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ДЛЯ ПОДАЧИ РУКОПИСЕЙ editor@vestnikrgmu.ru
ДЛЯ СОТРУДНИЧЕСТВА manager@vestnikrgmu.ru
АДРЕС РЕДАКЦИИ ул. Островитянова, д. 1, г. Москва, 117997

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COMPREHENSIVE ASSESSMENT OF POSTURAL CONTROL AS A CONCEPTUAL BASIS FOR OPTIMIZING REHABILITATION AND RECOVERY PROGRAMS IN SPORTS

Andreev DA[1]✉, Karmazin VV[2], Parastaev SA[1]

[1] Department of Rehabilitation, Sports Medicine and Physical Education, Faculty of Pediatrics, Pirogov Russian National Research Medical University, Moscow, Russia

[2] Federal Research and Clinical Center of Sports Medicine and Rehabilitation, Federal Medical-Biological Agency, Moscow, Russia

This literature-based review focuses on the basic physiological aspects of proprioception. Below, we describe and compare a number of biomechanical platforms used to measure postural control in high-class athletes. We define the primary goals of biomechanical assessment of postural problems, paying special attention to the functional performance of proprioceptors and proprioceptive control. We also provide a list of clinical and biomechanical indicators of proprioceptive damage and propose a diagnostic algorithm for assessing static and dynamic impairments in high-class athletes.

Keywords: biomechanics, proprioception, postural balance, functional asymmetry, stabilometry, unstable platforms, balance assessment, diagnostic algorithm, high-class athletes

✉ **Correspondence should be addressed:** Dmitry Andreev
ul. Ostrovityanova 1, Moscow, Russia, 117997; drada_rus@mail.ru

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ИНТЕГРАЛЬНАЯ ХАРАКТЕРИСТИКА ЭФФЕКТИВНОСТИ ПОСТУРАЛЬНОГО КОНТРОЛЯ КАК КОНЦЕПТУАЛЬНАЯ ПЛАТФОРМА ДЛЯ ОПТИМИЗАЦИИ РЕАБИЛИТАЦИОННЫХ И ВОССТАНОВИТЕЛЬНЫХ ПРОГРАММ В СПОРТЕ

Д. А. Андреев[1]✉, В. В. Кармазин[2], С. А. Парастаев[1]

[1] Кафедра реабилитации, спортивной медицины и физической культуры, педиатрический факультет, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Федеральный научно-клинический центр спортивной медицины и реабилитации, Федеральное медико-биологическое агентство, Москва

В обзоре проанализированы данные литературных источников по основным физиологическим аспектам системы проприоцепции. Проведена сравнительная характеристика используемого биомеханического оборудования для диагностики эффективности постурального контроля у спортсменов высокого класса. Определены первоочередные задачи биомеханического обследования при нарушении постурального баланса, среди которых приоритет отдается оценке функциональной состоятельности всех типов проприорецепторов и проприоцептивного контроля. Приведены клинико-биомеханические критерии проприоцептивных нарушений у спортсменов, а также разработанный авторами на их основе алгоритм диагностики статодинамических нарушений у спортсменов высокого класса.

Ключевые слова: биомеханика, проприоцепция, постуральный баланс, функциональная асимметрия, стабилометрия, нестабильные платформы, балансометрия, алгоритм обследования, спортсмены высокого класса

✉ **Для корреспонденции:** Андреев Дмитрий Александрович
ул. Островитянова, д. 1, г. Москва, 117997; drada_rus@mail.ru

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This review critically analyzes literature covering methodology for diagnosing and monitoring postural control disorders in top ranking athletes. Another point considered here are the design principles behind rehabilitation programs based on proprioceptive capabilities assessments.

Neurology, traumatology and orthopedics make quite an extensive use of medical equipment designed to diagnose and correct changes in speed and strength of movements. However, these indicators fail to fully describe the specifics of adaptation and compensation processes peculiar to various sports activities. Proprioception capabilities assessment and correction enjoyed less attention from the researchers; there is a number of applied methods that differ greatly from each other, especially in defining diagnostic approaches and establishing assessment criteria [1].

Physiological aspects of proprioception

Proprioception (deep or kinesthetic sensitivity) is the perception of body posture and movements, both as a whole and by segments. Understanding proprioception patterns (reception and regulation mechanisms in the first place) allows selecting diagnostic tools that would be effective in both clinical practice and sports biomechanics recognition.

There are three types of structurally and functionally different proprioceptors: muscle spindles, tendon and articular receptors [2].

Muscular spindles run parallel to skeletal muscle and consist of several striated intrafusal muscle fibers. They are attached to the connective tissue (perimysium) of the extrafusal muscle fibers bundle; when the muscle relaxes, receptors expand, which leads to their excitation [2, 3].

Tendon receptors, enclosed in the connective tissue capsule (Golgi body), lie sequentially in the skeletal muscles tendons. Their excitation occurs when the tendon stretches.

Muscle spindles send pulses to α-motoneurons of the spinal cord and excite them, which leads to the stretched muscle's contraction. As the muscle begins to contract, excitation of the muscle spindles disappears or weakens greatly; at the same time, impulses from the tendon receptors reach the spinal cord, Renshaw cells. The latter, when excited, inhibit α-motoneurons of the skeletal muscle, which relaxes. In other words, the muscle alternately contracts and relaxes following impulses the receptors send to its motoneurons [2–4].

Complex locomotions, like walking, imply synchronized contractions of flexors of one leg and extensors of the other. The contractions are also caused by afferent impulses from muscle and tendon receptors and, respectively, alternating excitation and inhibition of flexor and extensor centers [2]. Biomechanical methods provide explanations of peculiarities of this locomotion.

Joint receptors (mechanoreceptors) are in the capsule, cartilage, ligaments and pericapsular connective tissue. They are distinguished into types depending on their response to amplitude, speed and direction of movement in the joint.

For example, Ruffini endings (corpuscles), which can be found both in the joint's capsule and the surrounding connective tissue (including those lying deep in the dermis and subcutaneous fatty tissue), report articular angles, i. e. relative positions of elements of the joint. They send pulses while the angle remains unchanged, and the intensity of those pulses depend on the angle's value. These mechanoreceptors are considered to be particularly sensitive to extreme angles. Pacinian corpuscles reside in the joint capsule exclusively; they perceive direction and speed of change of its angle. The frequency of pulses they generate grows with that speed. Here, clinical biomechanics allows gathering exhaustive descriptions.

The sensation of movement, same as skin sensitivity (to touch, pressure), results from receptors sending pulses through two main pathways, lemniscus and spinotalamic tracts, which differ significantly in their morphological and functional properties. There is also a third pathway, the Morin lateral pathway, which resembles lemniscus in a number of characteristics.

As far back as in 1922, Miles [5] stressed the importance and versatile role movement control plays in maintaining vertical stability. In 1924, Magnus published his Body Posture, a fundamental work developing Sechenov's ideas on muscles own sensitivity ("dark muscle feeling") and those of Sherington, which pertain to the receptive fields. In the same paper, the Dutch scientist also scrutinized the special group of posture (adjustment) reflexes (Magnus–Klein tonic reflexes) that help maintain posture and balance and described other reflexes enabling animals to stand and walk normally [5].

In 1965, Gurfinkel et al. published the *Human Posture Regulation* paper that laid the foundation for instrumental assessment of proprioception system, which lead to introduction of stabilometry as a biomechanical diagnostics method. Thence, stabilometry helps clinicians assess functions of motor and nervous system, since postural balance tests allow assessing quality of proprioception in a closed kinetic chain [5]. It is the vertical posture maintaining strategy and somatosensory information coming from the foot contacting the support's surface that tell the most about balance control as proprioception indicators [5, 6].

Proprioception: biomechanical diagnostics methods

In the context of postural control rehabilitation, stabilometry allows objective functional monitoring of the progress made [1, 5]. Typically, the deficiency of postural control after trauma or with an orthopedic pathology in the background is considered to be the result of faults in the flow of afferent information generated by ligament and capsule mechanoreceptors. Current stabilometric systems include hardware and software and allow regulation of the degree of mobility of the support platform. Fig. 1 shows such a system.

Important diagnostic criteria describing vertical stability are the statokinesiogram area and the velocity of center of pressure (CoP), as well as the Romberg ratio (ratio of two statokinesiogram areas, one with eyes open and the other with eyes shut). This ratio reveals the functional ability of peripheral and vestibular links of the proprioception system to maintain vertical stability in the absence of visual clues, i.e. when visual posture control does not function.

We believe that current sports medicine does not fully appreciate the potential of the posture stereotype assessment, given the stabilometric diagnostics methods and principles adopted. However, stabilometry is the very tool that allows diagnosing functional postural asymmetries in athletes. Many authors believe that most sports have specific requirements to the athlete's musculoskeletal system and sensory organs; those requirements can imply special symmetry or asymmetry, and practicing those sports means their further development [8, 9]. Morphogenetic features and asymmetry determine how well an athlete can make special moves, i.e. each sport require special types of sensorimotor profiles.

Brain asymmetry's connection to vertical posture maintenance is of special importance. A person can remain upright for a long time when static momenta of all body parts are balanced, which requires adequate proprioceptive control all around.

It should be noted that some stabilometric indicators of functional postural asymmetry reveal special motor skills peculiar to this or that sport. The indicators are mean position and standard deviation of GoP in the frontal plane; they can be used in assessing the technique of performing specific locomotions [9].

Fig. 1. Stabilometric system [7]

At the same time, GoP velocity and area indicators are functional markers of the static position, which means they can help assess the various influences special types of physical activity have on all parts of the musculoskeletal system. Stabilogram, therewith, is an integral and complex method for evaluating the functional state of the motions regulation system. Clinical assessment of the muscles enabling sport-specific ("working") vertical stance is an essential part of the overall posture control evaluation in sports medicine.

Thus, the ability to stabilize to equilibrium in static (standing, sitting) positions and when moving (walking) is the most important motor-related aspect for the sports medicine. Testing and assessing this ability allows finding various proprioception deficiencies. In addition, rational interpretation of the stabilometric indicators and their comparison to the clinical tests of muscles enabling vertical posture help to improve rehabilitation programs designed for injured athletes and those suffering from musculoskeletal system disorders [10, 11]. Fig. 2 is an example of a stabilogram revealing a pronounced asymmetric stance shown by an athlete.

However, classical stabilometry has its limitations in assessing functions of the proprioceptive system: the latter makes use of the biological feedback principle, i.e. external stimuli lead to changes in the posture regulation strategy. Peripheral analyzer is the link fastest to respond to external stimuli. For the vertical stability regulation system, this analyzer is the ankle joint and the feet. If the support surface is stationary, it is impossible to assess how well does this peripheral analyzer functions when the posture responses are complex, much like those peculiar to the sport of records. In the 1960s, Freeman (trauma surgeon from the US) addressed this problem: unstable platform as part of the lower limbs injury rehabilitation program helped restore the peripheral analyzer's state to the optimal level through activation of foot and ankle receptors [10].

Currently, this or that variation of the unstable platform is widely used in rehabilitation of patients suffering the consequences of spinal cord traumatic disease, spine osteochondrosis complications, surgical treatment of hip, knee and ankle joints orthopedic pathology [10–12]. New methods that imply stimulation of muscles autochthonous to postural balance are developed and introduced. New exercisers are built around these methods; they have rigid and semi-rigid platforms that allow various degrees of angular displacement [12, 13].

However, mechanical exercisers were not designed to allow assessing postural control on an unstable surface. Diagnostics need platforms to have sensors recording athlete's response to their movements during examination. Current systems of this kind can have both a classical stabilometric platform and a less conventional balance rig incorporating a combined accelerometer-gyroscope that reports linear velocities and velocity-angle data against a system of coordinates. Balance metering (balancemetry) is the very method that produces accurate assessment of the functional activity of joint mechanoreceptors when that joint moves in space (Ruffini corpuscles), as well as record velocity of the joint angle change (Pacinian corpuscles). Balance metering systems equipped with an accelerometer and a gyroscope can register even minimal angular movements of the CoP and thus improve both proprioception diagnostics and stimulation during biofeedback sessions [14, 15].

From the point of view of diagnostics, balance metering systems add much value to the assessment of athletes' postural control of athletes. Such systems are also capable of targeted correction of postural disorders affecting biological response to proprioceptive, auditory and visual stimuli. Unlike classical stabilometric systems [16], unstable platform systems require active participation of the patient undergoing proprioceptive disorders treatment. Unstable platform means the patient needs to put effort into maintaining position of the body; the effort goes through muscles that stabilize posture, i. e. autochthonous, gluteal and hamstring muscles [11, 16]. The system's software registers body movements during diagnostics and treatment, which allows both verification of the primary postural control records and comprehensive rehabilitation monitoring [11, 17]. Besides, such systems offer extensive training sessions control tools, which gives the therapist an opportunity to design sessions taking into account the severity of the disease, compensatory reactions by central and peripheral nervous systems and musculoskeletal system, as well as the possible pathologies of these functional systems. Fig. 3 shows a mobile wireless balance metering platform.

Various hardware and software biomechanical diagnostics and correction systems apply the described principle of integral assessment of the proprioception system and feature an unstable platform. Fundamental research by Fellicetti,

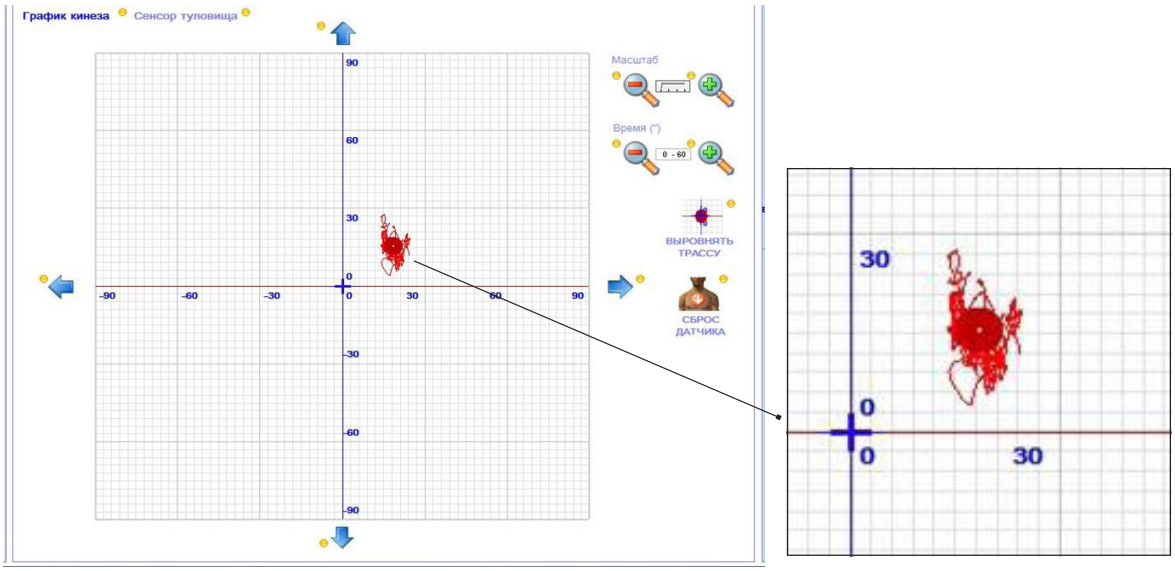

Fig. 2. Stabilogram of an athlete, GoP shifted towards the leading arm and leg. GoP velocity and area are significantly increased with clinical signs of muscular overstrain (right lower limb) in the background

Srivastava, Taly, Gupta, have proved this kind of equipment is highly efficient in treatment of proprioceptive disorders [19].

Baropodometry is one of the most promising methods of assessing postural control in athletes. Unlike stabilometry, baropodometry involves up to several tens of thousands strain gauges. These gauges register even the slightest movements of pressure exerted by feet and allow accurate assessment of CoP area and velocity (major postural diagnostics indicators) and dynamic changes of pressure peculiar to standing, walking, running and special dynamic tests [20].

Baropodometry systems make use of two types of gauges, capacitive and resistive. They register changes in electrical signal or medium resistance between the two plates. Capacitative gauges are more accurate, but their calibration is an intricate process, which is why they are only used in laboratory settings. Clinics find resistive gauge platforms more practical [21].

Baropodometry is developing rapidly. Researchers and designers remedy various faults found in early versions of gauges, like hypersensitivity, thermal sensitivity, unstable operation and insufficient robustness. Today, there are many variations of baropodometry platforms: compact systems for standing position assessment, walkways for gait analysis, treadmills, sensory insoles etc. Baropodometry also allows analysis of the feet's statodynamic function and gait. Baropodometry tests add much value to diagnostics of functional manifestations of flatfoot, monitoring rehabilitation from various neurological and orthopedic feet disorders. Such platforms form part of hardware and software systems designed to analyze movements and aide in manufacturing insole orthoses [20, 21].

Besides, some spine and autochthonous back muscles assessing methods grow more and more popular, including optical topography and regulated inclination trunk antigravitational muscles examination that requires a special set of hardware [22]. It should be noted that diagnostics of dynamic proprioceptive disorders in athletes is more accurate when biomechanical systems are used, those that ensure synchronization of different locomotion indicators registration methods (video analysis, myography), application of inertial systems making use of gyroscopes and accelerometers. We believe that wireless and inertial systems possess the greatest potential for comprehensive biomechanical examination of athletes in general and their proprioception systems in particular.

Another important aspect of the primary and dynamic assessment of postural control quality is local diagnostics of functions of ligaments and joints muscles. Electromyography and thermography are both good choices to this effect [23].

Biomechanical assessment of proprioception in athletes: methodology principles

The range of diagnostic equipment described above allows optimal and comprehensive assessment of athletes' postural stereotypes. In addition, such tools help reveal the symptoms of proprioception disorders, find proof backing clinical examination data, monitor proprioceptive data changes during the rehabilitation process [24]. However, our experience and various research efforts undertaken throughout the world suggest that the biomechanical equipment in question plays the most important role in designing rehabilitation programs [25].

Correct interpretation of clinical and biomechanical examination data require understanding of statodynamic peculiarities of various sports, preferences as to the arm or leg, physiological aspects influencing the supporting and dominating lower extremity [26].

Speaking of athletes, the main postural control diagnostics principles are:

- characterization of manifestation (degree) of asymmetry resulting from sports activities;
- vertical stability analysis - general, on one leg, when moving (motor coordination test);
- identification of the primary link in the proprioceptive disorders pathogenesis.

Postural asymmetry is a necessary component of an athlete's postural stereotype complex assessment. Signs of morphological and functional asymmetries can be found in major afferent elements, central and efferent posture control departments. Finding out the degree of asymmetry in athletes is closely related to ontogenetic features and the dominance of the "working" hand/leg in a particular sport [27–33].

In addition, it is necessary to assess the postural stereotype stability (control) both when the athlete takes the main stance and when he/she stands on one leg [28–30]. Gribble et al. [28] conducted a systematic comparative review of studies covering clinical and biomechanical aspects of athletes (competitive sports) and non-athletes doing the Star coordination test. This test is aimed at clinically assessing the vertical balance of the testee, who needs to stand on one leg and reach zones around him/her with the other leg. The postural biomechanical diagnostics data (stabilometry and baropodometry included) proved that the CoP shifts towards the dominating lower extremity when the testee takes the stance. Also, it was found that the testee's balance is better when he/she is standing on the dominating leg (applies to professional athletes, left for left-handed, right for right-handed) [28]. The results back the "working" asymmetry theory and the CoP shift toward the dominant lower limb as influenced by the functional requirements of the sport in question [34–36].

Fig. 3. Balance metering platform [18]

Biomechanical examination algorithm

Examination stage	Diagnostic method
Primary postural examination	• Classical stabilometry • Computer optical topography • Standard baropodometry
Identification of common proprioceptive balance disorders, vertical position	• Stabilometry (Romberg test) • Baropodometry (test on one leg)
Identification of statodynamic disorders of proprioception	• Balance metering (mono axis and multi axis tests) • Baropodometry (dynamic tests, frontal and sagittal directions) • Movements and gait analysis (video recorder, inertial wireless gauges, treadmill with baro-platform) • Examination of function of the trunk's antigravitational muscles, regulated inclination
Identification of functional local changes in muscles, ligaments and joints	• Electromyography • Thermography

These changes can be considered a manifestation of adaptive reorganizations of postural control. When physical overstrain is significant and also due to injuries, changes in practicing (different shoes, cover, position on the playing field), the posture regulation adaptation processes may be disrupted. Such a disruption may lead to disadaptation of intermuscular interactions, and if no correction measures are taken, appearance of compensatory changes. The latter up the risk of development of chronic musculoskeletal disorders in athletes [37–39].

Clinical and biomechanical criteria of disadaptation are:

1. pain in the overstrained part;
2. CoP shift towards the overstrained part;
3. functional deficiency of the muscles responsible for keeping the overstrained part's joints stable;
4. appearance of the secondary changes in parts undergoing compensatory changes.

Many years of clinical and biomechanical research in athletes allowed us to develop the following biomechanical examination algorithm (see table).

The results of the examination lead to the development of goals, structure, sequence of rehabilitation measures for athletes suffering from various proprioception system disorders.

CONCLUSION

We believe that the approach described above is the best option, since it takes into account the interrelationship between physiological capabilities of functional systems and training-related adaptation and compensatory processes specific to this or that nervous and musculoskeletal system pathology. It should also be emphasized that only complex biomechanical diagnostics allows obtaining meaningful data, which, in turn, can help to correctly assess the athlete's functional fitness and choose the most optimal way to stabilize and enhance it.

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AN UPDATE ON DEHYDRATION IN ATHLETES

Parastaev SA[1,2] ✉, Miroshnikova YuV[3], Pushkina TA[3], Kurashvili VA[4], Yashin TA[5], Vykhodets IT[1], Kupeev MV[1], Didur MD[6]

[1] Department of Rehabilitation, Sports Medicine and Physical Education, Faculty of Pediatrics, Pirogov Russian National Research Medical University, Moscow, Russia

[2] Medical Clinic of the National Agency of Clinical Pharmacology and Pharmaceutical Science, Moscow

[3] Federal Medical and Biological Agency, Moscow, Russia

[4] Federal Science Center for Physical Culture and Sport, Moscow

[5] Federal Research and Clinical Center for Sports Medicine and Rehabilitation, FMBA, Moscow, Russia

[6] N. P. Bekhtereva Institute of Human Brain, the Russian Academy of Sciences, Saint Petersburg, Russia

Fluid and electrolyte imbalances can compromise physical performance of professional athletes. We have conducted a study to understand how aware athletes are of their hydration status and how they deal with dehydration. First, we surveyed 51 athletes (mean age of 20.4 years) specializing in different sports, including ice hockey, water polo, tennis and figure skating, using a questionnaire. Next, we analyzed the anonymized results of the laboratory tests run on the samples of 30 athletes specializing in futsal. We focused on hemotocrit and sodium levels and urine specific gravity as indirect indicators of hydration status. Survey results demonstrated that 86 % of the participants lacked knowledge of wise approaches to replenishing fluid or electrolytes after physical exercise, did not adequately control fluid intake and developed various degrees of dehydration. We noticed that awareness of hydration status negatively correlated with professional qualifications of the participants. Retrospective analysis of laboratory tests showed that hypohydration prevailed among high-class athletes: at least 73 % of them showed signs of dehydration. We emphasize the need for elaborating unified clinical recommendations on rehydration for Russian athletes that should be further approved by doctors and coaches.

Keywords: hydration status, dehydration, rehydration, high-class athletes, carbohydrate-electrolyte solutions

✉ **Correspondence should be addressed:** Sergey Parastaev
ul. Ostrovityanova, d. 1, Moscow, Russia, 117997; sergeyparastaev@gmail.com

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К ВОПРОСУ ОБ АКТУАЛИЗАЦИИ ПРОБЛЕМЫ ОБЕЗВОЖИВАНИЯ В СПОРТЕ

С. А. Парастаев[1,2] ✉, Ю. В. Мирошникова[3], Т. А. Пушкина[3], В. А. Курашвили[4], Т. А. Яшин[5], И. Т. Выходец[1], М. В. Купеев[1], М. Д. Дидур[6]

[1] Кафедра реабилитации, спортивной медицины и физической культуры, педиатрический факультет, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Медицинская клиника Национального агентства клинической фармакологии и фармации, Москва

[3] Федеральное медико-биологическое агентство, Москва

[4] Федеральный научный центр физической культуры и спорта, Москва

[5] Федеральный научно-клинический центр спортивной медицины и реабилитации ФМБА России, Москва

[6] Институт мозга человека имени Н. П. Бехтеревой РАН, Санкт-Петербург

Нарушение водно-солевого баланса — это фактор, лимитирущий физическую работоспособность профессиональных спортсменов. Нами было проведено исследование с целью определения степени информированности атлетов по проблеме дегидратации в спорте. На первом этапе было проведено с помощью разработанного авторами опросника анкетирование 51 спортсмена (средний возраст — 20,4 года) со специализацией в различных видах спорта: хоккее на льду, водном поло, большом теннисе, фигурном катании. На втором этапе были проанализированы деперсонифицированные данные лабораторных исследований 30 спортсменов со специализацией в мини-футболе: оценивали косвенные признаки гидратационного статуса — гематокрит, содержание натрия в крови, удельную плотность мочи. По результатам анкетирования была констатирована низкая информированность 86 % спортсменов по вопросам рационального восполнения потерь жидкости и минералов вследствие физических нагрузок, что служит одной из важнейших причин неконтролируемого потребления жидкости и развития обезвоживания различной степени. Отмечена зависимость уровня информированности от спортивной квалификации атлета. При ретроспективном анализе данных лабораторного тестирования была ориентировочно установлена распространенность гипогидратации среди спортсменов высокой квалификации: вероятные признаки дегидратации имели место по меньшей мере в 73 % случаев. В России следует разработать и внедрить национальные клинические рекомендации по регидратации в спорте, которые были бы одобрены медицинским и тренерским сообществом.

Ключевые слова: гидратационный статус, дегидратация, регидратация, спортсмены высокой квалификации, углеводно-электролитные растворы

✉ **Для корреспонденции:** Парастаев Сергей Андреевич
ул. Островитянова, д. 1, г. Москва, 117997; sergeyparastaev@gmail.com

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The balance of fluids is as important for physical capabilities of athletes as their energy metabolism intensity [1, 2]. Various body fitness indicators depend on qualitative and quantitative characteristics of hydration, i.e. saturation of body with liquids. Hydration disorders, from subclinical hypohydration to dehydration, also affect them drastically. That is why hydration status can help assess physical and functional condition of athletes.

Effective rehydration solutions that help maintain and restore fluid balance in particular and water-salt balance in general, are essential for optimizing the recovery of athletes after strains of training and competition. Evidence-based studies found that carbohydrate-electrolyte solutions (CES) are superior to low-salinity water in rehydration, which means they can boost the mentioned recovery of athletes and possibly improve their performance [3]. According to the International Society of Sports Nutrition (ISSN), CES can be used to accelerate rehydration, restore the volume of electrolytes and maintain the endurance levels [4]. Nutritionists have defined the optimal composition of such solutions, which are classified as sports drinks. They should contain at least 2 carbohydrates and sodium, the only mineral that requires no mandatory replenishment [5]. Other minerals, especially potassium can join the composition, too, but that is an option. The reason behind the optional character of the inclusion is that the body retains acceptable volumes of potassium even when the strain is significant, i.e. lasts for 8 days in a row and results in up to 3 to 4 liters of sweat generated, and the potassium consumption is down to 30 % of the recommended daily intake. Secondly, there is no conclusive evidence backing the negative effect low levels of potassium, magnesium and calcium may have on physical endurance. There is significant number of Consensus Statements [5, 7–11] that regulate CES consumption and aim to develop the rational consumption algorithms.

Despite the attention coaches and medical doctors throughout the world pay to the water-salt balance problem, and regardless of the extremely wide range of commercial sports drinks available on the market, hypo- and dehydration is still a condition diagnosed quite often both in professional athletes and people regularly going in for sports. According to Sponsiello et al [12], only 37 % of the examined athletes were hydrated properly, and according to V.A. Kurashvili, [13], up to 91 % of professional competitive sports athletes (basketball, handball, football) begin their training session while dehydrated. This being said, athletes often disregard dehydration: 65 % of runners going long and super long distances did not attach any importance to the possible problem [14]. It should be noted that these data were obtained through interviewing 419 men and women participating in the Chicago Marathon, most of whom have been practicing long distance running for at least 10 years.

The level of hydration is an indicator defined by individual properties of the athlete's anthropometric data, instrumental and laboratory testing parameters, eating habits, social and cultural status, confessional identity [8]. However, the researchers behind paper [8] did not take into account the awareness of athletes of fluid deficiency caused by physical strain and ways to replenish that deficiency.

Designing this study, we aimed to identify the relationship between awareness about body hydration regimen rationalization as one of the characteristics of eating habits found in a quite specific social group (top tier athletes) and established laboratory methods of hydration status of athletes.

METHODS

There were two stages to the study.

First stage implied surveying 51 professional athletes with the help of a questionnaire we developed. There were slightly more men than women among the respondents: 53 % versus 47 %. Age-wise, the distribution was as follows: athletes 16–18 years old — 51 %, 19–21 years old — 18 %, 22–24 years old — 12 %, 25 years and older — 20 %. The mean age was 20.4 years. The athletes practiced various sports: ice hockey, water polo, tennis, figure skating. 9 respondents were Masters of Sport of International Class, 5 -- Honored Masters of Sports.

The questionnaire contained 19 questions grouped into 6 clusters.

- Cluster 1 (questions 1–5): general information about the athlete (sex, age, anthropometric data, practiced sport and skill level, current training focus and intensity);
- Cluster 2 (questions 6–7): self-assessment of the water-salt balance status during and after training sessions, awareness of the average fluid loss per a training session;
- Cluster 3 (questions 8–11): how does the athlete replenish fluid loss during training sessions;
- Cluster 4 (questions 12–15): how does the athlete replenish fluid loss after training;
- Cluster 5 (questions 16–18): how does the athlete replenish fluid loss during competitions;
- Cluster 6 (question 19): what brands of special water-salt balance normalizing sports drinks does the athlete prefer.

The second stage implied a retrospective analysis of the laboratory tests results that indirectly described the hydration status of athletes. We studied the depersonalized data obtained through in-depth medical examinations of 30 top tier athletes practicing futsal; none of those athletes filled the first stage's questionnaire. We took 3 indicators as markers of shifts in the water-electrolyte balance: hematocrit (volume of erythrocytes in blood) as recorded in the general clinical blood test; Na^+ content in blood; specific density of urine. The array of laboratory test results was provided by the Clinic of Sports Medicine of the Moscow Scientific and Practical Center for Medical Rehabilitation, Restorative and Sports Medicine of the Moscow Department of Health.

RESULTS

The survey revealed various levels of awareness of de/rehydration occurring when practicing sports, from an almost complete ignorance (sports requiring complex coordination efforts) to the decent levels of awareness (competitive sports). The key problem was the lack of information on the water-electrolyte balance assessment methods and data on how much fluid should an athlete consume before, during and after training sessions and competitions. That said, it should be noted that 86 % of athletes drink during long training sessions/competitions, which is a positive sign.

As a rule, the greater the qualification of an athlete is, the more he/she knows of the perspiration-related fluid loss and the better he/she can manage the hydration status. However, even top tier athletes generally underestimated the fairly simple ways of monitoring the body's moisture saturation, which are weighing before and after training session; grading the urine color against the template [15] published to the website of University of West Alabama's Athletic Training & Sports Medicine Center; identification of the urine's body composition and specific gravity.

Only 7 athletes of the 51 surveyed took time to establish the individual fluid loss after training sessions, which is 14 % of the respondents (Figure 1). It should be noted that members of this extremely small group, while training/competing, generally consumed 50 to 70 % of fluid lost to physical exertion. Figure 2 shows the actual fluid loss due to training activities, and figures 3 and 4 show that most athletes do not consume enough fluid to compensate for that loss, especially after the sessions.

The most popular fluid to restore the water-salt balance among athletes is drinking water. More often than not, it is coaches that insist coaches on such a choice; they are wary of CES due to their composition because of the risk of violation of anti-doping rules.

As for the special CES, the surveyed athletes prefer products procured by the management following orders placed by the team. The share of CES made in Russia is below 20 %.

Quite often, athletes take commercially available sports drinks (iso- and hypotonic types) with drinking water; some of them do this consciously, relying on some information (unfortunately, not always justified).

The results of analysis of the laboratory tests data that indirectly prove the water-electrolyte balance is broken were rather unusual.

The hematocrit reference value (47 %) was exceeded in one case only, while 23 out of 30 futsal players exhibited border values (44–46 %). Thus, in 80 % of athletes the liquid fraction of blood tended to grow smaller, which can be considered a sign of hypohydration.

Fifteen athletes had hypernatremia, which is a probable indicator of hyperosmotic hypohydration, i.e. prevalence of fluid loss over the mineral component (above 152 mOsmol/kg H_2O). 5 athletes returned border values (146–152 mOsmol/kg H_2O). Seven athletes had normal sodium content in their blood (135–145 mOsmol/kg H_2O); 2 athletes suffered from hyponatremia. These 2 athletes had a hypoosmotic condition, but there was no reason to assume hyperhydration ("water intoxication" resulting from consuming large amounts of drinking water) since the specific density of their urine was acceptable (1020).

In 5 athletes, the specific density of urine (due to excess concentration diluted substances) grew to 1025 and above, which may mean dehydration; 11 athletes had the density below 1020, which signals of the optimal level of hydration. Fourteen athletes had the density within the limits of normality (1020–1025). Thus, in about 2/3 of athletes the concentration of substances diluted in urine tended to rise; however, this fact cannot be taken as significant without information on the content of urea, which is more important to urine's osmolality than sodium.

DISCUSSION

Top tier athletes do not know much about fluid deficiency resulting from physical exertion, ways to identify that deficiency and remedy it. This is a factor putting their performance and endurance at risk [1–3]. Our survey proves that the low level of awareness means up to 86 % of athletes do not possess sufficient information to optimize their fluids consumption regimen and thus are unable to manage their hydration status.

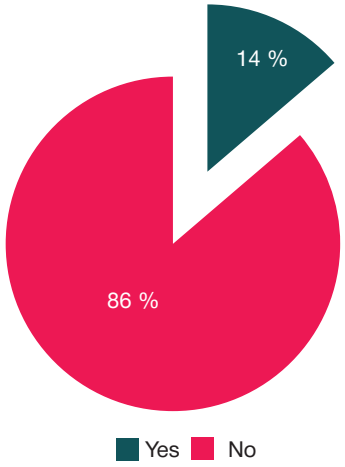

Fig. 1. Awareness of the actual fluid loss due to training activities. Results of surveying professional athletes (n = 51)

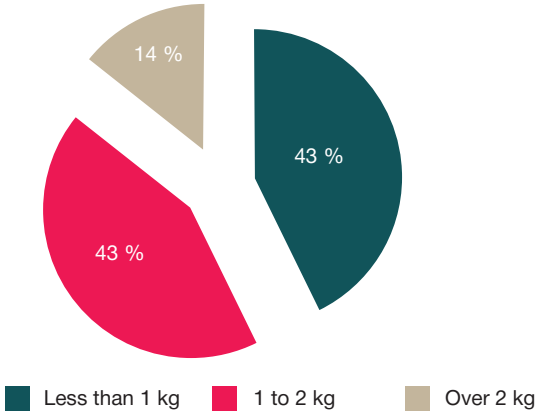

Fig. 2. The average volume of fluid lost during a training session (fact). Results of surveying professional athletes (n = 51)

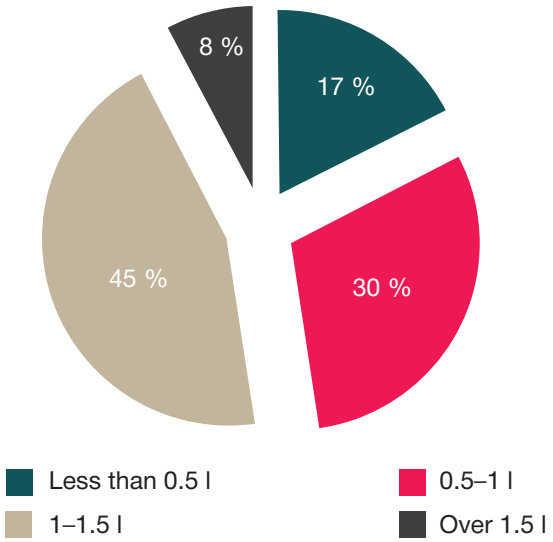

Fig. 3. The volume of fluid consumed during a training session. Results of surveying professional athletes (n = 51)

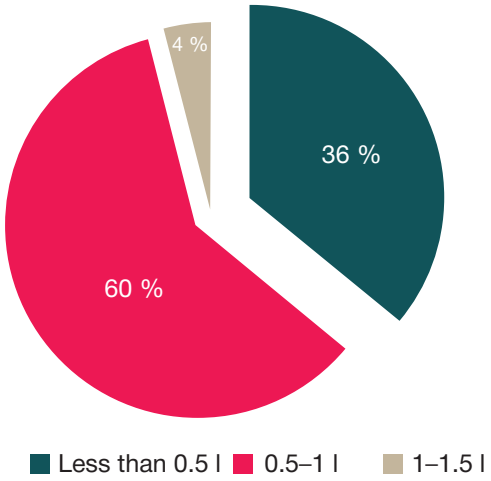

Fig. 4. The volume of fluid consumed after a training session. Results of surveying professional athletes (n = 51)

It should be noted that during training sessions/competition and after them the athletes should consume at least 150 % of fluids they have lost [3, 5]. Consequently, our observations show that no less than 2/3 of athletes, regardless of their drinking regimen motivation (thirst or directions issued by doctors), are at risk of developing hypo-and dehydration. That said, the surveyed athletes typically replenish 50 to 70 % of fluid lost during training sessions and competitions. Burke et al. report similar figures [16]. This means that the common problem is insufficient fluids consumption after physical exertion. All in all, the filled questionnaires we collected allow stating that 73 % of athletes do not consume enough liquids to compensate for the losses, regardless of sport practiced.

As for the results of the retrospective analysis of depersonalized laboratory tests data, 3 athletes exhibited 3 three signs of dehydration, 16 exhibited 2 signs and 8 athletes had just 1 sign. The optimal level of hydration (eughydration) was registered in 4 cases only, which makes 10 % of the sample. This is less than what other researchers found (see study [12], for example, where the share of properly hydrated athletes was 37 %). Our observations are paradoxical, since football in all its variations is a sport that takes nutrition and liquids intake seriously, which is proved by a regularly reissued set of rules [10].

The data obtained through this research signal of athletes underestimating the risk of dehydration and the negative impact it has on their performance; the athletes seem to lack understanding and knowledge of how to keep their water-electrolyte balance at the optimal level. On the national level, this problem is exacerbated by the lack of clinical recommendations on rehydration as applied to practicing athletes, the recommendations that would have been approved by doctors and the coaching community. Development of such recommendations and making them readily available to public could significantly improve the situation.

CONCLUSIONS

The survey conducted as part of this study was designed to achieve both research and didactic goals. The purpose-made questionnaire formed basis for a discussion with sports nutrition specialists; the discussion revolved around the possible reasons behind the great differences in answers, including those given to the question of rational rehydration (or maintaining the status at the desired level) and its role in improving the performance of athletes, most of which took it as an incentive to learn how to keep the water-salt balance in order. The retrospective

analysis of depersonalized laboratory tests data has objectively confirmed the problem exists.

The problem of irrational fluids consumption by practicing athletes is still extremely urgent. In this connection, the most important task to be solved as soon as possible is the development of various instruments to monitor hydration status, instruments that can be used both in laboratory settings (like stationary equipment to analyze body composition, segment-wise and not) and in the "field" (including test strips to determine the specific density of urine, electrolyte composition of sweat and viscosity of saliva, plus the urine color scale). Proper monitoring arrangements would allow development of personalized rehydration programs covering the yearly training cycle stages; such programs could be made up not just for the top tier athletes, who are few, but for the "reserve" sportsmen, too.

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CAN SPORTS BE SAFE? REALITY, CONCEPTS AND REGULATIONS

Didur MD[1] ✉, Vykhodets IT[2], Khokhlina NK[2], Zhuravleva AI[3], Polyaev BA[2]

[1] N. P. Bekhtereva Institute of Human Brain, Saint Petersburg, Russia

[2] Department of Medical Rehabilitation, Faculty of Continuing Professional Education, Pirogov Russian National Research Medical University, Moscow, Russia

[3] Department of Physiotherapy, Sports Medicine and Medical Rehabilitation, Russian Medical Academy of Continuing Professional Education, Moscow, Russia

A safe sport is a bit of an oxymoron: competition puts a severe strain on the vital systems of the organism that may be dangerous or uncontrollable, does not come in small doses and entails unpredictable results. Sports injury surveillance aims to estimate the impact of different factors that increase the risk of injuries and to elaborate wise and efficient measures to curb this risk. Accurate data on mortality rates in athletes help to improve approaches to health screening. Injury surveillance systems that also report injury-related deaths in athletes exist in many developed countries. This article talks about sports injuries in Russia and provides rationale for creating a nation-wide system of sports injury surveillance.

Keywords: sports, safety, injury, sudden death, statistics, risk factors

✉ **Correspondence should be addressed:** Mikhail Didur
ul. Akademika Pavlova, d. 9, Saint-Petersburg, Russia, 177376; didour@mail.ru

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БЕЗОПАСНЫЙ СПОРТ? РЕАЛИИ, ПОНЯТИЙНЫЕ И НОРМАТИВНЫЕ АСПЕКТЫ

М. Д. Дидур[1]✉, И. Т. Выходец[2], Н. К. Хохлина[2], А. И. Журавлева[3], Б. А. Поляев[2]

[1] Институт мозга человека имени Н. П. Бехтеревой, Санкт-Петербург

[2] Кафедра медицинской реабилитации, факультет дополнительного профессионального образования, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[3] Кафедра физической терапии, спортивной медицины и медицинской реабилитации, Российская медицинская академия непрерывного профессионального образования, Москва

Словосочетание «безопасный спорт» противоречиво: победа в соревновании требует напряжения систем жизнеобеспечения организма, но такое напряжение опасно, поскольку неконтролируемо, недозируемо и потому непредсказуемо. Статистический учет спортивных травм позволяет оценивать действие различных факторов риска получения повреждения и принимать взвешенные и эффективные меры по управлению ими. Оценка смертности спортсменов помогает совершенствовать подходы к скринингу здоровья атлетов. Такие системы получения информации о травмах и гибели спортсменов существуют во многих развитых странах. В статье анализируется ситуация по проблеме, сложившаяся в России, и обосновывается необходимость создания национальный системы учета спортивного травматизма.

Ключевые слова: спорт, безопасность, травматизм, внезапная смерть, статистический учет, факторы риска

✉ **Для корреспонденции:** Дидур Михаил Дмитриевич
ул. Академика Павлова, д. 9, г. Санкт-Петербург, 177376; didour@mail.ru

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"Safe Sport" is an established term widely used by professionals or various backgrounds. Russian legislation cites it, too, but this term finds different definitions in scientific, medical and sport circles.

Federal Law no. 329-FZ *On Physical Culture and Sport in the Russian Federation* defines sport as a social and cultural activity encompassing various sports and implying competitions training people for them [1]. The law prescribes ensuring safety of life and health of the participants of such competitions and states that it is not just the organizers who should put effort into that but sportsmen themselves as well: they must regularly undergo medical examinations and obey medical regulations when competing [2, 3]. Here lies the insoluble contradiction between the actual practice of sport and the basic laws of medicine [4]. Winning a competition always requires maximum effort, which puts body under an uncontrollable strain with possible unpredictable consequences. It is impossible to predict the number of situations when athletes practicing competitive and contact sports risk injury. It is also impossible to predict the outcome of a competition and the response a person's body may exhibit to a maximum load at any given time.

Thus, the legislative requirement to ensure safety of life and health of athletes is always violated. If this is the case, does it not make the "safe sport" phrase a dangerous illusion cherished by lawmakers that does not allow professional response to the problems of sports-related injuries? We believe that understanding the level of such injuries and reasons behind them can help build a foundation for safer competitions and sports in general.

Systematic registration and analysis of sports injuries

Competitions are always dangerous. This is a fact recognized by experts in all nations participating in international sports events. These experts have been studying the risk factors for over 50 years now; they distinguish between modifiable (controllable) and unmodifiable (uncontrollable) factors, as well as factors that cannot be accurately predicted at all [5, 6].

Sports injuries make up 2 to 5 % of all injuries suffered by people, including domestic, professional injuries etc. When determining the injury rate for this or that sport, it is common to calculate it for a thousand athletes practicing it. Such an approach allows offsetting the effect its general popularity produces on the value. This is the so-called intensive injury rate, which equals 188 for rugby, for example, and 18 for bodybuilding [7]. Foreign researchers also determine injury risks by calculating the number of injuries per a thousand training sessions or competitions/events (athlete exposure). In the US, this indicator brings box (5.2) and rugby (3.8) to the top of the list. The figures were obtained in 2003, when a study analyzed the examination data of 20.1 million athletes gathered in 2002 [7].

All foreign researchers acknowledge that sport is a dangerous activity; both on professional and state levels, they work to determine and measure the effect of controllable injury risk factors and minimize that of uncontrollable factors. For example, in 2007, the National Collegiate Athletic Association of USA (NCAA) provided data on 182,000 injuries from more than 1 million sports events reports submitted in seasons 1988/1989 to 2003/2004 (16 years). NCAA has been collecting standardized data on injuries suffered by student athletes in relevant competitions and training sessions since 1982. It was established that injury-wise, competitions mean greater risk (13.8 per 1,000 events) than training sessions (4.0 per 1,000 events); the difference is statistically significant [7]. Lower extremities suffered over half of all such injuries, and 15 % of those were sprained ankles.

Reports filed by medical institutions also help analyze sports injuries. In 2001–2012, emergency aid stations through the US received 3.42 million calls that had to do with brain injuries associated with sports activities [8]. Men suffered such injuries twice as often as women; almost 70 % of such patients were under 19 years of age. Cycling, football and basketball competitions and training sessions generated most of those injuries. Women commonly suffered them when practicing cycling and competitive sports. 89 % of men and 91 % of women received outpatient treatment at emergency aid stations. Another research paper analyzed calls to over 900 US hospitals (approximately 30 million people per year) that occurred in 2010–2013; the researchers found the number of competition-related eye injuries to be 120,847 [9]. 81.3 % of calls were made by men that got injured playing basketball, baseball or softball.

School reports is a yet another source of data. Online reports filed by US high schools in 2005–2014 present 59,862 sports injury cases [10]. Most of those that resulted in prolonged suspension from training activities occurred at competitions (60.4 %). The highest injury rate was seen in American football (26.5 per 100,000 athletes), then came gymnastics (18.6) and wrestling (17.9). In competitive sports, most injuries came through contacts (48.2 % of cases); knee joint suffered more often than other parts of body (33.7 % of cases).

Robust statistical tools allow effective monitoring of sports injuries and making adequate managerial and professional decisions. The above examples show the operation of sports injuries registration and analysis system developed in one of the leading sporting power, the US; the situation there is in stark contrast to that in Russia. The Sports Injury Notice (Form 092-u) was abolished more than 30 years ago. Since then, the sports injury statistics were not collected in our country in a centralized manner.

Sports-related sudden deaths registration and prevention

The world statistics knows of several thousand deaths that occurred during sports events, which conclusively proves the current life-threatening conditions prevention systems is ineffective. One of the first cases of sudden death of athletes dates back to 1976, when two basketball players from one of the American colleges died 8 weeks apart. One of them suffered from the Marfan syndrome, the other's condition was hypertrophic cardiomyopathy. Sudden deaths of athletes in subsequent years made the problem much more visible: the list includes names like Pete Maravich, Reggie Lewis, Corey Stringer, Jason Collier, Thomas Errion. Attention paid by professionals to sports-related deaths lead to better understanding of demographical factors, conditions and reasons behind those deaths, which include a variety of genetically determined cardiovascular diseases (most often — hypertrophic cardiomyopathy), blunt trauma or myocardial contusion, etc. A number of initiatives were launched; those initiatives aimed to develop consensus guidelines governing admission to sports activities and pre-professional screening designed to detect unforeseen cardiac abnormalities. Within three decades, the research efforts resulted in collection of great volumes of data, but they did not resolve all the contradictions in this area [11].

Screening for sudden death risk factors has originally been developed for athletes under 35, but recently attention has also been paid to the rapidly growing group of older athletes. The cardiac arrest risk they run in connection with physical activity is 10 times greater; its main cause is the coronary artery disease. Systematic review of 1,737 studies containing data on the effectiveness of various coronary artery disease imaging methods applied to athletes 35 and older showed that such sportsmen should also undergo CT angiography, echocardiography and MRI, otherwise the data to assess calcification of arteries and myocardial perfusion may be incomplete [12].

Efficacy of the sudden death prevention screening programs was appraised in a study carried out in Veneto (Italy). The study compared athletes and ordinary people aged 12–35; during the study, the number of sudden cardiovascular deaths among screened athletes was 55 (1.9 cases per 100,000 person-years) and that among unscreened ordinary people was 265 (0.79 cases per 100,000 person-years). The annual incidence of sudden cardiovascular death in athletes decreased by 89 %, from 3.6 cases in 1979–1980 to 0.4 cases per 100,000 person-years in 2003–2004 ($p < 0.01$). This is the result of introduction of mandatory athletes screening on the national level. Mainly, the number of sudden deaths caused by cardiomyopathies has decreased. This is a positive example of a professional approach to identifying life-threatening conditions in athletes and a real reduction in the number of sudden deaths.

Abroad, there are also special databases collecting data on all deaths occurring at the events organized by national sports associations [14]. Analysis of those data allows assessing mortality rates as they relate to specific reasons. For example, NCAA data analysis revealed that athletes belonging to the association run a high risk of sudden cardiac death, and male

athletes, black sportsmen and basketball players are exposed to a significantly higher risk of death [14].

Sports injuries management in Russia

Regretfully, the national sports injuries registration system at the level of Ministry of Health was lost almost 30 years ago. Statistical data provided by medical and sports clinics and departments are disembodied; they do not cover all sports and sporting events, never undergo systematization at the national level and, therefore, do not reflect the real state of affairs. There are no national registers of serious injuries and deaths resulting from sports activities. At the same time, official reports issued by the Russian Ministry of Sport state that the number of people going in for sports is constantly growing. National healthcare institutions have made a number of attempts to set up state-level regulation routines aimed at prevention of sudden cardiac death and other diseases in athletes. A decree issued by the Ministry of Health of Russia [3] prescribes that a person can only be allowed to train when he or she wields a medical examination note permitting practicing the sport in question. The note is a mandatory requirement for everyone, regardless of the specific sport chosen or sports school/club attended. At the same time, such medical examinations lack in substance and quality, and international admission screening recommendations produced no effect on the national laws so far.

The aforementioned decree issued by the Ministry of Health of Russia does have value, though: it is the first regulation to set standards for medical teams at sports events, prescribe the number of ambulance teams and doctors that should be present at competitions while taking into account the given sport's nature (injury-wise, too), number of participants and spectators.

We believe it is necessary to develop the national sports injuries registration system and follow the best international practices in this field.

CONCLUSIONS

Sports should be acknowledged as hazardous activity. The statistics clearly shows that injuries and deaths are inherent to sports nowadays, and their frequency will never dive below a certain level because it is impossible to reliably predict physical response to sport activities. However, foreign studies indicate that professional assessment of real injury and sudden death risks based on objective statistical data allows efficient control over them. It is necessary to restore the Russian sports injuries registration system that was lost several decades ago.

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DRUG-FREE TREATMENTS OF TENSION HEADACHES IN SCHOOL-AGE CHILDREN

Polunina VV[1] ✉, Sergeenko EYu[1], Yarustovskaya OV[2], Polunin VS[1]

[1] Pirogov Russian National Research Medical University, Moscow, Russia

[2] Russian Medical Academy of Continuous Professional Education, Moscow, Russia

Nowadays headaches are common among teenagers and children. This study aimed to assess effectiveness of reflexology, kinesiology taping and myofascial trigger point therapy in children with tension headaches and to compare these treatments with traditional drug-based modalities. The study recruited 37 children (19 boys and 18 girls) aged 9 to 14 years. The main group (n = 25) received 2 series of reflexology treatments separated by a month interval, kinesiology taping and trigger point massage, which was also taught to the patients and their parents. The control group (n = 12) received Ibuprofen and Mydocalm (the daily doses did not exceed 30 mg/kg and 2–4 mg/kg, respectively). Treatment duration in both groups was 4 months. Treatment effectiveness was assessed based on the evolution of patients' complaints and the impact of headache on children's daily activities, using the visual analog pain scale and the HIT-6 method, respectively. Within a month, headaches became 1.2 times less frequent and the attacks became 1.2 times shorter in the control group, while in the main group headaches became 2.5 times less frequent and the attacks became twice as short as they had been before Headache intensity did not change significantly in the control group, while in the main group it decreased 1.5 times ($p < 0.05$). The number of controls who experienced a severe impact of headache on their daily activities decreased 1.2 times after the treatment, while the main group reported no such impact at all. In the main group the number of patients who experienced only a slight impact of headaches on their daily activities increased 4.7 times, from 12 % to 56 %. Our findings demonstrate that drug-free treatments for tension headaches are more effective than drug-based regimens. Moreover, children benefit from drug-free regimens as they are not exposed to the negative effects of analgesics and muscle relaxants.

Keywords: children, tension headache, kinesiology taping, reflexology, drug-free treatment

✉ **Correspondence should be addressed:** Viktoria Polunina
ul. Ostrovityanova, d. 1, Moscow, Russia, 117997; vikt025@gmail.com

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НЕМЕДИКАМЕНТОЗНЫЕ СПОСОБЫ ЛЕЧЕНИЯ ГОЛОВНЫХ БОЛЕЙ НАПРЯЖЕНИЯ У ДЕТЕЙ ШКОЛЬНОГО ВОЗРАСТА

В. В. Полунина[1] ✉, Е. Ю. Сергеенко[1], О. В. Ярустовская[2], В. С. Полунин[1]

[1] Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Российская медицинская академия непрерывного профессионального образования, Москва

Головная боль очень распространена среди современных детей и подростков. Целью исследования являлось изучение эффективности рефлексотерапии, кинезиотейпирования и точечного массажа миофасциальных триггерных точек в лечении головных болей напряжения у детей в сравнении с медикаментозной терапией. В исследовании приняли участие 37 детей (19 мальчиков и 18 девочек) в возрасте 9–14 лет. В основной группе (n = 25) лечение включало 2 курса рефлексотерапии с перерывом между ними в 1 мес., кинезиотейпирование и точечный массаж, которому в том числе были обучены пациенты и их родители; в контрольной (n = 12) — получение ибупрофена (суточная доза — не более 30 мг/кг) и мидокалма (суточная доза — 2–4 мг/кг). Общая продолжительность лечения в обеих группах составила 4 мес. Эффективность лечения оценивали по динамике жалоб на головную боль с использованием визуальной аналоговой шкалы боли и степени влияния головной боли на повседневную активность детей с помощью методики HIT-6. В контрольной группе частота эпизодов головной боли за месяц и средняя продолжительность приступа в среднем уменьшились после лечения в 1,2 раза, а в основной группе частота эпизодов головной боли за месяц уменьшилась в 2,5 раза и средняя продолжительность приступа — в 2 раза. При этом обычная интенсивность головной боли в контрольной группе практически не изменилась, а в основной — уменьшилась в 1,5 раза ($p < 0,05$). В контрольной группе количество пациентов с сильным влиянием головной боли на повседневную активность уменьшилось после лечения в 1,2 раза, в основной же группе таких пациентов вообще не осталось, а количество пациентов с незначительным влиянием головной боли на повседневную активность увеличилось в 4,7 раза — с 12 до 56 %. Полученные результаты показывают, что немедикаментозное лечение головных болей напряжения у детей эффективнее медикаментозного, при этом важно, что дети не подвергаются негативным эффектам от употребления анальгетиков и миорелаксантов.

Ключевые слова: дети, головная боль напряжения, рефлексотерапия, кинезиотейпирование, немедикаментозное лечение

✉ **Для корреспонденции:** Полунина Виктория Валерьевна
ул. Островитянова, д. 1, г. Москва, 117997; vikt025@gmail.com

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According to various researchers, from 25 to 80 % of schoolchildren suffer from headaches [1–4]. Often, they are accompanied by attention deficit and hyperactivity. Eighteen to twenty-five percent of children and teenagers have tension headaches, i.e. repeated bilateral headaches of compressing, pressing and dull types [5–8]. They make 2/3 of all headaches suffered by this group [9].

The specific feature of tension headache pathogenesis in children and adolescents is the immaturity of their psychological defense mechanisms. They can develop tension headaches as a result of physical and mental fatigue, sleep deprivation, visual overstrain, problems with their group or classmates [1]. In addition, some researchers [10–12] point out that children suffering from tension headaches often have cerebrovascular disorders, including attention deficit, memory loss, increased irritability, affective outbreaks. These disorders may be caused by birth injuries to the CNS, somatic diseases, craniocerebral trauma, neuroinfections.

In some cases, headache is accompanied by the symptoms of autonomic dysfunction [13–15]. Timely detection of vegetative disorders allows effective correction of the clinical manifestations of the pathology, which translates into improved quality of life for patients [15]. It is especially important to diagnose vegetative imbalance in children and adolescents, because their age is the time when segmental and supra-segmental structures of their brain mature, and those structures influence appearance of pain syndromes, including cephalothia.

Trigger points, mainly those found around upper shoulder girdle and neck, play an important role in tension headache development. A trigger point is a cluster of electrically active sections of muscle fibers that are connected to the contracted ending of the motor nerve in the skeletal muscle. Constant tension of the trigger point fibers disrupts blood circulation in the corresponding parts of the muscle; metabolic products accumulate there and prolong the existence of those points, which can contribute to the reflected headaches of different locations. Massaging (and self-massaging, most importantly) the trigger points helps to somewhat remedy the headache. The alleviation is the result of disruption of chemical and neurological feedback loop that keeps the muscle contracted. Also, massaging improves the local blood flow and straightens the muscle fiber [16, 17].

Reflexology has been used to treat headaches for a long time [18, 19]. There are acupuncture points that help remedy the condition. Acupuncture combined with the trigger points massage may yield a long-term relief from a headache.

Kinesio taping is one of the pain management methods suggested by Kenzo Kase (Japan) in 1973. The method is effective as part of rehabilitation programs; it has been applied all over the world for more than 30 years now. Kinesio tapes are elastic bands of high quality cotton covered with acrylic hypoallergenic adhesive gel that activates at the body temperature. The elastic properties of tapes are close to those shown by skin. And since they are cotton, the skin is breathing and evaporation remains unhindered. Thus, the tapes can be left on the skin for 5–7 days and there is no need to skip showers. Clinical studies have shown that kinesio tapes normalize microcirculation in the skin's connective tissue and subcutaneous fatty tissue, alleviate pain, restore functional activity of the muscles and optimize afferent pulses at the segmental level [20]. Depending on the application method, a tape can relax or contract the muscle, reduce fascia tension, minimize swelling, help with the resorption of hematomas. Kinesio tapes at trigger points and zones reinforce the effect of reflexology and point massage.

The purpose of our study was to research the effectiveness of non-drug treatment of headaches in children, including reflexology and kinesio taping.

METHODS

The study was conducted at the premises of the Rehabilitation Department of Children's City Polyclinic no. 39 (Moscow). Thirty-seven children participated in the study: ages 9 through 14, 19 boys and 17 girls, suffering from tension headaches for 6 to 18 months. The average age of the children was 11.2 ± 1.6 years. Inclusion required the child to suffer from episodic tension headaches (at least once a month, but not more than 15 episodes a month) and have myofascial trigger points. Children with other types of headaches and of other ages were not included into the study.

Outpatient, all participants were examined by a neurologist, an oculist, a psychologist, a reflexotherapist; underwent ultrasound dopplerography of cerebral vessels, a general blood test, blood pressure checkup; had their neck and shoulder muscles assessed, as well as those of the back and upper limbs; filled questionnaires (their parents, too) and health diaries. Then the children were randomly divided into two groups, each receiving a different treatment.

The treatment group included 25 children (13 boys and 12 girls). A neurologist monitored them for 4 months; they had 2 reflexology courses with kinesio tapes and learned to massage myofascial trigger points. The control group included 12 children (7 boys and 5 girls); their state was monitored by a neurologist for 4 months from the day they applied for medical help; they received medication, analgesics and muscle relaxants: ibuprofen (daily dose — no more than 30 mg/kg) and midocalm (daily dose — 2–4 mg/kg).

Prior to the therapy, the patients kept health diaries for 1–2 months (aided by their parents). There, children registered days when they had headache, its intensity, duration and impact on general health and daily activities. Subsequently, based on those records we determined the pain intensity using the visual analogue scale (VAS): 0 points — "no pain", 10 points — "the pain is unbearable". HIT-6 was used to assess the impact headaches had on daily activities [21].

Treatment group received 2 two-week reflexology courses: 10–12 sessions per a course, on weekdays, each lasting

Characteristics of headache in the groups, before and after treatment

Headache characteristics	Treatment group		Control group	
	before treatment	after treatment	before treatment	after treatment
Frequency, average number of episodes per month.	12.9 ± 2.6	5.1 ± 1.2*,#	12.3 ± 2.1	10.1 ± 1.9#
Average duration per month, h	4.9 ± 1.4	2.4 ± 0.6*	5.2 ± 1.3	4.3 ± 0.9
Typical intensity per month, VAS score	6.2 ± 1.1	4.1 ± 0.9*	6.1 ± 1.1	5.6 ± 1.4
Impact of headache on daily activities, HIT-6 score.	53.5 ± 3.1	45.3 ± 2.7*	52.3 ± 4.9	49.5 ± 6.1

Note. * — p <0.05 when comparing results before and after treatment within a group, # — p <0.05 when comparing results after treatment between groups.

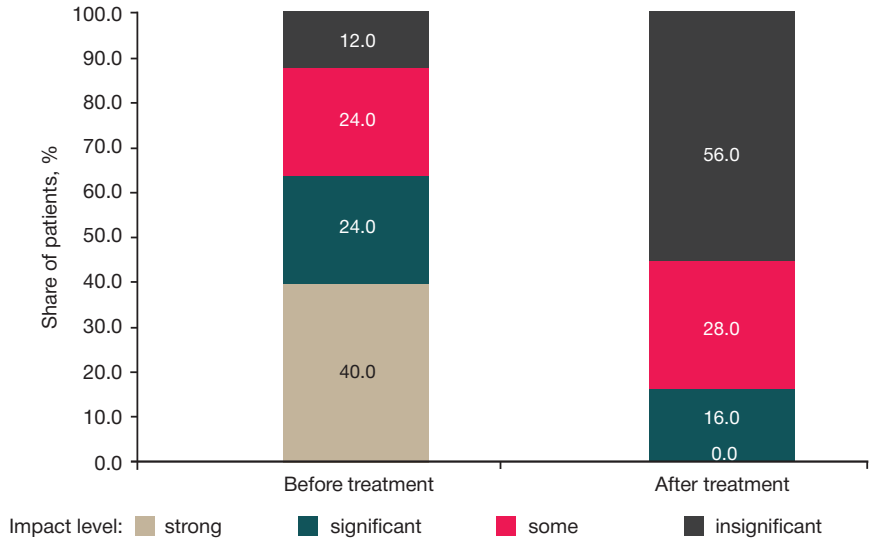

Fig. 1. Treatment group patients by impact of headache on their daily activities, before and after treatment (p <0.05)

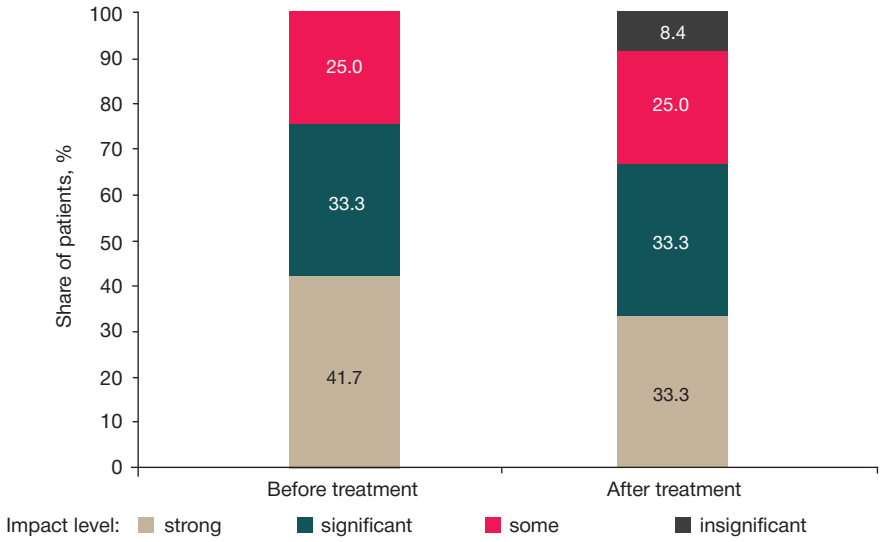

Fig. 2. Control group patients by impact of headache on their daily activities, before and after treatment (p <0.05)

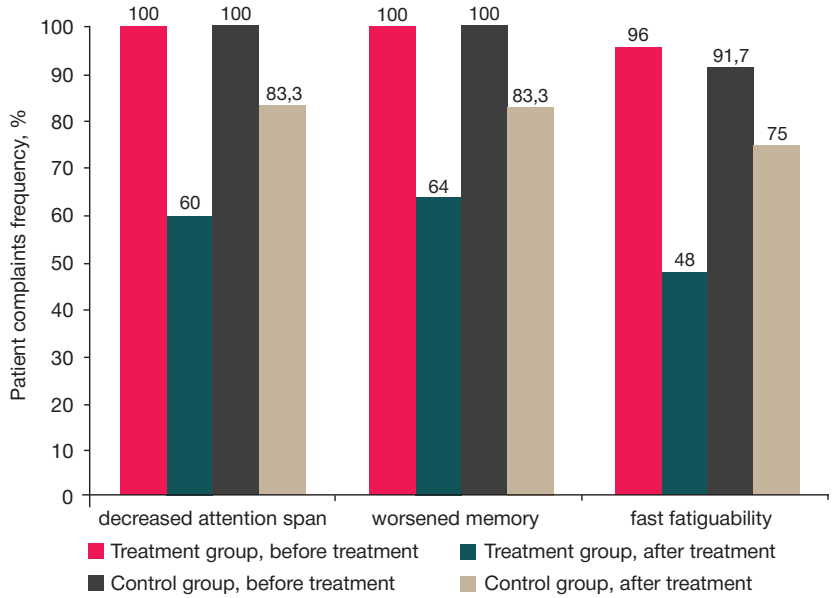

Fig. 3. Frequency of complaints of shorter attention span, poorer memory and rapid fatiguability among patients with tension headache, before and after treatment, calculated for 100 subjects (p <0.05)

10–15 minutes depending on the child's age. The second course was a month later. Reflexological treatment included acupuncture with a massage roller, corporeal and auricular acupuncture, acupressure. Five to seven corporal and two to three auricular points were affected in a session. The points were chosen following patient's complaints and results of examination.

After the reflexological treatment, children received kinesio tapes applied to their trapezius and hind neck muscles with the aim to relax them. Trapezius muscles received "Chinese lantern" applications, hind neck muscles — Y-shaped applications. No strain was put on the tapes.

In addition, therapists massaged trigger points found in the area of trapezoid, sternoclavicular-mastoid, belt, supraspinatus and pericranial muscles. Patients and their parents were trained to deliver point massage. They were recommended to massage at least twice a day, in the morning and in the evening, 1 minute to each point, and also every time after a long stay in uncomfortable poses.

MS Excel-2007 was used for statistical processing of the data. We calculated the mean value and the mean squared deviation of each parameter studied; Student's t-test was used to assess the confidence of differences revealed.

The study was approved by the ethics committee of the Children's City Polyclinic #39 (Minutes No. 1 of 21.03.2016). Parents of the patients signed voluntary informed consent forms and thus approved participation of their children in the study.

RESULTS

The effectiveness of treatment was assessed through analysis of the dynamics of patients' complaints, intensity of headache as reported at examination, during surveying and testing. The data considered were those obtained before treatment and 4 months after treatment.

The table shows characteristics of headache in the groups. In the control group, which received medications only, the frequency of headache episodes per month and their mean duration decreased on average 1.2 times, but the differences were insignificant. In the treatment group, which received non-drug treatment, the frequency of headache episodes per month decreased 2.5 times and they grew twice as short ($p < 0.05$). That said, the intensity of headache in the control group remained practically the same, while in the treatment group it decreased 1.5 times ($p < 0.05$). The same pattern applied to the impact headache had on the daily activity of patients. Thus, reflexology and kinesio taping alleviate headache better than drugs, with the difference being statistically significant.

The impact of headache on the daily life of patients is of great importance. This factor was assessed using HIT-6; Figures 1 and 2 show the results. In the control group, the number of patients whose daily life suffered greatly from their headaches decreased after treatment 1.2 times, and there were patients (8.4 %) whose activities were almost unhindered by the pain. But the differences were insignificant. In the treatment group, no patient reported any considerable impact of headache on the daily life after treatment, and the number of those who did feel a small-scale negative effect of headache in their daily lives increased 4.7 times ($p < 0.05$).

Patients suffering tension headaches also reported poor attention span, worsened memory and rapid fatiguability. Figure 3 shows that after treatment, participants from the treatment group complained of those symptoms significantly less often than children from the control group.

DISCUSSION

Tension headaches are primary headaches; children and adolescents develop this type of headache most often [1, 9]. The results we obtained through this research effort prove that reflexology and kinesio taping are effective methods of treatment of tension headaches with myofascial syndrome in children. It should be noted that they typically have no complaints about reflexology routines and its methods have no side effects. Treating children, it is very important to use guides when introducing acupuncture needles, since they help to keep associated pain to a minimum. It is also important to psychologically prepare children patients to acupuncture sessions and have calm music playing in the background. Teaching parents and children to massage myofascial trigger points and recommendations to do that regularly helped to successfully to stop headache and prevent the episodes.

Papers [22–24] had adults as participants, and they also show that reflexology is a valid headache treatment method, in part due to its capacity to decrease the consumption of analgesics and muscle relaxants. Compared to the drug treatment, reflexology had a more pronounced effect.

It is also important to note that successful treatment of tension headaches in children and adolescents largely depends on positive psychological atmosphere in family and school, adequate sleep, rest, daily schedule and nutrition regimens, appropriate physical and mental workload, limited computer and social networks time. In other words, the success of treatment largely depends on how strictly the patient follows the prescribed regimen.

CONCLUSIONS

In paediatrics, various reflexology techniques in combination with kinesio taping allow decreasing the frequency of headache episodes, their duration and intensity. Moreover, these non-drug methods also eliminate the associated symptoms such as attention and memory deficits and fatigue. Reflexology and kinesio taping offer a better therapeutic effect than drugs, with difference in results being statistically significant.

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DEPENDENCE OF MUSCLE STRENGTH ON BIOLOGICAL MATURATION RATES AND KEY VARIABLES OF PHYSICAL DEVELOPMENT IN TEENAGE BOYS

Milushkina OYu[1], Skoblina NA[1], Prusov PK[2,3], Bokareva NA[1]✉, Tatarinchik AA[1], Kozyreva FU[1], Moiseev AB[4]

[1] Department of Hygiene, Faculty of Pediatrics,
Pirogov Russian National Research Medical University, Moscow, Russia

[2] Department of Restorative, Sports and Health Resort Medicine and Physiotherapy,
Institute of Advanced Training of the Federal Medical-Biological Agency of the Russian Federation, Moscow, Russia

[3] Moscow Centre for Research and Practice in Medical Rehabilitation, Restorative and Sports Medicine, Moscow, Russia

[4] Department of Propedeutics of Childhood Diseases, Faculty of Pediatrics,
Pirogov Russian National Research Medical University, Moscow, Russia

Functional abilities of school-age children are affected by a variety of factors, including endogenous. Over the course of a few years, we studied physical development of 182 boys who underwent annual physical examination from the age of 11 to 17. We took basic anthropometric measurements, such as height and weight, tested hand muscle strength and assessed biological maturation and body build. Our study showed that muscle strength in school-age boys suffers a negative influence of such endogenous factors as delayed physical development, body mass deficit, short stature, and asthenic build. Excess weight and low skeletal weight also contribute to decreased muscle strength in teenage boys. Our results can be used to identify teenagers at risk who should be given special attention during PE classes at school or during training sessions before the GTO fitness test.

Keywords: physical development of school-age children, biological maturation rate, hand muscle strength, handgrip test, somatotype

✉ **Correspondence should be addressed:** Natalia Bokareva
ul. Ostrovityanova, d. 1, Moscow, Russia, 117997; nabokareva@mail.ru

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ЗАВИСИМОСТЬ МЫШЕЧНОЙ СИЛЫ ОТ ТЕМПОВ БИОЛОГИЧЕСКОГО СОЗРЕВАНИЯ И ОСНОВНЫХ ПОКАЗАТЕЛЕЙ ФИЗИЧЕСКОГО РАЗВИТИЯ У МАЛЬЧИКОВ-ПОДРОСТКОВ

О. Ю. Милушкина[1], Н. А. Скоблина[1], П. К. Прусов[2,3], Н. А. Бокарева[1] ✉, А. А. Татаринчик[1], Ф. У. Козырева[1], А. Б. Моисеев[4]

[1] Кафедра гигиены, педиатрический факультет,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Кафедра восстановительной медицины, ЛФК и спортивной медицины, курортологии и физиотерапии,
Институт повышения квалификации ФМБА России, Москва

[3] Московский научно-практический центр реабилитации, восстановительной и спортивной медицины, Москва

[4] Кафедра пропедевтики детских болезней педиатрического факультета, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

На формирование функциональных возможностей современных школьников влияют различные факторы, в том числе эндогенные. В статье представлены данные об особенностях физического развития 182 мальчиков, каждого из которых обследовали ежегодно с 11 до 17 лет. Изучали основные антропометрические показатели (длину и массу тела), функциональные показатели (мышечную силу кистей рук), показатели биологического развития и особенности телосложения. Исследование показало, что негативное влияние на формирование мышечной силы у мальчиков-подростков оказывает ряд эндогенных факторов: задержка биологического развития, дефицит массы тела, рост ниже среднего и астеноидный тип телосложения. На формировании мышечной силы мальчиков-подростков неблагоприятно сказываются также избыточная масса тела и низкая скелетная масса. Полученные данные позволяют выделить группу риска, детям из которой следует уделять особое внимание при занятиях физкультурой и спортом и при подготовке к сдаче норм ГТО.

Ключевые слова: физическое развитие школьников, темп биологического созревания, мышечная сила кистей рук, динамометрия, соматотипы

✉ **Для корреспонденции:** Бокарева Наталия Андреевна
ул. Островитянова, д. 1, г. Москва, 117997; nabokareva@mail.ru

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Hand grip strength reflects muscular and nervous health of an individual. Hand grip tests have long been used to assess the functional capacity and physical strength of children during regular medical checkups or pre-training consultations. Studies of physical capacity are becoming increasingly important in light of Order 172 of the President of the Russian Federation dated March 24, 2104 *On the Nationwide Fitness Program GTO* (GTO stands for Ready for Labor and Defense).

There is evidence of new trends in the physical development of children and teenagers towards a larger overall body size, accelerated biological maturation, earlier menarche, and overweight [1–9], as well as reduced functional capacities, including decreased muscle strength [10–13]. In the majority of studies dynamometry scores are analyzed in the context of social and environmental factors [14, 15]. However, the correlation between muscle strength and the physical development of children and teenagers accounting for the population variability remains understudied.

In this work we aimed to investigate how hand muscle strength correlates with physical development and the rate of biological maturation.

METHODS

This longitudinal study recruited 182 Moscow-born Caucasian teenage boys. The boys underwent physical examinations annually, from the time they were 11 till they turned 17 years of age. In terms of general health, the participants fell into health categories 1 and 2.

Physical development and biological maturity of the participants were assessed using a unified anthropometric method and standard techniques [16]. Basic anthropometric measurements were taken (body weight and height) and a functional right-hand grip test was conducted. To assess how balanced the physical development was, we did weight to height scaling using a modified regression technique [17]. Somatoscopy included visual assessment of biological maturity. Based on the maturation rate, the boys were classified as retarded in physical development (biological age lagged behind chronological age); normally developing (biological age coincided with chronological age); and accelerated in their physical development (biological age was ahead of chronological age).

Body build was classified using Darskaya's modification (1975) of the method proposed by Shtephko and Ostrovsky in 1929. Based on the visual assessment of the muscle bulk, bone skeleton, subcutaneous fat distribution, thorax shape, abdomen, back, and legs, we discriminated between the abdominal, thoracic, muscular, asthenic and mixed somatotypes [16].

Associations between muscle strength and muscle/fat mass were studied in 23 boys. Somatometric measurements were taken using conventional anthropometric methods and techniques. Body composition was analyzed on the InBody device (South Korea) for bioelectrical impedance analysis.

Statistical processing was performed using Statistica 6.0 (StatSoft, USA). To estimate significance of differences, Student's t test was applied. Correlations were studied between qualitative characteristics of physical development using Pearson's linear correlation coefficient r to describe correlation strength. At $r < \pm 0.3$, the correlation was either absent or weak; at r ranging from ± 0.5 to ± 0.7 the correlation was moderate; at $r > \pm 0.7$ the correlation was strong.

The study was approved by the Ethics Committee of Pirogov Russian National Research Medical University (Protocol 130 dated December 9, 2013). Informed consent was obtained from the parents and headmasters.

RESULTS

The correlation analysis showed that there was a statistically significant ($p<0.05$) moderate correlation between muscle strength and body height in 11-year old boys; the correlation between muscle strength and body weight also turned out to be significant in this age group. It should be noted that correlation strength declined as the boys grew older (Tables 1, 2).

More pronounced correlations were observed in impedance tests. The analysis revealed the presence of statistically significant ($p<0.05$) strong correlations between muscle strength and basal metabolism parameters ($r = 0.86$) and skeleton mass ($r = 0.86$). Moderate negative correlations were detected between muscle strength and fat mass ($r = –0.52$, $p < 0.05$).

Table 3 presents data on the hand muscle strength of boys grouped by their age. As the boys grew older, muscle strength increased from 15.25 ± 0.86 kg at 11 years of age to 38.66 ± 0.8 kg at 17 years of age, i.e. 2.5 times.

Figure 1 shows age-related dynamics of muscle strength in teenage boys depending on the rate of biological maturation.

The boys whose physical development was accelerated had better muscle strength at the age of 11, scoring even more by the age of 13, in comparison with their peers retarded in

Table 1. Correlations between the main parameters of physical development and hand muscle strength of 11-year-old boys (r; $p < 0.05$)

Parameter	Height	Weight	Right hand muscle strength
Height	1	0.75	0.53
Weight	0.75	1	0.47
Right hand muscle strength	0.53	0.47	1

Table 2. Correlations between the main parameters of physical development and hand muscle strength of 17-year-old boys (r; $p < 0,05$)

Parameter	Height	Weight	Right hand muscle strength
Height	1	0.56	0.47
Weight	0.56	1	0.39
Right hand muscle strength	0.47	0.39	1

Table 3. Age-related dynamics of muscle strength in boys aged 11 to 17 years (M ± m)

Age, years	11	12	13	14	15	16	17
Muscle strength, kg	15,15 ± 0,26	16,85 ± 0,30	19,93 ± 0,58	26,08 ± 0,69	31,49 ± 0,68	35,13 ± 0,60	37,50 ± 0,74

Table 4. Muscle strength in boys belonging to different somatotypes (M ± m)

Parameter	Somatotype				
	asthenic	thoracic	muscular	abdominal	mixed
	1	2	3	4	5
Right hand muscle strength	14.3 ± 0.2	17.04 ± 0.1	18.3 ± 0.2	18.6 ± 0.3	16.2 ± 0.2
p-value	$p < 0.05$ when comparing 1 and 3; $p < 0.01$ when comparing 1 and 4				

physical development. The highest scores in this group were seen at the age of 17. At 11 or 12 years of age, the boys whose development was retarded did not differ significantly from normally developing teenagers in terms of muscle strength, but at 13–15 years they scored less than normally developing or accelerating children. By the age of 16–17, these differences were leveled out and became unreliable.

Figure 2 shows how muscle strength depends on the physical development of the participants (body mass). In all age groups, no significant differences were observed in terms of muscle strength between normally developing and overweight children. The value of the muscle strength of boys with weight deficiency in all age groups except for 14 year old teenagers was significantly lower than that of harmoniously developing children ($p<0.01$, $p<0.05$, respectively).

We also discovered that muscle strength was dependent on body height. In all age groups, muscle strength of teenagers who were shorter than the average was significantly weaker than in other boys ($p < 0.01$, $p < 0.05$; see Table 3). The boys who were taller than the average or just tall scored better in

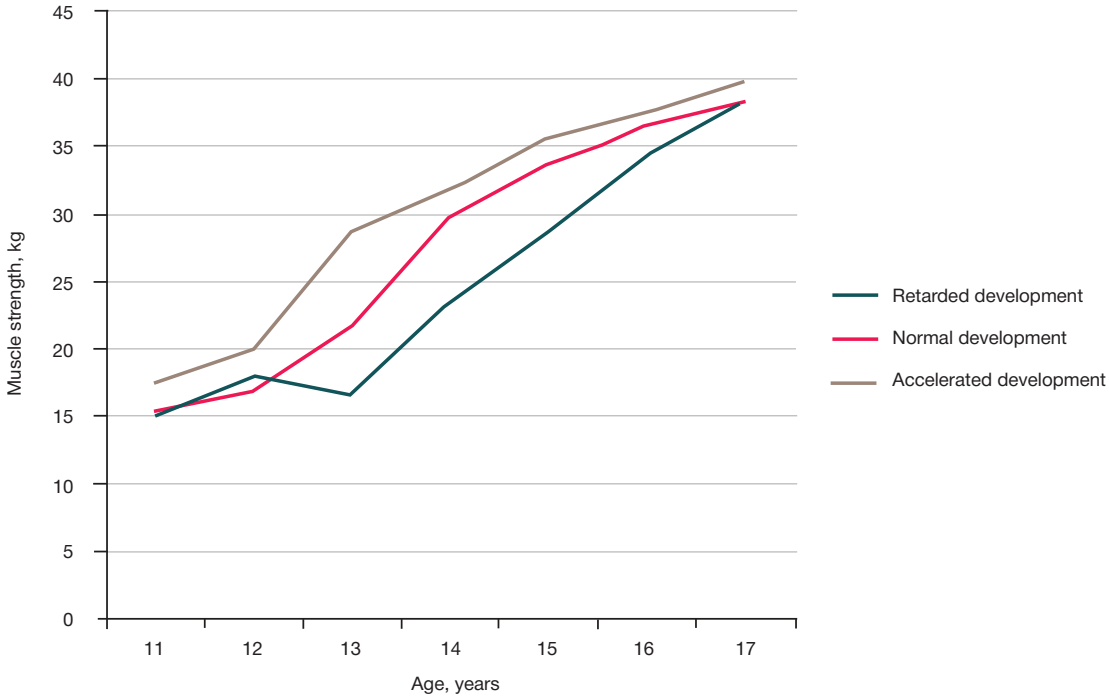

Fig. 1. Age-related dynamics of muscle strength measured in teenage boys with regard to their biological development

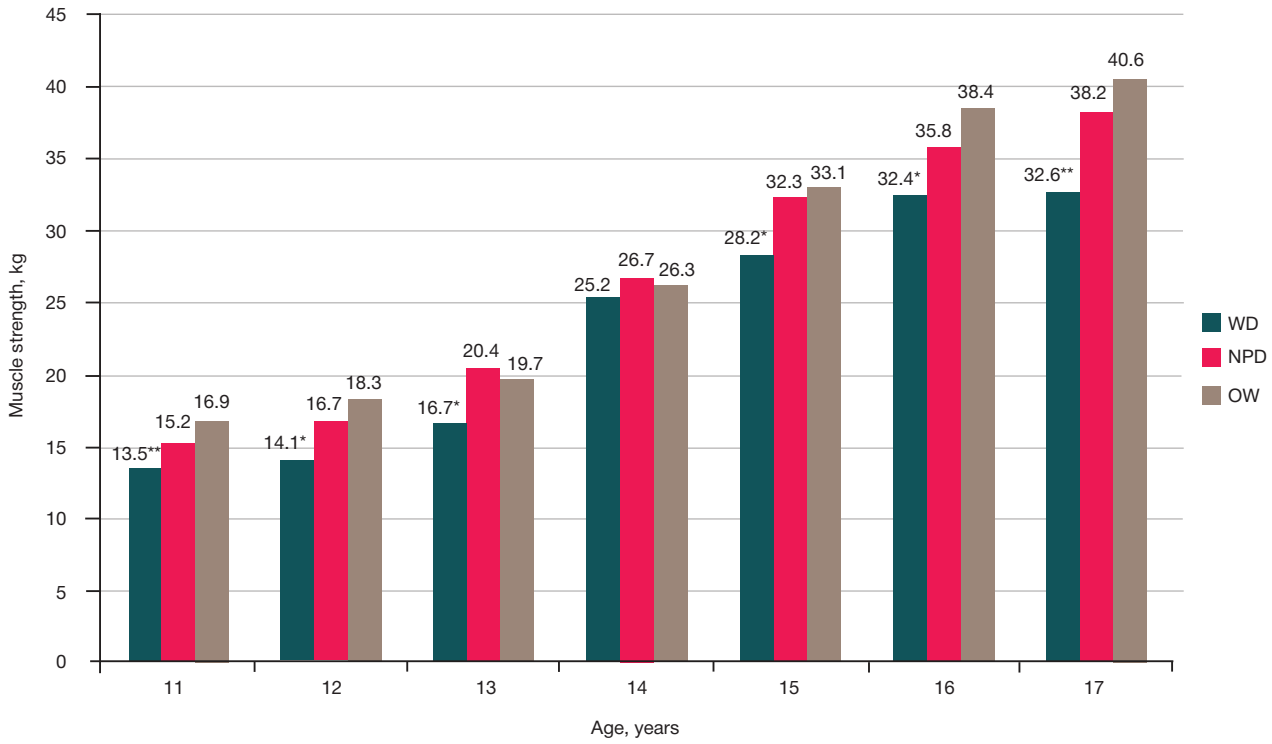

Fig. 2. Parameters of muscle strength of teenage boys depending on weight
* — $p < 0.05$, ** – $p < 0.01$
NPD — normal physical development, WD — weight deficiency, OW — overweight.

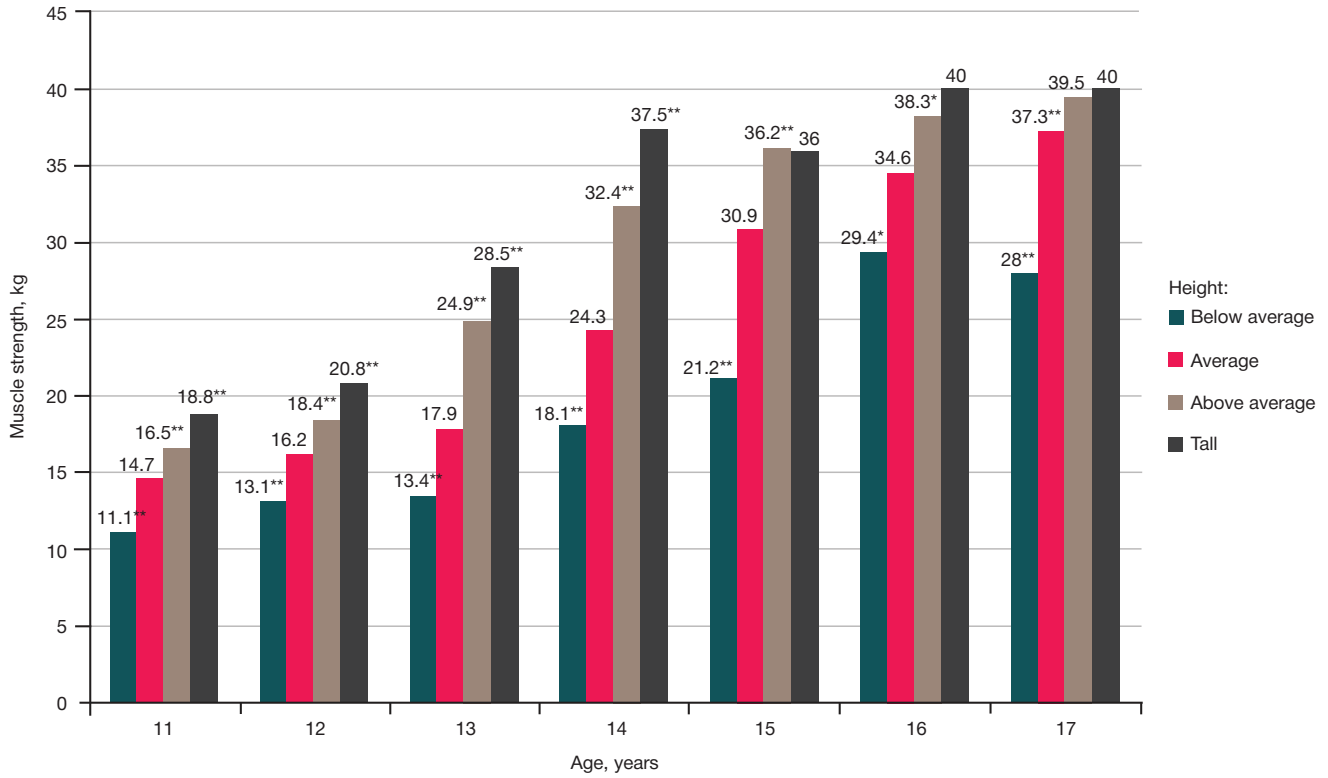

Fig. 3. Parameters of muscle strength of teenage boys depending on height
* — p < 0,05, ** — p < 0,01.

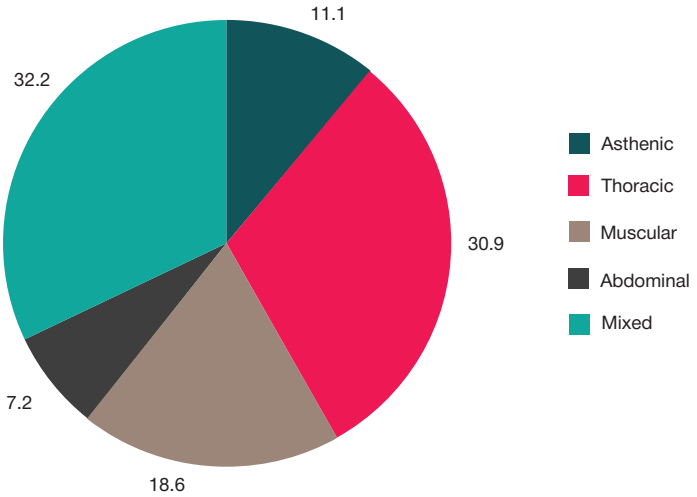

Fig. 4. Percentage of schoolchildren with different builds

hand grip tests than their peers at 11–16 years of age and 11–14 years of age, respectively (p < 0.01). In older age groups these differences were insignificant.

Somatotyping (Fig. 4) revealed that 42 % of boys belonged to the weak types (asthenic and thoracic); 25.8 % of the participants belonged to the relatively strong (muscular and abdominal) types; 32.2 % had mixed somatotypes. The analysis of muscle strength in children with different somatotypes showed that a somatotype significantly affects muscle strength. Asthenic children scored less than their peers who belonged to the muscular and abdominal types.

DISCUSSION

Studies conducted in different regions of our country are evident of a downward trend in muscle strength in modern children and teenagers. It has been established that in the Moscow region both boys and girls have worse dynamometry scores in comparison with the children tested in the 1960s and 1980s, and these differences are significant (p < 0.01) [15]. Low values of parameters reflecting the functional capacity of children mean that these children may not be able to meet the GTO requirements, risking their health or even life when attempting to pass this fitness test.

Among endogenous factors affecting muscle strength are the rate of biological maturation and body build [18–20]. Our findings demonstrate that decelerated rates of biological development and asthenic builds negatively affect muscle strength in teenage boys. In our study, average values of muscle strength in teenagers retarded in their physical development at the age of 13 to 15 were significantly lower than in other groups. At the same time, those boys had caught up with their peers in terms of muscle strength by the age

of 17. Dynamometric measurements in boys with the asthenic somatotype demonstrated significantly lower values than in those with the muscular and abdominal types.

While analyzing the influence of other endogenous factors on muscle strength in teenagers and children, we found out that (im)balanced physical development and height (stature) also affect the studied parameter. Boys with weight deficiency and shorter than average height in all age groups scored less than others in terms of muscle strength. In our sample there were no really short boys but we assume they also have reduced functional abilities.

Research studies suggest that about 40 % of all high-school children nowadays may not be able to pass the GTO fitness test [21–25], which brings the need for improving physical education at schools. Based on our findings, we can identify a group at a risk of reduced functional abilities. This group includes boys of asthenic body type, those with weight deficiency, short height and also teenagers retarded in their biological development at puberty. Teenagers at risk should receive special attention during PE classes at school and in the run up for GTO.

CONCLUSIONS

The conducted study has detected a negative effect of a few endogenous factors on the muscle strength of teenage boys, including retarded biological development, weight deficiency, short height, and the asthenic build. Muscle strength is also affected by high fat and low skeleton masses.

The obtained results have allowed us to identify a group at a risk of reduced functional capacities and to propose practical recommendations aimed at facilitating normal physical development of schoolchildren, that can be used by medical workers, teachers, parents and children themselves.

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WORKPLACE HYGIENE IN CORRECTIONAL FACILITIES: PROBLEMS AND SOLUTIONS

Polunina NV[1], Timerzyanov MI[2], Milushkina OYu[2], Pivovarov YuP[2], Polunin VS[1], Al Sabunchi AA[2], Kozyreva FU[2] ✉, Korolik VV[2]

[1] Department of Public Health, Healthcare and Health Economics, Faculty of Pediatrics, Pirogov Russian National Research Medical University, Moscow, Russia

[2] Department of Hygiene, Faculty of Pediatrics, Pirogov Russian National Research Medical University, Moscow, Russia

The state takes the responsibility of protecting the life, health and working ability of inmates of penitentiary institutions. This study aimed to explore working conditions at a correctional facility located in Tatarstan. Among the most significant workplace hazards were high noise and vibration levels, poor lighting, exposure to increased concentrations of harmful substances in the air, physical distress, constrained posture, sensory stress, and monotonous work. Health evaluation of 5,009 incarcerated individuals exposed to poor working conditions revealed that they were more likely to develop work-related diseases than their counterparts who worked in the office. Among the former skin and subcutaneous tissue diseases, hearing impairment, respiratory conditions and cardiovascular disorders were 2.1, 1.7, 1.5 and 1.3 times more frequent, respectively. Our study revealed the lack of medical examinations on admission, as well as regular medical checkups, and the reluctance of the inmates to use personal protection at work. Based on the study results, adequate measures were taken to improve working conditions, raise awareness of hygiene problems among the inmates and initiate routine medical checkups. The number of incarcerated individuals working under bad conditions plunged from 68 % to 19 %. Also, up to 82 % of inmates started to use personal protection.

Keywords: inmate, safety at work, working conditions, work environment, hard labor, work intensity, personal protection

✉ **Correspondence should be addressed:** Kozyreva Fatima
ul. Ostrovityanova, d. 1, Moscow, Russia, 117997; kf61@mail.ru

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ГИГИЕНИЧЕСКАЯ ОЦЕНКА УСЛОВИЙ ТРУДА В УЧРЕЖДЕНИЯХ ИСПРАВИТЕЛЬНОЙ СИСТЕМЫ: ПРОБЛЕМЫ И ПУТИ РЕШЕНИЯ

Н. В. Полунина[1], М. И. Тимерзянов[2], О. Ю. Милушкина[2], Ю. П. Пивоваров[2], В. С. Полунин[1], А. А. Аль Сабунчи[2], Ф. У. Козырева[2] ✉, В. В. Королик[2]

[1] Кафедра общественного здоровья и здравоохранения, экономики здравоохранения, педиатрический факультет, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Кафедра гигиены, педиатрический факультет, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

Государство берет на себя обязанность сохранить жизнь, здоровье и трудоспособность осужденных к отбытию наказания в учреждениях пенитенциарной системы. Целью исследования являлось изучение условий труда заключенных одного из исправительных учреждений в Республике Татарстан. К наиболее значимым вредным производственным факторам на рабочих местах по результатам их обследования были отнесены повышенный уровень шума, недостаточный уровень искусственной освещенности производственных помещений, повышенный уровень общей и локальной вибрации, превышение предельно допустимых концентраций вредных веществ в воздухе рабочей зоны, а также физические перегрузки, вынужденная рабочая поза, сенсорные нагрузки и монотонность работы. Анализ заболеваемости 5 009 осужденных, работавших во вредных условиях труда, показал, что среди них чаще в сравнении с лицами, работавшими в офисных помещениях, регистрировали заболевания, обусловленные неблагоприятными условиями труда, в том числе болезни кожи и подкожной клетчатки — в 2,1 раза, нарушения слуха — в 1,7 раза, болезни органов дыхания — в 1,5 раза, заболевания системы кровообращения — в 1,3 раза. Исследование выявило отсутствие предварительных и периодических медицинских осмотров, а также нежелание осужденных использовать средства индивидуальной защиты. По результатам исследования были проведены мероприятия по улучшению условий труда, была налажена санитарно-просветительная работа, внедрена система медицинских осмотров. Число работающих во вредных условиях труда снизилось с 68 % до 19 %. Средства индивидуальной защиты стали применять до 82 % заключенных.

Ключевые слова: заключенные, безопасность рабочих мест, условия труда, факторы рабочей среды, тяжесть труда, напряженность труда, средства индивидуальной защиты

✉ **Для корреспонденции:** Козырева Фатима Увжикоевна
ул. Островитянова, д. 1, г. Москва, 117997; kf61@mail.ru

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Occupational health and safety is still a concern faced by the members of some social groups, including inmates of correctional facilities (CF).

Labor is believed to be beneficial for physical and mental health in closed communities; it promotes strong bonding, encourages team spirit and respect for human dignity, and facilitates re-socialization. The penitentiary system gives inmates an opportunity not to lose their professional skills and learn a new profession that may aid further re-integration into the society. Through work inmates partially reimburse the expenses for their upkeep, pay fines imposed by court decisions, earn some pocket money and save up for the time when they will be released.

Working conditions for those serving sentences should be created taking into account the state of their health, work capacity, experience, availability of work skills and profession.

Working hours, health and safety requirements, sanitation and hygiene norms are established by the labor legislation of the Russian Federation. Labor protection is a system of preserving the life and health of workers in the process of work, including legal, socio-economic, organizational and technical, sanitary and hygienic, rehabilitation and other measures. Provision of acceptable working conditions will help to preserve the health of working convicts [1–3].

The aim of the study was to investigate the working conditions of those serving sentences in correctional institutions and to develop measures to optimize the labor process for preserving the health of convicts.

METHODS

The study was conducted in one of the penitentiaries of the Republic of Tatarstan. At the correctional facility, production enterprises have been set up, including foundry, woodworking, metalworking, slag-blocking, sewing industries and auto services, employing up to a third of all convicts. The assessment of sanitary and hygienic conditions of labor of the affected persons was carried out by carrying out laboratory-instrumental studies of physical factors in the workplace, determining the concentration of harmful substances in the air of the work area, studying the severity and intensity of the work process, and the safety of workplaces and the provision of prisoners with personal protective equipment in accordance with the guidance R 2.2.2006-05 *Guidance on hygienic assessment of working environment factors and labor process. Criteria and classification of working conditions* (Table 1).

Particular attention was paid to the study of the role of harmful production factors, the impact of which on the worker under certain conditions leads to illness or disability.

Assessment of the state of health was carried out on the basis of an analysis of data on the incidence of 5,009 working convicts obtained from the analysis of registration form No. 025-10/y-11. The comparison group included office workers (information and computing center, marketing department, logistics department, technical control department, technical department, a group of economists and accountants).

Individual protective equipment (IPE) plays an important role in the system of preventive measures aimed to ensure safe working conditions and to reduce occupational poisoning and diseases. To learn why IPE are ignored in the prison, we surveyed 5,009 inmates.

RESULTS

Noise sources in the conditions of foundry, blacksmith, metalworking and woodworking industries are working machines, manual power tools, electric machines, compressors, forging and pressing, handling and auxiliary equipment. The effect of high noise levels leads to a decrease in efficiency, development of fatigue, increase in morbidity and disability among workers [4]. Table 2 shows noise levels measured in the workshops of the correctional facility.

The table shows that the actual levels of production noise at the workstations of the spindle and forge areas exceeded the maximum permissible meanings. Unstable production noise in the workstations surveyed had a fluctuating character, with a continuous change in the sound level over time. The impulse noise was characteristic for the spindle and forging sections. The value of the equivalent noise level (in terms of the duration of the work shift) was calculated to estimate the possible harmful effect of noise of different levels and duration. The obtained data made it possible to classify the working conditions according to the level of effect of industrial noise on the spindle and forge areas to the harmful conditions of the 2nd degree (class 3.2).

Metalworking, woodworking machines, casting machines, press-forging equipment, transport are sources of general vibration. That is why the majority of working places, with the exception of places on forging and transport sites, were classified as places with harmful working conditions (class 3.1) of "general vibration" factor. Transport department workers are also exposed to local vibrations. The corrected acceleration of local vibration here was 118.3 ± 7.2 dB, which is acceptable (Class 2).

Inmates working in sewing workshops were exposed to the harmful effects of local vibrations produced by sewing machines. The acceleration of vibration was 134 ± 0.1 dB, exceeding the occupational standard of 126 dB; therefore, working conditions here were assigned to Class 3.2. High frequency vibrations of 30–125 Hz cause vascular, neural, muscular, bone and joint pathologies. The source of the general vibration in the sewing section is the engines, which most machines fasten directly to

Table 1. Assessment of labor conditions in the state penitentiary. Measurements taken

Measurement	Number of measurements/workplaces
Class of working conditions	296 workplaces
Noise levels	296 measurements
Vibration levels	204 measurements
Microclimate (in cold and warm seasons)	1, 776 measurements
Lighting	623 measurements
Air contamination	223 samples
Physical effort	2, 368 measurements
Stress	2, 368 measurements

the table top and do not have damping pads. Vibration is then transferred to the table top and machine body. The value of vibration increases with wear and malfunction of machines [5].

Hygienic assessment of production facilities showed that the total artificial illumination is significantly lower than the established norms at workplaces of turners, milling machines, in the area of processing colored castings; turners and thread-rollers of the assembly area of hulls and covers (Table 3). Insufficient lighting causes the development of eye fatigue, decreases work capacity and labor productivity, increases the number of defects and the danger of occupational traumatism [6].

As can be seen from the table 3, the total artificial illumination at the workplace of the machine operators is not sufficient, that's why working conditions for the "lighting" factor can't be recognized as acceptable. The lighting conditions on the mechanical section, the area of processing colored castings, the assembly of housings and covers, the spindle and forging areas belong to class 3.2, that means that they can cause persistent functional changes in the organs of vision. It was revealed the need to install additional lighting in general system of artificial lighting, replacement of lamps with more powerful ones, and installation of local lighting for machine operators.

Table 2. Industrial nose levels at production areas

Work areas	M ± SD. dB(a)	EL. dB(a)
Comparison group (office workers)	56.8 ± 8.4	60
Painting plot	66.0 ± 0.0	80
Mechanical processing area	78.6 ± 0.0	80
Nonferrous casting area	76.0 ± 0.0	80
Section for the assembly of housings and covers	64.7 ± 7.2	80
Spindle section	82.6 ± 12.0	80
Lock section	69.9 ± 14.9	80
Tool area	61.4 ± 13.6	80
Forging site	88.4 ± 0.0	80
Mechanical repair area	67.9 ± 13.7	80
Power-repair-mechanical section	58.2 ± 3.24	80
Transport area	67.1 ± 7.87	80
Railway section	60.6 ± 9.99	80
Oxygen substation	48.0 ± 0.0	80
Woodworking area	72.8 ± 11.0	80
Mounting area	65.0 ± 8.6	80
Sewing area	66.1 ± 4.77	80
Production-duty department (elimination of accidents)	64.2 ± 6.85	80

Note. EL — exposure limit

Table 3. Lighting in work areas

Work areas	M ± SD, lx	Minimum acceptable level, lx
Comparison group (office workers)	300.2 ± 128.5	300
Painting plot	204.7 ± 5.0	300
Mechanical processing area	186.2 ± 32.7	200
Nonferrous casting area	236.3 ± 20.5	200
Section for the assembly of housings and covers	148.2 ± 76.4	200
Spindle section	230.3 ± 43.6	200
Lock section	195.6 ± 75.4	200
Tool area	207.9 ± 99.1	200
Forging site	250.0 ± 0.0	200
Mechanical repair area	178.8 ± 111.5	200
Power-repair-mechanical section	248.6 ± 35.5	200
Transport area	145.0 ± 119.2	200
Railway section	101.5 ± 14.7	200
Oxygen substation	75.5 ± 0.71	200
Woodworking area	160.8 ± 15.5	200
Mounting area	244.7 ± 38.2	200
Sewing area	279.5 ± 130.0	400
Production-duty department (elimination of accidents)	123.0 ± 33.5	200

Assessment of air pollution in the working area showed that there was the dust in the air of the working area with an admixture of silicon dioxide in a volume of 2–10 %. Among the aerosols of predominantly fibrogenic action, the largest danger is dust containing free silicon dioxide [7]. The maximum permissible concentration (MPC) of such dust, depending on the content of silicon dioxide is 1 and 2 mg/m^3. For other types of dust, MPC is 2–10 mg/m^3. In our study, the proportion of samples with excess of hygienic standards was 84.4 %. Dust pathology can be manifested in the form of catarrh of the upper respiratory tract, dust bronchitis and pneumonia [8].

The share of samples with excess of MPC of mineral oils is 25.4 %. Lubricating oils, when inhaled, can irritate the mucous membranes of the upper respiratory tract. On the skin of workers may develop oily folliculitis and oily acne [2].

Gasoline fumes were detected in the air of the transport area. On average, their concentrations did not exceed occupational standards per shift. However, the share of non-standard samples was 33.3 %. The content of products of incomplete combustion of fuel did not exceed the maximum permissible values in samples of air in the breathing zone of workers in the transport section of shop No. 5. The studies were carried out taking into account the effect of summation.

The concentration of benzene, manganese in welding aerosols, lead-cadmium solder, acetone, white spirit, carbon monoxide, chlorine did not exceed the established standard values (according to the analysis of industrial air samples). In most cases working conditions could be assigned to Class 1 (third degree). Hygienic assessment of working conditions of convicts by chemical factor in office premises showed their compliance with class 2, that is, working conditions were acceptable.

Hygienic assessment of the microclimate of industrial premises showed that the air temperature in the workplace was within the acceptable range (Table 4). Relative humidity of air fluctuated in a range of 60–75 % with the speed of air movement from 0,1 to 0,3 m/s. Thus, according to the main parameters of the microclimate, working conditions were characterized as admissible (class 2).

The hygienic assessment of the working conditions of the convicts showed that the class of working conditions in all production facilities was harmful (Class 3.1-3.2, 1st to 2nd degree). In terms of stress, working conditions were either acceptable or harmful (Table 5).

The study showed that the majority (73.1 %) of the inmates exposed to harmful or dangerous factors did not use personal

Table 4. Air temperature in work areas

Work areas	Category of task depending on energy expenditure	Air temperature, C° (M ± SD)	
		Cold seasons	Warm seasons
Comparison group (office workers)	1b	23.4 ± 2.9	23.3 ± 2.8
Painting plot	2b	23.5 ± 0.5	23.5 ± 1.2
Mechanical processing area	2a	20.9 ± 0.2	20.3 ± 1.6
Nonferrous casting area	2b	24.9 ± 1.1	20.4 ± 0.8
Section for the assembly of housings and covers	2a	21.1 ± 0.5	21.0 ± 0.5
Spindle section	2a	23.0 ± 0.6	21.5 ± 1.1
Lock section	2a	20.2 ± 1.3	19.9 ± 1.4
Tool area	2a	20.3 ± 1.5	20.0 ± 1.1
Forging site	2b	24.8 ± 0.0	22.6 ± 0.0
Mechanical repair area	2b	20.0 ± 1.5	19.6 ± 1.5
Power-repair-mechanical section	2b	20.6 ± 0.7	20.3 ± 0.8
Transport area	2a	22.4 ± 0.9	21.0 ± 2.7
Railway section	2a	21.9 ± 0.7	16.7 ± 5.3
Oxygen substation	2a	22.5 ± 0.6	21.8 ± 0.2
Woodworking area	2b	22.1 ± 0.6	21.7 ± 2.2
Mounting area	2a	22.6 ± 0.1	22.1 ± 0.3
Sewing area	2a	24.3 ± 1.2	22.8 ± 1.9
Production-duty department (elimination of accidents)	2a	19.7 ± 1.6	17.2 ± 5.7

Table 5. Work classes depending on the physical effort required by and stress induced

Work type	Class of working conditions	Stress class
Sewing manufacture, seamstresses	3.2	3.2
Sewing manufacture, cutters	3.1	2
Foundry	3.2	3.1
Production of woodworking	3.1	3.1
Manufacture of metal machining	3.2	3.1
Construction industry	3.2	3.1
Painting production	3.2	2
Transport area	3.2	3.2
Production duty department (elimination of accidents)	3.1	3.1

protection equipment (PPE). The survey of persons who did not use PPE showed that 54.9 % of them do not know the means of individual protection; 47.6 % of those surveyed believed that their use made work difficult; 44.9 % noted the inconvenience of their use; 39.7 % did not know how to apply them; 25.9 % did not associate their health with work in harmful conditions; 17.1 % said they did not consider it necessary to use PPE. On average, every inmate provided 2 or 3 arguments against the use of personal protection.

Working conditions seriously affect workers' health [2, 9–14]. Our analysis revealed that in the inmates exposed to occupational hazards, morbidity rates were significantly higher than in those unexposed (1,267.2 ‰ vs 810.6 ‰, $p < 0.05$). Among the most common conditions were skin or subcutaneous tissue diseases (2.1. times more common), hearing impairment (1.7 times more common), respiratory diseases (1.5 times more common), cardiovascular diseases (1.3 times more common). It should be noted that the absence of medical examinations on admission and before working shifts, as well as regular medical checkups, prevented us from identifying those individuals who should not have been allowed to work in the harmful working conditions.

DISCUSSION

The study of working conditions in production facilities where convicts work allowed to identify violations of sanitary and hygienic requirements at individual workplaces in terms of noise level, vibration, illumination level, microclimatic parameters and chemical air pollution in the work area. The fact of evasion by working convicts from the use of PPE is established, which subsequently leads to an increase in the incidence among them. The use of personal protective equipment becomes necessary in cases where there are difficulties in ensuring the safety of technological processes and also in conditions of contact with factors harmful to health. Upon conducting a study, we proposed a number of measures for optimizing working conditions in the correctional facility aimed at reducing noise levels and total/local vibration and improving lighting conditions. These measures have been implemented. We also attempted to educate the inmates on the benefits of personal protection equipment and taught them how to use it. Based on the results of our study, preliminary and periodic medical examinations of convicts have been resumed before admission to work.

The study has also shown that the sanitary and hygienic conditions at the workplace have improved for the majority of convicts. The number of working in hazardous working conditions decreased from 68 % to 19 % (classes 3.1–3.2). During preliminary medical examinations 3.9 % persons who had a contraindication to work. During periodic medical examinations, 12.6 % of convicts were dismissed from work for health reasons, while performing medical examinations directly before the change — 10.2 % of convicts. The proportion of individuals using personal protection equipment is now 82 %.

CONCLUSIONS

The work of convicts takes place in certain production conditions, which can affect their health and work capacity, if hygiene requirements are not observed. Based on the results of our study, we have proposed and implemented measures aimed to eliminate occupational hazards, including optimization of manufacturing processes, automation, installation of modern equipment, reduction of the amount of manual labor, all of which have proved to be incredibly effective in a very short time.

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DEVELOPING AN ARTIFICIAL INTELLIGENCE-BASED SYSTEM FOR MEDICAL PREDICTION

Sakhibgareeva MV ✉, Zaozersky AYu

COMTEK LLC, Ufa, Russia

Diagnostic accuracy remains one of the central problems of medical care. In this work we attempt to apply artificial intelligence to solve this challenge. We propose an approach to medical prediction based on the intelligent analysis of patients' data from 200 different laboratory tests. The initial sample included 7, 918 cases falling into 4 nosological categories: D50 (iron deficiency anemia), E11 (non-insulin-dependent diabetes mellitus), E74 (other disorders of carbohydrate metabolism), and E78 (disorders of lipoprotein metabolism and other lipidemias), and was further divided into the training and testing datasets. Using gradient boosting, we constructed a machine learning model. The model demonstrated a recognition rate of 89 % (AUC-ROC) and a mean certainty in the diagnosis of 92 %. Our study proves feasibility of using machine learning in the analysis of this type of medical data. We are currently implementing a web-service for medical prediction as part of our *Healthcare* platform aiming at automation of clinical practice.

Keywords: artificial intelligence, analysis of medical data, machine learning, gradient boosting, laboratory diagnostics, nosological diagnosis, multiclass classification, iron deficiency anemia, lipidemia, carbohydrate metabolism disorders

✉ **Correspondence should be addressed:** Margarita Sakhibgareeva
ul. Bekhtereva 16, kv. 48, Ufa, Russia, 450047; margarita.vl2011@gmail.com

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РАЗРАБОТКА СИСТЕМЫ ПРОГНОЗИРОВАНИЯ ДИАГНОЗОВ ЗАБОЛЕВАНИЙ НА ОСНОВЕ ИСКУССТВЕННОГО ИНТЕЛЛЕКТА

М. В. Сахибгареева ✉, А. Ю. Заозерский

ООО «КОМТЕК», Уфа

В статье представлены результаты исследования по применению технологий искусственного интеллекта для решения одной из основных проблем здравоохранения — повышения качества диагностики заболеваний. Предложен подход к прогнозированию нозологических диагнозов путем интеллектуального анализа совокупности результатов лабораторных исследований (200 тестов), проводимых по каждому случаю заболевания пациентов. В общую выборку, разделенную впоследствии на обучающую и тестовую, включили данные о 7 918 случаях заболеваний по 4 нозологиям: D50 (железодефицитная анемия), E11 (инсулиннезависимый сахарный диабет), E74 (другие нарушения обмена углеводов), E78 (нарушения обмена липопротеидов и другие липидемии). Методом градиентного бустинга для них была построена модель машинного обучения. Точность распознавания моделью выбранных диагнозов составила более 89 % (ROC AUC) при средней уверенности модели в каждом прогнозируемом диагнозе в 92 %. Исследование показало принципиальную возможность применения методов машинного обучения для анализа данных такого рода. Система прогнозирования диагнозов заболеваний внедряется в виде веб-сервиса в программный комплекс «Здравоохранение», предназначенный для автоматизации работы медицинских учреждений.

Ключевые слова: искусственный интеллект, анализ медицинских данных, машинное обучение, градиентный бустинг, лабораторная диагностика, нозологический диагноз, многоклассовая классификация, железодефицитная анемия, липидемия, нарушения обмена углеводов

✉ **Для корреспонденции:** Сахибгареева Маргарита Владимировна
ул. Бехтерева, д. 16, кв. 48, г. Уфа, 450047; margarita.vl2011@gmail.com

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Development of information technologies aimed to facilitate the efficient delivery of medical care is one of the priority goals set for the Russian healthcare system. Increasing effort is being made to improve the quality of healthcare through the use of information systems, expedite transition from paper files to electronic medical records and employ data mining for the analysis of huge arrays of medical data [1, 2].

Collection of medical data still presents a problem, as noted in a number of works [3, 4], which seriously impedes their digitalization necessary for machine learning and delays development of analytical software. Our close collaboration with the Siberian Center for Information Protection and deployment of the original *Zdravookhranenie* software in a few regional medical centers allowed us to build a vast database of medical records and obtain authorization to process these data. It was a perfect opportunity to perform data mining using machine learning techniques.

The use of diagnostic information systems in clinical practice can be very beneficial for patients. High workload or the lack of expertise affects clinical decisions doctors make. Besides, a taking into account of a set of information about the patient is a basis for accurate diagnosis, prediction of disease progression and treatment planning; without it clinical decisions are mere approximations [5].

According to A. Chuchalin's report presented at the Second National Congress of GPs, every third case in Russia is

misdiagnosed [6]. Likewise, we have discovered a considerable number of diagnostic errors while analyzing the records of a few healthcare facilities that use our software. In the course of our analysis, we calculated the discrepancies between the definitive and preliminary diagnoses. Results are presented in Table 1 which features distribution of erroneous diagnoses across different departments of healthcare facilities and Table 2 showing the percentage of erroneous diagnoses in different nosological categories. Names of the healthcare facilities are not provided in this article for ethical reasons.

Not only patients becomes victims of wrong preliminary diagnoses and get useless treatments but also medical clinics incur considerable expenses: the Fund of Compulsory Health Insurance only subsidizes treatments based on a definitive diagnosis.

In view of this, we decided that prediction of nosological diagnosis should be a priority task in the development of an artificial intelligence-based system. The aim of this work was to test the feasibility of medical data mining using machine learning, to assess prediction accuracy that makes a machine learning model useful, and to enhance our *Zdravookhranenie* platform.

METHODS

Initial dataset

Medical decisions can be based on a medical history, physical examinations, and results of laboratory or complex functional tests. Lab tests provide the most objective information about patient's condition and are often used when other methods have failed to identify or confirm a pathology. These tests are especially useful in patients with anemia, lipidemia, hepatitis, seropositive rheumatoid arthritis, etc.

The source dataset consisted of disease cases with established definitive diagnoses. The feature space included patients' sex and age and the results of laboratory tests obtained from the data of prophylactic medical examination. The data were collected using our *Zdravookhranenie* software solution [7]. We chose 4 nosologies for the analysis, including D50, E11, E74, and E78, that can be suspected and diagnosed based on laboratory tests. The initial dataset was as follows:

- iron deficiency anemia (D50) — 778 cases (10 %);
- non-insulin-dependent diabetes mellitus (E11) — 1,392 cases (17 %);
- other disorders of carbohydrate metabolism (E74) — 163 cases (2 %);
- disorders of lipoprotein metabolism and other lipidemias (E78) — 5,585 cases (71 %).

In total, the dataset included 7,918 cases with results of 200 laboratory tests (blood and urine tests, cytologic examinations, etc.) that occurred during the period from 2005 to 2017 with patients aged 18 to 99 years, of whom 71 % of were females and 29 % were males. In some cases, the results of laboratory tests were recorded as "normal", "below the norm" and "above the norm".

Choosing a method of machine learning and performance metrics

Prediction of diagnosis based on the results of laboratory tests is a multiclass classification problem.

The data were analyzed using Scikit-learn [8], a Python-based open-source library for machine learning. We carried out a few preliminary tests involving such methods of machine learning as neuronal networks, decision trees, and gradient boosting. The last one showed the best results for our problem. It is a technique in which an ensemble of predictors is built sequentially, with every subsequent algorithm compensating for the mistakes of a previous predictor [9]. Gradient boosting over decision trees is believed to be the most effective universal method of machine learning. Decision trees also perform very well in classification tasks.

Considering the specifics of the problem and the fact that the initial dataset was imbalanced, we selected performance metrics with special care. The metrics will be described below in terms of a confusion matrix [9-10] with respect to multiclass classification using the one-against-all approach. This approach is based on reducing multidimensional classifications to a set of binary tasks in which a picked class is classified as 1, and the rest classes are classified as 0. For every picked class i the following parameters are determined:

- TP (True Positive) — the number of true positive instances correctly assigned to class i;
- TN (True Negative) — the number of true negatives instances correctly not assigned to class i and therefore assigned to class $j \neq i$;
- FP (False Positive) — the number of false positives instances incorrectly assigned to class i;
- FN (False Negative) — the number of false negatives instances incorrectly assigned to class $j \neq i$ that should have been assigned to class i.

Accuracy is the most intuitive performance metric showing a fraction of correct responses; however, is not suitable for imbalanced datasets.

Table 1. Percentage of wrong diagnoses in different units of several healthcare agencies based in Russia

Unit	Percentage of wrong diagnoses. %		
	Healthcare agency 1	Healthcare agency 2	Healthcare agency 3
Pulmonary	76.80	39.28	–
Anaesthetics and Intensive care	72.96	24.11	73.11
Cardiac care (>1)	57.88	23.00	46.43
Therapeutic	56.36	–	–
Gastroenterology	66.38	11.29	–
Trauma	32.19	–	60.64
Neurology	55.04	14.97	–
Urology	–	–	67.72

Table 2. Percentage of wrong diagnosis per nosological category in several Russia-based healthcare agencies

Nosology	Percentage of wrong diagnoses,%
Disorders of lipoprotein metabolism and other lipidemias	92.73
Cholera	88.89
Disorders of sphingolipid metabolism and other lipid storage disorders	88.72
Immunodeficiency with predominantly antibody defects	83.33
Sequelae of other and unspecified infectious and parasitic diseases	80.00
Evidence of alcohol involvement determined by blood alcohol level	80.00
Juvenile arthritis in diseases classified elsewhere	75.00
Other bacterial diseases, not elsewhere classified	66.67
Car occupant injured in collision with pedal cycle	66.67
Lactose intolerance	60.00
Pericarditis in diseases classified elsewhere	60.00
Trichomoniasis	50.00
Other intestinal helminthiases, not elsewhere classified	50.00
Viral agents as the cause of diseases classified elsewhere	50.00
Malignant neoplasms of lip	50.00
Carcinoma in situ of cervix uteri	50.00
Deficiency of other nutrient elements	50.00
Other diseases of inner ear	50.00
Intestinal malabsorption	50.00
Hypertrichosis	50.00
Other disorders of kidney and ureter in diseases classified elsewhere	50.00
Pre-existing hypertension with pre-eclampsia	50.00
Epidermolysis bullosa	50.00
Unspecified jaundice	50.00
Anomaly of leukocytes, not elsewhere classified	50.00
Glycosuria	50.00
Other and unspecified abnormal findings in urine	50.00
Other disorders of carbohydrate metabolism	20.70
Iron deficiency anemia	13.90
Non-insulin-dependent diabetes mellitus	3.240

$$accuracy = \frac{TP + TN}{TP + TN + FP + FN}.$$

Therefore, other metrics are often used instead, including:

• precision — a fraction of true positives instances among all predicted positives. In other words, it shows how many positive predictions were really positive:

$$precision = \frac{TP}{TP + FP};$$

• recall — a fraction of true negatives instances among all true and false positives. It is also known as a true positive rate (TPR):

$$recall = \frac{TP}{TP + FN}.$$

Recall is used to evaluate performance of a machine learning model when there is a need to reduce the number of false negatives (FN) and measure all positives [10]. This metric is preferred for medical diagnostic tasks when it is important not to miss a diagnosis. Although it is quite intuitive, it is not always good for imbalanced datasets.

Another metric used in our study was ROC AUC recommended in [10] for the evaluation of model performance on imbalanced datasets. AUC stands for area under [ROC] curve, ROC is receiver operating characteristic. This curve is constructed by plotting the true positive rate (TPR) against the false positive rate (FPR) and is a line connecting (0, 0) to (1,1):

$$TPR = \frac{TP}{TP + FN},$$

$$FPR = \frac{FP}{FP + TN}.$$

It is believed that the higher the ROC AUC value, the better the performance of the classifier. ROC AUC of 0.5 means the classifier makes random guesses. ROC AUC below 0.5 means that the classifier does the opposite of what is expected of it: if true positives were labeled as negatives, it would perform better.

Considering the above said, we used ROC AUC as a primary metric, but also accounted for recall.

Table 3. Performance of the machine learning model designed for diagnostic prediction

Diagnosis	Metric				Dimension of the test set, number of cases
	ROC AUC	Recall	Precision	Accuracy	
D50 (iron deficiency anemia)	0.98	0.66	0.83	0.95	44
E11 (non-insulin-dependent diabetes mellitus)	0.91	0.62	0.69	0.9	69
E74 (other disorders of carbohydrate metabolism)	0.89	0.21	0.6	0.97	14
E78 (disorders of lipoprotein metabolism and other lipidemias)	0.94	0.96	0.89	0.89	318

RESULTS

The diagnoses and the results of laboratory tests were divided into two sets: the training set (75 % of cases) and the test set (25 % of cases). The model was built for 4 nosological categories (D50, E11, E74, E78) using gradient boosting. For the test set ROC AUC was above 89 % (Table 3). Mean certainty in correct diagnoses included in a test sample was 92 %.

DISCUSSION

High ROC AUC values falling between 89 % and 98 % indicate that our model is feasible for the prediction of the studied diagnoses. Importantly, our dataset consisted of various data types, including the results of 200 different laboratory tests and such parameters as patients' sex and age. Among other strengths of the study is the use of enough large dataset accumulated over the course of a few years. For example, in [11] the analysis was carried out on the data collected over the period of just 3 months in a Boston hospital. The authors of the study attempted to predict ferritin blood levels. They also used ROC AUC as quality metric which turned to be as high as 97%. However, it should be noted that according to a number of research works [12–14] a focus on nosological categories may increase prediction accuracy. According to [15, 16], performance can be enhanced through the use of different methods for medical data preprocessing.

CONCLUSIONS

Our study has proved the feasibility of machine learning techniques for the analysis of our medical records. Currently, we are incorporating this model into our *Zdravookhranenie* software. We are working on a web service which will accumulate and analyze the results of all laboratory tests specified in a patient's medical history. The web service will "report" to the *Zdravookhranenie* platform the results of the analysis and returning the most probable diagnoses that a doctor may take into for appointment of a treatment regimen.

We are planning to include more nosologies into our model and improve its quality by designing separate models for each diagnosis. These models will account for the laboratory tests that affect the prediction outcome the most. Thus, we will be able to start developing a tool that can recommend the most relevant lab tests for the diagnosis of a particular condition.

We hope that our work will expedite transition to personal medicine [17, 18] based on the analysis of patient's unique medical records not limited to the results of the laboratory tests. This task can be solved using artificial intelligence for diagnostic prediction and generating personalized treatment recommendations. This will help to reduce the number of medical errors and increase clinical significance of prevention measures by monitoring patient's records.

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A FEW ASPECTS OF PLASTIC SURGEONS' PERFORMANCE

Manturova NE[1], Kochubey VV[2] ✉, Kochubey AV[3]

[1] Department of Plastic and Reconstructive Surgery, Cosmetology and Cell Technologies, Faculty of Continuous Professional Education, Pirogov Russian National Research Medical University, Moscow, Russia

[2] Department of Surgery No.1, Faculty of General Medicine, Yevdokimov Moscow State University of Medicine and Dentistry, Moscow, Russia

[3] Department of Public Health and Healthcare, Institute of Continuous Professional Education of the Federal Medical and Biological Agency, Moscow, Russia

In spite of accreditation programs, levels of professional skills vary among plastic surgeons: there are no requirements for the diversity and number of performed surgical interventions that a surgeon can specify in his/her portfolio. Rationale for elaborating such requirements can be explored by studying service reports of private medical practices certified to provide plastic surgery services to their in- and outpatients. In the course of out study we analyzed such reports using different statistical tools, including the variation coefficient, the Kolmogorov–Smironov, Mann–Whitney U and Kruskal–Wallis tests, and Spearman's correlation coefficient. Differences were considered statistically significant at $p < 0.05$. Surgical interventions were divided into 9 categories: skin/soft tissue plasty, rhinoplasty, breast plasty, blepharoplasty, otoplasty, lip and palate repair, craniofacial plasty, repair of urogenital defects, and hand surgery. On average, each surgeon performed a total of 112.3 ± 326.4 surgeries (Mo = 1). About 30.4 % of surgeons performed 1 to 10 interventions a year. None of the surgeons performed all types of interventions and hand surgery. We found that the diversity and number of interventions performed by a surgeon does not depend on the qualification or academic title ($r_S = –0.8$, $p = 0.2$ and $r_S = –0.2$, $p = 0.8$, respectively). Skin/soft tissue repair accounted for 51.1 % of all services provided by private medical practices. The number of post-operative treatment services was 0.017 per surgery.

Keywords: plastic surgery, plastic surgeon portfolio, plastic surgeon accreditation, continuous medical education

✉ **Correspondence should be addressed:** Valentin Kochubey
ul. Delegatskaya, d. 20/1, Moscow, Russia, 127473; kochoubey@gmail.com

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ХАРАКТЕРИСТИКИ ДЕЯТЕЛЬНОСТИ ПЛАСТИЧЕСКИХ ХИРУРГОВ

Н. Е. Мантурова[1], В. В. Кочубей[2] ✉, А. В. Кочубей[3]

[1] Кафедра пластической, реконструктивной хирургии, косметологии и клеточных технологий, факультет дополнительного профессионального образования, Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Кафедра факультетской хирургии № 1, лечебный факультет, Московский государственный медико-стоматологический университет имени А. И. Евдокимова, Москва

[3] Кафедра общественного здоровья и здравоохранения, Институт повышения квалификации Федерального медико-биологического агентства, Москва

Вводимая периодическая аккредитация не гарантирует поддержание одинаково высокого уровня квалификации врачей-хирургов, так как утвержденный формат портфолио не содержит требований к спектру и объему оперативных вмешательств. Целесообразность введения подобных требований можно обосновать, изучая деятельность пластических хирургов по сведениям отчетов о медицинских услугах по пластической хирургии, оказанных в медицинской организации частной системы здравоохранения, имеющей лицензию на выполнение работ и услуг по пластической хирургии в амбулаторных и стационарных условиях. В ходе анализа отчетов был проведен расчет коэффициента вариации, критериев Колмогорова–Смирнова, Манна–Уитни, Краскела–Уоллиса, коэффициента Спирмена. Статистически значимыми считали значения при $p < 0,05$. Оперативные вмешательства были разделены по 9 трудовым функциям: пластика покровных тканей; пластика носа; молочных желез (груди); век; наружного уха; губ и неба; краниофациальная пластика, урогенитальная пластика, хирургия кисти. Средний объем оперативных вмешательств по профилю составил 112,3 ± 326,4 на одного врача при Mo = 1. Причем 30,4 % хирургов выполнили за год 1–10 оперативных вмешательств. Ни один хирург не выполнял оперативные вмешательства по всем 9 трудовым функциям, а также по хирургии кисти. Спектр и объем оперативных вмешательств не зависит от категории или ученой степени ($r_S = –0,8$, $p = 0,2$ и $r_S = –0,2$, $p = 0,8$). Пластика покровных тканей составляет 51,1 % всех оказанных услуг. На одно оперативное вмешательство приходится 0,017 услуг по послеоперационному ведению.

Ключевые слова: пластическая хирургия, портфолио пластического хирурга, аккредитация пластического хирурга, непрерывное медицинское образование

✉ **Для корреспонденции:** Кочубей Валентин Владимирович
ул. Делегатская, д. 20/1, г. Москва, 127473; kochoubey@gmail.com

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Although plastic surgery is a relatively young field, there is already a lot of rigorous criticism regarding professional skills of plastic surgeons [1]. Plastic surgery as a strictly medical activity, should be provided in medical organizations [2]. Regardless of the form of business, those licensed centers must comply with the requirements for the quality and safety of provided services,

keep records of their activities, ensure effective internal control and undergo regular inspections and personnel performance evaluations carried out by authorized agencies [3]. Because the majority of plastic surgery clinics are private (not sponsored by the state), control over quality and safety of provided medical services should be stricter [4]. Ironically, quality assurance becomes a matter of discussion only when patient's health has been compromised and their life has been put at risk [5]. Such cases brought to the public eye by the media undermine reputation of the whole field.

The basis for ensuring the quality of medical care for plastic surgery is the order of delivery of medical care by types, profiles, individual diseases and conditions, as well as standards of medical care [6]. The orders of delivery of medical care have been approved by the Ministry of Health of the Russian Federation and are the same for all healthcare facilities registered in the country [7]. They are also a basis for the functional departmentalization of medical institutions. In turn, plastic surgeon's qualification, as well as doctors of other specialties, should be confirmed by certificates. the current system of certification, though, is a subject of criticism in the medical community [8]. A new accreditation system is hoped to encourage continuing medical education and help doctors attain an equally high level of professional skills [9] by training them in all subspecialties plastic surgeon receives full access to the activities of the specialty, and not to its part. An important part in accreditation is played by the specialist's portfolio, which should reflect the doctor's success in expanding the skills and improving professional skills. However, it is not obligatory to specify in the current model of portfolio the range or extent of performed surgeries and training programs that the surgeon has completed [10]. The lack of unified requirements for the portfolio diminishes the value of accreditation as a tool to ensure the same high level of qualification of plastic surgeons. Our study aimed to investigate a few aspects of plastic surgeons' performance, including the range and extent of surgical interventions, in order to provide rationale for unified requirements for the plastic surgeon's portfolio.

METHODS

Data were collected from official reports of private healthcare providers authorized to perform plastic surgeries at in- and outpatient facilities. The reports contained information about the number and diversity of medical services delivered to patients per year. We shortlisted data relevant for our study (pertaining to the delivery of plastic surgery). Because the computed coefficient of variance V was 257.2 %, which is above 33 % and suggests heterogeneity of the range, and the Kolmogorov–Smirnov test proved that distribution was non-uniform ($p < 0.001$), we estimated significance of differences between the samples using the Mann–Whitney U. Differences were considered significant at $p < 0.05$. Correlation between the rankings was considered significant if empirical Spearman's r_S was above the critical threshold at $p = 0.05$ and $p < 0.05$. The Kruskal–Wallis (K) test was used to determine differences in distributions; sample diversity was considered significant at $p < 0.05$. Data were analyzed using Microsoft Excel 2016 Analysis ToolPack and IBM SPSS Statistics 23.

The list of areas of expertise was proposed in another our ongoing study and included 9 categories: skin/soft tissue plasty, rhinoplasty, breast plasty (mammaplasty), blepharoplasty, otoplasty, lip and palate repair, craniofacial plasty, repair of urogenital defects, and hand surgery.

RESULTS

In total, 46 plastic surgeons conducted 5,184 medical procedures during the year, with a mean of 112.7 ± 289.9 procedures per surgeon. The minimal number of delivered services per surgeon was 1, the maximum — 1,760 (Mo = 2, Me = 10), with 36 (78.3 %) doctors performing below the average and 10 (21.7 %) doctors carrying out more than 112 procedures a year. On the whole, 4,329 (83.5 %) of all services provided to the customers during the year were done by 7 (15.2 %) surgeons.

All services were divided into 9 categories depending on the areas of surgeons' expertise. Skin/soft tissue plasty accounted for 51.1 % of all services provided. Blepharoplasty ranked second (5.7 %), and rhinoplasty ranked third (2.8 %). No hand surgeries were performed during the year (Table 1).

Among "other procedures" were initial consultations, application of aseptic dressings, management of clean wounds, follow-up examinations of postoperative patients, and removal of sutures (for patients who had received treatment at other healthcare facilities). These procedures made 36.5 % of the total services provided. It should be noted that 1,820 services (35.1 % of their total number) falling into this category were initial consultations, meaning that per one initial consultation of a plastic surgeon there were 1.8 invasive (including surgical) interventions. The total number of such procedures as clean wound management, application of aseptic dressings and postoperative follow-ups accounted for 55 (1.1 %), i. e. per one surgery there were only 0.017 follow-up care services.

Of 5,184 medical services delivered in total, 3,145 were surgical interventions, with a mean of 112.3 ± 326.4 procedures per plastic surgeon a year (Mo = 1, Me = 8). The smallest number of interventions per doctor was 1, the largest — 1,758. Of 46 surgeons, 14 (30.4 %) performed 1 to 10 surgeries a year, 4 (8.7 %) — between 14 and 50 surgeries, 6 (13.0 %) — between 64 and 134, 2 (4.3 %) — over 200, 1 (2.2 %) — 1,758 surgeries. Eighteen doctors conducted no surgical interventions at all in the studied period.

The majority of plastic surgeons (19 out of 46 people) dealt with skin/soft tissue plasty, 14 — rhinoplasty, 14 — blepharoplasty, 9 — mammoplasty, another 9 — otoplasty, 3 — craniofacial plasty, 3 — urogenital plasty, and 2 — lip plasty. None of the doctors covered the whole range of 9 types of operations. One doctor was able to perform 7 types of surgeries, another one — 6 types; 3 doctors were qualified in 5 types of surgical interventions, 3 doctors — in 3 types and another 3 — in 2 types; 4 surgeons were able to carry out

Table 1. Surgical interventions categorized depending on the area of surgeons' expertise

Area of expertise	Abs.	%
Skin/soft tissue plasty	2 649	51.1
Blepharoplasty	294	5.7
Rhinoplasty	147	2.8
Mammaplasty	131	2.5
Otoplasty	41	0.8
Urogenital defect repair	14	0.3
Craniofacial plasty	9	0.2
Lip and palate repair	8	0.2
Hand surgery	0	0
Other procedures	1 891	36.5
Total	5 184	100

Table 2. Distribution of surgical interventions among surgeons of different grades and academic titles

Parameter	Plastic surgeons				Other specialties
	No grade or academic title	Senior surgeons	Cand.Sc.	D.Sc.	
Average number of operation types	3.2	3.5	2.4	1.8	0.4
	2.7 ± 1.9				
Total number of operations	442	301	2222	148	32
Average number of operations	88.4 ± 88.0	100.3 ± 4.6	246.9 ± 543.7	37 ± 57.2	4.6 ± 4.2
	129.7 ± 370.0				

4 types of operations, and 13 surgeons — only 1 type. Table 2 presents data on the number of different types of operations performed by the surgeons with different academic titles and grades.

The calculated value of Spearman's coefficient was indicative of the absence of a statistically significant correlation between the average number of surgery types a surgeon was able to perform and the level of his/her professional skills (r_S = –0.8, p = 0.2); no correlation was also observed between the average number of operations per surgeon and the level of professional skills (r_S = –0.2, p = 0.8). Comparison of the average ranges of surgery types in different groups of surgeons and the average numbers of surgical interventions did not reveal any significant differences (K = 1.27, p = 0.2 and K = 1.9, p = 0.5, respectively). At the same time, differences between the average number of total surgeries (U_{emp} = 46.5, p = 0.014) and the number of surgery types (U_{emp} = 72.5, p =0.017) performed by plastic surgeons in comparison with other surgeons were statistically significant, with plastic surgeons being more versatile in their areas of expertise and performing more surgeries per year.

DISCUSSION

The reports on medical care services analyzed in the course of our study have revealed that plastic surgeries are performed not only by plastic surgeons, but also by the doctors of other specialties, such as maxillofacial surgeons, otolaryngologists, ophthalmologists, trauma surgeons, gynecologists, etc. Their narrower areas of expertise restrict the scope of surgical interventions they can perform; the average number of interventions they conduct is lower (U_{emp} = 46.5, p = 0.014) than that performed by plastic surgeons. The intrusion of other specialties into plastic surgery can be explained by the specifics of plastic surgery legislation [5]. Order 555n of the Ministry of Health of the Russian Federation dated October 30, 2012 allows delivery of plastic surgery services by surgeons who have been additionally trained in a chosen subspecialty of plastic surgery. However, we believe that this legal norm must be revised considering the received data, that surgeons of other specialties perform the extremely low average number of few surgical interventions per year and literary data that surgeons with plastic surgery residency training make fewer proven cases of medical errors [11–13].

We have discovered that soft tissue/skin plasty prevails in the range of all plastic surgery services offered to customers, indicating a demand for cosmetic surgery. It means that many plastic surgeons that have been practicing only cosmetic surgery for years lose the skills necessary to perform reconstructive surgery. Considering that a certified surgeon is allowed to conduct all types of plastic surgeries, his/her portfolio should include information about the number of reconstructive surgeries performed or he/she should only be allowed to provide a limited range of reconstructive surgery services based on the training he/she has received [14].

Restricting the range of interventions in the specialty of plastic surgery could be possible under a new accreditation system. The data obtained in the course of this study demonstrate that plastic surgeons perform 2.7 of 9 surgery types on average, which indicates the lack of versatility and proves the necessity of such restrictions [15].

Of particular interest are the data on the frequency of initial consultations and follow-up postoperative examinations with respect to the total number of surgical interventions. Considering that not every primary appointment ends with a surgical intervention, such a significant excess of the number of surgeries on the number of primary appointment can be explained either by holding consultations in previous years, indicating a long period of decision-making by the patient, or the determination of indications for several operations during one primary consultation.

It's troubling that the number of follow-up care procedures is ridiculously low: 0.017 per one operation. Inadequate postoperative management and underestimated health risks or patient's condition are considered medical errors in cosmetic surgery that affect the quality of medical care [16–18].

Interestingly, the range of operation types a plastic surgeon is qualified to perform and the number of operations conducted per year do not depend on the academic title or grade. But the lack of versatility and fewer surgeries performed by D.Sc. in comparison with other surgeons indirectly indicate a transition from clinical practice to research and teaching. Here, accreditation could stimulate professionals to keep their practical skills sharp [19].

CONCLUSIONS

The obtained data demonstrate a need for amendments to healthcare legislation regarding cosmetic surgery and professional training of surgeons. Considering that this study was based at only one medical facility, further research is necessary involving other private and state-funded medical institutions in order to obtain more accurate data and propose rational ideas concerning the evolution of plastic surgery in Russia.

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BREAST CANCER: ANALYSIS OF DRIVER SOMATIC MUTATIONS DETECTED BY NEXT-GENERATION SEQUENCING

Tsukanov KYu[1], Krasnenko AYu[1], Korostin DO[1], Churov AV[2], Stetsenko IF[1], Plotnikov NA[1], Zarubina SA[3], Belova VA[3], Kovyrshina AV[3], Vorotnikov IK[4], Mescheryakov AA[4], Ilyinsky VV[5,3,1] ✉

[1] Genotek Ltd., Moscow, Russia

[2] Karelian Research Centre of the Russian Academy of Sciences, Petrozavodsk, Russia

[3] Vavilov Institute of General Genetics of the Russian Academy of Sciences, Moscow, Russia

[4] N. N. Blokhin Russian Cancer Research Center, Moscow, Russia

[5] Institute of Biomedical Chemistry, Moscow, Russia

Breast cancer (BC) is one of the most common malignancies. There is a need for novel approaches to screening for genetic mutations in patients with BC that will help to reduce high mortality rates caused by this disease and improve treatment outcomes. In this study we employed next generation sequencing to screen a few key genes associated with the risk of breast cancer for mutations. We also evaluated their pathogenicity using the previously proposed bioinformatics-based algorithm and analyzed the associations between some of the detected mutations and the clinical manifestations of the disease. Our study recruited 16 female patients with BC (mean age was 50.7 ± 11.3 years). A total of 58 mutations were detected in the oncogenes *BRCA1*, *BRCA2*, *ATM*, *CDH1*, *CHEK2* and *TP53*. Bioinformatic analysis of the sequencing data revealed 14 mutations that affect the sequence of the encoded proteins. Most deleterious mutations were harbored by the genes *BRCA1/2*, *ATM* and *TP53*.

Keywords: breast cancer, next-generation sequencing, somatic mutation, oncogenes, *BRCA1*, *BRCA2*, *TP53*

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✉ **Correspondence should be addressed:** Valery Ilyinsky
Nastavnichesky per., d.17, str. 1, pod. 14, 15, Moscow, 105120; info@genotek.ru

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РАК МОЛОЧНОЙ ЖЕЛЕЗЫ: АНАЛИЗ СПЕКТРА СОМАТИЧЕСКИХ ДРАЙВЕРНЫХ МУТАЦИЙ С ПРИМЕНЕНИЕМ ВЫСОКОПРОИЗВОДИТЕЛЬНОГО СЕКВЕНИРОВАНИЯ

К. Ю. Цуканов[1], А. Ю. Красненко[1], Д. О. Коростин[1], А. В. Чуров[2], И. Ф. Стеценко[1], Н. А. Плотников[1], С. А. Зарубина[3], В. А. Белова[3], А. В. Ковыршина[3], И. К. Воротников[4], А. А. Мещеряков[4], В. В. Ильинский[5,3,1] ✉

[1] ООО «Генотек», Москва

[2] Карельский научный центр Российской академии наук, Петрозаводск

[3] Институт общей генетики имени Н. И. Вавилова РАН, Москва

[4] Национальный медицинский исследовательский центр онкологии имени Н. Н. Блохина, Москва

[5] Научно-исследовательский институт биомедицинской химии имени В. Н. Ореховича, Москва

Рак молочной железы (РМЖ) представляет собой одну из наиболее распространенных форм злокачественных опухолей. Развитие новых подходов к скринингу генетических изменений у больных с опухолями молочной железы поможет значительно снизить общую высокую смертность от рака этого типа и повысить эффективность противоопухолевой терапии. Целью настоящей работы являлось выявление методом высокопроизводительного секвенирования спектра мутаций в составе ключевых онкогенов при РМЖ оценка их патогенности с применением ранее разработанного биоинформатического алгоритма, а также оценка взаимосвязи некоторых мутаций с особенностями клинического проявления заболевания. В исследовании принялие участие 16 пациенток с РМЖ (средний возраст — 50,7 ± 11,3 года). Было обнаружено 58 мутаций в онокгенах *BRCA1*, *BRCA2*, *ATM*, *CDH1*, *CHEK2* и *TP53*. Среди выявленных генетических вариантов с применением биоинформатических подходов найдено 14 мутаций, оказывающих влияние на последовательность кодируемого белка. Большая часть патогенных мутаций идентифицирована в генах *BRCA1/2*, *ATM* и *TP53*.

Ключевые слова: рак молочной железы, высокопроизводительное секвенирование, соматическая мутация, онкогены, *BRCA1*, *BRCA2*, *TP53*

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✉ **Для корреспонденции:** Ильинский Валерий Владимирович
Наставнический пер., д. 17, стр. 1, под. 14, 15, г. Москва, 105120; info@genotek.ru

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Breast cancer (BC) is the second most common type of cancer and the second leading cause of death in women; it is also the most incident cancer worldwide [1]. The risk of BC increases with age: the majority of new cases are reported in women who are 60 to 65 years old. High BC mortality is explained by late diagnosis established when the disease has already progressed to the advanced stage. Metastatic BC is particularly dangerous, since it is resistant even to combination treatments based on chemotherapy, hormones and targeted drugs. The 5-year survival rate in patients with BC is 55 %. This brings the need for novel approaches towards more effective screening as well as targeted therapy of BC based on the molecular genetic profiling of tumors.

The rapid development of next generation sequencing (NGS) has yielded a bulk of information about genetic variants [2]. A lot of mutations are associated with BC, including somatic and germinal mutations in the genes *PIK3CA*, *STK11/LKB1*, *CDH1*, *ATM*, *CHEK2*, *BRIP1*, and *PALB2* and mutant variants of the highly penetrant genes associated with hereditary BC, such as *TP53*, *PTEN*, *MLH1*, *BRCA1*, and *BRCA2* [3].

The majority of tumor mutations are somatic; they have an important role in the pathogenesis of cancer and confer de novo resistance to treatment. Thus, a lot of ongoing studies utilize NGS in an attempt to profile mutant variants in tumors. As a result, it has been identified a significant amount of new mutations with unknown function. To describe these polymorphisms, mathematical algorithms are necessary that can automatically process huge data arrays, predict potentially pathogenic mutations and distinguish them from harmless variants. The resulting data can be used when developing screening or diagnostic tools (including liquid biopsy) and selecting adequate targeted therapies.

In this work we analyze a range of mutations identified in key BC oncogenes by NGS, using a previously developed bioinformatic pipeline for the functional annotation of mutations and assessment of their pathogenicity.

METHODS

We obtained tumor samples from 16 patients of Blokhin Russian Cancer Research Center, Moscow. The participants' age range was 27 to 76 years, with a mean of 50.7 ± 11.3 years. All patients had breast malignancies and received combination therapy. The inclusion criteria were as follows: age of 18 to 70 years, sex (all patients were females), histologically and cytologically confirmed breast cancer. The exclusion criteria were a medical history of other tumor types and pregnancy.

Disease stages were determined according to the TNM classification [4]. The study was carried out in the patients with stages T1–3N0–3M0–1.

All patients gave voluntary informed consent. The study complied with the principles of confidentiality. Patients' clinicopathologic features are summarized in Table 1.

DNA isolation and quality control

DNA was isolated from the samples of tumor tissue using DNeasy Blood and Tissue Kit (Qiagen, USA). Tumor tissue was cut into small pieces, and buffer ATL was added to the samples. The samples were then treated with proteinase K, incubated at 56 °C until fully lysed, and treated with RNase A. Next, we added 200 µl buffer AL and 96 % ethanol. The resulting mixture was transferred to spin columns and centrifuged at 8,000 g for 1 min. The samples were washed with AW1 and AW2 buffers to remove salts (guanidine and SDS). The columns were eluted twice with 30 µl Low-TE buffer; the samples were incubated and centrifuged according to the manufacturer's protocol. Quality control of the obtained DNA was performed on Qubit 3.0 (Thermo Fisher Scientific, USA). The samples were also run on 1 % agarose gel electrophoresis with ethidium bromide.

Sequencing of targeted oncogenes

DNA libraries were prepared using NEBNext Ultra DNA Library Kit for Illumina (New England Biolabs, USA). The libraries were dual-indexed by PCR using NEBNext Ultra DNA Library Prep Kit for Illumina and NEBNext Multiplex Oligos for Illumina (Dual Index Primers Set 1, New England Biolabs). Quality control of the obtained DNA libraries was performed on Agilent Bioanalyzer 2100 (Agilent Technologies, USA) using High Sensitivity Kit by the same manufacturer according to the official protocol.

For targeted enrichment of the coding regions of tumor genomes we used MYbaits Onconome KL v1.5 Panel (MYcroarray, USA). The enriched fragments were sequenced with 100 b. p. paired-end reads on HiSeq 2500 (Illumina, USA). Sample preparation and sequencing were done according to Illumina's protocols.

Bioinformatic analysis

Sequencing data were analyzed using an original algorithm developed previously [5]. First, the quality of reads was checked: sequences with read quality below 10 were removed from NGS data using Cutadapt software [6]. Then the reads were mapped to the reference genome hg19 (GRCh37. p13) using the Burrows–Wheeler Aligner algorithm [7]. PCR-duplicates were removed by running the rmdup command in SAMtools [8].

Mutations were called with MuTect [9]. DNA sequences covered by at least 12 reads were considered the most significant.

To assess the functional effect of the discovered mutations, they were annotated in SnpEff and their effect on the encoded protein was predicted based on the analysis of genomic coordinates [10].

RESULTS

Using Illumina-based NGS, we have screened 16 breast tumors for mutations harbored by cancer-associated genes *BRCA1*, *BRCA2*, *ATM*, *CDH1*, *CHEK2*, *MRE11A*, *NBN*, *PALB2*, *PTEN*, *RAD50*, *RAD51C*, *TP53*, and *SEC23B*. Out original bioinformatic algorithm has detected 58 point mutations in the genes *BRCA1*, *BRCA2*, *ATM*, *CDH1*, *CHEK2* and *TP53*, including 19 homozygous and 39 heterozygous variants. The list of unique mutations is provided in Table 2.

The figure below shows the frequency of mutations in the genes with the highest abundance of mutations, namely *ATM*, *TP53* and *BRCA1*. The most frequent mutations were c.376-283T>C (*TP53*), c.3994-193T>C, c.8010+186C>T (*ATM*), and c.5215+66G>A (*BRCA1*).

Based on the bioinformatic analysis and annotation of the identified polymorphisms, we selected those mutations that could significantly affect the regulatory or protein sequences. To assess pathogenicity and conservation of the mutations, we used data from COSMIC (Catalogue of Somatic Mutations In Cancer) [11] and dbNSFP [12]. Additionally, SIFT (Sorting Intolerant From Tolerant) and PolyPhen2 tools were used to

Table 1. Clinicopathologic features of patients with breast cancer (n = 16)

Parameter	Value. abs. (%)
Age. years	50.7 ± 11.3
Surgical intervention:	
yes	16 (100)
no	0 (0)
Stage according to TNM	
T1	10 (62.5)
T2	5 (31.3)
T3	1 (6.2)
Metastases in lymph nodes	
no. M0	10 (62.5)
yes. M1	6 (37.5)
Expression of estrogen receptors (ER):	
ER+	11 (68.8)
ER–	5 (31.2)
Expression of progesterone receptors (PR):	
PR+	10 (62.5)
PR–	6 (37.5)
Expression of HER2/neu:	
Her2+	9 (56.3)
Her2–	7 (43.7)
Expression of Ki-67:	
low (< 14 %)	13 (81.3)
high (≥ 14 %)	3 (18.7)
Adjuvant chemotherapy:	
yes	10 (62.5)
no	6 (37.5)
Adjuvant hormonal therapy:	
yes	9 (56.3)
no	7 (43.7)
Radiation therapy	
yes	0 (0)
no	16 (100)

predict pathogenicity of the mutations and assess their effect on the function of the encoded protein [13, 14]. Information about mutation frequencies was obtained from the 100 Genomes project and the Exome Aggregation Consortium [15, 16].

Altogether, we singled out 14 mutations affecting the protein sequence: *BRCA2* — c.4828G>A (p.Val1610Met), c.5070A>C (p.Lys1690Asn); *TP53* — c.524G>A (p.Arg175His), c.469G>T (p.Val157Phe); *CHEK2* — c.1289C>T (p.Thr430Ile); *ATM* — c.146C>G (p.Ser49Cys), c.4258C>T (p.Leu1420Phe), c.1192G>C (p.Asp398His); *CDH1* — c.790C>T (p.Gln264), c.1342C>T (p.Gln448); *BRCA1* — c.1865C>T (p.Ala622Val), c.384G>A (p.Met128Ile), and c.54G>T (p.Met18Ile).

DISCUSSION

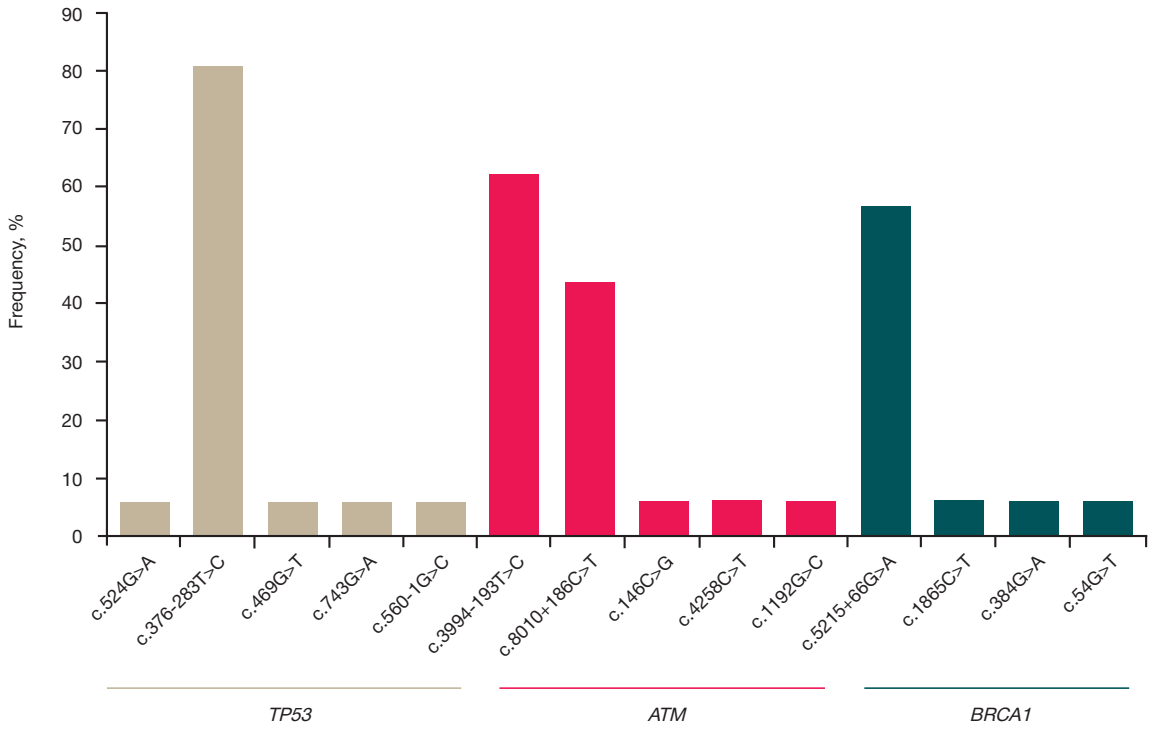
In Russia, the PCR-based methods for the detection of known mutations in BC-associated genes have become most widespread. However, today there are more advanced methods of genetic screening, the most promising being next generation sequencing that can be used for identifying genetic variants in malignant tumors and is especially suitable in exploring the variability of highly heterogeneous regions of tumor genomes. In this work we applied NGS to study a number of mutations of key oncogenes associated with BC and tested a previously developed algorithm for bioinformatic analysis of sequencing data.

One of the most well-studied genes playing a significant role in BC pathogenesis is *TP53*. It is involved in the regulation of the cell cycle, apoptotic activity and DNA repair. Mutations in *TP53* lead to the disruption of these regulatory mechanisms and may trigger formation of cancer. *TP53* is a tumor suppressor; mutant variants of this gene are detected in half of all cancers and in more than 30 % of BC cases. In turn, sporadic breast cancer is characterized by a varying frequency of *TP53* mutations between 25 % and 86 %, depending on the disease stage and the screening technique applied. The prognostic value of *TP53* mutations in BC has been sufficiently studied [17]. Among the mutations identified in our study the most frequent was c.376-283T>C discovered in 13 of 16 patients (81 %).

Patients with BC and with some of its types in particular have relatively high frequency of *BRCA1* and *BRCA2* mutations. *BRCA1* and *BRCA2* are involved in the regulation of many cell processes maintaining genomic stability and homologous

Table 2. Single nucleotide variants (mutations) identified in patients with breast cancer (n = 16)

Sample	Abundance of mutations in the sample, %	Mutations	Gene	Coordinates
1	18.7	c.4828G>A	*BRCA2*	Chr13:32913320
		c.3994-193T>C	*ATM*	Chr11:108158134
		c.-73G>A	*CHEK2*	Chr22:29137870
2	25.5	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.3994-193T>C	*ATM*	Chr11:108158134
		c.8010+186C>T	*ATM*	Chr11:108204881
		c.524G>A	*TP53*	Chr17:7578406
		c.376-283T>C	*TP53*	Chr17:7578837
3	29.4	c.8755-272A>G	*BRCA2*	Chr13:32953182
		c.376-283T>C	*TP53*	Chr17:7578837
4	26.5	c.3994-193T>C	*ATM*	Chr11:108158134
		c.8010+186C>T	*ATM*	Chr11:108204881
		c.376-283T>C	*TP53*	Chr17:7578837
5	26.5	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.3994-193T>C	*ATM*	Chr11:108158134
		c.8010+186C>T	*ATM*	Chr11:108204881
		c.1289C>T	*CHEK2*	Chr22:29091797
		c.376-283T>C	*TP53*	Chr17:7578837
6	20.6	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.3994-193T>C	*ATM*	Chr11:108158134
		c.8010+186C>T	*ATM*	Chr11:108204881
7	25.5	c.146C>G	*ATM*	Chr11:108098576
		c.376-283T>C	*TP53*	Chr17:7578837
8	25.5	c.3994-193T>C	*ATM*	Chr11:108158134
		c.790C>T	*CDH1*	Chr16:68844202
		c.376-283T>C	*TP53*	Chr17:7578837
9	28.5	c.5070A>C	*BRCA2*	Chr13:32913562
		c.-73G>A	*CHEK2*	Chr22:29137870
		c.469G>T	*TP53*	Chr17:7578461
10	37.3	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.4258C>T	*ATM*	Chr11:108160350
		c.376-283T>C	*TP53*	Chr17:7578837
11	26.5	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.1865C>T	*BRCA1*	Chr17:41245683
		c.384G>A	*BRCA1*	Chr17:41256196
		c.54G>T	*BRCA1*	Chr17:41276060
		c.3994-193T>C	*ATM*	Chr11:108158134
		c.-73G>A	*CHEK2*	Chr22:29137870
		c.743G>A	*TP53*	Chr17:7577538
		c.376-283T>C	*TP53*	Chr17:7578837
12	28.5	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.3994-193T>C	*ATM*	Chr11:108158134
		c.8010+186C>T	*ATM*	Chr11:108204881
		c.376-283T>C	*TP53*	Chr17:7578837
13	22.6	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.1192G>C	*ATM*	Chr11:108119786
		c.3994-193T>C	*ATM*	Chr11:108158134
		c.8010+186C>T	*ATM*	Chr11:108204881
		c.1342C>T	*CDH1*	Chr16:68849439
		c.376-283T>C	*TP53*	Chr17:7578837
14	22.6	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.376-283T>C	*TP53*	Chr17:7578837
15	26.5	c.5215+66G>A	*BRCA1*	Chr17:41215825
		c.560-1G>C	*TP53*	Chr17:7578290
		c.376-283T>C	*TP53*	Chr17:7578837
16	28.5	c.3994-193T>C	*ATM*	Chr11:108158134
		c.8010+186C>T	*ATM*	Chr11:108204881
		c.376-283T>C	*TP53*	Chr17:7578837



Distribution of mutation frequencies in the genes *ATM*, *TP53* and *BRCA1* in patients with beast cancer

recombination during repair of double-strand DNA breaks. Mutations occurring in these genes often disrupt their normal function and are a major causative factor of hereditary BC, increasing the risk of cancer in an individual. About a quarter of all hereditary BC cases are associated with mutations in *BRCA1/2* [17].

Mutations in *BRCA1* account for 80 % of all *BRCA1* and *BRCA2* mutations in Russians with BC. One of the most common mutant variants identified in Russian patients is 5382insC (*rs80357906*) that causes a reading frame shift and the loss of function of the encoded protein. The majority of the polymorphisms identified in our study were mutations in *BRCA1* and *BRCA2*, the most common being c.5215+66G>A (*rs3092994*) in *BRCA1*, detected in 9 of 16 patients (52.9 %).

Our findings on *ATM*, *TP53* and *BRCA1* mutations are on the whole consistent with the literature, which reports *TP53* variants to be the most common mutations in BC [17]. Our results of the diversity of *BRCA1/2* variants are also comparable with the literature data. Importantly, mutations in these genes are associated with poor prognosis and development of invasive ductal breast cancer. The existences of these mutations are considered at assessment of volume of surgical intervention [17]. In our study, of 12 patients with BC who had mutations in *BRCA1* and *BRCA2*, 8 were diagnosed with invasive ductal carcinoma. Of those 8, six had the mutation c.5215+66G>A in *BRCA1*.

We have analyzed next generation sequencing data using the original bioinformatic approach and discovered many driver mutations in the samples of malignant breast tumors. Using different databases, we have selected and annotated functionally significant mutations. Altogether, we have discovered 14 mutations affecting the amino acid sequence of the encoded proteins. Each of the studied samples had at least one such mutation. The original bioinformatic protocol allowed us to automatically process DNA sequencing data obtained with NGS.

CONCLUSIONS

A combination of next generation sequencing and modern algorithms for bioinformatic analysis is a good and clinically attractive method of screening for genetic polymorphisms and assessing the functional effect of mutations detected in the tumor. To date, NGS enables molecular classification of breast tumors and can be used to determine their subtypes depending on the spectrum of the identified mutations and the expression profiles of the studied genes. NGS data can facilitate the choice of adequate targeted therapies. One of the major tasks of cancer genetics is development of convenient tools for the detection of breast cancer biomarkers that can be used by clinicians for more accurate diagnosis and effective treatment. We believe that advances in the filed should include improvement of bioinformatic approaches, adoption of the systems for automatic analysis of tumor genetic profiles and introduction of NGS into clinical routine.

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ANALYSIS OF THE APOPTOTIC EFFECT OF ULTRAHIGH GAMMA DOSE RATES ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES *IN VITRO*

Grabovsky EV[1], Oleynik GM[1], Krastelev EG[2], Smirnov VP[2,3], Khmelevsky EV[4], Bozhenko VK[5] ✉, Shishkin AM[5], Ivanov AV[5], Kulinich TM[5]

[1] Troitsk Institute for Innovation and Fusion Research (TRINITI), Rosatom State Atomic Energy Corporation, Troitsk, Moscow, Russia

[2] Joint Institute for High Temperatures, Russian Academy of Sciences, Moscow, Russia

[3] Research Institute of Technical Physics and Automation, Rosatom State Atomic Energy Corporation, Moscow, Russia

[4] Hertzen Moscow Cancer Research Institute (affiliated branch of the National Medical Research Radiology Center), Moscow, Russia

[5] Russian Scientific Center of Roentgenoradiology, Moscow, Russia

Relative biological effectiveness of ionizing radiation is determined by a number of factors, including a dose rate. Radiotherapy equipment employs low dose rates of up to a few Gy per minute. But very little is known about the biological effect of high and ultrahigh (≥ 10^8 Gy/min) dose rate radiation. Our study aimed to investigate the apoptotic effect of ultrahigh gamma dose rates on human peripheral blood lymphocytes. Blood samples were collected from seemingly healthy donors. Lymphocytes were isolated by density gradient separation. Lymphocyte suspensions were irradiated with low-rate doses on the Rokus-AM gamma-ray machine for clinical use (Russia) and with 10^8 Gy/s doses on the experimental pulse generators Angara-5-1 and Mir-M (Russia). Apoptosis was measured by flow cytometry using annexin V and propidium iodide double staining. We established that in comparison with low dose rates, ultrahigh gamma dose rates (with doses ranging from 1 to 6 Gy) induced significantly more pronounced apoptosis in peripheral blood lymphocytes ($p < 0.05$) with fewer necrotic cells. Total radiation-induced cell death did not differ significantly between the therapeutic gamma machine and the experimental pulse generators. Further research is needed to assess biological and medical significance of our findings.

Keywords: ultrahigh dose rate gamma radiation, ultrahigh intensity X-rays, dose rate, cell death, apoptosis, necrosis, lymphocytes

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✉ **Correspondence should be addressed:** Vladimir Bozhenko
ul. Profsoyuznaya, d. 86, Moscow, Russia, 117997; vbojenko@mail.ru

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АНАЛИЗ ИНДУКЦИИ АПОПТОЗА ЛИМФОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ ЧЕЛОВЕКА СВЕРХИНТЕНСИВНЫМ ГАММА-ИЗЛУЧЕНИЕМ *IN VITRO*

Е. В. Грабовский[1], Г. М. Олейник[1], Е. Г. Крастелев[2], В. П. Смирнов[2,3], Е. В. Хмелевский[4], В. К. Боженко[5] ✉, А. М. Шишкин[5], А. В. Иванов[5], Т. М. Кулинич[5]

[1] Троицкий институт инновационных и термоядерных исследований (ТРИНИТИ), ГК «Росатом», Троицк, Москва

[2] Объединенный институт высоких температур РАН, Москва

[3] Научно исследовательский институт технической физики и автоматизации, ГК «Росатом», Москва

[4] Московский научно-исследовательский онкологический институт имени П. А. Герцена — филиал Национального медицинского исследовательского центра радиологии, Москва

[5] Российский научный центр рентгенорадиологии, Москва

Относительная биологическая эффективность ионизирующего излучения определяется рядом параметров, одним из которых является мощность дозы. В терапевтических лучевых установках используется облучение с мощностью дозы до нескольких Гр/мин. Эффект высоких и особенно сверхвысоких (10^8 Гр/мин и выше) мощностей дозы практически не изучен. Целью нашего исследования являлось определение влияния гамма-излучения, имеющего сверхвысокую мощность дозы, на индукцию апоптоза в лимфоцитах периферической крови человека. Лимфоциты получали из крови условно здоровых добровольцев выделением их на градиенте плотности. Образцы суспензии лимфоцитов при низкой мощности дозы облучали на установке «Рокус-АМ» (Россия), при мощности дозы около 10^8 Гр/с — на экспериментальных установках «Ангара-5-1» и «Мир-М» (Россия). Уровень апоптоза регистрировали методом проточной цитофлуориметрии с двойной окраской аннексином V и йодидом пропидия. Установили, что гамма-излучение со сверхвысокой мощностью дозы в диапазоне доз 1–6 Гр индуцирует апоптоз в лимфоцитах периферической крови достоверно выше, чем гамма-излучение с низкой мощностью дозы ($p < 0,05$), одновременно в меньшей степени индуцируя некроз. При этом общий уровень радиационной гибели лимфоцитов для терапетивческой и экспериментальных установок достоверно не различался. Дальнейшие исследования позволят уточнить биологическую и медицинскую значимость полученных результатов.

Ключевые слова: сверхинтенсивное гамма-излучение, сверхинтенсивное рентгеновское излучение, мощность дозы, клеточная гибель, апоптоз, некроз, лимфоциты

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✉ **Для корреспонденции:** Боженко Владимир Константинович
ул. Профсоюзная, д. 86, г. Москва, 117997; vbojenko@mail.ru

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Success of radiation therapy in patients with malignant tumors is determined by a number of biologic effects it induces, including cell death. Ionizing radiation causes molecular damage to cell organelles and compartments and has a particularly detrimental effect on the DNA structure. DNA damage either results in cell death or is repaired and cells subsequently recover their function. However, if mechanisms of DNA repair are affected by radiation, there is a risk of mutations and cancer formation [1].

There are a few types of cell death that can be induced by ionizing radiation [2, 3]. Of particular interest here is apoptosis, the programmed cell death that spares the surrounding healthy tissue, does not trigger inflammation and can be enhanced by radiation [4]. Certain cell types are resistant to radiation-induced apoptosis; others can modify their radiosensitivity depending on the stage of the cell cycle or microenvironment [5]. A lot of research is ongoing in the field aiming to find methods of control over apoptosis occurring in some cell types in response to radiation or chemotherapy in patients with malignant tumors [6, 7].

It has been proved that although many anticancer drugs promote apoptosis in tumors, they are not selective, which means they also attack healthy tissue [8]. Radiation therapy also has its downsides, including formation of necrotic lesions in the tumor and damage to the surrounding healthy cells. There is evidence that ionizing radiation stimulates apoptosis of lymphocytes, thymocytes and different precursor cells. Mature differentiated cells rarely become apoptotic in response to radiation but some authors report radiation-induced apoptosis in human breast cells, intestinal crypts and tonsils. In the experiment [9] apoptosis has been induced in mouse eggs and hepatocellular carcinomas [9].

High-current pulse electron accelerators capable of generating high-intensity bremsstrahlung radiation are employed by research studies aimed at developing new radiation technologies. It has been experimentally proved that these machines do have their drawbacks limiting their use in clinical practice, including unstable characteristics from pulse to pulse. At the same time, narrower electron beams are believed to be more sparing to healthy tissue, while high dose rates are expected to cause more profound damage to target areas [10]. Gamma machines used in clinical routine generate a therapeutic dose rate of 1.5–2 Gy/min allowing absorption of 100 quanta per second by a 10 μm cell at 100 keV energies. Intervals between bursts are as short as 1 to 10 ms, but cell repair mechanisms work faster. Increased radiation intensity can be achieved on high-current accelerators generating 1 to 100 GW relativistic electron beams, reducing intervals between bursts by up to 6 or 7 orders of magnitude and (hypothetically) modifying cellular response to irradiation. So far, experimental data on the impact of high dose rates on biologic objects remain controversial [11–15].

The aim of this study was to identify biologic effects induced by high dose rates generated by Angara-5-1 and Mir-M machines on human peripheral blood lymphocytes and to compare them to the effects induced by low dose rates generated by Rokus-AM gamma machine.

METHODS

The study included 3 stages:

1) preparation of mononuclear cell suspension;

2) irradiation of the obtained samples with gamma rays generated by Rokus AM machine and high-intensity bremsstrahlung radiation produced by Angara-5-1 and Mir-M;

3) determine the proportion of apoptotic cells in the irradiated samples using flow cytometry.

Preparing a suspension of human blood mononuclear cells

We used suspensions of healthy lymphocytes isolated from the blood of seemingly healthy donors. Blood samples (2.6 ml each) were collected into test tubes containing EDTA anticoagulant, diluted with phosphate buffered saline (PBS) in the ratio of 1 to 2, and then layered over the ficoll (ρ = 1.077; PanEco, Russia). The samples were centrifuged for 40 min at 400 g. The mononuclear layer containing 70 % to 90 % lymphocytes was collected from the interface, washed in PBS twice for 5 min at 200 g and transferred to RPMI 1640 medium with 10 % fetal bovine serum.

The obtained suspension of mononuclear cells was aliquoted in 12 portions (per sample): 2 controls were not irradiated; the remaining 10 were exposed to 5 different radiation doses in twos. The irradiated samples were incubated in the CO_2-incubator at 37 °C and 5 % CO_2. One sample from each pair was incubated for 24 h, another — for 48 h. Such time intervals were necessary to objectively estimate the level of radiation-induced apoptosis. All procedures from blood collection to incubation were performed at room temperature and took 3.5 to 4 h in total.

Irradiation of samples using Angara-5-1

Anagara-5-1 generates high-intensity bremsstrahlung radiation by means of 8 independent high-power sources [8]. They are activated simultaneously (the rms deviation is only 10 ns). The maximum output voltage at matched load is 1.5 MV. The voltage pulse is a half-sine in shape and has a duration of 40–60 ns at half of amplitude. The anode is 50 μm thick tantalum foil (Fig. 1).

Spectral analysis of bremsstrahlung radiation demonstrated that the majority of emitted quanta had energies ranging from 200 to 600 keV. The analysis was carried out on profiles of signals of AD3 diamond X-ray dosimeters (engineered at Dukhov Research Institute of Automatics, Russia) with various filters.

Dose measurements were taken using DPG-03 thermoluminescent dosimeters (TLDs) by Doza LLC, Russia, containing 3 polycrystalline magnesium borate detectors, and the KDT-02 TLD processing set (Electron Corporation, Ukraine). We experimentally evaluated dose dependence on the distance between the object and the diodes and estimated dose distribution depending on the position of the irradiated sample. According to our calculations, the generated dose was attenuated for every 1 cm of the sample's thickness by 10 % in the vertical direction and by 9 % — in the horizontal direction, relative to the center of the anterior surface of the object. We determined the desirable positions of the samples on the flange. The samples were placed in a duralumin cylinder with 7 mm thick bottom and walls. The bottom of the cylinder was positioned 57 mm away from the tantalum foil.

To adjust the doses, the samples were placed at different distances from the radiation source. The maximum dose varied from burst to burst, therefore, TLDs were installed in close proximity to the samples. Dose rates is also varied along with the dose, because pulse duration remained unchanged. For the maximum dose of 10 Gy its rate exceeds 100 MGy/s; for the minimum dose (less than 1 Gy) its rate was about 10 MGy/s.

Mononuclear cell suspensions were placed in plastic containers installed perpendicular to the electron beam

(Fig. 2, D). The liquid layer in the tubes was 4 mm thick. After irradiation the samples were transferred to culture dishes and loaded into the CO_2-incubator (see the details above).

Irradiation of samples using Mir-M

Mir-M is an experimental high-current nanosecond electron accelerator. Its peak output energy reaches 800 kV; half amplitude duration is 40 to 60 ns. The anode is made of 50–100 μm thick tantalum foil. Behind the foil an additional carbon composite Graflex filter is installed (0.5–1 mm thick) for capturing electrons that have passed through the foil. The flange of the exit window is made of 1 mm-thick aluminum. The studied dose rates were the same as for the experiment with Angara-5-1.

Two methods of dose measurements were used in the experiments.

Quick dose measurements were carried out using TLD dosimeters DTG-4 (Doza LLC) in the form of a monocrystalline tablet made of lithium fluoride activated with magnesium and titanium. The irradiated TLDs were read out using Doza-TLD measuring complex (Doza LLC) and DVG software of the same manufacturer. The margin of measurement error for the TLD/Doza-TLD complex was ± 30 %, with a confidence interval of 0.95. Therefore, for better accuracy the dosimeters were calibrated by irradiation using Rokus-AM machine (radiation source: cobalt 60, dose received: 7 Gy). Each irradiated TLD was read out and its individual characteristics were taken into account in the measurements.

For more accurate dosimetry we used Gafchromic EBT 2 films (Ashland, USA). The films were processed using DoseLab 6.5 (Moebius Medical Systems, USA). Because dose distribution along the beam axis was heterogeneous in the Mir-M experiment and the dose was attenuated 1.5–2 times for every 1 cm of the sample's thickness depending on the cathode, we tried to minimize the linear dimensions of the sample.

The samples were 0.6 ml stirred suspensions of mononuclear cells securely sealed in 2 mm deep wells of 16 mm in diameter. The suspension filled the well completely; thus the leukocytes were evenly distributed in the sample during irradiation.

The package for irradiation included: a 5 mm separator consisting of two 1 mm thick plastic sheets spaced at 3 mm intervals; a layer of 2 mm thick polyurethane foam with the sealed sample; 1 mm thick supporting plastic plate. Packages were installed along the same axis so that sample containers were precisely behind each other. The distance between the samples was 8 mm. The distance from the flange to the front-face of the first sample was about 1 cm. Dosimeters were placed behind the separator, adjacent to the anterior surface of the sealed sample (Fig. 1, A, C).

Irradiation of samples using Rokus-AM gamma machine

Rokus-AM is a 60Co-based therapeutic gamma machine. We used it to compare the effectiveness of low-intensity beams and high-intensity gamma radiation. The machine was operated at a dose rate of 0.9 Gy/min (15 mGy/s), which is 10^9–10^{10} times lower than that of nanosecond accelerators.

Cell suspensions were the same as in previous experiments; therefore, we did not have to account for individual characteristics of each sample.

Irradiation was performed using the same well as in the Mir-M experiment. The sample could be positioned horizontally, which allowed us to use an open 2 mm tall plastic container with thin walls, which fit in the well perfectly (Fig. 1, B). Since we had to ensure electron equilibrium, the sample was covered with a 5 mm thick plastic sheet and a 15 mm thick acrylic (plexiglass) sheet was put under the sample.

Irradiation parameters were as follows: distance from the source to the surface — 75 cm, field size — 10 × 10 cm. Irradiation was performed with a time shift of 1–1.5 h relative to the time of the irradiation on the high-intensity gamma sources.

Studying apoptosis in the samples

Following the incubation, we measured apoptosis in the samples of cell suspensions by flow cytometry. We applied two techniques: staining of unfixed samples using the

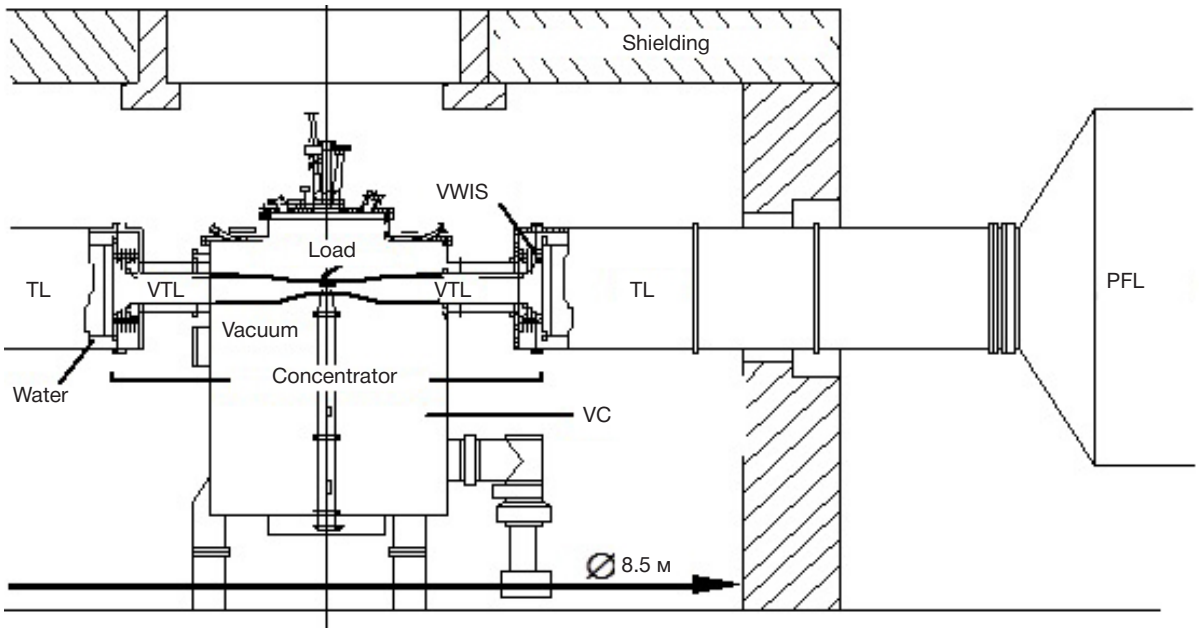

Fig. 1. Schematic of generation and delivery of the electromagnetic pulse by Angara-5-1 generator to the load in the vacuum chamber. PFL — double water pulse-forming line; TL — water transmission line; VWI — vacuum-water interface; VTL — magnetically insulated vacuum transmission line; VC — vacuum chamber with electromagnetic flux concentrator

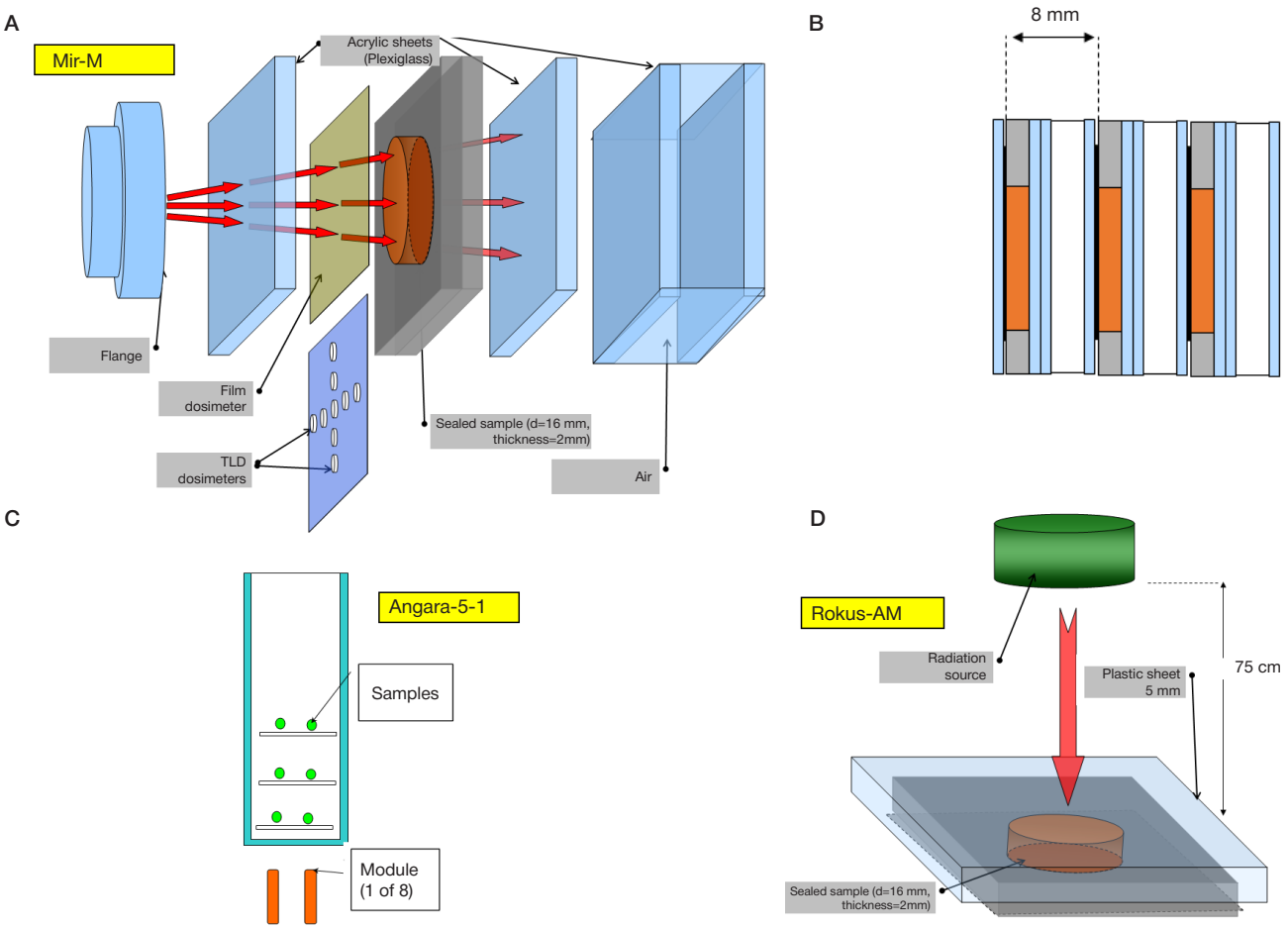

Fig 2. Position of the samples for irradiation by Mir-M **(A, B)**, Angara-5-1 **(C)** and Rokus-AM **(D)**

Annexin-V-FITC Kit (Beckman Coulter, USA) that contains annexin V and propidium iodide, and staining of fixed samples with propidium iodide.

For annexin V staining the irradiated and incubated samples were washed in PBS. A hundred μl of cell suspension containing 106 cells were stained using the Annexin-V-FITC kit according to the manufacturer's protocol. This staining technique helps to quantify the cells that have entered apoptosis (annexin V positive particles) and the cells that have died or are dying of necrosis (propidium iodide positive particles). It is also used to differentiate between the early apoptotic cells (annexin V positive) and the late apoptotic cells (annexin V positive, propidium iodide positive).

Apoptosis can also be detected by staining of fixed samples with propidium iodide: it appears as a subdiploid peak on the single-parameter histogram, which represents the amount of apoptotic bodies in the sample. The irradiated and incubated samples (incubation time was either 24 or 48 h) were left in 70 % ice cold ethanol for 72 h for fixation. Then the cells were washed twice in 1 ml PBS and resuspended in 100 μl PBS. To prevent propidium iodide from binding to RNA the suspension was incubated with 20 μl RNase (R4875, Sigma-Aldrich, USA) at 37 °C for 30 min. The incubated suspension was stained with 20 μl propidium iodide for 40 min at room temperature in the area protected from the light. Before flow cytometry was performed, the sample was replenished with 1 ml PBS. The final cell concentration was at least 10^6 cells.

Flow cytometry was performed on Cytomics FC 500 (Beckman Coulter). We measured the ratio of the subdiploid peak to the total number of cells.

RESULTS

We have established that apoptosis in the lymphocytes exposed to gamma rays generated by Rokus-AM linearly depends on the radiation dose (in the studied dose range). The longer the irradiated samples were incubated, the more apoptotic cells were there. The level of apoptosis increased by 8.0 ± 2.2 % (relative to the deemed 0 % in the non-irradiated samples) in the cells exposed to 5 Gy doses and subsequently incubated for 24 hours. But the samples incubated for 48 h showed a 10.0 ± 2.6 % increase in spontaneous apoptotic activity and a 27.0 ± 3.8 % increase in the proportion of apoptotic cells when exposed to the same 5 Gy radiation doses ($p = 0.004$) (Fig. 3).

The samples exposed to the beams generated by Angara-5-1 exhibited linear dependence of apoptotic activity on the dose; however, the line slope in this case was steeper in comparison with the line constructed for the Rokus-AM experiment (Fig. 3). Doses of 3 Gy generated by Rokus-AM induced apoptosis in 23.0 ± 3.1 % of cells in the samples incubated for 48 h, while high-intensity radiation (in doses comparable with 3 Gy) induced apoptosis in 31.0 ± 3.8 % of cells ($p = 0.050$) in the same sample. This means that high intensity bremsstrahlung radiation has a stronger proapoptotic effect than therapeutic doses of gamma rays.

Because differences between the two radiation types in terms of apoptosis induction in peripheral blood lymphocytes turned out to be significant, we went on to experiment with Mir-M (specially engineered for medical and biological research) in an attempt to better understand the apoptotic effect of high intensity radiation. We used markers of early and late apoptosis

and isolated a fraction of previously ignored necrotic cells (those cells appeared intact on the histogram).

Fig. 4. shows the changing levels of apoptosis (early apoptosis is shown in Fig. 4, A, late — in Fig. 4, B), necrosis (Fig. 4, C) and total cell death (Fig. 4, D) in the irradiated samples depending on the radiation dose and machine ability. We have compared the effect of "therapeutic" gamma radiation generated by Rokus- AM and high-intensity beams produced by Mir-M (only for the lymphocytes incubated for 48 h).

We have demonstrated that total cell death is the same for both machines, but the samples irradiated with 5 Gy doses generated by Rokus-AM tend to have higher apoptotic activity (Fig. 4, D). However, the analysis of cell death types reveals that the number of apoptotic cells (annexin V positive) is significantly higher ($p < 0.05$) in the samples irradiated with ≥ 4 Gy doses generated by Mir-M. The level of necrosis does not exceed 12.0 ± 2.2 % (6 Gy) for Mir-M and is 44.0 ± 8.1 % for Rokus-AM (6 Gy), at $p = 0.0029$.

DISCUSSION

Because Angara-5-1 and Mir-M are unique facilities (there are no other similar stations in the world), it is difficult to compare the results of our study with the findings of other researchers. A few authors studied the biologic effect of ultrahigh intensity photon beams [6, 7, 16], but they did not differentiate between various types of cell death induced by gamma rays and only estimated total cell death or compared anticancer effect of radiation in animals.

We have compared the apoptotic effect of high intensity bremsstrahlung radiation generated by Angara-5-1 and Mir-M and gamma rays produced by Rokus-AM on the peripheral blood lymphocytes of healthy donors and discovered that the former are more proapoptotic. Apoptosis increases linearly in the dose range below 6 Gy and shows dependence on post-irradiation incubation time (24 or 48 h). However, some authors report no differences in apoptosis levels stimulated by varying dose rates [16]. For example, Kotenko et al. have demonstrated that although higher dose rates cause more double-strand DNA breaks and affect mechanisms of DNA repair, the proportion of apoptotic cells remains stable. Similar results are reported by [17]. However, the authors studied therapeutic dose ranges, but never answered a question why disrupted mechanisms of DNA repair in combination with double-strand breaks do not enhance apoptosis. Probably the difference between those studies and our experiment lies in the specifics of the selected biologic models. We irradiated mononuclear cells isolated from peripheral blood, whereas in the studies [16, 17] human fibroblasts were used. Besides, Angara-5-1 and Mir-M generate extremely high dose rates of 100 MGy/s, which may have affected the outcome of our experiment. Perhaps, such dose rates "turn off" reserve DNA repair mechanisms that are still functional at 400 mGy/min (0.017–6.7 mGy/s) [17]. Maybe, double-strand breaks caused by such high dose rates have a specific spatial configuration that blocks repair mechanisms (the breaks are too "deep"). It should be noted that radiosensitivity of cells depends on the stage of the cell cycle and is the lowest for non-dividing cells, such as peripheral blood mononuclear cells (lymphocytes). Non-diving tumor cells constitute the largest population of radioresistant cells. In this light, our findings could be of some interest to the developers of radiation treatments based on the use of gamma rays with ultrahigh dose rates.

We have also discovered that necrosis induced by high intensity radiation is significantly less intense that necrosis induced by therapeutic gamma rays. In the dose range below 6 Gy, necrosis increases proportionally to the dose. The analysis of total cell death in peripheral blood lymphocytes shows that 2 different dose rates applied during one-time exposure to ≤ 6 Gy causes more or less the same number of cells to die, regardless of the type of particle accelerator. This is consistent

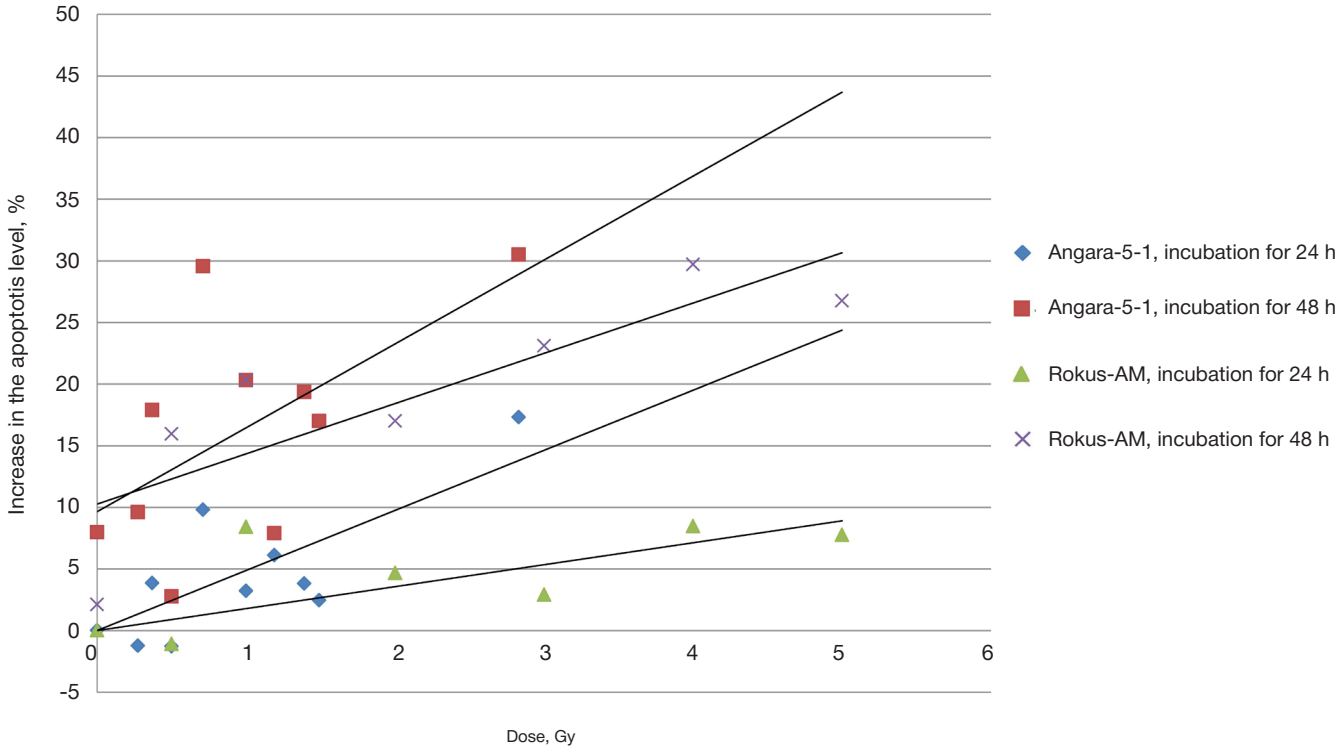

Fig. 3. Levels of apoptosis in the suspensions of peripheral blood lymphocytes irradiated by Rokus-AM and Angara-5-1 with doses from 0 to 5 Gy

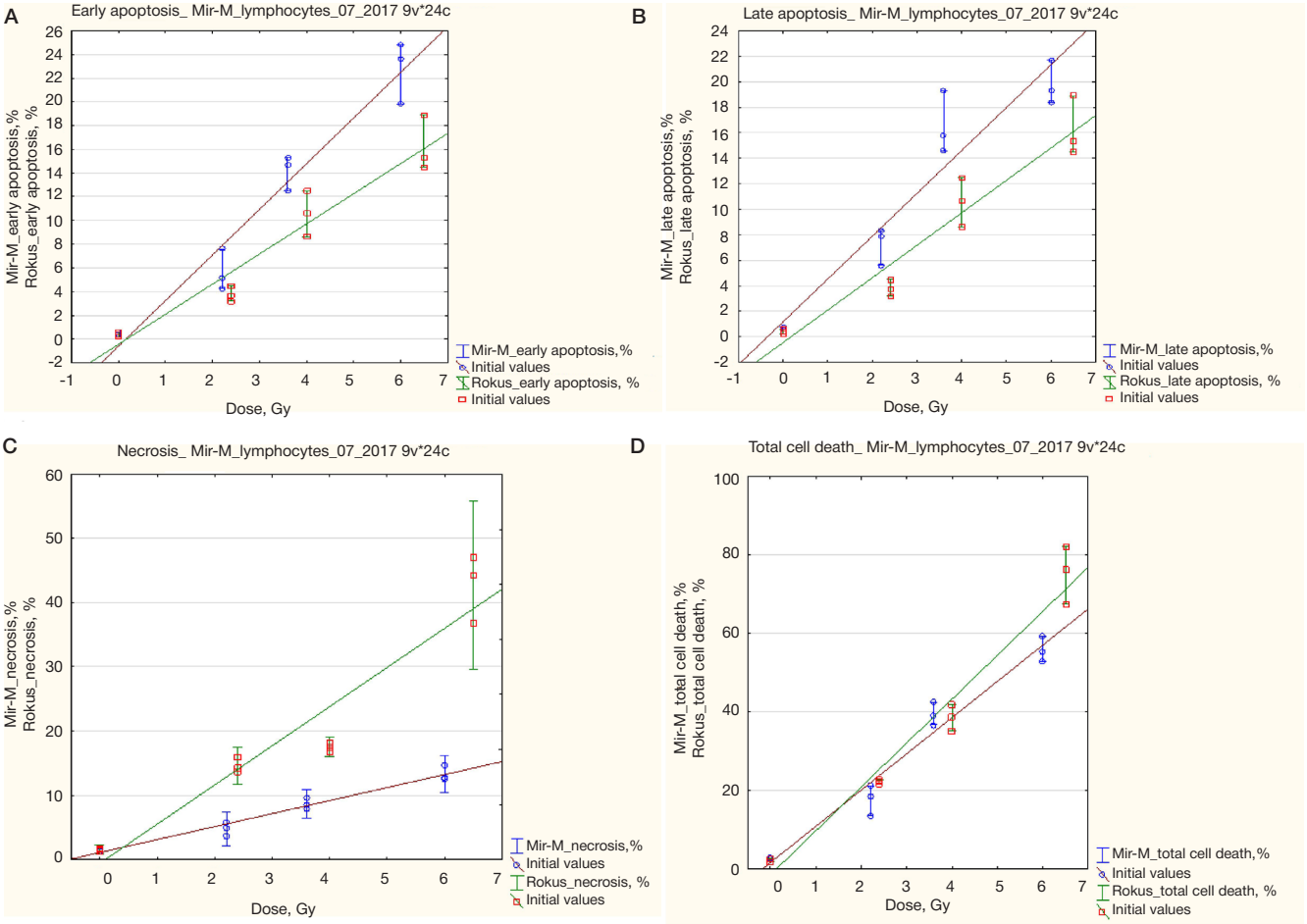

Fig. 4. Comparison of the effects induced by electron beams generated by high-current nanosecond electron accelerator Mir-M and gamma rays produced by Rokus-AM on human peripheral blood lymphocytes in the dose range from 0 to 6 Gy. **(A)** Early apoptosis (annexin V+/PI–); **(B)** late apoptosis (annexin V+/PI+); **(C)** necrosis (annexin V–/PI+); **(D)** total cell death. PI — propidium iodide. Positive and negative staining is represented by + and – respectively.

with the findings of other researchers. For example, in the work by Brüchne et al. [9] the depressing effects of therapeutic gamma rays and high intensity laser beams on KHT mouse fibrosarcoma were comparable.

CONCLUSIONS

In the dose range below 6 Gy, total cell death induced by high intensity gamma rays is comparable with that caused by therapeutic gamma machine. Higher dose rates induce apoptosis, while lower does rates induce necrosis.

Our findings suggest that higher dose rates could be more beneficial for patients than lower dose rates, because intense apoptosis is more “physiological” and will lead to fewer complications than massive necrotic cell death (tissue decay, intoxication, damage to healthy tissue, etc.). Further research is necessary to investigate mechanisms of apoptosis induction by ultra high dose rates in non-dividing (interphase) cells and to compare the effects of radiation at different stages of cell cycles.

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THE CHOICE OF ANESTHETIC TYPE AND CONDITIONS FOR 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE STAINING OF BRAIN SLICES IS IMPORTANT IN THE ASSESSMENT OF ISCHEMIC INJURY IN RATS IN THE EARLY STAGES OF PATHOLOGY

Bilan DS[1,2], Kelmanson IV[1], Belousov VV[1,2] ✉

[1] Laboratory for Molecular Technologies,
Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russia

[2] Department of Brain-Computer Interfaces, Research Institute for Translational Medicine,
Pirogov Russian National Research Medical University, Moscow, Russia

Studies of ischemic brain injury are an important area of modern biomedical research. So far, a lot of ischemic stroke models have been proposed, along with different imaging and staining modalities aimed to visualize the damaged tissue. In this work we use a rat model to investigate how the experimental setup affects the interpretation of experimental data obtained in the acute phase of ischemic stroke (5 hours after the occlusion of the middle cerebral artery). We show the association between the choice of the type of anesthesia and the severity of ischemic injury: in our experiments brain damage was the most pronounced in the animals anesthetized with a combination of chloral hydrate and Rometar; the least damage was observed for isoflurane. Staining was performed using the popular dye 2,3,5-triphenyltetrazolium chloride (TTC). We demonstrate that parameters of brain slices incubation in TTC also need to be accounted for when interpreting the results obtained during the acute phase of stroke, the optimum incubation time being 30 min and temperature 37 °C.

Keywords: stroke, ischemic injury, brain slices, 2,3,5-triphenyltetrazolium chloride, staining, anesthesia, rats

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✉ **Correspondence should be addressed:** Vsevolod Belousov
ul. Miklukho-Maklaya, d. 16/10, Moscow, Russia, 117997; belousov@ibch.ru

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ВЛИЯНИЕ ТИПА АНЕСТЕЗИИ И УСЛОВИЙ ПРОКРАШИВАНИЯ ТКАНЕЙ МОЗГА КРАСИТЕЛЕМ 2,3,5-ТРИФЕНИЛТЕТРАЗОЛИЕМ ХЛОРИСТЫМ (ТТХ) НА ОЦЕНКУ ИШЕМИЧЕСКОГО ПОВРЕЖДЕНИЯ МОЗГА КРЫС НА РАННИХ СТАДИЯХ ПАТОГЕНЕЗА

Д. С. Билан[1,2], И. В. Кельмансон[1], В. В. Белоусов[1,2] ✉

[1] Лаборатория молекулярных технологий,
Институт биоорганической химии имени академиков М. М. Шемякина и Ю. А. Овчинникова РАН, Москва

[2] Отдел нейро-компьютерных интерфейсов, НИИ трансляционной медицины,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

Изучение ишемического повреждения головного мозга является важным направлением современных медико-биологических исследований. К настоящему моменту разработано множество моделей ишемического инсульта, а также предложены различные способы визуализации поврежденных тканей мозга. В данной работе мы исследовали, как различные условия проведения эксперимента, моделирующего ишемический инсульт у крыс, влияют на интерпретацию результатов в острой фазе заболевания (5 ч с момента окклюзии средней мозговой артерии крыс). Мы показали, что на ранней стадии развития патологии существенное влияние оказывает выбор используемой анестезии животных. В наибольшей степени повреждение мозга было выражено при использовании для анестезии смеси хлоралгидрат/Рометар, в наименьшей — при использовании изофлурана. Для визуализации повреждения мозга животных мы использовали наиболее популярный краситель 2,3,5-трифенилтетразолий хлористый (ТТХ). Мы установили, что температура и время инкубации срезов мозга в растворе ТТХ также значительно влияют на интерпретацию результатов при оценке ишемического повреждения в острой фазе патологии. Оптимальными условиями окрашивания срезов мозга в растворе ТТХ являются 30-минутная инкубация срезов при 37 °C.

Ключевые слова: инсульт, ишемическое повреждение, срезы мозга, 2,3,5-трифенилтетразолий хлористый, окрашивание, анестезия, крысы

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✉ **Для корреспонденции:** Белоусов Всеволод Вадимович
ул. Миклухо-Маклая, д. 16/10, г. Москва, 117997; belousov@ibch.ru

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Ischemic stroke is one of the most serious neurological conditions and the second leading cause of death and disabilities worldwide after cardiovascular diseases [1–4]. So far, no effective treatment strategies have been proposed for this disease, and its pathogenesis remains understudied.

Of all currently existing models of ischemic stroke [5–12], monofilament occlusion of the middle cerebral artery stands out as the most common. First described by Koizumi et al. [13], it has been improved and adapted for use in different laboratory animals, such as rats [14] and mice [15].

Along with the variety of ischemic stroke models, there are different techniques allowing visualization of stroke-induced tissue damage. Infarcted zones of brain sections can be made visible using histological stains, such as traditional hematoxylin and eosin [16, 17], or Nissl staining and its modifications [18, 19]. Impregnation of nervous tissue with silver is reported to be helpful in detecting neuronal degeneration in the early stages of stroke [20, 21]. The same is true for Fluoro-Jade stains [22–24], but the exact mechanism of their action is still unknown. One of the simplest techniques to visualize ischemic lesions in brain slices is 2,3,5-triphenyltetrazolium chloride (TTC) staining [25]. Enzymes with dehydrogenase activity found in living cells reduce TTC to formazan, which stains healthy tissue deep red, whereas damaged tissue lacking healthy mitochondrial activity resists staining. Immunohistochemistry also has something to offer and can be employed to observe apoptotic cells in the lesion [26, 27]. Non-invasive techniques for stroke diagnosis include magnetic resonance imaging [28], positron emission tomography [29] and single-photon emission computed tomography [30]. The list of approaches to ischemic injury visualization is not limited to these modalities; detailed information is available in themed reviews [31].

Because approaches to studying stroke pathogenesis and developing treatment strategies are so different, the Stroke Therapy Academic Industry Roundtable (STAIR) has prepared a series of guidelines on ischemic stroke modeling [32–35], describing, in particular, a number of factors affecting its results and their interpretation, such as the selected model itself, the animal's breed, the type of an anesthetic, the visualization technique, etc.

Even protocols for standard interventions may vary greatly. For example, TTC staining, which is now the most common technique used to visualize ischemic areas in brain slices, was originally performed on rats' brain sections 24 hours after induced occlusion (the brain sections were incubated for 30 min at 37 °C) [25]. However, some authors were able to visualize infarcted tissue using TTC staining just a few hours after occlusion [21, 36–43]. Incubation time of brain slices in the TTC solution may vary from 5 min [44] to standard 30 min [25]. Some protocols warn that TTC is unstable when heated, therefore, staining should be performed at room temperature [45]. TTC is mainly used for staining brain slices, but sometimes animals are perfused with TTC transcardially [38, 46].

In this work we show that effective visualization of damaged tissue obtained from rats with acute ischemia depends largely on temperature and duration of incubation of brain slices in the TTC solution. These two factors can skew interpretation of the results. We also demonstrate that the type of an anesthetic affects the scope of ischemic injury in the early stage of stroke (5 hours after the occlusion), while in the later stages (24 hours after the occlusion) its role is insignificant.

METHODS

Experiments involving animals were carried out in compliance with the Directive 2010/63/EU of the European Parliament and the European Council, dated September 22, 2010. The study protocol was approved by the Animal Care and Use Committee of the Institute of Bioorganic Chemistry, RAS.

The study was carried out in male Wistar rats (weight ranging from 280 g to 330 g) purchased from Pushchino breeding facility. The rats were kept in the animal house of the Institute of Bioorganic Chemistry in plastic cages, 3 animals per cage. The animals had free access to water and food.

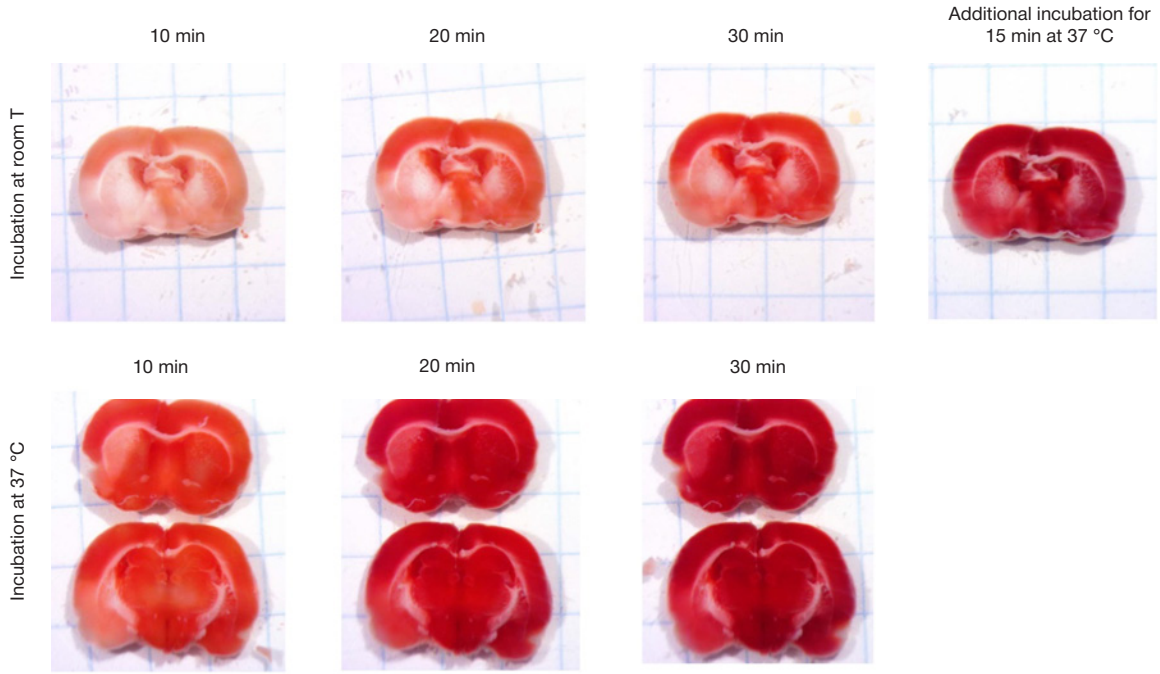

Fig. 1. Effects of different temperatures and duration of incubation of rat brain slices in 1 % TTC solution on visualization of ischemic injury 5 hours after the occlusion. The pictures show brain slices obtained from a Wistar rat with the occluded middle cerebral artery. One slice was stained at room temperature, another — at 37 °C. Samples were photographed at set time intervals. Anesthetic used: Zoletil/Rometar

Occlusion of the middle cerebral artery was induced according to the protocol [14]. We used three types of anesthetics:

1. isoflurane (marketed as Aerrane by Baxter, USA): a 5 % concentration for general anesthesia induction and a 1.5 % concentration for anesthesia maintenance.

2. tiletamine hydrochloride/zolazepam hydrochloride (Zoletil by Virbac Sante Animale, France; 40 mg/kg) + xylazine hydrochloride (Rometar by Bioveta, Czech Republic; 10 mg/kg), injected intraperitoneally;

3. chloral hydrate (Dia-M, Russia, 400 mg/kg).

The animals were analgesized with 5 mg/kg ketoprofen (Ketonal by Sandoz, Switzerland) administered subcutaneously; local analgesia was induced by administering 2 % Novocain.

In our study we used commercial middle cerebral artery sutures by Doccol (USA; catalog number 403756PK10Re) 0.185 mm in diameter.

The rats were decapitated after set time intervals, their brains removed and cut into 2 mm thick frontal sections, which were then placed in 1 % TTC solution (Sigma-Aldrich, USA). Staining was done at different temperatures (20 °C and 37 °C).

RESULTS

In an attempt to investigate how different TTC staining conditions affect visualization of ischemic lesions, we modeled middle cerebral artery occlusion in rats [14]. The occlusion was permanent, i. e. the vessel remained blocked throughout the experiment. The animals were anesthetized with a mixture of Zoletil and Rometar injected intraperitoneally. Five hours after the occlusion the brains were removed and cut into 2 mm thick frontal sections. Then, some slices were incubated in 1 % TTC solution at room temperature, while other were placed into TTC preheated to 37 °C. Photos of brain sections were taken at equal time intervals to assess how different temperatures and duration of incubation in the TTC solution affected visualization of ischemic tissue. Lesions became visible after 10 min of incubation at both temperatures: unlike the intact areas, they were weakly stained (Fig. 1). Further incubation in TTC at 37 °C produced a more intense color; after 20 min of incubation the color contrast between the healthy and ischemic tissues became less pronounced, as the damaged tissue developed an intermediate pink color. However, at room temperature the color contrast between the damaged and healthy tissues increased. Longer incubation at 37 °C produced a well-developed color throughout ischemic areas (Fig. 1). It is very important to control TTC staining conditions when only a short time has elapsed after occlusion induction, because damaged tissue may still contain living cells affecting color development. Twenty-four hours after the occlusion, the injury was clearly visible, and the color contrast between the lesion and the healthy tissue did not lose its intensity even after 2 hours of incubation at 37 °C.

Our next step was to find out how a choice of an anesthetic influences the scope of ischemic brain injury. Damaged tissue was visualized using TTC staining. In this series of experiments

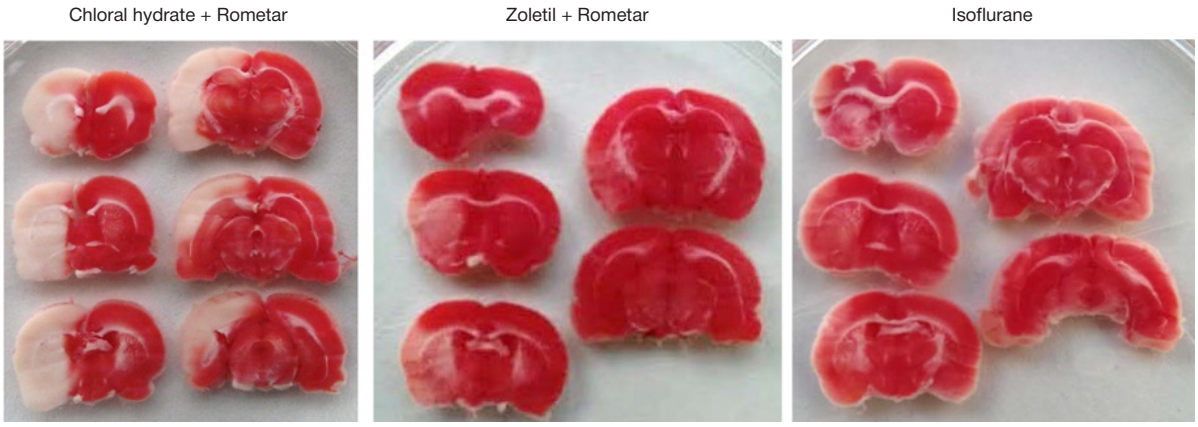

Fig. 2. Effects of different anesthetics on the scope of ischemic injury in rats with the permanently occluded middle cerebral artery (5 hours after the occlusion). Brain slices were incubated under identical conditions in 1 % TTC solution for 30 min at 37 °C

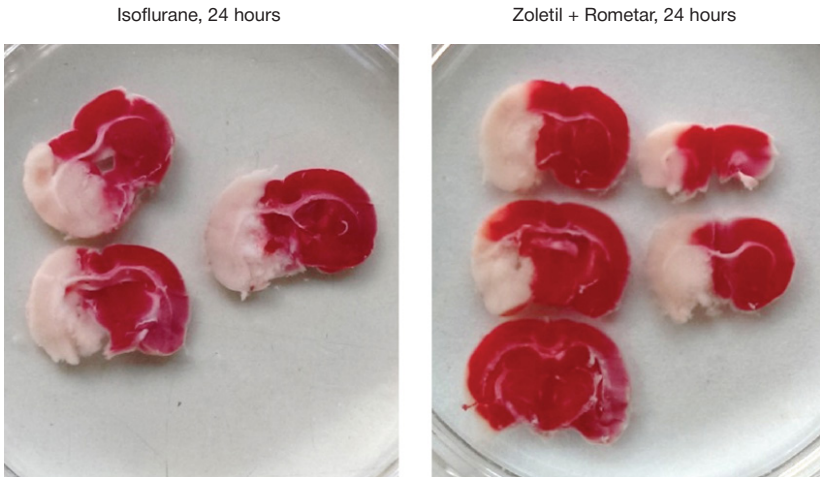

Fig. 3. Brain slices of rats anesthetized with different drugs 24 hours after the induced permanent occlusion of the middle cerebral artery. The slices were incubated under identical conditions in 1 % TTC solution for 30 min at 37 °C

we also modeled permanent middle cerebral artery occlusion in Wistar rats. The animals were anesthetized using three types of anesthetics: isoflurane (Aerrane), a mixture of Zoletil and Rometar injected intraperitoneally and a mixture of chloral hydrate and Rometar also injected intraperitoneally. Five hours after the occlusion the brains were removed, sectioned, and incubated in 1 % TTC solution at 37 °C for 30 min. The lesion size was the smallest in the animals who had received isoflurane (this was reliably demonstrated in 6 animals), and the color contrast between the damaged and healthy TTC-stained tissues was minimal. The most severe damage was observed in the animals who had received a mixture of chloral hydrate and Rometar (this was reliably demonstrated in 5 animals). The Rometar/Zoletil mix produced interesting results. Of 7 animals, only 2 developed massive stroke; in 5 other animals the lesions did not develop a contrasting color during staining (Fig. 2). To sum up, the choice of an anesthetic is an important factor that must be accounted for when studying acute ischemia. The underlying cause of the contributions made by anesthetics is not clear, though. The neuroprotective effect of isoflurane has been reported by a number of authors [47–49], but its mechanism remains unexplained. Interestingly, 24 hours after the occlusion of the middle cerebral artery in rats, the size of the lesion did not depend on the type of an anesthetic (Fig. 3).

DISCUSSION

We have analyzed how different factors affect the results of TTC staining of brain sections obtained from rats with induced permanent ischemia. Our study demonstrates that visualization of damaged tissue in the early phases of stroke (5 hours after the occlusion) is particularly sensitive to TTC staining conditions (incubation temperature and duration) and the type of an anesthetic. Therefore, we do not recommend TTC staining for assessing the size of the lesion in the early stages of ischemic stroke, regardless of the opinion expressed in a number of academic works.

Besides, TTC staining does not provide unambiguous evidence about the viability of cells in the ischemic tissue during the acute stage. TTC is an indicator of mitochondrial dehydrogenase activity. A number of studies confirm that mitochondrial dysfunction is one of the major consequences of ischemia [50, 51]. However, an intermediate color developed by tissue during staining raises a question of interpretation. Normally, in healthy tissue TTC is enzymically reduced to formazan, which stains the tissue deep red. In dead tissue this reaction does not happen, and the tissue remains white. But in our experiments the ischemic tissue developed an intermediate pink color whose intensity was growing as the incubation time and temperature of the environment were increasing. In the study [52] the researchers calculated the proportion of intact mitochondria in the brain sections that were subject to TTC staining and developed or did not develop a color. The study showed that about 5 % of mitochondria were intact in the areas that did not stain. Intermediate pink meant that the proportion of functioning mitochondria in the lesion was higher.

It is known that permanent occlusion does not necessarily cause immediate damage to mitochondria, and the latter remain intact for a few hours or even days, while other cell organelles, such as the nucleus, have already been destroyed [52]. In this case TTC-based visualization will not show tissue damage and, therefore, the real picture of progressing pathology will be blurred. A more traumatizing ischemia-reperfusion injury causes more rapid damage to mitochondria, which also should be accounted for when working with certain stroke models. Besides, TTC staining is not recommended for longer than 24 hours following artery occlusion because the lesions can accumulate inflammatory cells with intact mitochondria [52].

CONCSLUIONS

Our study conducted in rats with the permanently occluded middle cerebral artery demonstrates that estimates of the ischemic injury size in the early stages of stroke are affected by a number of factors, including the type of an anesthetic and staining conditions. Five hours after the occlusion, the least damage was observed in rats anesthetized with isoflurane; the most severe damage was observed in the animals who had received the chloral hydrate/Rometar mix. The optimum conditions for TTC staining of brain slices are 30 min incubation at 37 °C. Protocols that recommend a shorter incubation time and lower temperatures can yield incorrect results for the samples obtained in the early stages of stroke. But 24 hours after the occlusion damaged areas can be effectively visualized using TTC staining, regardless of incubation time/temperature and the selected anesthetic. Therefore, 24 hours are optimal for qualitative and quantitative TTC-based analysis of ischemic brain injury.

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TEMPORAL DYNAMICS OF CYTOKINES IN THE BLOOD OF RATS WITH EXPERIMENTALLY INDUCED AUTOIMMUNE ENCEPHALOMYELITIS

Pozdniakova NV[1], Turobov VI[2], Garanina EE[3], Ryabaya OA[1], Biryukova YuK[4], Minkevich NI[2], Trubnikova EV[5], Shevelev AB[6], Kuznetsova TV[7], Belyakova AV[4], Udovichenko IP[2,8] ✉

[1] Blokhin Russian Cancer Research Center, Moscow, Russia
[2] Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Pushchino branch
[3] Kazan Federal University, Kazan, Russia
[4] Chumakov Federal Center for Research and Development of Immunobiological Products, the Russian Academy of Sciences, Moscow, Russia
[5] Kursk State University, Kursk, Russia
[6] Emanuel Institute of Biochemical Physics of the Russian Academy of Sciences, Moscow, Russia
[7] Vavilov Institute of General Genetics of the Russian Academy of Sciences, Moscow, Russia
[8] Pushchino State Institute of Natural Sciences, Pushchino, Russia

In this work we explore the temporal dynamics of cytokines in Dark Agouti rats with experimentally induced autoimmune encephalomyelitis (EAE). The main group consisted of 11 animals who were injected with 100 µl (per leg) of spinal cord homogenate obtained from random-bred rats and combined with incomplete Freund's adjuvant to the hind footpads. The control group included 7 animals who received 100 µl of normal saline mixed with incomplete Freund's adjuvant. Blood samples (500 µl) were collected daily, starting from day 1 through day 7. We ran a Bio-Plex-based multiplex cytokine assay on the samples using the Bio-Plex Pro Rat Cytokine 24-plex Assay kit. EAE in rats was shown to simulate progression of multiple sclerosis in humans in terms of temporal dynamics of lymphoproliferative and hematopoietic factors IL-1b, IL-2, IL-4, IL-5, IL-6, and IL-7. The studied model satisfactory imitates the dynamics of factors stimulating migration of lymphocytes, monocytes and other immune cells, including IL-17, RANTES (CCL-5) and MCP-1 (CCL-2) but excluding GRO/KC (CXCL1), which shows a different dynamics. The model also resembles patterns of human multiple sclerosis in terms of factors affecting cytotoxic and apoptotic reactions, including IFNγ, IL-6 and IL-17, but excluding TNFα.

Keywords: multiple sclerosis, experimental autoimmune encephalomyelitis, myelin, immunization, multiplex cytokine assay

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✉ **Correspondence should be addressed:** Igor Udovichenko
Pr-t Nauki, d. 6, Puschino, Moscow oblast, Russia, 142290; iudovichenko1@yandex.ru

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ВРЕМЕННАЯ ДИНАМИКА ЦИТОКИНОВ В КРОВИ ПРИ ЭКСПЕРИМЕНТАЛЬНОМ АУТОИММУННОМ ЭНЦЕФАЛОМИЕЛИТЕ У КРЫС

Н. В. Позднякова[1], В. И. Туробов[2], Е. Е. Гаранина[3], О. А. Рябая[1], Ю. К. Бирюкова[4], Н. И. Минкевич[2], Е. В. Трубникова[5], А. Б. Шевелев[6], Т. В. Кузнецова[7], А. В. Белякова[4], И. П. Удовиченко[2,8] ✉

[1] Национальный медицинский исследовательский центр онкологии имени Н. Н. Блохина, Москва
[2] Филиал Института биоорганической химии имени академиков М. М. Шемякина и Ю. А. Овчинникова РАН, Пущино
[3] Казанский (Приволжский) федеральный университет, Казань
[4] Федеральный научный центр исследований и разработки иммунобиологических препаратов имени М. П. Чумакова РАН, Москва
[5] Курский государственный университет, Курск
[6] Институт биохимической физики имени Н. М. Эмануэля РАН, Москва
[7] Институт общей генетики имени Н. И. Вавилова РАН, Москва
[8] Пущинский государственный естественнонаучный институт, Пущино

Изучена динамика содержания цитокинов у крыс линии Dark Agouti с индуцированным экспериментальным аутоиммунным энцефаломиелитом (ЭАЭ). В экспериментальную группу включили 11 животных, которым в подушечки задних лап инъецировали гомогенат спинного мозга беспородных крыс, смешанный с неполным адъювантом Фрейнда. В контрольную группу включили 7 животных, которым в подушечки задних лап вводили по 100 мкл физиологического раствора, смешанного с неполным адъювантом Фрейнда. У животных ежедневно с 1 по 7 сутки отбирали по 500 мкл крови. Был выполнен мультиплексный цитокиновый тест с помощью набора реагентов Bio-Plex Pro Rat Cytokine 24-plex Assay на платформе Bio-Plex. Показано, что в контексте цитокинового профиля модель ЭАЭ у крыс отражает течение рассеянного склероза у человека в части динамики содержания системных лимфопролиферативных и гемопоэтических факторов: IL-1b, IL-2, IL-4, IL-5, IL-6 и IL-7. В части динамики факторов таксиса лимфоцитов, моноцитов и других клеток иммунной системы изученная модель удовлетворительно имитирует динамику содержания IL-17, RANTES (CCL-5) и MCP-1 (CCL-2), но отличается по динамике GRO/KC (CXCL1). В отношении факторов, влияющих на цитотоксические и апоптотические реакции, сходство модели с заболеванием человека было выявлено по таким ключевым факторам, как IFNγ, IL-6 и IL-17, но не по TNFα.

Ключевые слова: рассеянный склероз, экспериментальный аутоиммунный энцефаломиелит, миелин, иммунизация, мультиплексный цитокиновый тест

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✉ **Для корреспонденции:** Удовиченко Игорь Петрович
Пр-т Науки, д. 6, г. Пущино, Московская обл., 142290; iudovichenko1@yandex.ru

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Multiple sclerosis (MP) is a sever neurodegenerative autoimmune disorder. Due to its high prevalence and the severity of symptoms causing partial or complete loss of mobility, multiple sclerosis remains a pressing problem, prompting a search for new therapies. Most patients with MS completely loose the mobility 25 years after the onset of the disease. More than a half of MS patients become dependent on crutches 15 years after appearance of the first symptoms. To date, there is no effective causal treatment for MS.

Usually the disease strikes at young age: 70 % to 80 % of patients suffer the first symptoms of MS between 20 and 40 years of age [1]. MS is diagnosed by neurological examinations, magnetic resonance imaging of the central nervous system, and by biopsy or autopsy [2]. MS has numerous clinical manifestations indicating damage to the spinal cord, the brain, cranial nerves, the cerebellum, and cognitive function. Current diagnostics are insufficient for accurate estimation of MS severity. MRI, electroencephalography and lumbar puncture can still be inconclusive, in spite of providing valuable information about patient's condition. In patients with MS, many symptoms can be caused by infection, vascular pathology, or autoimmune comorbidities [3].

There are four types of MS: relapsing-remitting (RRMS, alternating periods of relapses and remissions) occurring in 80 % to 85 % of patients; primary progressive (PPMS) occurring in 10 % to 15 % of patients; progressive-relapsing (PRMS) — in 5 % of patients; and secondary-progressive (SPMS) [4, 5]. About half of patients with RRMS develop symptoms of SPMS 10 years after the onset of the disease. Over 90 % of patients with RPMS eventually demonstrate SPMS symptoms [6].

The hallmark of MS is destruction of the myelin sheaths of neurons in the central nervous system caused by clustering T- and B-cells. Another typical feature of this disease is accumulation of oligoclonal antibodies in the cerebrospinal fluid. It is not clear, though, how and where the clonal expansion of lymphocytes specific for myelin basic protein is initially triggered. We do not know yet whether it happens in the CNS, where the myelin sheath is directly involved, or outside of it, with autoreactive species migrating to the CNS from other places [7].

Development of effective MS treatments is impossible without animal models accurately replicating the course of the disease in humans, such as experimental autoimmune encephalomyelitis (EAE) of rats and mice. EAE is induced by injecting myelin or basic myelin protein (MBP) suspensions in incomplete Freund's adjuvant into the hind footpads of rodents [8]. One month after immunization the mice develop hind limb paralysis which lasts for 4–6 months [9]. In Dark Agouti (DA) rats, EAE progresses more rapidly (paralysis sets in on days 10–11 and lasts until day 14). The key difference of EAE in animals from MS in humans is full recovery of rodents, which is absolutely unattainable for humans at this point.

An interesting study [10] reports cytokine profiles of 19 patients with MS, including 16 patients with RRMS, 1 individual with PPMS, and 2 — with SPMS. The patients were distributed into groups based on disease duration from the moment of diagnosis: 4.2 ± 0.8 months in group 1 and 76.6 ± 14.3 months in group 2. The study showed that in earlier stages of MS (in comparison with later stages and the absence of the disease), interferon gamma (IFNγ) and the anti-inflammatory lymphokine IL-10 dominate in the cytokine profiles. In the late stage, the levels of IL-1RA, IL-8, IL-12(p70), CCL-3, CCL-7, CCL-11, CXCL-10, FGF, and IFNγ go down. Later stages are also characterized by elevated levels of IL-1a, IL-1b, IL-2RA, IL-3, IL-4, IL-7, IL-12(p40), IL-18, CCL-5 (RANTES), CCL-27, HGF, MIF, M-CSF and TRAIL. Interestingly, MS patients were shown to have elevated blood levels of IL-17, known to play a key role in triggering development of psoriatic skin lesions [11]. In addition, patients with RRMS exhibited elevated IL-22 levels. Dynamics of cytokine profiles in the cerebrospinal fluid drove the researchers [10] to the conclusion about the crucial role of the accumulating IFNγ and MIF (a key factor of joint capsule degeneration in osteoarthritis) and a few other factors stimulating migration of lymphocytes: CCL-5 (RANTES), CCL-2 and CCL-27, induced by IFNγ and MIF. The study also revealed accumulation of proapoptotic TNF-α and TRAIL-ligand in the cerebrospinal fluid (but not blood) of MS-stricken patients.

These data suggest a few patterns typical for MS, including increased long-term systemic activity of hematopoietic growth factors, in particular those targeting granulocytes, sustained Th1-response, and overrepresentation of lymphocyte/monocyte migration factors in the absence of pronounced proinflammatory response (factors stimulating production and taxis of neutrophils). The study [10] could provide an insight into how cytokine levels observed in the cerebrospinal fluid and blood change in patients with MS, but due to the limitations of the applied statistical methods, significance of the identified patterns is questionable.

Considering the above said, our study aimed to

1) investigate the short-term dynamics of cytokines in rats with rapidly progressing induced EAE;

2) compare the data on cytokine levels in patients with MS and in rats with induced EAE in order to assess the feasibility of the EAE rat model for testing anti-MS candidate drugs.

METHODS

Induction of EAE in rats

Experiments involving laboratory animals were carried out in compliance with the "Regulations for the use of Experimental Animals" (Addendum to Order 755 of the Ministry of Health of the USSR dated August 12, 1977) and the principles of the Declaration of Helsinki (2013).

Homogenates of the spinal cord of random-bred rats were prepared as described in [12]. Further *in vivo* experiments were carried out in Dark Agouti rats weighing 220–250 g. The main group included 11 animals. On day 0 the animals were injected with the spinal cord homogenate mixed with incomplete Freund's adjuvant in the ratio of 1 : 1 into the hind footpads. The total volume of the injected mixture was 100 µl per paw. The controls (n = 7) received 100 µl of normal saline mixed with incomplete Freund's adjuvant in the ratio of 1 : 1. From day 1 through day 7, except for day 6, blood samples were collected from the tail vein (500 µl of blood daily) and immediately used for serum preparation. Briefly, blood was placed into Vacuette Z serum sepclot activator vacuum test tubes and centrifuged for 15–20 min at 2,500 rpm and +4 °C. The obtained serum (about 100 µl) was transferred to microcentrifuge tubes and frozen at –20 °C. The animals were weighted daily, and the severity of the disease was assessed using the following scale: 0 points — no symptoms, 1 point — decreased tail tone, 2 points — impaired righting reflex, 3 points — partial paralysis, 4 points — complete paralysis, 5 points— moribund or dead. In borderline cases, a lower index value was opted. Clear signs of EAE appeared in the controls starting from day 8 to day 14 of the experiment. On days 11–14 the disease reached its peak, which lasted for 2–3 days.

Multiplex cytokine assay

Serum samples were analyzed on the Bio-Plex platform (Bio-Rad, USA) using the Bio-Plex Pro Rat Cytokine 24-plex Assay (Bio-Rad). This assay employs magnetic beads coated with monoclonal antibodies to rat cytokines. It was performed according to the manufacturer's recommendations and the protocol published in [13]. Serum was divided into 50 µl aliquots for the analysis. Mean fluorescence intensity of each sample was measured on Luminex 200 analyzer (Luminex Corporation, USA). Data were processed using MasterPlex CT and MasterPlex QT analysis software (Hitachi Solutions America, USA). For each analyte a calibration curve was constructed using 7 concentrations expressed as pg per 1 ml serum.

Statistical analysis

Two quartiles and median values of cytokine levels in each group were calculated daily for each cytokine. Then, significance of differences between the groups was tested using the nonparametric Mann-Whitney test and Statistica 8.0 for Windows. At p-value > 0.05 the differences were considered insignificant; we also used 3 significance thresholds: $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$.

RESULTS

The data on the short-term dynamics of cytokine levels in human and animal blood are still scarce. Multiplex assays are expensive, and daily blood tests in MS patients and lab animals can be technically challenging or raise ethical concerns. Data obtained from the controls in the course of our experiment demonstrate that although incomplete Freund's adjuvant injected into the footpads does not induce EAE, it still causes considerable fluctuations of cytokine levels in animals' blood, rendering less reliable the assessment of the impact of the spinal cord homogenate on the course of the disease. Therefore, special statistical methods are needed to analyze the dynamics of cytokine profiles.

All animals included in the main group developed paralysis of the hind legs. The rising phase of the disease was observed on days 11–13, while the decline — on days 12–17. By day 18 all animals had recovered from the paralysis. Blood was collected on days 1 through 7 in the absence of visible signs of EAE.

Tables 1 and 2 show that on day 1 of the experiment the levels of 13 of total 24 analytes (IL-1a, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12(p70), IL-17, IL-18, G-CSF, IFN-γ, RANTES (CCL-5), and MCP-1 (CCL-2)) were significantly higher (by up to 220 % for IL-4) in the main group than in the controls in terms of the second and third significance thresholds (Fig. 1). On day 2 no significant differences were observed for all studied cytokines. On day 3 differences were observed for IL-1b and VEGF (≤ 0.05), but on day 4 again no differences were found. On day 5 the main group demonstrated a considerable decrease in the levels of IL-1a, IL-1b, IL-13, and erythropoietin (Fig. 2). On day 7 the differences between the groups were observed for 14 of 24 studied cytokines. Those were practically the same cytokines that showed differences on day 1, although statistical significance was confirmed for IL-10 and erythropoietin GM-CSF only and was not confirmed for IL-12(p70) and G-CSF (Fig. 3) Of note, the levels of 13 of 14 cytokines in the main group were higher than in the controls. The only exception was GM-CSF that dropped from 8.17 pg/ml to 2.00 pg/ml.

DISCUSSION

A cytokine burst on day 1 of the experiment followed by a drop on day 2 should be interpreted as a manifestation of acute clonal nonspecific response to excess myelin outside the CNS. The response to the myelin manifested as simultaneous release of several lymphoproliferative factors is likely to be stimulated by hyperproduction of IL-1b originating from macrophages, dendritic cells and skin fibroblasts.

Increased cytokine synthesis on days 5 and 7 is, most probably, the result of the step-by-step accumulation of various clonal-specific lymphocytes, including those with autologous reactivity to myelin. Such longitude of the reaction is typical for the systemic clonal expansion of T-cells and eventually leads to visible physiological symptoms.

The most significant differences between the main and the control groups on day 7 were observed for the levels of IL-18 (2,475.85/4,182.05 pg/ml), RANTES (756.78/1,310.78 pg/ml), MCP 1 (CCL 2) (1,909.68/3,300.50 pg/ml) and IL-2 (743.52/1,091.57 pg/ml). Considering that IL-2 has been proved to induce production of other growth and hematopoietic factors [14], an assumption can be made that IL-2 triggers synthesis of such nonspecific immune factors as VEGF and erythropoietin, as well as IL-13, whose synthesis lagged in phase with respect to IL-2. Considering persistently high levels of IL-2 typical for patients with MS [10], this lymphokine seems to play a key role in the mass proliferation of lymphocytes

Table 1. Significance of differences between the main and the control groups of animals calculated by using the Mann–Whitney test with Yates's correction for continuity. Hypothesis tested: the absence of significant differences between the samples. The result is presented as Fisher's p with three significance thresholds: $p > 0.05$ — the difference is insignificant; $0.01 < p \leq 0.05$ — the first significance threshold; $0.001 < p \leq 0.01$ — the second significance threshold; $p \leq 0.001$ — the third significance threshold

Cytokine	Days of the experiment					
	1	2	3	4	5	7
IL-1a	3	–	–	–	2	2
IL-1b	–	–	1	–	2	-
IL-2	2	–	–	–	–	1
IL-4	3	–	–	–	–	2
IL-5	2	–	–	–	–	2
IL-6	3	–	–	–	–	1
IL-7	2	–	–	–	–	2
IL-10	–	–	–	–	–	2
IL-12	2	–	–	–	–	–
IL-13	–	–	–	–	2	–
IL-17	3	–	–	–	–	2
IL-18	3	–	–	–	–	2
Erythropoietin EPO	–	–	–	–	1	2
G-CSF	3	–	–	–	–	–
GM-CSF	–	–	–	–	–	3
GRO/KC	–	–	–	–	–	–
IFN-γ	2	–	–	–	–	1
M-CSF	–	–	–	–	–	–
MIP-3a	–	–	–	–	–	–
RANTES	2	–	–	–	–	2
TNFα	–	–	–	–	–	-
VEGF	–	–	1	–	–	–
Leptin	–	–	–	–	–	–
MCP-1	2	–	–	–	–	2

Table 2. Statistical analysis of changing cytokine levels in the main group of rats with induced autoimmune encephalomyelitis and the controls

Cytokine	Parameter	Day of the experiment											
		Day 1		Day 2		Day 3		Day 4		Day 5		Day 7	
		Controls	Main group	Controls	Main group	Controls	Main group	Controls	Main group	Controls	Main group	Controls	Main group
IL-1a	Mean	195	387	182	206	268	181	193	144	319	138	203	359
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	87	92	65	61	148	122	113	76	279	75	84	134
	Q25	132	304	133	170	160	68	98	83	182	90	139	218
	Median	188	347	175	204	218	200	195	155	184	121	184	411
	Q75	288	487	226	264	396	237	274	210	396	187	269	462
IL-1b	Mean	433	401	412	274	1033	493	469	265	819	250	319	571
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	386	382	244	184	427	362	432	157	861	164	180	288
	Q25	251	236	211	148	701	134	119	101	279	151	180	300
	Median	315	270	399	222	967	441	412	310	607	214	204	536
	Q75	411	367	550	370	1 477	782	553	409	730	307	504	821
IL-2	Mean	356	607	366	436	367	500	472	414	579	373	744	1092
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	103	138	127	207	209	303	292	180	286	215	245	348
	Q25	278	579	285	319	186	311	213	260	396	205	543	776
	Median	374	597	345	331	332	432	308	362	557	284	657	1145
	Q75	443	626	463	629	523	608	762	647	668	509	914	1368
IL-4	Mean	11	36	9	19	9	24	14	18	19	16	41	90
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	6	16	4	19	8	23	12	16	13	15	27	40
	Q25	6	27	5	5	4	4	4	5	9	4	17	57
	Median	11	30	8	8	6	17	8	12	15	14	33	76
	Q75	17	38	12	37	10	32	29	34	33	23	64	128
IL-5	Mean	77	128	59	69	56	88	69	74	91	68	136	193
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	25	27	33	51	47	59	62	45	43	49	46	34
	Q25	55	110	26	22	23	26	22	20	43	22	106	174
	Median	78	123	63	54	30	83	23	67	88	83	130	193
	Q75	97	147	78	121	113	133	144	106	118	106	176	212
IL-6	Mean	232	503	351	273	468	1595	515	540	600	316	668	1145
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	79	204	226	255	404	3960	572	743	284	269	292	496
	Q25	172	379	169	63	170	96	76	85	414	46	456	640
	Median	224	444	340	182	349	428	190	240	584	261	521	1240
	Q75	287	463	540	470	758	635	1 132	559	713	556	877	1624
IL-7	Mean	103	254	68	123	70	178	111	114	125	106	228	612
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	50	96	62	128	83	169	129	115	77	98	146	236
	Q25	58	181	14	16	12	13	11	16	50	10	119	428
	Median	102	226	63	72	23	144	20	64	109	108	161	724
	Q75	145	350	90	246	159	281	239	224	209	165	391	787
IL-10	Mean	149	240	105	180	121	215	163	157	208	132	403	634
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	70	80	53	131	93	200	129	99	70	106	160	226
	Q25	91	208	61	57	56	63	56	58	144	44	254	420
	Median	142	220	101	183	67	149	88	116	214	116	413	557
	Q75	217	280	129	284	237	277	294	272	287	208	550	821

Продолжение табл. 2

IL-12	Mean	46	125	35	56	43	81	58	54	68	47	155	288
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	29	62	35	61	56	102	68	61	50	59	98	143
	Q25	16	88	5	6	5	6	6	6	15	4	81	173
	Median	49	99	32	23	10	53	12	29	59	26	125	277
	Q75	74	133	46	123	98	118	135	70	104	81	212	425
IL-13	Mean	31	32	15	16	20	22	22	13	19	13	33	61
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	28	31	8	15	8	20	26	11	6	8	21	37
	Q25	18	16	9	6	13	9	7	6	15	6	19	29
	Median	21	20	14	9	20	13	13	9	18	11	23	44
	Q75	28	37	22	20	23	28	26	15	22	17	58	95
IL-17	Mean	24	55	17	25	19	36	26	27	36	25	67	119
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	12	20	15	28	24	36	32	23	22	24	38	43
	Q25	12	43	3	3	3	3	3	4	16	3	38	84
	Median	24	49	16	15	4	34	5	21	35	28	62	108
	Q75	37	56	24	44	50	52	52	42	58	45	102	168
IL-18	Mean	1110	2360	909	1480	976	2004	1351	1829	1628	1562	2476	4182
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	340	822	345	828	600	1513	895	1390	455	1557	774	1495
	Q25	846	1671	623	707	591	582	615	779	1494	426	1663	3219
	Median	1009	2167	877	1444	609	1677	1064	1157	1616	1152	2334	3808
	Q75	1462	2766	1094	2243	1701	3435	2055	2367	1831	2632	3217	4100
Erythropoietin EPO	Mean	202	263	186	242	197	310	238	246	281	175	342	745
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	68	60	77	141	62	240	71	95	86	91	117	345
	Q25	154	235	127	110	158	127	173	153	209	107	249	504
	Median	175	258	179	219	202	272	209	223	247	166	360	585
	Q75	235	287	225	346	241	352	300	346	373	197	415	1060
G-CSF	Mean	3	6	3	4	3	6	4	4	5	4	9	15
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	1	3	1	3	2	6	3	2	1	2	6	9
	Q25	3	4	2	2	2	2	2	2	3	2	4	8
	Median	3	6	3	3	2	4	2	3	4	3	6	12
	Q75	4	7	3	6	5	8	6	5	6	5	14	24
GM-CSF	Mean	5	7	5	5	6	9	8	5	6	4	8	2
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	3	3	3	3	4	10	6	2	4	2	2	1
	Q25	2	6	3	2	3	4	4	3	2	2	7	1
	Median	5	7	6	5	6	9	9	5	5	5	8	2
	Q75	6	10	8	8	9	10	10	7	11	5	10	3
GRO/KC	Mean	161	237	156	165	217	153	201	90	153	116	163	178
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	93	104	79	65	134	97	115	31	128	43	62	57
	Q25	72	124	101	135	75	64	84	62	82	74	103	153
	Median	142	267	140	157	239	133	198	96	90	119	155	170
	Q75	270	331	191	189	341	272	299	116	324	145	184	222
IFNγ	Mean	36	79	30	42	32	63	61	45	53	40	107	215
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	14	46	13	40	23	77	62	41	19	35	45	122
	Q25	27	52	20	14	17	14	17	15	36	12	65	87
	Median	30	64	27	20	20	43	24	28	52	27	98	206
	Q75	46	74	41	60	56	64	95	66	76	69	148	290

Продолжение табл. 2

M-CSF	Mean	132	174	92	89	91	121	100	79	93	104	113	164
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	42	44	46	38	70	83	116	49	54	60	55	74
	Q25	99	143	64	55	32	53	32	33	39	41	52	110
	Median	124	159	80	88	71	106	40	69	69	105	141	149
	Q75	175	205	122	111	174	183	164	121	147	164	165	234
MIP-3a	Mean	70	88	55	49	45	88	69	64	76	45	88	128
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	19	38	44	40	44	95	80	59	45	40	36	44
	Q25	52	47	19	13	12	21	14	13	25	11	65	107
	Median	76	94	41	31	21	53	19	51	91	34	83	139
	Q75	86	122	85	83	99	134	161	83	122	90	102	171
RANTES	Mean	685	1070	342	470	295	696	364	505	574	456	757	1311
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	230	247	277	422	306	541	403	381	208	389	147	388
	Q25	545	966	80	73	108	60	37	51	437	39	726	814
	Median	665	1052	334	387	139	679	57	545	634	660	758	1495
	Q75	805	1129	558	855	481	1212	821	777	687	735	846	1580
TNFα	Mean	58	39	25	30	29	33	30	28	33	27	54	82
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	26	10	15	18	18	19	20	14	11	19	26	42
	Q25	42	29	17	16	16	16	17	16	21	14	32	51
	Median	57	39	19	21	19	27	23	25	36	24	57	72
	Q75	72	49	26	47	46	44	49	34	39	29	82	106
VEGF	Mean	130	87	88	55	128	73	107	46	133	65	92	110
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	110	90	57	79	73	80	78	24	149	49	44	50
	Q25	41	52	42	20	53	37	54	32	33	29	57	57
	Median	119	60	88	35	129	44	78	36	97	46	84	100
	Q75	132	74	128	44	178	84	185	58	136	89	146	139
Leptin	Mean	158	217	59	131	121	264	129	90	231	213	239	460
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	161	240	77	184	163	255	202	167	219	199	237	358
	Q25	10	1	20	11	11	16	3	4	12	17	0	20
	Median	150	137	24	27	21	312	21	20	246	286	324	620
	Q75	301	437	83	273	304	482	375	30	447	419	404	745
MCP-1	Mean	1979	2662	2277	2317	2428	3097	2071	2468	2117	2254	1910	3300
	N	6	10	8	12	6	11	7	11	7	11	7	11
	SD	413	365	949	754	576	1120	830	1141	830	623	557	879
	Q25	1592	2353	1606	1729	2130	2126	1501	1575	1651	1889	1340	2986
	Median	2102	2606	2155	2063	2428	3108	1849	2192	2012	2448	1946	3411
	Q75	2317	2864	2909	3047	2904	3762	2369	3021	2189	2750	2405	3846

outside the CNS. Increasing levels of lymphoproliferative and hematopoietic IL-4, IL-5, IL-6, Il-7, and IL-13 in the backdrop of decreased GM-CSF can be described as a cascade induced with IL-2 participation.

Unlike MS of humans, EAE in rats is not accompanied by production of proapoptotic TNF-α, regardless of the increased synthesis of its classic inducers IL-12, IL-18 and IFNγ [14]. Therefore, elevated levels of TNF-α in patients with MS are rather a result and not the cause of myelin destruction. At the same time, TNF-α can contribute significantly to the damage of astrocytes and neurons in the late stages of MS.

According to the pattern described in [10], simultaneous increase and decrease of IFNγ and RANTES (CCL-5), respectively, in rats with EAE simulate similar processes occurring in humans with MS. The early stages of EAE in rats are not accompanied by an increase in GRO/KC (CXCL1) responsible for lymphocyte infiltration in the CNS, which renders the rat model different from MS in humans [10].

Both rats with EAE and humans with MS have hyperproduction of IL-17 which can contribute to the accumulation of specific lymphocytes in the CNS and activate their toxic function.

In spite of IL-1b hyperproduction, MS in humans shows no signs of neutrophil involvement in the pathology, which is also true for the factors regulating neutrophil taxis and activation. This pattern turned to be no different in the studied rat model.

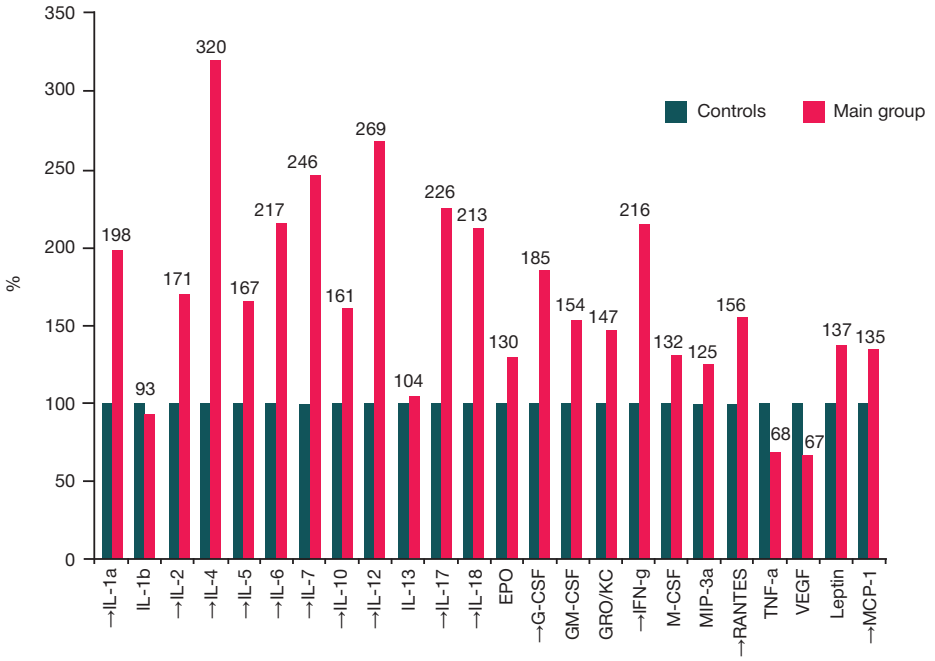

Fig. 1. Changes in cytokine levels in the blood serum of rats with induced EAE in comparison with the controls 1 day after the injection. Cytokine levels in the controls were taken as 100 %. Significant differences are marked with arrows

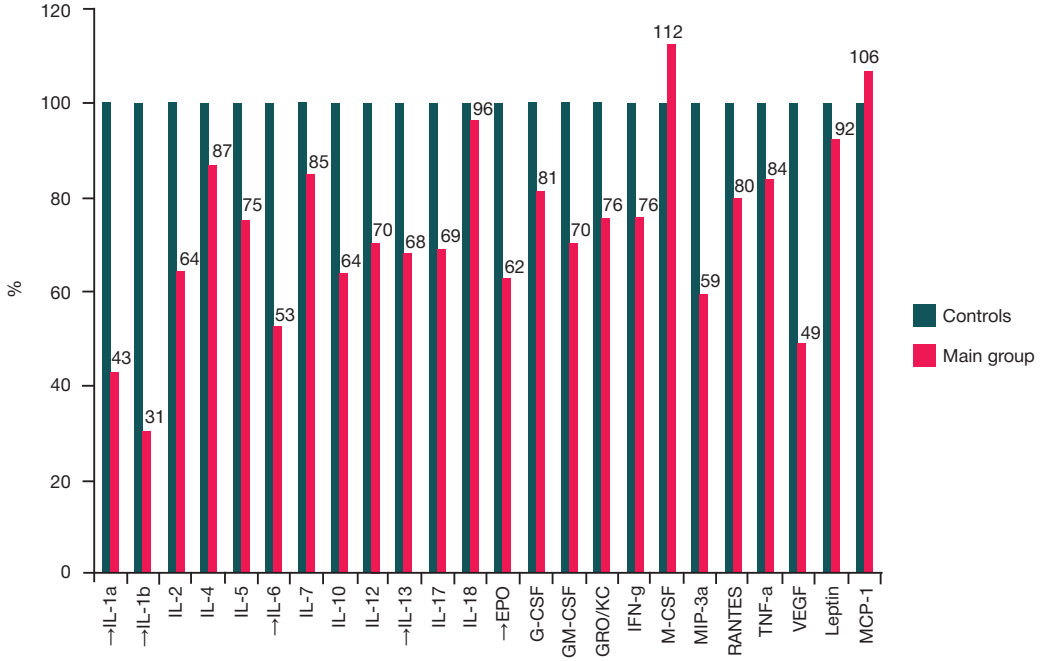

Fig. 2. Changes in cytokine levels in the blood serum of rats with induced EAE in comparison with the controls 5 days after the injection. Cytokine levels in the controls were taken as 100 %. Significant differences are marked with arrows

The levels of M-CSF stimulating proliferation of neutrophil precursors did not change throughout the experiment. The same pattern was observed for MIP-3a (CCL20) that protects mucosa from bacterial infection and for leptin that raises body temperature in infected individuals.

Hyperproduction of IL-4 and IL-10 in rats with EAE in the background of elevated IL-5, IL-13, and GM-CSF should be considered a factor stimulating proliferation of B-cells. In theory, this set of cytokines can trigger synthesis of oligoclonal antibodies, but this effect has not yet been described in the literature.

Our experiment proves that proliferation of myelin-specific lymphocytes can be triggered outside the CNS. However, the course of EAE in rats and the course of MP in humans differ considerably. We cannot rule out that the first event occurring at the onset of the disease is infiltration of the CNS by lymphocytes that do not undergo clonal expansion but do undergo further selection in the presence of excess myelin. Abnormal behavior of lymphocytes observed in the rat model can be a result of their primary clonal-nonspecific hyperproliferation triggered by systemic or local excess of lymphoproliferative factors or/and lymphotaxis factors originating in CNS. Another possibility is induction of abnormally rapid degradation of myelin in CNS leading to a massive release of degradation products into the systemic circulation. In this case the rat model seems to be quite adequate to the early stages of MS in humans.

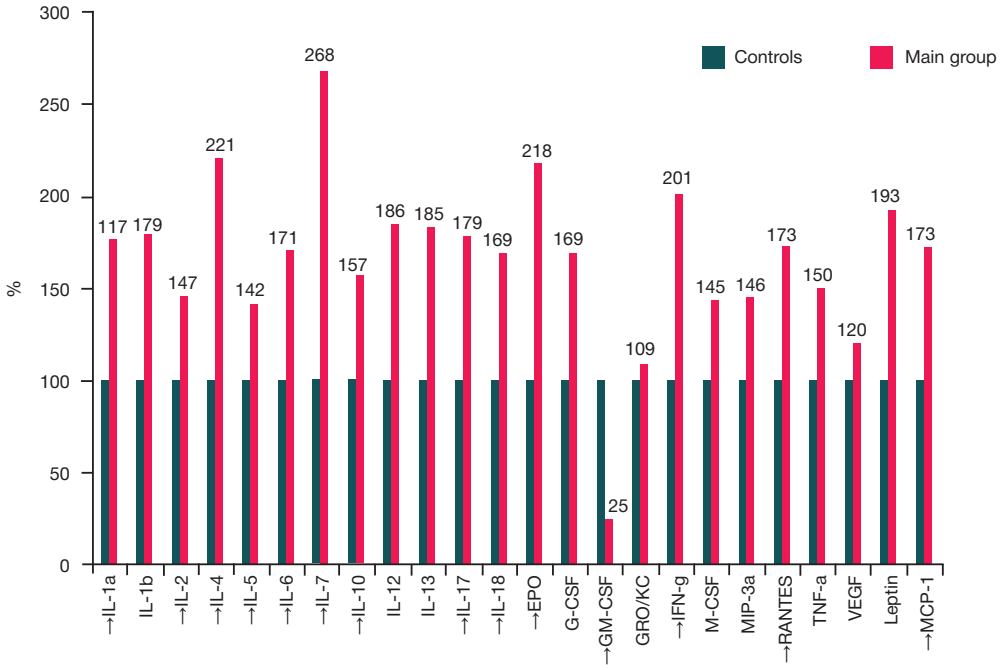

Fig. 3. Changes in cytokine levels in the blood serum of rats with induced EAE in comparison with the controls 7 days after the injection. Cytokine levels in the controls were taken as 100 %. Significant differences are marked with arrows

CONSLUSIONS

Data on the dynamics of cytokine production in rats with EAE obtained with the multiplex cytokine assay suggest that the rat model adequately imitates the course of MS in humans with respect to the levels of systemic lymphoproliferative and hematopoietic factors IL-1b, IL-2, IL-4, IL-5, IL-6 and IL-7. With respect to factors regulating taxis of lymphocytes, monocytes and other immune cells, the model fairly well imitates behavior of IL-17, RANTES (CCL-5) and MCP-1 (CCL-2), but exhibits a different dynamics for GRO/KC (CXCL1) levels. The model resembles the course of MS in humans in terms of IFNγ, IL-6 and IL-17 involved in cytotoxic and apoptotic reactions, but exhibits a different dynamics for TNF-α.

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