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PREVENTIVE MEDICINE IS A CORNERSTONE OF HEALTH PROMOTION

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Preventive healthcare encompasses a broad range of medical and social interventions aimed at protecting and promoting public health, averting diseases or reducing the risk of their development. Preventive measures seek to create conducive working conditions and good resting environment, promote physical exercise, healthy nutrition, personal hygiene and sanitation, and eventually improve the well-being of the population. This article reviews a number of research works into the impact of various factors on the health of adults and children. Based on their findings, we propose measures for reducing the effects of harmful factors and incorporating positive factors in our daily life. The model of preventive healthcare adopted in Russia is aimed at preventing adult and child morbidity, increasing life expectancy, promoting positive attitude to health, and creating safe environment.

Keywords: prevention, health factors, adults, children, healthy lifestyle

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ПРОФИЛАКТИЧЕСКАЯ МЕДИЦИНА – ОСНОВА СОХРАНЕНИЯ ЗДОРОВЬЯ НАСЕЛЕНИЯ

Н. В. Полунина, Ю. П. Пивоваров, О. Ю. Милушкина [✉]

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Профилактическая медицина — это комплекс медико-социальных мероприятий, направленных на охрану и укрепление здоровья населения, предупреждение возникновения заболеваний, устранение факторов риска их возникновения. Она включает как медицинские мероприятия, так и создание благоприятных условий труда и быта, отдыха и физического воспитания, организацию здорового и полноценного питания различных групп, оздоровление окружающей среды, повышение благосостояния и улучшение условий жизни населения. В статье представлен обзор научных исследований по оценке воздействия на здоровье населения, в том числе детского, различных факторов внешней среды, предложены конкретные мероприятия по профилактике, опирающиеся на эти исследования, и меры, которые разрабатываются по нивелированию негативных для здоровья факторов и активному внедрению в повседневную жизнь позитивных факторов. Профилактическая модель современного здравоохранения России направлена на предупреждение развития заболеваний детского и взрослого населения, увеличение продолжительности жизни граждан, формирование установок к здоровому образу жизни и создание безопасных условий окружающей среды.

Ключевые слова: профилактика, формирующие здоровье факторы, взрослое население, детское население, здоровый образ жизни

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Importance of joint socio-economic and clinical measures in preventive healthcare

One of the central challenges that our country has to address is protection and promotion of public health [1, 2]. In Russia, there has always been a focus on finding effective solutions to the problem of public health improvement. Among the principles underpinning the public healthcare system in Russia are prevention and social work aimed at maintaining the health of its young and old citizens [3].

Social and preventive healthcare encompasses a broad range of social, economic and clinical interventions aimed at protecting and maintaining public health. Prevention goes beyond clinical interventions, vaccination, good hygiene routine, adoption of sanitary acts, etc. It has many different aspects that should be promoted by the state, changing the mindset of the public in favor of healthy lifestyle.

Strategies for prevention should include social, economic, clinical, and organizational interventions. Prevention targets all aspects of social life, including working and living conditions, leisure, physical education, nutrition, environmental factors, and eventually the well-being of the population, contributing to the harmonious development of physical and mental capacities of an individual. Prevention transcends the boundaries of medicine and healthcare systems and becomes a social strategy of the state.

Among measures for diseases prevention are psychological, biological, social and economic interventions that reduce the risk of giving in to unhealthy choices, protect against occupational hazards and improve living conditions.

Classification of measures for disease prevention into socio-economic, psycho-social, socio-hygienic and medical is quite arbitrary. Much more important is the result of their application: good public health statistics that can be achieved by joining the

efforts of the state and society and implementing all aspects of the preventive healthcare strategy.

The history of preventive medicine

The concept of preventive healthcare emerged when society started to realize the significance of averting diseases and their complications. As knowledge was gradually accumulated, people established requirements for the quality of potable water, food, indoor air in residential and commercial buildings, artificial and natural lighting, elaborated radiation safety guidelines, standards of personal hygiene, recommendations for sleep-wake routine, controlled cold exposure, nutrition, physical exercise, etc. [4, 5]. Research has demonstrated a mutual impact between the environment and humans, as well as the role of social factors in maintaining public health and preventing diseases, which helped to identify the main areas of focus for preventive healthcare practiced in communities [6–8].

In March 1919 preventive healthcare defined as “a broad scope of recreational and sanitary measures aimed at preventing the risk of diseases” was declared a central challenge of Russian healthcare system. It still remains one of its major areas of focus. According to official documents, this strategy implemented as a complex of socio-economic and medical interventions is aimed at preventing and eradicating diseases, including infections, and increasing life expectancy [1].

Considering the deteriorating ecology, negative demographic trends and high incidence of non-infectious diseases among different subpopulations, development of measures for protecting and promoting public health remains a top-priority task.

In this light, prevention becomes a fundamental principle underlying the social policy of the state underpinning strategies for protecting and promoting public health.

Prevention (or prophylaxis, from Greek *prophylaktikos*) is a range of activities exercised by state agencies and healthcare providers aimed at identifying causes of diseases or injuries and neutralizing or eliminating their negative impact on humans. Prevention can be classified into personal and public; primary, which fights the actual cause of a disease, and secondary, which promotes factors reducing the risk of complications of an inflicted injury or disease.

The role of hygiene and social factors in public health

Preventive interventions can be implemented only after the role of different factors in promoting public health has been established. Factors that negatively affect public health should be neutralized and positive factors should be incorporated into everyday life. To this end, studies of social hygiene are conducted both abroad and here, in Russia, at the Faculty of Hygiene, Public Health and Healthcare Economics of Pirogov Russian National Research Medical University, and their findings are successfully implemented. Research into public health and the factors that affect it should be continuous and account for the constantly changing environment.

Based on the published literature, factors affecting public health can be distributed into four groups:

- 1) socio-economic situation and lifestyle;
- 2) environment;
- 3) biological factors;
- 4) activity of healthcare providers.

Lifestyle

Lifestyle has the most serious impact on human health, its contribution being as high as 50% in comparison with other

factors. The contribution of environmental pollution is 20–25%; biological factors, 15–20%, and 10–15% are the result of activities exerted by healthcare agencies and providers. These figures are consistent with the data reported by other authors [9].

The factors mentioned above can have both negative and positive impact on health increasing or reducing the risk of developing a disease.

Protection and promotion of health are important not only for individuals with compromised health [10, 11], but for the healthy ones as well [12–17] because projects seeking to improve the health of different subpopulations contribute to economic growth, ensure social stability and safety.

There are over 40 definitions of “lifestyle”, including those describing healthy and unhealthy behaviors. Most often, lifestyle is a cumulative concept that encompasses hygiene-related, social, economic, clinical and psychological aspects. There is another brief but very accurate definition of lifestyle as a public phenomenon closely related to the mode of production characterizing “a definite form of activity of ... individuals, a definite form of expressing their life, a definite mode of life on their part” [18].

In other words, lifestyle is a historically shaped activity exerted by an individual, a group of people, or a population in general in material and immaterial spheres of life. Lifestyle is studied by psychologists, psychiatrists, philosophers, legislators, sociologists, and doctors who look at human motivation and behavior from different perspectives noticing its direct impact on health.

Research studies yielded sufficient data to gauge the impact of social factors on the life of individuals and groups of people. A correlation has been established between social hygiene and human health. It was noticed that in the identical social settings the health of individuals and the activity they exert can vary, so the studies that followed focused on different aspects of lifestyle and their role in public health. Two types of activities (occupational and leisure) have been identified that can be further classified into professional, social, political, cognitive, cultural, educational, communal, and others. There are a lot of specific criteria that help to differentiate these activity types from one another, but at the same time the latter are closely related and complement each other, allowing us to perform a comprehensive analysis of lifestyle and identify its impact on the health of a studied cohort.

Work

Work is an essential part of our lives which affects both our lifestyle and health. Among the most important work-related factors that affect human health are the length of service, an atmosphere at workplace, and occupational hazards [12, 19–21].

There is a reliable direct correlation between the length of service and an employee's health ($r = + 0.639$; $m = \pm 0.08$); the length of service also correlates with the age of an employee ($r = + 0.714$; $m = \pm 0.09$) and an atmosphere at workplace ($r = + 0.513$; $m = \pm 0.07$). In older employees, the presence of chronic conditions correlates with their age ($r = + 0.924$; $m = \pm 0.03$; $p < 0.01$) and an atmosphere at workplace ($r = + 0.708$; $m = \pm 0.04$; $p < 0.01$).

Because an employee's health deteriorates with age and depends on the length of service, factors affecting occupational health should be thoroughly studied. The employees who work in a positive, friendly atmosphere are not susceptible to the negative impact of service length [17, 22, 23].

Exposure to occupational hazards also affect employees' health. On average, 25–35% of women and 57–63% of men

work in hazardous industries and are exposed to dangerous chemicals, extreme temperatures or radiation, have to spend a lot of time in constrained postures, etc. Long-term exposure to poor working conditions and occupational hazards is particularly dangerous for human health.

Intensive daily computer work lasting for over 6 hours can compromise an employee's health, too [22, 24]. Today, computers, mobile phones and other gadgets are ubiquitous, while their harmful effect on the organism is yet to be understood. Exposure to a combination of several occupational risk factors aggravates the situation. Of 100 respondents, 37 to 42 report exposure to 2 to 3 hazards in their workplace; men are exposed to a combination of occupational hazards more often than women.

Another serious problem faced by the society is gadget addiction. On a typical school day, schoolchildren spend 7 hours using electronic gadgets; male university students, 8.5 hours a day; and female university students, 10 hours a day. The time children and teenagers spend using their gadgets taking no breaks is 1.5 to 2 hours. Gadgets are often used outside the classroom, especially in public transport, school halls, recreational facilities, and other popular spots where wi-fi is available. As a result, both schoolchildren and university students complain of headache, eye strain, and blurred vision, i.e. asthenopia. University students tend to complain more of pain in the eyes and blurred vision ($p < 0.05$). Ocular fatigue depends on the frequency of computer (Pearson's contingency coefficient is 0.7; $p < 0.001$), laptop (Pearson's contingency coefficient is 0.7; $p < 0.001$) or tablet use (Pearson's contingency coefficient is 0.6; $p < 0.001$) [25].

Leisure

Time spent off work is an important part of our lives. It is time for family reunions, housework, rest, and other leisure activities.

The analysis of housing conditions, including the type of a flat or apartment, the number of people who live in it, its internal layout, density of occupation, availability of store rooms, public amenities and overall comfort shows that satisfaction with living conditions is an important component of a person's mental and emotional state. Among the respondents living in objectively poor conditions 21.3% are quite satisfied with their housing, while among those living in objectively good conditions 27.1% feel dissatisfied [13, 26–31]. The degree of satisfaction is the major factor that affects health since it is a component of a person's mental and emotional state ($r = + 0.416$; $m = \pm 0.06$; $p < 0,01$).

Studies demonstrate that only 18–23% of the respondents are satisfied with their income, whereas 26–32% are not happy with it. On the whole, 52–56% of working age respondents confess they live paycheck-to-paycheck. However, 70–74% of the surveyed families have a car, 68–72% have a country house, and 38–42% spend their vacations outside the area of residence. This means that more than half of the working-age population can be described as middle-income.

The mental and emotional state has a serious impact on our lifestyle and health and is a product of relationships within a family, family conflicts, traditions and rituals. It shows to what extent a person is satisfied with an atmosphere in their family. Financial status and living conditions also contribute to our emotional state. The majority of the surveyed families describe their mental and emotional state as good (28–32%) and satisfactory (49–53%). Families whose members complain of emotional distress report frequent arguments caused by poor living conditions, low income, disagreement on how to

raise children or grandchildren, alcohol abuse, and bad habits. Among younger respondents aged 30 to 40 years, 21–23% admit having problems they find hard to solve; however, only 18–19% of older respondents aged 40–50 years and 16–17% of people aged 50 to 60 years believe their problems are really difficult. There is a direct correlation between a person's health and his/her mental and emotional state ($r = + 0.328$; $m = \pm 0.05$; $p < 0.01$).

Regression analysis has revealed associations between medical, biological and social factors and the health of children and teenagers. The following standardized regression coefficients were computed: use of medications during pregnancy (0.36), number of births a woman had (–0.27), family composition (0.27), paternal education (0.25), conflicts in the family (0.24) [32].

Because the mental and emotional state has a profound impact on health, studies have been conducted to identify and analyze the factors that produce a positive effect. The results show that the majority of respondents list a stable job (80–84%), stable income (78–80%), good living conditions (70–74%), a good atmosphere in a family (64–68%), lack of conflicts in a family (52–56%), and satisfaction with leisure time activities (44–48%) among the factors they find uplifting. There are significant differences between men and women in terms of ranking the importance of the mentioned factors. Men think that a stable job and good relationships in a family are more important, while women rank stable income and good living conditions higher. Older respondents value good health more, as well as good relationship in a family, and believe the absence of conflicts is important. In general, the respondents give 2–3 reasons that promote positive atmosphere in a family.

Health awareness

The willingness of a person to use medical services and follow medical advice has a direct impact on health. Therefore, prevention should focus on the promotion of healthy habits and stimulation of positive attitude towards medicine in general. Taking care of your health implies regular medical checkups, adherence to medical regimens and recommendations, getting rid of bad habits, improving health literacy, etc. Health awareness is shaped by an attitude to nutrition, sports and physical exercise, alcohol, smoking, keeping fit, consultations with a doctor when feeling ill, undergoing regular medical checkups, hygiene, daily routine, and other things [31, 33–37].

These aspects of health awareness depend on a person's cultural background, education, outlook on life, living conditions, well-being, the state of public healthcare in a given country, etc. Among the factors that directly affect our health the leading role is played by health literacy, which is about being interested in obtaining information about health, risk factors that affect it, and disease prevention strategies.

Health literacy can be improved by reading specialized literature, watching or listening to TV or radio programs about health, risk factors or prevention strategies, or attending lectures delivered by healthcare professionals [20, 27, 28, 38–40].

Studies show that only 27–38 of 100 people are willing to seek such information. Among patients with chronic conditions this number is higher (65–70 individuals out of 100). Increased interest in medical knowledge is usually the result of a person's health status. Patients with chronic conditions read medical literature twice more often in comparison with individuals who do not have a chronic disease.

Of every 100 respondents who like to educate themselves about health-related issues, 68.1 people read popular science

literature, 9.4 people read specialized medical literature, 41.6 watch TV programs about health, 15.2 people listen to similar programs on the radio, 11.2 people ask their doctors, and 1.6 people attend specialized lectures. These data indicate the need for educational projects stimulating interest in popular science literature and TV or radio programs aimed at maintaining public health.

Health literacy largely determines a person's attitude to medical services, including the necessity to undergo regular medical checkups, willingness to follow medical advice, etc. Research demonstrates that the primary reason why patients present to a doctor is the symptoms of a disease; however, only 24–32% of patients seek medical advice on the first day the disease starts to manifest itself. Every second responder goes to a doctor when they need a sick leave or other medical certificates. Importantly, 24–30% of the working-age population undergo health checkups on their own initiative, but only 10–12% think it necessary to ask their doctor about disease prevention strategies. It has been established that only 64–68% of patients with chronic conditions visit their doctors regularly.

It is well known that a good night's sleep restores energy to the body and brain. However, research shows that only 10–18% of adults are not sleep-deprived. A lot of respondents (34–42%) report sleeping less than 6 hours a night. Another important characteristic of sleep is its quality. It has been established that 42–58% of the population are satisfied with their sleep quality and duration; there are 1.4–1.8 times more women who are satisfied with their sleep than men. Women also tend to take sleeping pills 2.0–2.5 times more often than men.

Lack of sleep in teenagers is becoming a serious concern. Most of them go to bed at 22:00–23:00 (45.9%), some go at 23:00–24:00 (35.6%) and at 00:00–01:00 (11.5%). The majority of university students go to sleep at 23:00–24:00 (60.7%), some go at 00:00–01:00 (34.1%). This means that nighttime sleep is reduced to 431.0 ± 70.1 min in schoolchildren (7 h on average) and 402.7 ± 89.5 min (6.5 h on average) in university students [41, 42]

A lifestyle a person leads, including diet and physical exercise, affects his/her weight. Both weight excess and deficiency can trigger diseases. About half of the respondents (42–48) watch their weight; women do it more 1.5–2.0 times more often than men. Patients suffering from chronic conditions start to control their weight if their health deteriorates (20–32%). Healthy individuals are more driven by the desire to have a slender figure (22–32%), which is more typical for younger people than for their older counterparts. Only 25–37% of overweight individuals try different methods of weight control, which indicates a generally low level of health literacy in the population. The survey shows that there are 1.5 more individuals ($p < 0.05$) watching their weight among those who follow medical advice.

Measures for weight control typically include new dietary regimens and physical activity. A study shows that 52–58% of adult population have bad eating habits, of them 34–38% eat irregular meals, 26–34% eat twice a day and 23–38% consume too much bread, salt, smoked foods, sweets, etc. The comparative analysis demonstrates that there are 1.9–2.4 times more individuals following an unhealthy diet among patients with chronic conditions ($p < 0.05$) than among healthy respondents.

Lack of movement not only leads to overweight, but also causes pelvic congestion, which in turn affects reproductive health and health in general ($r = -0.418$; $m = \pm 0.0022$;

$p < 0.0001$). Studies have revealed that the majority of the population (74–82%) live a sedentary lifestyle; of them there are 1.5–2.0 times more women than men ($p < 0.05$).

Smoking is another aspect of lifestyle that seriously affect human health. Smokers constitute 28 to 43% of the population. More than half of them (54–62%) have been smoking for 5–10 years and smoke up to 20 cigarettes a day on average. Passive smokers make up 12 to 37% of the population. On the whole, the majority of the population (both active and passive smokers) can be described as a group at risk of diseases caused by smoking. This is also true for the younger generation. Surveys show that among 15-year old teenagers 12.7% of girls and 15.1% of boys smoke regularly; 54.6% of girls and 48.1% of boys have already had their first cigarette. Boys typically start smoking at 14, girls at 13; the earliest age disclosed in the survey was 6 and 8 years for boys and girls, respectively. Alcohol is consumed once a month or less often by 32.0% teenage boys and 29.1% girls; 64.1% of boys and 58.1% of girls do not drink alcoholic beverages. The lifestyle of boys and girls does not differ significantly, and both sexes are susceptible to bad habits and unhealthy behavioral patterns. There are significant correlations between a handgrip and smoking ($r = -0.74$; $p < 0.05$), the age when the first cigarette was tried ($r = -0.33$; $p < 0.05$), time spent in front of the computer ($r = -0.39$; $p < 0.05$), and engagement in sports ($r = 0.34$; $p < 0.05$) [43].

The analysis of research works carried out at the Department of Public Health and Healthcare of Pirogov Russian National Research Medical University provided us with valuable data that was subsequently used to comprehensively assess the health awareness of the population. Based on the scored points, the respondents were distributed into 3 groups. The majority of the working-age participants (53–57%) constituted the group with average scores; one in every five or four participants (22–26%) represented the group of increased health risk; one in every five respondents (18–22%) had good scores for at least 70% of the studied parameters.

Considering the importance of health awareness, prevention strategies should pay more attention to encouraging the population to seek medical information related to their health. A lot depends on the willingness of patients to maintain good health and on their convictions. Therefore, projects of medical rehabilitation should include measures that can directly affect personal lifestyle and patients should be encouraged to develop a positive attitude to medical care.

Personal commitment to a healthy lifestyle, the active contribution of the state to promoting health among its citizens, and adequate resource provision are equally important [33]. Improving health literacy is a cornerstone in stimulating patients to change their lifestyle. It will help to eradicate bad habits and turn the patient to healthy practices. Instilling willingness to lead a healthy lifestyle should become the underlying principle of disease prevention, health protection and promotion [44–46].

The works we have analyzed reveal a significant increase in health awareness and literacy in the Russian population ($r = -0.523$; $m = \pm 0.0024$; $p < 0.0001$).

CONCLUSION

Promoting hygiene and sanitation is an essential component of preventive healthcare projects that should be implemented by healthcare professionals. It includes bedside conversations

with inpatients or conversations during home visits, lectures on health-related issues, TV or radio programs, advertisement, and educational films. Our healthcare system has sufficient resources to address the problems of disease prevention and health protection [47–50].

Prevention is a secret to a long and healthy life. It aims at averting diseases at early, middle and old ages, increasing life expectancy, promoting positive attitude to healthy lifestyles, and creating safe environments for studying, working, and leisure activities.

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DORSOPATHIES: ROUTINE CHECKUPS AS A PROCEDURE NECESSARY FOR EARLY DIAGNOSTICS, RISK FACTORS AND COMORBIDITIES IDENTIFICATION

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Early detection of dorsopathies is an urgent task for primary care physicians, since such conditions can combine with other chronic noncommunicable diseases (NCD) and adversely affect the course all comorbidities, consequently disimproving the quality of life of patients and increasing the frequency of their requests for medical assistance. This study aimed to determine the value of routine checkups in the context of detection of dorsopathies, NCD, and identification of risk factors (RF). We have retrospectively analyzed the patient records database of a rural outpatient clinic in the Tver region (years 2015 to 2017). The prevalence of dorsopathies and NCD RF were the subjects investigated. Fisher's exact test and Spearman's rank correlation coefficient (SRCC) were applied for the purposes of statistical processing of the results. We discovered that dorsopathy most often was a comorbidity to arterial hypertension and gastrointestinal tract diseases; it was strongly related to the NCD (SRCC = 0.506), age (SRCC = 0.383), slightly less so — to hypodynamia (SRCC = 0.146), type of the patient's occupation (intellectual or physical labor) (SRCC = 0.07). Routine checkups improve the rate of detection of dorsopathy: the more patients undergo such examinations, the more cases of dorsopathy are diagnosed. Thus, it is necessary to increase the number of working people attending the checkups in order to detect dorsopathies early and prevent them effectively.

Keywords: preventive medical examination, dorsopathy, risk factors, comorbidity, village resident.

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ДОРСОПАТИИ: АКТУАЛЬНОСТЬ ПРОФИЛАКТИЧЕСКИХ ОСМОТРОВ ДЛЯ РАННЕЙ ДИАГНОСТИКИ, ВЫЯВЛЕНИЯ ФАКТОРОВ РИСКА И КОМОРБИДНЫХ ЗАБОЛЕВАНИЙ

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Лечебно-профилактические мероприятия по ранней диагностике дорсопатий являются актуальной задачей врачей первичного звена здравоохранения. Это обусловлено тем, что сочетание дорсопатий с другими хроническими неинфекционными заболеваниями (ХНИЗ) формирует коморбидность, которая неблагоприятно влияет на их течение и исходы, увеличивает обращаемость населения за медицинской помощью, ухудшает качество жизни пациентов. Целью работы было определить значение профилактических осмотров в диагностике дорсопатий, сопутствующих ХНИЗ, и выявлении факторов риска (ФР). Проведен ретроспективный анализ базы данных амбулаторных карт пациентов сельской амбулатории Тверской области за 2015–2017 гг. Исследовали распространенность дорсопатий и ФР ХНИЗ. При статистической обработке применяли точный критерий Фишера и коэффициент ранговой корреляции Спирмена (КРКС). Было показано, что дорсопатия чаще всего сочеталась с артериальной гипертензией и заболеваниями желудочно-кишечного тракта, имеет сильную корреляционную связь с сопутствующими ХНИЗ (КРКС = 0,506); возрастом (КРКС = 0,383); более слабую с гиподинамией (КРКС = 0,146) и слабую с видом деятельности пациента (интеллектуальным или физическим трудом) (КРКС = 0,07). Профилактические осмотры улучшают диагностику дорсопатии — число выявляемых случаев заболевания растет с увеличением количества обследуемых в рамках профилактических осмотров пациентов. Таким образом, необходимо шире проводить профилактические осмотры для работающего населения с целью раннего выявления дорсопатии и эффективной ее профилактики.

Ключевые слова: профилактические осмотры, дорсопатия, факторы риска, коморбидность, жители села

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World Health Organization (WHO) announced prevention and treatment of noncommunicable chronic diseases (NCD) a priority project of the second decade of the 21st century, one aimed at improving the quality of life of the world's population [1]. There are two directions to the project: 1) healthy lifestyle education and promotion and 2) early detection of NCDs, learning the relevant risk factors (RF) and their timely correction. It is the healthcare systems of the countries that ensure progress in the second direction, with primary care physicians — district doctors, general practitioners (family doctors) — playing a special role: 30–40% of their working time should be dedicated to activities aimed at prevention [2].

Diseases of the musculoskeletal system and connective tissue (DMSCCT) are common; there are over 150 different conditions and syndromes belonging to the group, most of which usually cause pain and musculoskeletal system dysfunction. In the developed countries, the average age of people living in densely populated areas increases, which makes DMSCCTs a frequently registered trigger of disability. In 2000, WHO launched The Bone and Joint Decade 2000–2010, the goal of which was to increase the awareness of the existing problem throughout the world. Some of the initiatives undertaken within the decade implied studying medical, social and economic load born by the society in connection with the musculoskeletal system disorders, comprehensive understanding of RF affecting development and progression of musculoskeletal pathologies, as well as comorbidities [3]. According to the data published in 2016, every third European citizen complaining of pain in muscles, bones and joints, has limited ability to move the limbs, with the share of people affected ranging from 18% in Ireland to 46% in Croatia [4]. Lower back pain is the most common complaint (11% of all patients). People 55 years of age and older suffer pain twice as often. Women have the condition more often than men: 37% and 27%, respectively [4].

Demographic changes (population ageing) and lifestyle-related RFs of NCDs allow forecasting the further spread of the pathologies belonging to this group. DMSCCT are often accompanied by the cardiovascular diseases. Such comorbidities are always a complicated case, which is one of the reasons of the increased mortality rate among patients suffering them [5, 6].

This study aimed to determine the value of routine checkups in the context of early detection of dorsopathies, NCDs and their risk factors (RF).

METHODS

The subject of this research were reports made at Mokshino rural outpatient clinic and covering two districts (general practitioners) of Konakovo district of the Tver region. Namely, we studied the main indicators for 2011–2016: annual reports (forms 30, 12), "Outpatient medical history records" (UF #025/u); "Routine checkup control records" (UF #030/u);

"Additional checkup registration records (working citizen)" (UF #131/u-DD-10) and other record forms related to preventive and additional medical examinations of working population. We have retrospectively analyzed the 2015–2017 patient records database. 1203 records containing a dorsopathy diagnosis were selected for the purposes of analysis of age-related specifics of comorbidities' development. Inclusion criteria: men and women aged 18 years and older, diagnosed with dorsopathy, signed the informed consent.

Exclusion criteria: age below 18, no dorsopathy in the anamnesis. Time of first complaints, dorsopathy diagnosis date, presence of RF and comorbidities peculiar to these patients were analyzed.

The study protocol #10-16 of 11/09/2016 was approved by the local ethics committee of Sechenov University. Dividing the patients into groups and categories, we used the classification developed by WHO: young (18–44 years), middle-aged (44–60 years), senior (60–75 years), old-aged (75–90 years), long-livers (90+ years).

Diagnostic criteria recommended by Appendix #2 to the Order #1006n of 03.12.2012 issued by the Ministry of Health of Russia were referred to when determining the RF and pathological conditions and diseases.

Statistical processing

Planning the study, we determined the size of the sample to be taken from the Mokshino outpatient clinic's database with the help of the Sample Size modules of COMPARE 2 3.85 and DESCRIBE 3.18 programs of WinPEPI® 11.65 software package (author and copyright holder — J. H. Abramson). Fisher's exact test was applied in the context of statistical processing of the results. Investigating the relations between dorsopathy, RF, other NCDs, we used SRCC. The statistical significance threshold was set at 5%.

RESULTS

The number of patients diagnosed with dorsopathies grows proportionally to the number of those undergoing routine checkups, even with the number of visits to the outpatient clinic and the number of patients registered at the clinic decreasing (by 2.6% (159 people) and 1.6% (35 people), respectively) (see Table, Fig. 1 and 2).

Figure 3 shows the presence of NCDs RFs in lives of 1203 rural residents (540 men and 663 women). The most common RFs for men were smoking (73%) and hypercholesterolemia (17.3%). The predominant RF for women was excess body weight (39.8%).

Arterial hypertension (AH) was the most widely-spread condition: 516 patients (42.9% of all examined, 42,892.76 cases per 100,000 people) suffered from it, including 207 men (38.3% of all men examined) and 309 women (46.6% of all women examined). The gender differences are statistically significant

Table. Routine checkups and detected cases of dorsopathy in numerical terms, adults, Mokshino outpatient clinic, 2011–2016

Indicators	Reporting period, year					
	2011	2012	2013	2014	2015	2016
Number of people registered	2237	2216	2217	2206	2217	2202
Number of visits to the clinic	6122	6640	5587	5963	6784	5963
Number of routine checkups	1033	1128	1033	1493	1776	1493
Diagnosed dorsopathies (# of people)	257	253	281	299	327	347
Number of dorsopathy patients in follow-up care at the end of the reporting year	257	263	280	299	324	345

($p = 0.004$). From the point of view of prevalence, dorsopathy was next to arterial hypertension: it was detected in 347 patients (28.8% of all examined), which translates into 28,844.56 cases per 100,000 people; they were 147 men (27.2% of all men examined) and 200 women (30.2% of all women examined). Here, gender differences were not statistically significant ($p = 0.004$). Gastrointestinal tract diseases (except for gastric ulcer and duodenal ulcer) followed dorsopathies: such were detected in 141 patients (11.7% of all examined, 11,720.69 cases per 100,000 people), including 44 men (8.1% of all men examined) and 97 women (14.6% of all women examined). The gender differences are statistically significant ($p = 0.004$; Fig. 4). In 2016, dorsopathy was diagnosed in 82 young people (18–44 years old), which is 23.9% of all dorsopathy patients; in the middle-aged group (44–60 years), there were 127 cases (36.6%), and in the senior (60–75 years) and further groups dorsopathy diagnosis was announced to 137 (39.5%) persons.

In different years from 2011 to 2016, 99.1 to 100% of dorsopathy patients were observed by the Mokshino outpatient clinic's general practitioner (see Table).

In 31.7% of patients, dorsopathy was the only disease; in 28.5%, it combined with one other disorder, in 40% dorsopathy had two comorbidities (percentages of the total number of dorsopathy patients).

Two most frequent comorbidities were arterial hypertension (191 cases, 55.0% of all dorsopathy patients) and gastrointestinal tract diseases except for gastric ulcer and duodenal ulcer (67 cases, 19.3% of all dorsopathy patients). Less common diseases diagnosed together with dorsopathy were coronary heart disease (33 cases, 9.5% of all dorsopathy

patients), obesity (27 cases, 7.8% of all dorsopathy patients), diabetes mellitus (20 cases, 5.8% of all dorsopathy patients), gastric ulcer and duodenal ulcer (17 cases, 4.9% of all dorsopathy patients), chronic diseases of the respiratory system (15 cases, 4.3% of all dorsopathy patients). Women suffer the aforementioned comorbidities (except for the coronary heart disease) more often than men. Significantly more women had diabetes mellitus and obesity as comorbidities of dorsopathy than men: 8.0% against 2.7% with diabetes, 12.5% against 1.4% with obesity, respectively. Coronary heart disease was detected in patients with dorsopathy in equal proportions gender-wise (9.5%).

SRCC values for dorsopathy and NCDs' RF peculiar to the population group of rural residents studied are shown on Fig. 2 (2016). NCDs and age (SRCC values of 0.506 and 0.383, respectively) correlate with dorsopathy more often than any other factor. Hypodynamia's correlation is weaker (SRCC = 0.146), that of the type of the patient's occupation (intellectual or physical labor) — even less so (SRCC = 0.07). As for the inverse correlation ties, the strongest was with simple absence of any comorbidity (SRCC = -0.406), a weaker one — with alcohol abuse (SRCC = -0.006). In the studied sample of dorsopathy patients (347 people), gender differences were not statistically significant ($p = 0.004$).

DISCUSSION

In 2016, DMSCTs (as described by ICD 10: M00-M99, dorsopathy included) incidence in Russia was 2,953.2 cases per 100,000 people nationwide and 9,402.5 cases per 100,000

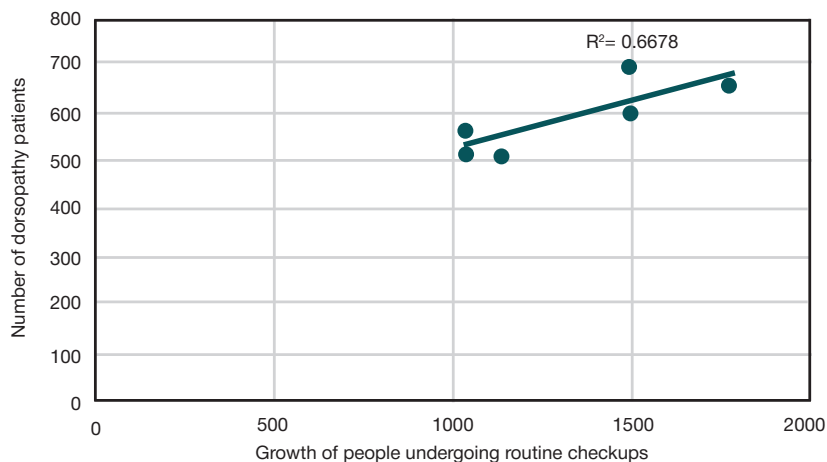


Fig. 1. Growth of the number of dorsopathy patients concurrent with the growth of people undergoing routine checkups, 2011–2016

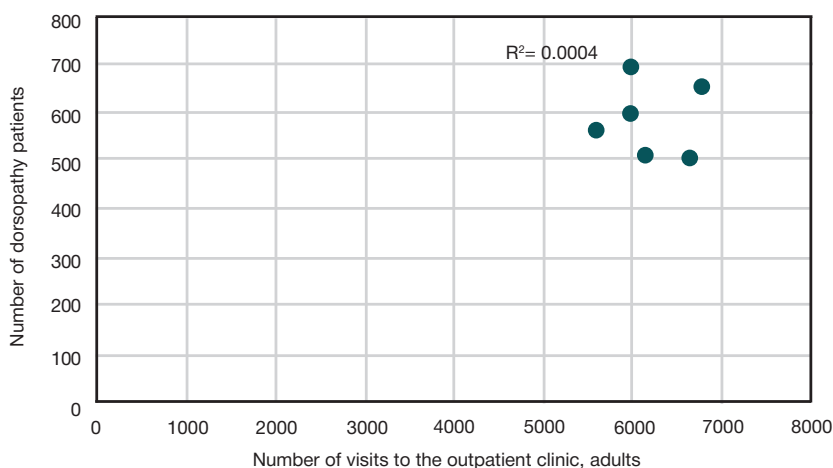


Fig. 2. Dot diagram reflecting distribution of the number of dorsopathy patients and number of visits to the Mokshino outpatient clinic (adults, 2011–2016)

rural residents. In the Central Federal District, the figure was 8,707.4 cases per 100,000 rural residents. In the Tver region in 2016 — 11,179.5 cases per 100,000 rural residents [7]. Thus, dorsopathy incidence as registered by the Mokshino outpatient clinic in 2016, which is 28,844.56 cases per 100,000 people, is 3.1 times higher than the statistically average incidence of DMSCTs detected in residents of the rural areas of Russia; as for the Central Federal District's rural population, the Mokshino figure is 3.3 times greater. The number of rural residents beyond employable age has grown by 2.5% in the period between 2004 and 2013 in Russia, and continues to grow [8]. Tver region is one of the national leaders by the number of such residents living there (in %); in the Central Federal District, it ranks 2nd (as of January 1, 2016) with 32.4% people living outside of cities being beyond working age [9]. Population ageing can be the explanation of high incidence of dorsopathy in the studied population group: out data indicates that age and dorsopathy have the strongest intercorrelation (SRCC = 0.383; Fig. 4).

Dorsopathy incidence as registered by the Mokshino outpatient clinic in 2016, which is 28,844.56 cases per 100,000 people, is 2.6 times higher than Tver region average as reported by Rosstat (DMSCTs, rural population). [7]. Such figures call for further research of the disease and identification of the causes of its spread in this population.

At the international level, there is no single generally accepted dorsopathy definition and assessment routine. Most

often, it is looked for in the context of examinations following complaints of chronic pain in the musculoskeletal system (pain persistent for at least three months of the past 12 months). The largest replicated cross-sectional studies with identical questions aimed at researching the prevalence of chronic pain in musculoskeletal system are the studies conducted in 1995–1997 ($n = 92,936$) and 2006–2008 ($n = 94,194$) in Norway, which enlisted adults aged 20 and over; these studies revealed that through the 11 years, the incidence of such diseases has grown significantly, with senior women (50 y.o. and older), smoking and overweight people facing a particular risk. Disorders of musculoskeletal system were spreading especially quickly among people 20–29 y.o. during those 11 years. The difference in incidence of such diseases as it grew through time was statistically significant for both genders; behind this growth was hypodynamia associated with extensive use of computers and mobile phones [10]. The data from this study coincide with the results we obtained from the Mokshino rural outpatient clinic in 2016.

In the Republic of Belarus, the prevalence of dorsopathy was $8,008.5 \pm 4,804.6$ cases per 100,000 people (depending on the region) in 2001 [11]. Even with the maximum registered in Belarus, which is 12,813.1 cases per 100,000 people, is 1.7 times smaller than the incidence according to the Mokshino rural outpatient clinic records of 2011, which is 21,366.34 cases per 100,000 people.

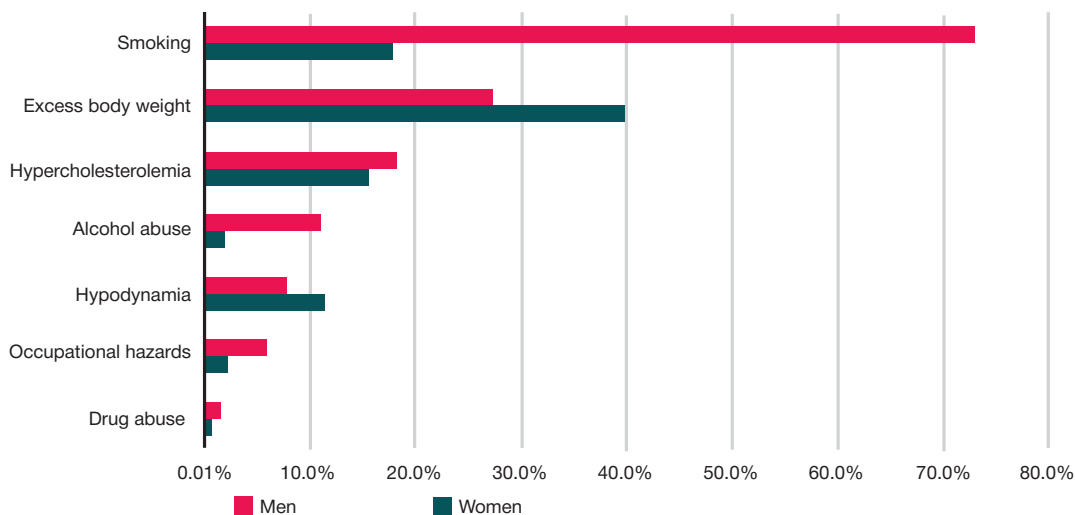


Fig. 3. Risk factors peculiar to 1203 rural residents, broken down gender-wise. 100% equals 540 men 663 women: HD — hypodynamia, HCL — hypercholesterolemia, AA — alcohol abuse, EBW — excess body weight

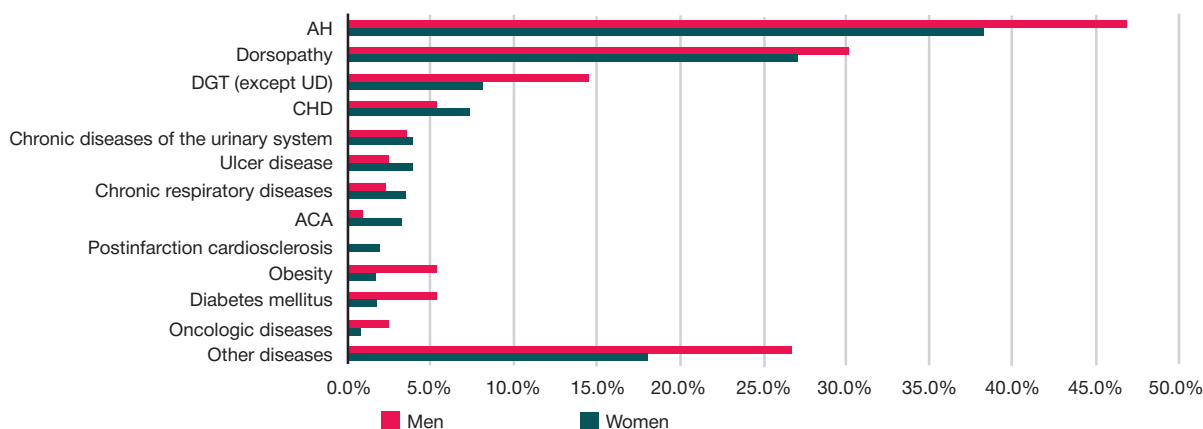


Fig. 4. Noncommunicable chronic diseases diagnosed in 1203 rural residents. 100% equals 540 men 663 women: AH — arterial hypertension, DGT (except UD) — diseases of the gastrointestinal tract, CHD — coronary heart disease, ACA — acute cerebrovascular accident, PCS — postinfarction cardiosclerosis, DM — diabetes mellitus, CDUS — chronic diseases of the urinary system, CRD — chronic respiratory diseases, UD — ulcer disease

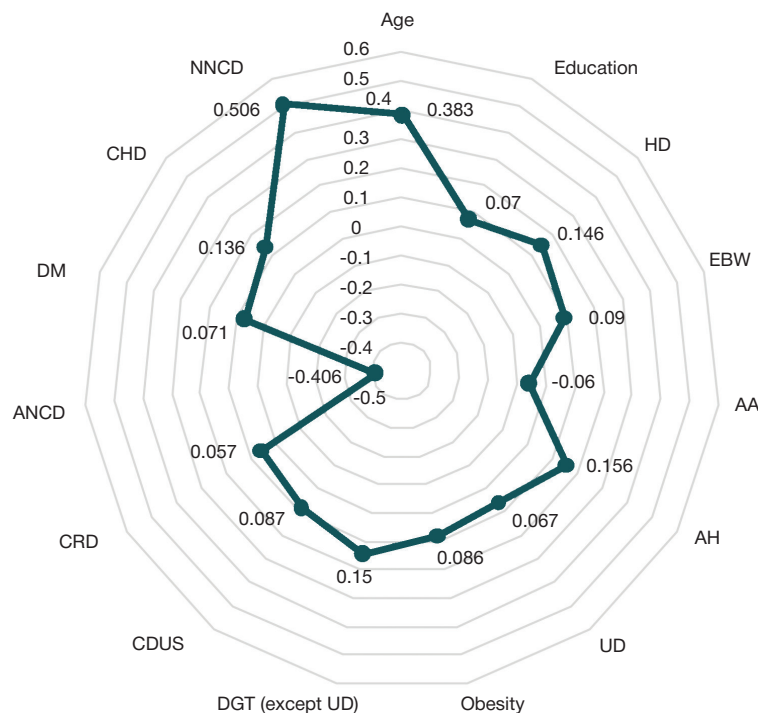


Fig. 5. SRCC values for dorsopathy and other factors peculiar to the population group of rural residents studied: AH — arterial hypertension, HD — hypodynamia, AA — alcohol abuse, DGT (except UD) — diseases of the gastrointestinal tract, CHD — coronary heart disease, EBW — excess body weight, NNCD — numerous noncommunicable chronic diseases, ANCD — absence of noncommunicable chronic diseases, DM — diabetes mellitus, CDUS — chronic diseases of the urinary system, CRD — chronic respiratory diseases, UD — ulcer disease

An interesting study was conducted by American researchers: they aimed to determine the trabecular bone structure tensor [12]. The study proves there is an association between DMSCs and hypodynamia; in the population group we studied, the SRCC between the two was 0.146. Citing the Wolff's law, the researchers have experimentally proven the need for moderate loads on musculoskeletal system as a prerequisite for correct development of the bone structure.

A recommended component of the secondary routines to prevent dorsopathies are special "schools" for people suffering back pain [13]. There, the patients should learn of the importance of moderate locomotor activity, minimization of NCDs RF in their lives [14, 15], treatment of comorbidities and physiotherapy. Such schools are open in many countries, e.g. in Australia [16].

CONCLUSIONS

Dorsopathy is the 2nd most common NCD found in the rural areas of the Tver region; the disease does not differentiate between types of occupations (heavy physical work and intellectual labor). The study shows that the number of

patients diagnosed with dorsopathies grows proportionally to the number of those undergoing routine checkups, even with the number of visits to the outpatient clinic decreasing; thus, routine checkups is an effective tool for primary and secondary prevention of dorsopathy.

Dorsopathy is strongly correlated to comorbidities (NCDs), age; less so — to hypodynamia and even less so — to the type of the patient's occupation. Significantly more women had diabetes mellitus and obesity as comorbidities of dorsopathy than men. Coronary heart disease was detected in patients with dorsopathy in equal proportions gender-wise.

General practitioners treating dorsopathy patients in a rural outpatient clinic are recommended to set up schools for patients suffering back pain, where they should learn of the importance of moderate locomotor activity, minimization of NCDs RF in their lives, treatment of comorbidities and physiotherapy. Curricula given to general practitioners should include specifics of observation and treatment of senior patients living in rural areas, as well as the effect sedentary lifestyle resulting from computerization of society has on the development of dorsopathy.

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EVALUATION OF EFFECTIVENESS OF MEDICAL AND ORGANIZATIONAL CARIES PREVENTION MEASURES DESIGNED FOR WORKING POPULATION

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Today, a dentist can make use of any of the multitude of methods to forecast treatment effectiveness, restoration lifetime, new caries lesions development risks. However, at the national level we see no studies dedicated to evaluation of clinical effectiveness of the majority of those methods. Having analyzed the available literature, we selected CAMBRA caries development risk assessment method and made it a goal of this study to evaluate its effectiveness while also seeking to develop the most effective treatment plan. We examined 126 young adults without concomitant somatic pathologies and divided them into treatment and control groups; treatment group was then further subdivided into 3 groups depending on the risk of caries development. All patients had their teeth and tissues of the oral cavity treated following the developed and the classic plans, accordingly. After 12 months, follow-up examinations of the treatment group revealed new caries lesions in 34 (43.5%) patients, while 40 (51.3%) were diagnosed with periodontal diseases. In the control group, we found new cavities on the teeth of 25 (52.1%) patients and periodontal pathologies in 28 (58.3%) study participants. This research shows that CAMBRA and the developed treatment plan can decrease the rate of caries development in young adults.

Keywords: caries risk assessment, CRA, CAMBRA, treatment prognosis, OHI-S, CPE, CPITN, filling material

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ОЦЕНКА ЭФФЕКТИВНОСТИ МЕДИКО-ОРГАНИЗАЦИОННЫХ МЕРОПРИЯТИЙ ПО ПРОФИЛАКТИКЕ КАРИОЗНЫХ ПОРАЖЕНИЙ У ЛИЦ ТРУДОСПОСОБНОГО ВОЗРАСТА

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В настоящее время в стоматологии существует множество методов, позволяющих спрогнозировать эффективность лечения, срок службы реставрации и риск появления нового кариозного поражения зубов, но в отечественной литературе нет результатов оценки клинической эффективности большинства методик. На основании анализа литературных данных была выбрана методология CAMBRA для определения риска развития кариеса. Целью исследования было определить эффективность данной методики, а также составить наиболее эффективный план лечения. Были обследованы 126 пациентов молодого возраста без сопутствующей соматической патологии, их разделили на основную и контрольную группы, основную группу разделили на 3 подгруппы, в зависимости от риска развития кариеса. Всем пациентам провели санацию полости рта по разработанной и классической методике соответственно. Через 12 месяцев на повторном осмотре в основной группе у 34 (43,5%) пациентов появился новый кариозный процесс. У 40 (51,3%) человек были выявлены заболевания пародонта. В контрольной группе у 25 (52,1%) пациентов появились кариозные полости, 28 (58,3%) имели патологию тканей пародонта. Исследование показывает, что CAMBRA и разработанная методика позволяют снизить прирост кариеса у пациентов молодого возраста.

Ключевые слова: риск развития кариеса, CRA, CAMBRA, прогноз лечения, планирование лечения, ИГР-У, КПУ, CPITN, пломбировочный материал

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The CFE ("caries-filling-extraction") index peculiar to the residents of Russia aged 35–44 years is 14.4, which is a high rate of incidence of caries. Moreover, despite the ongoing prevention programs, it tends to grow [1]. At the initial oral examination of patients with multiple caries lesions, CFE does not describe the real situation: patients that score the same values can have completely different clinical pictures. It cannot be reliably argued that after treatment the patient will not have recurrent or secondary caries [2].

According to some authors, an objective assessment of the oral cavity's condition requires determining the CFE index, oral hygiene index, severity of dental caries, analyzing the orthopantomogram (OPG), thorough instrument-aided examination of proximal surfaces for hidden cavities, as well assessment of the patient's periodontal status [3, 4].

Today, there is a multitude of methods to forecast the risk of the new caries development: software solutions that calculate the risk of caries development using the patient data

(Cariogram, Risk Profile); statistical methods (caries intensity index, CII); methods implying assessment of the saliva's physicochemical properties, i.e. its viscosity, pH, secretion rate (Salivary multi test meter) and buffer capacity (Saliva check-buffer); tests to approximate the number of bacteria that cause caries, e.g. Streptococcus mutans (Saliva check-mutans, Clinpro cario L-Pop (3M); Dentocult SM) or Lactobacilli (Dentocult LB); tests to determine the dental plaque's pH, maturity and volume (Plaque indicator kit) [5, 6]; a questionnaire (caries risk assessment, CRA) [7].

In addition to the questionnaire, some dentists use Clinpro cario L-Pop (3M), a rapid test deriving the number of cariogenic bacteria from the amount of sucrose processed by them, and Saliva check-buffer and Saliva check-mutans, diagnostic test kits (incl. special test strips and reagents) telling the physicochemical properties of saliva within 15–20 minutes. Plaque indicator kit occupies a place of its own: this is a set of 5 minute tests that enable learning the pH of dental plaque and its maturity, thus allowing the dentist to show the patient the "problematic areas" and assess the level of oral hygiene [5].

Some of these tests (CRA, CariScreen, OPG, clinical examination) are part of the CAMBRA (Caries Management By Risk Assessment) method developed by the California Dental Association in 2011. The method is widely used in Western countries (USA, UK, France, the Netherlands), but it is not popular in Russia [8–10].

This study aimed to compare the available caries lesions and caries risk assessment methods, determine which of them is the most effective, and develop the rational treatment plan that factors in the specific levels of risk.

METHODS

The study involved 126 young adults. The inclusion criteria were: 1) men and women 18–44 years old; 2) presence of carious cavities; 3) no registered concomitant pathologies. The exclusion criteria were: 1) ages other than 18 to 44; 2) no carious cavities; 3) presence of concomitant somatic pathologies. The design of this research was approved by the ethical committee of N. I. Pirogov Russian National Research Medical University (Minutes #15 of 2016.11.10). All participants filled the CRA questionnaire and signed the informed voluntary consent form; they were divided into two groups: treatment ($n = 78$) and control ($n = 48$). Each patient had his/her OHI-S, CII indices determined, medical history collected, oral cavity and OPG examined, treatment plan compiled. At the treatment stage, all patients were trained proper oral hygiene routines, had their teeth cleaned by a professional dental hygienist and caries lesions (incl. complications thereof) treated. The follow-up examinations were conducted 6 and 12 months after treatment.

To process and analyze the data obtained, we used Microsoft Excel 2010, vertically compared relative values by independent sets and observation series, applied ANOVA to quantity.

For the purposes of this study, we chose the CAMBRA method and adjusted it to our clinical conditions (see below). This method determines the risk of caries development (primary

and secondary) through clinical instrument-aided examination of the oral cavity, CariScreen dental plaque analysis, OPG examination, patient interview (to fill the medical history) and CRA questionnaire that the patient fills before the first visit to the dentist.

The CRA questionnaire included questions about diet, individual oral hygiene, somatic pathologies (cardiovascular diseases, endocrine system disorders, general metabolic and calcium and phosphorus metabolism disorders), bad habits, previous visits to the dentist, as well as the consent form confirming the patient's willingness to participate in the study. This questionnaire shows patients the plethora of different factors that affect the health of their oral cavity and should encourage them to proceed with the treatment.

Instead of the CariScreen system, which is not popular in our country, we used the OHI-S index by J. C. Green, J. R. Vermillion as modified by P. A. Leus. The index allows learning the amount of plaque and scale accumulated in the patient's teeth. The OHI-S procedure implies examining buccal surfaces of teeth 16, 11, 26, 31 and lingual surfaces of teeth 36 and 46. Dye (erythrosine, fuchsin) applied to those surfaces improves visualization of the plaque. Both soft and hard scale deposits are taken into account: the former on the tooth's surface, the latter supra- and subgingival. The index value is the the sum of codes describing each tooth divided by the number of teeth examined.

CFI index is the sum total of the number of carious teeth, number of filled teeth and number of extracted teeth. A value greater than 10 signals of intensive development of caries.

To determine if the patients had their periodontal tissues affected by any pathology, we calculated CPITN, Community Periodontal Index of Treatment Needs. The process implies using a special CPITN clinical periodontal probe to examine gingival sulcus, gum tissues for bleeding, as well as measuring sub- and supragingival plaque deposits and gingival pockets, if any. The treatment plan depends on the value of this index.

Apart from CPITN, the state of periodontal tissues can be determined with the help of OPG, which also helps discover hidden carious lesions. Examining the OPG taken with a system like Vatech Pax-i3D (Samsung; Korea) or Planmeca ProMax 3D (Planmeca; Finland), a dentist can find manifestations of periodontal diseases and caries lesions on proximal surfaces of the teeth, which are hard to diagnose in the context of the regular examination routine.

The survey and the methods described above allowed dividing the patients into groups by the caries risk factor, from very low to extremely high.

To determine the severity of caries, we used the Nikiforuk classification as modified by A.I. Nikolaev and L.M. Tsepov; this classification puts patients into three groups [2]:

- initial caries; clinical manifestations: signs of decay on fissures and proximal surfaces of molars (CFE index value below 8);
- moderate caries; clinical manifestations: lesions on fissures and proximal surfaces of molars and second premolars, single lesions on proximal surfaces of anterior teeth (CFE index value 9 to 12);
- severe caries; clinical manifestations: lesions on fissures

Table 1. Treatment group patients divided into subgroups by risk factor and caries severity

Risk group	Risk factor	Risk acc. to CRA	OHI-S value	Periodontal pathology	Caries severity
Subgroup I (low risk)		Low to moderate	0–1.2	No	Initial
Subgroup II (moderate risk)		Moderate to high	1.3–3.0	Gingivitis	Moderate
Subgroup III (high risk)		High to very high	Over 3.1	Periodontitis	Severe

and proximal surfaces of molars and premolars, lesions on proximal surfaces of anterior teeth, cervical caries (CFE index value 9 to 12).

CPITN helped assess the state of the patient's periodontal tissues.

Thus, applying the CAMBRA method and factoring in the examination data, we divided treatment group participants into 3 subgroups depending on the risk factor and caries severity (Table 1).

Different risk groups had different treatment plans. Patients in subgroup I had only initial caries lesions and exhibited a high OHI-S value; after professional teeth cleaning (soft plaque removal with air abrasion, hard plaque removal with a US scaler, teeth polishing), caries lesions in their teeth were removed mechanically, then prepared for adhesive restoration and restored with a nanohybrid light-cured composite resin following the ethanol protocol (prepared cavity treated with 2% chlorhexidine ethanol solution) [11], no liner.

OHI-S values peculiar to subgroup II patients were satisfactory; caries lesions discovered on their teeth were moderate. On the CPITN scale they scored 1 to 2 points, therefore, in addition to professional teeth cleaning, these patients were taught rational oral care routines, and a week

later they had the oral hygiene status checked again and brushed teeth under supervision. Caries lesions found in these patients were removed mechanically, teeth prepared for adhesive restoration and restored with a nanohybrid light-cured composite resin following the ethanol protocol; for the liner, we chose a dual cure glass ionomer cement (GIC), thus applying the "closed sandwich" technique. GIC releases fluoride ions, which makes liners made of this material bacteriostatic, i.e. capable of preventing secondary caries [2, 12].

Oral hygiene status of subgroup III patients was unsatisfactory, most of them had over 6 caries lesions on their teeth. The CPITN value was above 2, so we used Vector system to treat periodontal pockets in 4 patients with mild periodontitis. Vector (Durr dental; Германия) system was designed to enable administration of drugs in periodontal pockets, perform scaling and root planing. Other patients had their teeth cleaned by a professional dental hygienist. Carious lesions found on the proximal surfaces were removed mechanically, then the teeth were prepared for adhesive restoration, cavities filled with a nanohybrid composite resin over a GIC liner ("open sandwich" technique) [2, 13]. For decay found on occlusal surfaces we used a tri-cure GIC. Glass ionomer cements possess the "battery" power: they adsorb fluoride ions from special

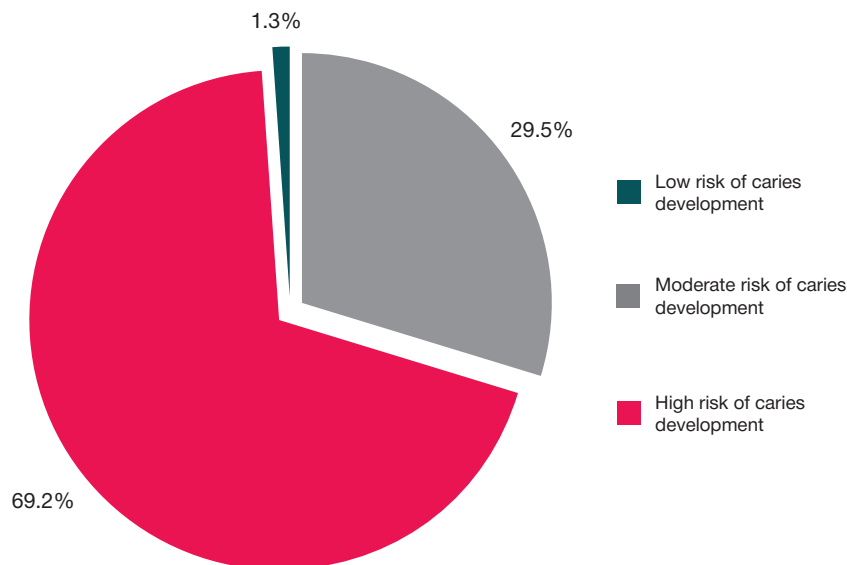


Fig. 1. Distribution of patients into risk groups

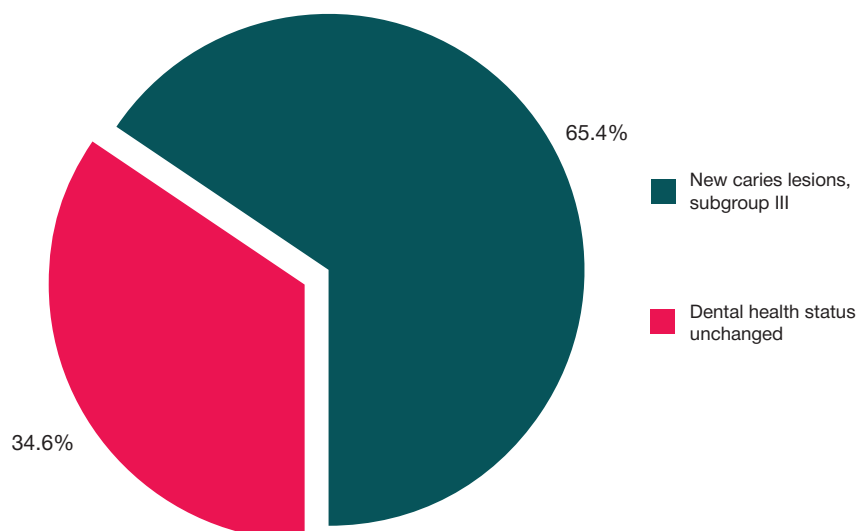


Fig. 2. Dental health status change, 6 months follow-up, treatment group

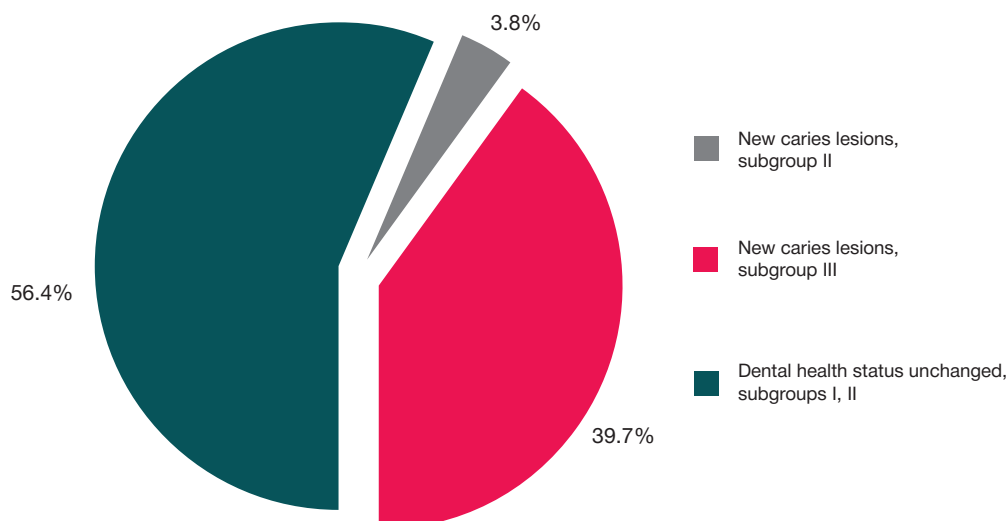


Fig. 3. Dental health status change, 12 months follow-up, treatment group

toothpastes and then release them when the pH level in the oral cavity goes down [14].

Control group patients underwent professional teeth cleaning, periodontal treatment, had their caries lesions removed and affected teeth prepared for adhesive restoration, then restored with a nanohybrid light-cured composite resin over a liner (standard technique, as decided upon by the attending dentist).

At the follow-up visits, 6 and 12 months after the initial treatment, new caries lesions in all participants (both control and treatment groups) were treated following the same routines as were applied during the first stage of the study.

RESULTS

Initial examination revealed that in the treatment group:

- 23 patients ran a moderate risk of new caries development (subgroup II);
- 54 patients ran a high risk of new caries development (subgroup III);

According to the CPITN:

- 77 patients had pathological changes in periodontal tissues (gingivitis in 93.5% of patients, mild periodontitis in 3.9%);
- 1 patient had healthy periodontal tissues (Fig. 1).

Initial examination of the 48 patients of the control group revealed that:

- their mean OHI-S value was 2.3 (satisfactory oral hygiene level);
- mean CFE index was 9.7 (moderate caries development intensity);
- 43 of them had pathological changes in periodontal tissues (gingivitis in 88.4% of patients, mild periodontitis in 11.6%);

At the 6 months follow-up examinations (Fig. 2), we discovered new caries lesions on intact or treated teeth of 27 (34.6%) patients, learned that 36 (46.1%) patients did not follow the oral care recommendations and registered no visible change in dental health status of 51 (65.4%) patient. Only the patients of subgroup III (high risk) exhibited new caries lesions. We registered no inflammatory periodontal diseases in 42 (53.8%) patients from subgroups I and II who observed the rational oral hygiene recommendations. Chronic catarrhal

gingivitis and mild periodontitis were diagnosed in 46.1% of the patients.

At the follow-up examination, 20 (41.7%) control group patients had new carious lesions and 27 (56.3%) — periodontal tissue pathologies.

After 12 months (Fig. 3), despite all measures taken, 31 (39.7%) patient from subgroup III and 3 (3.8%) patients from subgroup II had the new caries lesions developing. Only 44 (56.4%) participants had no visible changes to their dental health status; these patients belonged to all caries risk subgroups. In 38 (48.7%) patients from subgroups I and II that followed the rational oral hygiene recommendations we found no inflammatory periodontal diseases. Forty (51.3%) patients were diagnosed with chronic catarrhal gingivitis and mild periodontitis.

In the control group 25 (52.1%) patients had new caries lesions and 28 (58.3%) — periodontal tissue pathologies.

CONCLUSIONS

We discovered that 6 month after the first examination and treatment, 27 (34.6%) of subgroups II and III patients had new caries lesions; 12 months after, the figure increased to 34 (43.5%). In the control group, the new lesions were found in 20 (41.7%) patients after 6 months and in 25 (52.1%) patients after 12 months. The results allow a conclusion that the CAMBRA method combined with calculating the probability of caries development allows predicting remote treatment results, be it planned or performed.

Follow-up periods differ for different patients and depend on the specifics of the groups the patients belong to. Subgroup I patients can have the follow-up examination 12 months after treatment, while those belonging to subgroup II should visit the dentist 6 months earlier. Subgroup III patients should come to the dentist's office more often: higher caries risk requires dental health examinations every 3 months.

The choice of filling and restoration techniques also depends on the risk group the patient belongs to. Classic technique is good for those running low caries development risk, while moderate risk group would benefit from adding GIC as a liner, and patients whose caries risk level is high should have their teeth filled and restored following the "open sandwich" technique or with application of GIC.

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HYGIENIC ASSESSMENT OF HANDICAPPED ADOLESCENTS VOCATIONAL TRAINING CONDITIONS: PROBLEMS AND OPTIMIZATION OPPORTUNITIES

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In a number of occupations, there is a shortage of labor force that can be filled with economically active part of the population, including people with disabilities (handicapped, HC). Unfortunately, observance of hygienic requirements in the context of HP adolescents vocational training has not been studied sufficiently: most scholars have researched only the conditions of teaching healthy adolescents. This study aimed to examine the HC adolescents vocational training conditions and develop measures to improve their working conditions with pathologies factored in. We examined adaptation potential, anxiety level, mental efficiency, sick rate and personal well-being assessment in HC adolescents ($n = 120$) aged 16–18, not impaired intellectually, studying sewing equipment operation, shoe repairs in the boarding school. In the context of the study, we applied Smirnov hygiene criteria, Giessen Symptom Questionnaire, Spielberger's Test Anxiety Questionnaire, Sivkov scale, correction tables, Baevsky adaptation index. For the purposes of statistical analysis, we used parametric (Student t -test) and non-parametric (Mann–Whitney) criteria. The Spearman's correlation coefficient helped determine interrelationship of the studied parameters. We identified the following key adverse hygienic factors that have a significant impact on the students' health: intense character of labor performed, insufficient artificial lighting, noise level above the norm, high content of organic solvents and dust in the workshop's air. The timetable of the boarding school was also found to be inefficient. The results of this study allowed developing and introducing a software program to automatically compile the school's timetable with the aim to improve psychosomatic health of the students and halve the number of health-related complaints.

Keywords: handicapped students, vocational training, training and production environment factors, adaptation, classes timetable

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ГИГИЕНИЧЕСКАЯ ОЦЕНКА УСЛОВИЙ ПРОФЕССИОНАЛЬНОГО ОБУЧЕНИЯ ПОДРОСТКОВ С ОГРАНИЧЕННЫМИ ВОЗМОЖНОСТЯМИ: ПРОБЛЕМЫ И ПУТИ ОПТИМИЗАЦИИ

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Для восполнения недостатка трудовых кадров по ряду профессий представляется возможным использование экономически активной части населения страны, в том числе лиц с ограниченными возможностями (ОВ). К сожалению, соблюдение гигиенических требований в ходе профессионального обучения подростков с ОВ недостаточно изучено, поскольку в основном имеются данные об исследованиях условий подготовки только здоровых подростков. Целью работы были изучение условий профессионального обучения подростков с ОВ, разработка мероприятий по улучшению условий их труда с учетом имеющейся патологии. Подростки с ОВ (120 человек) в возрасте 16–18 лет с сохраненным интеллектом, обучающиеся в интернате по специальностям оператор швейного оборудования, обувщик по ремонту обуви, обследованы по показателям адаптационного потенциала, тревожности, умственной работоспособности, а также заболеваемости и субъективной оценке самочувствия. Использовали гигиенические критерии Смирнова, анкетирование с помощью Гиссенского опросника, тест Спилбергера, шкалу Сивкова, корректурные таблицы, методику Баевского. Для статистического анализа применены параметрический (t -критерий Стьюдента) и непараметрический (Манна–Уитни) критерии. Связь изучаемых параметров определена по коэффициенту корреляции Спирмена. Выявлены ведущие неблагоприятные гигиенические факторы, оказывающие значимое влияние на организм учащихся: тяжесть и напряженность трудового процесса, недостаточный уровень искусственной освещенности, превышение уровня шума, высокое содержание органических растворителей и уровня пыли в воздухе рабочей зоны. Определено нерационально составленное расписание учебных занятий в интернате. Результаты исследования позволили разработать и внедрить компьютерную программу автоматизированного составления расписания, позволяющую улучшить психосоматическое состояние здоровья и вдвое снизить количество жалоб на самочувствие.

Ключевые слова: учащиеся с ограниченными возможностями, профессиональное обучение, факторы учебно-производственной среды, адаптация, расписание занятий

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Preserving and strengthening health of adolescents is a topical problem nowadays and the most important task before the state [1, 2]. Overall, the state of health of children and adolescents worsens, which translates into the growing number of acknowledged disabilities that put health-related restrictions on the access to vocational education [3–6]. The law “On Social Protection of Disabled Persons in the Russian Federation”, adopted in 1995, guarantees social protection for people with disabilities, as well as unrestricted access to education. However, execution of HC adolescents vocational training is significantly limited. Moreover, training and production environment factors influence health of students learning various trades [7–10]. At the earliest stages, the influence manifests as disorders of adaptation mechanisms accompanied by morphofunctional disorders; these are the first signals of changes in health of students associated with vocational training [11, 12]. To make the training more effective, it is necessary to know how persons with disabilities adapt to the conditions this training occurs in. Thus, studying the HC adolescents' ability to adapt to learning trades in secondary vocational schools, the specifics considered, is a task of practical significance.

This study aimed to examine the conditions HC adolescents receive vocational education in and develop measures aiding their adaptation to training and production environment factors.

METHODS

The study was conducted at the Saratov Vocational Boarding School for Handicapped and Disabled Persons (State Autonomous Vocational Education Institution) in 2016–2017; it was approved by the Ethics Committee of V. I. Razumovsky State Medical University, city of Saratov (Protocol #3 of 07.11.2017). As prescribed by the Federal Law #323-FZ of 21.11.2011 “On the Principles of Protection of Health of Citizens in the Russian Federation” (revision of 03.07.2016), all participants gave the informed voluntary consent to the research activities; under Article 9 of the Federal Law #152-FZ of 27.07.2006 “On Personal Data”, they agreed to have their personal data processed.

We studied the training and production environment factors that affect adaptation of students. The study group included 120 HC adolescents aged 16 to 18; 58% of them had I–III class disabilities. Inclusion criteria: learning “sewing equipment operator”, “shoe repairman” trades in the boarding school; lack mental impairments. Exclusion criteria: severe mental disorders; low intelligence; proneness to conflict.

The training and production processes organization was studied through examining the chronometry (time and sequence of specific operations registered by seconds). Hygienic criteria suggested by N. K. Smirnov [13] enabled evaluation of the rationality of theoretical and practical classes; daily study load calculations helped estimate the soundness of weekly and daily load distribution pattern [14]. Form 086/u (approved by the Ministry of Health of the Russian Federation on 08.12.2014) provided the objective data describing state of the students' health. Analysis of the personal rehabilitation program forms (approved by Order #379n of 04.08.2008 issued by the Ministry of Health and Social Development of Russia) allowed studying the reasons and structures of the students' disabilities. We referred to classes and nosological entities described in ICD-10 when examining levels and structures of the diseases.

Giessen Symptom Questionnaire allowed the students to express their own view of the state of their health [15].

State-Trait Anxiety Inventory (Spielberger test) [16] enabled expression of personal and situational anxiety by the adolescents.

Baevsky adaptation index (as modified by A. P. Berseneva in 1995) [17] allowed calculating adaptation indices in the context of the personal adaptation capabilities assessment.

Correction tables by V. Ya. Anfimov (as modified by S. M. Grombakh) [18] were used to assess mental efficiency of the students.

Various criteria (Student *t*-test, Mann–Whitney test, means difference significance *p*) were used to establish significance of differences in the parameters studied. We considered the differences found significant at $p < 0.05$; $p < 0.01$; $p < 0.001$; the minimal significance level was 95%. Spearman's rank correlation coefficient (*r*) was used to determine closeness and significance of the relationship between the parameters. The data obtained was processed with the help of MS Excel Statistica 6.0 software.

RESULTS

Shoe repairman vocational training conditions evaluation

The labor component of the shoe repairman vocational education, as it is given to HC adolescents, is a heavy burden in itself: over 80% of the practical class implies sitting down in an awkward position forced by the specifics of operations performed. Artificial lighting of the workbenches was insufficient: in the training workshops, light intensity at the working surface of the benches was 226.5 ± 13.8 lx, while the norm is 300 lx. The air inside the students' working area contained excessive volumes of specific substances: acetone (up to 4.5 times the maximum allowable concentration), carbon dioxide (up to 1.5 times the maximum allowable concentration). As for the moderately hazardous industrial allergens (rosin, formaldehyde), their shift-average concentrations did not exceed the allowable levels. Taking into account the detected concentrations of chemicals in the working area air, the labor conditions of HC adolescents should be considered harmful. Moreover, examination of the educational process revealed irrationalities in length and sequence of various activities associated with the shoe repairman vocational training.

Sewing equipment operator vocational training conditions evaluation

Examining the conditions adolescents learn to operate sewing equipment in from the point of view of hygiene, we learned a number of factors affecting them:

1) labor intensity — working operations, which make up 82% of the lesson's duration, require a forced posture (sitting, body leaning forward)

2) strain — over 40% of the lesson the students had to concentrate on a single object;

3) lighting — the artificial illumination level registered in the context of using the sewing equipment was only 1456.2 ± 24.1 lx, while the norm is 2000 lx, $p = 0.15$; at ironing workbenches and manual operations desks the level was 350.7 ± 13.7 lx, while the norm is 600 lx, $p = 0.03$;

4) noise — during manual operations and ironing the sound pressure level in the workshop was 82.4 ± 2.4 dB; we have registered the excess of 9.5 ± 3.2 dB in the frequency range from 500 to 8000 Hz; in total, the students suffered from excessive noise 54.3% of time in class; the noise level reached 82.4 ± 2.4 dB, which exceeded the permissible values by 2 dB.

We connected the results obtained with improper organization of workplaces in the workshop: manual operations, ironing and sewing equipment zones were next to each other and in the single room. Sewing machine produced noise to the level of 87.1 ± 2.1 dB, while the chain weave machine's sound pressure level was 85.2 ± 3.5 dB. Both are in excess of the maximum permissible level of 80 dB.

Students sick rate assessment

In the first half of the school year, the sick rate was 5.23 cases per 1000 people (calculated from the registered visits to doctors). In the second half, the rate was greater than in the first (difference statistically significant, $p < 0.01$): 7.08 cases per 1000 students. The pathology index in the group was 199.5 ± 16.7 per 100 people. The most common disorders were those of the neuropsychic sphere (43.8%), musculoskeletal system (18.3%), circulatory system (16.5%).

Students working efficiency evaluation

We tested the students mental efficiency (ME) at the beginning and at the end of several lessons to cover all the subjects

studied (Table 1). According to the integral assessment, more than half of the adolescents ($64.2 \pm 3.1\%$) exhibited low levels of MC at the beginning of classes; only $14.2 \pm 2.1\%$ of students showed high MC. At the end of the class, there were more people with high MC ($35.4 \pm 3.3\%$) while the number of those who still remained at the low ME level decreased to $25.8 \pm 0.9\%$. The dynamics revealed result from the longer warm-up period, which is one of the functioning peculiarities of CNS of HC adolescents.

We applied the method suggested by P. V. Nefedov, N. B. Kutumova [14] when distributing the HC students according to their ME based on the data obtained through the week. The ME levels were determined with the daily study load coefficient taken into account (Table 2). On Mondays, the students' performance in studying was low, which may have been the result of changes in their daily routine (most of them left for home on Saturday and returned to the boarding school on Monday morning). The efficiency upturned sharply on Tuesday and Wednesday, then decreased gradually Thursday through Saturday, which indicates the predominance of inhibition processes in CNS as a result of accumulating fatigue.

A more detailed analysis of the efficiency levels demonstrated by HC adolescents through the school day

Table 1. Examination of mental efficiency of HC adolescents with the help of correction tables by V. Ya. Anfimov (as modified by S. M. Grombakh)

Subjects studied	Number of letters viewed		Student t-test	p	Average number of errors per 500 characters		Student t-test	p	Task performance accuracy (%)		Performance	
	Start of class	End of class			Start of class	End of class			Start of class	End of class	Start of class	End of class
Professional occupation	445.2 ± 9.1	479.3 ± 11.4	2.39	0.004	53.9 ± 2.7	49.9 ± 1.8	1.23	0.0001	89.1 ± 3.2	90.7 ± 2.4	4.9 ± 0.2	5.2 ± 0.5
Production technology	580.4 ± 10.5	452.1 ± 8.7	10	0.019	50.9 ± 1.3	73.0 ± 4.1	5.14	0.001	91.9 ± 1.5	86.0 ± 1.9	6.3 ± 0.9	5.2 ± 0.1
Equipment	544.3 ± 9.1	425.2 ± 7.6	10.44	0.017	49.8 ± 2.7	71.4 ± 3.6	4.8	0.001	91.6 ± 2.1	85.6 ± 1.1	5.9 ± 1.0	5.2 ± 0.3
Materials Science	520.4 ± 9.7	429.4 ± 7.2	7.98	0.013	53.1 ± 3.1	70.6 ± 2.3	4.53	0.0009	94.0 ± 1.8	85.8 ± 2.9	5.5 ± 1.2	5.0 ± 0.4
Industry and enterprise economics	600.4 ± 9.1	512.8 ± 8.6	7.31	0.013	56.6 ± 1.5	70.5 ± 1.3	5.06	0.0007	91.3 ± 2.2	87.8 ± 2.6	6.3 ± 0.9	5.8 ± 0.8
Fundamentals of artistic design	519.3 ± 8.6	459.3 ± 7.7	5.64	0.008	58.9 ± 1.6	67.1 ± 1.8	3.4	0.0004	88.5 ± 1.7	87.2 ± 2.9	5.8 ± 0.8	5.2 ± 1.1
Special drawing	587.1 ± 9.5	536.2 ± 8.1	4.24	0.008	52.1 ± 1.4	56.3 ± 1.4	2.2	0.0002	91.8 ± 1.5	90.4 ± 3.1	6.3 ± 0.3	5.9 ± 0.2
Native region history	603.8 ± 20.2	551.6 ± 8.4	2.41	0.008	54.7 ± 3.1	59.1 ± 1.6	1.26	0.0002	91.6 ± 3.2	90.3 ± 2.9	6.5 ± 0.2	6.1 ± 0.2
Ethics and culture of communication	498.7 ± 16.5	455.3 ± 7.5	2.46	0.022	56.8 ± 1.9	61.4 ± 1.5	1.9	0.0002	89.7 ± 1.9	88.1 ± 2.1	5.5	5.1
Basics of life safety	401.6 ± 6.9	463.6 ± 8.1	6.2	0.008	55.2 ± 2.4	49.8 ± 1.8	1.8	0.0002	87.9 ± 2.6	90.2 ± 3.5	4.9	5.1
Occupational safety	401.8 ± 5.4	445.3 ± 2.1	8.17	0.006	59.7 ± 2.4	54.3 ± 1.3	1.98	0.0002	87.0 ± 2.4	89.1 ± 4.0	4.6	5.1
Adaptive physical culture	481.1 ± 4.4	571.2 ± 9.1	9.14	0.013	54.1 ± 1.3	43.4 ± 5.4	1.93	0.0005	89.8 ± 2.8	92.9 ± 1.3	5.3	6.1

Table 2. Efficiency of students through the week

Days of week	Number of letters viewed	The total number of characters in the test	Load coefficient value (%)
Monday	121 ± 5.2	868	14
Tuesday	170 ± 1.9		19.7
Wednesday	171 ± 0.9		19.7
Thursday	149 ± 2.5		17.2
Friday	133 ± 1.3		15.4
Saturday	120 ± 6.2		14

revealed some differences from the classical daily performance curve. The efficiency level was at its highest on the 3-rd or 4-th hour of work, then it dropped, and the drop was followed by the final burst that has the performance peaking; compared to the classic efficiency level curve, the burst was shifted for 2–3 hours.

Assessment of adaptation capabilities

Assessing the students' adaptation capabilities, we learned that the adaptation index (AI) of $39.3 \pm 1.1\%$ of young men was 8.25 to 9.86, which corresponds to the unsatisfactory level of adaptation. As for the young women, $34.5 \pm 1.6\%$ of them had the adaptation index at unsatisfactory levels, and $26.5 \pm 0.7\%$ have exhibited signs of stressed adaptation mechanisms.

There were 26.3% more girls with satisfactory adaptation levels than boys. Increasing AI may signal of the health deterioration trend associated with decreased efficiency and growing fatigue.

It was found that adaptation of 48.1% of non-HC adolescents was unsatisfactory, while that of HC students was 41.6%.

As for the anxiety, the assessment revealed the average level of state anxiety to be low and that of trait anxiety moderate in the adolescents. Among the participating HC students, 76.2% exhibited low state anxiety values (< 30 points), which indicates that they are depressed, areactive and unmotivated to study. 23.8% of the students showed moderate state anxiety levels (33–44 points). A considerable share of adolescents (51.3%) studying to operate sewing equipment exhibited high trait anxiety, 52 ± 1.4 points; this is the result of their proneness to perceive a wide range of life situations as threatening and respond to them with a pronounced reaction. The trend reflected the peculiarities of personal patterns found in this group.

Correlation analysis allowed establishing that life in the dormitory is a positive example of successful adaptation of HC adolescents to the microenvironment of vocational education institution ($r = 0.32$). Besides, we have found the AI to be moderately associated with the trait anxiety level ($r = 0.36$), which characterizes peculiarities of personal patterns and those of body functioning under stress. Thus, adolescents with lower stress levels were able to adapt to the vocational training conditions in an educational institution better. State anxiety and AI were found to be inversely associated with each other, the association being moderate ($r = -0.38$). Those students whose motivation to study was low showed unsatisfactory adaptation values.

Classes timetable assessment

Vocational training implies greater studying-related loads, since the curriculum has special subjects (technology, materials science, vocational training etc.) added to it. Thus, one of the major challenges in educating HC adolescents when they start learning a trade is to compile the timetable in a rational manner.

The ranked subject difficulty scale developed by I. G. Sivkov [18] is one of the methods used to evaluate the timetable from the point of view of hygiene. However, in case of a vocational school this scale is inapplicable since it contains no specific profession-related subjects.

We assessed the difficulty of the curriculum subjects through monitoring functions of the students' CNS (ME indicators, correction tables). Analysis of dynamics of the CNS functions' indicators revealed certain trends governing changes in the parameters studied (Table 3).

Table 4 contains scores reflecting difficulty of the subjects as it was evaluated in the context of the research. We have compared this fatigue scale with the SanPIN 1186-03 Indicative Scale of Educational Subjects and found certain similarities

Table 3. Subjects scoring based on the dynamics of the students' CNS parameters

Evaluation criteria	Change in the number of viewed characters	Change in the number of errors	Points
Extremely low values (less than $M - 2\sigma$)	-27.0 – 18.1%	+27.0 + 18.1%	6
Low values ($M - 2\sigma$)	-18.0 – 9.1%	+18.0 + 9.1%	5
Below average ($M - 1\sigma$)	-9.0 – 0%	+9.0 + 0%	4
Average values ($M \pm 1\sigma$)	+0 + 9.0%	-0 – 9.1%	3
Values above average ($M + 2\sigma$)	+9.1 + 18.0%	-9.0 – 18.1%	2
High values (more than $M + 2\sigma$)	+18.1 + 27.0%	-18.0 – 27.0%	1

Table 4. Subjects ability to cause fatigue, ranked scale

Subject	Difficulty rank (points)
Shoe repairman profession	
"Shoe technology", "Equipment", "Materials science for shoe production"	6
"Industry and enterprise economics", "Fundamentals of art design and shoe design"	5
"Production training"	3
"Occupational safety"	2
"Physical culture"	1
Sewing equipment operator profession	
"Garments manufacturing technology", "Equipment", "Materials science"	6
"Design basics"	5
"Special drawing", "Native region history", "Ethics and culture of communication"	4
"Production training"	3
"Occupational safety", "Basics of life safety"	2
"Physical culture"	1

between the two. Thus, it was deemed possible to equate the compiled fatigue scale to the difficulty scale and call it accordingly.

Hygienic assessment of the sewing equipment operator curriculum timetable showed that the study load therein is not distributed evenly: it grows gradual Monday to Wednesday, then drops unevenly Thursday to Saturday while peaking on Friday.

The curriculum timetable for the shoe repairmen profession was as unbalanced: maximum study load from Monday to Friday, no gradual growth or drop. We established that the timetable in both groups is not rational, which lead us to developing measures to adjust and optimize it.

Application of the automated timetable compilation software

Since compiling the classes timetable is a labor-intensive process, it was decided to automate it. For this purpose, a software program was developed and registered ("Automated Timetable Compilation Program for Primary Vocational Education Institutions", certificate 2016612429, state registration date 26.02.2016). The core of the system and its interface were coded in CSharp 2010, operating system type and version — Microsoft Visual Studio.

The software program we have developed distributes subjects through the weekdays based on their difficulty score in points. Its purpose is to compile a classes timetable that would agree with the weekly performance curve and the daily study load index peculiar to the HC students.

Application of the software starts with inputting name of the educational institution, name of the trade taught, curriculum year, group code. Names of the institution and trade are plain text, year and group code — numeric symbols.

Next, the user inputs the weekly amount of classroom hours of each subject. The subjects themselves are pre-defined in the software as they are listed in the basis curriculum.

Finally, the program automatically compiles timetables factoring in the subjects' difficulty scores.

The automated timetable compilation software allowed compiling a classes timetable that agrees with the basis hygiene requirements and principles of biorhythmology.

After 6 months with the new timetable, we have registered changes in the participants' ME: the number of adolescents whose ME was average before the classes has grown by 21.3% ($p < 0.05$) and equaled $21.6 \pm 3.2\%$. The number of adolescents whose efficiency was low decreased by 7.7% ($p > 0.05$) and equaled 59.2%; the number of those with ME value above average has grown by 45.7% ($p < 0.05$) and amounted to 5.1% of the total number of students.

At the end of the classes, the students' performance was better, too: the share of those demonstrating average and above average efficiency increased by 40% and 3.1%, respectively ($p > 0.05$), while the number of students whose ME was below average dropped by 9.1% ($p \leq 0,05$).

Having analyzed the students' psychosomatic complaints again, we discovered that the share of non-specific complaints decreased by 52%, share of those reporting malfunctioning cardiovascular system and respiratory organs — by 46.6%. However, the students complained about problems with musculoskeletal system as often as they used to, since it was the timetable that changed and not the vocational training algorithms.

Analysis of the state anxiety levels revealed they have shifted towards moderate values. Trait anxiety did not change

significantly, which reflects peculiarities of personal characters of the students.

Automation of the timetable compilation routine improved its efficiency and clarity through information support; moreover, the software allowed optimization of utilization of the teaching workforce, which is limited.

DISCUSSION

Examining the conditions HC adolescents receive vocational education in, we found violations of sanitary and hygienic requirements in individual workplaces. The harmful physical factors peculiar to the workshops students learn the trades in are insufficient artificial lighting and excessive noise; the harmful chemical factors (polluting air in the working zone) are excessive dust content, presence and volume of organic solvents and allergens. Thus, the conditions were deemed hazardous, the labor itself — intense (as described in the labor classification). Our findings are in line with those of other researchers who revealed the discrepancy between the conditions of training and hygienic standards [19]. Moreover, harmful factors of the working environment and labor itself (1–3 degrees) accompany the subsequent employment of students, contributing to the development of occupational pathologies [20].

Comprehensive assessment of the educational process in the vocational boarding school for HC students revealed that the classes timetable is not rational in terms of duration of various activities and the frequency of their alternation. As a result, adolescents learning both trades researched exhibited: low mental efficiency, low levels of state anxiety (76.2%), unsatisfactory biological adaptation levels (44.85%), strain of the adaptation mechanisms (21.2%). The difference between the sick rates peculiar to second and first half-year periods (based on the registered requests for medical assistance) was statistically significant and equaled 7.08 and 5.23 per 1000 people, respectively ($p < 0.01$).

In the context of the study, we have developed a software program that automates compilation of the classes timetable while factoring in the functional peculiarities of HC adolescents' body systems. A 6-month trial of the hygienically rational timetable based on the subjects' difficulty scores confirmed its effectiveness. Introduced, the timetable secured a 52% decrease of non-specific complaints and a 46.6% decrease of those pertaining to problems with cardiovascular system and respiratory organs. At the same time, the state anxiety levels shifted toward moderate values and the number of students exhibiting average ME grew.

To further combat the adverse effects the factors peculiar to training and production environment have on the adolescents' organisms, the boarding school was given a developed set of guidelines approved by Rospotrebnadzor (Russian Agency for Health and Consumer Rights) in the Saratov region; the guidelines prescribe concrete measures:

- conducting occupation-related classes in rooms the floorspace and volume of which are sufficient for the number of students in a group or making that number smaller;
- installation of a fume hood (for adhesive operations) in the shoe repairs workshop;
- introduction of physical activity breaks, required due to the fact that over 80% of time at the practical lessons students have to maintain the same pose;
- installation of the local sources of light on the shoe repairman's workbenches and those designed for machine and manual operations part of the sewing machine operator training routines;

- use of the means of personal protection to counter the adverse effects of excessive noise (earplugs, helmets, headphones), vibration (mittens with inserts, mittens and gloves with reinforced palm covers, gripping pads and plates for vibrating arms and parts);
- control of performance of the ventilation system, its timely sanitary and technical maintenance in the workshops;
- motivating students to lead a healthy lifestyle.

CONCLUSIONS

The sanitary and hygienic conditions handicapped adolescents receive their vocational education in are suboptimal. Assessing the shoe repairmen training environment, we found that the air of workshops was polluted with chemicals (acetone — 4.5 MPC, carbon monoxide — 1.5 MPC), and the sewing

equipment operator training implied intense labor (over 40% of the lesson's time students have to concentrate on a single objects) and noise level 1.6 times the norm. The classes timetable was found to be not rational, which can be another factor adversely affecting adaptation of the handicapped people exercising their right to study and work. Thus, there is a need to optimize the training conditions in vocational schools educating this group of students. Based on the results of the study, we have developed and introduced a software program to automatically compile the school's timetable with the aim to improve psychosomatic health of the students and halve the number of health-related complaints, which translates into better alignment of professional training conditions and requirements imposed by the functional capabilities of people with disabilities.

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PREVENTION OF STAFF BURNOUT IN HUMANITIES TEACHERS OF HIGHER EDUCATIONAL INSTITUTIONS

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In their daily life teachers go through a lot of stress ensuing from abrasive relationships with the administrative staff, colleagues or students, and family conflicts. Long-term exposure to stressors leads to the syndrome of emotional burnout, which can be avoided if preventive care is taken. The aim of this study was to identify burnout symptoms in the humanities teachers working for higher educational institutions and to propose adequate preventive measures. We recruited 1,489 teachers who were offered to anonymously fill out a questionnaire and also collected information about their health from sickness leaves. Statistical analysis included calculation of means, their errors, intensive and extensive variables, and confidence intervals. Two in three teachers reported fatigue, regular back/chest pain, and headache. The use of a scoring scale allowed us to conclude that a lot of respondents (43.2%) had severe burnout symptoms. Every second teacher demonstrated poor health awareness. To prevent staff burnout, measures should be taken aimed at stimulating a positive attitude to medical care, improving health literacy and motivating teachers to lead a healthy lifestyle.

Keywords: teacher, staff burnout, professional occupation, health awareness, prevention

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ПРОФИЛАКТИКА СИНДРОМА ЭМОЦИОНАЛЬНОГО ВЫГОРАНИЯ У ПРЕПОДАВАТЕЛЕЙ ГУМАНИТАРНЫХ ВЫСШИХ УЧЕБНЫХ ЗАВЕДЕНИЙ

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Профессиональная деятельность преподавателей сопряжена с воздействием большого числа стресс-факторов, которые могут проявляться на работе, во взаимоотношениях с начальством, напряженной обстановкой в коллективе, вследствие различных социально-психологических переживаний на работе и дома. Длительное воздействие этих факторов способствует развитию синдрома эмоционального выгорания, профилактика которого просто необходима. Целью исследования было выявить симптомы синдрома эмоционального выгорания у преподавателей гуманитарных высших учебных заведений и разработать мероприятия по его профилактике. Обследовано 1489 преподавателей гуманитарных вузов с помощью составленных анонимных анкет для изучения профессиональной деятельности педагогов, а также выборочных карт на основании листов нетрудоспособности. Для статистического анализа рассчитывали средние величины и их ошибки, интенсивные и экстенсивные показатели; использовали метод сравнения относительных величин и определение достоверности различия. Выявлено, что у двух преподавателей из трех присутствуют чувство усталости, частые боли в спине и груди, головная боль. В результате балльной оценки степени выраженности данных симптомов обнаружено, что большая часть преподавателей (43,2%) оказалась в группе с высоким уровнем психоземotionalного выгорания. У каждого второго преподавателя наблюдается низкий уровень медицинской активности. Для профилактики синдрома эмоционального выгорания необходимо внедрение мероприятий, направленных на формирование позитивной медицинской активности, повышение медицинской грамотности и воспитание навыков здорового образа жизни.

Ключевые слова: преподаватель, синдром эмоционального выгорания, профессиональная деятельность, медицинская активность, профилактика

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Our everyday work affects our health and to a greater or lesser extent shapes our lifestyle, mentality and personality [1–3]. Extensive research into occupational risk factors reveals the need for special measures aimed at preventing work-associated health risks [4–6].

A lot of published articles focus on how work overload affects the health of white-collar workers causing stress and stress-related conditions [7, 8]. For example, a few research

studies have attempted to assess the impact of workload in an optimized work environment [9]. Some authors have proposed algorithms for improving the efficiency of intellectual labor and ease its burden based on cognitive semantics [10].

A number of authors have studied the occupational health of teachers. It has been shown that many teachers suffer from chronic diseases, but only 2 in 3 teachers undergo regular medical checkups [11, 12]. The most common chronic

disorders observed in teachers are hypertension, chronic laryngotracheitis, and varicose veins. Among the most prevalent acute conditions are respiratory infections, laryngitis, and acute laryngotracheitis. Many of these diseases are occupational and can be caused by voice overuse and serious psychological and emotional strain.

There are a lot of stressors intrinsic to the teaching job ensuing from the necessity to always be in contact with students encouraging them to acquire new knowledge and build professional skills. The teacher guides the student in his/her intellectual and emotional pursuits. In its essence, teaching is all about interacting with people, understanding them and helping them to channel their efforts towards achieving the goals set by the society and the state [13].

The modern teacher goes through a lot of stress trying to solve problems arising from the interaction with their colleagues, students and administrative staff. Stress affects the quality of teachers' work, their personality, relationships with colleagues, and takes a huge toll on his/her professional and private life. Working with people demands professional expertise, knowledge of psychology and sociology; it also poses a high risk of emotional distress and can eventually lead to the symptoms of emotional burnout.

The burnout syndrome (BS) is the body's response to long-term exposure to occupational stress factors. The term "staff burnout" was first used in 1974 by the American psychiatrist H. J. Fruedenberger to describe the emotional state of healthy individuals who were in long, close contact with emotionally unstable clients and patients. Fruedenberger saw this syndrome as a complex psychophysiological phenomenon implicated in emotional, mental and physical exhaustion that manifests itself as depression, fatigue, a feeling of being drained, a lack of energy and enthusiasm, an inability to value one's own accomplishments and achievements, poor workplace morale, and dissatisfaction with life [14, 15]. The social psychologist K. Maslac describes this syndrome as physical and emotional exhaustion characterized by low self-esteem, negativity, a lack of understanding and empathy to clients and patients. BS is a mechanism of psychological defense based on the complete or partial emotional withdrawal in response to a traumatic event [16].

The literature does not provide information about BS in teachers working for higher educational institutions, its causes and the impact it has on teachers' health. The aim of this study was to identify and investigate the symptoms of BS in humanities

teachers employed by higher educational institutions and to propose measures for preventing the negative impact of BS on health.

METHODS

To study BS manifestations and its effect on health, we recruited 1,489 teachers of both sexes aged from 25 years and older who had been working in their current jobs for at least 5 years. The participants were asked to anonymously fill out and immediately submit an electronic questionnaire containing 85 questions devised to assess their professional skills, psychological state and the severity of BS symptoms. Health-related information was obtained by analyzing sickness leaves. Statistical processing included calculation of means and mean errors, intensive and extensive variables and their errors, comparison of relative values, computation of confidence intervals and Pearson's correlation coefficients.

RESULTS

Among the teachers working for higher educational institutions the most common BS symptoms were fatigue, headache, chest or back pain, excess weight, low productivity, bad memory, difficulty concentrating, and sleep problems (Table 1).

On average, every teacher had 4 to 5 symptoms of burnout. Almost every second teacher complained of fatigue, headache, excess weight and sleep problems.

All symptoms typical for BS were rated by the participants depending on how pronounced they were on the scale of 0 (no symptoms) to 5 (severe symptoms). The minimum score was 6 points, the maximum score was 40 points. Depending on the total score, the teachers were distributed into 3 groups: mild burnout (0–12 points), moderate burnout (13 to 26 points), and severe burnout (27 to 40 points). Details are provided in Table 2.

The above listed symptoms of BS are often preceded by emotional distress. In our study almost every second teacher was emotionally labile (46.8%), moody, irritable, anxious, and had difficulty concentrating. About one in 3 teachers complained of feeling unwell. On average, every teacher had 1 or 2 symptoms indicative of emotional distress.

The comparative analysis of questionnaires revealed that teachers with severe BS complained of negative emotional symptoms more often than other participants (Table 3).

Table 1. Burnout symptoms in the teachers working for higher educational institutions

№	Burnout symptoms	Incidence per 100 participants
1	Fatigue	67.4
2	Chest or back pain	64.2
3	Headache	62.4
4	Excess weight	58.1
5	Reduced productivity	53.2
6	Sleep problems	50.9
7	Bad memory, difficulty concentrating	49.5
8	Sleepiness at daytime	47.1

Table 2. Distribution of humanities teachers into groups based on the severity of burnout symptoms

Severity of burnout syndrome	Points scored	Percentage of participants, %
Mild	> 12	19.4
Moderate	13–26	37.4
Severe	27–40	43.2
Total		100

In our study the sample was dominated by women (67.4%) aged 51.4 ± 0.7 years on average; 28.7% of the surveyed teachers were younger than 40; 31.7% of teachers were older than 60 years. The reported teaching experience was 23.1 ± 0.4 years on average; 24.2% of the respondents had 10 years of teaching experience while 36.4% of the respondents had been teaching for over 30 years. The majority of the surveyed teachers (62.4%) had only one job throughout their carrier.

The teachers older than 60 had BS symptoms more often than their younger (< 40 years) counterparts. Older teachers felt sleepiness at daytime, back and chest pains, and complained of deteriorating memory, difficulty concentrating, fatigue, and excess weight 2.8–2.4 times more often than other participants (Table 4).

The analysis has revealed a direct correlation between the symptoms of BS and the length of teaching experience ($r = 0.576$; $m = \pm 0.034$; $p < 0.05$) in the surveyed humanities teachers. Individuals who had over 30 years of teaching experience complained of SB symptoms more often than those who had less than 10 years of experience ($p < 0.05$) (Table 5).

Apart from BS symptoms we analyzed the atmosphere in the workplace and in the teachers' families.

We have established that the atmosphere in the family and in the workplace largely contributes to emotional burnout. Among the stressors we accounted for were strong heavy emotions, relationships with colleagues, students and relatives.

Every teacher was able to remember an episode in their professional or private life that led to a stressful or hostile atmosphere in the workplace or family. Among the most commonly reported problems were family conflicts with a spouse or relatives. One in five teachers had conflicts with colleagues, administrative staff and students. One in every five teachers reported a serious illness as a factor negatively affecting their morale (Table 6).

The comparative analysis showed that the teachers with severe BS symptoms reported stressors affecting the atmosphere in the workplace or family more often than those with mild symptoms of BS (Table 6).

The factors listed above contribute to psychological stress and compromise teachers' health.

Emotional burnout is a complex psychophysiological phenomenon defined as emotional, mental and physical exhaustion manifested as fatigue, depression, inability to notice and value one's own accomplishments and achievements, negativity, and dissatisfaction with life.

To neutralize the effect of negative factors, a teacher has to be able to switch off from work-related problems and engage in other types of activities in leisure time.

Our study demonstrates that the considerable proportion of teachers lack physical activity, eat unhealthy diet or take their meals irregularly, do not watch their weight, and use sleeping pills without doctor's permission (Table 7). Although the majority of the respondents had burnout symptoms, only one in every 4 teachers sought medical advice and only 39.6% of the respondents actually followed that advice.

On average, every teacher demonstrated 4.27 ± 0.32 signs of poor health awareness. The teachers with severe BS symptoms showed 4.96 ± 0.35 negative signs, while those in the group with mild BS symptoms showed only 3.12 ± 0.31 signs, which is significantly less.

The comparative analysis (Table 7) showed that the teachers with severe burnout syndrome did not see a doctor 3.1. times more often than others; slept less 1.9 times more often, took medications without consulting a doctor 1.7 times more often, did not watch their weight 1.5 times more often, tended to lead a sedentary lifestyle 1.5 times more often; besides they were not satisfied with night's sleep quality and did not follow medical advice.

The group with poor health awareness consisted of teachers who demonstrated 6 or more such signs. Among the teachers with severe symptoms of BS 68.5% demonstrated low health awareness; among those with mild symptoms of BS there were 1.9 more such individuals, which means that their attitude to medicine and health in general needs to be changed.

Table 3. Frequency of symptoms of psychoemotional distress in humanities teachers (per 100 participants)

Symptoms of psychoemotional distress	Per 100 teachers			Probability of accurate prediction p
	In total	Severity of burnout syndrome		
		mild	severe	
Emotional lability	39.3	29.8	38.8	< 0.01
Unstable mood	37.3	27.2	37.4	< 0.01
Reduced attention span	32.1	23.9	30.3	< 0.05
Irritability	29.7	19.9	29.5	< 0.01
Anxiety	24.2	15.9	22.5	< 0.05
Feeling of unwellness	29.1	21.7	26.5	> 0.05

Table 4. Burnout symptoms in humanities teachers depending on their age

№	Burnout symptoms	Incidence per 100 teachers		Probability of accurate prediction p
		< 40 years of age	> 60 years of age	
1	Fatigue	36.5	86.3	< 0.01
2	Chest or back pain	32.1	84.3	< 0.01
3	Headache	42.9	69.9	< 0.05
4	Excess weight	29.7	74.5	< 0.01
5	Reduced productivity	33.8	60.6	< 0.05
6	Sleep problems	34.9	54.9	< 0.05
7	Bad memory, difficulty concentrating	24.3	62.7	< 0.01
8	Sleepiness at daytime	21.6	60.6	< 0.01

DISCUSSION

In this work we attempted to study the symptoms of staff burnout in the humanities teachers employed by higher educational institutions. It has been established that about 2 in every 3 teachers suffer from fatigue, regular pain in the chest or back, and headaches. This leads to reduced productivity in the workplace, deteriorating memory and the inability to concentrate. Teachers often have sleep problems and complain of daytime sleepiness. As a rule, such teachers (58.1%) are overweight.

The use of a scoring scale allowed us to distribute the participants into 3 groups: severe symptoms of emotional burnout (27 to 40 points), mild symptoms (0 to 12 points), and moderate symptoms (13 to 26 points). Unfortunately, the majority of the respondents (43.2%) constituted a group with severe burnout syndrome. Only one in five teachers had mild symptoms of this condition.

Analysis of the social and psychological background of the teachers has revealed that our sample is dominated by women aged 51.4 ± 0.7 with a teaching experience of 23.1 ± 0.4 years. The majority of the teachers have had only one job in their professional life. The burnout syndrome is more often observed in the teachers older than 60 with a teaching experience of over 30 years.

A stressful atmosphere in the workplace or family also contributes to the development of burnout symptoms. Every teacher reported episodes that created a hostile or stressful atmosphere in their families or workplace. However, the teachers who suffered from severe BS symptoms reported stressful atmosphere more often than those whose symptoms were mild.

Special attention should be paid to health awareness demonstrated by the teachers. Almost every second teacher with severe BS symptoms has a negative attitude to their health. They lead a sedentary lifestyle, follow an unhealthy diet, eat irregularly, do not have enough sleep, rarely consult a doctor or follow medical advice, take unprescribed medications, and are overweight. Considering the fact that staff burnout is a sign of emotional, physical and mental exhaustion, we need to neutralize the effect of negative factors and to promote health awareness among teachers. The respondents demonstrate poor health literacy (69.7%), a lack of motivation to lead a healthy lifestyle (47.2%), poor knowledge of the roles of night's sleep quality (46.2%), physical activity (43.1%), and nutrition (39.7%), unwillingness to consult their doctors (39.6%) or follow medical advice (34.1%).

Preventive care should be provided by medical personnel and aim at improving health literacy, stimulating teachers to change their attitude to health and follow medical advice.

Table 5. Burnout symptoms in humanities teachers depending on their teaching experience

№	Burnout symptoms	Incidence per 100 teachers		Probability of accurate prediction p
		Less than 10 years of teaching experience	Over 30 years of teaching experience	
1	Fatigue	29.2	90.6	< 0.001
2	Chest or back pain	27.9	85.5	< 0.001
3	Headache	34.1	75.7	< 0.05
4	Excess weight	19.6	81.6	< 0.001
5	Reduced productivity	32.8	58.6	< 0.05
6	Sleep problems	35.1	51.7	< 0.05
7	Bad memory, difficulty concentrating	23.4	60.6	< 0.01
8	Sleepiness at daytime	21.8	57.4	< 0.01

Table 6. Stressors in the workplace or family per 100 teachers

№	Stressors in the workplace or family	Incidence per 100 teachers	Burnout syndrome		Probability of accurate prediction p
			Severe	Mild	
1	Frequent conflicts with a spouse	25.5	36.2	14.7	< 0.01
2	Frequent conflicts with close relatives	24.7	36.4	12.9	< 0.01
3	Frequent conflicts with colleagues or administrative staff	21.6	29.2	13.9	< 0.01
4	Frequent conflicts with students	21.2	31.9	10.4	< 0.01
5	Health problems	20.8	32.7	8.9	< 0.01
6	Serious illness or death of a close friend/relative	15.2	18.6	11.8	< 0.05

Table 7. Health awareness in the surveyed teachers (per 100 respondents)

№	Health awareness	In total	Burnout syndrome		Probability of accurate prediction p
			Severe	Mild	
1	Sedentary lifestyle	64.9	72.1	52.7	< 0.01
2	Unhealthy or irregular diet	57.1	66.4	42.8	< 0.01
3	Overweight	56.4	64.9	42.9	< 0.01
4	Dissatisfaction with sleep quality	54.7	60.7	43.7	< 0.01
5	No intention to normalize weight	47.3	54.7	34.9	< 0.01
6	Lack of night's sleep	46.8	58.2	30.4	< 0.01
7	Adherence to medical advice	39.6	43.6	30.6	< 0.01
8	Use of sleeping pills	37.2	44.1	25.3	< 0.05
9	Seeing a doctor due to health problems	23.4	31.7	10.1	< 0.01

Because the significant role in BS development is played by the psychological and emotional state, psychologists should be also involved in providing care for teachers employed by higher educational institutions.

CONCLUSIONS

More than half of the humanities teachers employed by higher educational institutions have symptoms of emotional burnout,

complain of fatigue, regular headaches, back or chest pain, deteriorating memory, and low productivity. Among the factors that contribute to the development of this condition are low health awareness, unwillingness to consult a doctor or follow medical advice, irregular or unhealthy meals, lack of physical activity, and use of unprescribed medications. To prevent BS, measures should be taken aimed at improving health literacy among teachers and motivating them to seek and follow medical advice.

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IDENTIFICATION OF DISTRICTS AT RISK OF NUTRIENT-RELATED DISEASES BASED ON THE LOCAL DIET

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The growing incidence of nutrient-related diseases is a global challenge. The aim of this work was to study consumption of staple food products and to estimate the incidence of nutrient-related diseases in Voronezh region. Food consumption was analyzed from the reports of the local branch of the Federal State Statistics collected over two 5-year periods (1995–1999 and 2012–2016). The incidence of nutrient-related diseases was estimated based on the reports providing information about patients' visits to healthcare facilities. The districts of Voronezh region were assigned to 5 ranks. Over the studied periods, the population of Voronezh region considerably changed its diet: consumption of fish and seafood, fresh fruits, meat, vegetables, gourds and melons, eggs, vegetable oil, milk and dairy products per person increased significantly. At the same time, consumption of sugar, breads and potatoes still exceeds the recommended intake 1.42–2.04-fold, which means that the main component of the diet is carbohydrates. We observed a significant 4.5-fold increase in obesity incidence, a 1.8–2.0-fold increase in the incidence of anemia and endocrine disorders and a 1.2 increase in the incidence of gastrointestinal diseases. In terms of prevention measures, the priority should be given to areas at a high risk of nutrient-related diseases.

Keywords: balanced nutrition, health risk of nutrition-related morbidity

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ОПРЕДЕЛЕНИЕ ТЕРРИТОРИЙ РИСКА ПО УРОВНЮ АЛИМЕНТАРНО-ЗАВИСИМЫХ ЗАБОЛЕВАНИЙ С УЧЕТОМ РЕГИОНАЛЬНЫХ ОСОБЕННОСТЕЙ СТРУКТУРЫ ПИТАНИЯ НАСЕЛЕНИЯ

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Проблема роста уровня алиментарно-зависимых заболеваний в настоящее время имеет глобальные масштабы. Целью исследования были изучение уровня потребления основных продуктов питания и оценка показателей алиментарно-зависимых заболеваний населения Воронежской области. Баланс потребления продуктов питания изучали по данным территориального управления Росстата по Воронежской области за два пятилетних периода (1995–1999 гг. и 2012–2016 гг.). Анализ алиментарно-зависимой заболеваемости выполняли по данным обращаемости населения за медицинской помощью с последующим ранжированием показателей на отдельных территориях на пять уровней. За два пятилетних периода произошли существенные изменения в характере питания: увеличилось потребление в расчете на одного жителя рыбопродуктов, свежих фруктов, мяса и мясных продуктов, овощей и бахчевых, яиц, растительного масла, молока и молочных продуктов. Вместе с тем наблюдается избыточное потребление сахара, хлебных продуктов, картофеля — от 1,42 до 2,04 раз, что свидетельствует о преобладании в рационе углеводной составляющей. По результатам оценки, отмечается достоверное увеличение показателей заболеваемости ожирением до 4,5 раз, анемией, болезнями эндокринной системы (в 1,8–2 раза) и болезнями органов пищеварения (в 1,2 раза). Приоритеты в профилактике алиментарно-зависимой заболеваемости должны быть отданы территориям риска.

Ключевые слова: сбалансированность питания, алиментарно-зависимое заболевание, структура питания

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The growing incidence of nutrient-related diseases is a global challenge. The majority of developed countries, including Russia, are addressing it at the statewide level [1]. Understanding the causes of nutrient-related diseases and assessing their incidence are important tasks that public

health monitoring and the risk-based approach to sanitary-epidemiological surveillance are expected to solve [2]. The results of data analysis and public health risk assessment are indicative of the growing incidence of nutrient-related diseases in some Russian regions, such as Samara

region [3], the Republic of Tatarstan [4], and the North of Russia [5].

The risk of developing a nutrient-related disease is especially high in certain cohorts of the population and certain regions, as shown by focused research. For example, questionnaires filled in by university students and their food records reveal that a combination of a poor diet, hereditary predisposition and an unhealthy lifestyle leads to anemia, obesity and type 2 diabetes mellitus [6]. Another research work demonstrates that industrial workers of the Arctic region are at increased risk of developing health problems because of their imbalanced diet [7]. A similar problem exists in the former USSR states. A study that included over 3,000 people residing in the towns and villages of Central and East Kazakhstan has revealed insufficient energy content in their daily meals (12–19%), low intake of vitamins C, B1, B2, and niacin (47–73% of the recommended daily dose) and deficit of retinol and tocopherol (50%) [8].

Nutrient-related morbidity varies across different Russian regions, necessitating research into regional nutritional patterns [9, 10]. Regional studies will help to elaborate measures aimed at providing the population with a balanced diet, ensuring adequate food quality control and preventing nutrient-related diseases [11].

The aim of this work was to study consumption of staple food products and assess the incidence of nutrient-related diseases in Voronezh region.

METHODS

The study was conducted in 32 districts of Voronezh region populated by 2.3 million people. Reports on the consumption of different food products collected over two 5-year periods (1995–1999 and 2012–2016) were provided by the local branch of the Federal State Statistics and reflected quantitative and qualitative changes in the consumption of 10 staple food categories: breads; potatoes; vegetables, melons and gourds; fresh fruits; sugar; meat; seafood and fish; milk and dairy products; eggs; vegetable oil. The data were compared with the values specified in the Recommended rational norms of food consumption that meet modern nutritional requirements (Order 614 of the Ministry of Health of the Russian Federation dated August 19, 2016).

The incidence of nutrient-related diseases was estimated from the data supplied by the Federal State Statistics Service (report form 12 providing information about the incidence of

diseases registered in patients residing in healthcare service areas) collected from 1995 through 1999 and from 2012 through 2016. The data were analyzed in Statistica 6.0 and Microsoft Office (Excel).

To rank the average long-term incidence of nutrient-related diseases, we applied an algorithm determining the upper and lower values for this parameter based on its average value in the studied region (M) and mean square deviation (σ); the obtained data were distributed into 5 sets. Values falling into the first set were ranked as low ($M - \sigma$ and lower); in the second set, below average (from $M - \sigma$ to $M - 0.5\sigma$); in the third set, average (from $M - 0.5\sigma$ to $M + 0.5\sigma$); in the fourth set, above average (from $M + 0.5\sigma$ to $M + \sigma$); and in the fifth set, high ($M + \sigma$ and above). Regions with high long-term incidence (rank 5) were considered to be at risk.

RESULTS

The analysis of data on the consumption of staple food products in Voronezh region in 1995–1999 and 2012–2016 revealed considerable changes in the diet of its population (Table 1). Consumption of fish and seafood per one person (kg/year) increased 3.14-fold; fresh fruits, 2.0-fold; meat and dairy products, 1.93-fold; vegetables, melons and gourds, 1.63-fold; eggs, 1.44-fold; vegetable oil, 1.36-fold; milk and dairy products, 1.13-fold. At the same time, consumption of potatoes and breads slightly decreased, while sugar consumption remained stable (49 kg per 1 person a year).

The population of Voronezh region consume 2.04 times more sugar than is recommended and 1.42 times more bread. At the same time, their diet lacks high biological value foods, such as milk and dairy products (sour cream, butter, cottage cheese, cheese, kefir and yoghurt), constituting only 83% of the recommended amount (i.e., consumption of these products is 1.21 times lower than it should be). Being an important source of vitamins and minerals, vegetables included in the population diet, such as cabbages, carrots, beetroot, onions, tomatoes, cucumbers, bell peppers, courgettes, green salads, etc., constitute only 92% of the advised intake (their consumption is 1.8 times lower than the recommended amount); fresh fruits, such as apples, pears, grapes, and citrus fruits, make only 75% of the advised intake (their consumption is 1.33 times lower than the recommended amount). This leads us to conclude that the main component of the diet in Voronezh region is carbohydrates.

Table 1. Consumption of staple foods in Voronezh region in 1995–1999 and 2012–2016 (per one person, kg/year)

Products	Recommended intake	Year					Average values (1995–1999)	Year					Average values (2012–2016)
		1995	1996	1997	1998	1999		2012	2013	2014	2015	2016	
Breads	96	137	137	138	142	142	139	136	136	137	137	135	136
Potatoes	90	122	115	137	141	141	131	126	127	127	127	127	127
Vegetables and gourds	140	79	76	77	81	83	79	127	129	130	130	130	129
Fresh fruits	100	39	40	38	43	22	36	74	76	75	74	74	75
Sugar	24	46	49	50	50	50	49	48	47	49	50	52	49
Meat	73	53	46	45	44	43	46	83	91	89	90	92	89
Fish	22	7	8	7	6	6	7	22	22	23	23	22	22
Milk and dairy products	325	255	227	238	238	237	239	267	269	270	270	271	269
Eggs	260	246	242	233	222	223	233	329	334	338	338	339	336
Vegetable oil	12	11	11	10	11	11	11	15	15	15	15	16	15

Imbalanced diet and negative environmental factors increase the risk of nutrient-related diseases. When comparing their long-term incidence in the two studied 5-year periods, we established that incidence per 1,000 population had increased 1.2–4.5-fold for almost every class of nutrient-related diseases except for peptic ulcers. The most significant 4.5-fold increase was observed for obesity (Table 2).

Using the data that describe the average long-term incidence of nutrient-related diseases in Voronezh region, we built a 5-level scale to rank its districts accordingly (Table 3).

Long-term statistics collected over 2012–2016 demonstrated that the incidence rate of diseases affecting blood and hematopoietic organs was 17.1 ± 1.22 cases per 1,000 population; anemia was diagnosed in 3.7 ± 0.26 individuals per 1,000 population. On the whole, this pathology tended to increase in incidence ($R^2 = 0.829$). The same situation was observed in some relatively “healthy” districts belonging to rank 1 (0.6–2.2 cases per 1,000 population): Nizhnedevitsky district had 1.6 cases per 1,000 population (the incidence growth rate was 111.0%); Verkhnekhavsky district had 1.4 cases per 1,000 population (the growth rate was 241.6%); in Khokholsky district 1.4 cases per 1,000 population were registered (the growth rate was 295.7%); in Ternovsky district, 0.6 cases per 1,000 population (the growth rate was 83.0%). Among the districts at risk of anemia (rank 5, 4.7–6.8 cases per 1,000 population) were Bogucharsky, Verkhnemamonsky and Kalacheevsky, exhibiting a slower growth rate (minus 33.3%, 11.9% and 30.8%, respectively) and the highest incidence rates of 4.7, 6.3 and 6.8 cases per 1,000 population, respectively.

Our analysis revealed that the incidence rate of endocrine diseases in Voronezh region had been gradually increasing since 2012 reaching its maximum in 2015 (73.3 cases per 1,000 population); their average long-term incidence was 69.3 ± 5.93 cases per 1,000 population. Five districts were ranked as high-risk areas (77.9–110.3 cases per 1,000 population), including Bobrovsky (110.3 cases per 1,000 population), Ramonsky (89.2 cases per 1,000 population), Pavlovsky, (81.4 cases per 1,000 population), Podgorensky (81.2 cases per 1,000 population), and Olkhovsky (80.1 cases per 1,000 population).

Obesity rates remained stable in only 8 districts of 32: Bobrovsky, Borisoglebsky, Buturlinovskiy, Vorobiovskiy, Kalacheevskiy, Novousmanskiy, Paninskiy, and Talovskiy. Among the districts ranked as high-risk (13.7–26.5 cases per 1,000 population) were Kashirskiy with 26.5 cases per 1,000 population (the growth rate of 637.5%), Verkhnekhavskiy with 20.3 cases per 1,000 population (the growth rate of 245.0%), Ternovskiy with 17.5 cases per 1,000 population (the growth rate of 394.1%), Ramonskiy with 17.2 cases per 1,000 population (the growth rate of 133.4%), Bobrovskiy with 17.1 cases per 1,000 population (the growth rate of 118.4%), and Khokholskiy with 16.9 cases per 1,000 population (the growth rate of 710.5%).

At present, the average long-term incidence of gastrointestinal diseases in Voronezh region is 97.4 ± 4.91 cases per 1,000 population showing a strong tendency to increase ($R^2 = 0.927$). Among the districts assigned to rank 5 on our scale (129.8–212.9 cases per 1,000 population) are Repievskiy with 212.9 cases per 1,000 population, Povorinskiy with 182.9 cases per 1,000 population and Talovskiy with 172.1 cases per 1,000 population. The lowest incidence (rank 1, 46.1–54.7 cases per 1,000 population) was observed in Verkhnekhavskiy (46.1 cases per 1,000 population), Olkhovskiy (52.9 cases per 1,000 population) and Ertilskiy (53.5 cases per 1,000 population).

Comparison of the two studied 5-year periods revealed that the incidence rate of peptic ulcers tended to decrease from 15.5 ± 0.03 to 12.9 ± 0.13 cases per 1,000 population (which is by 17%). Nonetheless, 4 regions were still assigned to rank 5 (the highest incidence) based on the average long-term disease incidence (2012–2016): Kalacheevskiy with 28.2 cases per 1,000 population, Talovskiy with 21.8 cases per 1,000 population, Kantemirovskiy with 21.7 cases per 1,000 population, and Ternovskiy with 17.8 cases per 1,000 population. But on the whole, in spite of high long-term growth rates, the incidence is decreasing.

Districts with the lowest incidence rates assigned to rank 1 included Verkhnekhavskiy, Nizhnedevitskiy and Repievskiy (6.7–8.5 cases per 1,000 population) and showed an alarming trend: they exhibited the highest growth rate of peptic ulcer

Table 2. Average long-term incidence of nutrient-related diseases in Voronezh region per 1,000 person, $M \pm m$

Classes/nosological categories	Average long-term incidence		Increase (+) or decrease (-) (-fold)
	1995–1999	2012–2016	
Diseases of blood/hematopoietic organs (in total)	9.8 ± 0.91	16.1 ± 1.22	+ 1.6
Anemia	2.0 ± 0.03	3.7 ± 0.26	+ 1.8
Endocrine disorders (in total)	33.3 ± 0.36	69.3 ± 5.93	+ 2.0
Obesity	2.5 ± 0.04	11.3 ± 2.58	+ 4.5
Diseases of the gastrointestinal system (in total)	78.6 ± 0.22	97.4 ± 4.91	+ 1.2
Peptic ulcers	15.5 ± 0.03	12.9 ± 0.13	- 0.8
Gastritis and duodenitis	15.1 ± 0.06	21.9 ± 1.08	+ 1.5

Table 3. District of Voronezh region ranked based on the incidence of nutrient-related diseases (upper and lower incidence values per 1,000 person)

Nosological categories	Rank 5 (high)	Rank 4 (above average)	Rank 3 (average)	Rank 2 (below average)	Rank 1 (low)
Diseases of blood/hematopoietic organs (in total)	22.3–25.7	20.3–22.2	14.2–20.3	8.1–14.1	4.6–14.0
Anemia	4.7–6.8	3.9–4.6	2.4–3.8	1.6–2.3	0.6–2.2
Endocrine disorders (in total)	77.9–110.3	68.9–77.8	51.1–68.8	42.1–51.0	26.4–42.0
Obesity	13.7–26.5	10.9–13.6	5.3–10.8	2.5–5.2	0.5–2.4
Diseases of the gastrointestinal system (in total)	129.8–212.9	110.9–129.7	73.5–110.8	54.8–73.4	46.1–54.7
Peptic ulcers	17.8–28.2	15.5–17.7	10.9–15.4	8.5–10.8	6.7–8.4
Gastritis and duodenitis	29.4–47.8	24.8–29.3	15.6–24.7	11.1–15.5	4.1–11.0

incidence in 2012–2016 (63.93%, 68.04% and 139.04%, respectively).

The average incidence of gastritis and duodenitis in Voronezh region is 21.9 ± 1.1 cases per 1,000 population; in 2012–2016 its growth rate was 16.6% ($R^2 = 0.879$). A few regions were assigned to rank 5 (29.4–47.8 cases per 1,000 population), including Talovsky with 47.8 cases per 1,000 population (the growth rate of 4.7%), Kamensky with 39.7 cases per 1,000 population (the growth rate of 6.8%), Repievsky with 32.6 cases per 1,000 population (the growth rate of 33.8%), Vorobievsky with 30.31 cases per 1,000 population (the growth rate of 100.1%), and Nizhnedevitsky with 29.5 cases per 1,000 population (the growth rate of 12.9%). The lowest incidence (rank 1) was observed in Verkhnekhavsky, Kashirsky, Liskinsky, and Paninsky districts (4.1–11.0 cases per 1,000 population), but the growth rate in these districts was the highest (60.02–203.21%).

DISCUSSION

The selective analysis of data on the consumption of staple food products in Voronezh region collected in 1995–1999 and 2012–2016 demonstrates considerable changes in the local diet: increasing intake (per 1 person) of fish and seafood, fresh fruits, meat, vegetables, melons and gourds, eggs, vegetable oil, milk and dairy products. However, consumption of sugar, breads and potatoes is well above the norm (1.42–2.04-fold), meaning that the diet is still dominated by carbohydrates.

A similar study was carried out in the northern regions of Russia demonstrating that their population follows an imbalanced diet poor in meat, fish, seafood, milk and dairy products, unsaturated fat and rich in saturated fat and breads [5]. The populations of the North of Russia and Voronezh region have been sharing the same trend for many years consuming too much breads [12].

Over the two 5-year periods studied in this work the population of Voronezh region has been showing a 4.5-fold increase in obesity incidence, a 1.8–3-fold increase in the incidence of anemia and endocrine disorders and a 1.2-fold increase in the incidence of gastrointestinal diseases in the backdrop of imbalanced diet. These troubling trends have been

highlighted in the works of some authors who studied nutrition and nutrient-related diseases and are typical for students who do not follow a balanced diet and do not have their meals regularly [13].

The study of the factors affecting the adaptation reserve of teenagers residing in the High North and the central part of Russia revealed changes in their immune status and blood chemistry caused by imbalanced diet; adaptive stress response was more pronounced in the teenagers of Russia's High North [14].

The growing incidence of nutrient-related diseases prompts the development of prevention measures and innovative approaches to food quality monitoring in the Russian Federation [15].

The incidence ranking for the districts in Voronezh region and identification of areas at risk are just the first step towards finding a solution to a problem of providing the population with safe quality food. We believe that priority in the prevention of nutrient-related diseases should be given to areas at risk. The diet offered to organized groups should include (if appropriate) products with a strong positive effect on health, as well as those boosting natural immunity [16]. Certain legal and technical aspects of specialized food production should also be improved [17]. So far, a few successful projects have been implemented in Voronezh region aimed at preventing iodine and fluorine deficiencies [12], reducing the level of food contamination based on the investigation of the associations between food quality and free radical oxidation [18]. At the same time, the problem of proper nutrition is complex and necessitates further elucidation of many of its aspects.

CONCLUSIONS

This study shows that the diet of the Voronezh region population is characterized by excess consumption of carbohydrates (breads and potatoes) and the lack of foods with high biological value (milk and dairy products, fresh fruits and vegetables). The incidence of nutrient-related diseases, such as endocrine disorders, obesity, and gastrointestinal diseases, is growing in the backdrop of imbalanced nutrition.

Our findings allowed us to identify the areas at risk of nutrient-related diseases and can be used to optimize the composition and quality of population diets.

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HYGIENIC CHARACTERISTICS OF THE CHEMICAL COMPOSITION OF GROUNDWATER IN RYAZAN REGION

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Among the factors that have a strong impact on public health the environment, living conditions, food and water quality are just as important as socio-economic forces. Providing the population with access to safe potable water has become a socio-economic priority in Russia. The aim of this work was to characterize the aquifers supplying the population of Ryazan region with water for personal and domestic needs and to compare their chemical composition. Sample collection was performed in cooperation with the Center for Hygiene and Epidemiology (Ryazan region). The obtained data were processed using ANOVA. The Kashirsky and Ozersko-Khovansky aquifers turn to be the most commonly used ones supplying water to 30.7% and 27.3% of the total artesian wells. The Oksko-Tarusky and Podolsko-Myachkovsky aquifers rank second, feeding 21% and 18.9% of the wells, respectively. The share of the Kasimovskiy aquifer in the total water supply is only 2.1%. Although the recommended lifespan of an artesian well is 25 years, two-thirds of the wells in Ryazan region have been in service for 26 to 50 years, and one in every 4 wells is over 50 years old. The chemical composition of the groundwater drawn from different aquifers is different. High concentrations (0.7 mg/l) of iron (Fe²⁺) are present in the water from the Ozersko-Khovansky aquifer ($p \geq 0.05$). Sulfates are found in abundance in the Podolsko-Myachkovsky and Ozersko-Khovansky aquifer. The water from the Oksko-Tarusky aquifer contains high concentrations of ionized ammonia.

Keywords: aquifers, interstitial waters, artesian waters, chemical composition of artesian waters, artesian wells

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ГИГИЕНИЧЕСКАЯ ХАРАКТЕРИСТИКА ХИМИЧЕСКОГО СОСТАВА ВОДЫ ПОДЗЕМНЫХ ВОДОИСТОЧНИКОВ РЯЗАНСКОЙ ОБЛАСТИ

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Среди факторов, влияющих на здоровье населения, кроме социально-экономических, большую роль играют состояние окружающей среды, условия быта, питание, водоснабжение. Обеспечение населения доброкачественной питьевой водой является важнейшим направлением социально-экономического развития России. Целью работы было охарактеризовать подземные водоносные горизонты, используемые для централизованного питьевого водоснабжения населения Рязанской области, и провести сравнительный анализ химического состава их артезианских вод. Материалом исследования служили данные о качестве воды водоносных горизонтов Рязанской области, полученные при совместной работе с ФБУЗ «Центр гигиены и эпидемиологии в Рязанской области». Использовали метод сравнительного анализа. Статистическую обработку проводили методом дисперсионного анализа. Чаще всего в районах Рязанской области для водоснабжения населения используются Каширский и Озерско-Хованский водоносные горизонты, на долю которых приходится соответственно 30,7 и 27,3% скважин от их общего количества. Окско-Тарусский и Подольско-Мячковский водоносные горизонты используются реже (21 и 18,9% скважин соответственно). Доля наиболее редко используемого Касимовского водоносного горизонта в целом по Рязанской области составляет 2,1%. Хотя рекомендуемый срок использования артезианских скважин составляет 25 лет, две трети из них эксплуатируются от 26 до 50 лет, а каждая четвертая скважина — более 50 лет. Вода различных горизонтов области отличается по химическому составу. По сравнению с другими водоносными горизонтами, Озерско-Хованский характеризуется более высоким содержанием железа (Fe²⁺), концентрация которого составляет 0,7 мг/л ($p \geq 0,05$), Подольско-Мячковский и Озерско-Хованский — сульфатов, а Окско-Тарусский — ионов аммония.

Ключевые слова: водоносные горизонты, межпластовые воды, артезианские воды, химический состав, артезианские скважины

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Among the factors that have a strong impact on public health the environment is just as important as socio-economic conditions; this is particularly true for drinking water [1, 2]. Providing the population with access to safe potable water has become a socio-economic priority in Russia [3, 4]. When it comes to public water supply, groundwater should be preferred over surface water [5–7]. This is because groundwater has a more stable chemical composition, is less likely to be contaminated with pathogenic bacteria and has better organoleptic properties [8, 9]. At the same time, groundwater sometimes contains high concentrations of chemical elements and compounds that exceed safety thresholds, depending on the sedimentary rocks that form an aquifer [10, 11]. In Ryazan region, the majority of residential areas rely on groundwater sources [12–15]. Therefore, it is vital to monitor the quality of groundwater intended for public use. The aim of this study was to compare the chemical composition of groundwater drawn from different artesian wells across Ryazan region, the share of individual aquifers in the total water supply and the age and depth of the wells currently in service.

METHODS

Sample collection was done in cooperation with the Center for Hygiene and Epidemiology (Ryazan region). Groundwater samples were collected from every artesian well across Ryazan region once per season from 2010 through 2015 in strict compliance with the Sanitary rules and regulations (SanPIN 2.1.4.1074-01) [14]. The samples were tested for the presence of iron, sulfates and ionized ammonia. The depth and age of the wells were compared. The data were processed in Statistica 6 using ANOVA.

RESULTS

At present, there are 291 artesian wells in the region drawing water from different aquifers. The most commonly used aquifers are the Kashirsky and Ozersko-Khovansky supplying water to 30.7% and 27.3% of the wells, respectively. The Oksko-Tarussky and Podolsko-Myachkovsky aquifers have

lower shares of 21% and 18.9%, respectively. The Kasimovsky aquifer is the most rarely used one feeding 2.1% of the wells.

The quality of drinking water largely depends on the depth of the well. We compared the depth of the currently used artesian wells across Ryazan region and found out that almost two halves of them were more than 150 m deep, every fourth well reached 100 to 150 m in depth, and only 15% of wells drew water from shallower depths (Fig. 1).

The deepest wells (over 100 m) are located on the territory of the Ozersko-Khovansky and Kasimovsky aquifers, making up 64% and 66% of the wells fed by each aquifer, respectively. Wells shallower than 50 m are typical for the Oksko-Tarussky aquifer (47.8%).

Well age is an important factor in assessing the sanitary condition of the well and groundwater quality. The recommended lifespan of an artesian well is 25 years [13]. Our analysis reveals that the majority (60%) of the wells for public water supply in Ryazan region have been in service for 26 to 50 years (Table 1). About one in every 4 wells is older than 50 years, while only 14.8% of the wells have been used for less than 25 years.

This trend is typical for the majority of aquifers except for Kasimovsky; two-thirds of the wells that draw water from this aquifer have been used for 25 years, while the rest are as old as 26 to 50 years.

The analysis of the chemical composition of the groundwater samples revealed that their salinity varied between 0.2 and 0.9 g/l. There were significant differences between the Ozersko-Khovansky, Oksko-Tarussky and Podolsko-Myachkovsky aquifers in the concentrations of certain chemical compounds and elements. For example, the average iron concentrations in the Ozersko-Khovansky aquifer were 0.7 mg/l ($p \geq 0.05$), which exceeds the threshold of 0.3 mg/l established by the Russian hygiene standard 2.1.5.1315-03. Iron concentrations in the studied samples ranged from 0.035 to 8.22 mg/l and were above the established norm in 40% of cases [5]. The average concentrations of ionized ammonia in the Oksko-Tarussky aquifer differed significantly from those in the Kashirsky, Kasimovsky and Ozersko-Khovansky aquifers. Importantly, the average sulfate levels in the groundwater samples from the Oksko-Tarussky aquifer were 113.9 mg/l, differing significantly



Fig. 1. The proportion of the artesian wells of various depth feeding from different aquifers

Table 1. The proportion of the artesian wells of various age feeding from different aquifers of Ryazan region

Aquifer	Proportion of artesian wells of various age, %		
	< 25 years	26–50 years	50 years and above
Kashirsky	16.3	60.4	23.3
Ozersko-Khovansy	16.9	60.0	23.1
Oksko-Tarusky	13.6	65.5	20.9
Podolsko-Myachkovsky	2.6	58.0	39.4
Kasimovsky	64.7	35.3	0

Table 2. The average concentrations of ionized ammonia, iron and sulfates in the studied groundwater sources

Aquifer	$\bar{x} \pm tm$, mg/ml		
	Ionized ammonia	Iron ions	Sulfates
Ozersko-Khovansy	0.2 ± 0.1	0.7 ± 0.2	125.1 ± 27.9
Podolsko-Myachkovsky	0.4 ± 0.1	0.3 ± 0.1	113.9 ± 20.4
Kashirsky	0.2 ± 0.1	0.3 ± 0.1	60.5 ± 8.1
Kasimovsky	0.2 ± 0.1	0.2 ± 0.2	43.4 ± 22.4
Oksko-Tarusky	0.6 ± 0.1	0.4 ± 0.1	54.5 ± 6.1

Note: \bar{x} is the average concentration, tm is confidence interval.

from the corresponding concentrations measured for other aquifers ($p \geq 0.05$). Sulfate levels reached their minimum at 0.3 mg/l and maximum at 810 mg/l. No significant differences between the aquifers were observed in the levels of fluorides, magnesium, manganese, bromine, beryllium, and chlorides.

To sum up, the concentrations of iron, sulfates and ionized ammonia differ significantly between the aquifers of Ryazan region (Table 2).

The levels of sulfates are higher in the Podolsko-Myachkovsky and Ozersko-Khovansky aquifers than in their counterparts. The average concentration of ionized ammonia in the Oksko-Tarusky aquifer was higher than in other aquifers.

DISCUSSION

Ryazan region relies on the groundwater stored in the carboniferous and Upper Devonian water-bearing layers of the Moscow artesian basin. The majority of Ryazan aquifers are part of the carboniferous aquifer system. The aquifers studied in this article were formed by water percolating through gypsum deposits; therefore, the groundwater in them is rich in sulfates and calcium.

Our study demonstrates that groundwater samples collected across Ryazan region are different in their chemical composition, which can be explained by the history of the corresponding aquifers [1]. The Ozersko-Khovansky aquifer has increased concentrations of iron (0.7 mg/l on average) exceeding those in other aquifers ($p < 0.05$); 40% of its groundwater samples do not meet the Russian hygiene standards of 0.3 mg/l (standard 2.1.5.1315-03). Such high figures mean that measures should be taken to deferrize the groundwater drawn from these artesian wells. The groundwater drawn from Ozersko-Khovansky and Podolsko-Myachkovsky aquifers contains more sulfates (125.1 and 113.9 mg/l, respectively) than other aquifers, which again can be explained by the specifics of their formation, such as gypsum dissolution [2]. Interestingly, the Oksko-Tarusky aquifer has the highest (0.6 mg/l) average concentrations of ionized ammonia ($p < 0.05$). However, the presence of ionized ammonia is not typical for this aquifer

and can indirectly suggest organic contamination resulting from the misuse of the wells [2]. The supposition about the organic origin of ionized ammonia is underpinned by the fact that the wells drawing water from this aquifer are mostly shallow (< 50 m in depth) ($p < 0.05$) and the majority of them (86.4%) have been in service longer than their recommended lifespan of 25 years. Obviously, shallower artesian wells are at a higher risk of surface water contamination. The large proportion of old (> 25 years) wells in Ryazan region prompts their renovation [16–18].

CONCLUSIONS

In Ryazan region, public water supply relies mainly on the groundwater of the Ozersko-Khovansky and Kashirsky aquifers. The deepest artesian wells (≥ 100 m) are located on the territory of the Kasimovsky and Podolsko-Myachkovsky aquifers where they also prevail. The Podolsko-Myachkovsky aquifer feeds the majority of the old wells (≥ 50 years). The highest concentrations of sulfates are found in the Podolsko-Myachkovsky and Ozersko-Khovansky aquifers, the highest levels of iron (Fe^{2+}) are observed for the Ozersko-Khovansky aquifer, and the highest concentrations of ionized ammonia are found in the Oksko-Tarusky aquifer. The groundwater drawn from the Ozersko-Khovansky aquifer does not meet the sanitary standards adopted in Russia (standard 2.1.5.1315-03) in terms of its iron levels, which are expected to be no higher than 0.3 mg/l, and requires deferrization. Because we covered a very limited range of chemical elements and compounds in our study and the sampling schedule was arbitrary, there could be some uncertainties in the results of the analysis. Therefore, we believe it reasonable to conduct another study in order to compare the concentrations of fluorine and molybdenum in the groundwater of the same aquifers. On the whole, our study demonstrates that the quality of water drawn from the aquifers with different chemical composition should be monitored on a regular basis. Measures should be taken to deferrize the supplied groundwater and to renovate old artesian wells.

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PHYSIOLOGICAL INDICATORS OF SCHOOLCHILDREN OF 7–12 YEARS OF AGE PECULIAR TO MENTAL ARITHMETIC SESSIONS COMBINED WITH ATTENTION SWITCHING PHYSICAL EXERCISES

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Present day education routines subject schoolchildren to considerable loads associated with studying. This research effort aimed to study the effect attention switching physical exercises (the type peculiar to the mental arithmetic curricula) have on heart rate, adaptation abilities, stress resistance, higher mental functions and conditioned reflex activity of children. Examining 124 schoolchildren aged 7–9 and 10–12 years, we applied the pedagogical experiment method that includes benchmarking, formation and control stages, as well as a number of psychological methods: short-term, associative and image memory diagnostics; Bourdon-Wiersma test; search for logical solutions; evaluation of motor skills. Varicard 2.51 was used to assess functions of the cardiovascular system and other physiological state indicators. At the benchmarking stage, we discovered the differences in higher mental functions conditioned by age and gender. Girls aged 7–9 had their memory, attention, ideation indicators 20–40% higher than boys of the same age. Boys aged 10–12 had their higher mental functions developed 10–30% better than girls of the same age. At the formation stage, we registered the optimizing effect attention switching physical exercises have on information processing speed and memorizing effectiveness: corresponding indicators increased 1.5–2 times (7–9 y.o., both genders) and 1.2–1.5 times (10–12 y.o., both genders), accordingly. It was concluded that adding the attention switching physical exercises to mental arithmetic curricula makes training sessions more effective.

Keywords: schoolchildren, cognitive sphere, cognitive activity, functional activity of CVS, age psychology, mental arithmetic

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ОСОБЕННОСТИ ФИЗИОЛОГИЧЕСКИХ ПОКАЗАТЕЛЕЙ ШКОЛЬНИКОВ 7–12 ЛЕТ ПРИ ЗАНЯТИЯХ МЕНТАЛЬНОЙ АРИФМЕТИКОЙ, ВКЛЮЧАЮЩИХ ФИЗИЧЕСКИЕ УПРАЖНЕНИЯ С ПЕРЕКЛЮЧЕНИЕМ ВНИМАНИЯ

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В условиях современного образования школьники подвергаются большим учебным нагрузкам. В связи с этим целью работы было изучить влияние физических упражнений с переключением внимания, используемых на занятиях ментальной арифметикой, на показатели сердечного ритма, адаптационные возможности, стрессоустойчивость, характеристики высших психических функций и условно-рефлекторной деятельности детей. При обследовании 124 школьников 7–9 лет и 10–12 лет использовали метод педагогического эксперимента, включающего констатирующий, формирующий и контрольный этапы, а также ряд психологических методик: диагностику кратковременной ассоциативной и образной памяти; корректурную пробу Бурдона; поиск логических решений; определение моторной одаренности. Для диагностики функционального состояния сердечно-сосудистой системы и других показателей физиологического состояния использовали программно-аппаратный комплекс «Варикард 2.51». На констатирующем этапе у школьников выявлены половозрастные различия высших психических функций. У девочек 7–9 лет показатели памяти, внимания, мышления были выше на 20–40%, чем у мальчиков этого возраста. У мальчиков 10–12 лет отмечался рост показателей высших психических функций по сравнению с девочками того же возраста на 10–30%. На формирующем этапе показано оптимизирующее воздействие двигательных упражнений с переключением внимания на скорость переработки информации и продуктивность запоминания, показатели которых увеличивались у детей обоего пола в 1,5–2 раза (7–9 лет) и в 1,2–1,5 раза (10–12 лет). Сделан вывод, что включение физических упражнений с переключением внимания повышает эффективность обучения ментальному счету.

Ключевые слова: школьники младших классов, когнитивная сфера, познавательная активность, функциональная активность сердечно-сосудистой системы, возрастная психофизиология, ментальная арифметика

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Present day school curricula imply constant intensification of studying and growing volumes of information schoolchildren have to process. Extended educational activities and supernormal educational techniques utilized therein add to the load. Such conditions call for better control over adaptation capabilities, their practical application and general health of schoolchildren [1–5]. Modern psychophysiology holds vegetative balance as an integrated indicator of adaptation in the context of various activities, including studying.

It was found that psychophysical stress can disbalance sympathetic and parasympathetic regulation links and bring sympathicotonia to the foreground, which signals of imbalance in the patterns autonomic nervous system (ANS) follows when reciprocally regulating cardiac system of a child. Such conditions lead to pronounced utilization of the cardiovascular system's compensatory mechanisms associated with the child's adaptation to physical and mental exertion [3, 6, 8, 9]. Mental and physical fatigue, emotional stress make the child psycho-emotionally strained and more vulnerable, which results in acceleration of the psychophysical and social deadadaptation processes [2, 4, 7, 8].

In a considerable number of children, one part of ANS is functionally dominant, especially when the pressure coming from the environment exceeds the child's ability to adapt [4, 5, 9, 10]. Changes in the regulatory action of higher nervous function and higher mental processes (HMP) that depend on the state of the central nervous system (CNS), brain and its cortex, alter the rate of conditioned reflex reactions, disrupt interaction between the first and the second signal systems, cause psychosomatic and behavioral deviations, etc. Children with such deviations belong to the group of conditionally healthy; their adaptation processes are considerably more intense than they normally are (premorbid, pre-nosological state), which can lead to a breakdown, primarily - disruption of regulatory links in the child's physiological processes, poor mental, physical and learning performance [1, 4, 6, 9, 11–14].

During ontogenesis, interaction patterns between the body's functional systems change dynamically, and the speed of such change grows due to acceleration. At the same time, the child develops mechanisms to adapt to physical and mental loads. Readjustment of the autonomic processes regulating body functions plays an important part in the development of these mechanisms [4, 8].

Understanding the age-related features of adaptation of children by their integrated psycho-physiological characteristics that change under the influence of learning activities would allow designing optimal educational environment and curriculum system for schoolchildren and enable development of the educational process psychological and pedagogical support methods. Such measures are called for by the major contradiction embedded into the current education system: accelerated intensification of studying (i.e., mental loads) leads to progression of deficit of physical activity (i.e., physical loads) initially caused by the changing regimens of children living in urbanized environments [4, 7, 15–17]. However, many integrative ontogenesis and age-related adaptation mechanisms influenced by studying and environment present day schoolchildren live in have not been researched sufficiently.

This study aimed to research the impact educational environment factors (mental arithmetic, physical exercise, attention switching) have on the development of physiological functions and cognitive processes in schoolchildren going through the most sensitive periods (ages 7–9 and 10–12) and studying intensely. The goals were: to assess the psychophysical development of younger schoolchildren and changes thereto

under the influence of physical exercises implying attention switching (counting, arithmetic); to reveal the gender-specific differences in cognitive capabilities and physical functions of children aged 7–12; to study the effect physical exercise has on cognitive processes of younger children practicing mental arithmetic with their physiological specifics factored in.

METHODS

Participating schoolchildren

124 schoolchildren attending Eureka Intellectual Development Center (city of Togliatti) participated in the study, which lasted October 2017 through May 2018. They were 30 boys and 32 girls aged 7–9 and 30 boys and 32 girls aged 10–12. Within each gender-age category, the children were divided into treatment groups (TG) and control groups (CG). While forming the groups, we aimed at making them relatively equal in terms of the initial psycho-physiological and cognitive characteristics.

Ethical review

The study was approved by the local ethics committee of Togliatti State University (protocol #1 of 08.29.17). Parents of the schoolchildren submitted the informed consent forms allowing their children to participate in the experiment.

Inclusion criteria: ages 7 to 12; no medical contraindications to learning and physical activities. Exclusion criteria: acute infectious diseases and other medical contraindications to learning and physical activities. Parts of the brain responsible for cognition develop intensively at the age 7 through 9 [13, 14, 18, 19]. 10 through 12, the process continues; this is also the period when the individual profile of lateral specialization of the brain hemispheres' functions completes its formation [11, 20].

Mental arithmetic program

All children were taught mental arithmetic for 6 months, 45 minute sessions twice a week. The skill was mastered with the help of abacus, a special arithmetic device; the children were doing the calculations mentally, without pen and paper. Individual characteristics and current psychophysical state defined how the participants of the experiment reacted to the mental stress. With the aim to optimize psychophysical indicators, TG children were also doing motor exercises implying attention switching (AS), an addition to the program not available to the CG participants.

Physical exercises with attention switching activities

The children were offered short breaks involving a combination of physical exercises and attention switching activities in the beginning, middle and end of their mental arithmetic lessons. The attention switching physical exercises were: 1) ball games; 2) counting while playing ball; 3) counting while jumping; 4) counting and answering questions while balancing on a board. The exercises help concentration, involve most muscles controlling the child's posture and strengthening leg tendons and ligaments, improve agility and motor coordination, promote development of perception and sensorimotor coordination. Mental arithmetic lessons combined with attention switching physical exercising (counting, doing arithmetic operations) stimulate psychomotor activity; the goal is to improve mental activity, extend the range and diversity of movements, develop dexterity, flexibility, speed, coordination ability.

Pedagogical experiment method

The cognitive activity indicators were registered at the three stages of the pedagogical experiment:

1) benchmarking stage (October 2017): registration of the baseline indicators of the cardiovascular system functional status, adaptation capabilities, cognitive processes development level;

2) formation stage (December 2017 - April 2018): cognitive tasks and physical exercises used to develop motor skills and mental processes;

3) control stage (May 2018): registration of the resulting indicators after a number of lessons involving exercising designed to stimulate the children's mental and physical activity.

Psychodiagnostic methods

The psychodiagnostic program included the following psychological techniques:

- 1) short-term memory diagnostics (memorization of 10 words);
- 2) associative memory diagnostics (10 pair associations);
- 3) image memory diagnostics (16 images);
- 4) Bourdon-Wiersma test;
- 5) search for analogies and logical solutions;
- 6) evaluation of motor skills (children aged 7–12, metric scale [21–23].

Attention switching physical exercises were part of the TG lessons exclusively.

Diagnostics of the functional parameters of the body

Varicard 2.51 software and hardware system (Ramena Institute for Implementation of New Medical Technologies, Russia) was used to determine functional state of the children's CVS. We applied the heart rate variability analysis to register all the major parameters, including:

- autonomic regulation of blood flow with assessment of load on the body's regulatory systems;
- autonomic cardiac rhythm regulation (baseline autonomic regulation level, autonomic reactivity, autonomic support to activity);
- functional state of the body and changes thereto as registered through the vegetative balance and neurohumoral regulation indicators.

Based on the assessment of the body's functional state, intensity of its adaptation responses and status of the regulatory systems, we have compiled prognostic conclusions and developed recommendations that were used in this study and suggested for educational process [24].

Methods of mathematical statistics

We have applied the following mathematical statistics methods when processing the data obtained: Kolmogorov–Smirnov test (nonparametric test of the equality of continuous, one-dimensional probability distributions) and parametric Student's *t*-test. The differences were considered statistically significant at $p < 0.05$.

Kolmogorov–Smirnov test revealed that the studied variables fall within the limits of normality, which allowed applying the Student's *t*-test for interrelated and independent samples.

SPSS 17.0 for Windows was used to process the experimental data.

RESULTS

At the benchmarking of the study, the functional indicators of development of children (functional status of regulatory systems) from TG and CG were almost similar. It is known that even minor changes to the vegetative balance significantly affect schoolchildren's health, which translates into deterioration of their stress resistance and capability to adapt to physical and mental stress associated with studying.

Primary school age is characterized by fluctuating intersystem and intersystem relations (psycho-emotional, autonomic and hormonal-metabolic mechanisms). This leads to instability and degradation of the functional and adaptive capabilities, a result of the constant load put on the regulatory systems by learning activities [4, 6, 8].

To study the vegetative balance, we have registered functional status of the CVS with the help of the heart rhythm variability method.

At the beginning of the study, the stress index (S_i) was growing; this index reflects the degree of predominance of central regulation mechanisms over autonomic mechanisms. In the TG, the index was 298 conditional units, in the CG — 486 conditional units, which signals of the intensive action of the body's regulatory systems and reduced functional reserves. These results show that the increasing learning-related loads lead to mental and physical fatigue that disturbs the balance between sympathetic and parasympathetic parts of the nervous system. Children participating in the study were 7 to 12 years old; at this age, morphofunctional structures of the CNS are still immature, thus the strength and the equilibrium of the nervous processes are relatively minor, which can translate into rapid depletion of the cerebral cortex cells, fatigue and poor mental performance. It is necessary to have the children switch to another type of activity in a timely manner when they start feeling physical and mental fatigue.

In this connection, a combination of physical and mental exercises was added to the beginning, middle and end of their mental arithmetic lessons.

Also, at the benchmarking and control stages of the pedagogical experiment we have studied the children's (boys and girls, ages 7–9 and 10–12) memorization ability, attention concentration and switching capabilities, ideation and motor skills. The results were compared for the following age groups (Tables 1–4):

- 7–9 y.o.: TG (15 boys, 16 girls) and CG (15 boys, 16 girls);
- 10–12 y.o.: TG (15 boys, 16 girls) and CG (15 boys, 16 girls).

The results of memory, attention and ideation diagnostics done at the benchmarking stage show that girls aged 7–9 have better (by 20–40%) short-term, associative and image memory, as well as concentration and attention switching capabilities than boys of the same age; see Tables 1, 2. These gender-specific differences result from the fact that ontogenetically, girls develop faster than boys [18, 19]. Girls have their first growth leap 0.5–1 year earlier than boys, so the development of their psychological capabilities, which is synchronized with morphofunctional development of the body, goes faster.

At the age of 10–12 years, boys are able to memorize, concentrate, switch attention and solve logic problems 10–30% better than girls (see Tables 3, 4); the difference is gender-specific, it is a result of the peculiarities of cerebral hemispheres lateral organization profiles [17, 18, 20], which, having developed completely, make the left hemisphere more active than the right, while in girls they are equipotential.

This growth of mental activity indicators, determined by age and gender factors, signals of the growing morphofunctional readiness of the brain and mental processes to solve spatial and temporal problems [5, 20].

Having studied two age groups, we also found that girls have better motor capabilities than boys, which is the result of the former having various links of the motor analyzer (including higher cortical sections) better developed at this stage of ontogenesis.

At the benchmarking of the study, the functional indicators of development of children (functional status of regulatory systems) from TG and CG were almost similar. It is known that even minor changes to the vegetative balance significantly affect schoolchildren's health, which worsens their stress

resistance and ability to adapt to physical and mental stress associated with studying [2, 6, 9, 10]. To study these specifics, we determined the functional status of CVS through measuring variability of the heart rhythm.

When the formation stage of the pedagogical experiment was over, we examined the children's regulatory systems again. The results of these examinations show that TG participants, provided their days are scheduled correctly, benefit from physical exercise: the functional status of their bodies improves.

Table 5 shows how the heart rhythm changed in TG and CG participants at the control stage of the pedagogical experiment.

The data presented in Table 5 reflect stabilization of the functional processes; Si index (regulatory systems load)

Table 1. Comparison of memory, attention, ideation and motor skills, in points, boys 7–9 y.o., TG and CG

Variables (functions)		Boys (benchmarking stage)			Boys (control stage)			Student's t-test
		Average	Standard deviation	Standard error of mean	Average	Standard deviation	Standard error of mean	
Short-term memory	TG	4.27	1.67	0.43	8.9	1.27	0.33	11.59**
	CG	4.20	1.64	0.42	6.3	1.29	0.33	2.18*
Associative memory	TG	11.07	4.46	1.15	17.09	3.41	0.88	6.87**
	CG	11.07	4.37	1.13	12.00	3.37	0.87	2.68*
Image memory	TG	7.60	1.68	0.43	15.60	1.08	0.28	11.77**
	CG	7.51	1.65	0.43	12.40	1.60	0.41	2.27*
Attention concentration	TG	13.79	6.72	1.74	20.79	3.02	0.78	5.87**
	CG	13.70	6.69	1.73	16.70	5.99	1.55	2.88*
Attention switching	TG	5.19	2.13	0.55	4.19	2.03	0.52	2.79*
	CG	5.20	2.11	0.54	5.00	2.01	0.52	1.69
Search for analogies	TG	6.60	3.11	0.80	9.60	3.31	0.85	5.35**
	CG	6.61	3.10	0.80	7.50	3.50	0.90	1.62
Logical tasks solving	TG	14.00	8.33	2.15	19.60	3.30	0.85	9.45**
	CG	14.01	8.90	2.30	16.00	5.90	1.52	2.17*
Motor skills	TG	1.80	0.94	0.24	4.1	0.54	0.14	11.56**
	CG	1.80	0.88	0.23	3.5	0.59	0.15	2.45*

Note: * — significance level $p \leq 0.05$; ** — significance level $p \leq 0.01$.

Table 2. Comparison of memory, attention, ideation and motor skills, in points, girls 7–9 y.o., TG and CG

Variables (functions)		Girls (benchmarking stage)			Girls (control stage)			Student's t-test
		Average	Standard deviation	Standard error of mean	Average	Standard deviation	Standard error of mean	
Short-term memory	TG	5.27	1.27	0.32	9.51	1.63	0.41	11.90**
	CG	5.20	1.09	0.27	7.20	1.67	0.42	2.21*
Associative memory	TG	15.00	3.85	0.96	19.00	2.85	0.71	4.61**
	CG	14.01	2.62	0.66	14.01	3.60	0.90	2.05
Image memory	TG	10.82	2.75	0.69	15.12	2.15	0.54	5.9**
	CG	10.90	2.64	0.66	11.00	2.34	0.59	2.34*
Attention concentration	TG	20.92	6.83	1.71	26.92	3.43	0.86	3.11**
	CG	20.80	6.87	1.72	24.80	5.87	1.47	2.52*
Attention switching	TG	4.75	2.39	0.60	3.60	1.39	0.35	2.71*
	CG	4.61	2.40	0.60	4.10	1.40	0.35	2.11
Search for analogies	TG	8.73	2.61	0.65	11.5	2.41	0.60	4.03**
	CG	8.62	2.51	0.63	8.70	2.50	0.63	1.86
Logical tasks solving	TG	18.82	6.28	1.57	26.80	3.28	0.82	7.09**
	CG	18.91	5.47	1.37	22.90	5.75	1.44	2.02
Motor skills	TG	2.82	0.75	0.19	3.40	4.50	1.13	8.91**
	CG	2.81	0.75	0.19	2.91	4.75	1.19	2.71*

Note: * — significance level $p \leq 0.05$; ** — significance level $p \leq 0.01$.

values and heart rate of the TG schoolchildren are close to the physiological norm. Regulation systems work normally, body copes with stress in an optimal way, its systems are stable, which proves that the combination of mental and physical exercising and lessons' regime are correct.

By the end of the study, CG participants had high Si, tachycardia, their regulatory systems were highly active and functional reserves of the body decreased (Table 5). In such a condition, the regulatory systems are overstressed. Prolonged overstress associated with studying can compromise physiological adaptation and trigger the pathogenic chain reaction: disadaptation — pre-existing disease — development of diseases — chronic pathologies. To avoid such situations, it is necessary to organize the child's day regimen correctly,

allocate mental and physical exercising rationally and introduce motor activities to the schedule properly, which is proved by the data shown in Tables 6, 7 and Figures 1, 2.

When the formation stage of the pedagogical experiment was over, we tested regulatory systems of the participants again. The results of these tests prove that introduction of physical exercising to the mental arithmetic lessons in TG translates into optimization of the schoolchildren's movements regime. Therefore, functional status of TG participants improves (Fig. 1, Table 6), as opposed to that of CG participants (Fig. 2, Table 2), with the applicable indicators growing accordingly. Here, it is important to note the effect attention switching physical exercises had on Si: it decreased 1.8 times. Figures 3, 4 show the data backing the results.

Table 3. Comparison of memory, attention, ideation and motor skills, in points, boys 10–12 y.o., TG and CG

Variables (functions)		Boys (benchmarking stage)			Boys (control stage)			Student's t-test
		Average	Standard deviation	Standard error of mean	Average	Standard deviation	Standard error of mean	
Short-term memory	TG	6.63	1.41	0.36	9.50	0.53	0.14	6.59**
	CG	6.50	1.53	0.40	7.60	0.89	0.23	2.13
Associative memory	TG	16.13	1.73	0.45	19.40	0.73	0.19	4.87**
	CG	15.08	1.74	0.45	15.09	0.84	0.22	1.96
Image memory	TG	10.38	2.45	0.63	15.40	0.74	0.19	7.17**
	CG	10.30	2.74	0.71	13.30	1.02	0.26	2.29*
Attention concentration	TG	27.25	7.36	1.90	34.00	4.31	1.11	4.84**
	CG	27.40	6.89	1.78	29.40	6.43	1.66	2.18*
Attention switching	TG	5.60	1.80	0.46	3.40	0.97	0.25	4.12**
	CG	5.80	1.94	0.50	4.20	1.14	0.29	2.69*
Search for analogies	TG	9.25	1.98	0.51	12.00	0.04	0.01	2.99**
	CG	9.40	1.90	0.49	10.5	1.01	0.26	2.62*
Logical tasks solving	TG	23.75	5.57	1.44	28.90	1.80	0.46	3.44**
	CG	23.88	5.81	1.50	24.60	2.09	0.54	2.19*
Motor skills	TG	2.25	0.71	0.18	4.10	0.14	0.04	5.71**
	CG	2.30	0.65	0.17	3.80	0.36	0.09	2.49*

Note: * — significance level $p \leq 0.05$; ** — significance level $p \leq 0.01$.

Table 4. Comparison of memory, attention, ideation and motor skills, in points, girls 10–12 y.o., TG and CG

Variables (functions)		Girls (benchmarking stage)			Girls (control stage)			Student's t-test
		Average	Standard deviation	Standard error of mean	Average	Standard deviation	Standard error of mean	
Short-term memory	TG	5.80	1.30	0.33	9.60	0.54	0.14	6.50**
	CG	5.71	1.55	0.39	6.60	1.05	0.26	2.34*
Associative memory	TG	14.40	2.61	0.65	19.60	0.54	0.14	5.98**
	CG	14.49	2.55	0.64	14.00	0.95	0.24	2.09
Image memory	TG	9.60	2.07	0.52	14.30	0.51	0.13	8.19**
	CG	9.70	1.55	0.39	10.10	0.75	0.19	2.19*
Attention concentration	TG	23.60	4.51	1.13	37.00	3.31	0.83	4.21**
	CG	23.50	4.95	1.24	30.60	4.45	1.11	2.59*
Attention switching	TG	4.80	1.36	0.34	3.10	0.69	0.17	3.71**
	CG	4.80	1.33	0.33	4.00	1.11	0.28	2.16*
Search for analogies	TG	9.80	1.64	0.41	12.00	0.06	0.02	5.13**
	CG	9.80	1.56	0.39	10.1	1.14	0.29	2.56*
Logical tasks solving	TG	22.00	3.74	0.94	28.40	0.54	0.14	9.01**
	CG	23.40	3.55	0.89	26.20	1.34	0.34	2.32*
Motor skills	TG	2.60	0.89	0.22	4.20	0.21	0.05	11.91**
	CG	2.70	0.84	0.21	3.60	0.83	0.21	2.82*

Note: * — significance level $p \leq 0.05$; ** — significance level $p \leq 0.01$.

By the end of the study, we registered differences in functional class of TG and CG schoolchildren. That of TG is second, which is the functional class peculiar to normal operation of systems supporting a developing organism. This fact confirms the necessity to switch types of activities during mental arithmetic lessons, namely — do attention switching physical exercises. Switch to other types of mental and physical activities: 1) activates CNS; 2) prevents monotony; 3) shifts attention and softens the tiring effect of studying (Fig. 3).

Functional class of CG participants is 6, which signals of the children's regulatory systems being overstressed (Fig. 4). Such a situation invites consideration of activity switching breaks as part of the lessons. Such breaks would help to prevent overstrain and depletion of the body's adaptation reserves, thus preserving health of schoolchildren while their academic loads grow.

Thus, the study confirms that incorporation of the attention switching physical exercises into mental arithmetic curricula improves mental performance and psychomotor capabilities of younger schoolchildren.

DISCUSSION

Many researchers, such as N. I. Shlyk, R. M. Baevsky, E. D. Sinyak, D. L. Sonkin and others, are studying physiological

adaptation of a child's organism to learning activities. Their research focuses on reasoning the methods of selection of physical movement loads adequate to both training and physical culture lessons with the children's physical abilities factored in. In this study, we have also considered the psychophysical indicators that allow allocating schoolchildren to certain functional classes. However, it is important to utilize the high playing potential of children [25]. With this in mind, we introduced the attention switching physical exercises into mental arithmetic lessons, which made the latter more exciting.

In the context of the study conducted, attention switching physical exercising added to the mental arithmetic lessons improved HMP of younger schoolchildren: they showed better memorizing abilities (short-term, associative, image memory), their voluntary attention, ideation and motor skills improved. It can be assumed that the positive dynamics of HMP are the result of prevention of monotony and optimization of balance of excitation/inhibition processes in the cerebral cortex.

Memory, attention and motor skills testing shows their improvement by a factor of 1.5–2 after incorporation of physical exercises into the mental arithmetic curricula. This growth of functional indicators under the influence of attention switching physical exercises confirms they contribute to psychomotor development of younger schoolchildren.

Table 5. Heart rhythm indicators, control stage, pedagogical experiment, TG and CG

Name	TG	CG	Norm
Heart rate (HR), beats/min.	71	92**	55–80
Standard deviation (SDNN), ms	55.8	51.4	30–100
Variation coefficient (VC), %	6.6	7.9	3–12
Stress index (Si), c.u.	114	227**	50–150
Index of centralization (IC), c.u.	1.3**	1.3**	2–8
Atyp index, c.u.	2	6*	1–3

Note: * — significance level $p \leq 0.05$; ** — significance level $p \leq 0.01$.

Table 6. Regulatory systems indicators, control stage, pedagogical experiment, TG

Functioning level	
Moderate bradycardia	-1
Regulation stability	
Normal rhythm stability	0
Vegetative homeostasis	
Normal vegetative balance	0
Sympathetic vascular center activity	
Normal activity of vascular center	0
Regulation centralization degree	
Central regulation levels — moderate decrease in activity	-1

Table 7. Regulatory systems indicators, control stage, pedagogical experiment, CG

Functioning level	
Severe tachycardia	2
Regulation stability	
Normal rhythm stability	0
Vegetative homeostasis	
Moderate predominance of sympathetic nervous system	1
Sympathetic vascular center activity	
Moderately increased activity of vascular center	1
Regulation centralization degree	
Central regulation levels — sharp decrease in activity	-2

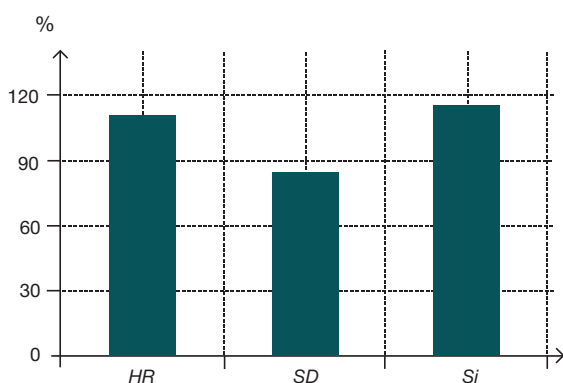


Fig. 1. Heart rhythm indicators, control stage, pedagogical experiment, TG: HR — heart rate, beats/min.; SD — standard deviation, ms; Si — stress index, c.u.

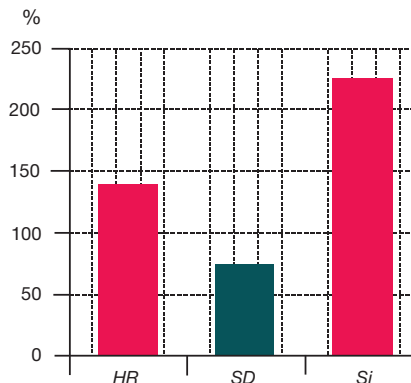


Fig. 2. Heart rhythm indicators, control stage, pedagogical experiment, CG: HR — heart rate, beats/min.; SD — standard deviation, ms; Si — stress index, c.u.

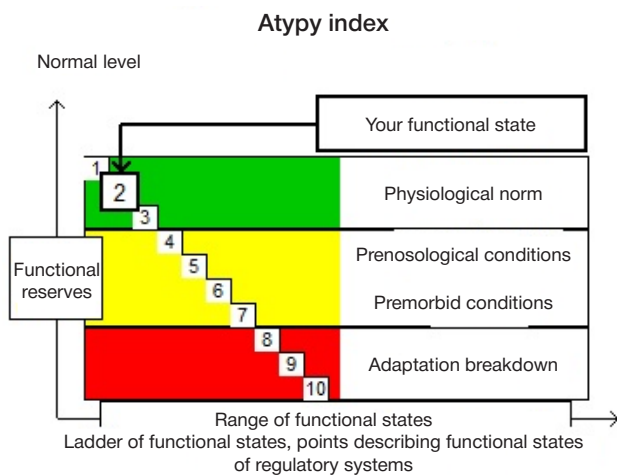


Fig. 3. Functional class diagram, TG, control stage, pedagogical experiment

Comparing the results obtained at benchmarking and control stages of the pedagogical experiment, we found the differences between them to be statistically significant ($p \leq 0.05$, all considered parameters).

Combination of mental and physical exercises prevented fatigue, normalized status of the body's regulatory systems and triggered restorative processes. The results show that TG participants, provided their days are scheduled correctly, benefit from physical exercise: the functional status of their bodies improves. A number of researchers stress the importance of fatigue prevention in children; in the long run, associated practices help to improve their health and learning performance, maintaining their CNS and ANS activity at the levels optimal for adequate functioning of the body on the whole in the context of studying [2–4, 8]. Another important observation made by the researchers is the correlation of functional status and disadaptation risk: the closer the former to the norm, the lower the latter.

Research of the causes of heterogeneity and instability of neurohumoral regulation of heart rate in children that results from constitutional genetic imbalance in AMS maturation at the certain stage of ontogenesis revealed that the mechanisms of adaptive reactions are best stabilized when the child's regime is in order (motor activity, day schedule, nutrition, sleep, psycho-emotional activity control) [1, 8, 10].

General state of the child's health should be taken into account when designing practices to improve his or her mental and physical performance. It is known that lessons without dynamic activities tend to tire children quicker. Thus, mental

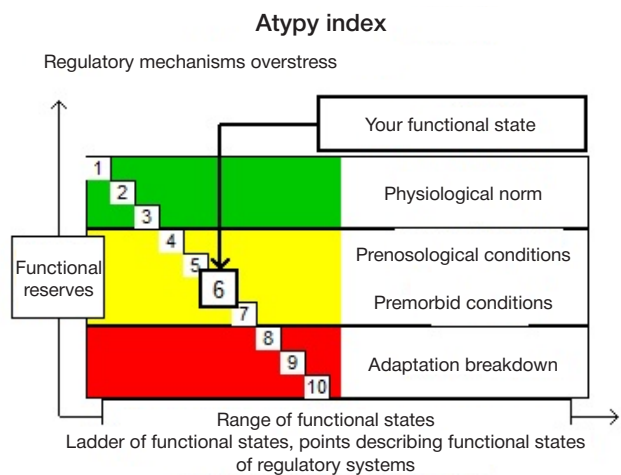


Fig. 4. Functional class diagram, CG, control stage, pedagogical experiment

arithmetic sessions should be short and supplemented by physical activities implying playing and attention switching, including ball games, jumps, balancing on a board, counting while playing with a ball and jumping a rope. The research conducted confirms that such exercising improves mental performance and psychomotor capabilities of younger schoolchildren.

CONCLUSIONS

Comparison of the HMP indicators at the beginning of the study revealed gender-specific differences in children aged 7–9. Cognitive processes and motor skills of girls, whose ontogenetic development is faster at this age, are 20–40% better than those of boys. At the age of 10–12 years, higher mental functions and motor skills of boys are 10–30% better than those of girls, which is the result of gender-specific activation of left hemisphere.

In TG, incorporation of attention switching physical exercises into mental arithmetic lessons improved the children's cognitive processes, attention, motor skills, adaptation and stress resistance abilities, and optimized functions of the body's regulatory systems; the improvement was statistically significant. In CG, which had mental arithmetic lessons without physical exercises, no pronounced improvement of cognitive processes and operation of functional systems of the body was observed. The results of this study allow recommending incorporation of the attention switching physical exercises into mental arithmetic lessons.

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URINARY DISORDERS AND BLADDER-BOWEL DYSFUNCTION IN CHILDREN: APPROACHES TO DIAGNOSIS, TREATMENT AND PREVENTION

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Pediatric urinary disorders manifested as urinary incontinence, compelling urges to urinate, etc. remain an important problem of contemporary healthcare. In spite of being extensively covered in the literature, urinary disorders, including enuresis, still present a diagnostic challenge to the physicians of primary healthcare facilities. Based on the findings of our retrospective study that revealed discrepancies between referral and final diagnoses of pediatric urinary disorders, we give recommendations to both physicians of primary healthcare facilities and their inpatient care colleagues that will help them to arrive at the correct diagnosis of a urinary disorder or concomitant bladder-bowel dysfunction using the proposed diagnostic algorithm.

Keywords: children, urinary disorders, urinary incontinence, enuresis, encopresis, diagnosis and treatment, diagnostic difficulties

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НАРУШЕНИЯ МОЧЕИСПУСКАНИЯ И СОЧЕТАННЫЕ НАРУШЕНИЯ ФУНКЦИИ ТАЗОВЫХ ОРГАНОВ У ДЕТЕЙ: ПОДХОДЫ К ДИАГНОСТИКЕ, ЛЕЧЕНИЮ И ПРОФИЛАКТИКЕ

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Нарушения мочеиспускания у детей, проявляющиеся недержанием мочи, нестерпимыми позывами к микции и т. д., являются на сегодняшний день одной из актуальных проблем отечественного здравоохранения. Несмотря на широкое освещение данной проблематики в отечественных и зарубежных источниках литературы, у врачей особенно первичного звена здравоохранения регулярно возникают сложности в правильной диагностике расстройств мочеиспускания, в том числе энуреза. По результатам ретроспективного анализа расхождения направительных диагнозов (из первичного звена здравоохранения) и заключительных клинических диагнозов у детей с нарушениями мочеиспускания даны рекомендации по правильной диагностике расстройств мочеиспускания, а также сочетанных нарушений функции тазовых органов; предложен алгоритм диагностики данных нарушений.

Ключевые слова: дети, нарушения мочеиспускания, недержание мочи, энурез, энкопрез, диагностика и лечение, трудности диагностики

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In the past 25–30 years urinary disorders (UD) in children have been a matter of serious concern for pediatricians, nephrologists and urologists. Of all urinary conditions affecting the urodynamics of the lower urinary tract, UD are the most prevalent and comprise a wide range of disorders caused by the impaired storage or voiding function of the bladder [1, 3–6].

Etiology and pathophysiology of urinary disorders in children

UD have different causes, including stress, impaired innervation, neurosis, sudden changes in the psychosocial environment, genetics, and infections of the urinary tract. A lot of attention has been recently paid to the psychosomatic aspects of UD [2, 7–10].

Micturition is a complex reflex act of excreting the accumulated urine from the bladder through the urethra; it is the final stage of eliminating urine from the body. In newborns,

infants and toddlers the bladder voids involuntary [4, 11, 12]. In older children, uncontrolled micturition signals the delayed development of the mature reflex and often results from late toilet training. Children are able to exert partial daytime control over their bladder at 2–3 years and achieve full dryness during the day by the age of 3–4 once their micturition centers in the brain and the spinal cord have matured and the adult myelination pattern of peripheral nerve fibers has been attained. By the age of 4–5 years children are able to stay dry through the night; the girls develop this ability earlier than boys. Full bladder control ensures adequate socialization. However, the mature micturition reflex is fully formed only when the child has learnt to voluntarily control the external periurethral sphincters, hold urine during the day and at night and intentionally halt its flow [3, 4, 8, 13].

Parental behavior plays a critical role in the formation of voiding habits. Parents are advised to start teaching their child good hygiene and introduce potty training at the age of

15–18 months encouraging socially acceptable behavior. The child quickly gets the hang of using the potty through positive reinforcement. Everything matters in toilet training, even the shape of the potty. Russian and foreign pediatricians usually recommend to start training at about 18 months [14]. The potty should have a simple shape, a solid color, a comfortable seat, be made of certified and easy-to-wash materials. Toy- or chair-shaped all-in-one potties in flashy colors should be avoided to prevent the child from associating the potty with playing, eating or sleeping [10, 15, 16]. The child gradually develops control over their bladder and bowel and feels satisfaction of becoming master of their own body. In this period the parents should be reassuring and patient, provide a positive feedback and create a friendly atmosphere in the family. Even the slightest stress or punishment for being wet or soiled can lead to diurnal/nocturnal enuresis, fecal incontinence and/or chronic constipation in 70 to 75% of children [1, 2, 10, 17]. Attaining continence is a milestone in the social, mental and psychosexual development of the child and a direct outcome of parental efforts [2, 17–19].

In the past 10 to 15 years absorbent hygiene products, such as disposable diapers, fitted briefs, pads, etc. have become increasingly popular among the parents of incontinent children [15, 18]. They are convenient, practical and make the life of such children more comfortable. But in fact, their continuous use aggravates the situation. The child gets used to being dry while being incontinent and stops controlling their bladder at all. This has a profound impact on the child's personality because delayed adaptation to social norms inevitably leads to immaturity. Although the materials diapers are made from are "breathable" and meant to prevent the genital area from overheating, their continuous use can still pose a threat to male health because the genitals stay too warm under a diaper [8, 11, 15, 20].

Based on our personal observations and the published literature, we recommend that parents of incontinent children should gradually eliminate diapers or other absorbent products as the first step towards recovery. It can be a real challenge because ironically it is usually the parent and not the child who benefits from diapers. Parents will often go in denial thinking that as long as the child stays dry wearing a diaper both at night and during the day there is no real problem; still the child keeps passing urine involuntarily unable to control the process. It can be much harder to persuade the parents to stop using diapers than to rehabilitate the child [12, 15, 21, 22].

Some research works in neurophysiology and urology link UD and impaired urodynamics to the developmental delay of higher brain centers involved in the autonomic regulation of the lower urinary tract. Substantial contribution to UD is made by the hypofunction of the hypothalamus–pituitary complex. Electroencephalography often detects abnormal electrical activity in the brain of incontinent children. Hypertonia and hyperactive sympathetic and parasympathetic divisions of the autonomic nervous system are another common cause of daytime incontinence. Disturbances in the regulation of the sleep/wake cycle by the limbic system and reticular formation and abnormal production of adrenal, growth or sex hormones can also result in UD [1, 4, 8, 10].

Some authors have demonstrated that UD severity is determined by the severity and character of nervous system pathology. Children who receive adequate treatment aimed at restoring the normal function of higher brain centers involved in the autonomic regulation of micturition make a complete recovery from incontinence. This is key to the effective therapy of UD in children [5, 9, 17, 23].

It has been established that about 90% of UD are functional meaning that they can be managed using a wide arsenal of effective conventional medical and nonmedical therapies, as well as psychological techniques. Importantly, though, neurological deficits that suppress maturation of centers exerting control over micturition resolve at different rates in different children. Therefore, treatment should be tailored to an individual patient to produce the desired outcome [1, 11, 24, 25].

Clinical manifestations of UD are very diverse: from different combinations of diurnal/nocturnal incontinence patterns to changes in the frequency or volume of the voided urine to urges to urinate. The most common UD are the urge syndrome (US) manifested as pollakiuria, a compelling urge to urinate and urge incontinence and enuresis. Its prevalence in children is estimated to reach 10%. In patients with renal and urologic diseases US prevalence is as high as 50–60%. Incontinence is not an exclusively pediatric condition; it also affects between 1–2% and 5–25% of adults [4, 13, 16, 25].

There is still an ambivalence about passing urine and stools felt by the modern society that often interferes with timely diagnosis and treatment of incontinence. On the one hand, people are constantly reminded that micturition and defecation are natural important aspects of human existence like sleeping, eating, etc. On the other hand, they are perceived as dirty or disgraceful, and discussing them (even with a physician) is believed to be inappropriate and vulgar. They are a taboo subject, an embarrassing issue that should be kept in secret. Therefore, patients do not realize that bladder or bowel disturbances they may be experiencing are a sign of pathology and do not rush to seek medical advice until it becomes a real problem. This explains the disparity in the results of randomized studies that report incontinence in 1–25% of adults caused by ethical and psychosocial issues: people find it difficult to tell the doctor that they have "accidents" [8, 13, 15, 23].

Enuresis is leakage of urine during sleep (more often at night time than during the day) at the age when full control over urination is expected to be achieved. According to Russian authors, full control over bladder should be attained by the age of 5 years. Foreign authors believe that 6 years is the right time. Enuresis is estimated to affect 2.3–30% of children between 5–6 and 15 years of age; of them the proportion of 5-year-old children is 15–20%; 6–8-year-old, 7–12%, and 15–18-year-old, 1.5–4%. In the past, the medical community (and ICD-10) distinguished between primary and secondary enuresis. Primary means that the child never had control of their bladder, secondary means that there was a period of dryness before the child became incontinent. At present, more attention is paid to how the condition progresses [1, 4, 19, 26]. The International Children's Continence Society (ICCS; 2011) has proposed to classify enuresis into monosymptomatic and nonmonosymptomatic. Monosymptomatic enuresis is involuntary voiding of discrete amounts of urine during nighttime sleep in the absence of daytime urinary symptoms suggestive of storage/voiding dysfunction of the bladder. Nonmonosymptomatic enuresis is a combination of nocturnal and diurnal wetting. Diurnal incontinence is wetting at daytime when the child is awake (at night the child stays continent). The reported prevalence of mono- and nonmonosymptomatic enuresis in children varies. Some authors estimate that monosymptomatic enuresis is found in 43% of children whereas nonmonosymptomatic, in 57% of children; others report that monosymptomatic enuresis occurs in 68.5% patients whereas nonmonosymptomatic, in 31.5% [3, 7, 8, 11]. According to our observations, monosymptomatic enuresis strikes 24% of children and

nonmonosymptomatic, 58%. Diurnal incontinence occurs in 18% of patients.

Agitation and stress are often accompanied by frequent urination, which in turn can trigger or aggravate incontinence. Usually, nocturnal enuresis can be kept secret, except when the child has to stay in other people's company round the clock. In contrast, involuntary urination at daytime often occurs in public places and is more emotionally traumatic for the child [2, 10, 18, 25].

Today, UD still remain a pressing concern for healthcare practitioners. The condition can be aggravated by social stereotypes, family attitude and the lack of medical awareness. In the majority of cases, voiding dysfunction is perceived as a shameful situation, lack of hygiene skills, "bad manners" or even a sign of mental retardation instead of being considered a serious health condition that should be reported to the doctor and treated adequately. Urinary disorders are usually swept under the carpet or ignored by parents who consider incontinence to be a "unique" feature of their child and a variation of the norm or punish the child or wait until the child will outgrow it and therefore do not seek medical advice [15, 20, 21].

Urinary disorders such as incontinence, pollakiuria, or frequent urges to urinate, are not life-threatening but have serious social implications. They significantly impair the psychological development and physical activity of a child, leave them feeling handicapped, instill guilt, prevent them from integrating into the society, and negatively affect the quality of life of the whole family [8, 22, 25]. Problems with peers, academic underachievement and family conflicts are common consequences of UD. About 30% of UD result in recurrent cystitis, vesicoureteral reflux, ureterohydronephrosis, pyelonephritis and subsequent nephrosclerosis or renovascular hypertension. UD and bladder-bowel dysfunctions are often accompanied by pelvic vein incompetence and weakness of pelvic floor muscles. In adults these conditions can cause sexual dysfunction (impotence, premature ejaculation or infertility in men and anorgasmia, vaginismus, prolapse, or miscarriage in women). Having an onset in childhood, UD and BBD debilitate economically active population groups and deteriorate their quality of life [8, 23, 27].

Difficulties in the diagnosis and treatment of urinary and defecation disorders in children

Extensive observation of our patients with UD has revealed a few negative trends in the primary healthcare system (mostly organizational problems) resulting in the delayed or misdiagnosis of UD and concomitant pelvic floor dysfunction in children.

Table 1. Symptoms of bladder-bowel dysfunction in the examined children

Symptoms of BBD	Boys		Girls		Total	
	Abs. number	%	Abs. number	%	Abs. number	%
Enuresis + US + chronic constipation + encopresis)	93	4.6	161	7.8	254	12.4
Enuresis + chronic constipation + encopresis	147	7.2	53	2.6	200	9.8
Enuresis + US + encopresis	53	2.6	67	3.3	120	5.9
Enuresis + encopresis	40	1.9	14	0.7	54	2.6
Enuresis + chronic constipation	27	1.3	12	0.6	39	1.9
Enuresis + US + chronic constipation	12	0.6	15	0.7	27	1.3
US + chronic constipation + encopresis	28	1.4	25	1.2	53	2.6
US + chronic constipation	3	0.1	49	2.4	52	2.5
US + encopresis	13	0.6	28	1.4	41	2
Total	416	20.4	424	20.7	840	41.1

In the past 20 years approaches to the diagnosis, treatment and rehabilitation of patients with functional UD and bowel comorbidities have changed dramatically both in Russia and abroad. Medical treatment has been largely replaced with nonmedical or combination therapies. Russian physicians have a great variety of treatment options at their disposal including anticholinergic drugs, surgical interventions to alleviate the symptoms (these are rarely used in children), physical therapy, acupuncture, psychotherapy, self-hypnosis affirmations, urotherapy (bladder re-education), and pelvic floor biofeedback therapy (BFT). Each of these effective techniques is actively promoted, making it hard for a physician to choose one the patient will benefit from the most. The first-line treatment of incontinence includes nonmedical and noninvasive interventions. They are popular among physicians due to their good diagnostic and rehabilitation potential. Among such treatment options is electromyographic biofeedback therapy, which is, in essence, computer-assisted pelvic floor muscle training (Kegels) [16, 28–30]. Nonmedical therapy also includes bladder re-education, scheduled voiding/defecation, psychotherapy and wetting alarms. If nonmedical modalities are ineffective, medications and physical therapy are introduced to the regimen alone or in combination [1, 8, 24, 26].

Treatment and rehabilitation of patients with UD will be ineffective if the diagnosis is inaccurate; therefore, it is important to discuss the problem of accurate diagnosis in detail. The lower urinary tract and the distal regions of the gastrointestinal tract are supported by the pelvic diaphragm; they share blood vessels and innervation and exert similar functions of storing, holding and excreting urine or feces. Disorders of the lower urinary tract (diurnal or nocturnal incontinence, US, etc.) and the intestinal tract (constipation, soiling) can co-exist aggravating each other [8, 20, 23, 27]. Patients with concomitant disorders of the lower urinary tract and the gastrointestinal system are often referred to in the foreign literature as having bladder-bowel dysfunction (BBD). This nosology is rarely seen in the medical records of Russian hospitals. Transition from the diagnostic approach based on the symptoms of one organ system, such as the urinary or gastrointestinal tract, to functional, which relies on the assessment of the integrated contribution of organs constituting different systems, is an important prerequisite for the accurate diagnosis and successful treatment of BBD. This interdisciplinary approach allows doctors with narrow specialist expertise, such as pediatricians, nephrologists, urologists, gastroenterologists, proctologists, neurologists, etc., to have a broader perspective of the problem, establish a timely and accurate diagnosis independent of each other, and choose an adequate treatment regimen for a patient with BBD.

The importance of the interdisciplinary approach is confirmed by the data obtained during the examination and treatment of 2,043 children aged 7 to 18 years who presented with nonorganic UD in 2003 through 2013 (881 boys and 1,162 girls, mean age of 9.8 ± 3.4 years, $p = 0.05$). The nonorganic (functional) etiology of UD was confirmed by the complete examination of the urinary system. The patients constituted 3 age groups: 7–10 years, 11–14 years and 15–18 years; each of the groups was divided into the subgroup of boys and the subgroup of girls. In 840 children (41.1%), of whom 416 (20.4%) were boys and 424 (20.7%) were girls, BBD manifested itself as a combination of enuresis, US, stress incontinence, chronic constipation, and encopresis (soiling) (Table 1).

Bladder-bowel dysfunction was prevalently manifested as enuresis (34%), chronic constipation (30.6%), US (26.8%), and a combination of chronic constipation and encopresis (24.8%).

The data demonstrating the relationship between a BBD symptom and a child's age are also interesting. BBD was manifested as concomitant enuresis, US, and encopresis predominantly in boys and girls between 7 and 11 years of age and also in girls aged 11–14 and 15–18. Co-existent symptoms of enuresis, US, encopresis, and chronic constipation were observed in all age groups, except for the boys between 15 and 18 years. A combination of enuresis, US and chronic constipation only occurred in children between 7 and 10 years, whereas enuresis + soiling occurred in children between 11 and 14 years. Concomitant enuresis, encopresis and chronic constipation were diagnosed in children aged 7–10 and in boys aged 11–14. US and comorbid encopresis were observed in children aged 7–10 and in girls of 11–14. A combination of US and chronic constipation occurred only in girls in all age groups, while US, soiling and chronic constipation were coexistent in boys between 7 and 10 years of age and in girls aged 11 to 14 years. The older the children were, the rarer and less varied the BBD symptoms became.

While conducting the retrospective analysis of pediatric medical histories, we found that the diagnosis established at a primary care facility that eventually referred the child to an inpatient facility was not always concordant with the final clinical diagnosis (Table 2).

The primary diagnosis of enuresis was established in 49.7% children, whereas its actual incidence was 2.2. times lower: it was verified only in 23% of the admitted inpatients ($p < 0.01$). Physicians of primary care facilities were often unable to notice other symptoms of urinary and defecation disorders. As a result, some children did not have a verified diagnosis before they were admitted to hospital: 20.9% of them were diagnosed with a neurogenic dysfunction of the bladder of unspecified etiology. Isolated US was not specified in the referral diagnosis, but as a final diagnosis it was established in 188 (9%) of the admitted patients. At primary care facilities BBD

was diagnosed in 29.4% vs 41.1% of in-hospital diagnoses ($p < 0.01$), meaning that its actual incidence was 1.4 times higher. The obtained data indicate that physicians at primary care facilities tend to overdiagnose enuresis and underdiagnose US and BBD in children with urinary and defecation disorders. Besides, many doctors erroneously think that any urinary disorder or incontinence is a case of enuresis. However, Russian authors and ICCS guidelines (2011) define enuresis as involuntary voiding of urine during sleep. In our retrospective study, urinary disorders, both isolated and concomitant with defecation disorders, were often mistaken for enuresis, which naturally resulted in the wrong treatment strategy and poor treatment outcome.

We believe that there are two major reasons underlying over-, under- and misdiagnosis of urinary disorders and BBD: doctors working at primary care facilities do not have sufficient medical knowledge of these conditions and are too pressed for time to take full medical history and analyze the presented problem. Patients are referred to different specialists again and again but doctors cannot arrive at the right diagnosis, therefore, treatment is delayed. Unfortunately, doctors often prescribe wrong treatments that do not help the patient, which causes frustration both in parents and their child as well as dissatisfaction with the quality of healthcare.

To improve the situation, physicians should be encouraged to maintain their certification. Information about pediatric UD and BBD should be included in the syllabus of professional training courses. At present, primary care facilities focus on improving the availability and quality of medical care. Better life quality for children is one of top priorities of modern healthcare that can be achieved, among other things, through timely diagnosis of urinary and defecation disorders. Adequate treatment will facilitate children's social adaptation allowing them to cope with the challenges of the current economic situation. Today patients are free to choose a medical care facility they want to seek advice at by channeling their health insurance payments to the preferable clinic. This stimulates competition between and among private and state-funded facilities.

The schematic algorithm below was developed by the authors to help doctors working at inpatient and primary care facilities make a successful clinical diagnosis of UD and BBD in children (Fig. 1).

CONCLUSIONS

UD and BBD have various causes. To establish an accurate diagnosis of UD or BBD, a multidisciplinary approach should be applied. It is important to rule out BBD and organic pathology in children with urinary incontinence prior to starting therapy. The first-line treatment of UD/BBD includes electromyographic biofeedback therapy, urotherapy, scheduled voiding/

Table 2. Concordance of the referral and final clinical diagnoses

Diagnosis	Referral		Final	
	Abs. number	%	Abs. number	%
Enuresis (isolated)	1015	49.7	470	23
Urge syndrome (isolated)	0	0	184	9
Enuresis and urge syndrome	0	0	268	13.1
Enuresis and stress urinary incontinence	0	0	198	9.7
Infrequent micturition	0	0	83	4.1
Concomitant bladder and bowel dysfunctions	601	29.4	840	41.1
Neurogenic dysfunction of the bladder of unspecified etiology	427	20.9	0	0
Total	2043	100	2043	100

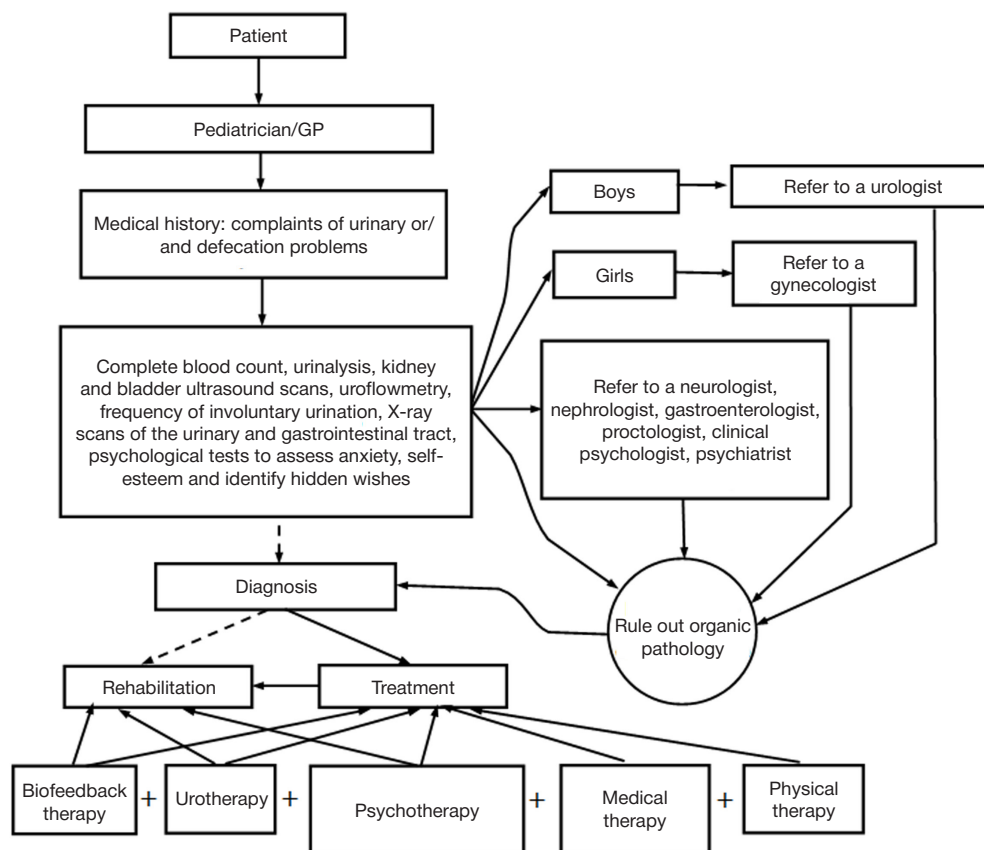


Fig. 1. The schematic algorithm for the diagnosis of urinary disorders and bladder-bowel dysfunction in children

defecation, psychotherapy, and the use of wetting alarms. Parents are advised to gradually eliminate diapers, fitted briefs or other absorbent hygiene products. If the first-line therapy does not produce the desired effect, medications, physical therapy, or a combination of both should be started. Treatment and rehabilitation outcomes can be significantly improved through maintaining the continuity of care for patients transferred from the outpatient to inpatient setting. To solve the problem of delayed diagnosis in children with UD/BBD, the following measures are proposed from the experience of

Russian healthcare practitioners and their foreign colleagues: 1) improving the quality of professional education and training; 2) increasing appointment time; 3) strengthening laboratory capacities affiliated with primary healthcare facilities and creating more hospital beds in day treatment units; 4) merging outpatient and inpatient facilities into bigger health centers that provide diagnostic, treatment and rehabilitation services to patients with UD and BBD. These measures can significantly improve diagnostic accuracy and treatment outcomes in children with UD and BBD.

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EVALUATION OF ABSORBED DOSE DISTRIBUTION IN MELANOMA B16F10 DURING CONTRAST ENHANCED RADIOTHERAPY WITH INTRATUMORAL ADMINISTRATION OF DOSE-ENHANCING AGENT

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Contrast-enhanced radiotherapy (CERT) is a binary treatment modality in which the absorbed radiation dose is not only determined by the parameters of the external radiation source but also affected by the concentration of a dose-enhancing agent (DEA) in the studied object. In this work we assessed the distribution of the absorbed dose in a murine B16F10 melanoma injected with a single dose of an aqueous Bi-DTPA solution. The mice were exposed to a single fraction of X-ray irradiation for 28.5 min. *In vivo* measurements of DEA concentrations were done on a micro-CT scanner using the radiopacity values of malignant tissues from the obtained CT images. We found that the presence of DEA enhanced the absorbed dose more than twofold in 6% of the tumor volume; in 29% of the tumor volume the absorbed dose increased more than onefold. The tumor growth delay time calculated for our model was 0.76 days (we only accounted for the damage caused directly by radiation), whereas in our previous research study tumor growth delay was 10 days. This discrepancy may indicate that in the tumors exposed to contrast-enhanced radiotherapy growth delay results from both the damage directly caused by radiation and other antitumor mechanisms.

Keywords: radiotherapy, contrast enhanced radiotherapy, melanoma B16F10, dose enhancement, CT, dose-volume histogram

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ИССЛЕДОВАНИЕ РАСПРЕДЕЛЕНИЯ ПОГЛОЩЕННОЙ ДОЗЫ ПРИ ФОТОН-ЗАХВАТНОЙ ТЕРАПИИ С ИНТРАТУМОРАЛЬНЫМ ВВЕДЕНИЕМ ДОЗОПОВЫШАЮЩЕГО АГЕНТА В МЕЛАНОМЕ B16F10

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В фотон-захватной терапии (ФЗТ) величина поглощенной дозы определяется не только параметрами облучения, но и концентрацией дозоповышающего агента (ДПА) в облучаемом объекте. В данной работе было проведено расчетно-экспериментальное исследование распределения поглощенной дозы на опухолевой модели мышинной меланомы B16F10, после однократной интратуморальной инъекции висмута в качестве ДПА в форме водного раствора комплекса Bi-DTPA. Оценку поглощенной дозы проводили для однофракционного рентгеновского облучения длительностью 28,5 мин. Количественное определение ДПА *in vivo* осуществляли при помощи микро-КТ, используя значения рентгеноплотности опухолевых тканей на полученных КТ-томограммах. В результате исследования установлено, что за счет присутствия ДПА в 6% объема опухоли поглощенная доза увеличивалась более чем в 2 раза и в 29% объема опухоли наблюдалось увеличение поглощенной дозы, отличное от 1. Время задержки роста опухоли, рассчитанное для полученного дозо-объемного распределения с учетом только непосредственного радиационного поражения опухолевых клеток, составило 0,76 суток, тогда как в ранее проведенных экспериментальных исследованиях данная величина равнялась 10 суткам. Полученное несоответствие может указывать на то, что торможение роста опухоли при ФЗТ с интратуморальным введением ДПА достигается за счет не только непосредственного радиационного поражения опухоли, но и иных противоопухолевых механизмов.

Ключевые слова: лучевая терапия, фотон-захватная терапия, меланомы B16F10, увеличение дозы, КТ, дозо-объемное распределение

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Fighting cancer is one of the top public health priorities. Radiotherapy is an effective treatment modality used in patients with different malignancies. However, its efficacy against some radioresistant tumors remains as low as 30–50% [1]. It can be improved by increasing the absorbed radiation dose at the cost of damage to healthy surrounding tissues. One of the methods of increasing the absorbed dose while sparing healthy tissues is contrast-enhanced radiotherapy (CERT), in which dose enhancement is achieved by injecting or otherwise delivering dose-enhancing agents (DEA) to the tumor. DEA are chemical elements with high Z numbers > 52, such as I, Gd, Au, Pt, Bi, etc. These elements readily absorb external X-rays and therefore can be used to enhance the dose absorbed by the tumor at the site of their uptake [2–4]. Unlike conventional radiotherapy in which tumor geometry is important, CERT precision is ensured by the tumor-tropic properties of DEA-based pharmaceuticals. The antitumor effect of CERT has been demonstrated in a number of research studies conducted in animals [5–11]. Unfortunately, the obtained results cannot be translated into clinical practice, because a treatment outcome cannot be predicted without establishing a correlation between the observed therapeutic effect and the absorbed radiation dose/its distribution in the tumor.

The hardest part of both research and clinical studies of CERT efficacy is dosimetry, which is also its least elaborated component. CERT is a binary modality; the absorbed dose and its distribution in the tumor volume are determined not only by the parameters of external radiation, but also by DEA concentration and distribution in the tumor volume.

The intratumoral route of DEA administration is used in the studies of CERT tumor suppressing efficacy both in lab animals [12–17] and in real patients in the clinical setting [18].

The aim of this work was to study the distribution of bismuth during CERT in the volume of a tumor grown from B16F10 murine melanoma cells following the intratumoral administration of its single dose and to analyze the dose-volume histogram data.

METHODS

The study was conducted in C57Bl/6 female mice weighing 20 to 22 g purchased from Stolbovaya breeding and nursery laboratory (Research Center for Biomedical Technologies of FMBA; Russia). The animals were housed in a conventional facility under natural lighting conditions. Murine melanoma B16F10 was used as a tumor model. The 14% cell suspension in 0.2 ml Hanks balanced salts prepared *ex tempore* was injected subcutaneously in the middle third of the right hind leg. DEA distribution was measured in the tumors of 6 animals once the malignancies reached ~700 mm³ in size. Tumor dimensions were measured with a caliper in three perpendicular planes and the tumor volume was calculated using the ellipsoid volume formula.

Bismuth diethylenetriaminepentaacetic acid (Bi-DTPA) used in our previous research study of CERT efficacy was chosen as DEA [15]. Properties of the Bi-DTPA aqueous solution are shown in the Table.

The animals received a single bolus intratumoral injection of 50 µl of the Bi-DTPA solution containing 5 mg of bismuth.

Distribution of DEA in the tumor volume *in vivo* was studied by micro-CT [19–21] performed on the IVIS Spectrum CT scanner (Perkin Elmer; USA). For the procedure, the mice were anesthetized with 2% isoflurane in air. The whole body scans were obtained before the injection, ~1 min after the injection, and then 3, 5, 10, 15, 20, 25, and 30 min following

the injection. Examples of CT tumor images obtained before and after intratumoral DEA administration are shown in Fig. 1. Once the scanning was completed, the anesthetized mice were euthanized by cervical dislocation.

DEA concentrations in the tumors were calculated from the radiopacity per each pixel of the tomographic image. Because the absolute radiopacity values for the homogenous object returned by the IVIS Spectrum CT scanner depend on the tomographic slice number and differ significantly between in the central and peripheral zones of a studied object [22], we calculated DEA concentrations from the difference between the radiopacity of tumor tissues measured before and after the DEA injection. Calibration curves were constructed from the scans of reference tubes containing solutions with known bismuth concentrations (Fig. 2).

To estimate the absorbed radiation dose per pixel on the CT image of the tumor, the dose enhancement factor (DEF) was calculated for the corresponding DEA concentration in this particular pixel measured in every studied time interval. DEF is a ratio of the dose absorbed by an object in the presence of DEA to the dose absorbed in the absence of DEA, irradiation parameters being the same. DEF is calculated by the formula:

$$DEF = D_{\text{DEA}}(C) / D_0 \quad (1)$$

where $D_{\text{DEA}}(C)$ is the absorbed dose at a point in an object containing the DEA concentration C irradiated with I_0 intensity at this particular point; D_0 is the absorbed dose at the same point in the same object with zero DEA concentration ($C = 0$) upon irradiation with the same intensity I_0 [23].

DEF was calculated from measured DEA concentrations based on experimental and theoretical data [2, 24]. DEF values were time-averaged in the 0–30 min interval; the same irradiation time was used in our previous work [15]. Then tumor voxels with the same average DEF values were added up to obtain dose-volume histograms. Because the presence

Table. The main physical and chemical properties of the Bi-DTPA solution

Parameter	Value
Gross formula	BiNa ₂ C ₁₄ H ₁₈ O ₁₀ N ₃
Molecular weight, Da	643.253
Metal content, wt%	34
Molarity M	0.5
Solution pH	7.4
Density at 20°C, g/cm ³	1.19
Complex stability, logK	31

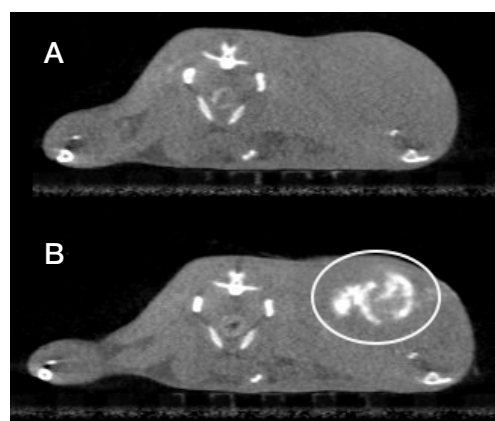


Fig. 1. The CT scans of a mouse with melanoma B16F10 before (A) and after (B) the intratumoral injection of 50 µL of the Bi-DTPA solution containing bismuth at a concentration of 104.5 mg/mL

of DEA leads to a local increase of the absorbed dose (the tumor or its part receives a radiation dose exceeding the one expected in the absence of DEA), we used modified cumulative dose-volume histograms different from those constructed for a conventional radiotherapy. The histograms demonstrate the dependency of DEF on the relative tumor volume expressed as percentages. In our study, each relative tumor volume was plotted against the minimal corresponding DEF. This type of dose-volume histograms is more informative for CERT because it visually represents the role of DEA in enhancing the radiation dose absorbed by the organ. The analysis of CT images, DEF computation and construction of dose-volume histograms were done in MATLAB (MathWorks; USA).

RESULTS

The analysis of Bi-DTPA distribution in the tumor injected with a single dose of DEA revealed that the half-life of Bi-DTPA in the tumor was 3 min. By minute 30 the tumor retained only 4% of the injected bismuth (Fig. 3).

The volume of Bi-DTPA distribution measured 1 min after the injection was $219 \pm 35 \text{ mm}^3$ ($24 \pm 1\%$ relative to the total tumor volume) (Fig. 4).

During the first two minutes after the injection there was a competition between Bi-DTPA distribution in tumor tissues and its elimination from the tumor, which kept the DEA-containing

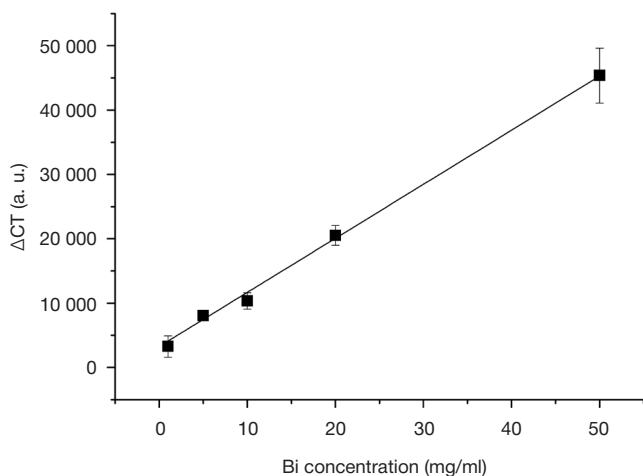


Fig. 2. The calibration curve showing the changes in radiopacity (ΔCT) detected on CT images produced by the micro-CT scanner IVIS Spectrum CT plotted against bismuth concentrations

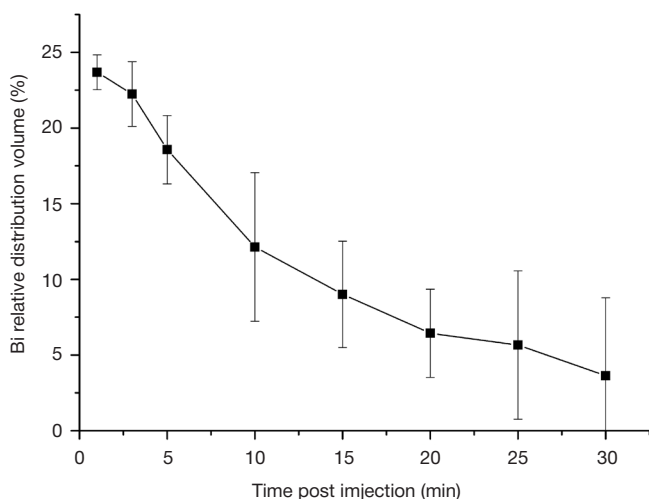


Fig. 4. The kinetics of DEA relative distribution volume in the tumor plotted against time elapsed after the injection

tumor volume unchanged. Then it started to decline gradually and by min 25 following the injection was as low as 1–7% of the total tumor volume. The modified dose-volume histograms are shown in Fig. 5.

As shown by the histograms, a twofold increase in the absorbed radiation dose was observed for 6% of the total tumor volume. Additional energy release exceeding the nominal radiation dose ($\text{DEF} > 1$) due to the presence of DEA was observed in 29% of the total tumor volume.

DISCUSSION

The obtained dose-volume histograms (Fig. 5) reveal a markedly nonuniform distribution of DEA in the tumor volume. The maximal predicted DEF value at the site of the maximum DEA concentration irradiated for 28.5 minutes is 4. This ensures an absorbed radiation dose of 80 Gy at the dose intensity of 0.7 Gy/min. However, such a significant increase in the absorbed dose was observed for only 0.1% of the tumor volume. A 1.5-fold or more dramatic increase in the absorbed dose ($> 30 \text{ Gy}$ at the same dose rate) caused by the presence of DEA was observed in 10% of the tumor volume.

In order to estimate the maximum growth delay for the irradiated tumor, the following assumptions were made:

1) the irradiated tumor grows exponentially, its doubling time being T_d [25];

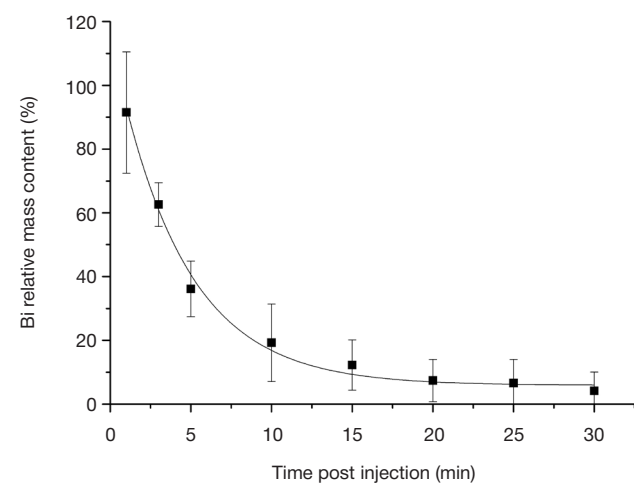


Fig. 3. The kinetics of bismuth relative mass content in the tumor plotted against time elapsed after the injection

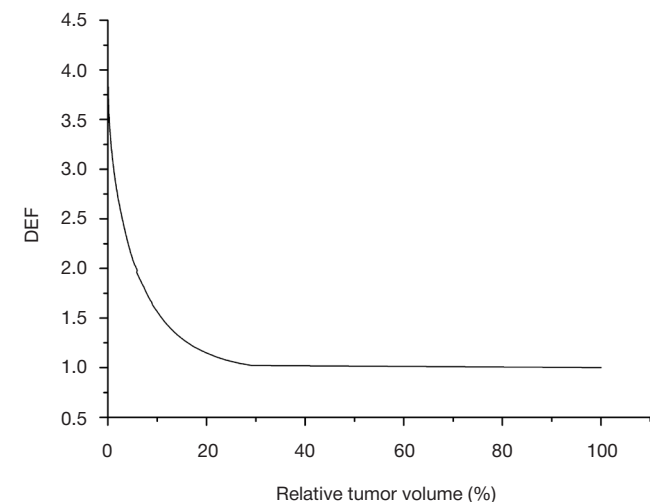


Fig. 5. The modified dose-volume histogram for B16F10 melanoma injected with DEA and subsequently exposed to X-rays for 28.5 minutes

2) the death of tumor cells is caused only by direct damage induced by radiation;

3) total cell death is observed in that 10% of the tumor volume which was irradiated with a dose over 30 Gy.

The last assumption was made to simplify the estimation of the maximum growth delay time. If some cells in the irradiated volume do survive, the growth delay will be shorter, but the minimum growth delay time will not be affected. If the volume of the cells that survive irradiation in the absence of DEA is taken as V_s , then the volume of the cells surviving irradiation in the presence of DEA injected into the tumor will be $0.9 V_s$. If the doubling time is the same in both cases and equals 5 days [17, 26] and the tumor grows exponentially, then the growth delay time will not exceed 0.76 days in the tumor exposed to CERT.

However, our previous experiments [15] demonstrated a longer 10-day tumor growth delay in tumors injected with DEA and exposed to X-ray radiation in comparison with those

irradiated in the absence of DEA. Obviously, the antitumor effect observed in the B16F10 melanoma injected with DEA and subsequently exposed to a single fraction of X-ray irradiation cannot be solely linked to the direct damage caused by radiation, but is supported by other mechanisms as well.

CONCLUSIONS

The analysis of DEA and absorbed dose distribution in the tumor volume revealed that a single intratumoral injection of DEA ensures its markedly nonuniform distribution in the tumor and enhances the absorbed radiation dose. To achieve a more uniform distribution of the dose-enhancing agent, multiple US/CT-guided injections are needed. Our previous findings [15] highlight the importance of studying the mechanisms of CERT tumor suppression efficacy following DEA delivery to the tumor by the same route of administration.

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INVESTIGATION OF THE LEVEL OF DNA DOUBLE-STRAND BREAKS AND MECHANISMS OF CELL DEATH UNDER IRRADIATION OF LUNG CANCER AND MELANOMA CELLS WITH ULTRA-HIGH DOSE RATE PHOTON RADIATION

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Research into the effects of radiation delivered at ultrahigh dose rates $> 1 \times 10^7$ Gy/min to biological objects is a new promising area of radiobiology. The unique characteristics of the high-current nanosecond electron accelerator Mir-M enable its use in medical and biological research, specifically in the experiments aimed at investigating the effect of therapeutic doses at a dose rate up to 100 MGy/s. In this work we study the effects of ultrahigh dose rate photon radiation on human lung carcinoma (A549) and melanoma (MelMtp-x) cells lines and compare them with those of the therapeutic gamma unit Rokus-AM. We show that ultrahigh dose rates induce more significant damage in the studied cell lines at doses between 2 and 7 Gy, radioresistant melanoma being more sensitive to photon radiation delivered at ultrahigh dose rates.

Keywords: photon radiation, gamma radiation, X-rays, ultrahigh dose rate, apoptosis, DNA double strand breaks

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ИССЛЕДОВАНИЕ УРОВНЯ ДВУНИТЕВЫХ РАЗРЫВОВ ДНК И МЕХАНИЗМОВ КЛЕТОЧНОЙ ГИБЕЛИ ПРИ ВОЗДЕЙСТВИИ НА КЛЕТКИ РАКА ЛЕГКОГО И МЕЛАНОМЫ ФОТОННОГО ИЗЛУЧЕНИЯ СВЕРХВЫСОКОЙ МОЩНОСТИ

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Изучение влияния фотонного излучения сверхвысокой мощности (мощность дозы $> 1 \times 10^7$ Гр/мин) на биологические объекты является новым и перспективным направлением радиобиологии. Экспериментальная установка «МИР-М» обладает уникальными характеристиками, позволяющими проводить на ней медико-биологические эксперименты и изучать влияние терапевтических доз при интенсивности дозы до 100 МГр/с. Целью работы было исследовать влияние фотонного излучения сверхвысокой мощности на клетки опухолевых линий рака легкого (A549) и меланомы (MelMtp-x), провести сравнение полученных эффектов с воздействием на клетки излучения терапевтической гамма-установки «Рокус-АМ». Показано, что излучение сверхвысокой мощности имеет большее повреждающее воздействие на клетки исследуемых опухолевых линий в диапазоне доз от 2 до 7 Гр, при этом радиорезистентная линия меланомы более чувствительна к фотонному излучению сверхвысокой мощности.

Ключевые слова: фотонное излучение, гамма-излучение, рентгеновское излучение, сверхвысокая мощность дозы, апоптоз, двунитевые разрывы ДНК

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Just like chemotherapy and surgery, radiation therapy is an important cancer treatment modality. Among the problems that have been receiving a lot of attention lately are individual sensitivity of patients to radiation and the choice of adequate radiation strategy [1–3]. The efficacy of treatment can be improved by applying ultrahigh dose rate radiation, which at the same time can reduce the adverse effects of radiotherapy.

However, some authors report that increased dose rates produce no biological effects, whereas others point to severe biological damage caused by radiation with ultrahigh dose rates [4–7]. Our previous *in vitro* study [8] has demonstrated that exposure of peripheral blood lymphocytes to photon radiation with dose rates of $\sim 10^9$ Gy/s entails some effects different from those of standard dose rates used in conventional radiation

therapy. Our findings suggest that ultra high-dose photon radiation may be more beneficial for the patient in terms of its therapeutic ratio and the mechanisms of damage induced. Photon radiation delivered at ultrahigh dose rates may one day become a new component of cancer treatment.

The aim of this work was to study the effect of ultrahigh dose rate photon radiation generated by the experimental Mir-M machine on human cancer cell lines *in vitro*.

METHODS

Photon pulses were generated by the experimental high-current nanosecond electron accelerator Mir-M developed at the Joint Institute for High Temperatures, RAS (Moscow). The dose rates ranged from 1×10^9 to 4×10^9 Gy/min. Standard therapeutic doses of 1 Gy/min used in patients with malignant tumors were generated by the therapeutic Co60-based gamma-ray unit Rokus-AM.

To study irradiation effects on a biological model *in vitro*, 2 cell lines were chosen: MelMtp-x (human melanoma cells from the collection of Blokhin Cancer Research Center, Russia) and A549 (human lung carcinoma 300114 from the Cell Lines Service repository). We assessed the cytotoxic effect of both radiation types by measuring the total number of killed cells, the proportion of apoptotic and necrotic cells, and the number of double-strand DNA breaks (DSBs).

The cells were thawed and cultured following standard protocols. A549 cells were cultured in the DMEM medium

(PanEco; Russia). MelMtp-x cells were cultured in RPMI 1640 (Gibco; USA). Both media were supplemented with 10% fetal bovine serum (S1800; BioWest; France).

Irradiation of the samples on the "Mir-M" and therapeutic gamma-unit "Rokus-AM" were produced by the described methods [8].

The cytofluorometric analysis was performed on the Flow Cytometer Cytomics FC 500 (Beckman Coulter; USA) equipped with an argon ion laser ($\lambda = 488$ nm).

The number of double-strand DNA breaks was estimated from the levels of phosphorylated H2A.X histone using the 17-344 H2A.X Phosphorylation Assay Kit for Flow Cytometry (Millipore; USA) according to the manufacturer's protocol.

Cell death pathways in the irradiated samples were studied 24 and 48 hours after irradiation with the help of the Annexin V-FITC Kit (Beckman Coulter; USA). The kit contains annexin V and propidium iodide (PI) and can be used to simultaneously estimate the proportion of both apoptotic and necrotic cells [8]. The significance of differences was assessed by Student's t-test. Differences were considered significant at $p < 0.1$.

RESULTS

The relative number of DSBs did not differ significantly between the A549 (human lung carcinoma) cells irradiated at standard therapeutic dose rates and those exposed to ultrahigh dose rate radiation (Fig. 1A). For MelMtp-x cells irradiated with standard therapeutic doses generated by Rokus-AM, the dose-effect

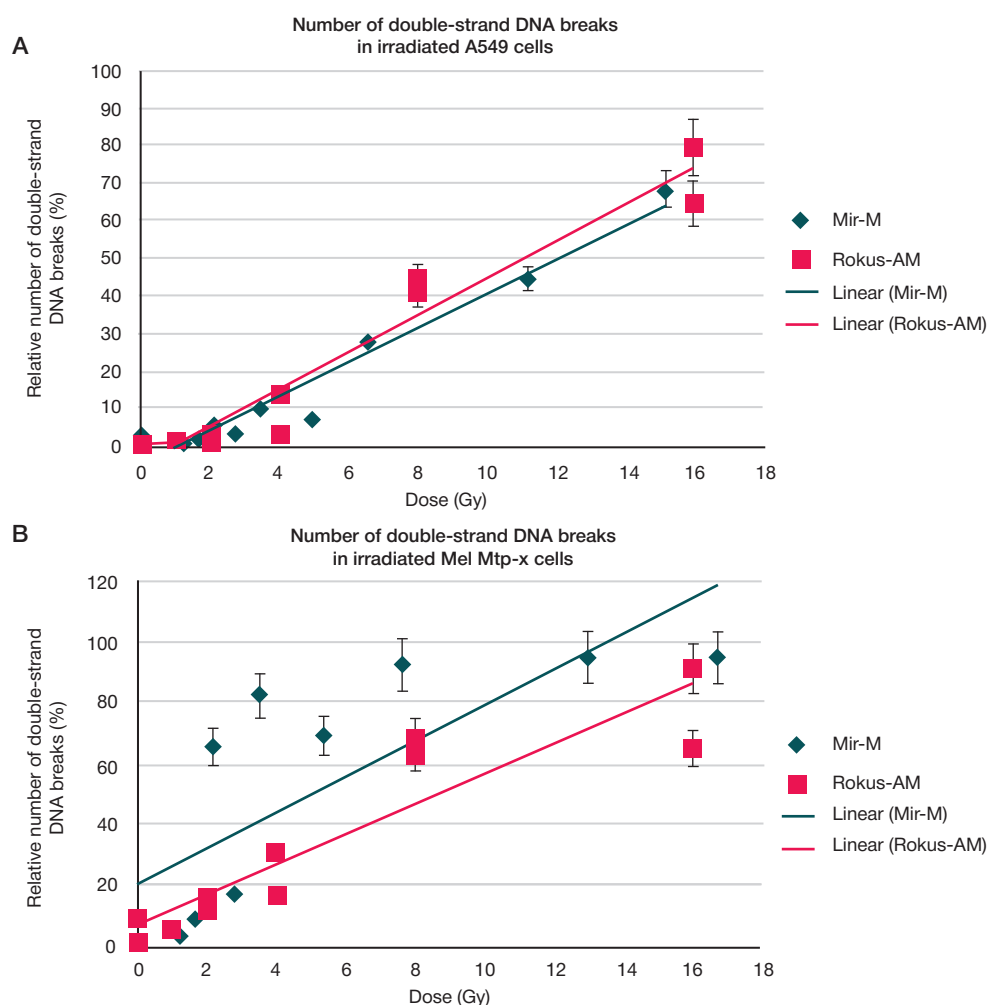


Fig. 1. Changes in the relative number of double-strand DNA breaks in A549 (A) and MelMtp-x (B) cells irradiated with therapeutic (Rokus-AM) and ultrahigh dose rate photon radiation (Mir-M)

relationship was linear, in contrast to MelMtp-x cells exposed to Mir-M pulses that demonstrated a nonlinear relationship (Fig. 1B). Photon pulses applied to MelMtp-x cells at doses from 2 to 5 Gy caused a sharp increase in the relative number of DSBs (65.7–80%; $p < 0.1$). For doses > 7 Gy, the levels of DSBs did not differ significantly between the two studied radiation types, reaching their maximum of 95%.

While analyzing the number of killed cells, we discovered a few different patterns possibly related to the radiation type applied and the specifics of the used cell lines (Fig. 2). The 24-h incubation of A549 cells irradiated with therapeutic gamma rays did not result in a significant increase in the number of killed cells; PI-positive cells made up only 6% of the total cells in the culture (Fig. 2A). But longer post-irradiation incubation time (48 h) caused a significant increase in the number of killed cells: 32.6% at 8 Gy and 41.2% at 16 Gy. Significant differences were also observed for the number of PI-positive cells between the MelMtp-x cultures irradiated at ultrahigh dose rates and subsequently incubated for 24 h, unirradiated controls and MelMtp-x irradiated with therapeutic gamma rays generated by Rokus-AM (Fig. 2B). The proportion of killed cells grew significantly at a dose of 1.4 Gy and higher (14.8%) reaching its maximum at 11.7 Gy (31.2%). At the same time, extended 48-h incubation of the samples irradiated by the Mir-M machine did not cause a significant rise in the number of killed cells. Interestingly, the differences in the proportion of killed cells between the cultures undergoing 48-h incubation and irradiated by different radiation sources were insignificant.

In MelMtp-x cultures irradiated by Rokus-AM the proportion of killed cells was no bigger than 7%; incubation time did not have any effect on cell mortality. Exposure to ultrahigh dose rates followed by 24-h incubation did not produce any significant therapeutic effect. But longer 48-h incubation led to a sharp rise in the proportion of killed cells in the sample: 13.4% at 2.5 Gy and 33.8% at 11.8 Gy.

The analysis of cell death pathways revealed that the contribution of apoptosis to cell death was the largest (Fig. 3). For A549 cells, significant differences in the levels of apoptosis induced by photon radiation as compared to the therapeutic gamma rays generated by Rokus-AM were observed at doses ≥ 1.4 Gy given that the cells were incubated for 24 h (Fig. 3A). When incubation time was increased to 48 h, the differences were leveled out. However, the rise in the number of apoptotic A549 cells was significant for both irradiation types: in A549 cells exposed to ultrahigh photon radiation doses of 11.7 Gy the level of apoptosis was $21.4 \pm 3.2\%$ after 24 h of incubation and $43.0 \pm 5.2\%$ after 48 h of incubation. In the culture exposed to 16 Gy doses generated by Rokus-AM and incubated for 24 h, the proportion of apoptotic cells was $4.8 \pm 0.7\%$; 48-h incubation resulted in the higher level of apoptosis ($38.4 \pm 4.6\%$).

The proportion of apoptotic MelMtp-x cells measured after irradiation with standard therapeutic gamma ray doses did not exceed 4%; incubation time did not affect cell mortality in the culture. Exposure to ≥ 5 Gy photon radiation followed by 48-h incubation led to a reliable increase in the proportion of apoptotic cells (Fig. 3B).

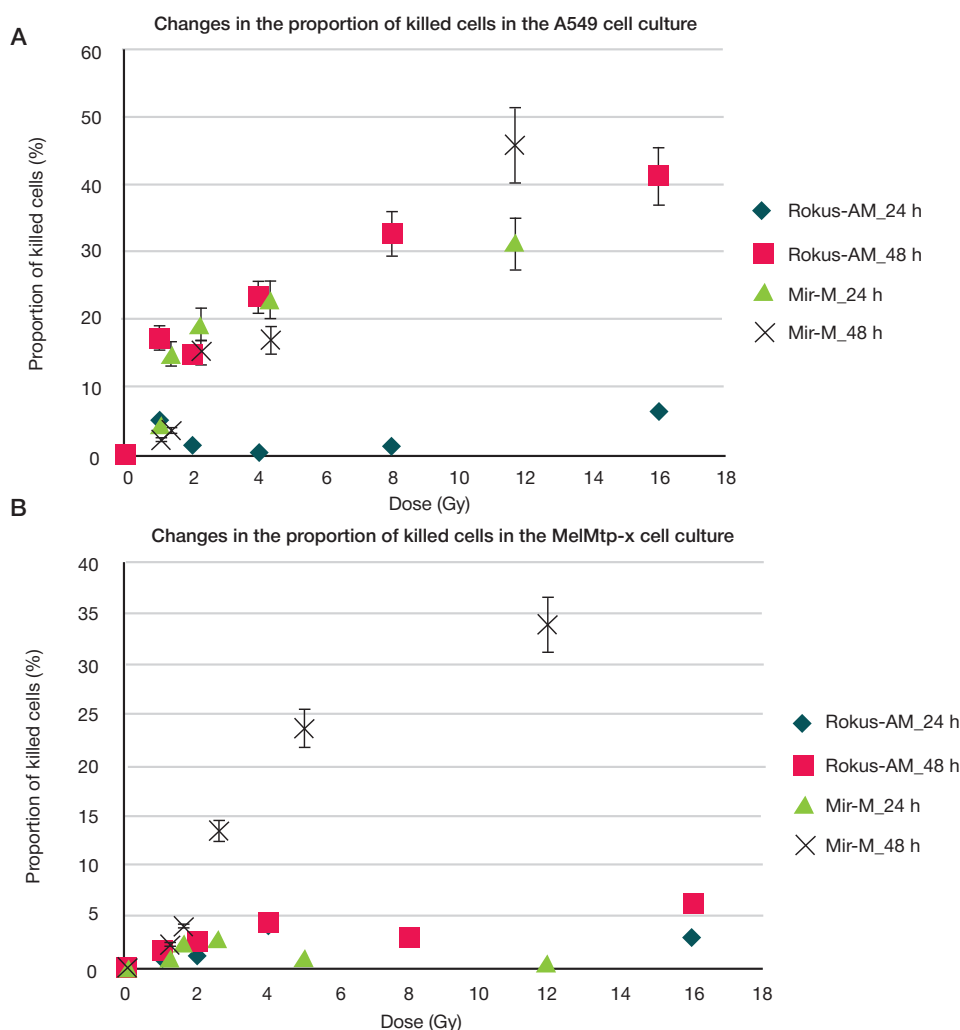


Fig. 2. Changes in the number of killed cells in A549 (A) and MelMtp-x (B) cell cultures irradiated with therapeutic (Rokus-AM) and ultrahigh dose rate photon radiation (Mir-M)

The proportion of necrotic A549 cells measured after irradiation with photon pulses generated by Mir-M was significant at 4.3 Gy (38.6% of the total killed cells in the culture) and at 11.7 Gy (30.6%) if the cells were incubated for 24 h (Fig. 4A). This proportion shrank to 7.1% and 6.1%, respectively, if the cells were incubated for 48 h. This leads to a supposition that in the A549 culture necrotic cells are eliminated within 48 hours while apoptosis induced by ultrahigh photon radiation doses goes on.

While analyzing the level of necrotic cells in the irradiated MelMtp-x culture, we found out that it did not differ significantly between the cells exposed to different radiation sources and incubated for 24 h (Fig. 4B). When incubation time was extended to 48 h, the proportion of necrotic cells increased in the samples irradiated by Mir-M with 1.58 Gy. At 2.6 Gy this proportion was $8.9 \pm 1.1\%$ making up 66.4% of total cell death; at 11.8 Gy the level of necrosis reached $17.5 \pm 2.1\%$ (51.8% of total cell death).

DISCUSSION

Our study demonstrates that cell mortality measured in irradiated A549 (human lung carcinoma) and MelMtp-x (human melanoma) cells is higher for the cells exposed to photon radiation generated by Mir-M, although the number of

induced DSBs is comparable between these two cell lines. The proportion of apoptotic cells is significantly higher in the A549 culture irradiated at ultrahigh dose rates.

The number of radiation-induced DSBs characterizes the DNA-damaging capacity of radiation and largely determines the fate of the affected cell [9]. The cell responds to this traumatic event by activating DNA repair pathways; if DNA integrity cannot be restored apoptosis is launched. Problems at any stage of DNA repair lead to chromosomal aberrations and eventually to cell death [10].

The number of DSBs estimated in our experiment is determined by the parameters of the radiation type and the state of DNA repair systems, in the first place. The relationships between the number of DSBs in A549 cells and the radiation type applied turned to be linear and almost identical, meaning that the damage caused by ultrahigh and standard therapeutic dose rates was comparable or that DNA repair mechanisms were intact in this cell line. In A549 cells irradiated at ultrahigh dose rates, apoptosis is induced 24 h after the exposure, whereas therapeutic gamma rays trigger it only 48 h after irradiation. One can assume that damage caused by the energies generated by Rokus-AM does not prevent A549 cells from activating their DNA repair mechanisms, while damage induced by Mir-M ultrahigh dose rates is soon identified by DNA repair mechanisms as

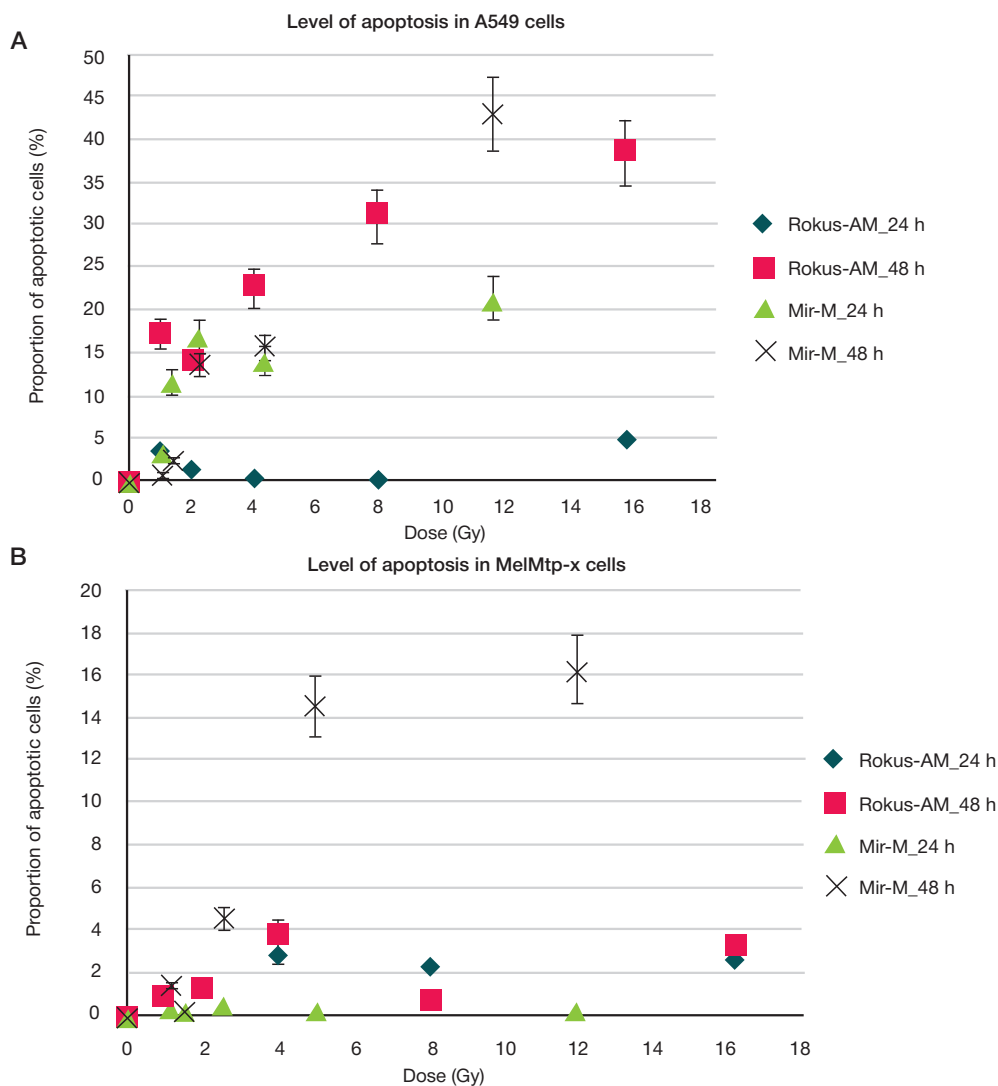


Fig. 3. Changes in the proportion of apoptotic cells in A549 (A) and MelMtp-x (B) cell cultures irradiated with therapeutic (Rokus-AM) and ultrahigh dose rate photon radiation (Mir-M). Staining: annexin V/ propidium iodide

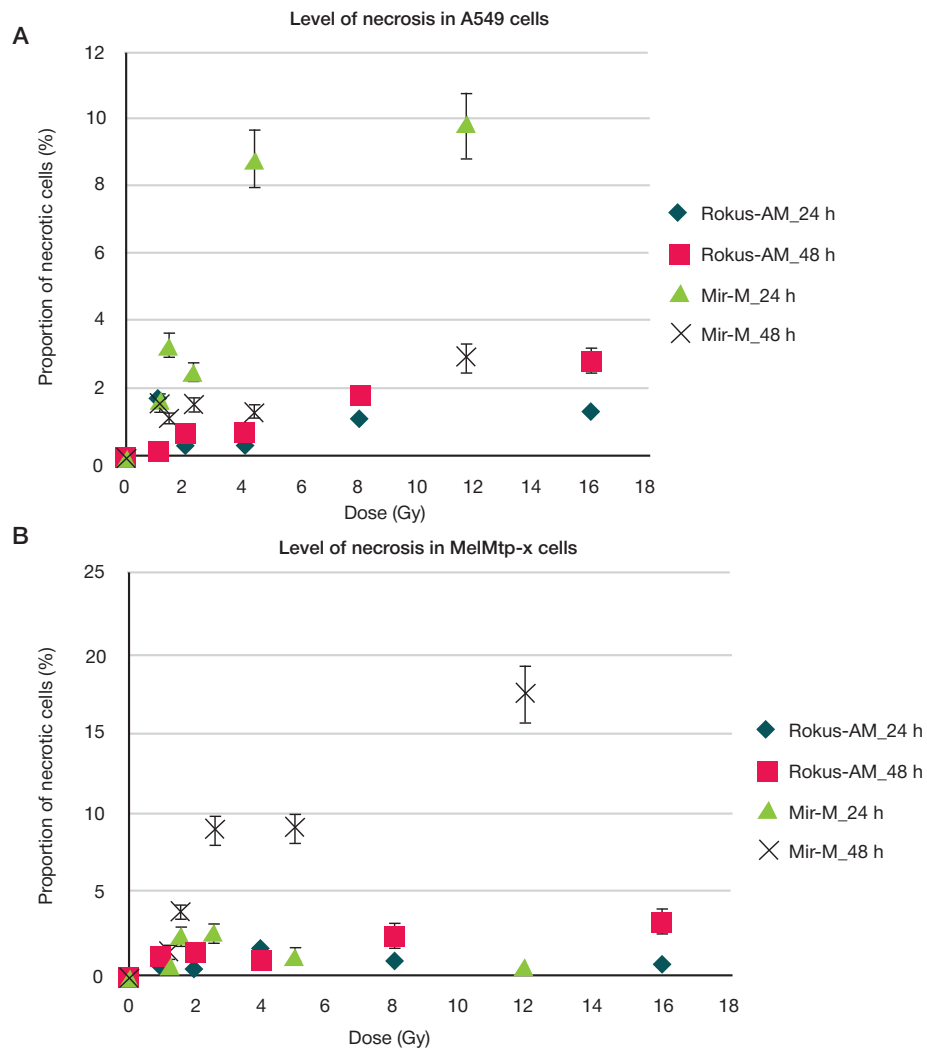


Fig. 4. Changes in the proportion of necrotic cells in A549 (A) and MelMtp-x (B) cell cultures irradiated with therapeutic (Rokus-AM) and ultrahigh dose rate photon radiation (Mir-M). Staining: annexin V/ propidium iodide

irreversible, and apoptosis is triggered as early as 24 h after the exposure. The proportion of apoptotic cells remains high 48 h after irradiation at ultrahigh dose rates but the level of necrosis drops.

The number of DSBs was significantly higher in the MelMtp-x culture irradiated with doses ranging from 2.15 to 7.6 Gy generated by the Mir-M machine. Possibly, at doses starting from ~2 Gy the type and extent of DNA damage in the cell prevent DNA repair systems from exerting their function. The results of cell death analysis in MelMtp-x cells are consistent with the reports of melanoma radioresistance [11, 12]; exposure to radiation generated by the therapeutic gamma ray machine Rokus-AM hardly induces cell death, which means that either apoptosis is not activated in response to DSBs [13], or DSB repair is effective [14]. Cell death was observed in melanoma cells irradiated at ultrahigh dose rates with the same doses (≥ 2.15 Gy) that caused an increase in the number of DSBs. Cell death was induced on day 2 after irradiation. Both apoptosis and necrosis pathways were equally involved. Perhaps, exposure of melanoma cells to ultrahigh dose rates causes their irreversible damage, which in some cases both triggers apoptosis and activates other death pathways. To sum up, we have demonstrated that radiation

generated by the experimental Mir-M machine kills significantly more cells than therapeutic gamma rays (Rokus-AM) in both studied cultures: A549 (human lung carcinoma) and MelMtp-x (human melanoma), although the number of induced DSBs is comparable between the cultures at the highest doses applied. In A549 cells irradiated by the Mir-M machine, apoptosis was more extensive.

The use of ultrahigh dose rate radiation holds promise for the treatment of radioresistant cancers and can minimize damage to the surrounding tissues when applied to solid tumors [15, 16].

Our findings may be interesting for clinicians looking for an alternative to conventional radiotherapy and for researchers studying the mechanisms of radioresistance and the ways to overcome it.

CONCLUSIONS

Our findings pave the way for further research of the effect of photon radiation delivered at ultrahigh dose rates on biological objects. This type of radiation may help to improve the efficacy of radiotherapy of radioresistant tumors and mitigate their detrimental effect on the surrounding healthy tissue.

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TESTING OF MONOCLONAL ANTIBODIES AGAINST THE T-CELL RECEPTOR ASSOCIATED WITH ANKYLOSING SPONDYLITIS

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In the last decade there has been a tendency to move away from the symptomatic treatment and embrace targeted therapies. This process is underpinned by the accumulated knowledge of the mechanisms underlying the pathogenesis of diseases and driven by the advances in biotechnologies. T-cell receptors with variable TRBV9 β -chain regions have been recently associated with spondyloarthritis including its subtype, ankylosing spondylitis. The aim of this work was to engineer a chimeric monoclonal antibody targeting the variable region of the T-cell receptor β -chain encoded by the TRBV9 gene segment and assess its specificity and cytotoxicity. Using flow cytometry and next generation sequencing, we demonstrate that the engineered chimeric antibody is highly specific and exhibits cytotoxic activity against its target. Approaches based on the use of therapeutic chimeric antibodies against pathogenic T-clones may hold great promise for the therapy of autoimmune disorders in general and AS in particular.

Keywords: autoimmune disease, ankylosing spondylitis, therapeutic antibody for autoimmunity treatment, T-cell receptor

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ТЕСТИРОВАНИЕ МОНОКЛОНАЛЬНЫХ АНТИТЕЛ К Т-КЛЕТОЧНОМУ РЕЦЕПТОРУ, АССОЦИИРОВАННОМУ С АНКИЛОЗИРУЮЩИМ СПОНДИЛИТОМ

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В последние десятилетия в лечении аутоиммунных заболеваний прослеживается тенденция к замещению симптоматической на молекулярно-таргетную терапию. Предпосылками для этого служат как установленные механизмы развития заболевания, так и прогресс в области биотехнологии. Недавно было показано, что Т-клеточные рецепторы, содержащие вариабельные участки β -цепи TRBV9, ассоциированы со спондилоартропатиями, включая анкилозирующий спондилит. Целью данной работы было получение, определение специфичности и оценка цитотоксичности химерного моноклонального антитела, взаимодействующего с вариабельным участком β -цепи Т-клеточного рецептора, который кодируется геномным сегментом TRBV9. С помощью цитометрического анализа, а также массивного секвенирования показано, что химерное антитело обладает высокой специфичностью и цитотоксической активностью. Получение лечебного антитела к потенциально патогенному Т-клону может быть перспективным подходом для терапии аутоиммунных заболеваний в целом и АС в частности.

Ключевые слова: аутоиммунные заболевания, анкилозирующий спондилит, терапевтические антитела, лечение аутоиммунных заболеваний, Т-клеточный рецептор

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Ankylosing spondylitis (AS, or Bekhterev's disease) is a chronic progressive autoimmune disease associated with HLA B2705 and characterized by the inflammation of the spine and sacroiliac, intervertebral and costovertebral joints that causes stiffness and eventually leads to spinal fusion. Treatment options include physical therapy and anti-inflammatories, mostly nonsteroidal, such as ibuprofen, diclofenac, or indomethacin. The use of steroids in patients with AS is only scarcely covered in the literature. Some authors report that high doses of prednisolone (50 mg a day) administered over the course of two weeks provide a good short-term fix [1]. Others describe a sustained symptomatic relief observed for an entire year following the intravenous injection of high doses of corticosteroids [2]. However, steroids also cause their typical side effects, including high blood pressure, elevated blood glucose and reduced bone mineral density. Patients with severe AS are prescribed phenylbutazone and opioids to ameliorate pain. The existing regimens bring partial relief but cannot prevent the disease from progressing and do not protect against ankylosis while causing complications, such as digestive tract problems, etc. [3].

The real breakthrough in the therapy of AS made in the last 15 years is linked to the use of biologic inhibitors of tumor necrosis factor alpha (TNF- α), among which are the monoclonal antibodies *infliximab*, *adalimumab*, *golimumab*, and *certolizumab pegol*. TNF is the major mediator of inflammation also involved in the progression of other autoimmune disorders [4]. In 2010 TNF inhibitors were added to the list of AS therapies recommended by the Assessment of Spondyloarthritis International Society (ASAS) [5]. Thus, *infliximab* suppresses inflammation and therefore alleviates pain and reduces stiffness [6]. However, clinical data show that only 50–60% of patients with AS respond to anti-TNF therapy [7]. Besides, although inhibition of TNF or its receptor reduces inflammation, it cannot stop the progression of the disease [8, 9].

It is known that autoimmune disorders are accompanied by overproduction of IL17, in which a few different cell types are involved, including CD4⁺ T helpers, gamma delta and KIR3DL2-expressing T-cells. A 2013 study investigating the effect of *secukinumab* (a monoclonal antibody against IL17) on AS symptoms demonstrated that the drug can mitigate the manifestations of the disease in patients with exacerbated AS [10, 11].

Anti-CD3 and anti-CD4 antibodies also may have a potential to treat autoimmune disorders in general and AS in particular due to their immunomodulating properties [12–14]. They are currently undergoing clinical trials and so far have been shown to reduce the levels of proinflammatory factors and induce production of the suppressor cytokines IL10 and TGF β [14, 15]. At the same time, anti-CD3 antibodies stimulate other T-cell populations besides Tregs and can trigger massive apoptosis of T lymphocytes [16]. Obviously, depletion of T lymphocytes is a traumatic event for the immune system that compromises the immunity, increases susceptibility to infections and cancer, and entails the risk of developing secondary autoimmune diseases.

T cells recognize antigens by means of their receptors (TCRs) consisting of two polypeptide chains (α and β). The chains are assembled during somatic recombination when V-, D- and J-segments of the gene are rearranged and juxtaposed and the terminal transferase adds random nucleotides at the site of their junction creating unique hypervariable regions (CDR). This process is key to the diversity of TCRs.

The IMGT nomenclature distinguishes between 26 different variable segments in the TCR β -chain and 41 different segments in the α -chain [17]. Normally, during the process of positive and negative selections occurring in the thymus autoaggressive T

cells are eliminated. Pathologic T-cell autoreactivity is a cause of many autoimmune conditions.

One of the approaches to the treatment of autoimmune diseases discussed in the literature is based on the use of antibodies that recognize all alpha/beta TCRs. It is described in the work [18] demonstrating that administration of an anti-TCR antibody right after the injection of myelin peptide MOG35-55 inducing experimental autoimmune encephalomyelitis (EAE) blocks the development of this condition. The authors did observe the predicted depletion of CD4⁺- and CD8⁺-cells, but these T-cell subsets behaved differently: CD4⁺ were the first to deplete and recover, while in CD8⁺ both processes were delayed. The authors noted the robust therapeutic effect of the treatment: in contrast to the CD3-based therapy, no signs of the disease were observed even after the T-cell population was completely restored. Although both therapies aim to deplete T cells, the intracellular pathways activated by the TCR-CD3-antibody complex are different and produce different effects. Monoclonal antibodies targeting variable domains typical for autoreactive TCRs seem to be a promising therapeutic tool. They cause selective elimination of T-cell subsets with pathogenic clones. The arrival of NGS technologies has given rise to new approaches based on the deep sequencing of TCR repertoires. The difficulty of such approaches lies in the great diversity of TCRs [19]. Identification of pathogenic clones is based on the comparison of TCR repertoires of healthy and diseased individuals and therefore becomes a very labor-consuming task. It is also important to account for the structural features of proteins constituting the major histocompatibility complex involved in antigen presentation as these features also affect the composition of a T-cell repertoire.

In spite of the obstacles, the T-cell clones potentially implicated in the pathogenesis of AS and the CDR3 consensus motif were finally identified in 2017 [20, 21]. The pathogenic clones were present in the synovial fluid and peripheral blood of patients with AS. Interestingly, the pathogenic TCRs were not detected in the samples of healthy individuals at the same sequencing depth regardless of whether the participants had the HLA*B27 allele or not. The identified AS-associated TCRs carried the gene segment TRBV9 (name specified in the IMGT database) coding for the variable domain of the β -chain. The obtained data suggest that anti-TRBV9 antibodies have a therapeutic potential for treating AS.

Elimination of T cells carrying a pathogenic TCR is an indispensable prerequisite for the effective therapy of autoimmune diseases. It is known that IgG1 antibodies, which have an Fc-region, induce death of the target cell by binding to it. There are two possible explanations of this phenomenon: antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In ADCC the antibody with a particular Fc binds its epitope on the surface of the target cell. An effector T cell that expresses an FcR receptor or a CD16 molecule recognizes the Fc domain and binds to it. Formation of this triple complex triggers a cascade of reactions inside the effector T cell resulting in the release of cytotoxic granules. Thus, the death of the target cell is mediated by the perforin-granzyme pathway. The effectiveness of Fc binding to the receptor of the effector T cell depends on the immunoglobulin allotype and the pattern of amino acid glycosylation. Therefore, the cytotoxic activity of the antibody can vary [22]. CDC is similar to ADCC but in CDC a complement complex is assembled on the cell surface triggering a cascade of reactions that eventually induce apoptosis of the target cell. The therapeutic antibodies modelled for the present work carried the same IgG1 Fc allotype as *rituximab*.

The aim of our study was to assess specificity and cytotoxicity of a few variants of engineered monoclonal antibodies against the variable TRBV9 domain of the TCR β -chain *in vitro*.

METHODS

Blood donors

Samples of peripheral blood were collected from two 53-year-old male donors 7 times at a minimum interval of 7 days between the procedures. The study was approved by the Ethics Committee of Dmitry Rogachev National Research Center of Pediatric Hematology, Oncology and Immunology (Protocol 2013–5/4).

Isolation of mononuclear cells from peripheral blood

Peripheral blood samples were collected into K2-EDTA-containing Vacuette tubes (4 ml of blood per tube) and diluted fourfold with PBS. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-urografen density gradient centrifugation (density of 1.077 g/cm³) (PanEco; Russia). Briefly, the diluted blood sample was layered onto the Ficoll-urografen solution at the ratio of 1:1. Then, the sample was centrifuged in the swinging bucket rotor at room temperature and 400 g for 30 min. The cell suspension was collected from the interface, washed twice in PBS, and centrifuged again at room temperature and 400 g for 10 min. PMBC count was done in the Goryaev chamber.

Chimeric antibodies and their kinetic screening by biolayer interferometry

Monoclonal antibodies MA-K1, MA-K2, MA-K3, and MA-K4 were manufactured by the biotech company Biocad and their nucleotide and amino acid sequences were published in the patent application. These antibodies contain chimeric heavy- (*H*) and light- (*L*) chains with a variable region of the rat immunoglobulin and a constant region of the human immunoglobulin. The degree of antibody humanization is 65%.

To measure the dissociation constant of the TCR/AB complex, we performed biolayer interferometry using the ForteBio Octet RED384 detection system (Pall Corporation; USA). The soluble TCR taken at a concentration of 20 μ g/ml was immobilized on the surface of AR2G sensors (ForteBio) and subsequently deactivated by 1 M ethanolamine (pH 8.5) according to the standard protocol supplied by the vendor. The temperature was set to 30 °C. The buffer used for the procedure was PBS supplemented with 0.1% Tween-20 and 0.1% BSA. After the baseline step, the sensors were dipped for 300 sec in the wells containing 67 nM antibody solutions where the TCR/AB complex was formed. Dissociation of the complex in the buffer was recorded for 600 sec. Reference subtraction was applied, and the binding curves were analyzed in Octet Data Analysis software (ver. 9.0) using the standard procedure and the 1:1 Global interaction model.

Table. Interaction of the studied MA-K1, MA-K2, MA-K3, and MA-K4 antibodies with different TCR complexes. The table features the dissociation rates and dissociation constants of the formed complexes. The measurements were done using ForteBio Octet RED384

MA	TRBV9 + TRAV26		TRBV9 + TRAV38		TRBV7 + TRAV38	
	Kd	Kdis	Kd	Kdis	Kd	Kdis
MA-K1	2,90E-10	1,79E-04	3,21E-10	2,26E-04	Does not interact	
MA-K2	<1.0E-12	<1.0E-07	<1.0E-12	<1.0E-07	Does not interact	
MA-K3	2,44E-10	1,63E-04	3,53E-10	1,23E-04	Does not interact	
MA-K4	<1.0E-12	<1.0E-07	<1.0E-12	<1.0E-07	Does not interact	

Flow cytometry analysis

To visualize their reactivity, the studied antibodies MA-K1, MA-K2, MA-K3, and MA-K4 were labeled with fluorescein isothiocyanate (Sigma; USA) following the manufacturer's protocol. The number of fluorochromes that reacted with antibodies was evaluated based on the ratio of the absorption spectra at 495/280 nm wavelengths.

A total of 10⁶ PMBC cells separated by Ficoll-urografen density gradient centrifugation as described above were incubated with each of the FITC-labeled monoclonal antibodies MA-K1, MA-K2, MA-K3, and MA-K4 taken at two different concentrations (3 μ g/ml (A) and 200 ng/ml (B)) and CD3-eFluor450 (clone UCHT1; eBioscience; USA) added in the quantity recommended by the manufacturer. The sample was transferred to 50 μ l 1X PBS supplemented with 0.5% BSA, incubated at room temperature for 20 min, and then washed in the same buffer solution.

Cell sorting and sequencing

PMBC (3 \times 10⁶) were combined with 100 μ l of 0.5% PBS/BSA solution, 6 μ l of anti-CD3-eFluor 450 (clone UCHT1; eBioscience; USA) and each of the studied FITC-labeled antibodies (one antibody variant per sample) taken at a final concentration of 100 ng/ml. The mixture was incubated at room temperature for 20 min and washed in 0.5% PBS/BSA solution.

Cell sorting and the analysis of cell subpopulations were carried out on FACSariaIII (BD; USA). To exclude debris and cells that did not meet the criteria for the size and granularity of live lymphocytes, forward and side scatter gating was applied. Two-parameter density plots were constructed to distinguish the populations of CD3⁺TRBV9⁺ and CD3⁺TRBV9⁻ cells.

To be sure of high sorting quality, we re-sorted the CD3⁺TRBV9⁺ subpopulation. The sorted cell population was 95% enriched in the target cells. The sorted cells were transferred to the RLT buffer (Qiagen; Germany) prior to RNA isolation. Total RNA was isolated using the Qiagen RNeasy mini kit #217004 according to the manufacturer's protocol. cDNA was synthesized from the obtained RNA template and the TCR beta chain fragment was amplified as described in [23]. PCR products were ligated to Illumina adaptors and then sequenced on MiSeq Illumina (Illumina; USA). Sequencing yield was analyzed in MiGEC, MiXCR and VDJtools [24] as described in [25]. Statistical processing was done in GraphpadPrism 3.0.

Cytotoxicity testing

The studied chimeric antibodies were tested for their cytotoxicity towards PMBC obtained from two donors. Samples of peripheral blood were collected into Vacuette K2-EDTA-containing tubes. Mononuclear cells were isolated as described above.

The mononuclear cell fraction was transferred to PBS supplemented with 0.5% BSA and 2 mM EDTA. The total cell number was inferred from the cell count in the aliquot. Cell viability was determined by Trypan Blue staining. To evaluate the cytotoxicity of the studied antibodies, $3-4 \times 10^6$ cells were incubated with the MA-K2 antibody taken at different concentrations (1 ng/ml, 10 ng/ml, 100 ng/ml, and 1 μ g/ml) for one hour. Following the incubation, the cells were washed twice in PBS, transferred to the RPMI media supplemented

with 10% human serum (BioIVT; UK) and incubated for 24 h in the CO₂ incubator. Then the cells were collected and stained with CD4-PE (clone RPA-T4; BD Bioscience; USA), CD8-FITC (clone SK3; eBioscience; USA), CD3-eFluor450 (clone UCHT1; eBioscience; USA), and TO-Pro3readyflow (ThermoFisher; USA) as described in the manufacturer's protocol. Staining was followed by the flow cytometry analysis on the FACS Aria III cell sorter (BD; USA).

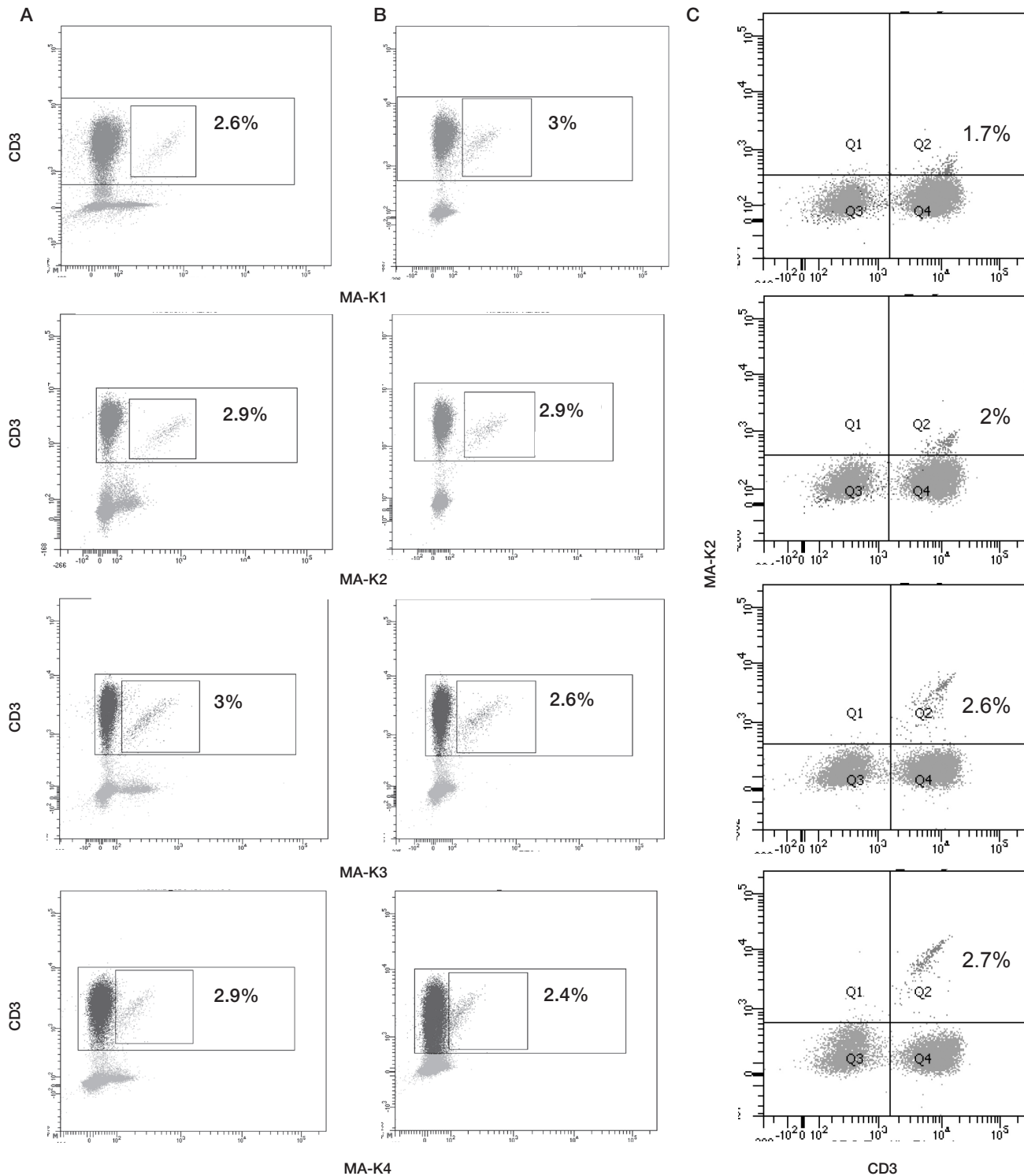


Fig. 1. Two-parameter density plots showing the distribution of mononuclear blood cells stained with anti-CD3-eFluor405 antibodies and 4 variants of FITC-labeled anti-TRBV9 antibodies (MA-K1, MA-K2, MA-K3, and MA-K4). Every tested anti-TRBV9 antibody was taken at two different concentrations: 3 μ g/ml (A) or 200 ng/ml (B) per 10^6 mononuclear cells. The CD3⁺TRBV9⁺ population is marked by a small square. The proportion of TRBV9⁺CD3⁺ cells is given relative to all CD3⁺ lymphocytes. C. Staining with different concentrations of the chimeric MA-K2 antibody: 2 ng/ml, 20 ng/ml, 50 ng/ml, 200 ng/ml (top to bottom)

RESULTS

Chimeric monoclonal antibodies MA-K1, MA-K2, MA-K3, and MA-K4 specifically bind the soluble TCRs carrying TRBV9

In this work we tested 4 variants of the monoclonal anti-TRBV9 antibody (MA-K1, MA-K2, MA-K3, and MA-K4) different in the amino acid sequence of their hypervariable CDR3 domain.

The dissociation and binding constants of the complexes formed by soluble TCRs and chimeric MA-K1, MA-K2, MA-K3, and MA-K4 were measured by biolayer interferometry (ForteBio; Pall Corporation; USA) and surface plasmon resonance (SPR) (see the Table). Each of the studied immunoglobulins exhibited high specificity and an ability to effectively bind the target TCR encoded by the TRBV9 gene segment; the antibodies did not react with TCRs that carried a variable domain encoded by a different gene segment (TRBV7). Specificity of the studied antibodies did not depend on the composition of the TCR alpha-chain in the complex. We tested two TCR complexes carrying TRBV9 and TRAV26 /TRAV38 only to reveal no

difference in the binding effectiveness. Based on the results of our experiments, we selected two antibody variants MA-K2 and MA-K4 for which dissociation constants and rates were the lowest ($k_D < 1.0E-12$ and $k_{dis} < 1.0E-07$ 1/s) (see the Table).

Affinity of cytotoxic antibodies often determines their *in vivo* effective concentrations, specificity and safety. The chimeric MA-K2 and MA-K4 bound at high affinity to their targets, which speaks in favor of their potential use as therapeutic agents.

Chimeric immunoglobulins specifically target the population of TRBV9+ lymphocytes

As part of the flow cytometry analysis, the obtained chimeric antigens MA-K1, MA-K2, MA-K3, and MA-K4 were conjugated to fluorescein isothiocyanate. Importantly, direct labeling significantly increases the informative value of the analysis.

The studied chimeric MA taken at a concentration of $3 \mu\text{g} / \text{ml}$ stained about 3% of the CD3^+ subpopulation (Fig. 1A). At 200 ng/ml (Fig. 1B) it became clear that $3 \mu\text{g/ml}$ exceeded the critical concentration; at this concentration the proportion of the

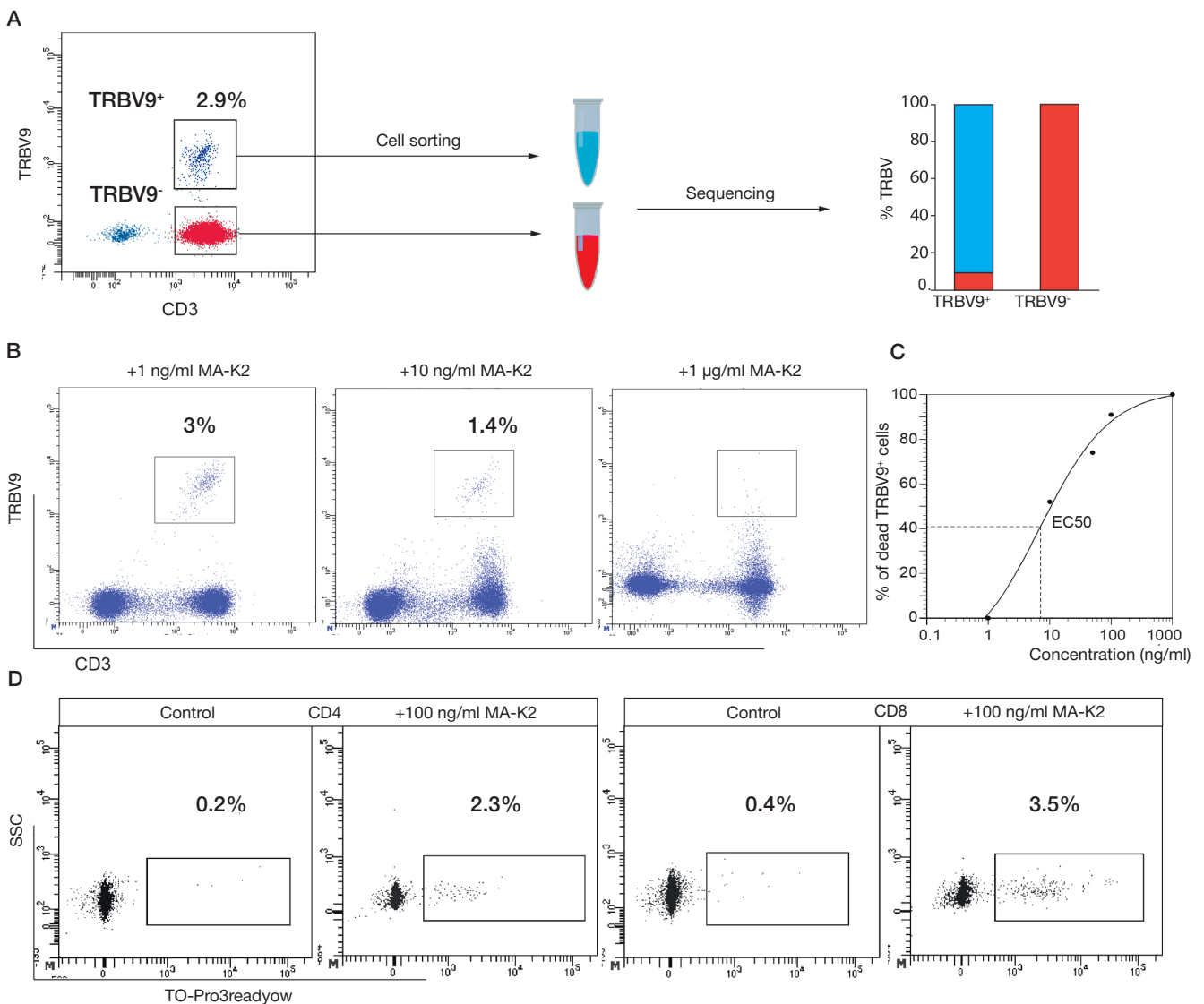


Fig. 2. A. The schematic of the experiment conducted to assess the binding specificity of the chimeric antibody MA-K2 to TCR using cell sorting and the analysis of the TCR repertoire of the sorted cells. Two T-cell populations were obtained: TRBV9⁺ (shown in blue) and TRBV9⁻ (shown in red) and their TCR repertoires were further analyzed. Results are presented as a proportion of all TCRs identified by sequencing. **B.** Flow cytometry cytotoxic activity analysis of the chimeric MA-K2 antibody. The panel shows the gated CD45⁺CD3⁺ population; the CD45⁺CD3⁺TRBV9⁺ population is marked by a rectangle. **C.** Half maximal effective concentration (EC₅₀) of MA-K2 estimated by the cytotoxicity test. **D.** The proportion of dead cells (%TO-Pro3readyflow+) upon addition of 100 ng/ml of MA-K2 to human PBMC and staining with TO-Pro3readyflow. The plots show gated CD4⁺CD3⁺ and CD8⁺CD3⁺ lymphocyte populations. The population of dead cells is marked by a black rectangle

stained TRBV9⁺ cells remained the same, but the percentage of nonspecifically bound CD3⁺ cells increased ($13 \pm 2\%$) (Fig. 1).

Based on the maximum ratio of CD3⁺TRBV9⁺ to TRBV9⁺CD3⁻ and the high intensity of the fluorescence signal indicating specific binding, we chose the MA-K2 antibody as the best candidate for further experiments. To determine the optimal antibody concentration, titration was performed at concentrations ranging from 200 to 2 ng/ml (Fig. 1C). For titration, PBMC of the healthy donor were used. The test determined the minimal MA-K2 concentration of 50 ng/ml applied to stain 10⁶ PBMC at which the TRBV9⁺ cells amounted to 3% of the entire CD3⁺ population and nonspecific binding was not observed.

MA-K2 specificity was confirmed by lymphocyte sorting and the subsequent sequencing-based analysis of the TCR repertoire (Fig. 2A). For sorting, we used PBMC isolated from the peripheral blood of the healthy donor and stained with fluorescently labeled antibodies CD45, CD3 and MA-K2 taken at concentrations of 100 ng/ml each (Fig. 2A). To analyze the repertoire of T cell receptors, we selected two populations of CD3⁺TRBV9⁺ cells, as well as a population of TRBV9⁻CD3⁺ cells. Sorting was carried out in two replicates, which was necessary to demonstrate its quality. Generally, when MA-K2 antibodies specifically bind to the TCRs representing the CD3⁺TRBV9⁺ subpopulation, the corresponding cDNA library is expected to be enriched in TRBV9 transcripts, whereas the CD3⁺TRBV9⁻ subpopulation is supposed to be free of the target sequences. Total RNA was isolated from all selected cell subpopulations and cDNA was synthesized using primers complementary to the constant region of the TCR beta chain. The libraries were amplified and sequenced by NGS (MiSEQ; Illumina; USA). The analysis of the obtained TCR beta chain repertoires revealed that the libraries obtained from the sorted MA-K2-stained cells were 93% enriched in the sequences encoded by the TRBV9 gene segment, whereas no TRBV9 sequences were observed in the repertoires of CD3⁺TRBV9⁻ beta chains.

This suggests high specificity and efficacy of the studied chimeric antibody.

Cytotoxicity of chimeric immunoglobulins

To assess the cytotoxic activity of the studied monoclonal antibody, we had to resort to a non-standard approach for a few reasons. First, the target population of cells constituted only a small proportion of the total cell pool (2.5% of CD3⁺ lymphocytes). Second, this population can be distinguished from the rest of the cells based on only one surface marker: the T cell receptor that has a unique variable beta chain domain. This obstructs the use of cell sorting for the purpose of enriching the target TRBV9⁺ population and complicates the classic cytotoxic test protocol (the target cell population + natural killers taken at various proportions).

Therefore, flow cytometry was employed to assess the cytotoxic activity of MA-K2. We used the mononuclear cell fraction as it contains both the target population and other cells that mediate the cytotoxic reaction (natural killers, etc.). The half maximal effective concentration (EC_{50}) of the MA-K2 antibodies was determined in a series of *in vitro* experiments. The cytotoxic effect was measured based on the progressively declining proportion of TRBV9⁺ cells in the population of CD3⁺ lymphocytes correlated with an increase in MA-K2 concentrations (Fig. 2B). At a concentration of 100 ng/ml complete elimination of TRBV9⁺CD3⁺ was observed. Thus, EC_{50} for MA-K2 was 7 ng/ml (Fig. 2C).

To measure the proportion of dead cells among the populations of CD4⁺CD3⁺ and CD8⁺CD3⁺ lymphocytes, we added TO-Pro3readyflow (ThermoFisher) to the cells incubated with MA-K2 (Fig. 2D). Twenty-four hours after incubation with MA-K2 taken at a concentration of 100 ng/ml, a significant increase in the proportion of dead cells was observed in comparison with the control (Fig. 2D). This experiment was conducted in 7 replicates, and every time we observed an increase in the proportion of dead cells depending on the concentration of MA-K2.

To sum up, the chimeric MA-K2 antibody exhibits high cytotoxic activity and target specificity *in vitro*.

DISCUSSION

The existing therapeutic antibodies are successfully used to manage severe disorders, such as multiple sclerosis, some cancers and retinal degeneration. Their development relies on the knowledge of targets they are expected to work against implicated in the pathogenesis of a disease. In a recent work published by our colleagues a correlation has been shown between AS and the T-cell clones carrying a variable beta chain region encoded by the TRBV9 gene segment [20, 21]. Involvement of certain T clones in the pathogenesis of AS is yet to be confirmed, but the T cell receptor itself is a promising candidate target for a therapeutic antibody.

The engineered cytotoxic chimeric (human-rat) antibody MA-K2 is the most important outcome of our work. It specifically and effectively binds the TCR beta chain region encoded by TRBV9T. To test its cytotoxicity, we used flow cytometry and TO-Pro3readyflow staining (ThermoFisher; USA), which enabled us to accurately separate dead and viable cells. This is not a common approach to assessing antibody-dependent cell-mediated cytotoxicity (ADCC). The literature describes a few methods of quantifying cells killed through an interaction with a cytotoxic antibody, but they all have their downsides. For example, the widely used method based on the detection of ⁵¹Cr [26] released in the course of cell lysis is not sensitive enough and cannot be employed to count dead cells in a cell population. This limits its application in cases when the target cell subset makes up only 3% or less of the total population. The approach exploited in this study was recently used to assess the toxicity of Trastuzumab detecting small proportions of dead cells (> 10%) in the cell culture and PBMC [27].

We hope that the engineered MA-K2 antibody has a good potential to be used *in vivo* to deplete the T-cell population associated with ankylosing spondylitis, thereby alleviating its symptoms. As noted previously, elimination of pathogenic clones is a promising approach to treating autoimmune diseases and another step towards precision medicine. The literature reports successful use of monoclonal antibodies against V β and for treating autoimmune diseases in model systems. In an experiment conducted in mice, all autoreactive T cell clones isolated from the animals with induced autoimmune encephalomyelitis carried a TCR domain encoded by the V β 8 segment (TRBV13). Encephalomyelitis was induced by the injection of the MBP peptide. The subsequent injection of the antibody specific to that domain had a protective effect and blocked the development of the disease [28]. Another monoclonal antibody against V β 8, KJ16, was used to protect mice against collagen-induced arthritis. Injections of this antibody significantly reduced the incidence of the condition [29].

Importantly, the proportion of cells carrying a TRBV9-encoded TCR on their surface is low and does not exceed

3%. This leads us to hypothesize that once an anti-TRBV9 is administered, no severe toxic effect will be observed caused by massive cell death and a cytokine storm, as is the case with some anti-CD3 monoclonal antibodies [30].

At present, targeted therapies rely on the use of monoclonal antibodies with different degree of humanization required to attenuate the immunogenicity of the drug. Three main types of therapeutic antibodies can be distinguished: chimeric (a constant domain of humans + a mouse variable domain), humanized (a human antibody + a mouse CDR) and fully human [31]. MA-K2 whose properties were studied in this work is 65% humanized. In the next stage of our research we are planning to humanize it further and to conduct a series of *in vivo* experiments in primates using the humanized antibody.

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CONCLUSIONS

We have characterized 4 chimeric monoclonal anti-TRBV9 antibodies (MA-K1, MA-K2, MA-K3, and MA-K4) different in the amino acid sequences of their hypervariable domain (CDR3). According to the literature, the TRBV9 family is associated with AS. The MA-K2 antibody was selected for further research based on its biochemical properties (Kd, Kdis, and specificity). It exhibited high specificity and cytotoxicity against the target. We are planning to further humanize the antibody and carry out a series of *in vivo* experiments in primates. If we succeed in eliminating the pathogenic T cell population carrying the TRBV9 gene segment, this monoclonal antibody will have the potential to become a drug candidate for AS therapy and a convenient tool for studying this disease.

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TARGETED SEQUENCING IN PATIENTS WITH CLINICALLY DIAGNOSED HEREDITARY LIPID METABOLISM DISORDER AND ACUTE CORONARY SYNDROME

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The actual prevalence of genetic variants causing familial hypercholesterolemia (FH) in every population remains unknown. The aim of this work was to determine the spectrum of pathogenic variants in patients with acute coronary syndrome (ACS) and clinically diagnosed FH using targeted sequencing. We selected 38 patients with ACS from the sample of 2,081 participants of two multicenter observational studies (2004–2007; 2014–2016) who had a clinical diagnosis of FH based on the Dutch Lipid Clinic Network score and Simon Broome criteria. The men and women included in the study were ≤ 55 and ≤ 60 years of age, respectively. Molecular genetic screening was done by targeted next-generation sequencing. We started by sequencing 3 genes associated with FH, including *LDLR*, *APOB*, and *PCSK9*. If no relevant variants were detected, the panel was expanded. Of 38 patients, 24 (63.2%) were shown to have mutations that could cause clinical manifestations of FH and premature coronary artery disease. All patients were heterozygous carriers. Mutations were detected in three “classic” genes *LDLR*, *APOB*, and *PCSK9* associated with FH, as well as in other genes involved in lipid metabolism, such as *APOE*, *ABCA1*, *ABCG5*, *ABCG8*, *LPL*, *ANGPTL3*, and *MTTP*. Five variants detected in our study sample had not been described previously: the pathogenic p.Val273_Cys313del variant of the *LDLR* gene, the likely pathogenic p.Arg160His variant in the *APOE* gene, two variants of uncertain significance p.Glu612Lys and c.*415G>A in the *PCSK9* gene, and the mutant variant p.Ala776Ser in the *LDLR* gene. We conclude that the use of clinical diagnostic criteria in patients with ACS and FH enables identification of carriers of both “classic” mutations associated with FH and rare genetic variants that can be phenotypically expressed as FH.

Keywords: familial hypercholesterolemia, acute coronary syndrome, genetic testing, lipid metabolism disorder, targeted sequencing

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ТАРГЕТНОЕ СЕКВЕНИРОВАНИЕ У БОЛЬНЫХ С КЛИНИЧЕСКИ ДИАГНОСТИРОВАННЫМ НАСЛЕДСТВЕННЫМ НАРУШЕНИЕМ ЛИПИДНОГО ОБМЕНА И ОСТРЫМ КОРОНАРНЫМ СИНДРОМОМ

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Истинная распространенность генетических вариантов, стоящих за развитием семейной гиперхолестеринемии (СГХС) и характерных для каждой популяции, остается неизвестной. Целью работы было определение спектра патологических генетических вариантов у больных острым коронарным синдромом (ОКС) с клинически диагностированной СГХС с помощью таргетного секвенирования. Были отобраны 38 из 2081 пациентов двух многоцентровых наблюдательных исследований больных ОКС (2004–2007; 2014–2016 гг.) возрастом ≤ 55 лет (мужчины) и ≤ 60 лет (женщины) с СГХС, клинически диагностированной по критериям Голландской сети липидных клиник и критериям регистра Simon Broome. Молекулярно-генетическое исследование проводили с помощью таргетного секвенирования следующего поколения: сначала секвенировали 3 гена, ассоциированные с СГХС: *LDLR*, *APOB*, *PCSK9*; при отсутствии значимых изменений панель расширяли. Из 38 пациентов у 24 (63,2%) были выявлены генетические изменения, которые могли обусловить клинические проявления СГХС и раннюю манифестацию ишемической болезни сердца. Все пациенты являлись гетерозиготными носителями генетических вариантов. Выявлены варианты в трех основных генах (*LDLR*, *APOB*, *PCSK9*), связанных с СГХС, и редкие варианты в других генах системы липидного обмена (*APOE*, *ABCA1*, *ABCG5*, *ABCG8*, *LPL*, *ANGPTL3*, *MTTP*). Пять генетических вариантов описаны впервые: патогенный вариант p.Val273_Cys313del гена *LDLR*; вероятно патогенный вариант p.Arg160His гена *APOE*; варианты неуточненной клинической значимости p.Glu612Lys и c.*415G>A гена *PCSK9*; вариант p.Ala776Ser гена *LDLR*. Таким образом, использование клинических критериев позволяет выявить среди пациентов с ОКС и СГХС носителей мутаций не только «классических» генов, связанных с СГХС, но и редких, способных приводить к фенотипическим проявлениям СГХС.

Ключевые слова: семейная гиперхолестеринемия, острый коронарный синдром, генетическое обследование, нарушение липидного обмена, таргетное секвенирование

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One of the known causes of premature coronary artery disease (CAD) is familial hypercholesterolemia (FH), a hereditary lipid metabolism disorder that increases the risk of developing cardiovascular disease twentyfold [1]. It is the most common monogenic autosomal-dominant lipid metabolism disorder characterized by elevated low-density lipoprotein (LDL) cholesterol. The majority (60–80%) of patients with clinically established heterozygous FH carry mutations in the gene coding for the LDL receptor (*LDLR*); the prevalence of each implicated mutation varies geographically. These mutant variants result in fewer LDL receptor molecules on the cell surface or their decreased activity. The *LDLR* gene is located on the short arm of chromosome 19 and consists of 18 exons transcribed and translated into 5 LDLR-forming domains [2]. About 5–10% patients with heterozygous FH have mutations in the *APOB* gene that are phenotypically expressed as a less pronounced elevation of cholesterol levels; these mutant variants are more common in Central Europe than in other regions [3]. The defect in the ApoB100 protein, a component of LDL particles, prevents it from binding to LDLRs. *APOB* has been mapped to chromosome 2p and comprises 29 exons [2]. Gain-of-function (GOF) mutations of the proprotein convertase subtilisin/kexin type 9 gene (*PCSK9*) are another cause of autosomal-dominant FH accounting for < 1% of all FH cases in the majority of the studied populations. GOF variants lead to rapid LDLR internalization and downregulate the number of LDLR molecules. The *PCSK9* gene sits on the short arm of chromosome 1p32 and is constituted by 12 exons and 11 introns [4, 5]. Mutations in other genes involved in lipid metabolism rarely cause FH [3]. Some patients carry two mutations in the genes associated with FH. Unfortunately, in 20 to 40% of patients with clinically confirmed FH the causative mutation cannot be identified.

The aim of this work was to determine the spectrum of pathogenic variants involved in lipid metabolism in patients with acute coronary syndrome (ACS) and a clinical diagnosis of FH by targeted sequencing.

METHODS

For this study we used the samples obtained from 2,081 patients with ACS who had participated in two multicenter observational studies carried out in 4 centers in Moscow, Kazan and Stavropol. The patients had been recruited in 2004 through 2007 and in 2014 through 2016. The study protocol was described in [6]. The study was approved by the Ethics Committee of the Medical Educational Research Center of the Department of Presidential Affairs of the Russian Federation (Protocol 14/14 dated October 20, 2014).

We selected 326 patients from the 1st and 374 patients from the 2nd recruitment periods. Over the first recruitment period the inclusion criteria were as follows: premature ACS (at ≤ 55 years of age in men and at ≤ 60 years in women); ACS with an onset at least 10 days before index admission to hospital; FH diagnosis based on the Dutch Lipid Clinic Network score and Simon Broome criteria [7, 8]; written informed consent to participate. During the second recruitment period the inclusion criteria were the same but additionally those patients were included who had indications for percutaneous coronary intervention (PCI), regardless of whether the latter was eventually performed or not.

The diagnosis of FH was based on the Dutch Lipid Clinic Network (DLCN) score and Simon Broome criteria. Generally, the DLCN set of criteria accounts for the patient's family history, early CAD onset in the patient, physical examination (tendinous

xanthomas, corneal arcus), and LDL levels. Patients who score more than 8 points are concluded to have definite FH; 6–8 points, probable FH; 3–5 points, possible FH; less than 3 points, unlikely FH [7]. The Simon Broome criteria account for the total cholesterol and LDL levels, the age of the patient and their kinship to a relative who also has elevated LDL and cholesterol. In the clinical setting, only possible FH can be detected; the diagnosis of definite FH cannot be established without identifying its causative mutation [8].

Molecular genetic screening was performed on the samples of 38 patients selected using the following algorithm. Patients enrolled during the first recruitment period were eligible for the genetic screening if they scored ≥ 5 points on the DLCN scale or scored 4 points on the DLCN scale and also had FH according to the Simon Broome criteria (a total of 10 people). Patients recruited during the second period were eligible if they scored ≥ 5 points on the DLCN scale or met the Simon Broome criteria and had a family history of cardiovascular disorders (a total of 24 individuals). This second sample also included 4 patients who did not have premature ACS at the time of hospital admission but still had an episode of early CAD manifestation according to their medical history (≥ 6 points on the DLCN scale or 5 points on the DLCN scale + a family history of cardiovascular disorders).

Sequencing was carried out in the ReadSense laboratory (Moscow). DNA was isolated from blood samples using the K-Sorb DNA isolation kit (Syntol; Russia) and spin columns. Preparation of DNA libraries was aided by the NEB Next Ultra kit (NEB; USA). Capture-based enrichment of target protein-coding regions was done using the NimbleGen panel of biotinylated probes (Roche; USA). Quality of the enriched DNA libraries was assessed by capillary gel electrophoresis on BioAnalyzer 2100 (Agilent; USA) and Qubit fluorometer (Invitrogen; USA). The libraries were sequenced using the 300-cycle v2 MiSeq reagent kit on the Illumina MiSeq next-generation sequencer (Illumina; USA) set up according to the manufacturer's recommendations.

First, 3 genes associated with FH were sequenced, including *LDLR*, *APOB*, and *PCSK9*. Then, if no relevant variants were detected, the panel was expanded to cover the entire range of genes involved in lipid metabolism: *APOA1*, *APOA5*, *APOC2*, *APOE*, *APOC3*, *ABCA1*, *ABCG1*, *ABCG5*, *ABCG8*, *ANGPTL3*, *CEL*, *CH25H*, *CPT2*, *CYP2D6*, *CYP3A4*, *CYP3A5*, *GPD1*, *GPIHBP1*, *INSIG2*, *LCAT*, *LDLRAP1*, *LIPA*, *LMF1*, *LPA*, *LPL*, *MTP*, *NPC1L1*, *PNPLA2*, *PPARA*, and *SAR1B*.

A number of steps were taken to evaluate the quality of the enrichment panel. The reads obtained for each sample were mapped to the reference human genome (GRCh37.p13 hg19). To understand whether sequencing coverage was sufficient, we analyzed the nucleotides used for probe synthesis that were covered at a minimum of 20x. Once detected, the target mutations were annotated using HGMD, COSMIC, ClinVar, 1000 GenomesProject, dbSNP, and ExAC databases. Conservation of substitutions was estimated by a number of prediction tools (POLYPHEN, SIFT, MUTATION TASTER, FATHMM, CADD, DANN, and EIGEN). Mutant variants were annotated as recommended by ACMG [9].

RESULTS

All patients who carried variants of the studied genes were heterozygous. Variants of the three "classic" genes implicated in FH were detected in 11 patients.

Among them were 3 pathogenic and likely pathogenic variants of the *LDLR* gene and one variant with uncertain

Table. Sequencing results in patients with premature ACS and clinically established FH

No	Sex	Age	Family history	LDL, mmol/l	DLCN score	FH. Simon Broome registry	Result	Pathogenicity	Population frequency
1	F	48	+	9.6	11	+	Gene <i>LDLR</i> p.Val273_Cys313del; c.817+303_940+943del	Pathogenic	Not described
2	M	53	-	9.1	11	+	Not detected	-	-
3	M	46	+	6.9	8	+	1. Gene <i>LDLR</i> p.Gly20Arg; c.58G>A 2. Gene <i>PCSK9</i> p.Glu612Lys; c.1834G>A	1. Likely pathogenic 2. Of uncertain significance	1. < 1% 2. Not described
4	F	51	+	5.9	6	+	Polymorphism of gene <i>APOE</i> p.Cys130Arg; c.388T>C; isoform apoE4	Modifying factor	> 1%
5	M	46	+	5.6	6	+	Not detected	-	-
6	M	49	+	5.1	6	+	Not detected	-	-
7	F	57	+	5.7	6	+	Gene <i>LDLR</i> p.Ala776Ser; c.2326G>T	Of uncertain significance	Not described
8	F	59	+	5.2	6	+	Not detected	-	-
9	M	52	+	6.2	6	+	1. Polymorphism of gene <i>APOE</i> p.Cys130Arg; c.388T>C; isoform apoE4 2. Gene <i>APOB</i> p.Ala4002Val; c.12005C>T	1. Modifying factor 2. Of uncertain significance	1. > 1% 2. < 1%
10	M	55	+	6.2	6	+	Gene <i>PCSK9</i> c.*415G>A	Of uncertain significance	Not described
11	M	54	+	6.5	6	+	Gene <i>ABCG8</i> p.Ala642Thr; c.G1924A	Of uncertain significance	< 1%
12	M	54	+	5.1	6	+	Not detected	-	-
13	M	36	+	5.6	6	-	Gene <i>APOE</i> p.Arg180Cys; c.C538T	Likely pathogenic	< 1%
14	M	34	N/A	5.4	5	+	Polymorphism of gene <i>APOE</i> p.Cys130Arg; c.388T>C; isoform apoE4	Modifying factor	> 1%
15	M	53	N/A	5.2	5	+	Not detected	-	-
16	F	56	N/A	5.5	5	-	Not detected	-	-
17	M	44	-	5.8	5	+	Gene <i>LDLR</i> p.Gly20Arg; c.58G>A	Likely pathogenic	< 1%
18	M	50	-	5.1	5	+	Not detected	-	-
19	F	58	-	5.0	5	-	Gene <i>APOE</i> p.Arg160His; c.479G>A	Likely pathogenic	Not described
20	M	55	-	6.3	5	-	Not detected	-	-
21	M	51	+	4.0	4	+	Polymorphism of gene <i>APOE</i> p.Cys130Arg; c.388T>C; isoform apoE4	Modifying factor	> 1%
22	F	57	+	4.0	4	+	Gene <i>ABCA1</i> p.Pro85Leu; c.C254T	Likely pathogenic	< 1%
23	M	43	+	4.9	4	+	Gene <i>LPL</i> p.Thr379Ile; c.C1136T	Likely pathogenic	< 1%
24	M	53	+	4.6	4	+	Gene <i>LDLR</i> p.Glu208Lys; c.G622A	Pathogenic	< 1%
25	M	47	+	5.4	6	+	Gene <i>ANGPTL3</i> p.Asn147*; c.431_434del	Pathogenic. familial hypobetalipoproteinemia	< 1%
26	F	54	+	5.0	6	+	Not detected	-	-
27	M	39	+	5.3	6	+	Gene <i>ABCG5</i> p.Gly27Ala; c.G80C	Likely pathogenic. sitosterolemia/hypercholesterolemia	< 1%
28	M	46	+	6.5	6	+	Gene <i>MTP</i> p.Leu838Phe; c.G2514C	Of uncertain significance	< 1%
29	M	45	+	5.0	6	+	Not detected	-	-
30	M	42	+	4.8	6	-	Not detected	-	-
31	M	51	N/A	5.2	5	+	Gene <i>ABCG8</i> p.Leu572Pro; c.T1715C	Pathogenic. sitosterolemia	< 1%
32	F	47	N/A	5.7	5	-	Gene <i>APOB</i> p.Val4265Ala; c.T12794C	Likely pathogenic	< 1%
33	M	48	-	4.9	5	-	Not detected	-	-
34	F	59	+	4.9	4	+	Gene <i>ABCA1</i> p.Val399Ala; c.T1196C	Pathogenic. congenital HDL deficiency	< 1%
35	M	56	+	6.5	7	-	Not detected	-	-
36	M	64	+	5.0	6	+	Gene <i>APOB</i> p.Ser3279Gly; c.A9835G	Likely pathogenic	< 1%
37	M	57	+	5.8	6	+	Gene <i>APOB</i> p.Asn2971Thr; c.A8912C	Of uncertain significance	< 1%
38	F	64	+	5.6	5	-	Gene <i>ABCA1</i> p.Arg496Trp; c.C1486T	Possibly pathogenic. congenital HDL deficiency	< 1%

clinical significance. Of them, one deleterious mutation (p.Val273_Cys313del) was previously undescribed. It is an in-frame deletion in exon 6 resulting in a missing class A7 domain at the N-terminus of the LDLR [10]. This mutation belongs to class 3 of *LDLR* mutations leading to the production of a defective receptor that cannot bind to LDL. A similar mutation g.11218068_11218190del was described by Usifo et al. [11]. Another mutation detected in our sample yet previously undescribed (p.Ala776Ser) is located in exon 16 and has uncertain significance. Such rare missense mutations observed in the general population are normally classified as non-pathogenic. However, we cannot completely rule out their contribution to hypercholesterolemia [12]. The likely pathogenic mutation p.Gly20Arg in the *LDLR* gene occurred in two patients. The pathogenic mutation p.Glu208Lys was detected in one patient.

Pathogenic and likely pathogenic *APOB* variants were observed in two patients. Two more patients carried mutant *APOB* variants of uncertain significance. The population frequency of all *APOB* mutations identified in our study is less than 1%.

Two patients carried previously undescribed *PCSK9* mutations of uncertain significance. One of them (p.Glu612Lys) is located in exon 11. *PCSK9* mutations resulting in the modification of the functional cysteine- and histidine-rich C-terminal domain are known to be associated with FH. Specifically, these genetic variants add to the severity of hypercholesterolemic phenotype in patients who also have mutations in the *LDLR* gene (our patient also had a likely pathogenic mutation p.Gly20Arg in this gene) [13]. The second *PCSK9* mutation c.*415G>A is located in exon 12. This region is untranslatable and contains elements responsible for *PCSK9* expression. However, it is impossible to predict the functional consequences of this genetic variation.

Patients with clinically established FH had mutations in other genes involved in lipid metabolism, such as *APOE*, which turned to be mutant in two participants. One of those patients had a previously undescribed and likely pathogenic mutation (p.Arg160His) located in exon 4. This genomic region is involved in the binding of the *APOE* lipoprotein to *LDLR*. Its mutant variants have been shown to be associated with autosomal-dominant familial dysbetalipoproteinemia [14]. Four participants carried the ε4 allele of the *APOE* gene resulting in the atherogenic isoform of apolipoprotein E (one of those patients also had the p.Ala4002Val variant of uncertain significance in the *APOB* gene).

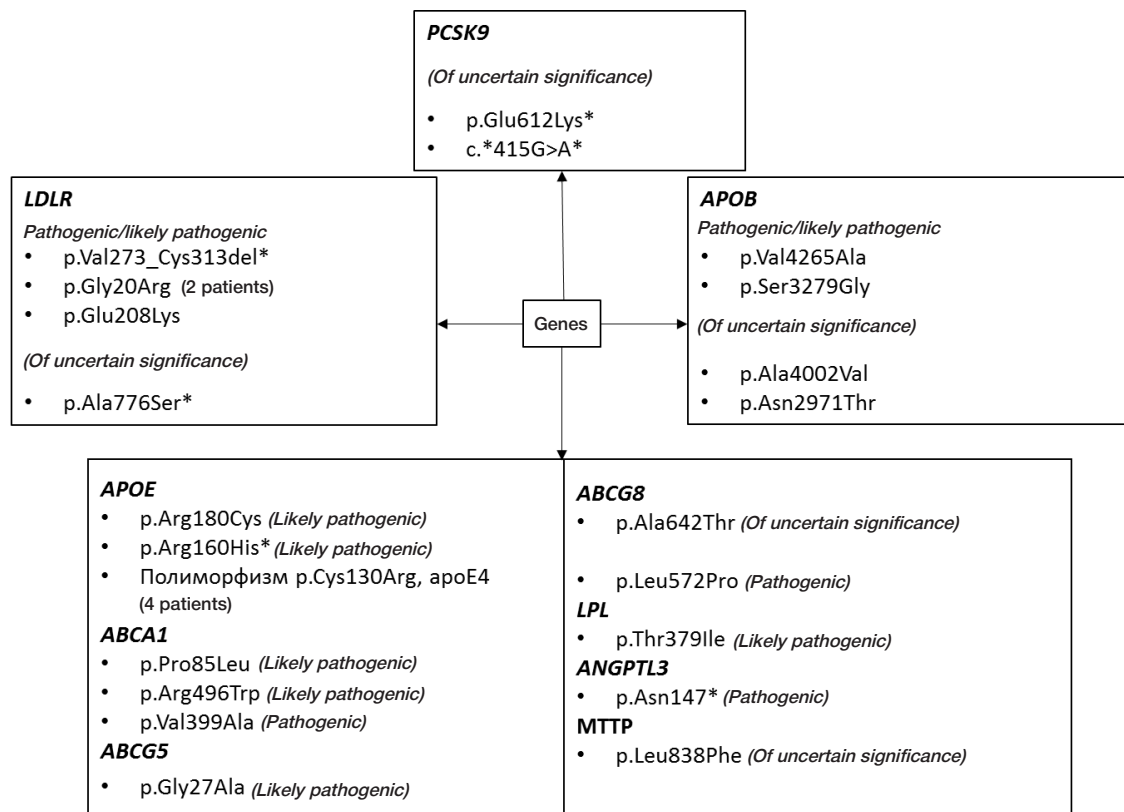
Three patients had *ABCA1* mutations; of them one was pathogenic and two likely pathogenic implicated in low HDL. Two patients were found to have *ABCG8* mutations thought to be associated with sitosterolemia: one was pathogenic and another one of uncertain significance [15]. One patient had a likely pathogenic mutation in the *ABCG5* gene; one patient carried a possibly pathogenic mutation in the *LPL* gene; one bore a pathogenic mutation in *ANGPTL3*; another, a mutation of uncertain significance in the *MTTP* gene.

Thus, of 38 patients with clinically established FH 24 (63.2%) carried variants that could be phenotypically expressed as FH or early CAD. Five of the identified variants were previously undescribed.

The results of our study are presented in the Table below and in Fig. 1.

DISCUSSION

The prevalence of FH in the general population is 0.2–0.5%, but in patients with ACS it can be as high as 8%. Therefore, its accurate diagnosis in this particular subpopulation is very important as it enables adequate lipid-lowering therapy and



* Previously undescribed variant

Fig. 1. Genetic variants associated with clinically diagnosed FH in patients with early ACS

initiate cascade screening [16, 17]. In Russia the first steps are being taken in investigating the prevalence of mutations implicated in FH [18]. However, we already know about the extreme genetic heterogeneity and the absence of the founder effect demonstrated by a research team from Saint Petersburg [19]. They screened a group of patients with FH who did not have ACS and found no carriers of mutant *APOB*. Another research work conducted in Northwest part of Russia also showed the absence of *APOB* mutations in the studied subpopulation [20]. In our sample of patients with premature ACS, *LDLR* and *APOB* mutant variants were equally represented (4 mutant variants of *LDLR* of which 3 were pathogenic or likely pathogenic and one was present in two patients; 4 mutant variants of *APOB* of which two were pathogenic or likely pathogenic). Interestingly, there were 2 patients in our sample with the FH phenotype who carried mutations of uncertain significance in the *PCSK9* gene. So far, only a few cases of *PCSK9* variants have been described in the Russian population [21]. All known deleterious mutations of *PCSK9* are very rare and have an allelic frequency of < 0.1% in the general population [22]. Distribution of SNPs in the general population only mildly affecting cholesterol metabolism demonstrates a balance between the alleles that up- and downregulate the levels of LDL. In the individuals at the extreme ends of this spectrum, alleles that increase LDL levels are hereditarily prevalent; together these alleles can cause elevated LDL characteristic of heterozygous FH. One of the most typical and well-studied examples of such single nucleotide polymorphisms is the $\epsilon 2/\epsilon 3/\epsilon 4$ polymorphism of the *APOE* gene. The risk of developing CAD increases in the carriers of the $\epsilon 4$ allele manifold [23, 24]. The $\epsilon 4$ allele of p.Cys130Arg

has been shown to be associated with FH in the Russian population [25], which is also confirmed by the present study that has identified this polymorphism in 4 patients. Importantly, the actual frequency of rare variants in other genes involved in lipid metabolism, including *APOE*, *ABCA1*, *ABCG5*, *ABCG8*, *LPL*, *ANGPTL3*, and *MTTP*, may be different from what we believe in the subpopulation of Russian patients with ACS and FH. Patient 1 (see the Table) carried a previously undescribed but definitely pathogenic mutation p.Val273_Cys313del in the *LDLR* gene [11]. The proband's daughter also had elevated total cholesterol and underwent a cascade screening procedure, which found no presence of the pathogenic variant in her genome. To sum up, of 38 patients 14 (36.8%) had pathogenic or likely pathogenic variants and 10 (26.3%) carried variants of uncertain significance. The literature does not provide us with the data on the frequency of FH-associated variants in patients with premature ACS. However, the authors who conducted target sequencing in 104 patients with a clinical diagnosis of FH revealed the presence of pathogenic variants both in the "classic" genes, including *LDLR*, *APOB*, and *PCSK9*, and rare genes involved in lipid metabolism in 67% of the participants with definite FH [26].

CONCLUSIONS

The use of clinical diagnostic tests followed by targeted sequencing helps to identify pathogenic variants not only in the "classic" genes implicated in FH but also in the "rare" genes that might be associated with the FH phenotype in patients with ACS and FH.

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BIOLUMINESCENT IMAGING: NEW OPPORTUNITIES

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Modern biomedical research technologies actively use bioimaging for studying cells, tissues and whole organisms. Multicolor bioimaging is applied when simultaneous observation of different events at the molecular and cellular level is needed. Bioluminescent imaging methods are the most sensitive, however, their use for multicolor labeling is complicated due to the insufficient number of available luciferin-luciferase pairs. Having a number of advantages compared to previously studied bioluminescent systems, the new bioluminescence systems of higher fungi and marine polychaete *Odontosyllis* could become a useful expansion of the bioimaging toolbox.

Keywords: bioluminescence, bioimaging, luciferin, luciferase

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БИОЛЮМИНЕСЦЕНТНЫЙ ИМИДЖИНГ: НОВЫЕ ВОЗМОЖНОСТИ

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Современные биомедицинские исследования активно используют методы биоимиджинга клеток, тканей и целых организмов. Многоцветный биоимиджинг находит свое применение в случае необходимости одновременного наблюдения разных событий на молекулярном и клеточном уровнях. Наиболее чувствительными являются методы биолюминесцентного имиджинга, однако их использование для многоцветного мечения сдерживается недостаточным количеством доступных пар люцифераза–люциферин. Удачным расширением палитры инструментов молекулярного имиджинга могут стать новые биолюминесцентные системы высших грибов и морской полихеты *Odontosyllis*, обладающие рядом преимуществ по сравнению с ранее изученными системами.

Ключевые слова: биолюминесценция, биоимиджинг, люциферин, люцифераза

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Bioimaging of tissues and whole organisms is an integral part of the current research routines aimed at studying molecular events in the context of the disease development investigations [1]. Fluorescent and bioluminescent proteins (luciferases) are a very popular vehicle in bioimaging. For the purposes of fluorescent bioimaging, there was developed a set of fluorescent proteins with various spectral properties, ranging from violet to far-red, photoactivated and photoconvertible, as well as derivative sensors [2]. Luciferases that can be used for bioimaging are significantly more scarce than fluorescent proteins, but the methods making use of bioluminescence deliver some of the most accurate results when applied to examine deep tissues.

Luciferase catalyzes luminescence through oxidation of luciferin, molecular substrate, which allows receiving the analytical signal without using an external source of radiation, an integral part of the routine with fluorescent proteins. Thus,

bioluminescent imaging *in vivo* is virtually free from background noise and offers unequalled sensitivity [3]. However, luciferases as reporter proteins are not flawless. The activity of some of them is strongly dependent on the number of cofactors and can be inhibited by intracellular components or medicines [4]. Still, despite bioluminescent systems being less practical because of the need for at least two components to trigger light emission, the relevant examination methods continue to evolve and gain popularity rapidly.

Tools for bioluminescent imaging

Multicolor bioimaging allows simultaneous observation of different events at the molecular level (e.g., gene expression or protein-protein interactions), which translates into a smaller number of animals used in the context of an experiment. There is a number of strategies to follow; some of them are: joint

use of luciferases of different organisms, mutant luciferases of one organism, use of "luciferase-fluorescent protein" hybrid constructs, etc. [5].

Some of the most popular luciferases used for bioimaging are those of *Photinus pyralis* (62 kDa) Fluc, a North American firefly, *Pyrophorus plagiophthalmus* (62 kDa) ($\lambda_{\text{max}} \approx 540\text{--}615$ nm), a click beetle, and Renilla reniformis Rluc (36 kDa) and *Gaussia princeps* Gluc (20 kDa) ($\lambda_{\text{max}} \approx 480$ nm), both of which are marine creatures [6]. Luciferases of terricole organisms catalyze the *D*-luciferin-oxygen reaction in the presence of ATP and Mg^{2+} ions; luciferases of marine organisms utilize coelenterazine as a sole substrate. NanoLuc (19 kDa) ($\lambda_{\text{max}} = 460$ nm), an engineered luciferase derived from the small domain of *Oplophorus gracilirostris* luciferase, has been very popular since 2012, when it was developed [7]. NanoLuc makes use of a different substrate, furimazine, a synthetic analogue of coelenterazine.

Apart from the purely historical reasons, luciferases catalyzing *D*-luciferin are popular because of their applicability to imaging the processes involving ATP molecule [8]. Also, there is a wide range of structural analogues of *D*-luciferin available [3], including those emitting in the more red portion of the spectrum, like the recently developed AkaLumine-HCl ($\lambda_{\text{max}} = 677$ nm) [9]. It is necessary to clarify here that shifting bioluminescence to the near IR range is important because the spectral window for *in vivo* examination of processes in deep tissues belongs there. In addition to the improved spectral characteristics, AkaLumine-HCl distributes through the cells better than *D*-luciferin and saturates firefly luciferase (Fluc) more effectively. Directed mutagenesis of Fluc produced Akaluc, an artificial luciferase, the synthetic substrate of which is more active than the natural one [10]. The developed AlaBLI system is almost 100–1000 times brighter than other available systems; it allows bioimaging of a single deep tissue cells of freely moving animals.

Technologically, a light filter and different bioluminescent systems used together allow analysis of signals from different processes; to this effect, the standard Promega DLR assay contains both *P. pyralis* and *R. reniformis* luciferase. Another option is to introduce several mutant proteins to a single bioluminescent system. DART, a recently developed examination method, is an example of the latter. The system makes use of green (PLG) and red (PLR1) firefly luciferase mutants together with *D*-luciferin and its benzothiofene analogue, respectively [11]. However, the techniques described above are not flawless: the former carries a risk of light signal inhibition by luciferases, the latter can produce cross-reactions of the related luciferases with similar substrates.

Development of the fully artificial luciferases that work with standard substrates (based on the genetic sequences of known proteins) is an interesting approach to remedying the flaws, one similar to the approach that produced the aforementioned AlaBLI system. An example of the product thereof is Aluc, a recent development that interacts with coelenterazine and analogues ($\lambda_{\text{max}} = 487\text{--}500$ nm) [12]. Chemical modification of coelenterazine by conjugation with fluorescent dyes allowed shifting the Aluc reaction's emission maximum to an even longer wavelength region [13]. Preliminary calculations in the context of an effort guided by a similar approach revealed

several orthogonal pairs "*D*-luciferin analogue–mutant Fluc luciferase"; their activity was further confirmed *in vivo* [14].

Absence of cofactors in the luminescence reaction simplifies analysis and makes marine luciferases usable in extracellular imaging, which makes them a convenient vehicle. NanoLuc, an engineered luciferase, is especially popular. Its miniature size simplifies development of the new "luciferase–fluorescent protein" fusion proteins used to expand the imaging palette with the help of the BRET technique. BRET is based on the Förster Resonant Energy Transfer (FRET) from luciferase to a fluorescent protein, which results in a shift of the emission maximum. NanoLuc luciferase and applicable proteins produced a whole range of chimeric proteins that have the emission maximum shifted to up to 680 nm with the help of various fluorophores [15]. The recently developed Rluc8-iRFPs chimeric proteins that also work in the long-wavelength portion of the spectrum are a similar example [16].

Expansion of the palette: new luminescent system

Studying new, previously unresearched luminescent systems is another approach to expanding the bioluminescent palette that shows promise. Some of the recent discoveries here include luciferin from *Fridericia heliota* ($\lambda_{\text{max}} = 480$ nm), an earthworm, and that from fungi ($\lambda_{\text{max}} = 530$ nm) [3]. Researchers have already obtained a recombinant luciferase for the latter [17]. The cost price of fungal luciferin is several orders of magnitude lower than that of *D*-luciferin while its stability is much higher. The new fungal bioluminescent system allows for a simple modification of luciferin structure, thus enabling production of the functional analogues emitting light in the longer wavelength range [18]; this property makes it a quite promising tool for bioimaging, even considering the membrane localization of the fungal luciferase, which may somewhat complicate its practical use.

In 2018, researchers isolated luciferase of *Odontosyllis undecimonta*, a marine polychaete [19]. This protein does not luminesce with the known luciferins of marine organisms (coelenterazine, *Cypridina* luciferin), which makes it the first marine luciferase belonging to a fundamentally new type of bioluminescent systems (i.e. orthogonal to all previously studied) described in a long time. *In vivo*, the bioluminescence of *Odontosyllis* peaks at around 510 nm. The reaction between *Odontosyllis* luciferin and luciferase requires no cofactors, which is also the case for other marine luminescent systems. As soon as the structure of *Odontosyllis* luciferin is deciphered and its synthesis method developed, this bioluminescent system will be actively used in bioimaging.

CONCLUSIONS

A huge variety of multicolor bioimaging techniques available allows choosing the one optimal for the particular task in the context of a specific medical research. The most sensitive are the bioluminescent imaging techniques, but in terms of multicolor labelling, their application is limited by the insufficient number of available luciferase-luciferin pairs. The recently discovered bioluminescent systems of higher fungi and *Odontosyllis*, a marine polychaete, which have several advantages over those that are currently popular, can extend the palette.

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INSTRUMENTAL PALPATION IN ENDOSCOPIC RENAL SURGERY: CASE REPORTS AND ANALYSIS

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Palpation is one of the classic examination methods in open surgeries. In minimally invasive surgery, intra-operational manual palpation is impossible to use for assessing tactile characteristics of tissues. In Russia, the only available instrument for intra-operational assessment and objective registration of tissue visco-elastic properties is the Medical Tactile Endosurgical Complex (MTEC). The aim of this work was to study the performance of MTEC in renal surgery. The study was performed during nine elective laparoscopic surgeries for clear cell renal carcinoma and simple renal cysts. We have found several differences in the use of MTEC in renal surgery, as compared to its use in gastrointestinal or lung surgeries. The key factor determining these differences was the inverse relations between tissue visco-elastic properties: the studied tumors were softer than the surrounding tissue. Detection of intraparenchymal tumors by tactile methods was impossible. For surface tumors, in one case out of nine it was possible to strictly locate the border of the tumor by tactile examination. We were able to quantitatively assess and determine the difference in hardness of tumors and intact tissue using MTEC. This allows studying the prognostic value of objectively registered tactile characteristics of renal tumors.

Keywords: renal surgery, instrumental mechanoreceptoric palpation, objective registration of tactile images, Medical Tactile Endosurgical Complex (MTEC), clear cell renal carcinoma, renal cyst

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ИНСТРУМЕНТАЛЬНАЯ ПАЛЬПАЦИЯ В ЭНДОСКОПИЧЕСКОЙ ХИРУРГИИ ПОЧЕК: ОПЫТ ПРИМЕНЕНИЯ

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Пальпаторная оценка — один из классических методов исследования при открытых хирургических вмешательствах. В малоинвазивной хирургии интраоперационная мануальная пальпация невозможна при оценке тактильных характеристик тканей. В России единственным доступным прибором для интраоперационной оценки и объективной регистрации вязко-упругих характеристик тканей является медицинский тактильный эндохирургический комплекс (МТЭК). Целью работы было изучить возможности применения МТЭК в хирургии почек. Исследование проводили в ходе девяти плановых лапароскопических вмешательств: по поводу светлоклеточного рака почки и простых кист почки. Выявлены особенности, отличающие использование МТЭК в хирургии почек от его применения на органах желудочно-кишечного тракта и легких. Ключевым фактором, определяющим наличие этих особенностей, является обратное соотношение вязко-упругих характеристик: исследованные опухоли оказались мягче окружающей ткани. Сделан вывод о невозможности выявления тактильными методами новообразований, расположенных в паренхиме. Для поверхностных новообразований в одном из девяти случаев механорецепторная пальпация позволила выявить четкое расположение границы опухоли. Применение МТЭК позволило количественно оценить и зафиксировать разницу в жесткостных характеристиках опухоли и неизменной ткани, что открывает возможность исследования прогностической значимости объективно регистрируемых тактильных характеристик новообразований почки на основании полученных цифровых данных.

Ключевые слова: хирургия почек, инструментальная механорецепторная пальпация, объективная регистрация тактильного образа, медицинский тактильный эндохирургический комплекс (МТЭК), светлоклеточный рак почки, киста почки

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Palpation, based on the sense of touch, is one of the basic parts of physical examination and is routinely used in open surgeries. During manual palpation, the visco-elastic properties of tissues and organs are assessed. This method is based on the evaluation of tactile characteristics, which change due to various pathologic processes. In particular, when malignant tumors form, the hardness of the tissue usually increases [1].

With the advent of minimally invasive surgery, intra-operational assessment of tissue tactile characteristics has changed. It is mediated by instruments and has taken the form of kinesthetic feedback in endoscopic surgery [2], and has practically disappeared in robot-assisted surgery [3, 4]. Information value of the feedback during manipulation strongly depends on the qualification and experience of the surgeon. It needs to be noted that even traditional palpation, despite its prolonged use in medical practice, is not a thoroughly standardized procedure, and the interpretation of its results depends significantly on the skill of the doctor [5, 6].

Development of instrumental tactile methods will help increase the awareness of the operator about tactile properties of the tissues during minimally invasive surgery and, in particular, will allow such assessment during robot-assisted surgery [7]. It will also help make palpation more objective, due to the possibility to save and reproduce the acquired information, among other features.

In medical practice, instruments for objective assessment of tissue tactile characteristics and tumor detection with tactile mechanoreceptor diagnostics are used in mammary glands and the prostate [8, 9]. An instrumental tactile vaginal examination method has been described for diagnosing pelvic organ prolapse [10]. Another instrumental palpation method has been described for detecting kidney stones during their laparoscopic extraction [11].

In Russia, the only commercially available instrument for intra-operational assessment of tissue tactile characteristics is the Medical Tactile Endosurgical Complex, MTEC-01 ("SPLAV"; Russia). MTEC is used, in particular, to detect and locate tumors in patients with peripheral lung cancers and gastrointestinal malignant tumors [12, 13].

Active implementation of minimally invasive treatment methods in renal surgery leads to an increased need for instrumental tactile examination. It is crucial for precise localization of the zone of pathological changes and additional

assessment of the spread of the pathological process during surgery. The aim of this work was to study the performance of MTEC in renal surgery.

METHODS

Patients

From March to May 2017, nine surgeries were performed using MTEC-01 in the City Clinical Hospital №52. The study was approved by the ethics committee of the hospital (protocol No. 0101/0117, January 25, 2017). Inclusion criteria were: indications for an elective, potentially organ-preserving laparoscopic surgery (cyst fenestration, kidney resection) or the diagnostic stage of a laparoscopic surgery on kidney parenchyma (in the course of nephrectomy). Exclusion criteria were: lack of the possibility and indications for a laparoscopic surgery. Nine patients took part in the study, four men and five women, aged 48–78 (average age 63.7 years). After the patients were informed about the possibility of using additional intra-operational diagnostic methods during surgery, all of them signed a voluntary informed consent form for the surgical procedure.

The surgeries included seven kidney resections, one nephrectomy and one elective nephradrenalectomy (see Table).

MTEC description

To perform an instrumental tactile examination during surgeries, we used MTEC-01 ("SPLAV", Russia). It comprises tactile mechanoreceptors (probes), a computer with special software, and an optional tactile display from which the surgeon can feel tactile images by hand. The instrument exists in two versions with different diameters of working part — 20 and 10 mm. On the working surface of the probe, depending on its diameter, 19 or 7 pressure sensors are located. They transfer data to the computer through a wireless connection, up to 100 times per second. Fig. 1 shows a probe with diameter 10 mm and seven sensors at the working surface. The results are shown on the tactile display in real-time, and also on a screen with the use of an adaptive color scale. With an average press force, softer tissues are shown green, harder tissues — red, and blue indicates intermediate results.

Table. Clinical characteristic of patients, type of surgery and results of histological analysis

Patient	Sex	Age	Diagnosis	Laparoscopic procedure	Histological description	Tumor size, mm
1	M	52	Renal cancer in left kidney, T3aN0M0. Chronic renal failure st. 1. Chronic kidney disease st. 3.	Left nephrectomy	Clear cell renal carcinoma, G2 according to Fuhrman	68
2	M	66	Renal cancer in left kidney, T1bN0M0	Left nephradrenalectomy	Clear cell renal carcinoma, G3 according to Fuhrman	54
3	F	78	Renal cancer in sole remaining right kidney, T1aN0M0	Resection of sole right kidney with tumor	Clear cell renal carcinoma, G1 according to Fuhrman	34
4	F	77	Cyst in the upper segment of right kidney Bosniak IIF	Resection of upper pole of right kidney with cyst wall	Simple tense renal cyst	62
5	F	48	Cyst in upper segment of right kidney Bosniak III	Resection of right kidney with cyst wall	Simple renal cyst	86
6	F	53	Cyst in lower segment of left kidney Bosniak IIF	Resection of left kidney with cyst wall	Simple renal cyst	79
7	M	63	Cyst in lower pole of left kidney Bosniak IIF	Resection of left kidney with cyst wall	Simple renal cyst	64
8	M	68	Cyst in middle segment of left kidney Bosniak III	Resection of left kidney with cyst wall	Simple renal cyst	57
9	F	68	Cyst in upper segment of left kidney Bosniak IIF	Resection of left kidney with cyst wall	Simple renal cyst	41

Surgical team training

When a new type of instrument is being introduced into clinical practice, theoretical and practical personnel training is necessary. Training with a simulator helps ensure reproducibility of results. All members of the surgical team (not only the operating surgeon) developed instrumental palpation skills with a box trainer. The aims of training were: to form a realistic vision of the method's capabilities, without unreasonably high expectations; to develop skills for understanding the adaptive color scale and assessing the amount of mistakes made when locating a hard object in soft tissues. Experienced practicing surgeons took part in the training. The length of the training was no more than 45 minutes. Later, during the study, one surgeon did all assessments, while assisting surgeons could also use the method if necessary. The training began with a brief explanation of the basics of the method and use of the instrument. Then the surgeons performed instrumental palpation on objects of different hardness, not hidden inside tissue. After that, they practiced palpating a metal ball placed inside soft spongy material. All surgeons mastered the method in 5 minutes, except one surgeon who needed a personal training session. Even taking this into account, six sessions with a laparoscopic box trainer were enough to achieve the goals of training.

The results of training have confirmed that information acquired by the surgeon with instrumental tactile examination is not analogue of information received during traditional palpation. While using MTEC, the surgeon combines kinesthetic sensations from contact with tissues, a visual image of the palpated organ, visualization of the tactile image based on the adaptive color scale and, optionally, a tactile image on the tactile display. The surgeon analyzes this information to find answers to the questions set by the aim of examination (for example, locating the border of the tumor to determine the necessary and sufficient resection margins).

Course of the study

MTEC was used for patients who, according to medical urological indications, had elective laparoscopic kidney resection, cyst fenestration, or nephrectomy. Tactile examination was performed by one surgeon in a stable laparoscopic surgical team. When planning the surgery, we took into account that the increase in surgery and anesthesia time cannot be more than 10–15 min. The palpation zone was controlled visually at all times. The paranephrium over the examination zone was removed according to the standard surgical protocol. Instrumental mechanoreceptor palpation and its results did not affect the initial surgery plan. To verify intra-operationally the location of the tumor and to verify the results of instrumental tactile examination, an intra-operational ultrasound examination was performed with an ultrasound apparatus Flex Focus (BK Medical; Denmark). There was no need to extend the stages of the surgery that require temporary cessation of blood flow (during resection). Thus, in the course of instrumental tactile examination the surgeon was not subjected to temporal stress due to the use of a new instrument. Additional laparoscopic ports, apart from the ones installed as a part of the standard laparoscopy protocol, were not used.

Conditions of instrumental palpation differed depending on tumor characteristics. Examination of visible tumors was performed starting from an arbitrary point closest to the renal hilum, clockwise until the tumor was fully localized. Two types of examination were used: static and dynamic. For static

examination, the tactile probe was applied to different parts of the tissue step by step. For dynamic examination, the probe was moved along the tissue under light pressure, while constantly preserving the contact between sensors and the tissue. When the border of the tumor was not visible, instrumental tactile examination was started from the upper pole of the assumed tumor location zone and continued clockwise. In the course of examination, (1) the possibility to locate the tumor border based on tactile mechanoreceptor data was analyzed; (2) a tactile characteristic of the tumor was given; (3) the kinesthetic sensation was recorded (soft-hard, softer or harder than healthy kidney tissue).

When visualizing the tactile image based on the adaptive color scale, the following templates were described:

- soft: the center is outside the palpation zone due to pressure on the outside perimeter of the mechanoreceptor working part (center is light green) (Fig. 2A);
- firm: the center is under pressure, the outside perimeter is partially outside the palpation zone (center is blue or red, perimeter color indicates lower pressure) (Fig. 2B);
- border: a border line without acute angles is visible, and on both sides of it the registered pressure values are close to uniform (but the pressure on different sides of the line is visualized with different colors) (Fig. 2C).

Due to the standard position of laparoscopic ports, the optimal contact angle (when the tactile probe is almost perpendicular to the examined tissue) could be reached when examining mainly the front, medial and, partly, other side surfaces of the kidney. Instrumental palpation of the back surface of the kidney required mobilizing the organ and rotating the renal pedicle. Because of this, in order to avoid ischemic injury, instrumental palpation of the back surface was performed only in the cases when kidney removal was planned.

RESULTS

During the study, the following points were considered most important:

- the possibility of “palpatory visualization” of the renal tumor for surface tumors and tumors located inside the parenchyma;
- how visco-elastic properties of the surrounding tissue (characteristics of the parenchyma) influence the information value of instrumental tactile examination;

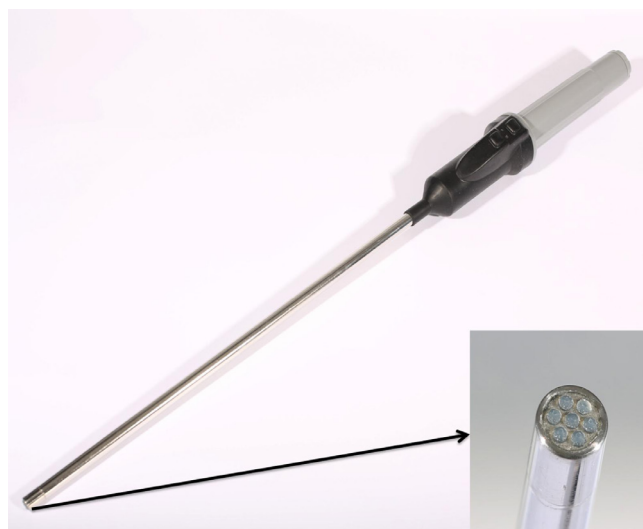


Fig. 1. A tactile probe with diameter 10 mm and seven pressure sensors on the working surface

– comparability of the results of instrumental tactile examination to information acquired from kinesthetic feedback.

The following results were obtained.

Patient Po., 52 years old. Macroscopically solid nodular tumor, up to 68 mm in size, located on the surface and visualized during laparoscopy (Fig. 3). Kinesthetically hard. According to kinesthetic sensation, the tumor was softer than intact tissue, which was consistent with the visualization of the registered tactile image based on the adaptive color scale (Fig. 3C). The border was convincingly detected by the instrumental tactile method. It needs to be noted that, due to the size of the tumor, the working angle of the tactile probe on the tumor was substantially different than on intact kidney tissue.

Patient Pe., 66 years old. Macroscopically cystic tumor, 54 mm in size, located on the surface and visualized during laparoscopy. Kinesthetically soft. According to kinesthetic sensation, kidney parenchyma is harder than the tumor. Instrumental palpation also showed that the tumor was softer than the parenchyma. Precise determination of the tumor border with instrumental tactile examination was deemed impossible.

Patient S., 78 years old. Tumor in the upper pole of the kidney, 34 mm in size (T1aN0M0). An organ-preserving resection of the sole remaining kidney was performed. The tumor was located under the renal capsule, was not visible and could not be located with instrumental tactile examination (Fig. 4). Resection became technically possible only after tumor visualization with an ultrasound 3D-reconstruction, which confirmed resectability and allowed determining the necessary operative procedure. At the same time, the tumor was found to be only 2 mm below the surface of the organ (Fig. 4A). The surgery lasted 140 mins. Temporary hemostasis control was obtained by endoscopically applying a bulldog clip on the whole renal pedicle. Warm ischemia time was 17 mins. Final hemostasis was reached by stitching the kidney wound.

Patient Sh., 77 years old. Macroscopically tense renal cyst, located on the surface, 62 mm in size, easily visible. Kinesthetically soft. According to kinesthetic sensation, the cyst was softer than kidney parenchyma. According to tactile characteristics visualized with the adaptive color scale, it did not differ significantly from healthy tissue and was difficult to localize. Under moderate pressure all fields were green.

The remaining five cases were presented with macro- and microscopically non-tense simple renal cysts and were analyzed together. In all five cases, strict localization of cyst borders based on the data from instrumental mechanoreceptoric examination was deemed impossible.

Cyst visualization yielded a template with an evenly colored perimeter. With this template, an analysis of cyst tension was possible by comparing the colors of the central and peripheral fields. A tense cyst yielded the firm template, and non-tense cysts yielded intermediate patterns between distinctly soft and distinctly firm templates.

DISCUSSION

When the first laparoscopic nephrectomy was described [14], the era of minimally invasive operations in renal surgery has started. Advantages of endoscopy, including less trauma, shorter postoperative period, and better visualization during surgery with instruments, come together with a shortage of available examination methods because palpating organs and tissues is rendered impossible. In endoscopic surgery, the only available feedback is kinesthetic, determined by the pressure

applied to muscles and ligaments [2]. Standard robot-assisted surgery lacks both tactile and kinesthetic feedback [3]. Studies with additional instruments creating tissue response have shown that these instruments allow reducing grasping power and, consequently, decreasing tissue damage [15].

In literature, several types of instruments have been described that help obtain kinesthetic and tactile information during laparoscopic surgeries, including robot-assisted surgeries [12, 13, 16–19]. The majority of these instruments performs specific tasks, such as controlling the grasping power, and does not allow an instrumental tactile examination. MTEC, on the contrary, is designed specifically for mechanoreceptoric palpation.

Apart from obtaining additional information during endoscopic surgery, with instrumental palpation it is possible to make the results of tactile examination more objective. This, in turn, increases the efficiency of examination for less experienced surgeons.

Implementing an objective method of tissue tactile characteristic assessment during surgery seems to be a promising but understudied aspect of renal surgery. In this work, we studied the performance of MTEC for instrumental mechanoreceptoric palpation in renal surgery. We found several features that differentiate the use of MTEC for instrumental mechanoreceptoric palpation in renal surgery from its use on gastrointestinal organs and lungs [12, 13]. The key factor determining these differences is the inverse relations between tissue visco-elastic properties of renal tumors and surrounding tissues. While most malignant tumors are harder than healthy tissue [1, 20–22], the studied renal tumors (according to histological examination, all studied tumors were clear cell renal carcinoma) were softer than the surrounding healthy tissue. These results consistent with observations showing that Young's modulus of healthy kidney tissue is significantly higher than Young's modulus of renal cell carcinoma [23]. The difference between direct and inverse relations of hardness in tumors and surrounding tissue is essential because, even with manual palpation, tissue softness is assessed not separately but together with other tissues and parameters. Because of this, intraparenchymal tumors, even at a small depth (2 mm), cannot be located with tactile methods

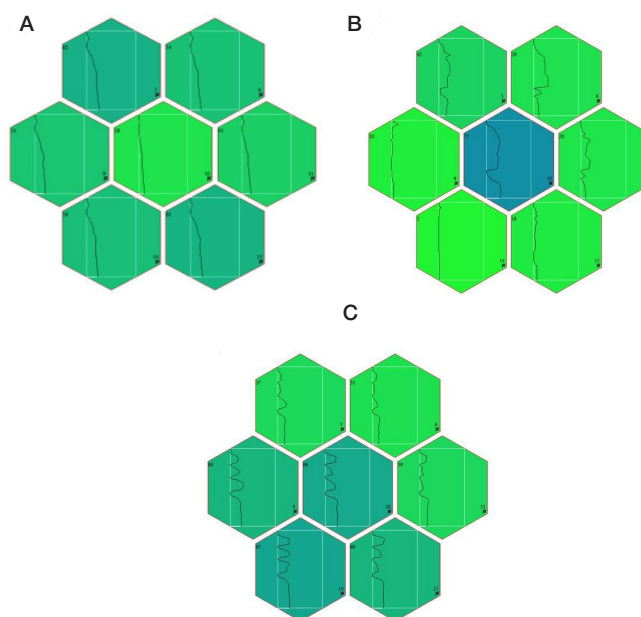


Fig. 2. Visualization of characteristic tactile frames in the adaptive color scale: soft template (A), firm template (B), border template (C)

due to their softness: the harder surrounding tissue conceals softer structures. We can propose an appropriate cooking analogy. When baking, the readiness of dough cannot be assessed with tactile characteristics, because the upper crust springs in the same way regardless of the state of inner parts. Other methods are used to determine if the product is ready. At the same time, surface tumors were identified with tactile examination. In one case, tactile examination made it possible to precisely localize the tumor border.

Cysts, according to their instrumentally registered tactile characteristics, did not differ significantly from healthy kidney tissue, which conformed well to results of instrument-mediated palpation and further manual palpation of removed specimens. Hardness of the tense cyst was slightly higher than in surrounding tissue, but it was impossible to locate its border with tactile methods.

Another important observation is that instrumental tactile examination with MTEC becomes more informative with dynamic rather than static palpation. The key feature of dynamic palpation is the "roll-over" of the instrument's working surface from the initial point over the study area. Because the probe was fixed inside a trocar, freedom of movement for dynamic palpation was limited but still sufficient for the manipulation. Static palpation, with pressure applied along the axis of the probe, was notably less informative.

During training with a metal ball inside spongy material, we discovered that with MTEC, localizing hard inclusions smaller than the working surface of the probe is easier than localizing larger inclusions. We propose that MTEC efficiency can be enhanced if the working surface area is increased without increasing the diameter of the probe itself.

For renal surgery, it is necessary to modify MTEC software to include a regime for localizing tumors softer than the surrounding tissue. Current software is aimed at searching for harder structures [24, 25]. A simple change in the adaptive color scale that would highlight softer zones during visualization will already help the surgeon read the image more naturally.

In most cases it was impossible to localize tumor borders based solely on the results of instrumental tactile palpation with MTEC. However, the study has shown differences in registered tactile templates for cases when tumors were harder or softer than the surrounding tissue. Standardization of instrumentally registered tactile information (for example, allocation of tactile frames which correspond to the pressure exactly determined by the probe's weight) raise a question of studying the prognostic value of instrumentally registered tactile characteristics for tumor staging. Standardization will also help specify surgical tactics. For several types of malignant tumors this connection was already described [22, 26, 27].

CONCLUSIONS

Malignant renal tumors show an inverse relations of tumor and healthy tissue hardness: tumors are softer than the surrounding kidney tissue. This feature is highly significant for instrumental tactile examination. Because of this, it is impossible to locate tumors situated inside the parenchyma, even at a small depth, with tactile methods. For surface tumors, in one of the cases, information from mechanoreceptoric palpation was sufficient to precisely locate the tumor border. In other cases, it was

impossible to locate the tumor based on the information from instrumental tactile examination. However, in these cases use of MTEC allowed a quantitative evaluation of the difference in hardness between the tumor and intact tissue. The advent of a technology that can perform such an evaluation opens the possibility to study the prognostic value of objectively registered tactile characteristics of renal tumors for intra-operational express diagnosis. In the course of this study, the methodology of tactile examination was improved and templates for use in diagnosing renal tumors were developed.

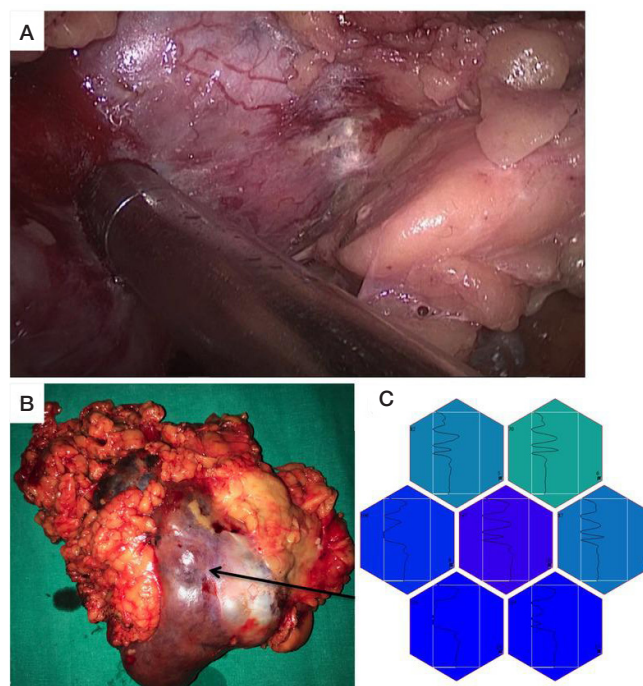


Fig. 3. Examining a tumor with a visible border. **A.** Image from a laparoscopic camera, examination with a tactile probe. **B.** Removed specimen; the visual border of the tumor is shown. **C.** Visualization of a tactile frame with the adaptive color scale

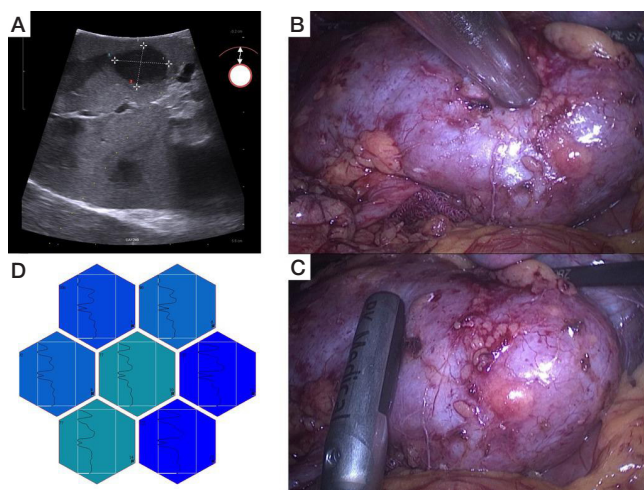


Fig. 4. Examining a non-visible tumor inside the parenchyma. **A.** Ultrasound visualization of the tumor; distance from the surface is shown. **B.** Examination with a tactile probe: image from a laparoscopic camera. **C.** Scanning with an ultrasound sensor; image from a laparoscopic camera. **D.** Visualization of a tactile frame with the adaptive color scale

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SYNTHESIS OF AU (III) POLYACRYLATES AND STUDY OF THEIR TUMORICIDAL ACTIVITY

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Aurumacryl is an incomplete metal salt of poly(acrylic acid) that exhibits hemostatic activity and inhibits the growth of transplantable carcinomas *in vivo*. The samples of aurumacryl synthesized following the original technique are insufficiently soluble, which complicates the study of the mechanisms involved in their synthesis and underlying their cytotoxic effect. The aim of this work was to study the impact of the following factors on aurumacryl properties: the molecular weight of the polyacrylate polymer in a range between 2 and 1,000 kDa, the presence of a counterion H⁺ or Na⁺, the molar ratio of AuCl₄⁻ to the polyacrylate polymer (1 : 5 and 1 : 10), the total concentration of the polyacrylate polymer during synthesis (0.1 and 3%), and the type of drying (lyophilization). By comparing the cytotoxicity of aurumacryl samples with significantly different molar ratio of gold ions to the polyacrylate polymer against human breast carcinoma cells (MCF-7), we established that the proportion of the polymer and its molecular weight in the sample do not affect the biological properties of the synthesized substance. Using UV spectroscopy, we revealed that the concentration of Au (III) ions in aurumacryl determines its cytotoxicity.

Keywords: aurumacryl, polyacrylate, chloroauric acid tetrahydrate, antitumor therapy, cytotoxicity, MCF-7

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СИНТЕЗ И ИССЛЕДОВАНИЕ ПРОТИВООПУХОЛЕВОЙ АКТИВНОСТИ ПОЛИАКРИЛАТОВ ЗОЛОТА (III)

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Аурумакрил проявляет гемостатическую активность и обладает онколитическим и онкостатическим действием в отношении перевиваемых карцином *in vivo*. Образцы аурумакрила, полученные по известной методике, недостаточно стандартизированы по растворимости, что не позволяет изучить механизмы реакции и цитостатического действия. Целью работы было исследовать влияние на свойства аурумакрила следующих параметров проведения реакции его синтеза: молекулярная масса полиакрилатного полимера (в диапазоне 2–1000 кДа), наличие в качестве противоиона H⁺ или Na⁺, соотношение иона тетрахлораурата и звена полиакрилатного полимера (1 : 5 или 1 : 10), общая концентрация полиакрилатного полимера в момент синтеза (0,1 или 3%), а также влияние лиофильного высушивания аурумакрила. Сопоставление цитотоксической активности полученных образцов аурумакрила со значительно различающимся соотношением ионов золота и полиакрилатного полимера в отношении клеток карциномы молочной железы человека MCF-7 позволило установить, что удельное содержание в препарате полиакрилатного полимера и его молекулярная масса практически не сказываются на биологических свойствах конечного продукта. Методом УФ-спектроскопии показано влияние концентрации ионов Au (III) в аурумакриле на их цитотоксическую активность.

Ключевые слова: аурумакрил, полиакрилат, золотохлористоводородная кислота, тетрахлораурат, противоопухолевое средство, цитотоксическая активность, MCF-7

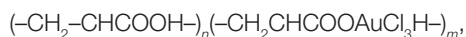
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Aurumacryl first described in [1] is an incomplete metal salt of poly(acrylic acid) (PAA) containing gold (III) ions. Based on the amount of the initial reactants consumed during aurumacryl synthesis, it was concluded that the final metal wt% in the substance would be 8.03% whereas the molecular weight of the polymer would fall within the range between 100 and 300 kDa. The chemical formula of aurumacryl is



where $n = 1,263$ and $m = 124$.

In the original study, aurumacryl was synthesized by combining aqueous PAA solutions and chloroauric acid tetrahydrate (HAuCl_4) at room temperature [1]. Briefly, HAuCl_4 was taken in the amount sufficient to produce 8.3% wt of gold in the resulting gold polyacrylate and added to a 5.7% PAA solution (the molar mass of the solute M_r was 100, 000). The reaction mixture was stirred, and the solution was then left in a vacuum desiccator. The resulting substance was a dense yellow film. Its physical and chemical properties can be inferred from the absorption bands of its IR spectra characteristic of carboxyl and carboxylate groups at 1,720 and 1,570 cm^{-1} wavelengths, respectively. However, the actual content of aurumacryl components in the resulting compound, including polyacrylic acid, gold ions (III), gold ions (I), elemental gold, and other anions and cations, cannot be deduced from the presented data.

The aim of this work was to improve the technique for aurumacryl synthesis in order to obtain a chemically stable standard preparation sufficiently soluble in water and to elaborate a method for testing aurumacryl quality thereby predicting its biological activity. The substance was synthesized using commercially available PAA and sodium polyacrylate (PANa) with known molecular weights. The molar ratio between AuCl_4^- anions and PAA in the reaction mixture was 1 : 5 and 1 : 10.

Unlike our colleagues, we thoroughly desalted the PAA polymer by continuous-flow dialysis, which took a few days, and dried the solutions of the obtained polymer-gold complexes in a freeze dryer instead of leaving them to air-dry. The lyophilized aurumacryl samples were cryopreserved in liquid nitrogen and vacuumized.

To evaluate our improved method of synthesis, we tested aurumacryl for solubility and cytotoxicity against MCF-7 cells, measured the amount of gold salts in the produced samples by UV-spectroscopy, and estimated the content of gold nanoparticles in aurumacryl using transmission electron microscopy.

METHODS

Reagents

Commercially available PAA and PANa (Sigma Aldrich; USA) with different polymerization degrees (i.e., the average number of monomers per molecule) of 23; 80; 160; 1,389; 1,944; 3,472; and 13,889 were used as starting materials. The average degree of polymerization and polymer dispersion properties were specified by the manufacturer. Another component used for aurumacryl synthesis was dry chloroauric acid (Aurat; Moscow) with 48% gold content.

Preparing polyacrylic polymers for synthesis

For synthesis we chose PAA with an average molecular weight of 1,633 Da, 11,360 Da, 100,000 Da, 140,000 Da, 250,000

Da, and 1,000,000 Da (the molecular weight was specified by the manufacturer) and sodium polyacrylate (SigmaAldrich; USA) with an average molecular weight of 2,100 Da, 15,000 Da, 132,350 Da, 185,370 Da, 330,980 Da, and 1,324,000 Da.

Prior to synthesis, all PAA samples were additionally purified by flow dialysis continued for several days and thoroughly dried by freezing.

Then, weighted amounts of the polymers (50 mg) were dissolved at room temperature in 50 and 1.67 ml of water to obtain 0.1% and 3% wt polymer solutions, respectively. During the 12-h process the polymers were continuously stirred on the magnetic stirrer.

Aurumacryl synthesis

Chloroauric acid was added to the solutions of pre-deionized polymers as described below:

- 50 μL of the aqueous solutions containing 23.8 and 47.6 mg of chloroauric acid were added dropwise to 0.1% wt PAA solutions;

- 30 μL of the aqueous solutions containing 23.8 and 47.6 mg of chloroauric acid were added dropwise to 0.3% wt PAA solutions;

- 50 μL of the aqueous solution containing 18.4 and 36.7 mg of chloroauric acid were added dropwise to 0.1% wt PANa solutions;

- 30 μL of the aqueous solution containing 23.8 and 47.6 mg of chloroauric acid were added dropwise to 0.3% wt PAA solutions.

Synthesis was carried out at room temperature and took 24 h. The reaction mixture was continuously stirred on the magnetic stirrer at 60 rpm.

Low molecular weight components were separated from the obtained solutions by continuous-flow dialysis using MWCO 15,000 dialysis tubing. The solutions of gold-polymer complexes (1.7–20 ml) were poured into the tubing and dialyzed against water for 3 h at room temperature.

After that, the solutions were frozen at -70°C and lyophilized, except for a few samples that were not freeze-dried but stored at $+4^\circ\text{C}$ under aseptic conditions instead.

Determining solubility of aurumacryl samples

Solubility was determined for each of the obtained dry aurumacryl samples. Briefly, 100 μL water aliquots were added one by one to 1 mg of the sample at room temperature and its behavior was monitored for 30 min. If the sample did not dissolve, another water aliquot was added to it. The procedure was repeated until complete dissolution was achieved.

Measuring the conversion of gold ions in concentrated aurumacryl solutions

The conversion of gold ions was measured in 100 mM HCl at $\lambda = 226.5$ nm. For the measurements, we took a 10 μL aliquot of a concentrated solution obtained in the previous solubility assessment step and 10 μL of a solution that was not dried immediately after the synthesis. The aliquot was introduced into 3 ml of a 100 mM HCl solution and then its absorbance $A_{226.5}$ at 226.5 nm was measured. The conversion of gold salts was determined for each sample based on the calculated $A_{226.5}$ ($\approx[\text{HAuCl}_4]$, μM) relative to the total solids concentration (the residual moisture in the dry sample was ignored). For further toxicity experiments in MCF-7 cells, concentrations of all samples were normalized based on absorbance at $\lambda = 226.5$.

Studying the cytotoxic activity of aurumacryl samples in the MCF-7 cell line with an MTT assay

Five thousand cells were seeded onto the Costar 96-well plate containing 180 μL of the DMEM medium (Paneco; Russia) per well supplemented with 10% fetal bovine serum. The cells were cultured at 37 °C and 5% CO_2 for 24 h. Then 20 μL of the aqueous aurumacryl solution with the normalized concentration (absorbance at 226.5 nm) were added into each well and continued incubation at 37 °C and 5% CO_2 for 24 h. After that, 10 μL of the MTT reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (item 57360-69-7; Sigma Aldrich; USA) were added at a final concentration of 0.5 mg/ml to the culture, and the cells were incubated for 3 h. Absorbance from the wells A_{570} at 570 nm was measured by the Multiskan Go spectrophotometer (Thermo Fisher Scientific; USA).

Measuring the proportion of gold nanoparticles in aurumacryl samples by transmission electron microscopy

A 3 μL aliquot of the aqueous aurumacryl solution containing 1–5 μg of the gold-polymer complex was applied onto the Fine 200 Copper Grid Mesh (SPI Supplies; USA) with a diameter of 3.5 mm, air-dried for 5 min and vacuumized. The analysis was carried out using the JEM-100B microscope (JEOL; Japan) at the accelerating voltage of 120 kV. The microscope was equipped with an accessory for X-ray phase-contrast imaging.

The images of the particles detected within the field of view were analyzed in ImageJ (a free software by the National Institutes of Health; USA): the square area of each dark spot was calculated allowing for the zoom factor. At least 10 randomly chosen fields of view were analyzed for each sample. Once the linear sizes of the particles and their number was identified, we calculated the proportion of the particles falling within each of 5 diameter ranges of 1–10, 11–20, 20–50, 50–100, and >100 nm in Windows Excel.

RESULTS

We synthesized 23 aurumacryl samples from PAA and sodium polyacrylate with various molecular weights at 2 different molar ratios between AuCl_4^- and PAA of 1:5 and 1:10; the initial polymer concentration in the reaction mixture was 3% or 0.1%.

A total of 10 freeze-dried samples were re-dissolved in water showing good solubility.

Chloroauric acid is known to undergo gradual hydrolysis in aqueous solutions with neutral and alkaline pH; the reaction produces hydrochloric acid, which increases the acidity of the medium [2]. At $\text{pH} < 1$ this reaction is impossible. In our experiment, the aqueous solution of chloroauric acid in 100 mM HCl had an absorbance peak at $\lambda = 226.5$ nm, the extinction coefficient ϵ being $3.5 \times 10^4 \text{ l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. These values are consistent with the published literature [2]. For the polyacrylates synthesized in the course of our experiment the extinction coefficient was below $10^6 \text{ l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$. Gold nanoparticles had zero absorbance at $\lambda = 226.5$ nm, except for the light scatter effects. Therefore, we measured the proportion of gold ions in the synthesized substance at $\lambda = 226.5$ nm in 100 mM HCl (see *Methods*).

To test a hypothesis that the cytotoxic activity of aurumacryl is determined by the proportion of Au (III) in the substance, the solutions of the synthesized aurumacryl samples and the control sample prepared following the original technique (sample 1) were normalized to the Au (III) concentration calculated from absorbance at $\lambda = 226.5$ nm. The solutions were tested for

cytotoxicity at concentrations of 9.1 mU/ml (the IC_{50} of the control sample) and 91.0 mU/ml, which is 10 times higher than the IC_{50} of the control sample. The results were averaged based on 3 independent measurements and are presented in Table 1.

Although concentrations of Au ions expressed as $\mu\text{mol}/\text{mg}$ differed between the solutions, all normalized aurumacryl samples exhibited the same cytotoxic potency as the control aurumacryl sample prepared following the original technique. The biological activity of the normalized aurumacryl samples was identical both at concentrations equaling IC_{50} of the control sample and at those 10 times higher than IC_{50} . This proves that *in vitro* cytotoxicity of aurumacryl is determined by Au ions. The polymer component of the compound seems to work as a shield protecting Au ions from the interaction with other organic molecules that can reduce them to inactive gold nanoparticles.

We hypothesized that lower conversion of gold ions in the process of aurumacryl synthesis is due to their spontaneous reduction to elemental gold that aggregates into nanoparticles. To test this hypothesis, we studied a few synthesized aurumacryl samples by transmission electron microscopy (TEM) (Table 2).

The bigger was the molecular weight of the polymer used for aurumacryl synthesis, the higher was the proportion of gold nanoparticles sized > 10 nm, which in essence are aggregates of smaller particles under 10 nm in size. Regardless of PAA molecular weight, the proportion of aggregated nanoparticles, as compared to the proportion of unaggregated, increased dramatically when the molar ratio of Au (III) to PAA was reduced from 1 : 5 to 1 : 10. When PANa was used instead of PAA as the starting component for aurumacryl synthesis, the average size of gold nanoparticles increased and more pronounced aggregation of gold nanoparticles was observed. For example, in the samples synthesized from PANa with polymerization degrees of 3,472 and 13,889 at the 1 : 10 molar ratio between gold ions and PANa, the proportion of gold nanoparticles sized ~100 nm was 75%. In other samples synthesized from PANa, such nanoparticles were not detected. The control aurumacryl sample synthesized following the original technique contained a remarkably high proportion of gold nanoparticles with an average diameter of 20–50 nm, which exceeded the proportion of such nanoparticles in other tested samples several-fold.

To validate our hypothesis about the cytotoxicity of aurumacryl samples being dependent on the proportion of Au ions and independent of the proportion of polyacrylate, elemental gold and other components, the most active samples 3, 10 and 17 (Table 1) were scrutinized for their cytotoxic activity against MCF-7 cells. The aurumacryl solutions were normalized to ion gold concentrations based on absorbance at $\lambda = 226.5$ nm in 100 mM HCl. Aurumacryl synthesized following the original technique was used as a control sample. The results of the test are shown in Fig. 1.

The correlation between the cytotoxic activity of the sample and the Au (III) concentration was the same for samples 3 and 10, as well as for the control sample, although the samples were different in their composition and the applied method of synthesis. Sample 3 was synthesized from PAA with an average molecular weight of 1.6 kDa (low polymerization degree) and was freeze-dried immediately after synthesis. Sample 10 was synthesized from PAA with a molecular weight of 250 kDa and was stored as a solution. The molar ratio of gold ions to PAA in the reaction was 1 : 32 for the control sample and 1 : 10 for samples 3 and 10. Thus, the tested samples had different solubility and a different molar ratio of Au (III) to PAA. Table 2 suggests that the samples may have contained

different proportions of gold nanoparticles. However, after the concentrations of the tested samples were normalized to Au (III), they exhibited identical cytotoxicity against the MCF-7 cells.

The correlation between the cytotoxic activity of sample 17 synthesized from PANa with an average molecular weight of 5.75 kDa and spared from freeze-drying and the concentration of gold ions in it implies that sample 17 has some properties missing by other samples. At low concentrations in the solution (0–10 mU/ml) its cytotoxicity is higher than that of the samples synthesized from PAA whereas at higher concentrations of 25

and 90 mU/ml its cytotoxic potency is worse. It means that the interactions between chloroauric acid and PAA and between chloroauric acid and PANa produce substances in which the biological activity of Au ions is slightly different.

Aurumacryl synthesized from PANa (as opposed to PAA) can hardly be used in clinical practice because of its insufficient chemical stability during storage. Regardless of the molecular weight of the polymer, both solutions and lyophilisates have a tendency to the spontaneous reduction of Au ions that subsequently form gold nanoparticles, which causes a dramatic decline in their cytotoxicity.

Table 1. Synthesis conditions, physical, chemical and biological properties of aurumacryl samples

Sample ID	Concentration of the initial polymer during synthesis, %wt	Mr of the initial polymer, kDa	Counterion	Molar ratio between Au (III) anions and PAA or PANa in the reaction mixture, mol:mol	Max concentration of aurumacryl solution based on total solids concentration, mg/ml	A _{226.5} of the concentrated aurumacryl solution, U/ml	Concentration of Au ions, mmol/mg of solids	Proportion of surviving MCF-7 cells at aurumacryl concentration A _{226.5} expressed as ml (MTT assay)	
								9.1 mU	91.0 mU
1	5.7	100–300	H	1 : 32	2.7	0.91	12.5	5	49
2	3.0	1.6	H	1 : 10	29.4	2.40	32.8	5	51
3	3.0	1.6	H	1 : 10	28.3	3.00	41.0	5	47
4	3.0	11.5	H	1 : 10	29.4	1.00	15.5	5	21
5	3.0	100	H	1 : 10	29.4	1.53	21.0	4	45
6	3.0	100	H	1 : 10	33.3	3.00	41.0	5	46
7	3.0	140	H	1 : 5	29.4	4.80	65.5	5	42
8	3.0	140	H	1 : 10	29.4	2.00	23.6	4	51
9	3.0	140	H	1 : 10	47.0	4.30	58.7	5	44
10	3.0	250	H	1 : 10	29.4	2.00	24.2	4	60
11	3.0	250	H	1 : 10	16.7	0.96	13.2	5	54
12	0.1	250	H	1 : 10	4.2	0.77	10.5	6	43
13	3.0	1000	H	1 : 10	29.4	0.56	7.7	5	44
14	0.1	1000	H	1 : 10	5.0	0.69	9.4	6	49
15	3.0	1.6	Na	1 : 10	29.4	1.27	17.4	5	45
16	0.1	1.6	Na	1 : 10	27.3	0.39	5.4	6	41
17	3.0	5.75	Na	1 : 10	29.4	1.00	14.8	6	39
18	3.0	100	Na	1 : 10	29.4	1.00	18.4	4	39
19	0.1	100	Na	1 : 5	12.6	2.58	35.3	6	46
20	3.0	140	Na	1 : 10	29.4	1.33	18.2	11	42
21	3.0	250	Na	1 : 10	29.4	1.30	17.8	6	44
22	0.1	250	Na	1 : 10	13.3	0.37	5.2	7	41
23	3.0	1000	Na	1 : 10	29.4	0.37	5.2	11	49
24	0.1	1000	Na	1 : 10	10.0	0.54	7.5	9	38

Note: Freeze-dried samples dissolved in water are shown in grey; other samples were not lyophilized but stored as solutions instead.

Table 2. Distribution of gold nanoparticle sizes in aurumacryl samples synthesized from PAA and PANa

M _r of the polymer, kDa	Counterion	Molar ratio of Au (III) ions to PAA or PANa in the reaction mixture, mol:mol	Proportion of nanoparticles of a given diameter, %				
			1–10 nm	11–20 nm	20–50 nm	50–100 nm	>100 nm
1.6	H	1 : 5	76.0	14.0	10.0	0.0	0.0
1.6	H	1 : 10	57.5	25.0	15.0	2.5	0.0
11.5	H	1 : 10	84.0	12.0	4.0	0.0	0.0
250	H	1 : 10	42.9	48.6	8.6	2.9	0.0
1000	H	1 : 10	36.0	50.0	2.0	0.0	12.0
11.5	Na	1 : 5	82.0	8.0	6.0	4.0	0.0
140	Na	1 : 5	78.0	16.0	6.0	0.0	0.0
140	Na	1 : 10	0.0	2.5	0.0	22.5	75.0
100–300	H	1 : 0.0018	46.7	23.3	30.0	0.0	0.0

DISCUSSION

The tumoricidal effect of aurumacryl was investigated both *in vivo* and *in vitro* in the study [3]. The authors report that a single intraperitoneal injection of aurumacryl at a dose of 20 mg per 1 kg mouse weight causes 80–90% growth inhibition of solid tumors in mice, including Lewis lung carcinoma, large intestine adenocarcinoma ACATOL and Ca-755 adenocarcinoma, in comparison with the controls. In the experiments *in vitro* the aurumacryl concentration of 1 mg/ml kills ~70% of the MCF-7 cells (human breast carcinoma) by triggering both necrosis and apoptosis [3]. The mechanism of the selective tumoricidal effect of aurumacryl *in vivo* is yet to be elucidated; it is known, though, that gold ions do not selectively accumulate in the tumor following the intraperitoneal injection of aurumacryl but are evenly distributed in the body of the mouse and eliminated through excretion [3].

Over the past 3 years there have been reports that acrylic acid polymers can effectively deliver different cytotoxic and antiproliferative agents to cancer cells *in vitro* and *in vivo* (see below), meaning that polyacrylic particles of different molecular weight can selectively transport such agents to cancer cells and enhance their activity inside the cancer cell. Importantly, polyacrylates can selectively accumulate in dendritic cells and macrophages. This can improve the presentation of antigens carried by a polyacrylate agent to lymphoid cells as shown in the successful test of a polyacrylate conjugated to the peptide antigen mimetic of the HPV-16 oncoprotein 7 [4]. Polyacrylate particles immobilized inside the virus-like particles composed of the hepatitis B HBs-antigen enhanced the cytotoxic effect of doxorubicin [5]. There is evidence that peroxovanadate delivered to A495 lung carcinoma cells can effectively inhibit their growth [6]. Another study demonstrates a possibility of using theranostic polyacrylate-calcium phosphate complexes as carriers of gold nanoclusters in the therapy and diagnostic imaging of solid tumors [7].

Polyacrylic clusters can be used to deliver titanium oxide nanoparticles, ferromagnetic contrast agents, and salts and oxides of other metals to the tumor to increase its sensitivity to radiotherapy, achieve a cytotoxic effect or facilitate bioimaging based on the principles of fluorescence or magnetic resonance

[8–11]. Polyacrylate can be employed to deliver methylene blue used as a photosensitizer for contrast-enhanced radiotherapy to human breast carcinoma cells (MCF-7) [12]. All data mentioned here indirectly suggest that polyacrylates are capable of selectively targeting cancer cells *in vivo*. The underlying mechanism is unclear but the existing evidence leads us to hypothesize that PAA itself cannot exhibit cytotoxic activity against cancer cells. It is probably Au ions (III) carried by PAA that make the complex toxic for cancer cells.

This supposition can be confirmed by the findings of the study [13] of Au (III) compounds dichloro(ethylenediamine)aurum and dichloro(S-methyl-L-cysteine)aurum that exhibit pronounced cytotoxicity against human lymphoid leukemia cells. The authors of the study prove that just like cisplatin Au (III) ions can form DNA adducts in living cells causing their death.

The insufficient solubility of aurumacryl synthesized as described in [1] complicates the testing of its safety and therapeutic activity against cancer cells in biological models, as well as its use in clinical practice. The effective aurumacryl dose in the mouse model is 20 mg per 1 kg body weight. The required concentration of aurumacryl in the solution would be 1 mg/ml [3]. Given that a mouse weighs about 20 g, the volume of the injected solution would be 200 μ L per mouse, approaching the acceptable threshold: further increase in the injection volume would threaten the survival of mice regardless of the composition of the injected drug. Besides, dry samples stored at +4 $^{\circ}$ C for 6 months show a sharp decline in their solubility.

By varying the parameters of the initial polymer and the molar ratio of PAA or PANa to chloroauric acid, we were able to study the impact of these factors on the reaction yield. We revealed that PAA concentrations in the produced aurumacryl and its molecular weight had almost no effect on the biological properties of the obtained substance. In contrast, the concentration of Au (III) ions measured from the absorbance of aurumacryl solutions ($\lambda = 226.5$ nm) turned to be an accurate predictor of their *in vitro* cytotoxic activity in the cell culture.

However, the molar ratio of the polymer to AuCl_4^- anions, the molecular weight of the polymer and the absolute concentration of the polymer solution have a profound impact on the gold ion concentration in the synthesized sample and

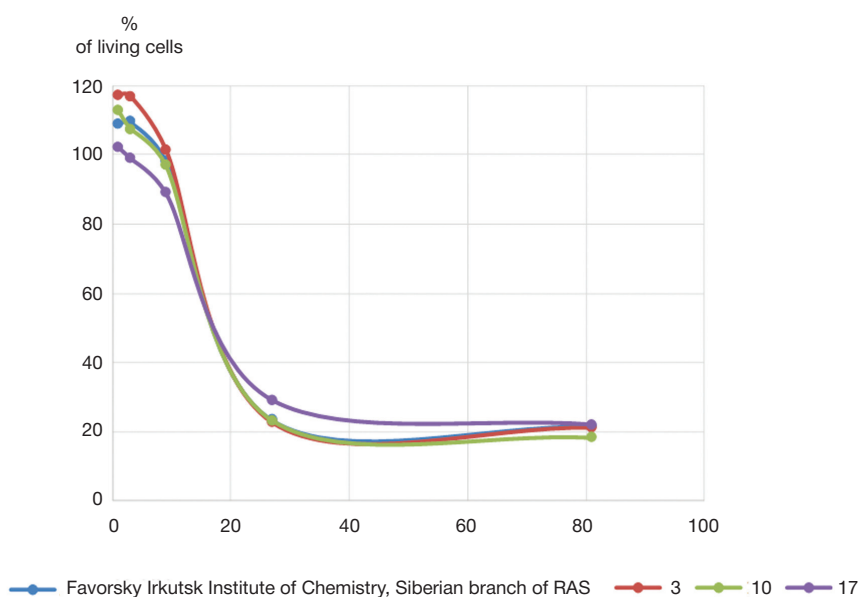


Fig. 1. The graph shows a correlation between the Au (III) concentration in the tested aurumacryl samples and their cytotoxicity against human breast carcinoma cells (MCF-7). Concentrations at $A_{226.5}$ expressed as mU are plotted on the X axis. The proportion of the surviving cells measured by the MTT assay is plotted on the Y axis. The curves were constructed for samples 3, 10 and 17 synthesized in the course of this work and for the control sample synthesized at Irkutsk Institute of Chemistry (see Table 1).

the solubility of the latter. In our experiment, the solubility of the sample was the highest when aurumacryl was synthesized from the polymers with polymerization degrees of 1,389 and 1,944. The use of the polymers with higher molecular weight resulted in a slightly worse solubility of the aurumacryl sample if the initial polymer had the polymerization degree of 3,472, and caused a dramatic drop in the solubility of the sample if the degree of polymerization was 13,889. The use of the low molecular weight polymers with polymerization degrees of 23, 80 and 160 enhanced the solubility of the synthesized substance in some cases, but because the produced substance tended to increasingly form nanoparticles, the reproducibility of the synthesis technique remained poor. The polymer with a polymerization degree of 1,389 was slightly less beneficial for the solubility of the sample than the polymer with a polymerization degree of 1,944.

The 1 : 5 molar ratio of chloroauric acid to the polymer with an average molecular weight of 140 kDa ensured a slightly better reaction yield than the 1 : 10 ratio. The concentrated solution of the polyacrylate polymer (3%) significantly increased the gold ion conversion in comparison with the diluted solution (0.1%). The presence of Na^+ as a counterion for the carboxyl group of the polyacrylate polymer reduced the gold ion conversion by 10%. Besides, the presence of Na^+ in the sample negatively affected the chemical stability of the samples during storage. The samples synthesized from PANa showed the signs of steady spontaneous reduction of Au (III) ions to metal nanoparticles regardless of the PANa molecular weight when stored at +4 °C for 5–30 days. Thus, pre-deionization of the polymer aimed at eliminating the cations of alkali metals is required to ensure the suitability of the polyacrylate polymer for aurumacryl synthesis.

Importantly, we revealed that the interaction between AuCl_4^- anions and the polyacrylate polymer can cause reduction of gold atoms to elemental gold aggregating into nanoparticles. This reaction results in the low gold conversion. We were able to come to this conclusion using transmission electron spectroscopy, which facilitates observation of electron-opaque particles securely attached to the polymer. The presence of gold nanoparticles of 1–100 nm in diameter in aurumacryl samples does not affect the cytotoxic activity of aurumacryl against MCF-7 cells at the studied range of concentrations: it only indirectly suggests that the proportion of bioactive gold ions decreases in such samples.

Based on the correlation between the concentration of gold ions measured from the absorbance at 226.5 nm and the cytotoxic activity of the obtained aurumacryl samples, we developed a method for assessing the quality of the synthesized aurumacryl. We applied this method to demonstrate that regardless of the synthesis technique and the molar ratio of Au ions to the polyacrylate polymer, the cytotoxicity of aurumacryl against the MCF-7 cell line was proportional to its $A_{226.5}$. It means that aurumacryl quality assessment should include measurements of the absorbance of the saturated solution in the UV spectra at $\lambda = 226.5$ nm. To avoid the impact of hydrolysis on the measurements, the analysis should be performed in the acidic media (such as 100 mM of HCl). Using this principle, we were able to assess the reaction yield and optimize the conditions of the reaction.

The fact that the concentration of gold ions in the sample is a predictor of aurumacryl toxicity against MCF-7 cells regardless of the concentration of the polyacrylate polymer in it, leads us to conclude that the polyacrylate polymer does not have a critical role in transporting gold ions into cancer cells or at least does not limit the efficacy of gold permeating the cell membranes *in vitro*. Still, it is possible that in *in vivo* experiments the role of the polyacrylate polymer in delivering the gold particles to the tumor is quite important. In order to investigate the properties of polyacrylate polymer carriers in biological systems, aurumacryl cytotoxicity needs to be studied first against other cell lines and primary cell cultures *in vitro*. Their nonspecific toxicity and tumoricidal effects should also be compared *in vivo*.

Unlike our colleagues who resorted to air-drying and used a vacuum exicator, we freeze-dried the obtained aurumacryl samples immediately after synthesis. This type of drying significantly improves the solubility of aurumacryl under physiological conditions up to 30 mg/ml of solids and ensures chemical stability necessary for long-term storage.

By optimizing the synthesis technique, we were able to obtain an aurumacryl solution with $A_{226.5} = 4.8$ (equivalent to the optical path length of 1 cm), which is 5.3 times higher than the best result demonstrated by the aurumacryl samples synthesized according to the original technique ($A_{226.5} = 0.9$). The cytotoxicity of our samples increases proportional to $A_{226.5}$. The use of concentrated solutions will facilitate the testing of aurumacryl biological activity *in vivo* and *in vitro* and promote the application of the compound as an tumoricidal agent in the future.

We believe that aurumacryl applications in medicine should not be limited to adjuvant chemotherapy of carcinomas. TEM findings suggest that aurumacryl is capable of forming gold nanoparticles when interacting with reductants. Considering the big atomic radius of gold, such particles can serve as radiosensitizers to enhance the effect of ionizing radiation on tumor cells [14]. This approach is underpinned by the affinity of aurumacryl to cancer cells discovered in this work and in [3], leading us to expect that gold nanoparticles will tend to selectively accumulate in malignant cells instead of healthy tissue.

CONCLUSIONS

By comparing the cytotoxicity of aurumacryl samples with significantly different molar ratio of gold ions to the polyacrylate polymers, we established that the proportion of the polymer and its molecular weight in the sample do not affect the biological properties of the synthesized substance. On the contrary, the concentration of Au (III) ions measured from the absorbance of aurumacryl solutions at $\lambda = 226.5$ nm is an accurate predictor of aurumacryl cytotoxicity against the MCF-7 cells *in vitro*. To synthesize the sample with the highest Au (III) conversion and the best solubility in water, free PAA should be thoroughly deionized prior to the synthesis. The recommended molecular weight of PAA is 140 kDa; its recommended concentration is 3%. The molar ratio of chloroauric acid to PAA should be 1 : 5. It is important to freeze-dry the solution immediately after synthesis.

We hope that our findings will be useful for planning a preclinical trial of aurumacryl.

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EVALUATION OF THE RIVAROXABAN-INFLUENCED EFFECT OF *ABCB1* AND *CYP3A5* GENE POLYMORPHISMS ON PROTHROMBIN TIME IN PATIENTS AFTER TOTAL HIP OR KNEE REPLACEMENT SURGERY

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Rivaroxaban is a safer and more effective alternative to warfarin. However, there are reports of some cases of major hemorrhagic complications associated with rivaroxaban that significantly impair the patients' quality of life and can lead to a fatality. Personalized therapy, including pharmacogenetic testing, may help prevent such adverse events. This study aimed to investigate how *ABCB1* 3435C>T (rs1045642) and *CYP3A5* 6986A>G (rs776746) gene polymorphisms, when carried by a patient taking rivaroxaban to prevent thrombosis after total hip or knee replacement surgery, affect prothrombin time (PT). Sixty-five patients participated in the study. Their genotypes were identified by PCR in real time. To learn PT peculiar to each patient, we collected venous blood on the 5th day of their anticoagulation therapy, 1 hour before they took rivaroxaban and 3 hours after. Having calculated %ΔPT, we divided the patients into 2 groups: 1) %ΔPT ≤ 0 (*n* = 7; 10.8%); 2) %ΔPT > 0 (*n* = 58; 89.2%). Regarding the distribution of rs1045642 polymorphism, we determined the difference between the groups to be statistically significant ($\chi^2 = 6.64$; *p* = 0.027). As for rs776746 polymorphism, the difference was insignificant ($\chi^2 = 0.101$; *p* = 1.0). We discovered that rs1045642 polymorphism has a significant effect on PT variance in patients taking rivaroxaban to prevent thrombosis after total hip or knee replacement surgery.

Keywords: rivaroxaban, pharmacogenetics, prothrombin time, hip replacement surgery, knee replacement surgery, thromboprophylaxis

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ОЦЕНКА ВЛИЯНИЯ ПОЛИМОРФИЗМОВ ГЕНОВ *ABCB1* И *CYP3A5* НА СТЕПЕНЬ ИЗМЕНЕНИЯ ПРОТРОМБИНОВОГО ВРЕМЕНИ ПОД ВЛИЯНИЕМ РИВАРОКСАБАНА У ПАЦИЕНТОВ ПОСЛЕ ЭНДОПРОТЕЗИРОВАНИЯ КРУПНЫХ СУСТАВОВ НИЖНИХ КОНЕЧНОСТЕЙ

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Несмотря на высокую эффективность и безопасность применения ривароксабана по сравнению с варфарином, в клинической практике наблюдаются редкие случаи крупных геморрагических осложнений, которые могут значительно ухудшать качество жизни пациентов или быть летальными. Остается открытым вопрос, насколько фармакогенетические тесты позволят профилактировать развитие таких неблагоприятных событий. Целью работы было оценить влияние носительства полиморфизмов *ABCB1* 3435C>T (rs1045642) и *CYP3A5* 6986A>G (rs776746) на изменение протромбинового времени (ПВ) у пациентов, принимающих для тромбопрофилактики ривароксабан после эндопротезирования крупных суставов нижних конечностей. В исследование были включены 65 пациентов. Генотипирование проводили с помощью ПЦР в реальном времени. Для определения ПВ венозную кровь отбирали на 5 сутки приема антикоагулянта 2 раза: за 1 ч до приема ривароксабана и через 3 ч после приема. Вычислив %ΔПВ, пациентов делили на 2 группы: 1) %ΔПВ ≤ 0 (*n* = 7; 10,8%); 2) %ΔПВ > 0 (*n* = 58; 89,2%). Между группами %ΔПВ относительно распределения генотипов полиморфизма rs1045642 была определена статистически достоверная разница ($\chi^2 = 6,64$; *p* = 0,027). Относительно распределения генотипов полиморфизма rs776746 статистически значимой разницы между группами %ΔПВ обнаружено не было ($\chi^2 = 0,101$; *p* = 1,0). Выявлено статистически значимое влияние полиморфизма rs1045642 на характер изменения ПВ у пациентов, принимающих с целью тромбопрофилактики ривароксабан после эндопротезирования крупных суставов нижних конечностей.

Ключевые слова: ривароксабан, фармакогенетика, протромбиновое время, эндопротезирование тазобедренного сустава, эндопротезирование коленного сустава, тромбопрофилактика

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Deep vein thrombosis (DVT) and pulmonary embolism (PE) are some of the most important problems encountered by practitioners. PE risk group includes patients after hip or knee replacement surgery. About 50–60% of THR (total hip replacement surgery) and TKR (total knee replacement surgery) patients that receive no thrombosis prevention therapy have DVT after surgery [1]. Approximately 1 in every 500 THR patients can have a fatal PE [2]. The number of THRs in Russia is increasing every year; currently, about 25 persons in each 100,000 have THRs [3].

Direct oral anticoagulants (DOACs) have recently been approved for the prevention of venous thromboembolism (VTE) in patients after elective hip or knee arthroplasty. DOACs have demonstrated acceptable efficacy and safety profiles. These drugs require no laboratory control. This article focuses on rivaroxaban, which is a direct factor Xa inhibitor. The anticoagulant got the FDA approval in 2011 as a medication to prevent thrombosis in patients after elective hip or knee arthroplasty [4].

About 18% of the rivaroxaban dose is metabolized by CYP3A4/5, 14% — by CYP2J2.

Approximately 36% of the dose is excreted via kidney in form of the unchanged drug, involving the active transporter-mediated secretion by P-glycoprotein (P-gp) and BCRP (Breast Cancer Resistance Protein) [5].

P-gp is a large membrane protein that transports drugs from inside the cell. It is found on the surface of epithelial cells lining small and large intestines, pancreatic duct, in the liver's bile vessels membrane, proximal kidney tubules and adrenal glands, as well as in endothelial cells of blood-tissue interfaces (brain-blood, blood-follicle, blood-testis, and blood-placental barriers) [6]. P-gp is encoded by the *ABCB1* gene located on chromosome 7 (7q21.12) [7]. The most common single nucleotide polymorphisms (SNPs) of the *ABCB1* gene are 1236T>C (rs1128503), 2677T>G/A (rs2032582) and 3435T>C (rs1045642) [8]. In this study, we focused on the impact of the *ABCB1* 3435C>T polymorphism (rs1045642).

Jointly, CYP3A4 and CYP3A5 proteins account for about 30% of hepatic cytochrome P450; about half of the drugs metabolized by cytochrome P450 are CYP3A substrates. *CYP3A4* and *CYP3A5* are expressed in liver and intestines, with *CYP3A5* expression seen mostly in extrahepatic tissues. The *CYP3A5* gene is located on chromosome 7 (7q22.1); it encodes the protein of 502 amino acids. The most common SNP of the *CYP3A5* gene is 6986A>G (rs776746). It should be noted here that *CYP3A5* 6986GG genotype carriers do not fully express the *CYP3A5* isoenzyme [9].

According to the RECORD 1-4 research program [10–13], despite the good efficacy and safety profiles of rivaroxaban when prescribed to prevent thrombosis after hip or knee replacement surgery, 2.87% of patients exhibited nonmajor clinically relevant bleeding, including hematomas in the area of surgery that may be infected. Such complications require removal of the endoprosthesis, which worsens the patient's quality of life and translates into an additional financial burden for the health care system. Currently, practitioners resort to the personalized therapy, including pharmacogenetic testing, to prevent such adverse events.

This study aimed to investigate how the *ABCB1* 3435C>T (rs1045642) and *CYP3A5* 6986A>G (rs776746) gene polymorphisms, when carried by a patient taking rivaroxaban to prevent thrombosis after total hip or knee replacement surgery, affect prothrombin time (PT).

METHODS

The study protocol was reviewed and approved by the local ethics committee of I. M. Sechenov First Moscow State Medical

University (Sechenov University) (meeting minutes #03-17 of 2017.04.19). The inclusion criteria were: any gender; age ≥ 18 years; primary TKR or THR performed; thrombosis prevention therapy — 10 mg rivaroxaban OD; informed voluntary consent. The exclusion criteria were: atrial fibrillation with anticoagulant therapy; hemorrhagic diathesis; acute intracranial disease or hemorrhagic stroke recorded in the past three months; gastrointestinal bleeding, hematuria, peptic ulcer or duodenal ulcer recorded in the last 6 months; severe liver disease; liver transaminases (GPT and GOT) ≥ 2 upper limits of the norm in the last month; severe renal impairment (CK < 30 ml/min); advanced stage of cancer; pregnancy, lactation; age < 18 years.

Sixty-five persons participated in the study, 19 THR patients (29.2%) and 46 TKR patients (70.8%). They were 48 (73.8%) women and 17 (26.2%) men aged 24 to 83 years (mean age — 59 ± 12 years). Following the instructions detailing the use of the medicine for the purposes of post-surgery thrombosis prevention, the patients took 10 mg rivaroxaban once a day. THR patients received the medicine for 35 days, TKR patients — for 14 days [14].

We purified DNA from the patients' venous blood. For the purposes of genotyping, the blood was collected into 4 ml Vacuette® vacuum tubes with EDTA-K3 anticoagulant. Real-time polymerase chain reaction (PCR) allowed genotyping *ABCB1* 3435C>T (rs1045642) and *CYP3A5* 6986A>G (rs776746) polymorphisms; we used the CFX96 Touch™ Real-Time PCR Detection System DNA amplifier (Bio-Rad Laboratories, Inc.; USA) at Research and Development center of the Russian Medical Academy of Continuous Professional Education of the Ministry of Health of Russia.

PT was the indicator reflecting the rivaroxaban's pharmacodynamic properties; to learn it, we collected venous blood of the patients on the 5th day of their anticoagulation therapy, 1 hour before they took the medicine (PT₁) and 3 hours after (PT₂), into 2.7 ml BD Vacutainer® vacuum tubes with 3.2% sodium citrate. PT was determined manually, using Tekhplastintest (Tekhnologiya-Standard; Russia) in accordance with the manufacturer's instructions.

The formula $\% \Delta PT = (PT_2 - PT_1) / PT_1 \times 100\%$ allowed finding the differences between PT values through calculating $\% \Delta PT$, which governed the division of patients into two $\% \Delta PT$ groups: 1) patients with $\% \Delta PT \leq 0$; 2) patients with $\% \Delta PT > 0$.

The studied polymorphism genotypes frequency distribution was tested for compliance with the Hardy–Weinberg equilibrium using an online calculator [15]. To determine the difference between the groups as conditioned by the impact of *ABCB1* 3435C>T (rs1045642) and *CYP3A5* 6986A>G (rs776746) polymorphisms, we ran the chi-square test for independence. PASW Statistics 18 (2009) software was used to process the results.

RESULTS

Based on the *ABCB1* 3435C>T (rs1045642) polymorphism genotype, the patients were divided into 3 groups: 1) 3435CC genotype, $n = 17$ (26.2%); 2) 3435CT genotype, $n = 27$ (41.5%); 3) 3435TT genotype, $n = 21$ (32.3%). As for the *CYP3A5* 6986A>G (rs776746) polymorphism genotypes, we found 7 patients (10.8%) with 6986AG genotype (10.8%) and 58 patients (89.2%) with 6986GG genotype (Table 1).

The *ABCB1* 3435C>T (rs1045642) and *CYP3A5* 6986A>G polymorphism genotypes distributions were within the Hardy–Weinberg equilibrium, ($\chi^2 = 1.79$; $p = 0.409$ and $\chi^2 = 0.21$; $p = 0.9$, respectively).

Among the patients participating in the study, the mean PT₁ value (measured 1 hour before administration of rivaroxaban) was 15.5 ± 4.1 seconds, and the mean PT₂ value (measured 3 hours after administration of rivaroxaban) was 19.1 ± 3.2 seconds. Table 2 shows other mean PT values through the lens of their dependence on the *ABCB1* 3435C>T (rs1045642) and *CYP3A5* 6986A>G (rs776746) polymorphism genotypes.

Case-wise analysis of the PT variability revealed 7 patients (10.8%) that had a paradoxical reaction, i.e. after administration of rivaroxaban the PT in them either grew smaller or did not change (%ΔPT ≤ 0). In the remaining 58 patients (89.2%) rivaroxaban made the PT greater, as expected (%ΔPT > 0). 6 patients (85.7%) were carrying the *ABCB1* 3435CT genotype and none — the *ABCB1* 3435TT genotype in the group with %ΔPT ≤ 0. The chi-square test for independence revealed a statistically significant difference between %ΔPT groups as conditioned by the distribution of the *ABCB1* 3435C>T (rs1045642) polymorphism genotypes ($\chi^2 = 6.64$; $p = 0.027$) (Table 3).

Patients with the *ABCB1* 3435CT genotype contributed the most to the difference between %ΔPT groups. Table 4 presents "manual" determination of reliability of differences in the set of frequencies, which allows seeing the reasons behind the main difference.

As for the *CYP3A5* 6986A>G (rs776746) polymorphism genotypes, there were no significant differences in their distribution among %ΔPT groups ($\chi^2 = 0.101$; $p = 1.0$) (Table 5).

DISCUSSION

Drugs as P-gp substrates are widely used in the routine clinical practice, which is why studying the effect *ABCB1* gene

polymorphisms have on pharmacodynamic and pharmacokinetic qualities of medications is a matter of great interest.

Today, many researchers investigate the impact of *ABCB1* gene polymorphisms on the pharmacodynamic properties of various medicines. One of the efforts was aimed at studying the relationship between *ABCB1* 3435C>T polymorphism and response to antiretroviral therapy in HIV-1 patients ($n = 123$) that received efavirenz or nelfinavir [16]. The authors of that research found that patients with the 3435T allele respond to antiretroviral therapy better. Another work revealed no link between the *ABCB1* 3435C>T (rs1045642) polymorphism and virological, immunological responses to antiretroviral therapy [17].

P-gp also transports antiepileptic drugs [18]. Genotyping epilepsy patients ($n = 315$) by the *ABCB1* 3435C>T (rs1045642) polymorphism, the researchers discovered that those resistant to antiepileptic pharmacotherapy are more likely to carry the *ABCB1* 3435CC genotype as opposed to the patients whose response to such therapy was positive [19].

CYP3A5 gene polymorphism can contribute to the variability of *CYP3A5* substrates clearance. Studying the effect *CYP3A5* expression has on the pharmacological response of statins, the researchers have shown that lovastatin, simvastatin and atorvastatin are significantly less effective in *CYP3A5* expressors than in non-expressors [20].

In the context of our work, which also addressed the impact of *ABCB1* and *CYP3A5* genes polymorphisms on the pharmacodynamic properties of a drug — rivaroxaban, in our case, — we have revealed a statistically significant difference in distribution of *ABCB1* 3435C> T (rs1045642) polymorphism genotypes between %ΔPT groups, one with %ΔPT ≤ 0 and another with %ΔPT > 0. The difference mainly originates with

Table 1. Distribution of *ABCB1* 3435C>T (rs1045642) and *CYP3A5* 6986A>G (rs776746) polymorphisms genotypes in THR and TKR patients receiving rivaroxaban

Polymorphisms	Genotype	Number of patients	
		absolute	relative, %
<i>ABCB1</i> 3435C>T (rs1045642)	<i>ABCB1</i> 3435CC	17	26.2
	<i>ABCB1</i> 3435CT	27	41.5
	<i>ABCB1</i> 3435TT	21	32.3
<i>CYP3A5</i> 6986A>G (rs776746)	<i>CYP3A5</i> 6986AG	7	10.8
	<i>CYP3A5</i> 6986GG	58	89.2

Table 2. Mean PT values by group and as conditioned by the *ABCB1* 3435C>T (rs1045642) and *CYP3A5* 6986A>G (rs776746) polymorphism genotypes in THR and TKR patients receiving rivaroxaban

	Mean PT ₁ , s	Mean PT ₂ , s	p	Mean ΔPT, s	Mean %ΔPT, %
Participating patients ($n = 65$)	15.5 ± 4.1	19.1 ± 3.2	$t = 9.185$ $p = 2.7 \times 10^{-13}$	3.6 ± 4.9	27.4 ± 26.3
<i>ABCB1</i> 3435CC	14.8 ± 2.1	19.8 ± 3.7	$t = 5.48$ $p = 5.03 \times 10^{-5}$	4.95 ± 3.3	34.7 ± 24.5
<i>ABCB1</i> 3435CT	16.0 ± 5.7	18.4 ± 3.0	$t = 4.074$ $p = 3.85 \times 10^{-4}$	2.4 ± 6.8	22.8 ± 32.9
<i>ABCB1</i> 3435TT	15.5 ± 2.8	19.5 ± 2.7	$t = 6.652$ $p = 1.77 \times 10^{-6}$	4.02 ± 2.2	27.3 ± 15.8
<i>CYP3A5</i> 6986AG	14.3 ± 1.9	20.6 ± 3.1	$t = 5.358$ $p = 0.002$	6.3 ± 4.2	47.3 ± 34.8
<i>CYP3A5</i> 6986GG	15.7 ± 4.3	18.9 ± 3.1	$t = 7.798$ $p = 1.49 \times 10^{-10}$	3.2 ± 4.95	25.0 ± 24.3

Table 3. Distribution of *ABCB1* 3435C>T (rs1045642) polymorphism genotypes, %ΔPT groups, THR and TKR patients receiving rivaroxaban ($n = 65$)*

	%ΔPT ≤ 0	%ΔPT > 0
<i>ABCB1</i> 3435CC	1 (14.3%)	16 (27.6%)
<i>ABCB1</i> 3435CT	6 (85.7%)	21 (36.2%)
<i>ABCB1</i> 3435TT	0 (0%)	21 (36.2%)

Note: * $\chi^2 = 6.64$; $p = 0.027$.

Table 4. Determination of significance of differences between observed and expected distribution of *ABCB1* 3435C>T (rs1045642) polymorphism genotypes, % Δ PT groups

Observed genotypes distribution, % Δ PT groups			
	% Δ PT \leq 0	% Δ PT > 0	Total
<i>ABCB1</i> 3435CC	1	16	17
<i>ABCB1</i> 3435CT	6	21	27
<i>ABCB1</i> 3435TT	0	21	21
Total	7	58	65
Expected genotypes distribution, % Δ PT groups			
	% Δ PT \leq 0	% Δ PT > 0	Total
<i>ABCB1</i> 3435CC	2	15	17
<i>ABCB1</i> 3435CT	3	24	27
<i>ABCB1</i> 3435TT	2	19	21
Total	7	58	65
Significant difference between observed and expected genotypes distribution			
	% Δ PT \leq 0	% Δ PT > 0	Total
<i>ABCB1</i> 3435CC	0.50	0.07	0.57
<i>ABCB1</i> 3435CT	3.00	0.38	3.38
<i>ABCB1</i> 3435TT	2.00	0.21	2.21
Total	5.50	0.66	6.16

Table 5. Distribution of *CYP3A5* 6986A>G (rs776746) polymorphism genotypes, % Δ PT, THR and TKR patients receiving rivaroxaban ($n = 65$)*

	% Δ PT \leq 0	% Δ PT > 0
<i>CYP3A5</i> 6986AG	1 (14.3%)	6 (10.3%)
<i>CYP3A5</i> 6986GG	6 (85.7%)	52 (89.7%)

Note: * $\chi^2 = 0.101$; $p = 1.0$

the patients carrying the *ABCB1* 3435CT genotype (85.7%) that belong to the % Δ PT \leq 0 group; this may point to the necessity to adjust the dose for such people, since the standard dose of rivaroxaban may have no clinical effect in them.

The majority (89.2%) of patients participating in our study had the *CYP3A5* 6986GG genotype; the *CYP3A5* isoenzyme was not fully expressed in their bodies. As for the distribution of *CYP3A5* 6986A>G (rs776746) polymorphism genotypes in the % Δ PT groups, the difference between them was insignificant, which presumably signals of the role of *CYP3A5* isoenzyme in rivaroxaban pharmacokinetics being inferior to that of the P-gp membrane transporter.

Considering the discovered effect *ABCB1* 3435C>T (rs1045642) polymorphism has on % Δ PT under the influence

of rivaroxaban, we believe it is important to research not only pharmacodynamics, but also pharmacokinetics of rivaroxaban in patients with different genotypes.

CONCLUSIONS

We discovered that *ABCB1* 3435C>T (rs1045642) polymorphism has a statistically significant effect on PT variance in THR and TKR patients taking rivaroxaban to prevent thrombosis. There was found no statistically significant dependence of % Δ PT on *CYP3A5* 6986A>G (rs776746) polymorphism. We believe it is necessary to study both pharmacodynamics and pharmacokinetics of rivaroxaban in patients with different genotypes.

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