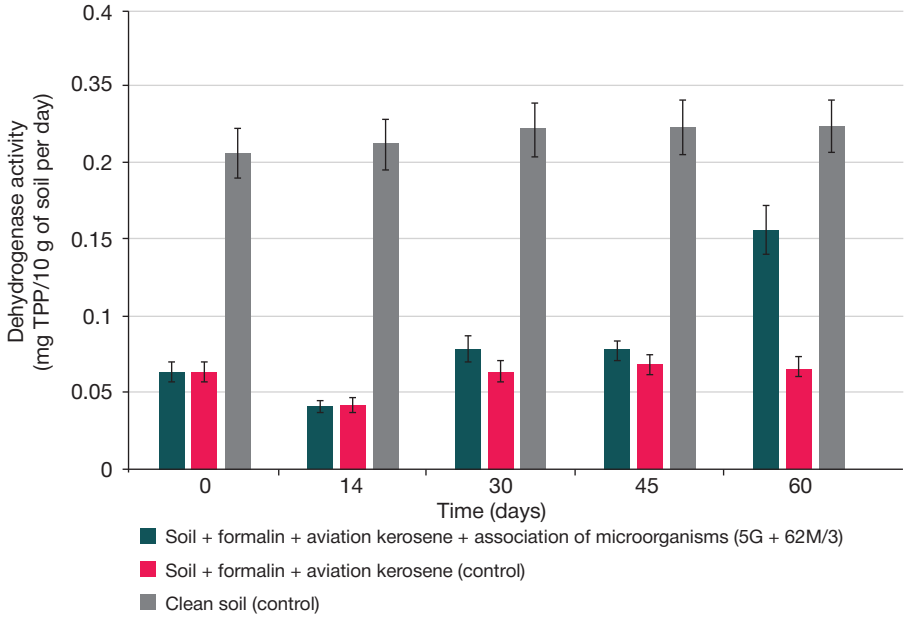

Fig. 6. Dehydrogenase activity of the soil in the field experiment

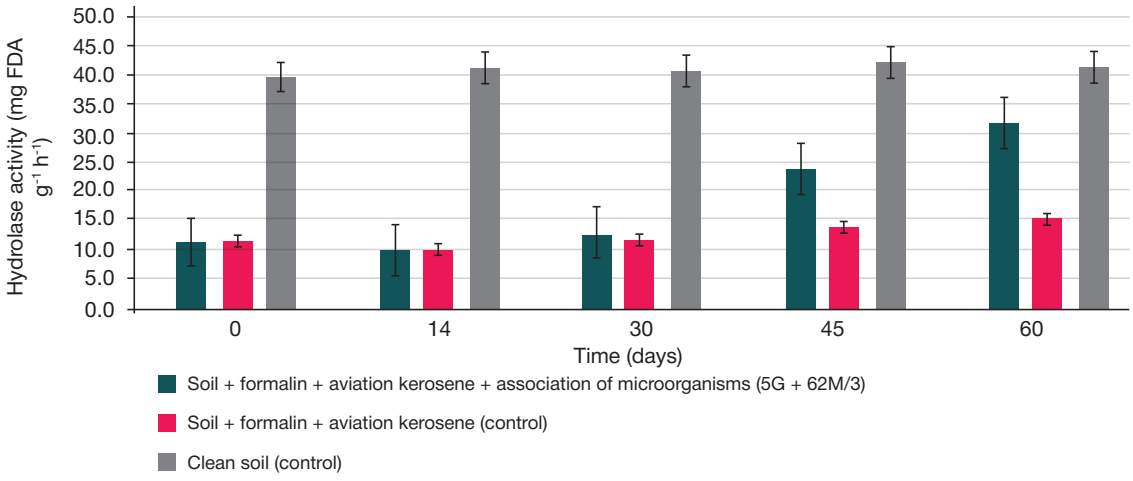

Fig. 7. Hydrolase activity of the soil in the field experiment

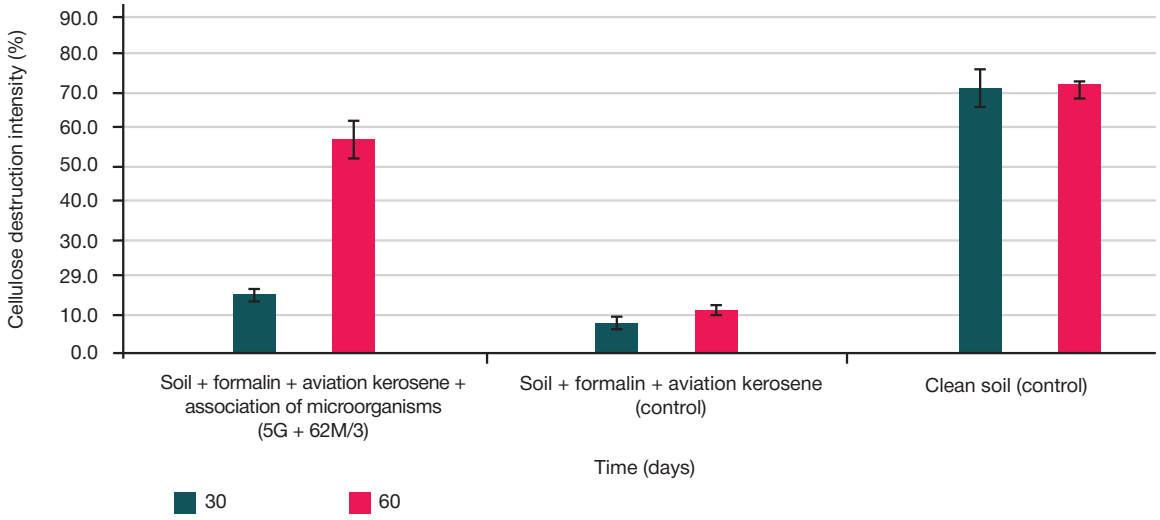

Fig. 8. Cellulase activity of the soil (%) in the field experiment

and a corresponding decrease of the substrates essential for the biodestructor existence. Bacteria counts of saprophytic bacteria reached their maximum on day 60 of the experiment. Microbial cell counts of saprophytic bacteria in the clean soil remained almost the same throughout the experiment.

Soil treated with formalin and aviation kerosene at the start of the experiment was highly toxic to Daphnia (Table 5). Contaminated soil, that was treated with microorganisms, became non-toxic by day 7 of the experiment.

The concentration of aviation kerosene in the soil gradually decreased during bioremediation (Fig. 5).

Soil enzyme activity changed considerably during bioremediation (Fig. 6–8).

The findings showed that biological activity of the soil significantly decreased after adding formalin and aviation kerosene. Thus, on day 7 of the field experiment, dehydrogenase and hydrolase activity of soil in the studied variants was 19% and 24%, respectively, compared to the clean soil (control). The increase in biological activity was observed in the contaminated soil, treated with microorganisms-destructors, starting from day 30 of the experiment. After 60 days the values of soil enzyme activity significantly exceeded that of the contaminated soil not subjected to microbial remediation: dehydrogenase activity increased by 2.4 times, hydrolase activity by 2.1 times, and cellulase activity by 5.1 times.

Phytotoxicity of the soil contaminated with formalin and aviation kerosene was determined during the field experiment. The decrease in phytotoxicity of the recultivated soil to oat seeds to the level of the clean soil was revealed by day 60 based on such parameters, as the “sprouts length” and the “number of ungerminated seeds” (Table 6).

The field experiment revealed a decrease in the acute toxicity and phytotoxicity of the cleaned soil, as well as an increase in the soil biological activity (levels of dehydrogenases, hydrolases, cellulases).

Microorganisms-destructors of RFC *Ps. putida* (strain 5G) and *Rh. erythropolis* (strain 62M/3) were deposited in the All-Russian Collection of Microorganisms and assigned the numbers BKM Ac-2933D and BKM B-3636 D.

DISCUSSION

We isolated microorganisms capable of degrading dimethylhydrazine and aviation kerosene from soils that had been contaminated with these substances for a long time. When performing laboratory testing, we selected two active

Table 6. Soil phytotoxicity to oat seeds on day 60 of the field experiment

Experiment variants	Measurement	Units	Mean value (M ± 6)	Number of seeds per iteration, *n*	Number of iterations
Control (clean soil)	Roots	mm	76.9 ± 45.3	25	3
	Sprouts	mm	49.8 ±18.7		
	Weight (roots and sprouts)	g	4.2 ± 0.1		
	Number of ungerminated seeds	*n*	6		
Soil + formalin + aviation kerosene	Roots	mm	72.2 ± 37.3	25	3
	Sprouts	mm	46.2 ± 16.9		
	Weight (roots and sprouts)	g	3.9 ± 0.1		
	Number of ungerminated seeds	*n*	14		
Soil + formalin + aviation kerosene + microbial association (5G + 62M/3)	Roots	mm	78.4 ± 29.0	25	3
	Sprouts	mm	53.4 ± 20.4		
	Weight (roots and sprouts)	g	4.4 ± 0.1		
	Number of ungerminated seeds	*n*	7		

Note: M — mean, 6 — standard deviation.

strains of microorganisms: biodestructor of formaldehyde *Ps. putida*, strain 5G, and biodestructor of aviation kerosene *Rh. erythropolis*, strain 62M/3. The conditions for the separate cultures and co-culture were worked out.

The results of toxicity testing performed in white mice and rats showed that *Ps. putida*, strain 5G, and *Rh. erythropolis*, strain 62M/3, were not pathogenic (harmless) to warm-blooded animals based on their virulence, toxicity, toxigenicity, and dissemination patterns. Microorganisms can be used for bioremediation of contaminated soils without limitation.

Laboratory experiments showed that treatment of the soil, contaminated with formalin and aviation kerosene, with the association of microorganisms-destructors of RFC for 30 days resulted in the reduced soil contamination, as well as in the decreased soil integral toxicity and phytotoxicity. Soil enzyme activity gradually increased to reach the level of the clean soil during the microbial remediation.

The field experiment showed that the association of microorganisms consisting of the formalin (heptyl) biodestructor *Ps. putida*, strain 5G, and the aviation kerosene biodestructor *Rh. erythropolis*, strain 62M/3, was suitable for cleaning of soils contaminated with RFC. Microorganisms-destructors of RFC added to the contaminated soils are not suppressed by indigenous microflora and grow actively.

The isolated strains of microorganisms-destructors of RFC can be used at the Russian cosmodromes and Baikonur Cosmodrome (Kazakhstan) to clean up the polluted areas.

The package of measures aimed at detoxication of soil contaminated with RFC will reduce the risk of occupational disorders in the employees engaged in bioremediation of the soil and disposal of space rockets.

CONCLUSIONS

Laboratory and field testing of the association of microorganisms-destructors of propellant components (RFC) *Pseudomonas putida*, strain 5G, and *Rhodococcus erythropolis*, strain 62M/3, showed its high efficiency for decontamination of RFC, as well as its environmental and toxicological safety. The association of microorganisms *Ps. putida*, strain 5G, and *Rh. erythropolis*, strain 62M/3, can be recommended for practical use, i.e. for bioremediation of soil contaminated with RFC.

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CURRENT ISSUES OF THE OCCUPATIONAL SENSORINEURAL HEARING LOSS EVALUATION IN AIRLINE PILOTS

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Occupational hearing loss in the civilian flight crew members prevails in the structure of occupational hearing loss. The study was aimed to assess errors made in forensic evaluation of flight personnel with hearing loss. A total of 213 definitive diagnoses of occupational hearing loss established in 2015–2021 were assessed. Expert errors were found in 73% of cases. The most common, typical errors were reported that were evident in 12 cases submitted for forensic evaluation. The main errors were as follows: incorrect assessment of the parameters of noise inside the aircraft cabin, lack of knowledge about the clinical and audiological features of noise-induced hearing loss, incorrect assessment of audiological indicators, underestimation of the role of comorbidities, etc. The findings showed that high proportion of occupational hearing loss in the civilian flight crew members was in large part due to imperfection of expert solutions resulting from the lack of knowledge about the basic criteria of the diagnosis and the relationship between the noise-induced hearing loss and profession. The major requirements for evaluation of the relationship between the noise-induced hearing loss and profession and the issues related to professional suitability set out in the current regulatory documents issued by the Ministry of Health of the Russian Federation and seminal publications were not taken into account. The cornerstones of expert work on the issues of the relationship between the hearing organ disorder and profession in the civilian flight crew members were underestimated. This resulted in unsubstantiated professional unsuitability, disability among working-age people, and economic losses.

Keywords: airline pilots, occupational sensorineural hearing loss, expertise, professional suitability

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АКТУАЛЬНЫЕ ПРОБЛЕМЫ ЭКСПЕРТИЗЫ ПРОФЕССИОНАЛЬНОЙ НЕЙРОСЕНСОРНОЙ ТУГОУХОСТИ У ПИЛОТОВ ГРАЖДАНСКОЙ АВИАЦИИ

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Показатели профессиональной тугоухости представителей летных профессий гражданской авиации (ГА) превалируют в структуре профессиональных потерь слуха. Целью исследования было проанализировать ошибки в экспертно-диагностической работе с летным персоналом, имеющим потери слуха. Проведен анализ 213 заключительных диагнозов профессиональной тугоухости за период 2015–2021 гг. Экспертные ошибки выявлены в 73% случаев. Описаны наиболее часто встречающиеся типичные ошибки, которые были явно выражены в 12-ти случаях, представленных на судебно-медицинскую экспертизу. Основными ошибками были некорректная оценка параметров внутрикабинного шума, незнание особенностей клинико-аудиологической картины «шумовой» тугоухости, неправильная оценка аудиологических показателей, недоучет роли коморбидной патологии и др. По результатам исследования установлено, что высокий удельный вес показателей заболеваемости профессиональной тугоухостью у лиц летного состава ГА в значительной степени обусловлен несовершенством экспертных решений, связанным с незнанием базовых критериев диагностики и связи потерь слуха, вызванных шумом, с профессией. Не были учтены основные требования проведения экспертизы связи потерь слуха, вызванных шумом, с профессией и вопросы профессиональной пригодности, изложенные в действующих нормативно-регламентирующих документах Минздрава РФ и основополагающих публикациях. Имел место недоучет опорных моментов экспертной работы по вопросам связи заболевания органа слуха с профессией у представителей летного состава ГА, который приводил к необоснованной потере профессиональной пригодности, инвалидизации работников в трудоспособном возрасте, экономическим потерям.

Ключевые слова: пилоты гражданской авиации, профессиональная нейросенсорная тугоухость, экспертиза, профессиональная пригодность

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Currently, there has been a steady increase in the reported rate of occupational sensorineural hearing loss, that holds first position the structure of occupational morbidity and constitutes over half of all occupational diseases caused by physical factors of working environment, in the Russian Federation (Fig.).

For its part, among all cases of occupational hearing loss almost one third is registered in the civilian flight crew members [2–4].

This is a worrying trend for medical professionals and employers, since civil aviation is one of the leading economic sectors of the country. The hearing ability of the flight crew members is of great professional importance for medical flight safety support [5]. It is worth noting that there is almost no such problem in the world, despite using the same types of aircraft [6–7].

For more than 20 years, 80% of the civil aircraft fleet in the Russian Federation have been represented by foreign-made airliners, the noise levels in the cabins of which do not exceed the sanitary and hygienic standards established in Russia. This means that there is no direct a priori risk of the noise-induced damage to the pilot's hearing organ [8]. However, according to foreign authors, even the noise levels of 90–100 dBA cause no increase in the hearing thresholds of military pilots [9].

The study was aimed to assess errors made in forensic evaluation of flight personnel with hearing loss.

METHODS

The group of experts that included Chief Occupational Health Physician of the Ministry of Health of the Russian Federation (MHRF), Chief Otorhinolaryngologist of the MHRF, two board-certified otorhinolaryngologists-audiologists having a specialization in occupational health and safety, Head of the NGO of the Occupational Health and Safety of ENT Organs, and board-certified occupational health physician provided forensic analysis of 213 definitive diagnoses of occupational hearing loss established in 2015–2021. Expert errors were found in 73% of cases. A total of 12 cases to be submitted for forensic evaluation were selected based on reviewing the evidence used to establish the diagnosis amd criteria for the relationship between the hearing organ disorder and profession (assessment of sanitary and hygienic characteristics of working conditions, clinical and hearing history data, clinical manifestations, features of audiogram graphs provided by the audiogram archive, etc.). The article reports the most common, typical errors that were evident in all of 12 evaluated cases.

Level of evidence C (level 5 evidence).

RESULTS

The earlier publications [10] provide the review of all expert errors observed when performing evaluation of the relationship between hearing loss and working conditions in employees engaged in various “noisy” professions based on analysis of 213 definitive diagnoses of occupational sensorineural hearing loss. This article provides the main errors made in forensic evaluation of civilian flight crew members with impaired auditory perception. A total of 12 forensic evaluations were assessed that had been submitted for peer review in order to determine the validity of the definitive diagnosis of occupational hearing loss.

A thorough analysis of all submitted documentation justifying the relationship of the hearing organ disorder allowed us to identify a number of the most common fundamental errors that had contributed to inaccuracy and insufficient evidence base of the definitive diagnosis in all of 12 cases considered in forensic evaluation.

The most common errors are as follows:

– the main clinical and pathogenetic characteristics that are pathognomonic for noise-induced hearing loss are not taken into account;

– poor quality audiograms or the use of audiograms that show discrepancies between hearing tests performed in different health institutions;

– establishing the definitive diagnosis without taking into account the main criteria for the relationship between the hearing organ disorder and profession set out in contemporary regulatory and methodological documents issued by MHRF;

– incorrect assessment of the parameters of sound pressure inside the aircraft cabin, underestimation of noise attenuation properties of aviation headsets;

– cardiovascular comorbidity, neurological and systemic diseases, lipid disorders not taken into account;

– misrepresentation (or concealment) of the diagnosis in the medical record of employee when performing checkup: establishing various diagnoses, such as eustachitis, tubootitis,

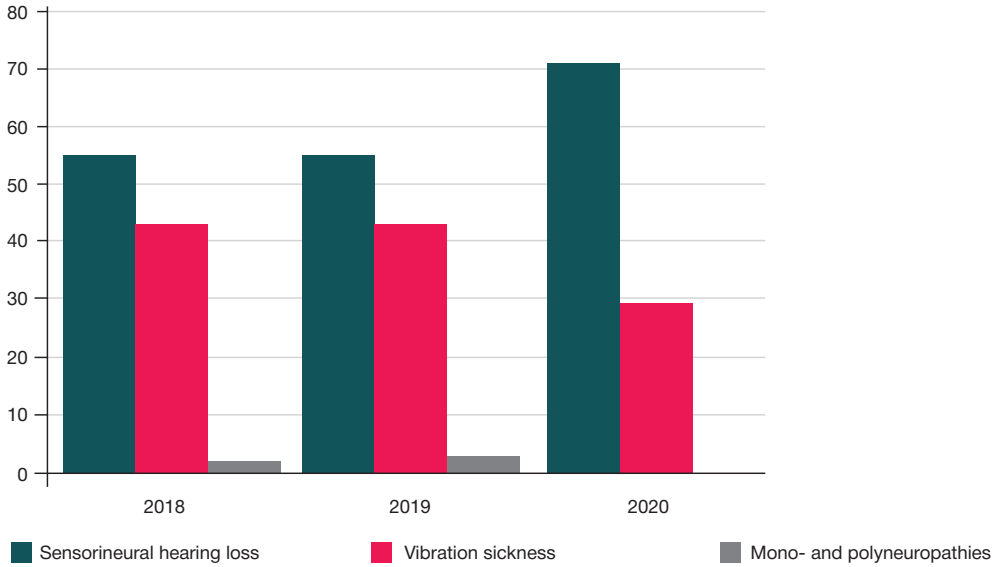

Fig. The occupational sensorineural hearing loss share (%%) in the structure of occupational disorders resulting from exposure to physical factors in the Russian Federation, 2018–2020 [1]

etc., when there are audiograms that show characteristic features of impaired auditory perception;

– inappropriate patient referral for evaluation of the relationship between the hearing organ disorder and profession;

– incorrect estimation of the hearing loss degree as a criterion of professional suitability/unsuitability when suspending the employee from work;

– deontological mistakes consisting in unreasonable patient's fixation on the rental aim due to occupational nature of hearing loss.

DISCUSSION

The main clinical and pathogenetic characteristics of the development and course of hearing loss in airline pilots are discussed in contemporary guidelines [10–12] and articles published by authors that are focused on the issues related to evaluation of the relationship between the hearing organ disorder and profession in employees in leading economic sectors [13–15]. The main issues are as follows: the length of service under exposure to industrial noise exceeding the permissible levels at least 8–10 years, obligatory bilateral onset of hearing loss, gradual worsening of hearing loss, no dissociation between bone-conducted and air-conducted sound perception, dynamic changes in the development of hearing loss and the lack of worsening in patients with industial noise-induced hearing loss during the post-exposure period of working in noisy environment.

However, even the co-authors of methodological guidelines for healthcare practitioners, that contain algorithms for the diagnosis and evaluation of the relationship between the hearing organ disorder and profession, make mistakes, while being members of medical advisory committees. In all the discussed cases, diagnostic errors were due to the use of poor quality audiograms or discrepancies between the hearing tests performed in different health institutions. In such cases, it is necessary to take into account the results of the comprehensive in-depth audiological evaluation performed in the specialized clinic. It should also be borne in mind that discrepancies between the results of the hearing threshold estimation could be due to fluctuations (instability) in the course of auditory perception impairment, which are not typical for noise-induced damage to the hearing organ.

When addressing issues related to possible occupational cause (noise exposure) of auditory perception impairment, the type of audiogram graph is important. Horizontal curve with no features typical for noise-induced hearing loss (dip at 4,000 Hz or less often at 3,000 Hz, significant differences between the mean indicators of speech perception and high-frequency hearing, as well as slow gradual hearing loss) cannot be considered as associated with the noise exposure of the hearing organ. Diagnostic errors result from underestimation of the important role of cardiovascular comorbidity, neurological and systemic diseases, lipid disorders, i.e. the conditions that can cause impaired auditory perception or enhance damaging effects of noise. Discrepancies between the acumetry data and the data of audiometric testing of hearing thresholds are uncharacteristic for noise-induced hearing loss.

Correct assessment of the parameters of sound pressure inside the aircraft cabin is one of the fundamental provisions for evaluation of the relationship between the hearing organ disorder and profession in airline pilots. Such work should be based on the guidelines of Rospotrebnadzor updated in 2022 [16]. According to the guidelines, analysis of the “Sound pressure levels record” for the entire period of flight activities of certain employee with the outlined aircraft type and noise attenuation properties of the used aviation headsets is necessary for evaluation. Only these data can be used when specifying noise characteristics along with the sanitary and hygienic characteristics of the employee's workplace. Unfortunately, the data are being presented based on the parameters of industrial noise for several months of the year showing sample months when the highest levels that exceeded permissible exposure limits (PEL) are observed. It is also necessary to take into account the flight experience requirements currently used in civil aviation: in 1990s, flight load significantly decreased, that is why pilots were protected against noise inside the cabin by the reduced time of exposure [17].

When undergoing medical examination (Medical Flight Expert Commission) according to regulations of industry requirements [18], pilots often conceal their complaints due to fear of losing their job, and present with complaints of hearing loss (often even exaggerating) only when they are declared unsuitable for flight duties. In such cases, physicians, who perform medical examination of employees, give in to the employees' intense desire to pursue their professional activity and therefore report “masking” diagnoses (eustachitis, tubootitis, etc.) in medical records regardless of the fact that there are audiograms that show characteristic features of impaired auditory perception.

Detailed documentation of the hearing loss degree as a criterion of professional suitability/unsuitability when suspending the employee from work based on the correct assessment of the hearing loss degree is the basis of the professional suitability evaluation in patients with noise-induced hearing loss.

We should also note deontological aspects of incorrect expert solutions that result in the patient's rental behavior due to occupational disorder. Unsubstantiated, rash and intolerant comments not supported by objective evidence or documentary proof, claims or promises regarding the fact that employee's hearing loss is of occupational origin result in the patient's unwarranted confidence in occupational nature of the disorder, as well as in rental aim to receive legal redress. This gives rise to medical and social conflicts, as well as to litigiousness, which, in turn, worsen the patient's health condition.

It must be remembered that current regulations for establishing the diagnosis of occupational disease dictate working in two phases that involve making a preliminary diagnosis and a definitive diagnosis [19]. Furthermore, the latter can be made only by the medical commission of the specialized occupational health institution having a license to provide “Occupational Health Service”, “Evaluation of the Relationship Between the Disease and Profession”, and “Evaluation of Professional Suitability” [20].

Resolving the issue of establishing a relationship between the hearing organ disorder and profession is a complex process that requires the engagement of a team of experts: public health physician or occupational health specialist, clinicians (otorhinolaryngologist, general practitioner, neurologist, ophthalmologist), and, if necessary, other specialists being the members of medical advisory committee who have appropriate occupational health and safety certificates of the specialized occupational health institution having a license to provide “Evaluation of the Relationship Between the Disease and Profession” and “Evaluation of Professional Suitability”.

Underestimation of the cornerstones of expert work results in unsubstantiated professional unsuitability, disability among working-age people, economic losses, protracted conflicts initiated by patients or employers that adversely affect the quality of life of the patient, physician and society in general.

Medical ethics and deontology, the main formal logic principles of conducting evaluation suggest that each subsequent evaluation

of the challenging conflict case, especially the one related to the socially significant problem of occupational disorders, should be conducted by more qualified experts. This is necessary to exclude one of the main sources of expert errors, in particular insufficient basic knowledge of methods and inability to apply the theoretical positions of methods in practice [21].

CONCLUSIONS

The high proportion of occupational hearing loss in the civilian flight crew members is in large part due to imperfection of expert solutions resulting from the lack of knowledge about the basic criteria of the diagnosis and the relationship between the noise-induced hearing loss and profession. The main errors are as follows: incorrect assessment of the parameters of noise inside the aircraft cabin, lack of knowledge about the clinical and audiological features of impaired auditory perception, incorrect assessment of the hearing loss audiological indicators, underestimation of the role of comorbidities, etc. The main requirements for evaluation of the relationship between the noise-induced hearing loss and profession and the issues related to professional suitability set out in the current regulatory documents issued by MHRF and seminal publications are not taken into account. Underestimation of the cornerstones of expert work on the issues of the relationship between the hearing organ disorder and profession in the civilian flight crew members results in unsubstantiated professional unsuitability, disability among working-age people, and economic losses.

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TRANSCRIPTIONAL ACTIVITY OF DNA-METHYLTRANSFERASE GENES IN THE CHRONICALLY EXPOSED RESIDENTS OF THE URAL REGION

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In addition to damaging the genetic apparatus of the cell, ionizing radiation can cause epigenetic alterations. DNA methylation that plays a vital part in regulation of cellular processes is a common epigenetic modification. DNA methylation ensured by DNA methyltransferases occurs in the CpG-rich sequences. The study was aimed to assess mRNA expression of genes encoding DNA methyltransferases (*DNMT1*, *DNMT3A*, *DNMT3B*) in the chronically exposed individuals who live along the River Techa over a long-term period. A total of 112 people were examined more than 65 years after the beginning of chronic exposure. The average accumulated dose to red bone marrow (RBM) was 782.0 ± 82.3 mGy, and the average accumulated dose to thymus and peripheral lymphoid organs was 93.2 ± 13.6 mGy. The subjects' age at the time of examination was 67.9 ± 0.8 years (54–83 years). The relative mRNA levels for the studied genes were assessed by real-time polymerase chain reaction (real-time PCR). mRNA expression of *DNMT1* correlated positively with the dose to RBM ($p = 0.04$), thymus and peripheral lymphoid organs ($p = 0.02$), as well as with the dose rate in these organs ($p = 0.05$, $p = 0.04$, respectively) during the period of the highest levels of radiation exposure. In individuals exposed in the high dose range (over 1000 mGy) there was a significant increase in the expression of *DNMT1* mRNA compared to the comparison group ($p = 0.02$). The findings may indicate the *DNMT1* gene involvement in epigenetic alterations that occur in the chronically exposed people in the long term.

Keywords: gene expression, chronic radiation exposure, DNA methylation, Techa River, low doses

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ТРАНСКРИПЦИОННАЯ АКТИВНОСТЬ ГЕНОВ ДНК-МЕТИЛТРАНСФЕРАЗ У ЖИТЕЛЕЙ УРАЛЬСКОГО РЕГИОНА, ПОДВЕРГШИХСЯ ХРОНИЧЕСКОМУ РАДИАЦИОННОМУ ВОЗДЕЙСТВИЮ

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Помимо повреждения генетического аппарата клетки, ионизирующее излучение способно приводить к эпигенетическим изменениям. Распространенной эпигенетической модификацией является метилирование ДНК, играющее важную роль в регуляции клеточных процессов. Метилирование ДНК происходит в последовательностях, богатых CpG-динуклеотидами, и осуществляется при помощи ферментов ДНК-метилтрансфераз. Целью работы было изучить экспрессию мРНК генов ДНК-метилтрансфераз (*DNMT1*, *DNMT3A*, *DNMT3B*) в отдаленные сроки у лиц, подвергшихся хроническому радиационному облучению на р. Теча. Обследование 112 человек было проведено спустя более чем 65 лет после начала хронического облучения. Средняя накопленная доза облучения красного костного мозга составляла 782,0 ± 82,3 мГр, а средняя накопленная доза облучения тимуса и периферических лимфоидных органов — 93,2 ± 13,6 мГр. Возраст людей на время проведения обследования составил 67,9 ± 0,8 лет (54–83 года). Оценку относительного содержания мРНК исследуемых генов проводили с использованием метода полимеразной цепной реакции в реальном времени. Установлена прямая корреляция между экспрессией мРНК гена *DNMT1* и дозой облучения красного костного мозга ($p = 0,04$), тимуса и периферических лимфоидных органов ($p = 0,02$), а также мощностью дозы облучения этих органов ($p = 0,05$, $p = 0,04$ соответственно) в период максимального радиационного воздействия. У облученных лиц в диапазоне больших доз (более 1000 мГр) наблюдается значимое увеличение экспрессии мРНК гена *DNMT1* относительно группы сравнения ($p = 0,02$). Полученные результаты могут свидетельствовать о вовлеченности гена *DNMT1* в изменение эпигенетического статуса у людей, подвергшихся хроническому радиационному воздействию в отдаленные сроки.

Ключевые слова: экспрессия генов, хроническое облучение, метилирование ДНК, река Теча, малые дозы

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Genome-wide DNA methylation profile is a dynamic feature capable of changing during ontogenetic development or under the influence of environmental factors. Methylation that involves enzymes DNA methyltransferases (DNMT1, DNMT3A, DNMT3B, DNMT3L) occurs in the CpG-rich sequences [1]. Methyltransferases DNMT3A and DNMT3B display de novo methylation activity. Methyltransferase DNMT1 ensures restoration and maintenance of the previously established methyl labels. Methylation levels of each DNA segment result from two opposing processes, methylation and demethylation, which generally depend on the activity of DNA methyltransferases and DNA demethylating enzymes [2].

The ionizing radiation-induced alterations in expression of DNA methyltransferases were studied in cells cultured in vitro: in some cases these alterations correlated with DNA methylation levels [3]. The experiments with mice and human thymocytes that involved the use of the combined radiation regime (exposure to an initial low dose administered prior to a subsequent higher radiation dose) revealed the decrease in expression of *DNMT2*, *DNMT3B*, *DNMT3L* in murine thymocytes and *DNMT2*, *DNMT3A* in the exposed *in vitro* human thymocytes [4]. The increased expression of DNA methyltransferases was observed in surgeons who had been practicing interventions for more than three years [5]. Protein p53 that binds directly to DNA is one of methyltransferase expression regulators in response to the ionizing radiation exposure. Direct binding of the protein is reduced upon exposure, which results in the increased transcritional activity of *DNMT1* [6].

It is shown that radiosensitivity of cells (including stem cells) is affected by the genome-wide DNA methylation levels and methyltransferase activity. This is due to the fact that methyltransferase activity and methylation of certain DNA sites can potentially alter secretion of such factors, as TNFα, NO, and TGFβ [7]. Furthermore, the ionizing radiation-induced methylation and methyltransferase gene expression alterations contribute to induction of genome instability [8].

Thus, the study was aimed to assess mRNA expression of genes encoding DNA methyltransferases (*DNMT1*, *DNMT3A*, *DNMT3B*) in the chronically exposed individuals (mostly exposed to low-dose radiation) over a long-term period.

METHODS

The object of the study were peripheral blood samples collected from 112 residents of the villages located along the Techa River, who were cronically exposed after the discharge of liquid radioactive waste from PA Mayak. Internal exposure occurred due to radionuclides that entered the body through consuming river water and locally manufactured food products, and external gamma exposure resulted from radionuclide contamination of bottom sediments and floodplain soils.

Massive discharge of radioactive waste started in 1950. The short-lived radionuclides were the main source of exposure during the first few years. Then, as a result of protective actions and the decay of short-lived radionuclides, external and internal dose rate in soft tissues significantly decreased: after 1960 it did not exceed 10^{-5} Gy/year in all people living along the river banks. The features of red bone marrow (RBM) exposure were slightly different, since the main contribution to the radiation dose was made by the long-lived osteotropic radionuclide ^{90}Sr, which provided chronic exposure with the monotonically decreasing dose rate that became less than 10^{-5} Gy/year by 1985 in all the exposed people [9].

Inclusion criteria: permanent residence in one of 41 villages located along the Techa River between January 1, 1950 and December 31, 1960; availability of reconstructed absorbed doses to RBM, thymus and peripheral lymphoid organs, calculated using the Techa River Dosimetry System-2016 (TRDS-2016) [10]. Exclusion criteria: autoimmune disorders; acute or chronic (exacerbation) inflammatory diseases; taking antibiotics, glucocorticoids or cytostatic drugs during a period of six months prior to blood sample collection.

The study participants were divided into the following groups based on the absorbed dose to RBM: the comparison group (67 individuals), where the doses to RBM did not exceed 70 mGy throughout the subjects' life, and the group of chronically exposed people (45 individuals), whose doses exceeded 70 mGy.

The average accumulated dose to RBM in the cronically exposed individuals was 782.0 ± 82.3 mGy (dose range: 77.8–3179.7 mGy), and the average dose rate in RBM during the period of the highest levels of radiation exposure (years 1950–1951) was 145.7 ± 16.3 mGy/year (dose rate range: 0.1–542.6 mGy/year). The average accumulated dose to thymus and peripheral lymphoid organs was 93.2 ± 13.6 mGy (dose range: 2.8–644.8 mGy), while the average dose rate during the period of the highest levels of radiation exposure was 42.8 ± 6.8 mGy (dose range: 0.1–320.9 mGy/year). The average accumulated dose to RBM in the comparison group was 20.7 ± 2.7 mGy (dose range: 1.3–63.2 mGy), and the average accumulated dose to thymus and peripheral lymphoid organs was 8.8±1.6 mGy (dose range: 0.2–33.5 mGy).

The average age of chronically exposed individuals was 72.2 ± 0.7 years (63–83 years), and the average age of people in the comparison group was 63.7 ± 1.0 years (54–79 years). The vast majority of samples were obtained from females in both groups. Thus, in the group of chronically exposed people women accounted for 70.1% (47 individuals), and in the comparison group they accounted for 68.9% (31 individuals).

To assess the relative mRNA levels of methyltransferases, 3 mL of blood were collected from the cubital vein in sterile vacuum Tempus Blood RNA Tubes (Thermo Scientific; USA). RNA was isolated by the column-based extraction method using the GeneJET Stabilized and Fresh Whole Blood RNA Kit (Thermo Scientific; USA). Qualitative and quantitative characteristics of the isolated total RNA samples were

Table 1. Olygonucleotide sequences of primers and probes

Gene	Olygonucleotide sequences
DNMT1	Forward: 5'-CCTTCACGTTCAACATCAAGC-3' Reverse: 5'-GCTCTGGGTACAGGTCCTCATC-3' Probe: FAM-BHQ1 - 5'-CCAGTCCCGTGAAACGCCCA-3'
DNMT3A	Forward: 5'- GGCTCCAGATGTTCTTCGCTA-3' Reverse: 5'- GGATGGGCTTCCTCTTCTCA-3' Probe: FAM-BHQ1 - 5'-CACGACCAGGAATTTGACCCTCCA-3'
DNMT3B	Forward: 5'- GAATCAAGGAAATACGAGAACAAGAC-3' Reverse: 5'- CTTCATCCCCTCGGTCTTTG-3' Probe: FAM-BHQ1 - 5'-CGACTCAGCCACCTCTGACTACTGCC-3'

Table 2. Relative mRNA levels (Me) of DNA methyltransferase genes (RU) in peripheral blood cells of chronically exposed people over a long-term period

Group	*n*	*DNMT1*	*DNMT3A*	*DNMT3B*
Comparison group	45	1.18 (0.78–1.67)	0.71 (0.58–0.82)	0.78 (0.18–1.37)
Chronically exposed individuals	67	1.43 (0.99–1.67)	0.71 (0.55–0.83)	0.46 (0.20–1.18)

Note: in parentheses are 25th–75th percentile; *n* — sample size.

assessed with the NanoDrop 2000C Spectrophotometer (Thermo Scientific; USA). Sample purity was determined based on absorption values at wavelengths of 260 nm and 280 nm (A260/280). The reverse transcription reaction was performed as a separate step using the MMLV RT Kit (Evrogen; Russia). The relative mRNA levels were defined by real-time polymerase chain reaction (real-time PCR) using the Real-Time CFX96 Touch system (Bio-Rad Laboratories; USA). Olygonucleotide sequences of primers and probes were synthesized by LLC DNA-Synthesis (Russia) (Table 1).

Real-time PCR was carried out as follows: initial denaturation at 95 °C for 5 min, cycles of denaturation at 95 °C for 20 s, primer annealing and elongation at 65 °C for 60 s (50 cycles). Each sample was tested three times.

The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression [11]. *ACTB* housekeeping gene was used as an endogenous control. Calculations were performed with the software installed in the Real-Time CFX96 Touch system (BioRad; USA).

Statistical processing of the results was carried out with the SPSS Statistics 17.0 (IBM; USA) and Graph Pad Prism 8.4.3 (GraphPad Software Inc.; USA) software packages. Distributions of indicator values were tested for normality using the Kolmogorov–Smirnov test. Mean values (M), standard error of the mean (± SE), and the range of values (min–max) were used to characterize samples with normal distribution. The indicators with non-normal distribution were presented as median (Me), 25th–75th percentile (Q_1–Q_3). Samples were compared using the Mann–Whitney U test, since the majority of values had non-normal distribution. Correlation analysis for assessment of the effects of dose characteristics on the relative mRNA levels of methyltransferases was performed by calculating Spearman's rank correlation coefficients (*R*). The differences were considered significant at $p < 0.05$ in all tests. When $0.05 < p < 0.1$, the difference was considered as a trend towards significant difference.

RESULTS

When comparing two samples, no significant differences in the relative mRNA levels of the *DNMT1*, *DNMT3A*, and *DNMT3B* methyltransferase genes were observed (Table 2).

Correlation analysis of the combined sample revealed a weak correlation of the relative mRNA levels of *DNMT1* with the accumilated dose to RBM ($R = 0.19$; $p = 0.04$), thymus and peripheral lymphoid organs ($R = 0.19$; $p = 0.05$), and the dose rate in RBM ($R = 0.21$; $p = 0.02$), thymus and peripheral lymphoid organs ($R = 0.20$; $p = 0.04$) during the period of the highest levels of radiation exposure (Table 2).

The relationship between the relative mRNA levels of *DNMT1* and the dose characteristics was assessed by regression analysis of the combined sample. The analysis confirmed the correlation between the changes in expression on the *DNMT1* gene mRNA and the dose rate in RBM ($R = 0.20$; $p = 0.03$), thymus and peripheral lymphoid organs ($R = 0.19$; $p = 0.04$) during the period of the highest levels of radiation exposure. No correlations were found between the relative *DNMT3A* and *DNMT3B* mRNA levels and the dose parameters (Table 3).

In individuals with the accumulated dose to RBM exceeding 1000 mGy (1044.8–3179.7 mGy) the significant increase in the expression of the *DNMT1* gene mRNA (the average value: 1659.0 ± 155.7; $p = 0.02$) compared to the comparison group was observed (Fig. A).

Furthermore, it was found that relative mRNA levels of *DNMT1* were significantly higher ($p = 0.04$) in individuals with accumulated doses to thymus and peripheral lymphoid organs of 103.9–644.8 mGy (average value: 200.9 ± 30.3 mGy) compared to individuals with accumulated doses not exceeding 10.0 mGy (average value: 2.5 mGy) (Fig. B).

DISCUSSION

DNMT1 is one of the main DNA methyltransferases in mammalian cells. It is a highly dynamic enzyme with multiple regulatory functions that is capable of controlling DNA methylation. In particular, DNMT1 expression is necessary for maintaining the pattern of DNA methylation during mitosis. Furthermore, DNMT1 plays a direct role in the recovery of epigenetic information during the DNA repair [8].

DNMT1 gene upregulation is often associated with global DNA hypomethylation [12]. It is worth noting that such an effect of genome-wide hypomethylation was observed in employees of nuclear facilities exposed to the combination of high- and low-LET radiation. The researchers [13] noted that

Table 3. Spearman's rank correlation (*R*) between the relative mRNA levels of DNA methyltransferase genes and the values of dose and dose rate during the period of the highest levels of radiation exposure

Gene	Dose to RBM, mGy	Dose rate in RBM during the period of the highest levels of radiation exposure, mGy/year	Dose to thymus and peripheral lymphoid organs, mGy/year	Dose rate in thymus and peripheral lymphoid organs during the period of the highest levels of radiation exposure, mGy/year
	R (*p*)			
DNMT1	0.19 (0.04)	0.21 (0.02)	0.19 (0.05)	0.20 (0.04)
DNMT3A	–0.03 (0.74)	–0.07 (0.45)	–0.04 (0.65)	–0.04 (0.65)
DNMT3B	–0.13 (0.17)	–0.17 (0.08)	–0.14 (0.16)	–0.15 (0.11)

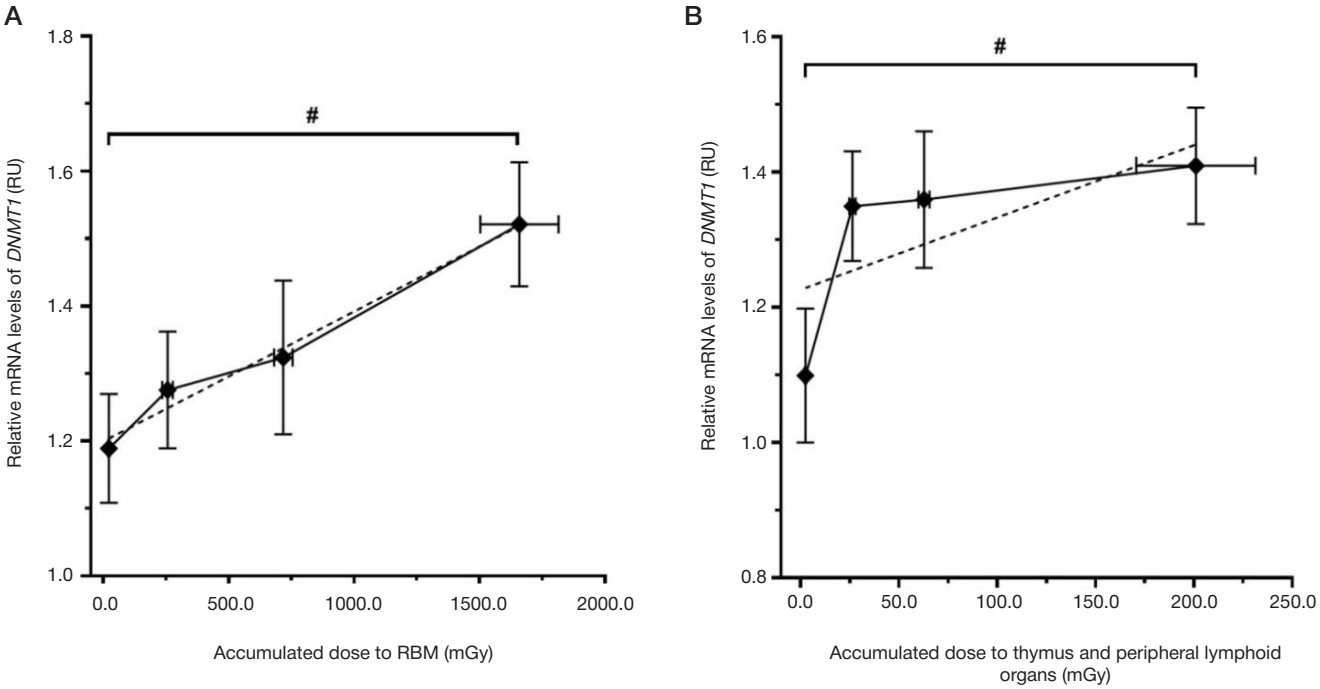

Fig. Relative mRNA levels of *DNMT1* (RU) in exposed individuals as a function of dose to RBM (**A**), thymus and peripheral lymphoid organs (**B**): the *dotted line* indicates linear approximation (trend line); # indicates significant differences in *DNMT1* gene expression between the comparison group and the group of chronically exposed people; vertical error bar corresponds to the error of the mean of the relative mRNA levels of *DNMT1*; horizontal error bar corresponds to the error of the mean of the accumulated dose to RBM (**A**), thymus and peripheral lymphoid organs (**B**)

workers whose total cumulative dose exceeded 103.14 mSv, had significantly higher global methylation levels compared to workers with lower doses (below 103.14 mSv), which indicated a differential response of epigenome to the effects of exposure to low and high doses.

The other study reported by the same authors showed reduced total levels of 5-methylcytosine in leukocytes of employees exposed to gamma ray and X-ray radiation [14].

DNA methyltransferase expression alterations are often associated with the locus-specific changes in methylation of genes responsible for maintaining cell homeostasis. The paper [15] reports CpG island hypermethylation in promoters of some genes (particularly, *p16/INKA* and *GSTP1*) in normal leukocytes of individuals who were exposed to radiation long time ago. Further examination of the PA Mayak employees with available reconstructed absorbed doses from external exposure to gamma ray radiation or combined exposure to external gamma- and internal alpha-radiation performed using the extended range of the studied loci revealed the combination of genes *p16/INKA*, *p53*, *GSTP1*, *SOD3*, *ATM*, *ESR1*, hypermethylation of which was associated with radiation exposure [16].

Our previously published studies revealed a positive correlation between the promoter methylation levels of the *ATM* gene and the dose to RBM, thymus and peripheral lymphoid organs in exposed individuals [17]. Moreover, *ATM* gene expression was significantly reduced in the group of chronically exposed people with the doses to RBM exceeding 1000 mGy [18].

It is possible that alterations in expression of human gene *DNMT1* in the long term after the beginning of chronic exposure in case the doses to RBM exceed 1000 mGy could be involved in induction of epigenetic alterations. To answer the question whether DNMT1 is involved in epigenetic mechanisms, it is necessary to study the impact of the gene transcriptional activity on the promoter methylation levels of genes involved in regulation of repair, cell proliferation and death in exposed individuals.

CONCLUSIONS

The significantly increased expression of the human *DNMT1* mRNA in the long term after the beginning of the low-dose radiation exposure with the dose range exceeding 1 Gy has been revealed. Positive correlations of the *DNMT1* mRNA expression with the dose to red bone marrow, thymus and peripheral lymphoid organs, and the dose rate in these organs during the period of the highest levels of radiation exposure (years 1950–1951) are observed in the chronically exposed individuals.

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STATUS OF FACTORS OF INNATE IMMUNITY IN EXPOSED PEOPLE WHO SUBSEQUENTLY DEVELOPED CANCER

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Currently, cancer is the major cause of mortality and disability among the working age population of the developed countries. Early diagnosis of tumors, that involves monitoring the health of people exposed to radiation, is one of the most pressing challenges faced by radiation medicine. The study was aimed to perform quantification and functional assessment of the system of neutrophil granulocytes, monocytes and natural killers (NK cells) in people who were diagnosed with tumors after chronic radiation exposure. Certain factors of innate immunity were assessed in 104 people, chronically exposed to low-dose radiation over a wide dose range. Of them 34 exposed individuals were later diagnosed with malignant tumors (MTs). We assessed the number of white blood cells, neutrophil granulocytes, eosinophils, basophils, monocytes and NK cells ($CD16^+/CD56^+$ lymphocytes) in peripheral blood, as well as phagocytic, lysosomal activity and intracellular oxygen-dependent metabolism of neutrophils and monocytes. Individuals, chronically exposed a few years before the development of MTs, showed a significant increase in the phagocytosis rate of monocytes (median 10.50 AU vs. 6 AU; $p = 0.05$) and lysosomal activity of neutrophils (median 482 AU vs. 435.5 AU; $p = 0.03$) compared to patients with no MTs. Assessment of the the dose–response relationship in exposed people, who subsequently developed cancer, revealed a significant increase in the phagocytosis rate of monocytes as a function of the accumulated dose to thymus and peripheral lymphoid organs ($\rho = 0.45$; $p = 0.009$), and the increase in phagocytic activity of neutrophils with the increase in the accumulated dose to red bone marrow ($\rho = 0.44$; $p = 0.01$).

Keywords: chronic radiation exposure, carcinogenesis, neutrophils, monocytes, natural killers

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Compliance with ethical standards: the study was approved by the Ethics Committee of the Urals Research Center for Radiation Medicine (protocol № 4 of 23 August 2022). All patients submitted the informed consent to study participation.

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СОСТОЯНИЕ ФАКТОРОВ ВРОЖДЕННОГО ИММУНИТЕТА У ОБЛУЧЕННЫХ ЛИЦ, ВПОСЛЕДСТВИИ ЗАБОЛЕВШИХ РАКОМ

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В настоящее время онкологические заболевания являются одной из ведущих причин смертности и инвалидизации среди зрелого трудоспособного населения в развитых странах. Одной из актуальных задач радиационной медицины является ранняя диагностика опухолевых заболеваний на основе мониторинга состояния здоровья людей, подвергшихся радиационному воздействию. Целью исследования было оценить количественные и функциональные показатели системы нейтрофильных гранулоцитов, моноцитов и натуральных киллеров у лиц, подвергшихся хроническому радиационному воздействию и впоследствии заболевших опухолевыми заболеваниями. Проведено исследование отдельных факторов врожденного иммунитета у 104 человек, подвергшихся хроническому низкоинтенсивному радиационному воздействию в широком диапазоне доз. Из них у 34 облученных лиц позднее были диагностированы злокачественные новообразования (ЗНО). Проведена оценка количества лейкоцитов, нейтрофильных гранулоцитов, эозинофилов, базофилов, моноцитов и НК-клеток ($CD16^+/CD56^+$-лимфоциты) в периферической крови, а также фагоцитарная, лизосомальная активность и интенсивность внутриклеточного кислородозависимого метаболизма нейтрофилов и моноцитов. У лиц, подвергшихся хроническому радиационному воздействию за несколько лет до развития ЗНО, наблюдалось значимое повышение интенсивности фагоцитоза моноцитов (медиана — 10,50 усл. ед. против 6 усл. ед.; $p = 0,05$) и лизосомальной активности нейтрофилов (медиана — 482 усл. ед. против 435,5 усл. ед.; $p = 0,03$) по сравнению с пациентами без ЗНО. При анализе дозовых зависимостей у облученных лиц, впоследствии заболевших онкологическими заболеваниями, обнаружены увеличение интенсивности фагоцитоза моноцитов в зависимости от дозы облучения тимуса и периферических лимфоидных органов ($\rho = 0,45$; $p = 0,009$), а также повышение активности фагоцитоза нейтрофилов с увеличением накопленной дозы облучения красного костного мозга ($\rho = 0,44$; $p = 0,01$).

Ключевые слова: хроническое радиационное воздействие, канцерогенез, нейтрофилы, моноциты, натуральные киллеры

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Вклад авторов: Е. А. Блинова — обобщение первичного материала, анализ и обсуждение результатов, подготовка текста статьи; А. И. Котикова — статистическая обработка первичных данных; А. А. Аклеев, А. В. Аклеев — планирование исследования, редактирование текста, подготовка окончательного варианта статьи.

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It is believed that genetic and epigenetic alterations induced by exposure to ionizing radiation may contribute to the development of malignant tumors (MTs). There is no doubt that the functional state of the body's defense systems, such as DNA repair, cell cycle arrest, antioxidant system and anti-tumor immunity, plays a vital part in malignant transformation of cells. When the defense mechanisms are effective, radiation exposure may result in no pathological changes, however, inefficiency of a particular defense system results in the risk of cancer.

Systemic immunity plays an important role in body's defense against cancer [1]. In particular, neutrophils, monocytes and natural killers (NK cells) are able not only to recognize, lyse, and eliminate tumor cells and mutant cells from the body, but also to regulate the function of other immunocompetent cells (macrophages, T and B cells, eosinophils, basophils) due to production of chemokines, pro- and anti-inflammatory cytokines, prostaglandins, leukotrienes [2, 3]. Furthermore, activated macrophages can exhibit antitumor activity due to lysing enzymes and free radicals that damage tumor cells, and produce TNFα, the antitumor cytokine [4].

In the residents of coastal villages located along the Techa River (South Ural), who had been exposed to low-dose radiation due to the discharge of liquid radioactive waste in the Techa River by the Mayak Production Association for a long time, the changes in their immune system were detected in the form of reduced white blood cell count (mainly due to neutrophils and lymphocytes), increased neutrophil lysosomal activity, certain suppression of monocyte intracellular oxygen-dependent metabolism, and the shift in cytokine balance towards pro-inflammatory response [5, 6]. Furthermore, exposed individuals with obligate precancerous conditions showed the increased absolute and relative natural killer blood cell counts ($CD16^+/CD56^+$ cells) compared to exposed patients with no precancerous conditions [7]. Epidemiological studies of the cohort of people exposed on the Techa River revealed the increased risk of morbidity and mortality from malignant neoplasms and leukemia [8, 9].

The study was aimed to perform quantification and functional assessment of the system of neutrophil granulocytes, monocytes and natural killers in people who were diagnosed with cancer after chronic radiation exposure.

METHODS

The system of neutrophil granulocytes, monocytes and natural killers was assessed in 104 people, chronically exposed to low-dose radiation due to the discharge of radioactive waste in the Techa River by the Mayak Production Association in the 50–60-ies of the XX century. The exposure pattern is detailed in the book [10].

The inclusion criteria for the studied groups were as follows: permanent residence in one of 41 villages located along the Techa River between 1 January 1950 and 31 December 1960; availability of individual absorbed doses to red bone marrow (RBM), thymus and peripheral lymphoid organs calculated using the Techa River Dosimetry System-2016 (TRDS-2016) [11]. Exclusion criteria: no information about the residence on the territory of radioactive contamination; autoimmune, acute or chronic (period of exacerbation) inflammatory disorders, hematologic cancers, kidney or liver failure diagnosed at the time of examination, acute cerebrovascular accident in the last three months, cancer (for comparison group); taking drugs capable of affecting the studied parameters (antibiotics, glucocorticoids, cytostatics).

All the examined individuals were divided into two groups: the index group included 34 exposed people who were later diagnosed with neoplasms (immunology tests were performed once, 1–7 years before the onset of the disease in 2007–2014), the comparison group included exposed individuals with no oncology disorders (70 people). In the index group consisting of exposed people, small intestine and colorectal cancer (five cases), gastric cancer (two cases), bladder cancer (three cases), skin cancer (seven cases), cancer of the orbital connective tissue and retro-ocular space (one case), lip cancer (one case), breast cancer (two cases), cancer of female reproductive organs, including cervical cancer (three cases), cancer of male reproductive organs (three cases), bronchial and lung cancer (four cases), osteosarcoma of the skull (one case), and MTs of unspecified site (two cases) were diagnosed in 2009–2017.

The studied groups were comparable in gender, ethnicity, age at the time of examination, and radiation dose. Characteristics of the studied groups are provided in Table 1.

Venous blood of the individuals in the studied group (10 mL) was collected from the cubital vein into a syringe with heparin. The white blood cell, neutrophil granulocyte, basophil, and monocyte counts in peripheral blood were defined with the Pentra 120 DX automatic hematology analyzer (HORIBA ABX S.A.S.; France). NK cells ($CD16^+/CD56^+$ lymphocytes) were counted using the fluorochrome-labeled monoclonal antibodies to appropriate CD receptors (conjugated antibody $CD3^-$FITC/$CD16^+$CD56-PE, Beckman Coulter; USA). Cell number was assessed using the Navios flow cytometer (Beckman Coulter; USA).

Table 1. Characteristics of studied groups

Parameters of the groups		Index group	Comparison group	*p*
		n = 34	*n* = 70	
Age at the time of examination, years: M ± SE (min–max)		69.09 ± 0.78 (60–78)	68.96 ± 0.53 (58–79)	0.89
Gender, *n* (%)	Male	14 (41.2)	24 (34.2)	0.49
	Female	20 (58.8)	46 (66.8)	
Ethnicity, *n* (%)	Slavs	14 (41.2)	30 (42.9)	0.87
	Turks	20 (58.8)	40 (57.1)	
Accumulated dose to RBM, mGy: M ± SE (min–max)		852 ± 116 (5.37–3507)	826 ± 72.40 (6.03–3394)	0.92
Accumulated dose to thymus and peripheral lymphoid organs, mGy: M ± SE (min–max)		140 ± 20.7 (2.45–466)	127 ± 13.70 (0.55–460)	0.58

Note: *n* — number of surveyed people; M ± SE — mean ± standard error of the mean; *p* — significance levels for intergroup differences.

Table 2. Quantity of innate immune cells in blood of examined individuals

Parameter	Index group Me (Q_1–Q_2)	Comparison group Me (Q_1–Q_2)	Significance level
Basophils, %	0.85 (0–1)	0.60 (0–1)	0.9
Eosinophils, %	2.00 (1–4)	2.25 (1–3.75)	0.78
Band neutrophils, %	5 (2.50–6.50)	3 (2–5.50)	0.08
Segmented neutrophils, %	53 (43.25–61)	52 (43.20–57)	0.95
Leukocytes, 10^9/L	6.36 (5.26–7.38)	6.58 (5.29–7.76)	0.66
Lymphocytes, %	31 (26.65–39.25)	34 (29.10–40.75)	0.71
Lymphocytes, 10^9/L	1.83 (1.60–2.57)	2.14 (1.83–2.74)	0.22
Neutrophils, %	57.50 (52–63.50)	54 (48.25–59.95)	0.29
Neutrophils, 10^9/L	3.62 (2.81–4.76)	3.53 (2.76–4.37)	0.64
Monocytes, %	6 (3–7.25)	6.9 (4–9.15)	0.21
Monocytes, 10^9/L	0.35 (0.20–0.51)	0.38 (0.27–0.61)	0.35
Natural killers ($CD16^+/CD56^+$ lymphocytes), %	16.25 (12.08–23.90)	14.40 (9.20–19.70)	0.09
Natural killers ($CD16^+/CD56^+$ lymphocytes), 10^9/L	0.30 (0.23–0.53)	0.31 (0.17–0.46)	0.24

Note: Me (Q_1–Q_2) — median (25^{th}–75^{th} percentile range).

Phagocytic, lysosomal activity, as well as neutrophil and monocyte intracellular oxygen-dependent metabolism were determined by standard methods [12, 13]. The following parameters were assessed: phagocytic activity of neutrophils and monocytes (PAN, PAM), phagocytosis rate of neutrophils and monocytes (PRN, PRM), phagocytic numbers for neutrophils and monocytes (PNN, PNM), the levels of neutrophil and monocyte intracellular oxygen-dependent metabolism in both spontaneous and induced variants (NBT-test of neutrophils, NBT-test of monocytes), lysosomal activity and total lysosomal activity of neutrophils and monocytes (LAN, LAM, TLAN, TLAM). The details of the methods are provided in previously published articles [7]. In all cases the Axio Imager A2 light microscope (Carl Zeiss; Germany) was used for recording of reactions.

Statistical processing of primary data, that involved the use of Mann–Whitney U test to compare two data sets, was performed with the SigmaPlot software, ver. 12.5 (SYSTAT Software; USA). Pearson and Spearman correlation analysis, as well as linear regression analysis were performed. The results with the significance levels below 0.05 were considered significant.

RESULTS

Peripheral blood cell counts and parameters of neutrophil and monocyte functional activity in the studied groups were compared. The results are presented as median values (Me) and 25^{th}–75^{th} percentile ranges (Q_1–Q_2) (Tables 2, 3).

The study revealed no significant differences in the indicators of cellular immunity between exposed people, who subsequently developed cancer, and exposed people with no cancer.

Assessment of the neutrophil granulocyte and monocyte system functional characteristics revealed the significantly increased phagocytosis rate of neutrophils and lysosomal activity of monocytes in individuals who subsequently developed cancer compared to the group of people with no MTs.

Table 3. Functional characteristics of innate immune cells in the examined individuals

Parameter	Index group Me (Q_1–Q_2)	Comparison group Me (Q_1–Q_2)	Significance level
PAM, %	4.50 (2.25–10)	4 (2–5.50)	0.27
PRM, AU	10.50 (3.25–21.75)	6 (3–12)	0.05**
PNM, AU	1.88 (1.04–2.95)	1.50 (1–2)	0.11
NBT-test of monocytes, spontaneous, %	53 (39.25–60.75)	52 (42–58.50)	0.66
NBT-test of monocytes, induced, %	55.50 (42.25–63.75)	52 (42.50–58)	0.53
LAM, AU	292 (214.50–373)	306.50 (254.50–370.25)	0.45
TLAM, AU	1.06 (0.44–1.53)	1.19 (0.62–1.94)	0.11
PAN, %	5 (3–7)	4 (2–6.50)	0.42
PRN, AU	9 (5.25–18)	8 (4–11)	0.14
PNN, AU	1.78 (1.34–2.65)	1.80 (1.16–2.26)	0.48
NBT-test of neutropils, spontaneous, %	56 (40.25–62)	50 (40.50–55)	0.61
NBT-test of neutropils, induced, %	56 (40.25–62)	50 (40.50–55)	0.61
LAN, AU	482 (408.50–613.50)	435.50 (362–491)	0.03**
TLAN, AU	15.96 (11.27–25.28)	15.88 (11.19–19.51)	0.45

Note: Me (Q_1–Q_2) — median (25^{th}–75^{th} percentile range); ** — significant differences between the studied groups.

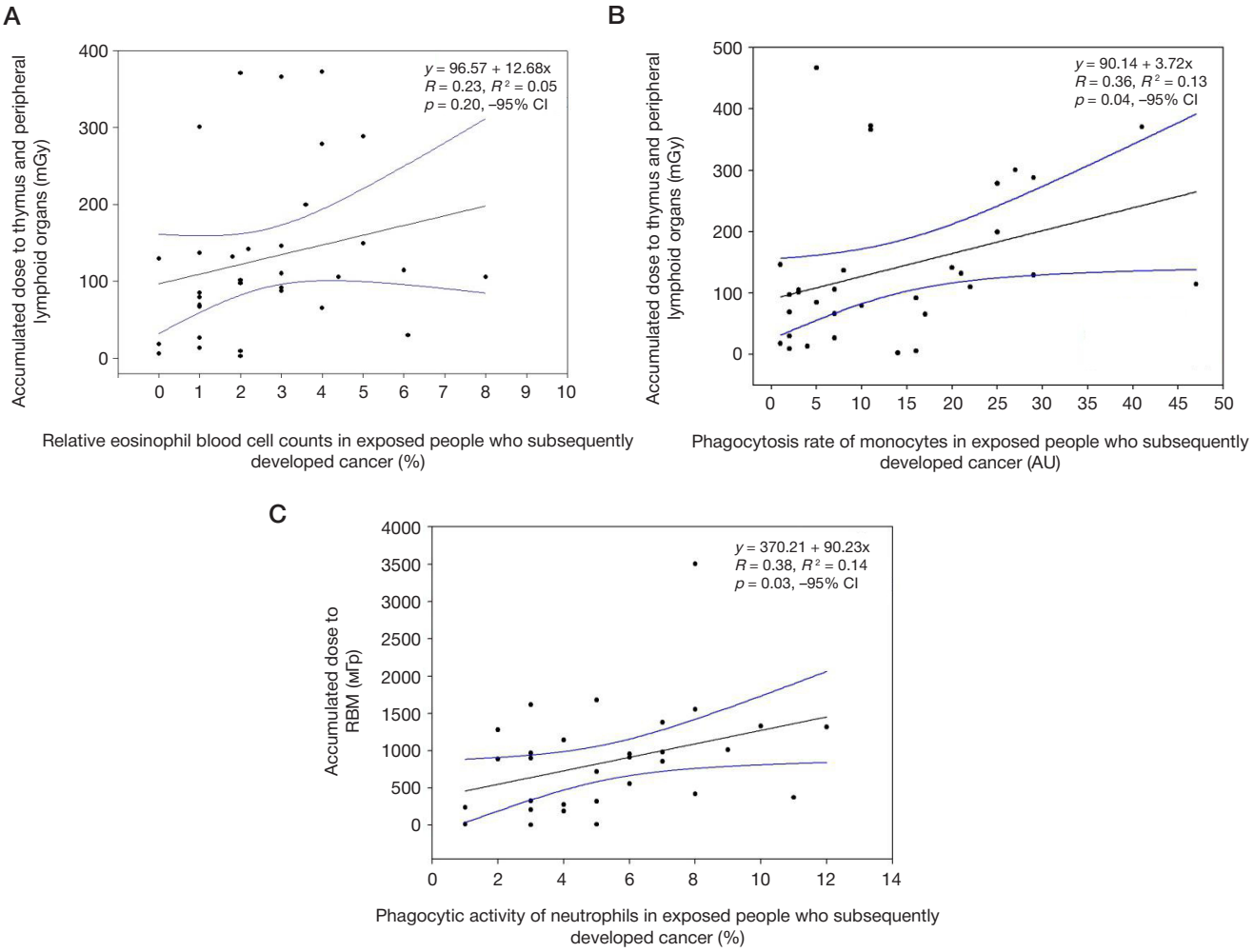

Fig. 1. Linear regression analysis of the dose–response relationships in the group of exposed people who subsequently developed cancer for the following indicators: **A** — relative eosinophil counts, **B** — phagocytosis rate of monocytes, **C** — phagocytic activity of neutrophils

When assessing the dose–response relationship in exposed people who subsequently developed cancer, a significant increase in the percentage of eosinophils in blood as a function of the dose to thymus and peripheral lymphoid organs was revealed (Spearman correlation analysis: ρ = 0.38, p = 0.03). However, linear regression analysis showed no reliable results (Fig. 1A). The dose-dependent increase in the phagocytosis rate of monocytes with the increase in the accumulated dose to thymus and peripheral lymphoid organs was also noted (Spearman correlation analysis: ρ = 0.45, p = 0.009; Pearson correlation analysis: r = 0.36, p = 0.04), along with the increase in phagocytic activity of neutrophils with the increase in the accumulated dose to RBM (Spearman correlation analysis: ρ = 0.44, p = 0.01; Pearson correlation analysis: r = 0.38, p = 0.03). The results of linear regression analysis for these indicators are provided in Fig. 1B, C.

Different relationships were found in the comparison group: there was a significant decrease in relative basophil counts with the increase in the accumulated dose to RBM (Spearman correlation analysis: ρ = –0.26, p = 0.03; Pearson correlation

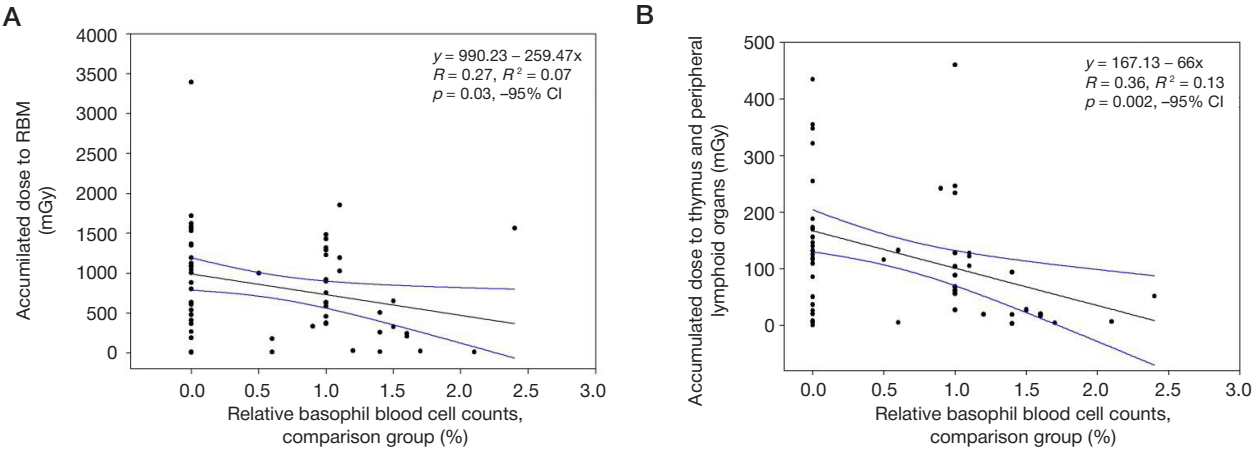

Fig. 2. Linear regression analysis of the dose-dependent decrease in basophil counts for: **A** — accumulated dose to RBM, **B** — accumulated dose to thymus and peripheral lymphoid organs

analysis: $r = –0.27$, $p = 0.03$) and the accumulated dose to thymus and peripheral lymphoid organs (Spearman correlation analysis: $\rho = –0.42$, $p = 0.0005$; Pearson correlation analysis: $r = –0.32$, $p = 0.009$). The results of linear regression analysis for these indicators are provided in Fig. 2.

There was also a significant increase in the phagocytic activity of monocytes with the increase in the accumulated dose to thymus and peripheral lymphoid organs (Spearman correlation analysis: $\rho = 0.27$, $p = 0.03$). However, linear regression analysis showed no reliable results (Fig. 3).

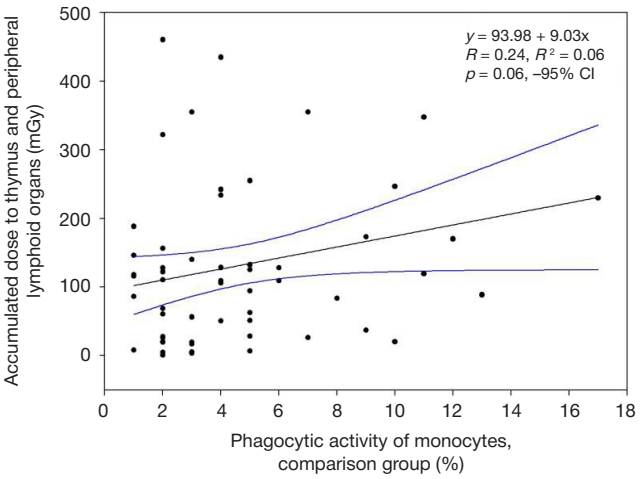

Fig. 3. Linear regression analysis of the dose-dependent increase in the monocyte phagocytic index, comparison group

DISCUSSION

The study has shown that more than 60 years after the beginning of chronic radiation exposure, years before the diagnosis of cancer, the significantly increased phagocytosis rate of neutrophils and lysosomal activity of monocytes are observed in exposed individuals compared to patients who have not developed MTs. Furthermore, the dose-dependent increase in the phagocytosis rate of monocytes as a function of the accumulated dose to thymus and peripheral lymphoid organs was revealed, along with the increase in phagocytic activity of neutrophils as a function of the accumulated dose to RBM.

Several studies provide evidence of the important role of neutrophils in antitumor immune response. Thus, experiments with SR/CR mice have shown that neutrophil granulocytes are the first immune cells to migrate into tumor tissue, and that these cells are involved in realization of the phenomenon of spontaneous regression of tumors of different histological types [14]. The mechanism underlying the neutrophil cytolytic effect on tumor cells is associated with the production of the reactive oxygen and nitrogen species by these cells, although necrosis plays a key part in the tumor cell death [14].

On the other hand, neutrophils and monocytes may promote invasive tumor growth, vascularization and metastasis [15, 16], while the selectin-mediated adhesion of atypical cells to the membrane of neutrophil granulocytes may result in their hematogenous dissemination [17]. Furthermore, the abundance of infiltrating innate immune cells, such as macrophages, mast cells and neutrophils, in the tumor stroma is related to both increased tumor angiogenesis and adverse outcome [18].

It is important to note that the previously conducted retrospective dynamic studies of the peripheral blood cellular composition in individuals exposed on the Techa River, who later developed chronic myeloid leukemia or acute leukemia, showed that neutrophil counts and composition were predictors that made it possible to predict the increased risk of radiation-induced leukaemia in the early period after the start of exposure [19]. Perhaps, the changes in neutrophil and monocyte functional activity observed in chronically exposed people a few years before the onset of the clinically diagnosed cancer could be considered the response to the increased number of transformed cells. However, it is worth noting that phagocytosis rate of monocytes and lysosomal activity of neutrophils may be also affected by non-radiation factors, such as harmful habits and lifestyle.

That is why it is currently impossible to draw definitive conclusions about the possibility of treating the indicators identified as candidate biomarkers of MTs in the long term after the start of exposure. To draw a conclusion about allocation of these indicators to predictors of MTs, further research focused on assessing sensitivity and specificity is required.

CONCLUSIONS

The study of people exposed over a wide dose range in the long term after chronic radiation exposure, years before the development of MTs (MTs diagnosed between 2009 and 2017), revealed a significant increase in the phagocytosis rate of monocytes and lysosomal activity of neutrophils compared to people with no MTs and comparable exposure doses. In the long-term period, the dose-dependent changes in the phagocytosis rate of monocytes and phagocytic activity of neutrophils were observed in chronically exposed individuals.

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EVALUATION OF THE USE OF SOFT SKILLS BY DEPARTMENT HEADS

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Successful management cannot take place without soft skills. The study was aimed to assess the frequency of using soft skills by heads of clinical departments based on feedback from physicians working at the departments. For that an online survey of 433 physicians was conducted with the use of the questionnaire taken from the 360 Sample Competencies Guide. The questionnaire included 20 statements divided into five items: self-awareness, drive for results, leadership, communication, teamwork. The respondents were given five response options per statement: from "always" to "never". The clue referred the assessed individual to one of five groups based on the frequency of using soft skills: leaders, key management, typical management, underachievers, outsiders. Nonparametric methods of analysis were used for data processing. Based on the survey data, 8.6% of assessed individuals joined the group of leaders, while 15.8% were referred to the group of key management, 23.4% to the group of typical management, 30.3% to the group of underachievers, and 21.9% to the group of outsiders. The scores of items correlated with each other ($0.973 \le R \ge 0.967$; $p < 0.001$). The respondents' age, years of service and gender ($p > 0.05$), as well as the assessed individuals' gender ($p > 0.05$) provided no significant differences between the scores. The scores obtained for "communication", "teamwork", emotional control, setting challenging goals, and prioritizing work were lower in the hospital-based physicians than in those who worked in outpatient settings ($p < 0.05$). The questionnaire can be recommended as a feedback tool. Soft skills of the department heads require further development. Soft skills that belong to the items "drive for results", "leadership", and "self-awareness" are worst affected.

Keywords: heads of clinical departments, soft skills, feedback, staff evaluation

Author contribution: Kochubey AV — concept, design, research coordination, conclusions, manuscript writing; Yarotsky SYu — analysis, research planning, data interpretation, discussion; Kochubey VV — literature review, data analysis and interpretation, manuscript writing; Evdoshenko OA — statistical analysis and data interpretation.

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ОЦЕНКА ПРИМЕНЕНИЯ ГИБКИХ НАВЫКОВ ЗАВЕДУЮЩИМИ ОТДЕЛЕНИЯМИ

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Успешное управление невозможно в отсутствии гибких навыков. Целью работы было оценить частоту применения гибких навыков заведующими отделениями на основании обратной связи от врачей отделений. Для этого провели заочный опрос 433 врачей по анкете из руководства «360 Sample Competencies». В анкете 20 утверждений, разбитых на пять блоков: самоконтроль и критичность к своим действиям, достижение результата, лидерство, общение, командная работа. Все утверждения имеют пять вариантов ответа от «всегда» до «никогда». Ключ расшифровки относит оцениваемого в зависимости от частоты использования гибких навыков к одной из пяти групп: лидеры, ведущие, типичные, отстающие, аутсайдеры. Для обработки результатов применили непараметрические методы анализа. В результате опроса в группу лидеров вошли 8,6% оцениваемых, в группу ведущих — 15,8%, в группу типичных — 23,4%, в группу отстающих — 30,3%, в группу аутсайдеров — 21,9%. Обнаружена корреляция оценок по блокам ($0,973 \le R \ge 0,967$; $p < 0,001$). Возраст, стаж и пол респондентов ($p > 0,05$), а также пол оцениваемого ($p > 0,05$) не дают значимой разницы оценок. Оценки «общения», «командной работы», контроля эмоций, постановки сложных целей, расстановки приоритетов ниже у врачей стационаров, чем врачей поликлиник ($p < 0,05$). Опросник можно рекомендовать как инструмент обратной связи; гибкие навыки заведующих отделениями нуждаются в развитии и сильнее страдают гибкие навыки в сферах «достижение результата», «лидерство» и «самоконтроль и критичность к своим действиям».

Ключевые слова: заведующие отделениями, гибкие навыки, обратная связь, оценка персонала

Вклад авторов: А. В. Кочубей — идея, дизайн, координация исследования, формулирование выводов, подготовка рукописи; С. Ю. Яроцкий — анализ, планирование исследования, интерпретация данных, обсуждение результатов; В. В. Кочубей — работа с литературой, анализ и интерпретация данных, подготовка рукописи; О. А. Евдошенко — статистический анализ и интерпретация результатов.

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Managing structural units is the responsibility of the department heads [1]. Successful management cannot take place without soft skills, which include communication skills, interpersonal skills, organizational skills, and leadership qualities [2]. It is clear that all healthcare professionals must, for example, control their emotions when interacting with patients. However, heads of clinical departments not only interact with patients and their family members, colleagues, subordinates, seniors, supervisors and experts, but are often obliged to play a role of dampener in conflicts between parties.

In recent years the term "soft skills" has become more widely used to cover various qualities, features, values, and traits [3].

Soft skills complement hard skills, increase productivity of employees in any field, including healthcare [4], such skills are necessary for personal growth [5]. Soft skills improve self-confidence, social competence and compassion, optimism, and provide energy for personal and professional success [6].

Healthcare professionals, who have been trained to improve empathy skills, show better communication with patients [7–10].

Those, who have improved their communication skills, are better at helping patients to overcome fear of therapy [11]. Identification and prioritization of interpersonal skills improve the quality of work in physicians and healthcare managers [2, 12].

Soft skills or social skills, which are related to personality traits [13] and lifestyle, could be improved by appropriate training [14–16]. Unfortunately, soft skills are poorly taught in the traditional system of medical education [17–19] and are acquired through trial and error [20].

The Russian Scientific Electronic Library elibrary.ru finds a total of 279 scientific papers published in 2017–2022 by keywords “soft skills”. Two of those are about the development of soft skills in physicians and students of medical schools [21, 22]. No studies of soft skills in heads of clinical departments or heads of medical institutions have been found in elibrary.

The importance of soft skills for heads of clinical departments and the lack of such studies in the national database have defined the aim of research: to assess the use of soft skills by the department heads based on feedback from physicians working at the departments.

METHODS

The study involved an online (e-mail) survey of physicians, who received supplementary professional education at the Academy of Postgraduate Education of Federal Scientific and Clinical Center of Specialized Types of Medical Care of FMBA. The survey was conducted with the use of the questionnaire taken from the 360 Sample Competencies Quickstart Guide [23]. The questionnaire includes five items: self-awareness (№ 1–4), drive for results (№ 5–8), leadership (№ 9–12), communication (№ 13–16), teamwork (№ 17–20). The total number of statements is 20. The response options are the same for all statements: always / often / in half of the cases / rarely / never.

The questionnaire was translated into Russian by two professional translators working independently of each other. They performed harmonization of the direct translations and did the reverse translation. The basic version of the questionnaire was tested on a focus group of 14 people. We made sure that the translated items had the original meaning during the face-to-face oral interview. Internal consistency of the questionnaire met the current requirements; Cronbach's alpha (α) was 0.947. Test-retest reliability was tested 74 days later, significance of the intraclass correlation coefficient was <0.001. Internal consistency of the questionnaire together with the test-retest reliability were tested on the group of 107 people that included physicians (median age 48 years, 39 males (36.4%)). Among the respondents, 68 people (63.6%) worked in hospital settings.

According to the available clue, the assessed individuals can be divided into five groups: leaders (those who “always” demonstrate the skill), key management (those who “often” demonstrate the skill), typical management (those who demonstrate the skill in “half of the cases”), underachievers (those who demonstrate the skill “rarely”), outsiders (those who “never” demonstrate the skill).

Characteristics of respondents

A total number of valid questionnaires to be assessed was 433, which corresponded to the sample for the study with improved accuracy to be conducted by the method proposed by K.A. Otdelnova [24]. Characteristics of the respondents are provided in Table 1.

The respondents were also asked to specify gender of the assessed department head. There were 250 men (57.7%) and 183 women (42.3%) among the surveyed people. The characteristics were selected based on the results of third-party and our own research on the impact of the respondent's age, years of service, gender, workload, as well as the surveyed individual's gender on the feedback results [25–27].

Statistical processing of the results was performed with the SPSS software, ver. 23 (IBM Company; USA). Since the distribution of the values of age, years of service, scores per item or statement was significantly different from normal, the median was used together with nonparametric methods of analysis. The differences in median values were assessed using the median test for two independent samples and the Wilcoxon test for dependent samples. Spearman rank correlation was used to define the correlation of scores with age and years of service. Frequency analysis was also performed.

RESULTS

The survey of physicians showed that the majority of the department heads not necessarily used soft skills that belonged to the items “self-awareness”, “drive for results”, “leadership”, “communication”, “teamwork”. Figure presents the total number of the department heads divided into five groups based on the frequency of using soft skills and the list of statements in the following way: 8,660 assessments = 20 statements multiplied by 433 assessed heads of clinical departments.

The group of leaders who always use distinct soft skills constitutes 8.6% in the assessment structure; key management constitute 15.8%, typical management account for 23.4%, underachievers constitute 30.3%, and outsiders who never use their soft skills account for 21.9%.

The percentage of the department heads who always demonstrate certain soft skills does not exceed 10% (Table 2). Up to 20% of the department heads often use various soft skills, up to 33.9% use soft skills in half of the cases, up to 38.3% use soft skills rarely. Up to 35.8% of the assessed individuals have never used soft skills.

The maximum positive score for each statement was 5.

The median score for the “self-awareness” item was 3.50, while the scores for individual statements of the item did not exceed 3.68. In the first item, statement of the department head controlling his/her emotions was assigned the lowest

Table 1. Characteristics of respondents

Characteristics	Number, abs. (%)	Median age (years)	Median years of service
All respondents	433	48	23
Women	294 (67.9)	48	22.5
Men	139 (32.1)	48	23
Worked in inpatient settings	276 (63.7)	49	24
Worked in outpatient settings	157 (36.3)	45	20

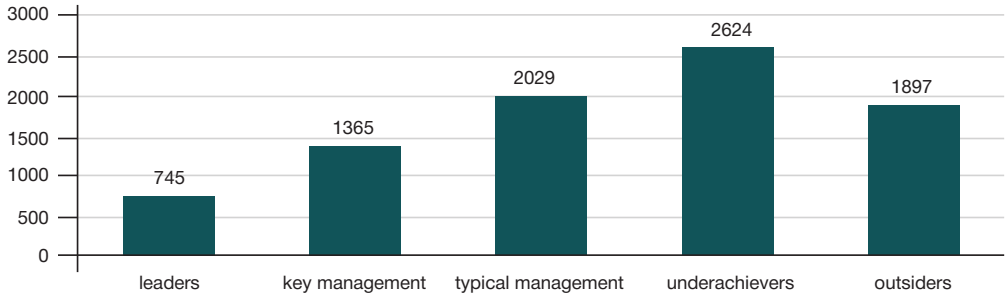

Fig. Size of groups for the entire list of statements

score. The median scores for the item (p = 307) and individual statements were the same in the respondents of both genders ($0.929 \leq p \geq 0.211$), and these for the item (p = 0.256) and individual statements were the same in assessed individuals of both genders ($0.928 \leq p \geq 0.150$). The median scores for item 2 (p = 0.051), statements 2 (awareness of how his/her actions and decisions affect others) and 4 (seeking feedback from others) were the same in the respondents working in both hospital and outpatient settings ($0.425 \leq p \geq 0.258$). The median scores for statements 1 (emotional control) and 3 (learning from mistakes) were higher in the respondents who worked in outpatient settings (p = 0.029; p = 0.047). No correlations of the scores for the "self-awareness" item and individual statements of the item with age or years of service were found ($0.355 \leq p \geq 0.098$).

The median score for the item "drive for results" was 3.75, the scores for individual statements of the item did not exceed 3.66. Statement 6 of the item (helping others to achieve their objectives) was assigned the lowest score. The respondent's ($0.408 \leq p \geq 0.083$) and assessed individual's gender ($1.000 \leq p \geq 0.216$) had no effect on the scores for the item (p = 0.408; p = 0.897) and individual statements of the item. Physicians who worked in the hospital settings gave the department heads a lower rating for items 5 (setting challenging goals; p = 0.001) and 7 (prioritization; p = 0.009) than those working in outpatient settings. No significant differences in the scores for the entire item (p = 0.230), statements 6 (helping others to achieve their objectives; p = 0.230) and 8 (achieving objectives even when faced with obstacles and challenges; p = 0.075) between the hospital-based physicians and physicians working in outpatient settings were found. The respondents' age and gender had no impact on their scores ($0.364 \leq p \geq 0.083$) for this item.

The median score for the "leadership" item was 3.50. Among all statements of the item, statement 10 (taking into account team members' ideas and opinions) was assigned the lowest score. The scores for the item (p = 0.574) and individual statements of the item were the same in the respondents of both genders ($0.630 \leq p \geq 0.400$). The assessed individual's gender also had no effect on the score (item, p = 0.908; statements, $0.908 \leq p \geq 0.598$). The respondents who worked in the hospital settings scored statement 9 (translating strategy; p = 0.009) lower than physicians working in outpatient settings. No differences in the scores for the entire item (p = 0.309) and other statements between the respondents working in the in-patient and outpatient settings were found ($0.309 \leq p \geq 0.127$). Age and gender had no impact on the scores ($0.270 \leq p \geq \geq 0.180$).

The median score for the item "communication" was 4.0. Among all statements of the item, statement 14 (tailoring communication to the needs of the audience) was assigned the lowest score. Men and women scored statement 14 different (p = 0.035), however, the scores for the entire item (p = 0.124) and other statement s ($0.452 \leq p \geq 0.082$) were the same. Male and female heads of clinical departments were rated the same based on the entire item (p = 0.111) and individual statements ($0.752 \leq p \geq 0.106$). The hospital-based physicians gave the department heads a lower rating for the entire item (p = 0.003) and individual statements of the item ($0.033 \leq p \geq \geq 0.002$). No differences in the "communication" scores between the respondents of various age and years of service were found ($0.174 \leq p \geq 0.064$).

The "teamwork" item had a median score of 4.0. The department heads' effective working in a team was assigned the lowest score. The respondents of both genders rated the entire item (p = 0.556) and individual statements ($0.556 \leq p \geq 0.167$) the same. "Teamwork" of the department heads of both genders was rated the same by the respondents based both on the entire item (p = 0.556) and individual statements ($0.556 \leq p \geq 0.167$). Female heads of clinical departments were rated the same as male department heads based on the entire item (p = 0.224) and individual statements ($0.559 \leq p \geq 0.106$). The hospital-based physicians gave their department heads a lower rating for the "teamwork" item (p = 0.018) and individual statements of the item ($0.018 \leq p \geq 0.002$) than physicians who worked in outpatient settings. Age and years of service had no effect on the scores ($0.167 \leq p \geq 0.059$) of the department heads' teamwork.

The "self-awareness" item was rated by the respondents the same as the "leadership" item (p = 0.884). However, the scores of the "self-awareness" item and items "drive for results" ($p < 0.001$), "communication" ($p < 0.001$) and "teamwork" ($p < 0.001$) were different. The scores of the item "drive for results" were significantly different from the scores of other items ($p < 0,001$). The scores of the items "leadership" differed from the scores of the items "communication" and "teamwork" ($p < 0.001$). The scores of the items "communication" and "teamwork" were different ($p < 0.001$). Strong positive correlations between all items were found ($0.973 \leq R \geq 0.967$; $p < 0.001$).

DISCUSSION

The survey showed that the majority of the assessed department heads had soft skills in the areas of "self-awareness", "drive for results", "leadership", "communication" and "teamwork", and showed those during work. However, most of the department heads, 53.7% (30.3 ± 23.4%), use these skills in half of the cases or more rarely and could be therefore assigned to the group of typical management or underachievers. Only 24.4% (15.8% ± 8.6%) use their soft skills often or always and are therefore assigned to the group of leaders or key management. Outsiders occupy the second place (21.9%). We would like to point out that the group of outsiders includes heads of clinical departments, who "never" use certain soft skills.

In our opinion, strong significant positive correlations between the scores of different items are the important finding.

Table 2. Results of the survey of physicians about the department heads

Statements	Answers ± m_p (%)					Median (years)
	Always	Often	In half of the cases	Rarely	Never	
Item 1. Self-awareness						3.5
1. Controls his/her emotions, even in high-pressure situations	9.0 ± 0.458	18.7 ± 0.433	27.9 ± 0.408	24.9 ± 0.416	19.4 ± 0.431	3.31
2. Demonstrates an awareness of how his/her actions and decisions affect others	2.8 ± 0.474	17.6 ± 0.436	33.9 ± 0.391	25.9 ± 0.414	19.9 ± 0.430	3.42
3. Treats mistakes and setbacks as learning opportunities	6.7 ± 0.464	14.8 ± 0.444	26.8 ± 0.411	30.5 ± 0.401	21.2 ± 0.427	3.53
4. Actively seeks feedback from others on his/her performance	6.5 ± 0.465	17.8 ± 0.436	15.2 ± 0.443	38.1 ± 0.378	22.4 ± 0.423	3.68
Item 2. Drive for results						3.75
5. Sets challenging goals for him/herself	7.4 ± 0.462	12.7 ± 0.449	22.6 ± 0.423	33.7 ± 0.391	23.6 ± 0.420	3.66
6. Helps others achieve their objectives	9.9 ± 0.456	20.6 ± 0.428	30.3 ± 0.401	19.4 ± 0.431	19.9 ± 0.430	3.18
7. Prioritizes his/her work based on the needs of the organization and its customers	8.8 ± 0.459	15.7 ± 0.441	22.6 ± 0.423	30.3 ± 0.401	22.6 ± 0.423	3.54
8. Achieves his/her objectives even when faced with obstacles and challenges	9.5 ± 0.457	16.9 ± 0.438	21.2 ± 0.427	33.0 ± 0.393	19.4 ± 0.431	3.48
Item 3. Leadership						3.5
9. Translates the organization strategy into concrete actions/plans	8.8 ± 0.459	16.4 ± 0.439	24.0 ± 0.419	29.3 ± 0.404	21.5 ± 0.426	3.48
10. Takes team members' ideas and opinions into account when making decisions	8.1 ± 0.461	13.2 ± 0.448	31.9 ± 0.397	26.6 ± 0.412	20.3 ± 0.429	3.44
11. Helps team members resolve work-related problems	8.1 ± 0.461	12.5 ± 0.450	30.9 ± 0.399	26.3 ± 0.413	22.2 ± 0.424	3.49
12. Holds team members accountable for achieving their objectives	7.4 ± 0.462	11.8 ± 0.451	27.0 ± 0.411	34.4 ± 0.389	19.4 ± 0.431	3.56
Item 4. Communication						4
13. Actively listens to others	8.5 ± 0.460	14.1 ± 0.445	21.5 ± 0.426	33.9 ± 0.391	21.9 ± 0.425	3.6
14. Tailors his/her communication to the needs of the audience	9.5 ± 0.457	15.0 ± 0.443	18.7 ± 0.433	36.5 ± 0.383	20.3 ± 0.429	3.59
15. Communicates clearly and concisely	7.9 ± 0.461	12.9 ± 0.449	16.4 ± 0.439	38.1 ± 0.378	24.7 ± 0.417	3.77
16. Conveys credibility and expertise when he / she communicates with others	8.1 ± 0.461	11.8 ± 0.451	23.8 ± 0.420	32.1 ± 0.396	24.2 ± 0.418	3.65
Item 5. Teamwork						4
17. Works effectively in a team	9.5 ± 0.457	14.3 ± 0.445	18.7 ± 0.433	35.1 ± 0.387	22.4 ± 0.423	3.63
18. Gives constructive and helpful feedback to others	7.9 ± 0.461	13.6 ± 0.447	14.5 ± 0.444	38.3 ± 0.377	25.6 ± 0.415	3.8
19. Treats others with respect	9.7 ± 0.457	14.8 ± 0.444	18.0 ± 0.435	33.3 ± 0.392	24.2 ± 0.418	3.64
20. Values and respects differences among team members	5.3 ± 0.468	9.5 ± 0.457	22.4 ± 0.423	27.0 ± 0.411	35.8 ± 0.385	3.97

Taking into account the fact that soft skills in various areas are uneven [5], the correlation identified proves that each group consists mainly of the same heads of clinical departments, i.e. those, who "always demonstrate self-awareness", always or often use their soft skills in such areas as drive for results, leadership, communication, teamwork. On the contrary, heads of clinical departments, who have been assessed by the respondents as "never using self-awareness skills", join the group of underachievers or outsiders based on the scores of other items.

The lack of correlation of the scores with the respondents' age and years of service, as well as with the respondents' and assessed individuals' gender, is a good sign that confirms objectivity of the questionnaire used in feedback polls.

The scores for the number of statements and items revealed differences between the physicians who worked in in-patient and outpatient settings. The hospital-based physicians rated their department heads significantly lower in terms of emotional control, setting challenging goals, prioritizing, translating strategy, active listening to others, tailoring communication to the needs of the audience, communicating clearly and concisely and conveying expertise, effective working in a team, giving constructive and helpful feedback, treating others with respect, and respecting differences among team members.

Taking into account our data on the correlation between the department heads' psychological well-being and working in various healthcare settings with varying workload, it is impossible to outline the background of different estimates for soft skills obtained during the study [27]. Presumably, hospital-based physicians work in more close cooperation with heads of clinical departments: achieving the result relies more heavily on the team efforts and the role of the department head, than in outpatient clinics. That is why the requirements related to communication skills, teamwork, emotional control, prioritizing,

and translating strategy for hospital-based physicians are higher. However, this assumption needs to be verified and can make the hypothesis for further research.

Attention should be paid to the differences between the median scores of the items. These differences allow us to say that the department heads' soft skills in various fields are uneven.

We would like to remind that the median scores for the items and statements are not tethered to standard values. That is why it is a mistake to assume that the median value over 3 points is the evidence of the assessed person's soft skills being above satisfactory levels. The division of the assessed individuals into five groups shows the true state of their soft skills.

CONCLUSIONS

The questionnaire can be recommended for use as part of the performance appraisal system tailored for physicians who serve as heads of clinical departments. Soft skills of the department heads require further development. The skills that belong to the items "self-awareness", "leadership", and "drive for results" are worst affected. The findings are valuable for construction of the performance appraisal systems in medical institutions, including as part of the internal control of the quality and safety of care, as well as for educational institutions when developing the additional professional education programs and heads of clinical departments when choosing the path for professional development.

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PNEUMOLABYRINTH AS A POSTOPERATIVE COMPLICATION OF STAPEDOPLASTY

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Pneumolabyrinth is characterized by the presence of air in the inner ear due to intrusion from the tympanic cavity. It is a rare complication of stapedoplasty. Currently, there is no clear algorithm for treatment of this complication. The paper reports two cases of pneumolabyrinth being the short-term and long-term postoperative complications. In the first case, the patient, who had undergone stapedoplasty in the left ear, suddenly developed rapid hearing loss and tinnitus in the left ear with dizziness three weeks after physical activity. Physical examination revealed no evidence of the tympanic membrane defect. Audiometry revealed left-sided IV degree of sensorineural hearing loss. Pneumolabyrinth was detected on the temporal bone CT scans. In the second case, vestibulocochlear symptoms developed three days after stapedoplasty in the right ear. Pure tone audiometry revealed right-sided IV degree of mixed hearing loss. CT scan of the temporal bone confirmed the diagnosis of pneumolabyrinth. In both cases the correct position of the stapedial prosthesis, "empty" vestibule and perilymphatic fistula were found during revision tympanotomy. The prosteses were removed during surgery, Dexamethasone solution was introduced into the vestibule; stapedoplasty with autocartilage on the perichondrium was performed. After surgery, vestibular symptoms disappeared, and hearing improved.

Keywords: pneumolabyrinth, stapedoplasty, perilymphatic fistula, vestibulocochlear symptoms, pneumatization, sensorineural hearing loss

Author contribution: Diab KhMA — research design, surgical treatment of patients, manuscript editing; Daikhes NA — manuscript editing; Pashchinina OA, Zukhba AG — manuscript writing; Kokhanyuk SV, Pirogova NE — literature review.

Compliance with ethical standards: the study was approved by the Ethics Committee of the National Medical Research Center for Otorhinolaryngology of FMBA (protocol № 03/22 dated June 20, 2022). The patients submitted the informed consent to surgery and personal data processing.

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ПНЕВМОЛАБИРИНТ КАК ОСЛОЖНЕНИЕ ПОСЛЕ СТАПЕДОПЛАСТИКИ

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Пневмолабиринт характеризуется наличием воздуха во внутреннем ухе вследствие его проникновения из барабанной полости. Он является редким осложнением стапедопластики. В настоящее время нет четкого алгоритма лечения этого состояния. В статье рассмотрены два случая пневмолабиринта, возникшего как раннее и позднее осложнения после операции. В первом случае у пациентки, перенесшей стапедопластику на левом ухе, через три недели после физической нагрузки внезапно развились резкое снижение слуха на левом ухе, шум в левом ухе и головокружение. При физикальном осмотре не было обнаружено никаких признаков дефекта барабанной перепонки. На аудиометрии диагностирована левосторонняя сенсоневральная тугоухость IV степени. По данным компьютерной томографии (КТ) височной кости выявлен пневмолабиринт. Во втором случае развитие вестибулокохлеарной симптоматики имело место спустя три дня после стапедопластики на правом ухе. На аудиометрии обнаружена правосторонняя смешанная тугоухость IV степени. Данные КТ височной кости подтвердили диагноз пневмолабиринта. В обоих случаях во время ревизионной тимпанотомии был обнаружен стапедиальный протез, установленный проксимальным концом в преддверие и дистальным концом закрепленный на длинном отростке наковальни, а также перилимфатический свищ. В ходе операции протез удалили, в преддверие ввели раствор дексаметазона, выполнили стапедопластику по методике «аутохрящ на надхрящницу». После операции вестибулярные симптомы исчезли, слух несколько улучшился.

Ключевые слова: пневмолабиринт, стапедопластика, перилимфатический свищ, вестибулокохлеарные симптомы, пневматизация, сенсоневральная тугоухость

Вклад авторов: Х. М. А. Диаб — разработка дизайна исследования, выполнение хирургического лечения пациента, редактирование текста статьи; Н. А. Дайхес — редактирование текста статьи; О. А. Пащинина, А. Г. Зухба — написание текста рукописи; С. В. Коханюк, Н. Е. Пирогова — обзор литературы.

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Pneumolabyrinth is a rare condition characterized by substitution of perilymph that fills the labyrinth with air due to abnormal communication between the middle and inner ear. In other words, pneumolabyrinth is the presence of air in the inner ear that results from communication between the inner ear and middle ear that is filled with air. Clinical manifestations of the condition are associated with such vestibulocochlear disorders, as sensorineural hearing loss, “ringing” in the ears and/or head, ear fullness, as well as vertigo and nausea [1, 2]. The final diagnosis is established based on the data of high resolution computed tomography (HRCT) of the temporal bone by the presence of air in the inner ear.

The term “pneumolabyrinth” was introduced into the scientific literature by M.F. Mafee et al. [3]. Etiological factors that lead to the development of pneumolabyrinth conditionally can be divided into traumatic, iatrogenic and inflammatory factors. According to the statistical analysis of data obtained from electronic databases (PubMed, MEDLINE, EMBASE, Cochrane Library, Scopus), temporal bone fractures (31.8%), stapes surgery (18.2%), penetrating trauma (15.9%), and barotrauma

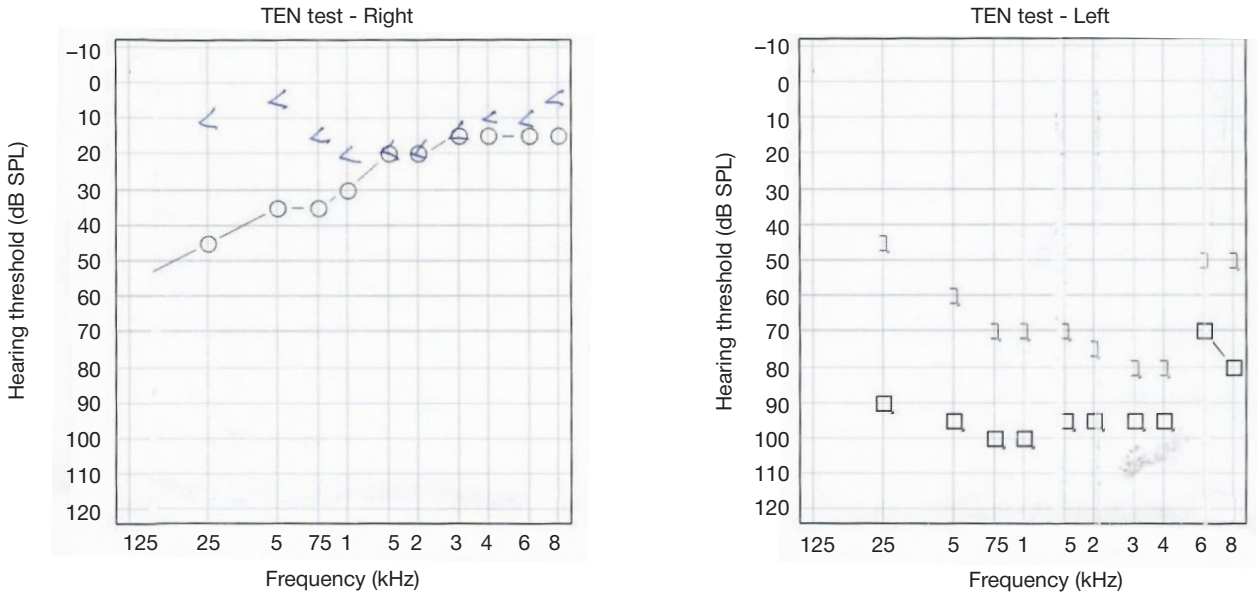

Fig. 1. PTA data of patient K., 32 years old, at admission (before revision tympanotomy)

(11.4%) are the most common causes of pneumolabyrinth. Based on the temporal bone CT scans, pneumolabyrinth, both as a solo disorder and in combination with other disorders, is most often found in the vestibule (95.3%), followed by cochlea (40.2%) and semicircular canals (23.4%) [4].

Long-term pneumolabyrinth is a rare complication of stapedectomy related to the prosthesis displacement or luxation. The condition should be considered in patients with vestibulocochlear symptoms, even many years after surgery. It should be noted that pneumolabyrinth that occurs within the first week after surgery suggests the presence of perilymphatic fistula. In the majority of cases reported in scientific literature, pneumolabyrinth occurred within weeks or months after stapes surgery. The article presents two cases of pneumolabyrinth.

Clinical cases

Pneumolabyrinth as a late complication of stapedoplasty

Female patient K. aged 32 was admitted to the Department of Ear and Skull Base Pathology of the National Medical Research Center for Otorhinolaryngology of FMBA with complaints of vertigo when changing body position and walking, tinnitus and hearing loss in her left ear. According to the history of the disease, the patient underwent left piston stapes surgery with the use of the SPL 03.44S prosthesis (Audio Technologies; Italy) (prosthesis length: 4.5 mm) on September 2, 2020; the perforation diameter was 0.6 mm. The patient was discharged from the hospital on September 7, 2020 with improved hearing in the operated ear. During the postoperative period (October 2020), she noted left-sided rapid hearing loss, tinnitus in her left ear, vertigo when changing body position and walking that developed after excercise. The patient received conservative therapy in the local clinic. She was referred to the outpatient clinic of the National Medical Research Center for Otorhinolaryngology of FMBA and then admitted to the Department of Otology and Skull Base Disorders due to persistent complaints.

Physical examination showed that both tympanic membranes were pearly grey, translucent, with clear margins, mobile, had no defects. Examination revealed no spontaneous nystagmus, a negative Rinne's test in the left ear, and sound lateralization to the right ear in the Weber's test. Pure-tone audiometry (PTA) showed right-sided mixed hearing loss, degree I, and left-sided sensorineural hearing loss, degree IV (Fig. 1).

The temporal bone CT scans revealed CT signs of bilateral fenestral otosclerosis, the condition after left stapedoplasty. Pneumatized antrum, mastoid cells and tympanic cavity were observed. Air was found in the cochlear duct and vestibule of the labyrinth on the left (Fig. 2).

The following clinical diagnosis was made based on the patient's complaints, disease history, PTA and the temporal bone CT scan: fenestral otosclerosis; condition after surgery (stapedoplasty) performed in the left ear on September 2, 2020; pneumolabyrinth; left-sided sensorineural hearing loss, degree IV.

The committee of physicians decided to perform left revision tympanotomy under local anesthesia (sol. Lidocaini 2% — 12 mL). Intrameatal approach through Rosen's incision performed with microsurgical technique was used under microscope. Meatotympanic flap was separated to the level of annulus fibrosus, tympanotomy was performed. Exploration of the tympanic cavity revealed the long process of the incus and malleus along with no stapes structures. The Audio Technologies SPL 03.44S prosthesis (4.5 mm) was installed with its proximal end in the vestibule and the distal end fixed on the long process of the incus. The oval window perilymphatic fistula was found. The prosthesis was removed.

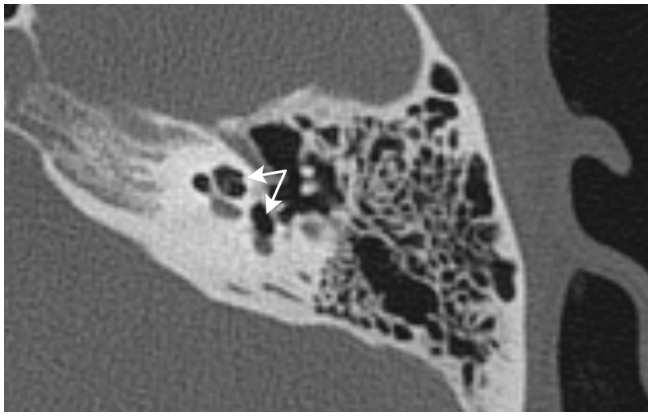

Fig. 2. CT scan at admission, left temporal bone of patient K. aged 32; the *arrows* indicate the presence of air in the cochlea and vestibule

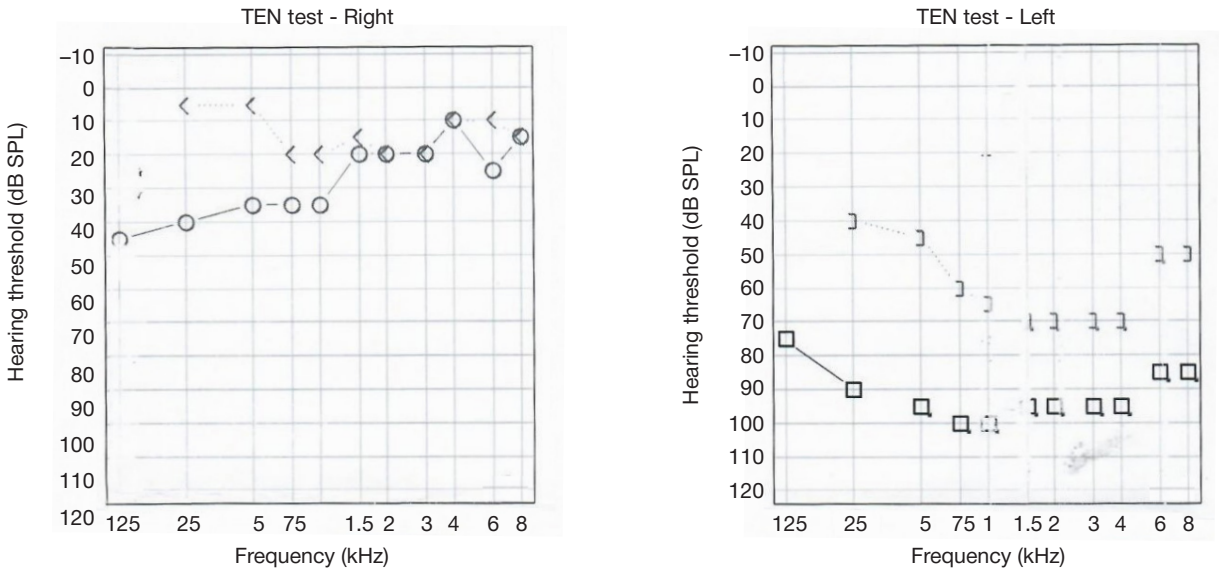

Fig. 3. PTA data of patient K., 32 years old, obtained in the short-term postoperative period

Autocartilage graft with perichondrium was taken from the auricle. Dexamethasone solution and normal saline were injected in the vestibule. A perichondrium flap was put on the area of vestibule at the level of window frame. An autocartilaginous columella with the length of 3.5 mm was installed with its proximal end on the above-mentioned peace of perichondrium and its distal end under the lenticular process of the incus. Meatotympanic flap was put back. Tamponade of the external auditory canal with the antibiotic-loaded (ceftriaxone) hemostatic sponge was peformed.

The patient was prescribed a course of conservative therapy: glucocorticoid drug (dexamethasone 24–20–16–8–4 mg + NaCl 0.9% 200.0 mL, intravenous infusion once a day); Betahistine (Betaserc) 24 mg three times daily; vitamin therapy (vitamins B1, B6 — 1.0 mL, intramuscular injection every other day); vascular therapy (actovegin 10.0 mL + NaCl 0.9% 10.0 mL, intravenous infusion); Choline Alfoscerate (Noocholin Rompharm) 4.0 mL (250 mg/mL) + NaCl 0.9% 100.0 mL, intravenous infusion.

The control PTA performed in the postoperative period revealed right-sided mixed hearing loss, degree I, and left-sided sensorineural hearing loss, degree IV (Fig. 3).

Another CT scan of temporal bones performed on December 4, 2020, revealed CT signs typical for the condition after left stapedoplasty (Fig. 4).

Four days after revision surgery, the patient noted no dizziness and improved hearing in her left ear. No signs of paresis of the mimic muscles or spontaneous nystagmus were revealed. The Weber's test showed sound lateralization to the left ear. It was decided to discharge the patient from the hospital for further ENT follow-up in the community clinic.

Pneumolabyrinth as a short-term complication of stapedoplasty

Female patient S. aged 37 was admitted to the Department of Ear and Skull Base Pathology of the National Medical Research Center for Otorhinolaryngology of FMBA with complaints of right-sided hearing loss, tinnitus in the right ear, and vertigo. Physical examination showed that both tympanic membranes were pearly grey, translucent, with clear margins, mobile, with no defects. There was no spontaneous nystagmus. The tuning fork tests showed a negative Rinne's test in the right ear, and sound lateralization to the right ear in the Weber's test. PTA revealed right-sided mixed hearing loss, degree III (Fig. 5).

The temporal bone CT scan performed on August 17, 2020 revealed CT signs of fenestral otosclerosis.

The following clinical diagnosis was made based on the patient's complaints, disease history, PTA and the temporal bone CT scan: fenestral and cochlear otosclerosis; right-sided mixed hearing loss, degree III.

On November 17, 2020, the patient underwent right piston stapedoplasty performed with the use of the Audio Technologies SPL 03.44S prosthesis (prosthesis length 4.5 mm) under local anesthesia. She noted hearing improvement in her right ear during the operation. Three days after surgery, the patient noted rapid hearing loss and tinnitus in her right ear, vertigo when changing body position, nausea, and high-amplitude horizontal right-jerk nystagmus that developed due to patient non-compliance (physical exercise).

The patient got the consultation of otoneurologist, and underwent PTA that revealed right-sided mixed hearing loss, degree IV, during the early postoperative period. The temporal bone CT scan was performed that revealed CT signs of bilateral fenestral otosclerosis, the condition after right stapedoplasty (air was found in the cochlear duct, vestibule of the labyrinth, and lateral semicircular canal) (Fig. 6).

The committee of physicians decided to perform revision tympanotomy. Intrameatal approach through Rosen's incision, performed through microsurgical technique, was used, that was controlled with the surgical microscope. Meatotympanic flap was separated to the level of annulus fibrosus, tympanotomy

Fig. 4. CT scan performed during the short-term postperative period, left temporal bone of patient K. aged 32: the *arrows* indicate the cochlea and vestibule filled with liquid content

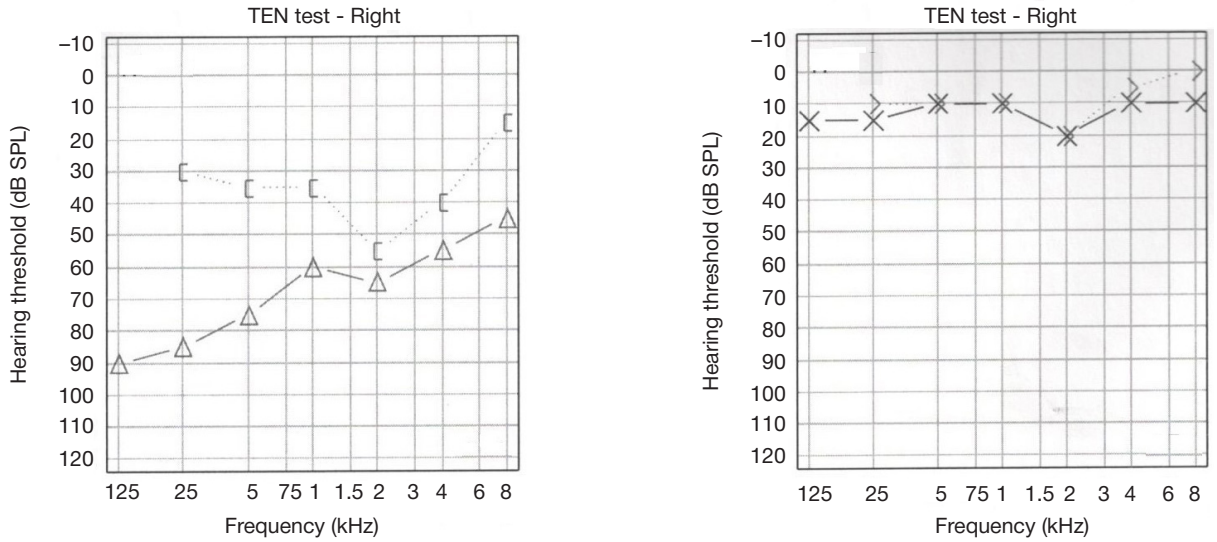

Fig. 5. PTA data of patient S., 37 years old, at admission

was performed under local anesthesia (sol. Lidocaini 2% — 12 mL). Exploration of the tympanic cavity revealed no stapes structures. The Audio Technologies SPL 03.44S prosthesis (4.5 mm) was installed with its proximal end in the vestibule and the distal end fixed on the long process of the incus. The prosthesis was removed. Autocartilage graft with perichondrium was collected from the auricle. Dexamethasone solution and normal saline were injected up to the level of the vestibule window frame. A piece of perichondrium was put on the area of the oval window niche. An autocartilaginous columella with the length of 3.5 mm was installed (Fig. 7). The meatotympanic flap was put back. Tamponade of the external auditory canal with the antibiotic-loaded (ceftriaxone) hemostatic sponge was peformed. A dry sterile auricular dressing was applied. No dizziness or spontaneous nystagmus was observed immediately after surgery.

The patient received a course of conservative therapy: dexamethasone 20–16–12–8–8–4 mg + NaCl 0.9% 200.0 mL, intravenous infusion; betahistine (Betaserc) 16 mg, three times daily; vitamin B12 250 mg, intramuscular injection once a day; choline alfoscerate (Noocholin Rompharm) 4.0 ml (250 mg) + NaCl 0.9% 100.0 mL, intravenous infusion; actovegin 10.0 mL + NaCl 0.9% 100.0 mL, intravenous infusion.

On 6 day after surgery, the patient noted improved hearing in her right ear, reduced dizziness, and reduced tinnitus in the right ear (Fig. 8).

Another CT scan of temporal bones revealed CT signs of fenestral otosclerosis, the condition after right stapedoplasty (Fig. 9).

It was decided to discharge the patient, who showed an obvious improvement, from the hospital for further ENT follow-up in the community clinic and subsequent consultations in the Center.

Clinical case discussion

Pneumolabyrinth is a rare condition associated with air trapped in the inner ear, that is usually caused by the temporal bone injury or develops after stapedoplasty. In some cases the condition is complicated by the oval window fistula [5]. Several cases of pneumolabirynth have been reported in literature: the majority of cases were diagnosed in patients who had undergone primary stapedoplasty. The other causes are the stapes footplate fracture or dislocation, penetrating trauma, temporal bone injury, barotrauma, and cochlear implantation [6].

In the majority of reported cases, pneumolabirynth occurred within weeks or months after stapedectomy. This complication usually manifests itself as a combination of complaints of vestibular (dizziness, bouts of nausea) and cochlear disorders (rapid sensorineural hearing loss, ear fullness and tinnitus).

High-resolution CT is essential for the diagnosis of the pneumolabyrinth. Air in the inner ear and possible stapedial prosthesis displacement indicate the presence of pneumolabirynth [7].

There is no clear algorithm for treatment of the condition due to the small number of cases reported in literature. In patients, who experience rapid hearing loss and vestibular symptoms in the short-term postoperative period (from a few

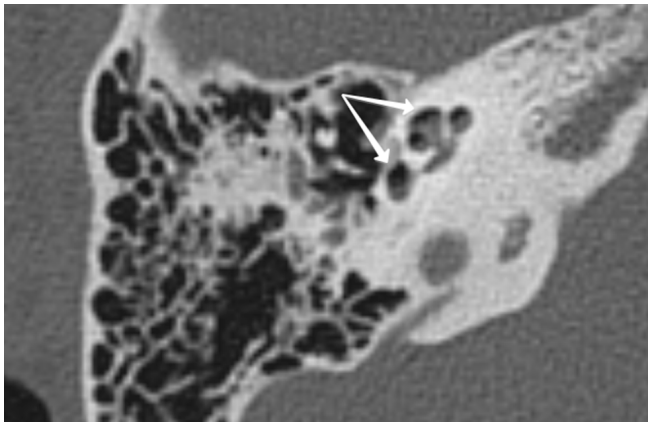

Fig. 6. CT scan performed during the early postperative period, right temporal bone of patient S. aged 37: the presence of air in the cochlea and vestibule

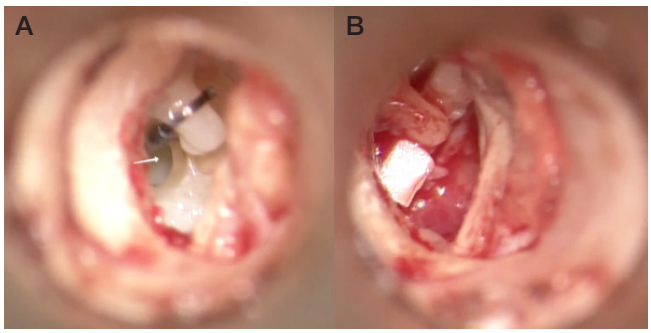

Fig. 7. A. The figure shows the stapedial prosthesis in the correct position that is fixed on the long process of the incus: the *arrow* marks the "open" fistula of the oval window, the "empty" vestibule. **B**. The figure shows the correct position of the autocartilage columella (sesamoid bone is located in the notch of the autocartilage distal end) at the final stage of the surgery

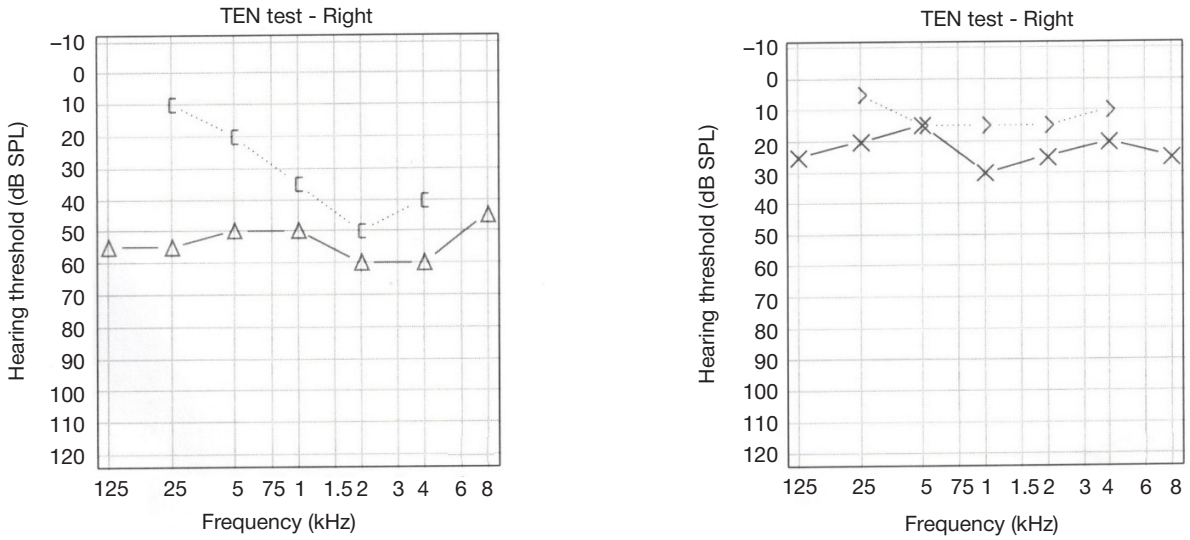

Fig. 8. PTA data of patient S., 37 years old, obtained in the short-term postoperative period

days to three months) after stapes surgery, the treatment strategy (conservative therapy vs. revision tympanotomy with mandatory elimination of the cause) is selected based on the diagnosed type and nature of injury [8]. In cases when pneumolabirynth occurs as a delayed complication of stapedoplasty (within months or even years after surgery), the decision on treatment strategy is less certain [9, 10]. In the reported cases of the stapes surgery postoperative complications, revision tympanotomy was mostly used that involved sealing the defects with autogenous tissue grafts: perichondrium of auricular autocartilage (as in cases presented in our article); ear lobe fat graft; temporal fascia graft [11]. The cases reported in the article confirm the efficiency of this method for surgical treatment of pneumolabirynth and repair of the defect (perilymphatic fistula) with the autogenous tissue graft (autogenous perichondrial graft). Vestibular symptoms disappeared after surgical treatment in almost all the reported cases, supposedly due to vestibular compensation.

Thus, the outcomes of the vestibular system adaptation after surgery are definitely positive, regardless of the lesion type and severity, or the type of previous intervention. In contrast to vestibular symptoms, the results related to hearing repair are not that clear. Thus, in the 1st reported case of delayed pneumolabirynth, we failed to restore hearing to the socially significant levels, despite the fact that improved hearing (by one degree) in the operated ear was observed. In the 2nd reported case of early pneumolabirynth, hearing results were more successful.

The recent review [11] proposed three prognostic factors to predict hearing improvement after correction of pneumolabirynth: bone conduction hearing test at the onset of the disease, time interval from injury to surgery, and traumatic damage to stapes.

In the reported cases, in was decided to perform surgery in patients K. and S. due to pneumolabirynth (confirmed by the temporal bone CT scans), rapid hearing loss, and profound vestibular disorder. Perilymphatic fistula, that enabled communication between the middle ear and inner ear allowing air to enter the vestibule, was found during revision surgery.

Air intrusion to the perilymphatic space due to perilymphatic fistula is one of the mechanisms of hearing loss. The transition of the complex hydrodynamic system of the inner ear from the "closed" to the "open" state, associated with partial substitution of perilymph with air, dramatically alters normal physiological and physical parameters of the labirynth causing the decrease in the cohlear microphonic response, complex action potential, and endocochlear potential [12]. Removal of air from the labirynth by replacing air with liquid together with closure of the defect bring the system of the inner ear to the situation of normal physiological communication. In some cases this results in improvement (or even restoration) of cochlear microphonic response amplitude and action potential, and, consequently, in improved hearing [6].

The cases reported in this article support the theory proposed by the authors who earlier addressed the issue of the development of pneumolabirynth and treatment strategy. Both patients had vestibular disorders and profound hearing loss that significantly improved after surgery in patient S. and improved, in patient K. (although did not get back to baseline levels). It should be remembered that in case of pneumolabirynth due to stapedial prosthesis subluxation the patient should be aware that the prosthesis removal with subsequent installation in the correct position may not improve hearing, but significantly improve or completely eliminate the vestibular symptoms [13, 14].

We believe that in the reported cases it is advisable to carefully remove the remaining footplate, remove the prosthesis to inject dexamethasone solution, and perform stapedoplasty with autocartilage on the vein. A total of 400 operations (stapedoplasty) are performed annually at the Department, and only two cases of pneumolabirynth have been identified within three years. In both cases, pneumolabirynth occurred due to patient non-compliance in the postoperative period, there was no prosthesis displacement (see the images obtained during surgery). According to our observations, the reported complications are not related to the type of prosthesis.

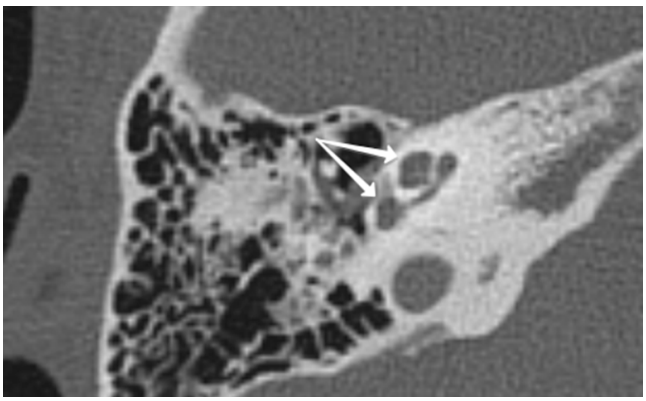

Fig. 9. CT scan performed during the short-term postperative period, left temporal bone of patient S. aged 37: the *arrows* indicate the cochlea and vestibule filled with liquid content

The cases reported in the article highlight the importance of surgical intervention for management of patients with pneumolabirynth and sensorineural hearing loss confirmed by audiograms.

CONCLUSION

Pneumolabirynth is a rare postoperative complication of stapes surgery. It should be suspected in patients with vestibulocochlear symptoms that emerge both in a short time and many years after stapedoplasty. In case of suspected pneumolabirynth it is necessary to conduct an examination in order to confirm the presence and circulation of air in the affected structures of the inner ear. The temporal bone CT is the best imaging tool used to confirm the diagnosis. Revision tympanotomy is recommended to patients diagnosed with pneumolabirynth, sensorineural hearing loss and/or constant dizziness.

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CASE OF *STREPTOCOCCUS MASSILIENSIS* ISOLATION FROM BLOOD OF PATIENT WITH MENINGOENCEPHALITIS

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To date, *Streptococcus massiliensis*, the representative of human oral normobiota, was detected in the patients' blood only twice (which was confirmed by the reports published in 2004 and 2015). The patient with the demyelinating disease of the nervous system was routinely admitted to the neurological department for further examination and treatment. The diagnosis of meningoencephalitis was established based on the laboratory and instrumental tests. Meningoencephalitis was later complicated by bilateral multilobar pneumonia, systemic inflammatory response syndrome, and multisystem organ failure. After sharp deterioration of patient's health in the form of the decreased level of consciousness and brainstem symptoms, cerebrospinal fluid was tested by PCR for markers of viral and bacterial infections, and blood was cultured. Bacterial growth was detected in blood culture, and then *Streptococcus massiliensis*, susceptible to benzylpenicillin, vancomycin, levofloxacin, linezolid, sulfamethoxazole/trimethoprim, and erythromycin, was identified by time-of-flight mass spectrometry. Thus, it seems necessary to focus attention on this case of the *Str. massiliensis* isolation from blood due to the near total lack of data on the *Str. massiliensis* biological role and the more frequent isolation of bacteria of the genus *Streptococcus* from sterile human body fluids.

Keywords: meningoencephalitis, *Streptococcus massiliensis*, MALDI-TOF mass spectrometry

Author contribution: Lyang OV — literature review, manuscript writing; Pakhilova-Popova AV — bacteriological testing, manuscript writing; Kabaeva AR — writing clinical parts of the manuscript; Boyko OV — writing clinical parts of the manuscript; Shamalov NA — concept, manuscript editing.

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СЛУЧАЙ ВЫДЕЛЕНИЯ *STREPTOCOCCUS MASSILIENSIS* ИЗ КРОВИ У ПАЦИЕНТА С МЕНИНГОЭНЦЕФАЛИТОМ

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На сегодняшний день *Str. massiliensis*, представитель нормобиоты полости рта человека, был обнаружен в крови пациентов всего дважды (что подтверждают публикации 2004 и 2015 гг.). В неврологическое отделение для проведения дообследования и лечения в плановом порядке поступил пациент с демиелинизирующим заболеванием нервной системы. По результатам лабораторных и инструментальных исследований был выставлен диагноз «менингоэнцефалит», позднее осложненный двусторонней полисегментарной пневмонией, синдромом системной воспалительной реакции и полиорганной недостаточностью. После резкого ухудшения состояния пациента в виде угнетения сознания, нарастания стволовой симптоматики было проведено исследование ликвора на маркеры вирусных и бактериальных инфекций методом ПЦР и выполнен микробиологический посев крови. В крови обнаружен рост и затем методом времяпролетной масс-спектрометрии идентифицирован *Streptococcus massiliensis*, чувствительный к бензилпенициллину, ванкомицину, левофлоксацину, линезолиду, сульфаметоксазолу / триметоприму, эритромицину. Таким образом, ввиду практически полного отсутствия данных о биологической роли *Str. massiliensis*, а также в связи с повышением частоты выделения бактерий рода *Streprococcus* из стерильных жидкостей человека представляется необходимым заострить внимание на данном случае выделения *Str. massiliensis* из крови.

Ключевые слова: менингоэнцефалит, *Streptococcus massiliensis*, MALDI-TOF масс-спектрометрия

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Bacteria belonging to the genus *Streptococcus* are one of the most widespread groups of microorganisms that colonize various areas of the human body. Besides the well-known and well-studied species *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus mutans*, attention has been increasingly paid to other representatives of the genus in the last decade. The emergence of modern and high-tech methods for identification of microorganisms, such as matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry, had a critical impact. All these methods have greatly facilitated the task of identifying bacteria. However, further challenges, faced by both medical microbiologists and doctors of other specialties, have arisen when performing the microbiological study data interpretation and assessing the clinical significance of various bacterial pathogens. The clinical case reported is dedicated to one of such pathogens, *Streptococcus massiliensis*. Because of the small number of publications on the topic, attention should be paid to this pathogen, especially in case of isolation from the normally sterile sites.

Clinical case

Patient D., 41 years old, diagnosed with the demyelinating disease of the central nervous system, was routinely admitted to the Federal Center of Brain Research and Neurotechnologies

of FMBA on 8 July 2021 for further examination and treatment. It was known from the case history that he had an acute disorder, severe headache arose on 19 May 2021. Hyperthermia (up to 39 °C) developed within a week, however, the patient tested negative for COVID-19 (PCR), and his chest CT was normal. The patient started to notice the decline in short-term memory. He was admitted to hospital with a diagnosis of acute ischemic cerebrovascular accident on 8 June 2021 due to severe speech disorder. Ten days after the improvement the patient was discharged. However, weakness in his arms and legs increased, cerebral symptoms and disorientation developed and increased within two weeks of staying at home. After readmission the diagnosis of hemorrhagic necrotizing encephalitis was established based on the MRI scan, the patient was discharged again and subsequently routinely admitted to the neurological department of the Federal Center of Brain Research and Neurotechnologies of FMBA of Russia. Clarification of past medical history showed that the patient had no chronic infections, including those involving paranasal sinuses, oropharynx, and nasopharynx.

On admission, the patient's condition was assessed as of medium severity. The patient was in a state of clear consciousness, he had severe cognitive, speech, and bulbar impairment. The results of routine laboratory tests (complete blood count, urinalysis, biochemical profile, coagulation profile) showed no significant deviations. Assessment of cerebrospinal fluid revealed cytosis (up to 19 cells/μL), moderate increase in the levels of protein (up to 0.95 g/L) and glucose (up to 4.1 μmol/L). MRI scan was performed. MRI features were typical for encephalitis involving bilateral mediobasal temporal lobe with posthemorrhagic component and areas of postinflammatory encephalomalacia on the left. The diagnosis of meningoencephalitis was established.

Worsening of the condition occurred on 12 July 2021: meningeal signs and oculomotor dysfunction emerged. On the next day, 13 July 2021, the loss of consciousness to the level of sopor occurred, and brainstem symptoms increased. No bacterial growth was detected in the blood and cerebrospinal fluid samples collected on 13 July 2021 for bacteriological testing. The patient was transferred to the department of anesthesiology and intensive care, where the loss of consciousness to the level of coma 1 occurred, oxygen saturation decreased, and bradycardia developed two days later. The patient underwent intubation and was put on a ventilator. Laboratory tests revealed hyperglycemia (up to 9.8 μmol/L), uric acid levels up to 10.5 mmol/L, leukocytosis (up to 13.4 × 10^9/L with further increase up to 20.4 × 10^9/L), aPTT reduced to 19 s, D-dimer levels increased up to 2,925 ng/mL, and the C-reactive protein levels up to 56 mg/L. Regardless on the increasing levels of inflammatory markers, the patient's body temperature was within the range of 36.1–36.8 °C. PCR tests for herpes virus types 1, 2 and 6, *Mycobacterium tuberculosis*, Epstein–Barr virus, and cytomegalovirus, performed on 15 July 2021, were negative; no growth was also detected. Such complications of the underlying disease, as bilateral multilobar pneumonia (*Acinetobacter baumannii*, *Corynebacterium striatum*, *Klebsiella pneumoniae* were found in bronchoalveolar lavage fluid), systemic inflammatory response syndrome, and multisystem organ failure were revealed, complex antimicrobial therapy was prescribed.

Because of the patient's serious condition and persistent loss of consciousness, on 19 July 2021 blood and cerebrospinal fluid were sampled for culture for the second time in the BactAlert Aerobic and BactAlert Anaerobic bottles, 10 mL of blood and 2 mL of cerebrospinal fluid per bottle. The bottles were transferred to the bacteriology laboratory within an hour. The BactAlert 3D automated microbial detection system was used to incubate the bottles. Growth was observed in blood contained in the BactAlert Anaerobic bottle in 72 h.

Primary isolation from blood samples was performed on the ready-to-use solid culture media (Biomedia; Russia): Columbia agar with sheep blood, chocolate agar with growth factors, chromogenic agar medium for detection and enumeration of uropathogens, MacConkey agar, Sabouraud agar. Incubation was carried out under anaerobic conditions. Primary growth was observed after 48 h of incubation at a temperature of 37 °C on the Columbia blood agar and chocolate agar. Tiny gray matte colonies were found.

Microorganisms were identified with the Microflex MALDI-TOF mass spectrometer (Bruker; Germany) using the method of the direct application of pure culture onto the target [1]. *Streptococcus massiliensis* was identified by this method. *S. massiliensis* was isolated from biomaterial obtained from the patient admitted to the Federal Center of Brain Research and Neurotechnologies of FMBA of Russia for the first time in 2.5 years.

Clinical case discussion

Streptococci are a group of the spherical-shaped aerobic Gram-positive microorganisms that include numerous facultative anaerobes. Streptococci inhabit diverse environments in the human body not only as pathogens, but also as a part of normal microbiome. Based on the ability to induce hemolysis when grown on blood agar, streptococci are divided into alpha-hemolytic, beta-hemolytic and gamma-hemolytic. Alpha-hemolytic streptococci (generally referred to as the viridans streptococci) produce a greenish zone due to partial hemolysis [2]. The viridans streptococci constitute part of normal microflora of the human oral cavity, respiratory, reproductive and digestive tracts [3].

S. massiliensis, that is curerently considered to belong to the viridans group, was first isolated from blood of the patient admitted to the La Timone hospital (Marseille) with a gunshot wound to the head in June 2004. In 2006, the researchers, who had identified the new microorganism, published the paper reporting the details of the identification process [3]. The strain was named massiliensis in honor of Massilia (Ancient Greek and Roman name of Marseille, the city, where the strain was isolated). The colonies grown on blood agar were described as surface colonies, round-shaped, white to grayish, glossy, raised, with a diameter of 1–2 mm within 48 h of incubation at 37 °C in the CO_2-enriched atmosphere.

The second currently available scientific report that described *S. massiliensis* was issued in 2015. The group of researchers performed phylogenetic analysis of 17 *Streptococcus species*, including *S. massiliensis*, in order to define their habitats. It was shown that *S. massiliensis* found in human blood was a common inhabitant of human oral cavity [4].

S. massiliensis was assessed with the Vitek 2 Compact microbial detection system (bioMerieux SA; France) using the Vitek 2 AST-ST03 card in order to define antibiotic susceptibility. AST cards are used to determine minimum inhibitory concentration (MIC) of antibiotic by the turbidimetric technique, and the results are analyzed using the specialized AES software. Data interpretation was performed in accordance with the criteria of EUCAST-2021. Susceptibility to the following antibacterial drugs was revealed: benzylpenicillin, vancomycin, levofloxacin, linezolid, sulfamethoxazole/trimethoprim, and erythromycin. Ethiological role of this microorganism is

uncertain, and the risk of possible contamination is minimized by using the standard procedure, developed based on the Russian Practice Guidelines for the Preanalytical Phase of Microbiology Testing [5], when collecting venous blood for culture.

The control blood culture and cerebrospinal fluid culture tests, performed on 28 July 2021 and 2 August 2021, were negative. The patient in fair condition was transferred to the neurology department in 16 August 2021, then transferred to the department of medical rehabilitation, and discharged on 9 September 2021 due to significant improvement of neurological symptoms.

CONCLUSION

In recent years the reports of the *Streprococcus* species isolation from human blood and other sterile body fluids have become more frequent in domestic and foreign literature. This is probably due to the growing number of immunocompromised patients with primary and secondary immunodeficiencies. There are also cases of streptococci isolation from blood of patients with various neurological and cardiovascular disorders [6–8]. That is why the cases of isolating rare *Streprococcus* species require close attention of both medical microbiologists and the doctors of other specialties.

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THE COMPONENT OF PATHOGENESIS OF SUDDEN NOCTURNAL DEATH IN PATIENTS WITH HEART FAILURE

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Patients with chronic heart failure (CHF) constitute the bulk of the group at the highest risk of sudden death (SD). The majority of SDs occur at night. However, CHF grade and ejection fraction do not always determine the risk of SD in the outcome of the disease. The following view has been expressed based on the research on the topic and the described mechanisms underlying SD: impaired QT interval adaptation ("hyperadaptation": QT/RR slope > 0.24) to HR in patients with CHF who show maximum QT interval prolongation during the night, capable of triggering life-threatening ventricular tachyarrhythmias that trigger the mechanism of SD associated with CHF, can play some role. It is possible that identification of QT interval hyperadaptation in patients with CHF makes it possible to form the group at high risk of SD associated with HF and can become an additional indication for implantation of cardioverter-defibrillator.

Keywords: sudden death, heart failure, night, QT dynamics, Holter monitoring

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ЗВЕНО ПАТОГЕНЕЗА НОЧНОЙ ВНЕЗАПНОЙ СМЕРТИ ПРИ СЕРДЕЧНОЙ НЕДОСТАТОЧНОСТИ

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Основную часть группы с наибольшим риском внезапной смерти (ВС) составляют больные с хронической сердечной недостаточностью (ХСН). В большинстве случаев ВС происходит в ночное время. При этом степень ХСН и фракция выброса не всегда определяет риск именно ВС в исходе заболевания. Основываясь на анализе исследований по теме и описанных механизмов ВС, высказано мнение о возможной роли нарушенной адаптации («гиперадаптации»: QT/RR slope > 0,24) интервала QT к ЧСС у больных с ХСН, с максимальным удлинением интервала QT именно в ночное время, что может приводить к запуску жизнеугрожающих желудочковых тахиаритмий, запускающих механизм ВС при ХСН. Возможно, что выявление «гиперадаптации» QT у больных с ХСН может формировать группу повышенного риска по ВС при СН и быть дополнительным показанием в имплантации кардиовертера дефибриллятора.

Ключевые слова: внезапная смерть, сердечная недостаточность, ночь, QT-динамика, холтеровское мониторирование

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Sudden death (SD) is one of the main challenges for modern cardiology. People with chronic heart failure (CHF) associated with coronary artery disease (CAD) are at the highest risk of SD. Up to 20% of patients in this group die suddenly [1]. Analysis of the PRAISE (Prospective Randomized Amlodipine Survival Trial) study results has shown that patients with ischemic heart failure significantly more often (44%) died during sleep between 4 and 8 hr am compared to other four-hour blocks of the day [2].

QT interval prolongation is a well-known independent risk factor of SD. Studying the circadian variation in QT dispersion showed that patients with CHF had a more prolonged QT interval compared to both healthy people and patients with CAD and no CHF [3]. However, the study revealed no significant differences in the values of QT dispersion between nighttime and daytime. The method for assessment of the QT interval/heart rate (or QT interval/RR interval) relationship based on the Holter monitoring data known as QT dynamics has been relatively recently widely implemented in clinical practice [4]. When using the method, it is assumed that the main indicator of QT dynamics, the linear regression coefficient (slope) between QT and RR intervals, defines the degree of QT variability related to changes in heart rate (Fig. 1). In the adopted interpretation of QT dynamics, the steep and flat QT/RR slopes are distinguished with the low or high values of the linear regression slope [4].

The criteria for physiologically normal QT dynamics (QT/RR slope = 0.13–0.24) were previously identified, the approach to clinical interpretation of QT dynamics was proposed that defined the concepts of the QT interval "hyperadaptation" and "hypoadaptation" [5–7]. QT interval hyperadaptation is determined when QT/RR slope > 0.24 and is characterized by excessive QT prolongation associated with bradycardia and QT shortening associated with tachycardia. QT interval hypoadaptation (QT/RR slope < 0.13) is characterized by insufficient QT interval adjustment to heart rate with any heart rate changes. These criteria for QT dynamics interpretation were included in the National Russian Guidelines on Application of the Methods of Holter Monitoring in Clinical Practice [8]. This approach was pioneered in healthy neonates with age-related sinus tachycardia [5]. However, when assessing the proposed interpretation of QT dynamics, M. Malik, one of the world's leading experts on electrocardiology, noted its prospects: "Their concept or hypo- and hyperadaptation of QT interval to heart rate might be worthy of further studies in different populations…." [9]. We also found in promising to use this approach in assessing possible mechanisms underlying SD in patients with CHF. We did not assess groups of patients with CHF, although a few isolated cases of nocturnal SD in patients with CHF were available (Fig. 2 and 3). We have found no Russian reports of using the QT dynamics method in

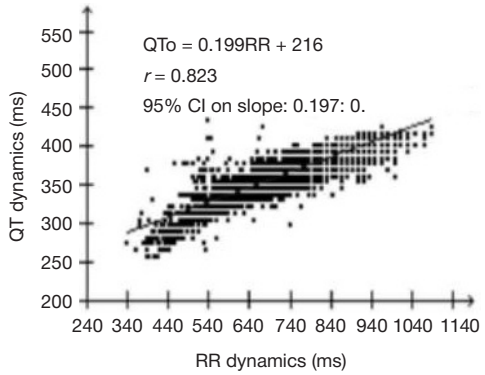

Fig. 1. Assessment of the 24-hr QT dynamics in healthy male aged 22 using the modern Holter monitoring system. QT/RR slope (QTo) = 0.199

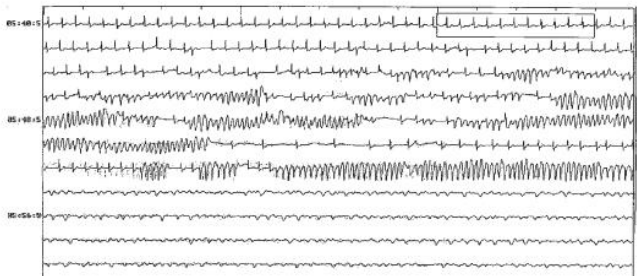

Fig. 2. Ventricular tachyarrhythmia that occurs at 05:44 am, ventricular fibrillation and sudden death that occur at 05:56 am in female patient aged 77 with ischemic cardiomyopathy and ejection fraction of 38%. No option for assessment of QT dynamics was installed in the system

patients with CHF, but, in our opinion, some global studies have provided interesting results. Thus, when predicting the risk of SD in patients with ischemic cardiomyopathy during the European Myocardial Infarct Amiodarone Trial (EMIAT), it was shown that patients with CHF, who died suddenly, had the significantly higher QT/RR slope values compared to those who survived (0.26 vs. 0.16, respectively) [10]. According to other researchers, QT/RR slope exceeding 0.28 was a strong independent predictor of SD in patients with CHF (relative risk 3.47; 95% confidence interval 1.43–8.40; $p = 0.006$) [11]. Both studies showed that QT hyperadaptation to heart rate with disproportionate QT prolongation associated with bradycardia during night-time was typical for patients with CHF who died suddenly.

The records of implantable cardioverter-defibrillators obtained from patients with CHF showed that the decrease in ejection fraction below 30% resulted in the higher incidence of ventricular tachycardia during the second half of the night compared to the first half [12]. This period of sleep is characterized by the daily minimum heart rate [13] and therefore by maximum QT interval prolongation in case of QT hyperadaptation. Higher abundance of REM sleep is typical for the second half of the night [13]. Ventricular extrasystoles are more often registered in patients with CHF during the REM sleep phase (163.0 vs. 118.4) [14], despite the fact that this phase accounts for only 20% of total sleep. It could be this combination of factors (prolonged QT interval and increased arrhythmogenic electrical instability of myocardium) that makes this period the most vulnerable to fatal arrhythmias in patients with CHF.

As explained in the opening lecture of the ISHNE Sudden Cardiac Death World Wide Internet Symposium, "… despite the fact that congenital long-QT syndrome is rather rare compared to other cardiovascular disorders, we could understand the whole problem of sudden cardiac death by studying the underlying mechanisms of arrhythmias". In support of this thesis, it can be noted that QT interval hyperadaptation is typical for patients with the molecular genetic type 3 long-QT syndrome (Fig. 4), who most often die during sleep [15]. This may indicate the common mechanisms underlying pathogenesis of SD in patients with the same main cause of the disease.

CONCLUSION

Based on the above, we have formulated the following hypothesis:

– QT interval hyperadaptation in patients with CHF associated with disproportionate prolongation of the QT interval and higher incidence of ventricular tachyarrhythmias in the night-time makes this group more susceptible to life-threatening nocturnal arrhythmias and SD.

– Identification of QT interval hyperadaptation in patients with CHF makes it possible to form the group at high risk of SD associated with HF and become an additional indication for implantation of cardioverter-defibrillator.

Confirmation or refutation of this hypothesis requires targeted research, which obviously pose no practical differences, since there is sufficient number of Holter recordings obtained from patients with CHF in numerous studies.

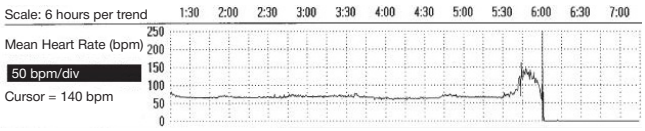

Fig. 3. Nocturnal heart rate trend with sudden death occurring at 05:56 am (see Fig. 1) during the period of the increased heart rate variability that corresponds to REM sleep phase according to conventional somnography [8, 13] in the 77-year-old female patient with ischemic cardiomyopathy

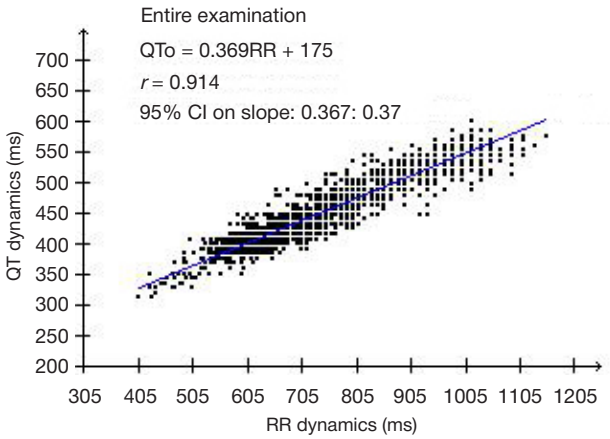

Fig. 4. QT hyperadaptation in male patient with type 3 long QT syndrome. QT/RR slope (QTo) = 0.369 (normal range: 0.13–0.24)

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