BULLETIN OF RSMU

MAY-JUN 2016 (3) ISSN 2070-7320
VESTNIKRGMU.RU

GENOME EDITING REVIEW

BULLETIN OF RUSSIAN STATE MEDICAL UNIVERSITY

Scientific Medical Journal of Pirogov Russian National Research Medical University

ВЕСТНИК РОССИЙСКОГО ГОСУДАРСТВЕННОГО МЕДИЦИНСКОГО УНИВЕРСИТЕТА

Научный медицинский журнал Российского национального исследовательского медицинского университета имени Н. И. Пирогова

The journal is included in the list of the leading peer-reviewed scientific journals and publications approved by the Higher Attestation Commission

The journal is available in Google Scholar.

IF RSCI 2014: 0,134.

Approved for print June 30, 2016.

Address: ul. Ostrovityanova, d. 1, Moscow, Russia, 117997
For papers submission: editor@vestnikrgmu.ru
For collaboration: manager@vestnikrgmu.ru

The mass media registration certificate no. 012769 issued on July 29, 1994
Subscription index in the Rospechat catalog: 46826
Printed by Premium Print
www.premium-print.ru

BULLETIN OF RSMU 3, 2016
ВЕСТНИК РГМУ

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Rebrikov DV[1,2,3] ✉

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

[2] Kulakov Federal Research Center for Obstetrics, Gynecology and Perinatology, Moscow, Russia

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

The rapidly evolving genome editing techniques are steadily moving from research laboratories to clinical practice. Fundamentally new methods of editing the genome of human embryos in the early stages of development have been developed. Tools for correction of genetic disorders in people of any age have also been created. In fact, the doctor is becoming a corrector of genetic instructions on construction and functioning of the human body. This review generalizes the data on the current state of genome editing techniques and existing approaches to applying them in clinical practice.

Keywords: genome editing, gene therapy, hereditary diseases, oncology, HIV

Correspondence should be addressed: Denis Rebrikov
ul. Ostrovityanova 1, Moscow, Russia, 117997; drebrikov@gmail.com

Recieved: 23.06.2016 **Accepted:** 25.06.2016

РЕДАКТИРОВАНИЕ ГЕНОМА ЧЕЛОВЕКА

Д. В. Ребриков[1,2,3] ✉

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

[2] Научный центр акушерства, гинекологии и перинатологии имени В. И. Кулакова, Москва

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

Быстро развивающиеся технологии редактирования генома из научно-исследовательских лабораторий уверенно переходят в клиническую практику. Разработаны принципиально новые методы изменения генома человеческих эмбрионов на ранних стадиях развития. Создан инструментарий для исправления генетических нарушений у людей в любом возрасте. Врач, по сути, становится корректором генетической инструкции по построению и функционированию организма человека. В обзоре обобщены сведения о современном состоянии технологий редактирования генома и существующих подходах к их использованию в клинической практике.

Ключевые слова: редактирование генома, генотерапия, наследственные заболевания, онкология, ВИЧ

✉ **Для корреспонденции:** Денис Владимирович Ребриков
117997, г. Москва, ул. Островитянова, д. 1; drebrikov@gmail.com

Статья получена: 23.06.2016 **Статья принята в печать:** 25.06.2016

Genome changes occur constantly in living organisms, determining the course of their evolution. Man started interfering in this process thousands of years ago, selecting successfully cultivated plants and producing breeds of pets. Genetic engineering, which emerged a little more than half a century ago, made it possible to create transgenic organisms: gene transfer between genomes or gene manipulation within a single genome. The idea of using DNA imported from the outside for treatment of human genetic diseases originated in the early 1970s [1]. In the 1980s, improvement in gene handling techniques and creation of eukaryotic vectors opened up a real opportunity for correction of human genetic material for therapeutic purposes. However, the first successful result was only reported in 1990 [2]. That same year, researchers used a retrovirus to inject a working adenosine deaminase (ADA) gene into the cells of four-year and nine-year old female patients with severe combined immunodeficiency. From 1993, gene therapy was regularly used to treat newborns with ADA deficiency, by delivering the gene into undifferentiated cells of the umbilical cord blood.

We live in the genomics era, and the term ‘genomic therapy’ is increasingly found in science literature today. Perhaps, the use of the terms ‘gene therapy’ and ‘genomic therapy’ needs to be clarified. Since terminology issues are not fundamental, these terms can either be considered as interchangeable or one can consider the term ‘genomic therapy’ as a variant of ‘gene therapy’ in which nuclear genome (chromosomal DNA) changes. The point is that gene therapy may not have anything to do with chromosome — the delivered gene may operate as an extrachromosomal element (plasmid) or can be injected in the form of messenger RNA (mRNA); moreover, mitochondrial DNA may be subjected to modification.

Between 1989 and 2016, over 2,300 clinical trials had been conducted worldwide [3]. To date, there are more or less effective approaches to gene therapy for treatment of over 50 genetically determined diseases in humans, including severe combined immunodeficiency [4], hemophilia [5, 6], hemoglobinopathies [7–13], cystic fibrosis [14,15], achromatopsia [16], Leber’s congenital amaurosis [17–19], epilepsy [20], osteoarthritis [21, 22], Parkinson’s disease [23–25], and a wide range of cancers [26–32].

For the past few years, with the emergence of qualitatively new directed genome change techniques (ZFNs, TALENs, CRISPR/Cas9), areas of applications for clinical trials of gene therapy drugs have skyrocketed in number like an avalanche. Thanks to the simplicity and accuracy of new techniques

deployed to introduce changes in the genomic DNA of eukaryotic cells, a new term 'genome editing' even arose — after all, DNA change may in the future be used not only for therapeutic purposes but also for less important tasks.

Genomic therapy can be applied for: treatment of hereditary (usually monogenic) diseases, treatment of diseases caused by somatic mutations (mostly cancer), and attempts to treat HIV infections by destroying the copies of the virus integrated into the genome or receptor genes allowing the virus to enter the cell. Genomic therapy is one of the variants of personalized medicine, when the approach used is selected individually to the patient's disease (and sometimes even to his genome).

Russia's Federal Law No. 86-FZ dated 5th July 1996 "On State Regulation in the Field of Genetic Engineering" defines gene therapy as a set of genetic engineering (biotechnological) and medical techniques aimed at making changes in the genetic apparatus of human somatic cells for the purpose of treating diseases. With the advent of Federal Law No. 180-FZ dated 23 June 2016 "On Biomedical Cell Products", the number of clinical trials of innovative gene therapy products in Russia is expected to increase.

Genome editing techniques

Although there are a variety of methods for directed change of complex eukaryotic genomes, only a few of the methods are currently used in practice:

a) non-break induced homologous recombination [33];

b) site-specific recombination (recombinase and transposase) [34, 35];

c) repair induced by site-specific nuclease, where the following are used as the nuclease:

– artificial (hybrid, designer) nucleases with zinc finger nucleases (ZFNs) [36–38],

– natural or hybrid endonucleases of gene conversion or meganucleases (homing endonucleases, HEs) [39],

– artificial (hybrid, designer) nucleases with transcription activator-like effector nucleases (TALENs) [40],

– natural RNA-guided nucleases (RGNs), in particular, clustered regularly interspaced short palindromic repeats / CRISPR-associated nuclease 9 (CRISPR / Cas9) with designer 'guide' RNA [41],

– a combination of various nucleases [42–44].

Fig. 1 shows a timeline indicating the evolvement of genome editing techniques [45].

To date, the most promising techniques are those based on the use of artificial (so-called hybrid or designer) site-specific nucleases: ZFNs, TALENs and CRISPR/Cas9 [46]. Although, the term 'hybrid' (or 'designer') nuclease was initially fully applied to 'protein' techniques ZFN and Talen, today the CRISPR/Cas9 technique can be confidently categorized under the same class since the RNA in this system is the designer component (similarly to the 'guide' blocks of the ZF or TALE domain) (fig. 2) [47].

In general, each of these genome editing tools consists of three components: 'guide' specific to DNA sequence (indicating where to cut), cutting DNA 'scissors' (endonuclease) and the actually introduced DNA sequence (not always necessary). Delivery into cells a 'genetic patch' (a DNA fragment to be substituted) is required in those cases where it is necessary to add or substitute a genomic fragment. However, in some cases, only part of the sequence needs to be removed. Table 1 shows the features of the most popular genome editing systems.

We use the 'guide' to indicate endonuclease, where a cut needs to be made in the DNA molecule. The cut is stitched usually through intracellular repair systems (such as double-stranded break repair or homologous recombination).

Since genome editing techniques — based on homologous recombination, recombinase and transposase — have been actively used in clinical practice for over 30 years and they are well described in literature (a Nobel Prize was even awarded in 2007 for homologous recombination techniques), we will not delve into details in this review. Rather we will elaborate on relatively new approaches based on hybrid nucleases and meganucleases, as well as directed double-stranded break repair.

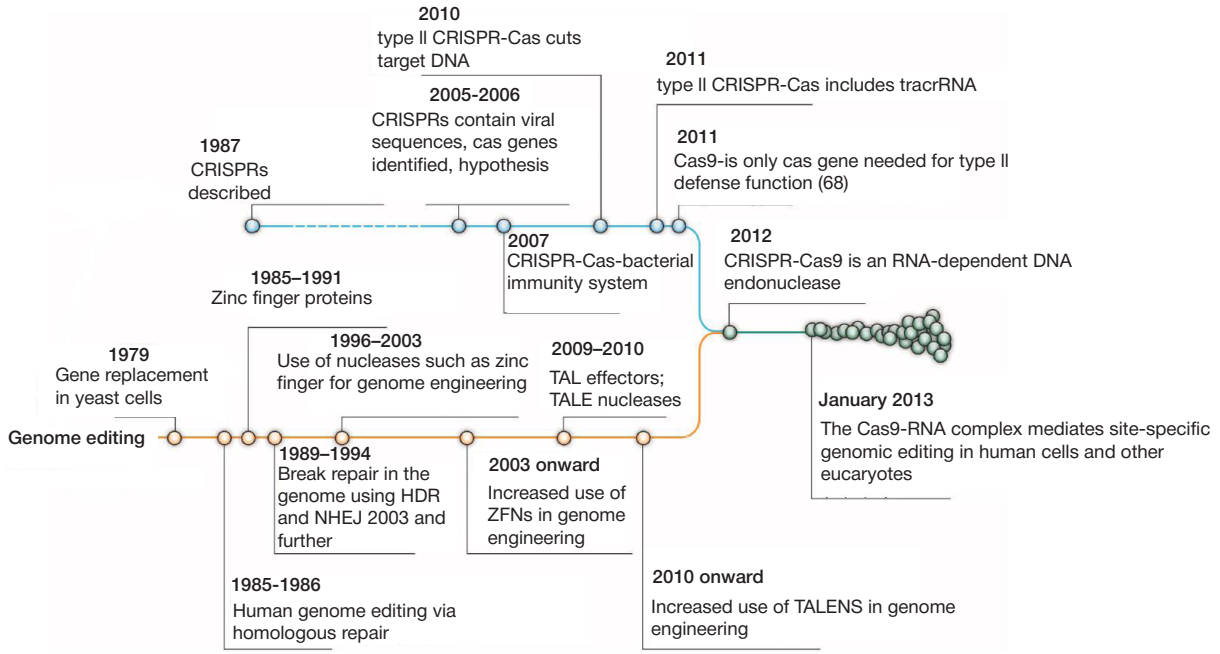

Fig. 1. Timeline indicating the evolvement of some genome editing systems (Doudna, Charpentier [45])

Guidance of nuclease using zinc fingers (ZFNs)

Small domains stabilized by one or more zinc ions and known as 'zinc fingers' were identified around the middle of the 1980s (for the first time as part of *Xenopus laevis* transcription factor TFIIIA). These domains can bind DNA, RNA, proteins and lipids effectively and quite specifically [48]. It turned out that a zinc finger specifically binds a triplet of nucleotides. If 3–6 zinc fingers with known specificity are combined into a single protein, sufficiently accurate recognition of DNA sequence in 9–18 base pairs can be achieved. Here, any endonuclease (the most commonly used is non-specific endonuclease and FokI catalytic domain from *Flavobacterium okeanokoites* which introduces a single-stranded break) is added to the zinc fingers, you get targeted endonuclease. To get a double-stranded break, it is necessary to create two such enzymes recognizing neighboring regions on opposite DNA strands (fig. 2).

Since the beginning of the 2000s and up till now, zinc-finger systems had been successfully used in a wide range of practical modifications of genomes both on plant and animal models, and on therapeutic approaches (table 2). The advantage of the method is the versatility of its nuclease targeting technique. The disadvantages include the relatively high complexity of genetic engineering assembly of the enzyme gene; the need to create two enzymes for each of the DNA strands; toxicity associated with lack of specificity of this type of systems [49]; risk of immunogenicity of foreign proteins [50]. In this regard, the use of zinc-finger systems is gradually being replaced by new approaches.

Hybrid meganucleases

In 2003, Epinat et al. proposed a genomic editing technique based on the so-called meganucleases [39] (fig. 2). Meganucleases were found in archaea, bacteria, phage, yeast, algae and some plants, and they are endodeoxyribonuclease — small proteins, mirror monomers or homodimers, characterized by a very long double-stranded DNA recognition site: from about 10 to 40 base pairs. Usually, a site of such length is seen only once in the genome or even never. For example, the I-SceI meganuclease recognition site, which is 18 base pairs in length, theoretically occurs once in the genome, exceeding the length of the human genome by 20 times. Typically, they form a part of introns or transposable elements of the genome. The biological function of meganucleases is unclear.

Representatives of the LAGLIDADG meganuclease family, found in the mitochondria and chloroplasts of unicellular eukaryotes, are the most widely used tool for genomic editing. Advantages of the technique involve the fairly high site-specificity and spontaneous dimer assembly. The disadvantage — high limitation on impact site selection.

TALEN (transcription activator-like effector nucleases) technique

The history of development of the TALEN system is connected with the study of the *Xanthomonas* bacteria genus. The reason for the long-term study of this group of bacteria was their pathogenic effects on crops, including tomatoes, peppers, rice and others. It was found that *Xanthomonas* secrets regulatory proteins — transcription activator-like effectors (TALE) — into the plant cell cytoplasm. These proteins increase the susceptibility of cells to the pathogen. Upon further study of the mechanisms of action of these proteins, they were found to be capable of binding to DNA and activating the expression of certain genes, mimicking host cell transcription factors [51, 52].

It was found that the TALE of a particular site in the DNA is recognized with the help of a series of small domains, each of which recognizes a single nucleotide in the site. Researchers figured out quite quickly the specificity of domains to specific nucleotides. This allowed to collect from them 'packs' precisely recognizing a specific sequence of bases in DNA.

Thus, the principle of use of TALEN system is similar to that of the system described above using triplet specific zinc-finger domains. The only difference is that nucleotide-specific domains (transcription activator analogs), connected in series by 12–20 pieces each are used as the 'guide', while the proven FokI catalytic domain is used as the nuclease. For double-stranded break, it is necessary to create two such enzymes (the target landing sites of 'guide' TALEs) that should be on the opposite DNA strands and separated by a site of about 20 base pairs (fig. 2). Advantages of the method: versatility of the nuclease guiding technique and versatility of the designer nuclease assembly technique. Disadvantages: high complexity of genetic engineering assembly of the enzyme gene and the need for creation of two enzymes for each of the DNA strands.

There are attempts to cross individual elements of different techniques. For example, hybrids of 'guide' TALEs and meganucleases (megaTALs) are described [42]. There are attempts to attach enzymes to meganucleases (by one way or another), which process (for example, destroy) the ends of the double-stranded break in order to strengthen the mutagenic effect of this break, and achieve other effects [43, 44].

In 2012, the Nature Methods journal named high-precision genome editing methods the methodical discovery of the year. TALEN was included in the methods.

CRISPR/Cas9 (nuclease associated with regularly interspaced short palindromic repeats) technique

The CRISPR/Cas9 system — proposed just a few years after TALEN — is a fundamentally different system when it comes to mechanism for guiding a nuclease to the target. The system differs from TALEN by the fact that as a 'guide', it uses not

Table 1. Composition of the components of the main enzyme of genome editing systems, and some features of the systems

Technique	Sequence	«Guide»	Scissors	Double-stranded break caused by:
ZFNs	Almost none	3–6 zinc finger protein domain	Non-specific endonuclease (for example, FokI)	Artificial heterodimer
HEs	Limited set of sites	Meganuclease	Meganuclease	Natural mirror monomer or homodimer
TALENs	Almost none	12–20 protein domains from transcription activator	Non-specific endonuclease sequence (for example, FokI)	Artificial heterodimer
RGNs (CRISPR/Cas9)	None	RNA of about 40 nucleotides in length	Cas9 nuclease	Artificial heterodimer

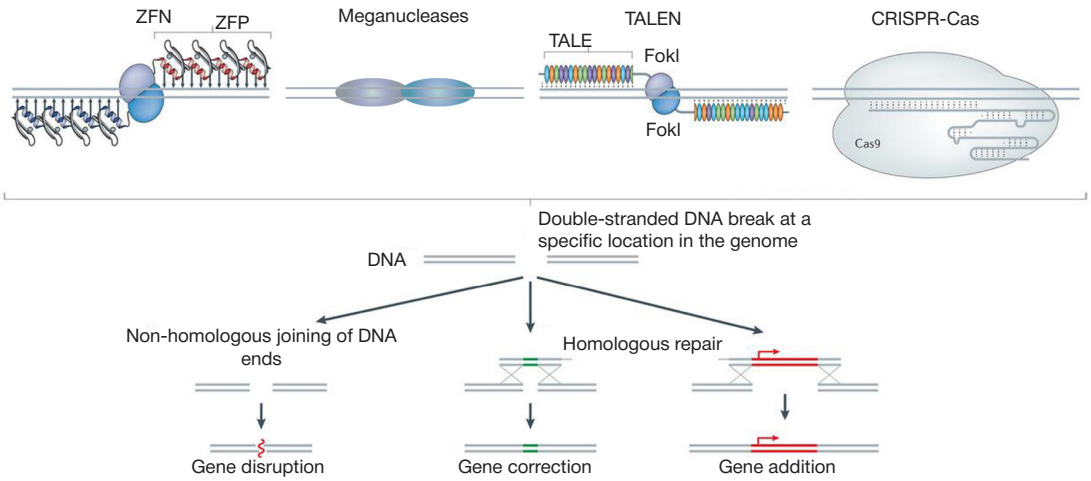

Fig. 2. Scheme of genomic editing based on zinc-finger hybrid nucleases, meganucleases, TALE hybrid nucleases and CRISPR/Cas9 (Yin et al., 2014 [47], as amended)

protein domains but RNA molecule (subgenomic RNA, sgRNA) of about 40 nucleotides in length, consisting of two parts: 'guide' crRNA and adaptor (trans-activating) tracrRNA. CRISPR elements were discovered in bacterial and archaeal genomes in the late eighties. It turned out that this is apeculiar bacterial 'immune system' element protecting the immune system against foreign DNA (such as bacteriophage penetration) by reading from complementary DNA phage of replicates of RNA molecules, which, in association with specific nuclease, disrupt the phage genome. Moreover, bacteria are able to remember the DNA sequences that infected their phages in order to continue to use them for reading 'guide' RNA [53].

It was also found that the sequence of these 'guide' RNAs can be changed, making them complementary to any DNA region without losing the nuclease activity of the Cas9 enzyme (fig. 2). Moreover, the RNA itself can be used as a genetic patch donor if the corresponding sequence is built in it [54].

At the moment, the CRISPR/Cas9 looks the most promising genome editing tool because it is versatile, fairly simple to apply and has high site specificity.

The method has several important advantages: versatility of the nuclease guiding method; there is no need for genetically engineered assembly of enzyme — only the 'guide' RNA changes; ability of Cas9 to cut both DNA chains; ability to integrate a genetic patch into the 'guide' RNA. Disadvantage: potential immunogenicity of a foreign protein.

Genomic therapy algorithms

Therapeutic uses of genome editing systems can be divided into three groups: 1) changing the genome of gamete/zygote/blastomeres for the purpose of obtaining a whole organism from one modified cell (fetal gene therapy); 2) changing the genome of individual somatic cells selected from the body for the purpose of subsequently returning the modified cells to the organism (somatic cell gene therapy); 3) changing the genome of individual groups (or all) of somatic cells in a multicellular organism directly (tissue somatic gene therapy).

The first two approaches involve manipulation of cell cultures in the lab (for which the broadest technology base is currently being developed). For the third approach, special systems (preferably tissue-specific systems) should be used for delivery of genetically engineered constructs into the body's cells.

Genetically engineered constructs

Hybrid nucleases and genetic patches (genetic material to be replaced) are typically delivered into the cell in the form of genetically engineered constructs from which the corresponding RNA and proteins are developed inside the cell. Variants of direct introduction into the mRNA cell are described, particularly for the CRISPR/Cas9 system [55].

A typical genetic construct for site-specific designer nuclease system contains a nuclear localization signal, an artificial guide unit (zinc fingers, TALE or 'guide' RNA), nuclease catalytic domain (for example FokI) and, if required, fragment to be replaced.

Gene delivery systems

Various viral and non-viral systems recognizing a large number of potential target tissues (skin, muscle, lung, brain, colon, spleen, liver, blood cells, and so on.) have been designed for delivery of 'therapeutic' genes or genetic constructs. The delivery system should ensure high efficiency of absorption of the genetic construct by the target cells, immunity to intracellular destruction during transportation to the nucleus and maintenance of necessary expression level.

Non-viral systems include direct introduction of DNA constructs into cells and tissues (e.g. electroporation), liposomes, cationic polymers, and others. Among viral systems, the most common are systems based on retroviruses, lentiviruses, adenoviruses, adeno-associated viruses and herpes simplex virus. Targeted delivery is determined by the presence of specific molecules (recognizable by target cell receptors) on the surface of viral particles or on the liposome's membrane. Such molecules could be viral capsid proteins, antibodies to surface cell antigens (inserted into the liposome's membrane), folic acid molecule (strongly captured by tumor cells), and others.

There are much attempts to use viral and non-viral delivery methods to deliver vectors with hybrid nucleases [56].

Genomic therapy of genetic disorders

As mentioned above, therapeutic approaches based on addition of genetic material into a cell with the help of viral vectors have been in use since the beginning of the 1990s.

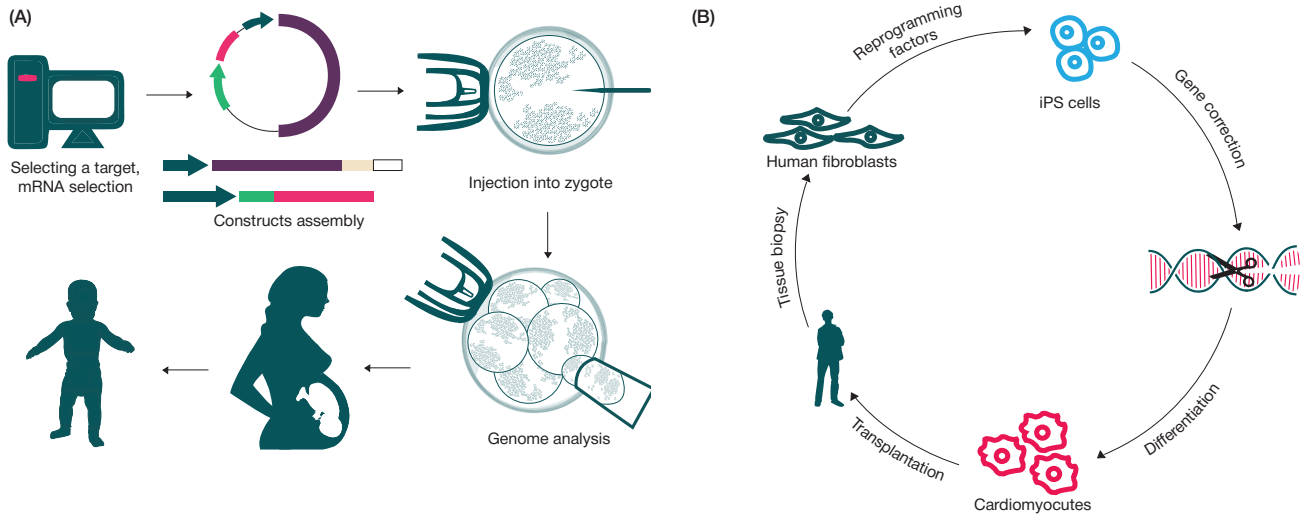

Fig. 3. Fetal **(A)** and somatic cell **(B)** gene therapy algorithms

These techniques restore synthesis of protein, whose gene is dysfunctional in both copies in the chromosome. However, changing or deleting DNA regions has long been an extremely complex and non-reproducible approach. With the advent of designer nucleases, researchers began to rapidly develop methods of directed DNA change directly in the chromosome structure. At present, there are treatment options fro retinitis pigmentosa, glaucoma, hemoglobinopathies, muscular dystrophies (table 2).

Fetal gene therapy is the most actively developing direction. In 2015-2016, many laboratories in the US, China, UK and several other countries, as well as some biotech companies, namely Ovascience (USA), Editas Medicine (USA), etc announced their plans for modification of human embryo genomes using CRISPR/Cas9-mediated gene editing techniques. If for a given pair of individuals, a potentially healthy genotype cannot be selected from 'natural' variants of embryos, genome editing methods aimed at adding/correcting a pathogenic allele in the zygote stage can be used.

Liang et al. published their work in April 2015, in which the CRISPR/Cas9 system was used at the zygote level to repair a mutant beta-globin gene. Of the 86 zygotes taken for experiment, only 4 cases were repaired [7].

Somatic gene therapy

"Chemotherapy will be obsolete within 20 years," said Prof Jeremy Farrer, head of the Wellcome Trust Sanger Institute. "We will look back in 20 years time and the blockbuster chemotherapy drugs that gave you all those nasty side effects will be a thing of the past and we will think 'gosh what an era that was'. Just as today, we are terrified by examples of electricity treatment at the beginning of the last century. Understanding humanity's genetic code is going to be fundamental to the medicine of the future. In rare congenital disease, in cancer and in infections, genomic insights are already transforming diagnosis and treatment" [57].

Designer nucleases can be used to effectively and accurately alter the DNA to correct any mutations. This opens up great opportunities for their use for correction of disorders that caused tumorigenesis [29, 30]. Variants for use of the CRISPR/Cas9 system for treatment of sarcoma and lung cancer have been proposed [31, 32]. For example, correction or deletion of mutant version of the *EGFR* gene using the CRISPR/Cas9 system delivered by virus has been proposed for lung cancer [32].

Antiviral therapy

HIV gene therapy

The fight against HIV is another direction of therapeutic use of hybrid nucleases. There are two directions in this fight: deleting HIV copies from the genome of the HIV carrier and altering the receptor genes through which the virus penetrates the T-lymphocytes, particularly the *CCR5* gene (table 2). By destroying the proviral DNA copies in the genome, it is theoretically possible to completely neutralize the virus and

Table 2. Examples of diseases treated using genomic editing based on designer nucleases

Area of application	Mechanism	Technique	Reference
Genetic eye dieseases	Gene disruption	TALENs, CRISPR/Cas9	[59–63]
Hemoglobinopathies (sickle cell anemia, β-thalassemia)	Insertion of working β-globin gene	ZFNs, TALENs, CRISPR/Cas9	[7–13]
Muscular dystrophy	Insertion of working dystrophin gene or deletion of bad exon in the existing gene	ZFNs, TALENs, CRISPR/Cas9	[64–71]
Oncology	Removal or correction of mutant gene variant	TALENs, CRISPR/Cas9	[29–32]
HIV	Cutting off DNA copies of virus from the human genome or deletion of receptor gene through which virus enters the cell	ZFNs, TALENs, CRISPR/Cas9	[72–82]
Hepatitis B virus	Virus genome destruction	CRISPR/Cas9	[58]
Genetic doping	Adding the desired gene allele	TALENs, CRISPR/Cas9	[83–87]
Reprogenetics	All kinds of changes	TALENs, CRISPR/Cas9	[83–87]

prevent it from reactivating in the patient's cells. Another approach — altering the receptor gene — does not allow the virus to infect the lymphocytes, thus leading to restoration of the patient's T cell population.

One of the problems in the development of gene-editing antiviral drugs is the ability of the virus to very quickly change sequence and thereby leave from the 'guide', specific to a particular sequence of the attack site. However, with proper legislative regulation of the release of modifications of gene therapy drugs, the release of new 'antivirus' can easily overtake the pace of HIV.

Fight against non-integrated viruses

The research world is also trying to apply genome editing systems to fight against viruses that do no integrate genetic material into the cellular genome. Their destruction mechanism is the same as in the case of HIV, but hybrid nuclease attacks free viral genome. The use of CRISPR/Cas9 to fight hepatitis B virus is described in [58].

Non-therapeutic genomic editing objectives

Genetic doping

Genetic doping is a variant of non-therapeutic use of genome editing to enhance athletic performance. It is no secret that maximum sports performance is largely determined by the individual's genetic component. Athletes from Kenya or Ethiopia almost always win marathons because genetically determined glucose metabolism pathway, which determines the ability to quickly run a marathon, is most developed in the African population of these countries.

At present, athletic success is linked with over 150 polymorphic positions in the DNA out of which 93 are associated with endurance and 62 with power load [91]. The spectrum of potential genes for effecting an influence by means of genomic editing is very wide: erythropoietin, insulin-like growth factor 1, human growth hormone, myostatin, endothelial growth factor, fibroblast growth factor, endorphins, enkephalins, cytoskeleton protein genes, etc. Approaches have already been developed for some of these genes, while clinical trials on introduction of specific alleles into the human genome have been carried out [85].

Reprogenetics

In classical interpretation, reprogenetics involves the selection of human embryos with certain properties from the resulting 'natural' variants. However, genome-editing technology helps to expand the features of the approach by creating variants that are impossible for a given pair of parents [88]. This raises a lot of ethical issues that mankind is yet to address [90].

Genomic editing: ethical and regulatory issues

Despite the fact that genome editing techniques via designer nucleases have enormous potential for creation of an effective therapy for patients suffering from genetic diseases, their use for therapeutic purposes is still in its infancy. In this regard, development of an ethical and legal framework that would ensure the effectiveness and safety of using genomic editing is an extremely important factor [92].

While developing such a framework, ethics committees and authorized government bodies need to establish and clarify aspects influencing the clinical implementation of genome editing techniques. These bodies should propose such a road map for development and implementation of genomic editing techniques that would allow to safely and quickly transfer the latest techniques into clinical practice.

Rapid development of innovative medical technologies does not allow the legislator to work on the legal framework for the use of these technologies in the same way as before. Presently, there is a change in the paradigm of legislative regulation on introduction of new medical technologies from research laboratory to the clinic. Globalization has made innovation to be spread around the world literally at the speed of light. New promising medical technology, no matter where it has been developed, is inevitably developed and primarily used in countries with more flexible and liberal laws. Such countries receive a head start on early introduction of innovative approaches, despite the risks involved in such approaches. Many legal restrictions on 'research-to-medicine' transition in certain countries do not make sense because technologies quickly spread to the rest of the world from 'research offshores', attracting clients to their territory.

From the very start, some countries try to prohibit the use of designer nucleases for human genome editing. However, Such countries are forced to quickly change position to avoid being at the tail of technological leaders. After a Chinese team announced in 2015 that it has carried out experiments on editing of genomes of human embryos through the CRISPR/Cas9 method, a group of British scientists were in February 2016 granted permission to genetically modify human embryos through CRISPR/Cas9 and related designer nuclease methods [93].

Public opinion amidst the introduction of techniques in separate jurisdictions is changing rapidly and starting to put pressure on their own legal regulation.

CONCLUSION

Genome editing methods created over the last few years are an improvement of gene therapy approaches existing at the end of the last century. However, it can be confidently argued that today the paradigm in the field of genomic medicine is shifting. The beginning of the second decade of the 21st century witnessed several technological breakthroughs with a strong synergistic effect – improvement in directed cell differentiation techniques, considerable reduction in cost, routine application of genome sequencers and creation of the described genome editing systems. All of these combined will inevitably give birth to new-quality personalized genomic medicine in the next 3–5 years. Directed genome alteration techniques will be a new tool for doctors.

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MODIFICATION OF THE METHOD FOR ANALYSIS OF GENOME EDITING RESULTS USING CRISPR/CAS9 SYSTEM ON PREIMPLANTATION MOUSE EMBRYOS

Dimitrieva TV[1]✉, Reshetov DA[1], Zhernovkov VE[1], Vlodavets DV[3], Zotova ED[1], Ermolkevich TG [1,2], Deykin AV [1,2]

[1] Marlin Biotech, Moscow, Russia

[2] Molecular Biology Core Facility,
Institute of Gene Biology, Russian Academy of Sciences, Moscow, Russia

[3] Department of Neurology, Neurosurgery and Medical Genetics, Faculty of Pediatrics,
Pirogov Russian National Research Medical University, Moscow, Russia

Genetically modified animals are an important tool for biomedical research. The CRISPR/Cas9 editing genome system is increasingly being used for production of such animals. Through microinjection, complex with guide RNA and Cas9 protein is delivered in fertilized eggs from which the animal subsequently develops with a modification in the genome. Generally, analysis of the specificity and efficiency of the system in each case is carried out after obtaining a progeny with the likely mutation. However, analysis at the preimplantation stage would allow reducing the time of the experiment, as well as understanding the reason for the birth of a small number of transgenic animals, or even lack of them in the offsprings. The paper proposes a modification of the method of preparation of total DNA from mouse blastocysts. The modification allows to easier and faster detect the results of microinjection of the CRISPR/Cas9 complex in the zygote. Having applied the method described in this paper, we successfully identified short deletions in intron 34 of dystrophin gene (DMD) in 12 out of 13 treated embryos and insertion in the break site in intron 8 of the DMD gene in 11 out of 21 samples analyzed. Using for analysis the total DNA prepared by the method proposed, you can analyze up to 20 different sites in the mouse embryo genome at the blastocyst stage without the need for full genomic amplification.

Keywords: genome editing, CRISPR/Cas9, short nucleotide insertions, nucleotide deletions, mouse embryos, Duchenne muscular dystrophy

Acknowledgement: authors thank the Shared Resource Center of the Institute of Gene Biology of Russian Academy of Sciences for the equipment provided for this research.

✉ **Correspondence should be addressed:** Tatiana Dimitrieva
ul. Vavilova, d.34/5, Moscow, Russia, 119334; dimitrieva.ta@gmail.com

Received: 16.06.2016 **Accepted:** 21.06.2016

МОДИФИКАЦИЯ МЕТОДА АНАЛИЗА РЕЗУЛЬТАТОВ РЕДАКТИРОВАНИЯ ГЕНОМА С ПОМОЩЬЮ СИСТЕМЫ CRISPR/CAS9 НА ПРЕДИМПЛАНТАЦИОННЫХ ЭМБРИОНАХ МЫШИ

Т. В. Димитриева[1]✉, Д. А. Решетов[1], В. Е. Жерновков[1], Д. В. Влодавец[3], Е. Д. Зотова[1], Т. Г. Ермолкевич[1,2], А. В. Дейкин[1,2]

[1] Marlin Biotech, Москва

[2] Центр коллективного пользования,
Институт биологии гена Российской академии наук, Москва

[3] Кафедра неврологии, нейрохирургии и медицинской генетики, педиатрический факультет,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

Генно-модифицированные животные — важный инструмент биомедицинских исследований. Для их получения все чаще используют систему редактирования генома CRISPR/Cas9. С помощью микроинъекции комплекс РНК-гида и белка Cas9 доставляется в оплодотворенную яйцеклетку, из которой впоследствии развивается животное с модификацией в геноме. Как правило, анализ специфичности и эффективности системы в каждом случае проводят после получения потомства с вероятной мутацией. Однако анализ на предимплантационной стадии позволил бы сократить время эксперимента, а также понять причину рождения малого числа или даже отсутствия трансгенных особей в потомстве. В статье предложена модификация метода подготовки тотальной ДНК из бластоцист мыши, позволяющая проще и быстрее детектировать результаты микроинъекций комплекса CRISPR/Cas9 в зиготу. Применив описанный в статье метод, мы успешно идентифицировали короткие делеции в интроне 34 гена дистрофина (*DMD*) в 12 из 13 обработанных эмбрионов и вставку по месту разрыва в интроне 8 гена *DMD* в 11 из 21 проанализированных образцов. Используя приготовленную предложенным способом тотальную ДНК, можно анализировать до 20 различных сайтов в геноме мышиного эмбриона на стадии бластоцисты, не прибегая к полногеномной амплификации.

Ключевые слова: редактирование генома, CRISPR/Cas9, короткие вставки нуклеотидов, делеции нуклеотидов, эмбрионы мыши, миодистрофия Дюшенна

Благодарности: авторы выражают благодарность Центру коллективного пользования Института биологии гена РАН за предоставленное для экспериментов оборудование.

✉ **Для корреспонденции:** Димитриева Татьяна Владимировна
119334, г. Москва, ул. Вавилова, д. 34/5; dimitrieva.ta@gmail.com

Статья поступила: 16.06.2016 **Статья принята к печати:** 21.06.2016

Genetically modified organisms are an indispensable tool in the study of gene functions and non-coding sequences, interactions between regulatory sequences in the genome and recombinant protein expression. They are also vital for human disease modeling. Until recently, obtaining genetically modified animals was very time-consuming and expensive, thus making the process practically inaccessible for many research groups. However, all these obstacles have been eliminated by the advent of new genome editing systems. In 2013, the first paper on the use of CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9) was published — techniques that can be used to inactivate several genes at a go [1].

The system includes an RNA containing clustered regularly interspaced short palindromic repeats (CRISPR), trans-activating RNA and Cas9 nuclease. This complex, which naturally acts as bacterial immunity against parasitic phages [2], was adapted for DNA engineering both in vitro and in mammalian cells [3]. The Cas9 protein generates double-stranded breaks at 3 nucleotides from the PAM (protospacer adjacent motif) site — NGG, which is located just behind the sequence complementary to the 19-nucleotide guide RNA [4]. The cell repair system of the genome makes short deletions or insertions in the site of the break. Addition of constructs containing sequences homologous to the region around the break leads to homologous repair and possibly insertion of the desired fragment into the genome at a particular site.

This approach is used to knock in expression cassette into a site where a break has been introduced. The discovery of CRISPR/Cas9-mediated genome engineering system has revolutionized the generation of genetically modified animals and shortened the experimental time from several years to several months. Genetically modified mice [6, 7], rats [7], monkeys [8], and other animals were obtained through pronuclear injections into zygotes by Cas9/RNA-mediated gene targeting.

The CRISPR/Cas9 system has been repeatedly applied effectively for creation of mouse model for human diseases [9–11]. In creating such models, it is important that modification is done strictly at a specific site, without disrupting other genes. The issue of non-specific modifications is a very urgent one. Despite the existence of many bioinformatics programs that can pick up guide RNAs for given sites and assess their effectiveness and specificity, there is still the probability of introducing unwanted mutations in off-target sites [6]. Predicted off-target sites are usually analyzed for the presence of mutations. The analysis is done after the birth of transgenic offspring (at least three weeks after microinjection), while analysis at the blastocyst stage would allow to in advance assess the specificity of modifications being introduced.

However, the methods most commonly used for DNA amplification of preimplantation embryos cannot analyze several regions of the genome [12]. At present, whole genome amplification is used for analysis of multiple sites in mouse embryonic genomes before staging a polymerase chain reaction (PCR) or two PCR rounds are carried out [13]. Such an approach leads to both false positive and false negative results due to the low DNA content in the original sample [14]. Moreover, the high cost of whole genome amplification reagents compels researchers to analyze fewer embryos.

In this paper, we describe a method for preparation of total DNA from one mouse embryo at the blastocyst stage, which simplifies, speeds up and reduces the cost of the process of obtaining genetically modified animals.

METHODS

In the first phase of the study, the efficiency of several blastocyst lysis methods was assessed. Experiments were carried out in triplicates from the moment fertilized eggs were obtained. At least 20 blastocysts were used for each method. We concurrently investigated the influence of blastocyst-preparation method on lysis. The minimum amount of lysate for amplification (three experiments) was also determined. Then, experiments were performed to analyze the efficiency of CRISPR/Cas9-mediated genome engineering (detection of short insertions and deletions, identification of target insertions in the genome) using the lysis method that yielded the best results in the previous experiments. Guide RNAs were selected for introns 8 and 34 of the dystrophin gene (*DMD*) mutations in which triggers Duchenne muscular dystrophy.

Selection and synthesis of guide RNAs

Guide RNAs were selected using online resource CHOPCHOP [15]. For guide RNA synthesis, two partially complementary oligonucleotides — SgR (containing the T7 promoter) and Sg31 (encodes the g31 site in the genome) were used or SgR and Sg34 (encodes the g34 site in the genome) were used. DNA research company Evrogen (Russia) synthesized the primers. Their sequences are presented in the table.

To obtain DNA matrix, oligonucleotides were mixed in equimolar ratio and amplified in thermal cycler T100 (BioRad, USA) based on the program: 95 °C — 1 min; 30 cycles of 95 °C — 30s, 65 °C — 30s, 72 °C — 30s; 72 °C — 5 minutes, using reagent kit GenPack PCR-core kit (Isogene, Russia). The matrix was purified from reaction products using reagent kit CleanUp Kit (Evrogen, Russia) based on the manufacturer's protocol. Guide RNA was synthesized using reagent kit RiboMax express (Promega, USA) and was isolated from the reaction mixture by phenol-chloroform extraction, and was then precipitated in isopropanol [16]. Guide RNA was dissolved in free of nucleases water. The concentration was measured on NanoDrop 8000 (Thermo Fisher Scientific, USA). Guide RNA aliquots were stored at –70 °C.

In conducting experiments to detect an insertion at the break site, we synthesized repair template — a 70-nt single stranded oligonucleotide composed of two overlapping restriction sites for NcoI and BamHI enzymes, surrounded by 30-nt homology arms on each side. In the PAM site, replacements were made to prevent the binding of guide RNA to the repair template (sg31-HDRt, the sequence is shown in the table). Synthesis was performed by DNA research company Evrogen.

Oligonucleotides sequences

Name	5'-3' sequence
g31	GTGGCAGACTAGTAGTTTG
g34	GTAAAGACTCGGCAGTTAAG
SgR	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
sg31-HDRt	GTAGATAGAATAGTTTATTGGTGATCTCAACCATGGATCCACTACTAGTCTGCCACTGAGAAAAGAGAAG

Microinjections into the pronucleus of zygotes and embryo cultivation

In immature female mice (C57BL6 x CBA), weighing 12-13 g, superovulation was induced by introducing at first 5 units of pregnant mare serum gonadotropin (PMSG) and then after 46–48 hours — 5 units of human chorionic gonadotropin (hCG). After injection, the female mouse was immediately placed in a cage with a male mouse for mating. Ovulation occurred within 11–14 hours after hCG injection. Fertilized eggs were surgically washed out within 12–13 hours after copulation (middle of the dark illumination period), i.e. 25–27 hours after hCG injection.

Genome editing complex was microinjected predominantly into the male pronucleus of mouse zygotes at the two-pronuclear stage. The zygotes were placed in a chamber consisting of two coverslips, fixed one above the other such that the upper and lower droplet edge of the M2 medium (MTI-GlobalsStem, USA) were flat and parallel. Pronuclei were visualized by differential interference contrast microscope Axiovert 200 (Carl Zeiss, Germany). The microinjection needles were made from G100 glass capillaries (Narishige, Japan) on P97 puller (Shutter Instruments, USA), while holder capillaries were made from GD1 capillaries (Narishige) on PC-10 puller (Narishige) and MF-900 Microforge (Narishige).

For microinjection, guide RNA (50 ng/ml) was mixed with Cas9 (0.1 pM; NEB, UK) in TE buffer (10 mM Tris-HCl, pH 7.4; 0.1 mM EDTA) and, depending on the experiment – with repair matrix (3 pM; Evrogen, Russia). The components were mixed immediately prior to microinjection and incubated for 5 min at 37 °C to form a complex.

After microinjection, the zygotes were cultured for 2–3 hours in CO2 incubator 150 IGO (Thermo Electron Corporation, France) at a 5 % carbon dioxide content in air and 100 % humidity. They were then assessed visually and those that were in satisfactory condition were left for 3 days in KSOM buffer (MTI-GlobalStem, USA) to form a blastocyst. Cultivation took place in Petri dishes (35 mm in diameter) in 50–60 µl droplets. The droplets were covered with embryo tested light mineral oil. All the preparatory procedures were performed in a laminar flow hood.

Preparing total blastocyst DNA samples and PCR amplification of target fragment

Under a microscope, each embryo was transferred sequentially through 3 drops of water for PCR (Evrogen) using automatic pipette and tips with filters and placed in a 1 µl volume on a 0.2 ml tube wall. 1 µl of water from the last drop was used as the PCR negative control. When necessary, samples at this stage were frozen until further analysis and stored at –20 °C or unwashed blastocysts were used, for which embryos were transferred in a small volume of the incubation medium (less than 1 µl) using glass capillary on a tube wall for analysis.

Several methods were used for lysis of blastocysts: direct addition of blastocyst to a small volume in the reaction mixture, i.e. alkaline lysis (200 mM NaOH, 50 mM DTT) [17]; repeated freezing and thawing in water for PCR, lysis with lauryl sarcosine [18]; Proteinase K processing with detergent using the Sakurai et al. technique [19]. The last method was modified by deleting yeast tRNA from the buffer and increasing the amount of buffer to the sample to 20 µl. 20 µl of buffer was added to the test tubes for lysis. The buffer consisted of proteinase K (125 µl/ml), Tris-HCl (100 mM, pH 8.3), KCl (100 mM), gelatin 0.02 %, and Tween-20 0.45 %. The tubes were incubated for 10 min at 56 °C, then for 10 min at 95 °C to inactivate proteinase. PCR analysis was performed immediately after sample preparation or lysate was stored at –20 °C.

The target region around the recognition site of guide RNA sg31 was amplified using primers g31-434F (5'-TCAAACAAAAGGCAGAAGAGTAAG-3') and g31-434R (5'GGTCCAAAGTAGGCCTCGTA-3'), guide RNA sg34 – using primers g34-505F (5'-CAGTGCCCCACACACATACA-3') and g34-505R (5'-AGCAAAAGTTATTTTAGGGCATACT-3'). From 1 to 10 µl blastocyst lysate or the appropriate control sample was added to the reaction. Reagent kit GenePack PCR core (Isogene, Russia) was used for PCR. Amplification program: 95 °C — 1 min; 40 cycles of 95 °C — 30 sec, 60 °C — 30 sec, 72 °C — 30 sec; 72 °C — 5 min. PCR products were analyzed by electrophoresis in 2% agarose gel using intercalating dye.

Mutational analysis using bacteriophage T7 endonuclease I

Short deletions and insertions which are formed after double-stranded break repair were detected using bacteriophage T7 endonuclease I (T7EI; NEB, UK). For this purpose, PCR product was mixed with control template, amplified under the same conditions (5 µl of reaction mix for each fragment) and with buffer NEB2 (NEB). In the final 9 µl volume, oligonucleotides were annealed at a temperature of 95 to 25 °C at a speed of 0.1 °C/sec. Then, 0.1 units of enzyme activity were added to each tube. The reaction was conducted for 1 hour at 37 °C. Reaction products were separated by electrophoresis in a 2 % agarose gel using intercalating dye. The deletion boundaries and insertion sizes were determined by Sanger sequencing of PCR products (Genome Center, Russia).

Checking for the presence of insertion in the restriction genome at the BamHI site

Single-stranded DNA fragment used as the repair template encoded two recognition sites for two endonucleases: NcoI and BamHI. PCR products obtained by amplification of the intron 8 site of injected and control embryos were mixed with buffer FastDigest Green Buffer 10X and added with 0.1 units of activity of BamHI (Thermo Fisher Scientific, USA). The reaction was conducted for 1 hour at 37 °C. Reaction products were separated by electrophoresis in a 2 % agarose gel using intercalating dye. The presence of an insert was confirmed by Sanger sequencing of PCR products (Genome Center, Russia).

RESULTS

Choosing a PCR technique in blastocysts

In a series of experiments using various lysis methods, samples containing PCR product were in the greatest amount if lysate was prepared by Sakurai et al. technique [19] with modifications indicated methods (results not shown).

It was further found that preparation of blastocysts for lysis is vital to the reproducibility of PCR results since the components of the medium can inhibit lysis and polymerase later on. In parallel experiments, it was shown that with other things being equal, embryo transfer in the incubation medium worsens the reproducibility of PCR results (an average of 11 successful reactions on 20 samples) compared to the use of embryos washed from the medium components (20 successful reactions on 20 samples). In this and subsequent experiments, lysis was performed by Sakurai et al. method with modifications.

Experiments were carried out to determine the minimum amount of lysate that can be used for amplification. For 40 PCR cycles, 434 bp DNA segment was successfully amplified using 1 to 10 µl of lysate as the matrix (fig. 1).

Thus, the proposed method can analyze up to 20 different sites in a mouse embryonic genome at the blastocyst stage.

Searching for short insertions and deletions in the g34 site of the *DMD* gene of mouse embryos microinjected with guide RNA and Cas9 protein

Guide RNA sg34 with Cas9 protein was microinjected into the pronucleus of the zygote. 13 embryos at the blastocyst stage were lysed by Sakurai et al. method with modifications. 505 bp *DMD* gene fragment was amplified using injected and control embryos lysates as a template. PCR fragments were successfully amplified in all samples (results not shown).

Next, PCR fragments were hybridized with the control fragment and processed with endonuclease I. In 12 out of 13 samples, there was cleavage into two fragments — 300 bp and 250 bp (fig. 2, A). Four PCR fragments were selected to check for the presence of mutations in the gRNA binding region by Sanger sequencing. The results are shown in fig. 2, B. 12-nt deletion (from –5 to 7, where nucleotide N in triplet NGG of the PAM site was adopted as position +1) was found in sample 1. In samples 2 and 3, the deletion size was 17 nt (from –11 to +6), while in sample 4, the size was 38 nt (from –7 to +31).

Detection of insertion after homologous recombination at break site

Single-stranded oligonucleotide sg31-HDRt was injected together with the gRNA sg31/Cas9 complex into the pronucleus of zygotes. 21 embryos at the blastocyst stage were used for analysis. The intron 8 site of the 434 bp *DMD* gene was amplified with injected and control embryos that were preliminarily lysed using the chosen method. PCR products were treated with restriction endonuclease BamHI. The results are shown in fig. 3. In 11 out of the 21 treated samples, partial or complete cleavage into two fragments was detected. Sample 2 was shorter than the others by about 50 nucleotides, indicating sufficiently large deletion. Sample 5 was lost during restriction analysis (fig. 3A). Five samples (6, 12, 15, 19 and 20) were selected to confirm the presence of insertions in the sg31 recognition site by Sanger sequencing. An insertion was detected in all sequences. In samples 6, 15 and 19, apart from copies of the gene with insertion, a normal copy was identified (fig. 3, B).

DISCUSSION

The DNA of newborn offspring is most often used to determine the success of manipulations in generating genetically modified organisms [12]. However, this approach requires replanting of fertilized embryos after microinjection into the recipients and total or partial incubation of offspring, which takes most of the time allotted for the experiment. Analysis of embryos at the blastocyst stage described by us helps to quickly determine whether it is possible to obtain genetically modified organisms using a selected guide RNA. At the same time, thanks to the fact that results are obtained rapidly, the need to test guide RNAs on cell cultures in advance is eliminated. In addition, statistical analysis is also possible. In cases where replanting of fertilized eggs after microinjection does not result in the birth of genetically modified offspring, the proposed method can help determine the causes of fetal death by analyzing the non-specific gRNA binding sites or test the effectiveness of the gRNA-Cas9 complex.

Clonal selection is characterized by the problem of insufficient amount of DNA in the starting material after transfection (using plasmid encoding the nuclease complex) of primary cell cultures, immortalized cell lines or stem cell. In this case, selection of genomic DNA using commercial reagent kits is expensive and inefficient, while the proposed method for obtaining total DNA can help to analyze a greater number of clones. This is especially essential during insertion into the genome via homologous recombination matrix since the efficiency of this process is much lower than in formation of short deletions or insertions.

The modified method of obtaining total DNA from mouse embryos eliminates the need for whole genome amplification, use of a large number of cycles or conduct of several PCR rounds. Analysis of embryos at the blastocyst stage will not only save time and money for the researcher but is also more humane than postnatal analysis of offspring since the number of animals involved in the experiment is reduced.

The total DNA prepared — by the proposed method — from mouse embryos at the blastocyst stage is suitable for analysis of short deletions or insertions in the Cas9 cutting site using bacteriophage T7 endonuclease I and other similar enzymes, as well as specific hydrolysis by restriction endonucleases with existence of appropriate site. Bacteriophage T7 endonuclease I recognizes and cleaves single-stranded sites in the heteroduplex composition. It can be used to detect the presence of short deletions and insertions at the double-stranded break site after hybridization of the analyzed fragment with control amplicon. Some studies question the applicability of bacteriophage T7 endonuclease I in identification of short deletions or insertions in mouse embryos [7, 12]. Using the described sample preparation method, T7EI and Sanger sequencing, it was established that CRISPR/Cas-mediated modifications in mouse genome using a single guide RNA are highly efficient. This finding is consistent with previous results [1]. Thus, it was established that amplification of the target fragment from one blastocyst, followed by processing with bacteriophage T7 endonuclease I is a reliable method for mutation detection after microinjection of gRNA/Cas9 into a fertilized egg. It was shown that the method involving sample preparation of total DNA from one blastocyst is suitable for detection of insertions at the break site via restriction analysis. It was also established

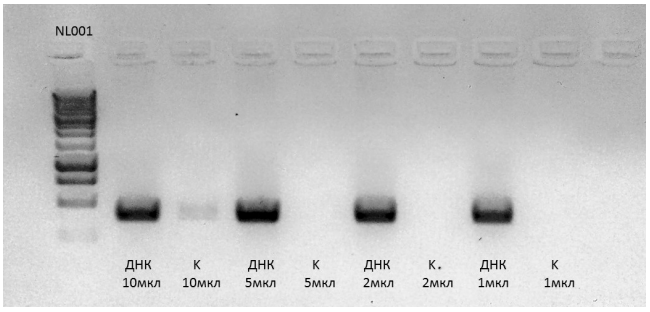

Fig. 1. Amplification of *DMD* intron 8 using different amounts of matrix

Blastocysts were lysed by Sakurai et al. method [19] with modifications in a final volume of 20 µl. 1 to 10 µl of total DNA solution was used for polymerase chain reaction (PCR). The appropriate volume of water from the final wash, treated similarly was used as the control for each sample. PCR products were separated in a 2 % agarose gel using intercalating dye. NL001 (Evrogen, Russia) was used as the DNA fragment length marker.

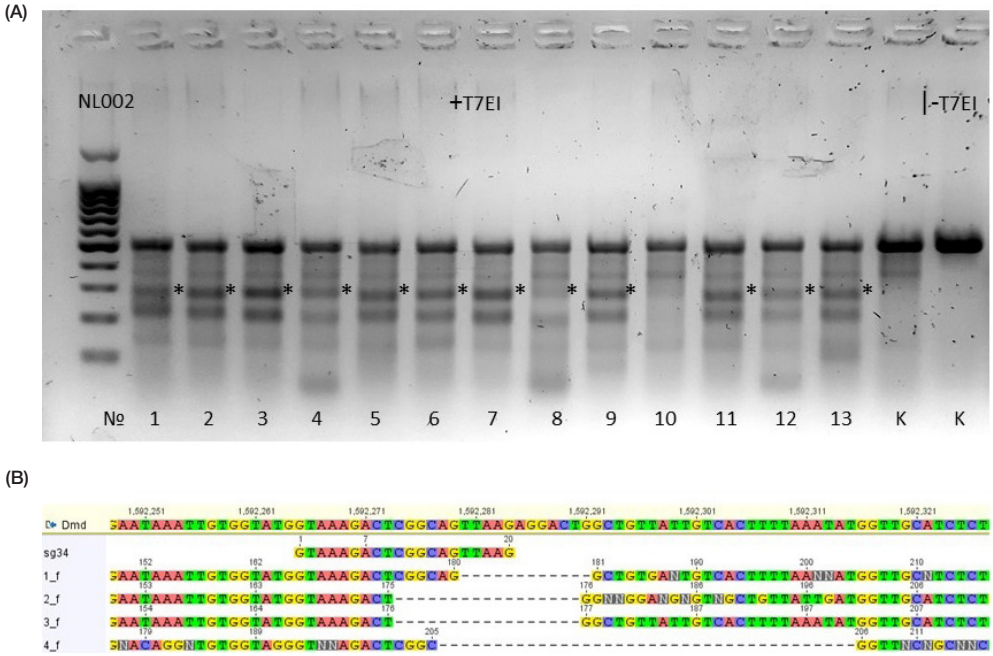

Fig. 2. Detection of insertions and deletions in *DMD* intron 34 after microinjection of the sg34/Cas9 complex

The intron fragment around recognition site g34 was amplified with injected and control embryos. PCR products were annealed in control amplicon and processed with bacteriophage T7 endonuclease I. Cleavage into two fragments occurred in samples that contained insertions and deletions. Such samples are marked in the figure with an asterisk * **(A)**. The boundaries of insertions and deletions in selected samples were determined via Sanger sequencing **(B)**. PCR products were separated in a 2 % agarose gel using intercalating dye. NL001 (Evrogen, Russia) was used as the DNA fragment length marker.

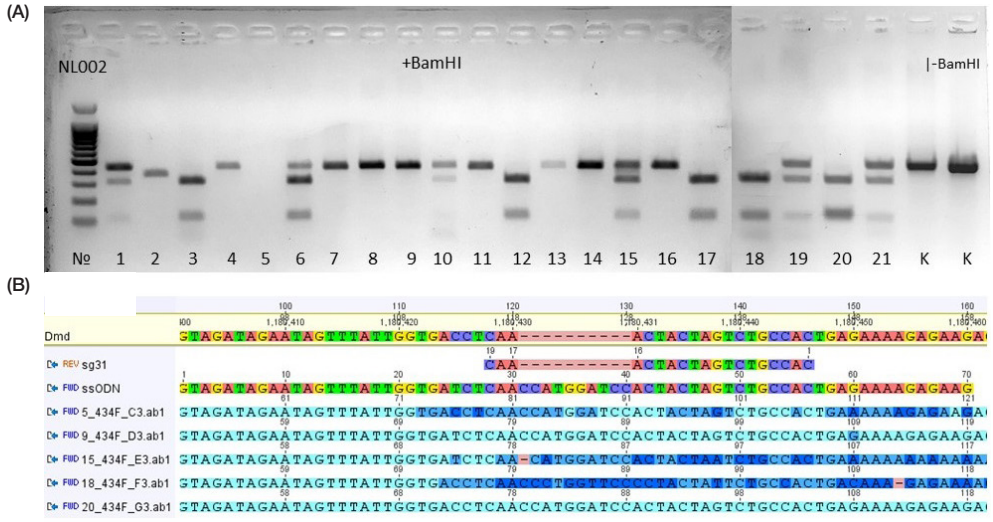

Fig. 3. Detection of insertion at the break site in *DMD* intron 8 after microinjection of the sg31/Cas9 complex with repair matrix

The intron fragment around recognition site g34 was amplified with injected and control embryos. PCR products were treated with restriction endonuclease BamHI, whose recognition site was encoded in repair matrix. Cleavage into two fragments occurred in samples that contained insertions and deletions. Such samples are marked in the figure with an asterisk * **(A)**. The presence of an insertion in selected samples was confirmed by Sanger sequencing **(B)**. PCR products were separated in a 2 % agarose gel using intercalating dye. NL001 (Evrogen, Russia) was used as the DNA fragment length marker.

that homologous recombination at the guide RNA recognition site occurs less efficiently than non-homologous connection of ends in a double-stranded break (11/21 vs 12/13).

CONCLUSIONS

The article describes a modification of a method for obtaining total DNA from mouse embryos at the early development stage. It was shown that sample preparation process is equally of great importance as a correctly selected lysis technique. Unlike the presently used method, the proposed method is simple and eliminates the need for rare and expensive reagents. Using embryo lysate as the matrix at the blastocyst stage, it is possible to amplify the desired DNA fragment in a single PCR round, significantly reducing the probability of result distortion and contamination. Moreover, the mixture components do not inhibit PCR and enzymatic reactions, and the method allows for analysis of up to 20 different independent fragments of the mouse genome.

The proposed modification of the analysis method can be useful for amplification of DNA regions after using other genome engineering systems, such as TAL effector nucleases or zinc fingers nucleases.

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ANALYSIS OF PHENOTYPE EXPRESSIONS OF DELETIONS IN THE DYSTROPHIN GENE IN TERMS OF EFFICIENCY OF EXON SKIPPING AS A METHOD FOR TREATMENT OF HEREDITARY DYSTROPHINOPATHIES

Zotova ED[1,2] ✉, Reshetov DA[1], Zhernovkov VE[1], Vlodovets DV[3], Dimitrieva TV[1], Deykin AV[1,2]

[1] Marlin Biotech, Moscow, Russia

[2] Molecular Biology Core Facility,
Institute of Gene Biology, Russian Academy of Sciences, Moscow, Russia

[3] Department of Neurology, Neurosurgery and Medical Genetics, Faculty of Pediatrics,
Pirogov Russian National Research Medical University, Moscow, Russia

Duchenne muscular dystrophy (DMD) is a common genetic disease caused by a mutation of the dystrophin gene. It leads to death in childhood. At the time of writing this paper, patients had access to supportive therapy only. However, DMD treatment methods are actively being developed. Exon skipping is a promising method. Exon skipping involves restoration of the reading frame within a gene by inducing alternative splicing. This leads to synthesis of truncated but still functional dystrophin. The paper assesses the functionality of the truncated forms of dystrophin resulting from correction of nonsense mutations and internal exon indels by exon-skipping technique. The assessment was made based on data on the phenotype of carriers of mutations in the dystrophin gene taken from the Leiden Open Variation Database (LOVD). It was revealed that the same mutation could manifest itself as a variety of phenotypes. This, perhaps, is as a result of the patients having different genetic background. For example, deletion of exon 48, for which there is 97 records in LOVD, resulted in asymptomatic diseases in 2 % of cases, Duchenne muscular dystrophy in 60 %, Becker muscular dystrophy (characterized by milder symptoms than DMD) in 12 % and intermediate phenotype in 26 % of cases. High phenotypic variability of mutations of the dystrophin gene raises the issue of limits of applying exon skipping for treatment of inherited myopathies.

Keywords: muscular dystrophy, Duchenne muscular dystrophy, Becker muscular dystrophy, exon skipping

Acknowledgement: authors thank the Shared Resource Center of the Institute of Gene Biology of Russian Academy of Sciences for the equipment provided for this research.

✉ **Correspondence should be addressed:** Evgeniya Zotova
ul. Vavilova, d. 34/5, Moscow, Russia, 119334; zotova@fbb.msu.ru

Received: 17.06.2016 **Accepted:** 24.06.2016

АНАЛИЗ ФЕНОТИПИЧЕСКИХ ПРОЯВЛЕНИЙ ДЕЛЕЦИЙ В ГЕНЕ ДИСТРОФИНА В КОНТЕКСТЕ ЭФФЕКТИВНОСТИ ПРОПУСКА ЭКЗОНОВ КАК МЕТОДА ТЕРАПИИ НАСЛЕДСТВЕННЫХ ДИСТРОФИНОПАТИЙ

Е. Д. Зотова[1,2] ✉, Д. А. Решетов[1], В. Е. Жерновков[1], Д. В. Влодавец[3], Т. В. Димитриева[1], А. В. Дейкин[1,2]

[1] Marlin Biotech, Москва

[2] Центр коллективного пользования,
Институт биологии гена Российской академии наук, Москва

[3] Кафедра неврологии, нейрохирургии и медицинской генетики, педиатрический факультет,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

Мышечная дистрофия Дюшенна (МДД) — распространенное наследственное заболевание, развивающееся вследствие мутации в гене дистрофина и приводящее к смерти в детском возрасте. На момент написания статьи пациентам была доступна только поддерживающая терапия, однако подходы к лечению МДД активно разрабатываются, и перспективным является метод пропуска экзонов. Его суть заключается в восстановлении рамки считывания гена путем индукции альтернативного сплайсинга. В результате синтезируется укороченный дистрофин, который в той или иной степени сохраняет функциональность. В работе дана оценка функциональности укороченных форм дистрофина, получающихся при коррекции нонсенс-мутаций и внутриэкзонных инделов по методике пропуска экзонов. Оценка производилась по данным о фенотипе носителей мутаций в гене дистрофина, взятых из базы LOVD (Leiden Open Variation Database). Было обнаружено, что одни и те же мутации способны проявляться как различные фенотипы, что, возможно, объясняется разным генетическим фоном пациентов. Так, делеция экзона 48, для которой в LOVD есть 97 записей, в 2 % случаев приводила к бессимптомному течению заболевания, в 60 % — к миодистрофии Дюшенна, в 12 % — к миодистрофии Беккера (отличается более мягкой симптоматикой, чем МДД), в 26 % случаев — к промежуточному фенотипу. Высокая фенотипическая вариабельность мутаций в гене дистрофина ставит вопрос о границах применения методики пропуска экзонов для терапии наследственных миопатий.

Ключевые слова: мышечная дистрофия, миодистрофия Дюшенна, миодистрофия Беккера, пропуск экзонов

Благодарности: авторы выражают благодарность Центру коллективного пользования Института биологии гена РАН за предоставленное для экспериментов оборудование.

✉ **Для корреспонденции:** Зотова Евгения Дмитриевна
119334, г. Москва, ул. Вавилова, д. 34/5; zotova@fbb.msu.ru

Статья поступила: 17.06.2016 **Статья принята к печати:** 24.06.2016

Duchenne muscular dystrophy (DMD) is an X-linked recessive hereditary disease that occurs in about 1 out of every 3,600 male infants and it is the most common hereditary disease that causes death in childhood [1, 2]. Skeletal, cardiac, and smooth muscle failure can occur in DMD. The disease can also cause damage to the digestive and excretory systems. But, the skeletal muscle is affected the most. Apart from progressive muscle weakness, development delay, speech and breathing problems and heart failure are all observed in patients [3]. While diagnosed at 5 years average, DMD progresses to wheelchair dependency by the age of 13 years (95 % of patients). The mean age at which the patient dies not receiving special treatment is 19 years [4].

Since DMD is an X-linked disorder, it affects men in most cases, who are hemizygous by X-chromosome. The disease is usually inherited by the son from the carrier mother with X-chromosome. However, it may also occur de novo as a result of mutation in the dystrophin gene (*DMD*) [5]. The dystrophin gene is considered the longest in the human genome, measuring 2.4 million base pairs [6], and has 79 exons. The encoded protein contains approximately 3,700 amino acid residues [7]. Dystrophin mRNA requires about 16 hours to be synthesized [8].

Dystrophin is a structural protein which makes part of the costamere complex, responsible for connecting acto-myosin complexes (sarcomeres) to plasma membrane (sarcolemma) and proteins of extracellular matrix [9]. Dystrophin has an elongated shape. One of its ends binds actin, and the other — the membrane dystroglycan complex, also anchored to the spectrin cytoskeleton inside and to extracellular matrix proteins outside the cell.

In the absence of dystrophin, complexes of proteins associated with it lose their stability. It is known that two protein complexes are part of costameres: dystrophin-glycoprotein complex and vinculin-talin-integrin complex, apparently performing similar functions. Damage to various links of this complex and non wholly understood system can lead to different types of hereditary myopathies [10, 11]. However, apparently due to the complex structure of the system and duplication of the functions among costamere proteins, even complete absence of dystrophin expression leading to DMD does not completely break the link between the actomyosin complexes, membrane and extracellular matrix, but only strongly weakens its strength. Because of this, fragile sarcolemma is subjected to mechanical damage during muscle contraction. Loss of sarcolemmal integrity leads to muscle fiber necrosis and inflammation. In the patient's first years of life, his muscle fibers are regenerated through pool of myosatellites — muscular differon stem cells. However the pool of myosatellites gradually get depleted, resulting in muscle degeneration and fibrosis [12].

The human dystrophin contains 4 structural domains: N-terminal actin-binding domain; central rod domain containing 24 spectrin-like repeats and 4 hinges; cysteine-rich domain, binding β-dystroglycan; C-terminal domain binding dystrobrevin and syntrophin. The central rod domain has a strongly extended form, and since each spectrin-like repeat consists of three α-helices, it is apparently sufficiently flexible and elastic [12]. According to data obtained from analysis of known mutants, a small change in the number of spectrin repeats caused by frameshift deletions does not have much impact on the normal operation of the protein [13].

A shift in the reading frame in the dystrophin gene typically leads to DMD. The shift causes premature termination of translation and nonsense-mediated decay of mRNA, nonsense mutations, as well as large deletions in the gene regions encoding the N- and C-terminal domains of dystrophin. This results in completely disrupted binding of either with actin or with membrane dystroglycan complex. Non-frameshift deletions of average size in the middle of the gene are usually associated with other myopathy: Becker muscular dystrophy, which has less severe symptoms — many patients retain in adulthood the ability to walk independently [14].

A range of DMD therapies aimed not only at suppressing inflammation and fibrosis and reducing the toxic effects of excess of calcium in the cytoplasm, but also at restoring dystrophin expression are being developed [15]. One of the most promising therapies is exon skipping. In this method, natural oligonucleotides or their synthetic analogs are used to eliminate some exons from mature mRNA during splicing due to steric hindrance in spliceosome. Thus, with the presence of frameshift deletions or nonsense mutations, exclusion of one or more exons can translate all the subsequent part of the gene in the correct reading frame [16]. Translation of trancated but partially functional protein can significantly improve the condition of DMD patients, especially if treatment is started at early age [1].

One of the first studies on exon skipping as a correction therapy for DMD involved four patients — carriers of the dystrophin gene with frameshift deletion of one or more exons: 50, 52, 49–50 and 48–50. In all the four cases, exon 51 skipping induction is able to restore the reading frame in theory. Therapy was carried out by intramuscular injection of antisense oligonucleotides, and although exon skipping efficiency was 46–90 %, results of standard physiological tests have not been improved [17].

However, according to Aartsma-Rus and van Ommen [18], exon skipping can help most DMD patients. Exceptions will be mutations located between exons 64 and 70, which appear to be necessary for the functioning of dystrophin, as well as deletions disrupting actin-binding domains in the N-terminal region or affecting the first or last exon, and large chromosomal rearrangements such as translocations. However, the above-mentioned mutations are rare and together account for less than 10 % of all the described mutations in the dystrophin gene. Thus, theoretically, expression of functional or partially functional protein can be restored via exon skipping in about 90 % of DMD patients.

Prediction and analysis of the efficiency of exon skipping therapy are an important applied task since due to the large size of gene, the frequency of mutation in it is very high and about every third DMD patient has a de novo mutation [13]. An important theoretical aspect of the applicability of the method is assessment of the functionality of the dystrophin, truncated by exon skipping. Such assessment was the aim of this work. To make such assessment, it was necessary to decide which mutations can be analyzed, how they can be corrected (i.e. skipping of which exons would restore dystrophin expression) and which methods of analysis of phenotypic expression of mutations can be used. We studied and summarized literature data on these aspects of the study.

The simplest case for *DMD* gene correction is the presence of a nonsense mutation or frameshift insertions and deletions (indels) within a single exon. Theoretically, such mutations can be corrected by skipping one or a group of adjacent exons whose removal from mature mRNA will not lead to frameshift. In this case, 7 exons at most can be skipped — this is the exact number allowed to be excluded from the mature mRNA for the method to remain efficient [18].

Out of 79 exons of the dystrophin gene, 34 can be removed without disrupting the reading frame. Mutations in 29 exons

can be corrected by removing exons together with one of its neighbors. Moreover, 8 out of these 29 exons may be removed together with an upstream or a downstream heighboring exon (fig. 1). Errors in the remaining 14 exons can be corrected by removing a greater number of exons: exons 6–8 without frameshift are removed only together, as well as exons 76–78; exons 61 and 71 are removed only with two upstream exons, while exon 67 is removed with both upstream and downstream neighboring exons; exons 72 and 73 are removed only together with all the upstream exons up to exon 69 (four and five exons respectively); exon 74 can be removed in the same way or in a unit consisting of exons 70–75 (six exons in both cases), while exon 75 — only with the five upstream exons. Correction of exon 2 requires removal of six exons. However, Wein et al. [19] claim that exon 2 deletion leads to a very weak or asymptomatic disease course since translation starts from the internal ribosome entry site in exon 5, and in this case, truncated dystrophin retains functionality. Correction of mutations in the first and last exons is considered impossible (though this may be incorrect considering the fact that an internal ribosome entry site exists in exon 5). Thus, theoretically, mutations in 75 exons can be corrected by skipping no more than 6 exons, corresponding to the stipulated limit for the exon skipping method.

The number of possible corrections of single nucleotide substitutions in the dystrophin gene is extremely high. Experimental checking of all combinations in animal models is hardly possible. Besides, mouse model of DMD does not reproduce human DMD phenotype too well [20]. A more realistic approach to solving this problem is to analyze a large amount of clinical data of patients with mutations in the dystrophin gene that lead to DMD or BMD. Obtaining a sufficient sample from literary sources is a time-consuming process complicated by the fact that not all mutations have been published. Fortunately, there are databases of genetic variations. One of them — Leiden Open Variation Database (LOVD) [21] — contains records of different mutations in the dystrophin gene, information on the gender and population affiliation of their carriers, analysis method and most importantly, information on phenotype to which this or that mutation in a given patient leads to: DMD, BMD or asymptomatic course. Users around the world can add records to LOVD on their own. This is both an advantage (data vastness) and a disadvantage (possible errors) for the database. As of June 2016, LOVD contained over 16,000 non-unique records of mutations in the dystrophin gene relating to one or more exons. Deletions account for 83 % among them.

METHODS

Data on mutations were found using LOVD's built-in text search system [22]. A search query was introduced in the field 'Exon'. The query was created according to the database nomenclature: N-1i_Ni for exon N, which means that the mutation should affect the introns adjacent to the exon. For example, to search for exon 3 deletion, the query 2i_3i was created. For deletion of an exon group, the query was made in similar fashion. Only records containing information about full deletions of targeted exons (exons with which nonsense mutation or internal exon indels can be corrected) were taken for analysis. Duplications, as well as mutations carried by women were excluded from this list.

RESULTS

The distribution of phenotypes corresponding to the exon combinations selected for reading frame correction is shown in fig. 2. For 25 deletions no record was found in LOVD. Most of such deletions were located in the central domain region, as well as in the region of exons 65–78. Only in 11 combinations the number of cases found in the database exceeded 5. Distribution of these 11 combinations by dystrophin domains was very uneven: 7 of them were combinations correcting exons 45–55 (C-terminal part of the central rod domain), 1 combination — exon 13 deletion — affects the N-terminal region of the central domain, while 3 combinations affect the N-terminal actin-binding domain.

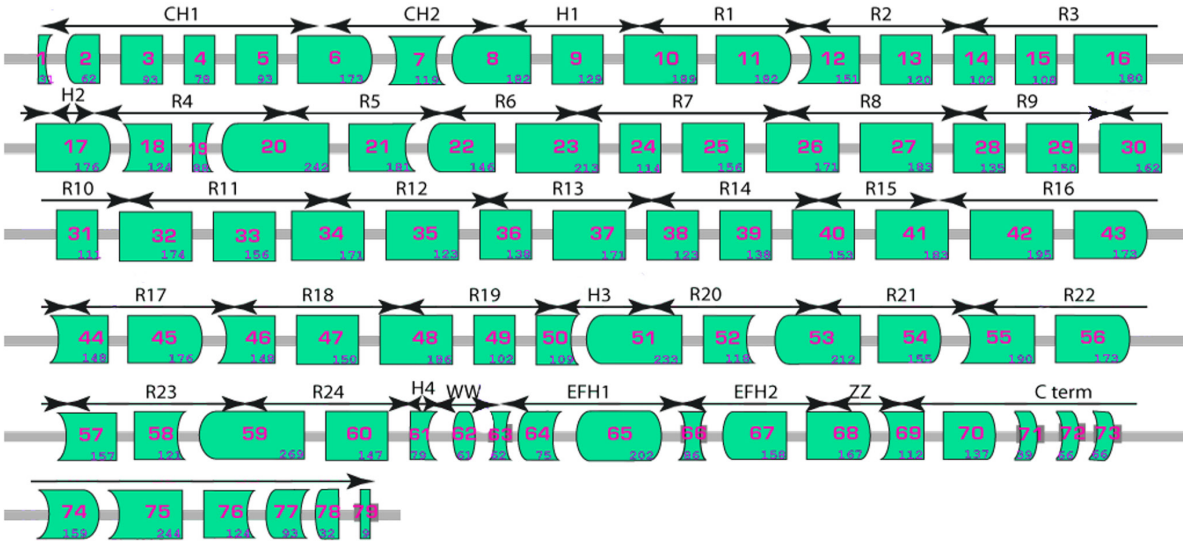

Fig. 1. Exon structure of the dystrophin gene

Green blocks indicate exons; exon size (in base pairs) is indicated in the bottom right and its sequence number in the middle. Three types of joints (straight, concave and convex) indicate the three reading frames within which exon boundaries fall. Arrows indicate the gene region borders encoding the structural domains of dystrophin: CH1 and CH2 — elements of the N-terminal actin-binding domain, R1–R24 and H1–H4 — spectrin repeats and hinges of the central rod domain, respectively, WW, ZZ, EFH1 and EFH2 — structural elements of the cysteine-rich domain, and C term – a C-terminal domain unique for dystrophin and its homologues (Nicolas et al., 2012 [29]).

BMD or asymptomatic course were observed in 45 % of all the cases (corresponding to the deletions we chose for analysis) represented in the database, whereas larger frameshift deletions of exons manifest as BMD in less than 4 % of cases, indicating the milder nature of muscular dystrophy in patients with non-frameshift deletions.

It was also established that the same mutation could manifest itself as a variety of phenotypes. Specifically, exon 48 deletion, which is found 97 times in the database, may be asymptomatic (2 % of cases) or result in BMD (60 % of cases), DMD (12 % of cases) or intermediate phenotype (26 % of cases). Even more interesting are deletions of exons 50–51 and 51–52, for which 14 and 11 cases are described respectively: there are 6 (43 %) total of BMD or asymptomatic course for the first, and none for the second, although both deletions do not affect anything except the hinge region and one of the spectrin repeats of the central domain, and as it would seem, should not have significant effect on the protein structure. Exon 47 deletion is another frequent non-frameshift deletion (13 cases), which also does not affect anything except spectrin repeats, but manifests as DMD or mixed phenotype in 100 % of cases.

DISCUSSION

Records on patients (carriers of different deletions) in the database quite strongly vary in number. The reasons for this may be both unequal frequency of mutations in different regions of the gene and under-representation of a group of mutations in the database. Indeed, such distribution can be explained by the presence of meiotic recombination hot spots in the regions of exons 7 and 44 [23]. Absence of information on deletions in the central rod domain region can be attributed largely to asymptomatic manifestation of such deletions which is likely to greatly reduce the probability of such mutations being detected. However, it is surprising that the database does not contain any combinations for correction of mutations in exons 65–78. One possible explanation for that is a large size of exon combinations needed to be deleted to correct mutations at C-end.

According to Aartsma-Rus et al. [24], 91 % of mutations in the LOVD database are in agreement with the following rule: frameshift mutations and translation of truncated non-functional dystrophins are the cause of DMD, while non-frameshift mutations usually almost do not affect the functionality of dystrophin and they cause BMD, if they do not affect the key domains located on the protein's N- and C-ends.

A confirmation of this rule apparently is the case described in the article Passos-Bueno et al. [25]. It is about a patient with BMD induced by a major non-frameshift deletion (from exon 13 to 48). This deletion captures 18 out of 24 spectrin repeats of the central domain, but does not touch the N- and C-ends. Of protein amino acid sequence only about a half remains, which, according to the authors, is enough for dystrophin to remain partially functional. However, this rule has some interesting exceptions. For example, Takeshima et al. [26] describe a case of large N-terminal non-frameshift deletion (from exon 3 to 41), which manifests itself in the form of an intermediate phenotype between DMD and BMD. The absence of an N-terminal domain makes dystrophin dysfunctional. Nevertheless, the patient's phenotype is not consistent with the classical picture of DMD course.

Another exception is nonsense mutation in exon 76 described in Suminaga et al. [27], which leads to synthesis of truncated form of dystrophin, detectable by immunohistochemistry on muscle biopsy preparations. At the same time, except elevated levels of creatine phosphokinase in the blood (which is one of the key markers of DMD and BMD), no other muscular dystrophy symptom was detected in a patient. The authors write that they cannot explain the picture obtained: another nonsense mutation described in the literature, which cuts the protein only into 10 amino acids closer to the beginning than the mutation found in [27], leads to a typical picture of DMD course [28]. The work tested different assumptions on the possible causes of such phenotype: somatic mosaicism, restoration of reading frame through alternative splicing, compensatory high level of expression of utrophin, the dystrophin homologue protein. However, all the assumptions were rejected.

As seen from analysis of the database and the cases mentioned above, same mutations in the dystrophin gene can lead to different phenotypes. The reasons for this phenomenon are not fully understood, but it is probably due to patients' different genetic background. This is a problem since even if effective skipping of exon or exons, resulting in reading frame restoration, is induced in the patient and synthesis of truncated dystrophin is observed, there is still no guarantee that the patient's condition will improve. Besides, due to insufficient knowledge of the problem, there is no reliable method of

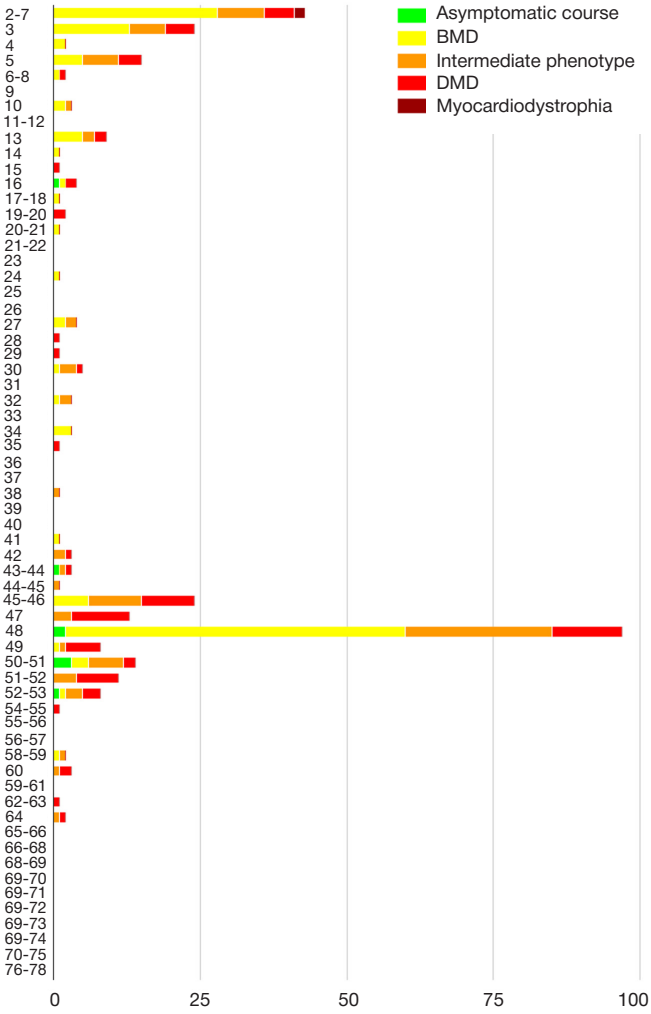

Fig. 2. Distribution of phenotypic manifestations of deletions in the dystrophin gene in exon skipping to restore the reading frame (based on LOVD data)

The vertical axis indicates the numbers of exons that were included in the deletion, while the horizontal axis is the number of records containing data on a particular deletion, which are contained in the LOVD database. Sections of horizontal bars correspond to representation of individual phenotypes.

predicting whether frame restoration would help a particular patient. Statistical analysis in this case is also difficult because phenotypic data are sufficient for only a small number of deletions.

Our work did not consider some variants of truncated dystrophins obtained by exon skipping correction of mutations that are more complex for analysis: large deletions affecting at least one exon entirely and inducing frame shift, and duplications of one or more exons. Some of them can be corrected by skipping the same exons as those used for correction of intraexonic mutations, for example, duplication of exons, deletion of which causes no frameshift, or deletion of a whole exon, which can be corrected by deletion of a neighboring one. So, in skipping both copies of exon, duplication of exon 13 is reduced to deletion of exon 13, while exon 51 deletion can be corrected by skipping exon 50 or exon 52. Correction of some duplications of two or more exons and large deletions, leading to a frameshift and affecting two or more exons, is also feasible. So, deletion from exon 7 to exon 34 can be corrected by skipping exon 6. The resulting deletion will not disrupt the reading frame. However, it is unknown whether this form of dystrophin would be functional. Moreover, LOVD contains no data on this particular mutation. As a result, it was decided not to carry out detailed analysis of such mutations due to the large number of combinations and lack of data on the functionality of the dystrophin forms resulting from correction of the mutations.

CONCLUSIONS

Analysis of data on phenotypic mutations in the dystrophin gene showed that the same mutations can manifest various phenotypes. This indicates that the success of exon skipping for treatment of Duchenne muscular dystrophy is probabilistic in nature even in the case of highly efficient induction of alternative splicing. Accurate assessment of the probability of success for a particular therapy is difficult due to lack of data. However, one can hope that the situation will change with the emergence of higher-throughput and cheaper DNA sequencing methods.

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GADOLINIUM- AND CURCUMIN-LOADED MICELLES BASED ON α-FETOPROTEIN FUNCTIONALIZED AMPHIPHILIC BLOCK COPOLYMERS

Pozdniakova NV[1]✉, Grigorieva EYu[1], Shevelev AB[2]

[1] Laboratory of Radionuclide and Beam Technologies in Experimental Oncology, N. N. Blokhin Russian Cancer Research Center, Moscow

[2] Laboratory of Light Sensitization, N. M. Emanuel Institute of Biochemical Physics, RAS, Moscow

This article describes a method of obtaining curcumin- and gadolinium-loaded micelles based on triblock amphiphilic polyethylene glycol and polypropylene glycol copolymers Pluronic F-127 and Pluronic P-123 (Sigma-Aldrich, USA) superficially functionalized with recombinant human α-fetoprotein. The size of nanoparticles was measured using dynamic light scattering and amounted to an average of 50 to 100 nm. The micelles were stable: stored at +4 °C for 10 days, they exhibited no changes in their properties that would not fall within the standard error of measurement for the methods used for the analysis. Preliminary *in vivo* experiments conducted on mice showed no conspicuous toxicity of micelles with the maximum possible concentration of gadolinium, which enables their use in tumor tissue imaging *in vivo.*

Keywords: imaging, contrast agents, gadolinium, curcumin, micelles, Pluronic F-127, Pluronic P-123

Funding: the study was supported by the Ministry of Education and Science of the Russian Federation (Grant Agreement no.14.607.21.0133 dated October 27, 2015, Project ID RFMEFI60715X0133).

✉ **Correspondence should be addressed:** Natalia Pozdniakova
Kashirskoe shosse, d. 24, Moscow, Russia, 115478; natpo2002@mail.ru

Received: 23.06.2016 **Accepted:** 27.06.2016

МИЦЕЛЛЯРНЫЕ КОМПОЗИЦИИ НА ОСНОВЕ ФУНКЦИОНАЛИЗИРОВАННЫХ α-ФЕТОПРОТЕИНОМ АМФИФИЛЬНЫХ БЛОК-СОПОЛИМЕРОВ, СОДЕРЖАЩИХ ГАДОЛИНИЙ И КУРКУМИН

Н. В. Позднякова[1]✉, Е. Ю. Григорьева[1], А. Б. Шевелев[2]

[1] Лаборатория радионуклидных и лучевых технологий в экспериментальной онкологии, Российский онкологический научный центр имени Н. Н. Блохина, Москва

[2] Лаборатория процессов фотосенсибилизации, Институт биохимической физики имени Н. М. Эмануэля РАН, Москва

Предложен метод получения мицеллярных композиций куркумина и ионов гадолиния на основе трехблочных амфифильных сополимеров полиэтиленгликоля и полипропиленгликоля Pluronic F-127 и Pluronic P-123 (Sigma-Aldrich, США), поверхность которых функционализирована рекомбинантным производным α фетопротеина человека. Методом динамического светорассеяния определили размер наночастиц, и он составил в среднем 50–100 нм. Композиции отличались устойчивостью: в течение 10 суток хранения при +4 °C характеристики мицелл изменялись в пределах стандартной ошибки измерения для выбранных аналитических методов. Предварительные эксперименты *in vivo* на мышах показали отсутствие явно выраженной токсичности композиций при максимально возможной концентрации гадолиния, что делает возможным их дальнейшее использование для визуализации опухолевых тканей *in vivo*.

Ключевые слова: визуализация, контрастные средства, гадолиний, куркумин, мицеллярная композиция, Pluronic F-127, Pluronic P-123

Финансирование: исследование поддержано Министерством образования и науки Российской Федерации (Соглашение о предоставлении субсидии № 14.607.21.0133 от 27.10.2015, уникальный идентификатор RFMEFI60715X0133).

✉ **Для корреспонденции:** Позднякова Наталья Владимировна
115478, г. Москва, Каширское шоссе, д. 24; natpo2002@mail.ru

Статья получена: 23.06.2016 **Статья принята в печать:** 27.06.2016

Due to its unique paramagnetic properties, gadolinium (Gd^{3+}) holds a firm place in medical diagnostics (magnetic resonance imaging, MRI) and therapy (secondary thermal radiation used for eliminating tumor cells) [1]. Electron spin-lattice relaxation time T1 of gadolinium-based contrast agents is about 0.1 ns, i.e., an order of magnitude shorter than proton relaxation time in water [2]. Since Gd^{3+} is very toxic even at low concentrations that are routinely used for enhanced MRI, it must be chelated with agents that have a high effective association constant, such as diethylenetriaminepentaacetic acid [3]. Nanoparticles containing Gd^{3+} chelates are widely used in clinical practice for performing contrast-enhanced MRI of various tissues on living patients, including scans of brain structures [4], vascular plexuses [5], myocardium and coronary arteries [6].

Kang et al. [7] have described a method of preparing gadolinium oxide nanoparticles $Gd@SiO_2$-DO3A and $Gd@SiO_2$-DO2A-BTA of 50-60 nm in size. Nanoparticles were synthesized from tetraethyl orthosilicate (TEOS) and (3-aminopropyl) triethoxysilane (APTES); then, aminopropylsilane groups were functionalized with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) or 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid conjugates of benzothiazole (DO3A-BTA). The obtained nanoparticles were highly soluble in water; their colloid solution was stable. Their relaxivity r1 was much higher than r1 of low molecular weight contrast agents; the ratio r2/r1 [8] was close to 1, which indicates that the investigated nanoparticles can be used as MRI contrast agents [9]. Biodistribution analysis showed that $Gd@SiO_2$-DO2A-BTA is excreted in bile and urine. The nanoparticles also accumulated in tumor cells and were found efficient for *in vivo* elimination of such continuous cell lines as SK-HEP-1, MDA-MB-231, HeLa and Hep 3B. Although some authors report cumulative toxic effects of nanostructured gadolinium-based contrast agents [10, 11], including renal toxicity [12], the latter remain an attractive subject for research, as they can be used in MRI and cancer therapies [13].

The aim of this work was to elaborate a technique for obtaining a Gd^{3+} — based low-toxic micellar formulation for visualizing tumor tissues *in vivo*.

METHODS

To ensure that the system is low toxic and has affinity to tumor cells and to prevent its non-specific binding to normal tissues, three solutions were suggested.

First, we decided to use curcumin as a complexing agent. Curcumin is a non-toxic natural compound (fig. 1). It is abundant in the roots of Indian saffron *Curcuma longa*. Some of its properties come in handy for our research [14]: it can form stable coordinate bonds with transition metals [15], it is not toxic at usual concentrations, and its hydrophobicity allows accommodating its compounds in the micelle core and ensures micelle stability in aqueous solutions.

Second, we obtained a micellar formulation of the compound. Methods of preparing micellar formulations have already been employed by biotechnology [16] and can possibly find their way into pharmaceutical industry [17]. Micellar formulations have some advantages over other types of carriers: in aqueous solutions, they self-assemble into structures with a hydrophobic core and a hydrophilic surface, which makes it possible to accommodate hydrophobic agents inside the micelle and thus protect them from inactivation in biological media. Micelles are small (<100 nm), easy to formulate and have long blood circulation time [18]. Among the advantages of block copolymer (Pluronic)-based micelles are low toxicity, weak immunogenicity and simplicity of surface modification with functional groups necessary for obtaining certain properties. For this work, we chose amphiphilic triblock copolymers of polyethylene glycol and polypropylene glycol Pluronic F-127 and Pluronic P-123 (Sigma-Aldrich, USA) (fig. 2)

Third, we improved the technique of conferring target specificity to a micelle by immobilizing to its surface a recombinant vector protein (a fragment of human alpha-fetoprotein with high affinity to tumors of various types) [19].

The following reagents by Sigma-Aldrich, USA, were used in the study: gadolinium(III)nitrate hexahydrate, triblock copolymers Pluronic F-127 and Pluronic P-123, triethylamine, curcumin, 1,1′-carbonyldiimidazole, N-hydroxysuccinimide, 2,4,6-trinitrobenzenesulfonic acid, tetrahydrofuran, dimethyl sulfoxide, methanol, dimethylformamide, ethanolamine, cobalt thiocyanate, barium hydroxide. The protocol for obtaining human alpha-fetoprotein-based vector protein was described previously [19].

Preparation of curcumin – gadolinium complexes

Complexes were prepared in the organic solvent tetrahydrofuran by mixing gadolinium nitrate, curcumin and triethylamine at molar ratio of 3:1:1, heating the mixture to 50 °C for 30 minutes. Then, triethylamine and the solvent were removed in the rotary evaporator. Details are given below. 100 µl of 0.5 M curcumin solution in tetrahydrofuran were mixed with 300 µl of 0.5 M gadolinium nitrate solution in tetrahydrofuran. Then, 100 µl of 0.5 M tryethylamine solution in methanol were added dropwise over 1–2 minutes while stirring the mixture vigorously. The mixture was heated to 50 °C for 30 minutes while stirring. Solvents and triethylamine were removed from the solution at 50 °C using the rotary evaporator; the solids were washed in methanol and air-dried.

Qualitative assessment of Gd^{3+} complex formulation was performed on the Cary 50 Scan UV-Vis spectrophotometer (Agilent Technologies, USA) with a scan range set to 300–650 nm, in a 1 cm-pathlength disposable cuvette (Sigma-Aldrich, item no. Z330418). Dry aliquots (weight ranging from 1 to several mg) were collected with a spatula. Mother liquor was diluted down with dimethyl sulfoxide (DMSO) to obtain the optical density of 0.5 to 1.5 at 450 nm. To determine complex concentration, the peak height was measured at 455 nm (with free curcumin the peak is absent). To find the peak height [20], we deducted spectral data obtained from free curcumin solution in DMSO from the spectral data of the reaction mix.

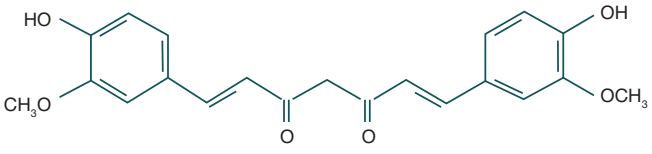

Fig. 1. Chemical structure of curcumin

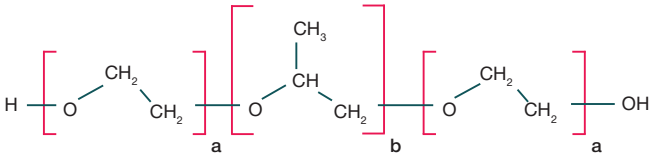

Fig. 2. Structure of amphiphilic triblock copolymers Pluronic F-127 and Pluronic P-123 (Sigma-Aldrich, USA), where a represents the number of hydrophilic monomers and b represents the number of hydrophobic monomers. For Pluronic F-127, the a-b-a formula is 98–67–98. For Pluronic P-123, the a-b-a formula is 20–70–20.

Preparation of a modified copolymer Pluronic F-127 with NHS end groups for vector protein immobilization to micelle surface

Pluronic F-127 was modified in dimethylformamide (DMFA) in two steps. At first, hydroxyl end groups were activated by carbonyldiimidazole (CDIz). Next, a reaction with N-hydroxysuccinimide (NHS) was carried out (fig. 3). Briefly, 204 mg of Pluronic F-127, 19 mg of NHS and 26 mg of CDIz were dry-mixed. Then, DMFA was added to bring the final volume of the reaction mixture to 500 µl. The mixture was stirred for 1 hour at 37 °C until CO_2 gas bubbling ceased. Excess CDIz was removed by adding 50 µl water to the reaction mixture. After excess CDIz was hydrolyzed, the modified copolymer was extracted several times with diethyl ether. The solids were air-dried.

The number of NHS end groups in the modified Pluronic F-127 was measured by performing a reaction with excess ethanolamine in 10 mM borate buffer (pH 8.5) with subsequent titration of unreacted amino groups by 2,4,6-trinitrobenzenesulphonic acid (TNBS), as described previously [21].

Micelle formulation

Various copolymer-based micellar systems were prepared as described below. Weighted amounts of the curcumin/gadolinium complex and modified pluronics were dissolved in tetrahydrofuran and mixed together. The amount and the ratio of the components were different in various micellar systems (see the table). Then, the solution was poured into a rotary evaporator flask, and the organic solvent was removed at 50 °C while the flask was rotating. Then, distilled water was added and the mixture was stirred vigorously for 15 minutes and centrifuged. Supernatant was removed; the mixture was dialyzed against the phosphate-buffered saline (pH 7.5) at +4 °C for 24 hours.

To assess how efficiently the investigated substance was encapsulated into the micelles (%), the following formula was applied:

$$E_{enc} = \frac{M_{enc} \times 100}{M_{init}},$$

where M_{enc} is the mass of the encapsulated curcumin-gadolinium complex, and M_{init} is the initial mass of the curcumin-gadolinium complex.

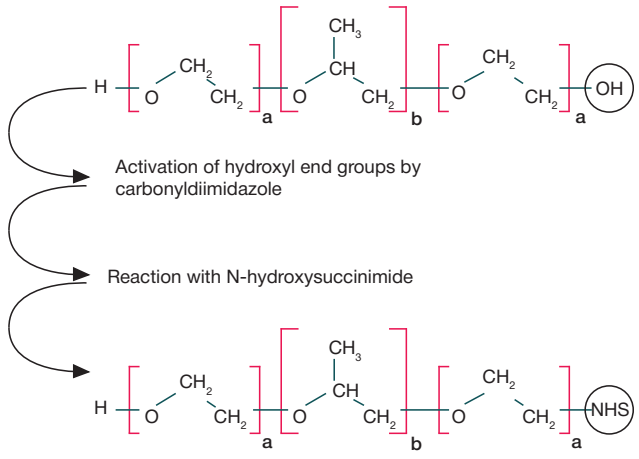

Fig. 3. Chemical schematic of Pluronic F-127 modification

Concentration load (% m/m) was calculated according to the following formula:

$$L = \frac{M_{enc} \times 100}{M_{enc}+M_{pol}},$$

where M_{pol} is the copolymer mass.

Chemical modification of micelle surface

Micelles were conjugated to protein by mixing the copolymer-based micellar solution and the protein solution, in molar excess of the modified copolymer (NHS groups) to the protein. For functionalization, we used recombinant domain 3 of human alpha-fetoprotein [19]. The detailed protocol is below. 1 ml of the micellar solution was mixed with 200 µl of 0.1 mM protein solution (concentration of 3 mg/ml) in the phosphate-buffered saline (pH 8.0) and incubated for 1 hour at room temperature. To deactivate excess reactive NHS groups, excess ethanolamine was added after incubation, until its concentration in the solution was 10 mM; the mixture was then incubated at room temperature for 2 hours and dialyzed against the phosphate-buffered saline (pH 7.2) at +4 °C for 24 hours. The obtained substance was stored at +4 °C for one week.

Qualitative detection of protein in the obtained micelle composition was performed in a polyacrylamide gel by electrophoresis with subsequent staining for 10 minutes in a 5% aqueous solution of barium iodide [22].

Evaluation of micelle size

The average size of the micelles and size distribution were evaluated by dynamic light scattering on ZetasizerNano ZS (Malvern Ltd., UK) in thermostatic plastic microcuvettes ZEN0040. Measurements were performed using the helium-neon laser with a wavelength of 633 nm and a power level of 4 mW at 25 °C. Prior to measurements, samples were diluted tenfold with deionized water. Measurements for each sample were performed in replicates of 3. Mean root square deviation was calculated.

Determination of gadolinium content in micelles

Measurements were performed on the X-ray fluorescence analyzer X-art M (Komita, Russia). The signal from the studied samples was compared to the signal from the solutions of known gadolinium concentrations.

Determination of curcumin content in micelles

To determine curcumin content, absorption spectra of the micelles and calibration solutions of known concentration were measured in a disposable cuvette (Sigma-Aldrich, item no. Z330418) at 430 nm wavelength on the Cary 50 Scan UV-Vis spectrophotometer.

Measuring polymer dry matter content

Dry matter content was measured using a previously described method [23] with slight modifications. The reagent was prepared as follows: 0.2 ml of 2.6 M aqueous solution of cobalt thiocyanate (CoSCN) were mixed with 0.8 mL of 0.8 M barium hydroxide aqueous solution. A 10 µl aliquot of pluronic-containing solution was mixed with 10 µl of the reagent, stirred vigorously and centrifuged; supernatant was removed; the solids were air-dried. Then, 100 µl of DMSO and 5 µl of 5 M HCl

were added to the solids. Absorption was measured at 630 nm on the Titertek Multiscan® Plus reader (LabX, Canada) in 96-well plates. Calibration solutions of known concentration were measured in parallel. Using a calibration curve, concentration in the analyzed sample was calculated.

Determination of total protein content

Total protein content was evaluated by a modified Lowry method using bicinchoninic acid (Sigma-Aldrich). A standard bovine serum albumin solution was used as a calibration solution [24].

Assesment of toxicity of curcumin-gadolinium micelle formulation

To verify that further *in vivo* research is possible, we investigated animal tolerance to the obtained micelle formulation. We used 10 C57/black female mice with an average weight of 25 g. The animals were administered the micellar formulation composed of 10 mM Gd^{3+}, 10 mM curcumin, 5 mM Pluronic F-127 and phosphate-buffered saline (pH 7.0). Micelle diameter was 20 nm. The animals received a single 200 µl injection into the tail vein. Dosage was selected based on the pluronic concentration optimal for delivering the cytostatic drug Docetaxel to lung tumor cells *in vivo* in the mice model [25]. The animals were observed for 2 months. Then, they were sacrificed; autopsy and histological analysis were performed subsequently.

RESULTS

The process of curcumin-gadolinium complex formation was controlled by recording spectral changes, such as the additional peak occurring at 455 nm [20] (fig. 5).

The copolymer:NHS ratio in the modified pluronic F-127 was about 1:1. In total, we obtained about 100 µl of dry modified copolymer containing one NHS group per molecule.

Encapsulation efficiency of the method applied for micelle formulation varied from 40 to 80 % and load concentration varied from 10 to 25 % m/m. As a result, we obtained a number of micelle solutions with various amounts of basic components (see the table).

Curcumin/gadolinium ratio in the micelles implies vacant valence orbitals in a gadolinium atom that are presumably occupied by water molecules, which is essential for preserving contrasting properties during MRI scans [26]. As far as micelle stability goes, we should note that evaluation of micelle sizes by dynamic light scattering and measurements of visible absorption spectrum typical for the curcumin-gadolinium complex were repeated after the micelles had been stored at +4 °C for 10 days. All values remained unchanged. Differences fell within the standard error of measurement.

The size of the obtained micelles was approximating (but no exceeding) 100 nm. It allows micelles to circulate in the blood for a long time without being captured by the hepatic portal and reticuloendothelial systems [27]. This property is important for ensuring that micelles are delivered to tumor tissues, owing to the immobilized functional protein agent on their surface [28].

During the experiment on mice, no visible signs of toxicity of the micelle system were detected. We did not observe symptoms of heavy metal poisoning [1], such as collaptoid reactions or bleeding. The animals had a good appetite and were active; there were no signs of pathological thirst or photophobicity.

DISCUSSION

A number of works [14, 29] prove that curcumin can be used as a complexing agent that suppresses gadolinium toxicity *in vivo*. Patil et al. [29] used such complexes for detecting amyloid plaques in Alzheimer's patients. The researchers exploited the natural affinity of curcumin to β-amyloids. Our work is the first to demonstrate the possibility of encapsulating hydrophobic curcumin complexes into micelles formed by pluronics (block copolymers of polyethylene glycol and polypropylene glycol). Such micelles exhibit stability in aqueous media, therefore, curcumin pharmacokinetic properties no longer play a key role in distributing Gd^{3+} ions in body tissues, because the chelated complex remains highly stable.

Other authors [7] worked on an almost identical task, but found a different solution to it: they encapsulated Gd^{3+} ions

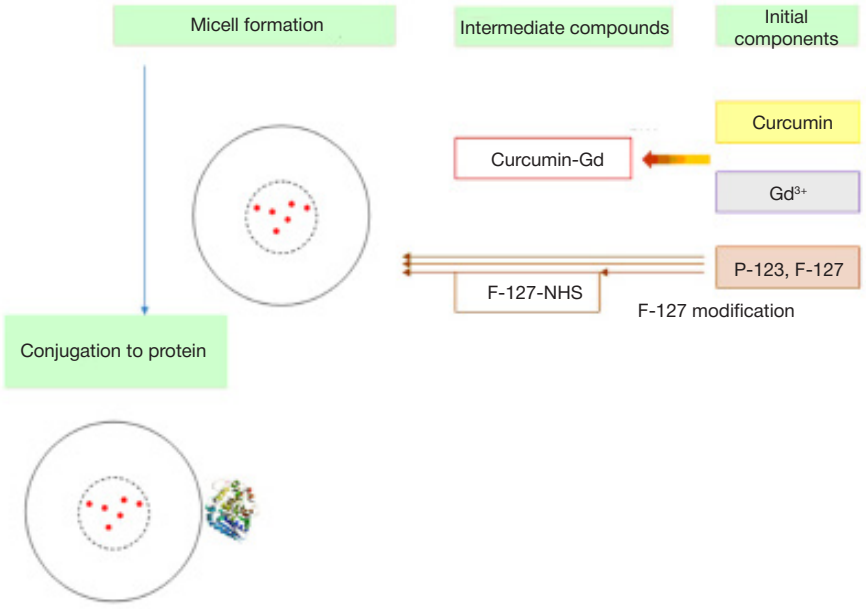

Fig. 4. Schematic of obtaining gadolinium-containing functionalized micelles

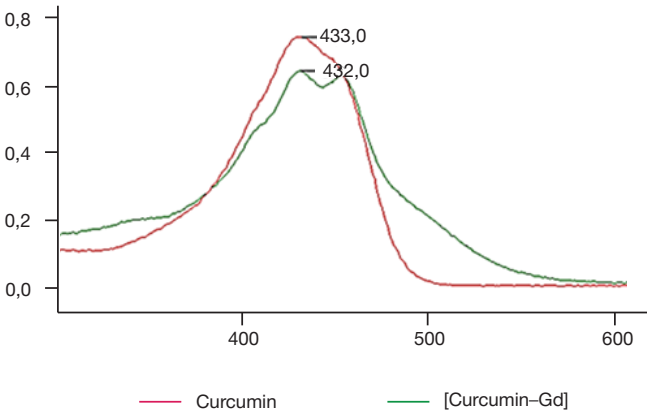

Fig. 5. Spectral data of curcumin and curcumin-Gd^{3+} complex

into silicon. It proves that the task itself is of high practical significance. The technique we suggest has an advantage over the one described in work [7]: it can be implemented in a biochemical laboratory using commercially available reagents and standard equipment, with almost 100% yield and without recruiting professional synthetic chemists. This technique also allows both small and commercial scale production of Gd^{3+} containing nanoparticles. Despite its simplicity, the absolute size of particles, size distribution and the amount of the encapsulated substance do not differ from the corresponding values suggested in [8]. It became possible due to those curcumin properties that make it a natural Gd^{3+} chelator.

Micelles have an important advantage over hard silicon oxide nanoparticles. They can circulate in the blood for a long time without being destroyed by liver monocyte derivatives [9]. At the same time, defective or leaky tumor vasculature allows micelles to be retained in tumor tissues [30].

Additional enhancement of target specificity of Gd^{3+} containing micelles can be achieved through using various target-specific functional groups. Domain 3 of α-fetoprotein employed for this work has a high affinity to receptors of various tumor types, since, like albumin, it uses them as a nutrient [19]. The suggested technique can efficiently immobilize any protein, peptide or other target-specific groups to micelle surfaces.

CONCLUSIONS

We have demonstrated that curcumin-gadolinium complexes are efficiently encapsulated and strongly retained in micelles formed by polyethylene glycol and polypropylene glycol copolymers (pluronics). Stored at +4 °C for 10 days, the micelles exhibited no changes in their properties that would not fall within the standard error of measurement. The micelles have a standard size of 50-100 nm depending on the ratio of their components and a prolonged blood circulation time.

We suggest a technique for modifying the surface of pluronic mycelles containing curcumin and Gd^{3+} complexes using target-specific molecules, such as domain 3 of human α-fetoprotein.

Due to its simplicity, high efficiency and a possibility to work with scant amounts of components, this method is intended primarily for studying distribution of macromolecules and their complexes *in vivo*. It can also be used for screening functional target-specific groups that deliver biomacromolecules and their complexes to tissues and cells of various types, including tumors.

Micelle composition

№	Components	Concentration of components	Mean micelle diameter, nm
1	Gd^{3+} – Curcumin – P-123	Gd^{3+} – 12 мМ Curcumin – 12 мМ P-123 – 3 мМ	57.0 ± 1.2
2	Gd^{3+} – Curcumin – F-127	Gd^{3+} – 10 мМ Curcumin – 10 мМ F-127 – 5 мМ	20.0 ± 0.9
3	Gd^{3+} – Curcumin – P-123 – F-127	Gd^{3+} – 9 мМ Curcumin – 21 мМ P-123 – 2.7 мМ F-127 – 2.7 мМ	83.0 ± 1.4
4	Gd^{3+} – Curcumin – F-127 – Protein	Gd^{3+} – 57 мМ Curcumin – 30 мМ F-127 – 4 мМ Protein – 0.7 мг/мл	32.0 ± 2.2
5	Gd^{3+}– Curcumin – P-123 – F-127 – Protein	Gd^{3+} – 18 мМ Curcumin – 8.4 мМ P-123 – 2.5 мМ F-127 – 2.5 мМ Protein – 1.1 мг/мл	69.0 ± 1.3

Note: P-123 — Pluronic P-123, F-127 — Pluronic F-127 (Sigma-Aldrich, USA).

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IMMUNOMODULATOR IMUNOFAN AFFECTS CELL PROFILE OF MORPHOFUNCTIONAL ZONES OF RAT THYMUS AND DELAYS ITS AGE-RELATED INVOLUTION

Bobrysheva IV ✉

Department of Histology, Cytology and Embryology, Faculty of Stomatology,
Lugansk State Medical University, Lugansk, Ukraine

The immunomodulatory agent Imunofan (Bionox, Russia) is widely used in clinical practice. It affects the immune and endocrine systems and enhances cell-mediated and humoral immunity. The aim of this study was to investigate the cell profile (lymphoblasts, small, medium and large lymphocytes, macrophages, mitotic cells and damaged cells) in the subcapsular and inner zones of the thymic cortex and thymic medulla of random-bred male albino rats with conspicuous age-related changes after stimulating their immune system with Imunofan. The animals in the experimental group (n = 30) were administered to intramuscular injections of the drug (0.7 mg/kg) on the 1st, 3rd, 5th, 7th and 9th days of the experiment; the controls (n = 30) were administered to the equivalent amount of normal saline on the same days. Rats were decapitated on the 1st, 7th, 15th, 30th and 60th days after the final injection. Thymic sections were studied using Olympus CX-41 microscope, Olympus SP 500UZ camera (Olympus, Japan) and Morpholog software (Ukraine). Thymic morphology was similar in the experimental and control groups; however, cell profiles were different. On the 7th, 15th and 30th days, lymphoid cells and macrophages prevailed over damaged cells, the number of which decreased ($p < 0.05$). Similar statistically significant trends were found in the inner zone of the thymic cortex. The number of medium lymphocytes was statistically higher on the 7th, 15th and 30th days of the observation, while the number of small lymphocytes was also higher on the 60th day of the observation. The number of damaged cells was significantly lower on the 15th and 30th days ($p < 0.05$). The obtained results indicate conspicuous thymic response in rats with conspicous age-related changes to Imunofan administration, and partial temporary delay of age-related thymic involution.

Keywords: thymus, age-related involution, immune stimulation, immunomodulator, Imunofan, albino rats

✉ **Correspondence should be addressed:** Inessa Bobrysheva
ul. 15-ya Liniya, d.18, kv. 8, Lugansk, Ukraine, 91016; inessa_lug@mail.ru

Received: 31.05.2016 **Accepted:** 22.06.2016

ИММУНОМОДУЛЯТОР «ИМУНОФАН» ВЛИЯЕТ НА КЛЕТОЧНЫЙ СОСТАВ МОРФОФУНКЦИОНАЛЬНЫХ ЗОН ТИМУСА КРЫС И ЗАМЕДЛЯЕТ ЕГО ВОЗРАСТНУЮ ИНВОЛЮЦИЮ

И. В. Бобрышева ✉

Кафедра гистологии, цитологии и эмбриологии, стоматологический факультет,
Луганский государственный медицинский университет, Луганск, Украина

В клинической практике применяется иммуномодулятор «Имунофан» («Бионокс», Россия), воздействующий на иммунную и эндокринную системы и усиливающий клеточный и гуморальный иммунитет. Целью исследования являлось изучение содержания лимфобластов, малых, средних и больших лимфоцитов, макрофагов, митотически делящихся и деструктивно измененных клеток в субкапсулярной и внутренней зонах коркового вещества и мозговом веществе паренхимы тимуса беспородных белых крыс-самцов периода выраженных старческих изменений при иммуностимуляции «Имунофаном». Животным опытной группы (n = 30) вводили препарат внутримышечно из расчета 0,7 мг/кг в 1, 3, 5, 7 и 9-е сутки эксперимента, а животным контрольной группы (n = 30) — эквивалентный объем физиологического раствора в те же сроки. Декапитацию осуществляли на 1, 7, 15, 30 и 60-е сутки после последней инъекции. Срезы изучали с помощью микроскопа Olympus CX-41, фотоаппарата Olympus SP 500UZ (Olympus, Япония) и программного пакета Morpholog (Украина). Морфологические особенности органа в опытной и контрольной группах были схожими, но клеточный состав зон различался. В субкапсулярной зоне на 7, 15 и 30-е сутки было больше клеток лимфоидного ряда и макрофагов при одновременном снижении числа клеток с признаками деструкции ($p < 0,05$). Аналогичные статистически значимые закономерности были выявлены для внутренней зоны. В мозговом веществе содержание средних лимфоцитов было достоверно выше на 7, 15 и 30-е сутки наблюдения, а малых лимфоцитов — также и на 60-е сутки. Количество деструктивно измененных клеток значительно уменьшилось на 15 и 30-е сутки ($p < 0,05$). Полученные результаты свидетельствуют о заметной реактивности тимуса крыс периода выраженных старческих изменений на введение «Имунофана» и временном частичном замедлении его возрастной инволюции.

Ключевые слова: тимус, возрастная инволюция, иммуностимуляция, иммуномодулятор, имунофан, белые крысы

✉ **Для корреспонденции:** Бобрышева Инесса Венедиктовна
91016, Украина, г. Луганск, ул. 15 линия, д. 18, кв. 8; inessa_lug@mail.ru

Статья поступила: 31.05.2016 **Статья принята к печати:** 22.06.2016

Thymus is a primary organ of the immune system. It largely contributes to the intensity of immune response and maintains immune homeostasis. Morphological changes in the thymus triggered by a variety of factors, including administration of immunotropic drugs, are accompanied by changes in the cellular microenvironment and cytoarchitecture [1–7].

The immunomodulatory agent Imunofan is widely used in clinical practice. It positively affects the immune and endocrine systems and enhances cell-mediated and humoral immunity [8]. It is an immunoregulatory hexapeptide (arginil-alfa-aspartil-lysil-valil-tyrosil-arginine) synthesized from a thymopoietin fragment that contains amino acid residues of its active site. Pharmacological effects of Imunofan include fine-tuning of the immune system and elimination of oxidative/antioxidative imbalance. Drug action starts 2–3-hours after the injection and lasts up to 4 months. The time course of the drug action can be divided into three phases. During the first phase (2–3 days after the injection), a detoxifying effect of Imunofan is observed: the drug normalizes lipid peroxidation and inhibits breakdown of cell membrane phospholipids and arachidonic acid synthesis. During the second phase that lasts for 7–10 days, phagocytic activity increases followed by the death of intracellular bacteria and viruses. During the final phase that lasts up to 4 months, impaired functions of cell-mediated and humoral immunity are restored.

Effects of Imunofan on different animal and human body systems have been studied widely [1, 3, 9–12]. However, little attention has been paid to the changes in the thymic cytoarchitecture induced by Imunofan in subjects of various age. The aim of this study was to investigate the cell profile of thymic parenchyma in aging rats after stimulating it with Imunofan.

METHODS

The study was conducted in 60 random-bred male albino rats (age of 20 months, weight of 300–330 g). The animals were housed at 20–25 °C, humidity below 50 %, 12 h light (from 8:00 to 20:00) in standard plastic cages, 6 rats per cage, with free access to food and water [13]. According to daily observations, all animals were active and healthy.

The animals were divided into two groups, of 30 rats each. The experimental group received 0.7 mg/kg IM Imunofan (Bionox, Russia; Registration Certificate UA/0318/01/01) once a day on days 1, 3, 5, 7, and 9 of the experiment (the route of administration was chosen according to manufacturer's recommendations; human dosage was converted to animal dosage). The controls were administered to sodium chloride 0.9 % IM in the same amount and on the same days. In each group, rats were sacrificed in sixes 1, 7, 15, 30 and 60 days after the final injection of the drug or sodium chloride solution (the animals were anesthetized with ether and decapitated).

The object of our study was the thymus. Sampling, fixation and paraffin block preparation were performed according to standard techniques for lymphoid tissue processing [14]. To study thymic structural components, 4–6 μm thick paraffin sections were stained with hematoxylin and eosin; for cell identification, azure II and eosin were used. Histological analysis was performed on Olympus CX-41 microscope, using Olympus SP 500UZ digital camera (Olympus, Japan) and Morpholog software (Ukraine) [15]. Microphotographs were taken in various magnification modes, using objective lenses PlanC N x10/0.25∞/–/FN22, PlanC N x40/0.65 ∞/0.17/FN22, PlanC N x60/0.80∞/0.17/FN22, with zoom 132 and 142. From each thymus, six sections were obtained; six fields of view were analyzed in each case, which is sufficient for obtaining a representative sample [16].

We calculated percentages of lymphoblasts, small, medium and large lymphocytes, macrophages, mitotically active cells and damaged cells per 100 cells in the thymic parenchyma, including the subcapsular and inner zones of the cortex and the medulla. Small, medium and large lymphocytes were distinguished based on the morphometric parameters of the nuclear area. According to Kriventsov [17], lymphocytes with the nuclear area of 6 to 14 μm^2 are classified as small, 14 to 22 μm^2 are considered medium and 22 to 30 μm^2 are large.

Data were statistically processed using Student's t-test ($p < 0.05$). Data distribution was normal. Distribution type was identified using Kolmogorov-Smirnov test. Arithmetic mean and standard error ($M \pm m$) were computed.

The experiment was conducted in compliance with the regulations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and approved by the Bioethics Committee of Lugansk State Medical University (Protocol no.1 dated January 19, 2013).

RESULTS

Age-related involution of the thymus was confirmed by the comparative histological analysis of the thymus of the controls and the pubertal rats (those data were obtained prior to this experiment [18]).

Thymic lobules looked smaller than in younger animals (fig. 1). They were separated by thick connective tissue septa. The border between the cortex and the medulla was blurred; medulla size was increased. Lobule parenchyma was partially replaced with white adipose tissue. Similar age-related changes in the thymus of 12-month old rats were described by Moroz [19]; similar changes in 6–10-month old rats were described by Moskvichev et al. [6].

Morphologically, the thymus of the experimental animals did not differ from that of the controls throughout the experiment. We observed capsule- and septa-forming connective tissue overgrowth and partial replacement of the parenchyma with adipose tissue. But at a higher magnification, microscopic images revealed changes in the parenchyma cell profile.

The stroma of the subcapsular zone of the cortex parenchyma is formed by a network of epithelial reticular cells and macrophages. In the stromal area, several layers of round lymphoid cells were observed. The majority of those cells were small and medium lymphocytes, but large lymphocytes and lymphoblasts were also present. Mitoses were rare. Epithelial reticular cells were flat and irregularly shaped, larger than lymphocytes and had a paler cytoplasm. Macrophages were large, irregularly shaped with branching projections and a typical foamy cytoplasm. Damaged lymphoid cells were also observed (those contained hypercondensed chromatin in the shrunk nucleus). We observed an increased number of lymphoid cells and macrophages, compared to the controls, and a reduced number of damaged cells (fig. 2). However, the figures were statistically significant only for the animals sacrificed on days 7, 15 and 30 after the final injection of Imunofan. The number of epithelial reticular cells did not differ significantly from that of the controls in any of the experimental subgroups.

The inner zone of the cortex demonstrated a maximum cell density with several layers of medium and small lymphocytes, some of them mitotically active, integrated into the network

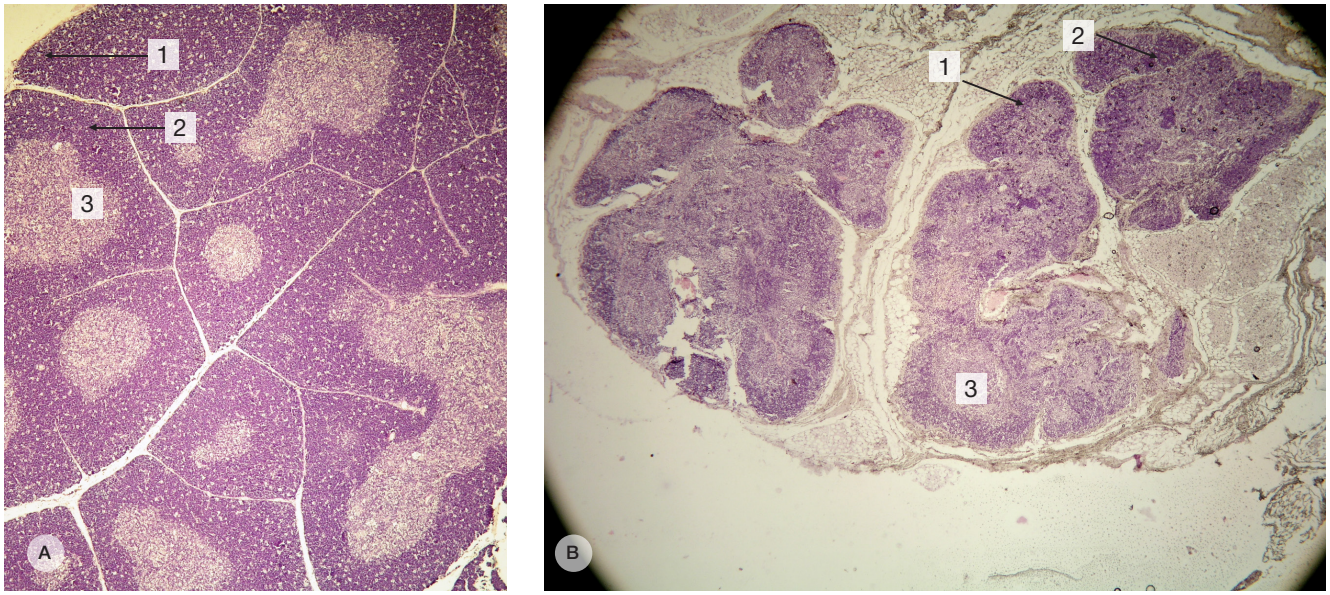

Fig. 1. Age-related involution of the thymus in random-bred albino male rats

Microphotographs of thymic sections obtained from pubertal male rats **(A)** and 20-month-old rats **(B)** one day after the administration of sodium chloride solution. 1 — the subcapsular zone of the cortex; 2 — the inner zone of the cortex; 3 — the medulla. Staining: hematoxylin-eosin. Objective lens: PlanC N x10/0,25 ∞/–/FN22. Zoom 132.

formed by epithelial reticular cells and macrophages. Lymphoid cells with degrading nuclei and cytoplasm were also observed. Changes in the cell profile here were similar to those in the subcapsular zone. One day after the administration of Imunofan, the number of medium lymphocytes increased by 8.5 % and the number of damaged cells dropped by 48.0 % ($p < 0.05$) (fig.3). After decapitation performed on day 7, an increased number of medium and, small lymphocytes, mitotically active cells and macrophages was observed, while the number of epithelial reticular stromal cells and damaged cells dropped by 15.8 and 62.2 %, respectively. On day 15, the same trend was observed as during the previous week, but the increase in the number of medium lymphocytes was no longer statistically significant. On day 30, the number of small lymphocytes and macrophages was higher, compared to the controls (by 11.3 and 51.3 %, respectively). There were 14.9 % less epithelial reticular cells and 23.1 % less damaged cells, compared to the controls. We found no statistical difference in cell profiles on day 60 after the final injection.

The density of lymphoid cells (mainly small and medium lymphocytes) in the medulla was reduced, compared to the cortex. We observed a thicker network of epithelial reticular cells grouped as strands and clusters. We found no lymphoblasts and large lymphocytes in the medulla; the number of medium lymphocytes was significantly higher on days 7, 15 and 30 of the observation (by 6.1, 9.3 and 7.5 %, respectively). The number of small lymphocytes remained increased on days 7 (8.6 %), 15 (10.0 %) , 30 (11.0 %) and 60 (10.4 %) of the observation (fig. 4). The number of mitotically active cells was insignificantly higher only on day 7 of the observation, compared to the controls. The number of damaged cells was significantly lower on days 15 and 30. The difference between the number of epithelial reticular cells and macrophages was insignificant.

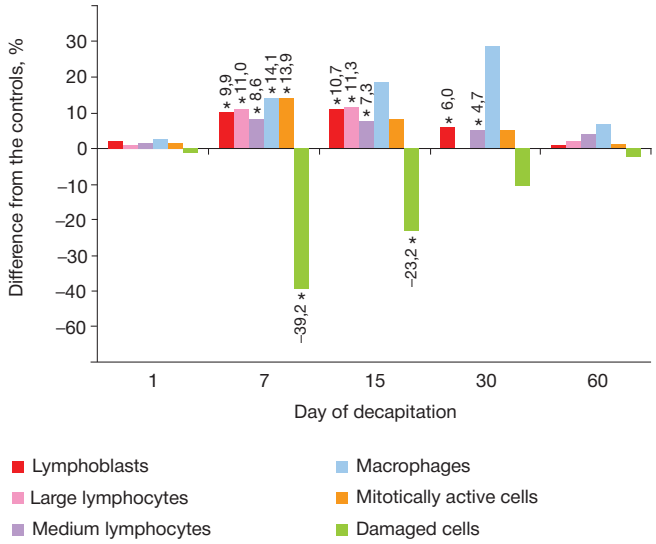

Fig. 2. Cell profile of the subcapsular zone of the thymic cortex of aging rats at various time points after injections of the immunomodulatory agent Imunofan

* — $p < 0.05$ when comparing the experimental and the control groups

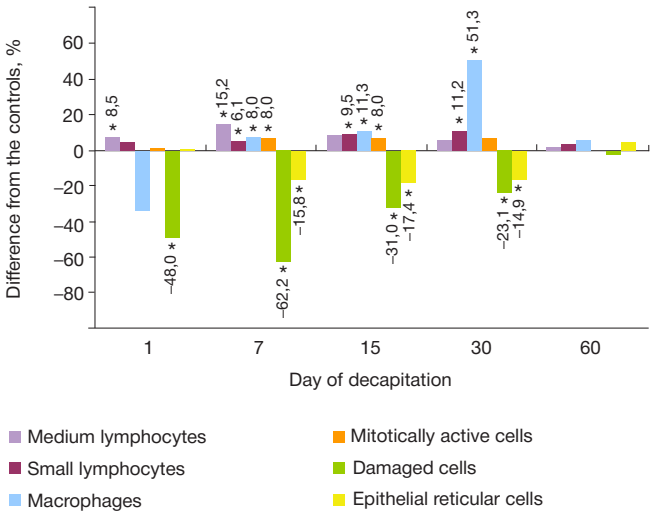

Fig. 3. Cell profile of the inner zone of the thymic cortex in aging rats at various time points after injections of the immunomodulatory agent Imunofan

* — $p < 0.05$ when comparing the experimental and the control groups

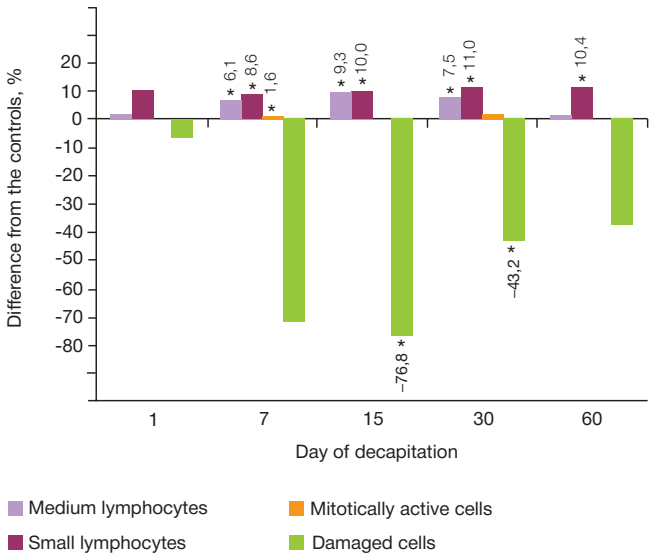

Fig. 4. Cell profile of the thymic medulla in aging rats at various time points after injections of the immunomodulatory agent Imunofan

* — p <0.05 when comparing the experimental and the control groups

By the end of the experiment (day 60), the number of small and medium lymphocytes, young cells, mitotically active cells and macrophages decreased and was about the same as in the controls.

RESULTS

The obtained results indicate a conspicuous thymic response to the administration of Imunofan in aging rats. Changes in cell profile of the subcapsular and inner zones of the thymic cortex were similar, which indicates that cytoarchitecture of the whole cortex was affected. The medulla is the most areactogenic zone of the thymic parenchyma [17], but its cell profile also changed in the experimental group. The possible mechanism of immunostimulating action of Imunofan (including temporary partial delay of age-related involution) can be associated with maintaining and/or restoring lymphoid cell population and its microenvironment, including macrophages. The number of epithelial reticular cells in the subcapsular cortex and the medulla did not differ significantly, which leads us to conclude that Imunofan does not suppress thymic reserve capacities.

Changes to the cytoarchitecture of the thymic parenchyma, namely, a higher cell population density in all thymic zones studied during the experiment and an increased number of lymphoid cells, including young cells, can indicate a more active inflow of precursors from the red bone marrow into the thymus and increased proliferation of lymphocytes. Such stimulating effect on the thymus was observed by some authors when using another immunomodulator, Polyoxidonium [4, 6]. Zakharov [3] describes slower age-related involution in the thymus of adult laboratory rats administered to Imunofan.

CONCLUSIONS

The thymus of aging rats was highly responsive to the administration of Imunofan. Injections of therapeutic doses of the drug contributed to the restoration of the thymic structure and delayed age-related involution of the thymus. A close interaction of various cellular components of the thymus indicates the importance of studying the balance between thymic lymphocytes and their microenvironment treated with immunomodulatory agents.

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ANALYSIS OF TLRs GENES EXPRESSION AND *DEFB1* POLYMORPHISMS ASSOCIATION IN CHILDREN WITH BRONCHIAL ASTHMA

Zaitseva MA[1]✉, Bragvadze BG[1], Svitich OA[1,2], Namazova-Baranova LS[3], Gankovskaya LV[1]

[1] Department of Immunology, Biomedical Faculty,
Pirogov Russian National Research Medical University, Moscow, Russia

[2] Laboratory of Molecular Immunology,
Mechnikov Research Institute of Vaccines and Sera, Moscow, Russia

[3] Scientific Center of Children's Health, Moscow, Russia

Bronchial asthma (BA) is one of the most common respiratory system diseases. The role of innate immunity components in the pathogenesis of bronchial asthma is studied widely, with particular focus on the antimicrobial peptides. Those include beta defensins that prevent pathogen intrusion into the respiratory tract mucosa, the most active of such pathogens being β-defensin-1 (human beta defensin-1, HBD-1) encoded by the *DEFB1* gene. We studied the association of three single nucleotide polymorphisms in the 5'- untranslated region of the gene, namely, *rs11362*, *rs1799946* and *rs1200972*, with bronchial asthma in children. We also evaluated gene expression of toll-like receptors *TLR2*, *TLR4* and *TLR9*. The experimental group included 48 patients of 3 to 7 years of age with BA and 70 healthy children. The AA genotype of the *rs11362* polymorphism and the CC genotype of the *rs1799946* polymorphism were reliably associated with the disease, while the GG genotype of the *rs1799946* polymorphism and the AA genotype of the *rs120097* polymorphism were found protective. Also, the AA genotype of the *rs11362* polymorphism was associated with the reduced expression of *DEFB1*, the human beta defensin-1 encoding gene, while the AG genotype was associated with its increased expression. In children with BA, *TLR2* expression increased 19.5 times in comparison with the controls; *TLR9* expression increased 9.5 times, while *TLR4* expression increased 8.3 times.

Keywords: bronchial asthma, human beta defensin-1, toll-like receptors, *DEFB1*, *TLR2*, *TLR4*, *TLR9*, single nucleotide polymorphism, polymorphic marker

✉ **Correspondence should be addressed:** Margarita Zaitseva
ul. Svyazistov, d.10, kv. 68, Krasnoznamensk, Moscow oblast, Russia, 143090; astice@list.ru

Recieved: 14.06.2016 **Accepted:** 23.06.2016

АНАЛИЗ ЭКСПРЕССИИ ГЕНОВ TLRs И АССОЦИАЦИИ ПОЛИМОРФИЗМОВ ГЕНА *DEFB1* У ДЕТЕЙ С БРОНХИАЛЬНОЙ АСТМОЙ

М. А. Зайцева[1]✉, Б. Г. Брагвадзе[1], О. А. Свитич[1,2], Л. С. Намазова-Баранова[3], Л. В. Ганковская[1]

[1] Кафедра иммунологии, медико-биологический факультет,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

[2] Лаборатория молекулярной иммунологии,
Научно-исследовательский институт вакцин и сывороток имени И. И. Мечникова, Москва

[3] Научный центр здоровья детей, Москва

Бронхиальная астма (БА) — одно из наиболее распространенных заболеваний органов дыхания. Активно исследуется роль элементов врожденного иммунитета в патогенезе бронхиальной астмы, в частности, противомикробных пептидов. К ним относятся β-дефенсины, предотвращающие вторжение патогенов в слизистую оболочку респираторного тракта, наиболее активным из которых является β-дефенсин-1 (human beta defensin-1, HBD-1), кодируемый геном *DEFB1*. В исследовании была изучена ассоциация трех однонуклеотидных полиморфизмов в 5'-нетранслируемой области гена — *rs11362*, *rs1799946* и *rs1200972* — с бронхиальной астмой у детей. Также оценивали уровень экспрессии генов toll-подобных рецепторов *TLR2*, *TLR4* и *TLR9*. В опытную группу включили 48 пациентов в возрасте 3–7 лет с БА и 70 здоровых детей. Генотип АА полиморфизма *rs11362* и генотип СС полиморфизма *rs1799946* достоверно ассоциированы с заболеванием, а генотип GG полиморфизма *rs1799946* и генотип АА полиморфизма *rs120097* являются протективными. Генотип АА полиморфизма *rs11362* также ассоциирован с пониженной экспрессией, а генотип AG — с повышенной экспрессией гена β-дефенсина-1 *DEFB1*. У детей с БА выявили повышение уровня экспрессии гена *TLR2* в сравнении с контрольной группой в 19,5 раз, *TLR9* — в 9,5 раз, *TLR4* — в 8,3 раза.

Ключевые слова: бронхиальная астма, β-дефенсин-1, toll-подобные рецепторы, *DEFB1*, *TLR2*, *TLR4*, *TLR9*, однонуклеотидный полиморфизм, полиморфный маркер

✉ **Для корреспонденции:** Зайцева Маргарита Алексеевна
143090, Московская область, г. Краснознаменск, ул. Связистов, д. 10, кв. 68; astice@list.ru

Статья получена: : 14.06.2016 **Статья принята в печать:** 23.06.2016

Bronchial asthma (BA) is a chronic inflammatory disease of the upper respiratory tract accompanied by bronchial obstruction and hyperresponsiveness. It manifests itself through shortness of breath, wheezing, coughing and choking episodes. It's prevalence is increasing fast in high- and middle-income countries. According to the Russian Respiratory Society, asthma affects as many as 10 million people in Russia; over 20 % of them are children [1].

It was observed that respiratory infections have a more severe course in patients with BA than in healthy individuals [2, 3]. Acute infections of the upper respiratory tract frequently trigger asthma exacerbations: about 85 % of exacerbations in children and 50 % in adults are caused by respiratory viruses [2]. Pathogens damage ciliated epithelium of the respiratory tract mucosa making it more vulnerable for allergens and toxins and maintaining bronchial hyperresponsiveness. Acute exacerbations can be life-threatening regardless of the BA grade of severity [3].

A lot of contemporary research studies focus on the in-depth analysis of BA pathogenesis, including the role of innate immunity components. Of particular interest is a new class of effector molecules (antimicrobial peptides), such as β-defensins. Antimicrobial properties of the latter are due to the electrostatic interactions between negatively charged surface components of the bacterial membrane, such as lipopolysaccharides of gram-negative bacteria and teichoic or lipoteichoic acids of gram-positive bacteria, and a positively charged β-defensin molecule. Critical concentrations of β-defensin on the surface of the target cell trigger pore formation in its membrane followed by cell lysis. Besides, β-defensins exhibit immunoregulatory activity, participating in chemotaxis and adaptive immunity activation, inducing dendritic cell maturation, etc. [4].

The key role in protecting respiratory tract mucosa is played by human β-defensin-1 (HBD-1) synthesized by epithelial cells [5]. β-defensin-1 is encoded by the *DEFB1* gene located on the short arm of chromosome 8 (8p23.1) in a highly polymorphic cluster. Due to gene mutations, its expression can be decreased; in turn, insufficient secretion of β-defensins facilitates bacterial adhesion to and invasion of the mucosa and triggers inflammation [6, 7].

Toll-like receptors (TLRs) of the epithelial cells of the respiratory tract mucosa are another important element of the innate immunity. They recognize pathogen-associated molecular patterns (PAMP) of microorganisms and their metabolic byproducts, transmit the signal into the cell and boost leukocyte functional activity, increase pro-inflammatory cytokine and interferon gene expression. The majority of bacterial and viral pathogens are recognized by TLR2, TLR4, and TLR9 that can activate the local mucosal immunity in the respiratory tract.

The aim of this work was to give a comprehensive assessment of the innate immunity markers, namely, the level of expression of the *TLR2*, *TLR4*, *TLR9* and *DEFB1* genes, and to study the association of some single nucleotide polymorphisms (SNPs) in the 5'-untranslated region of the *DEFB1* gene with bronchial asthma in children. Three SNPs were studied: *rs1799946*, *rs1800972* and *rs11362*. They are associated with HIV infection and infections caused by *Candida albicans*, *Pseudomonas aeruginosa* and other microorganisms and sepsis development [8, 9], but there are no reports on their association with allergies.

METHODS

The study was carried out in patients of the Rehabilitation

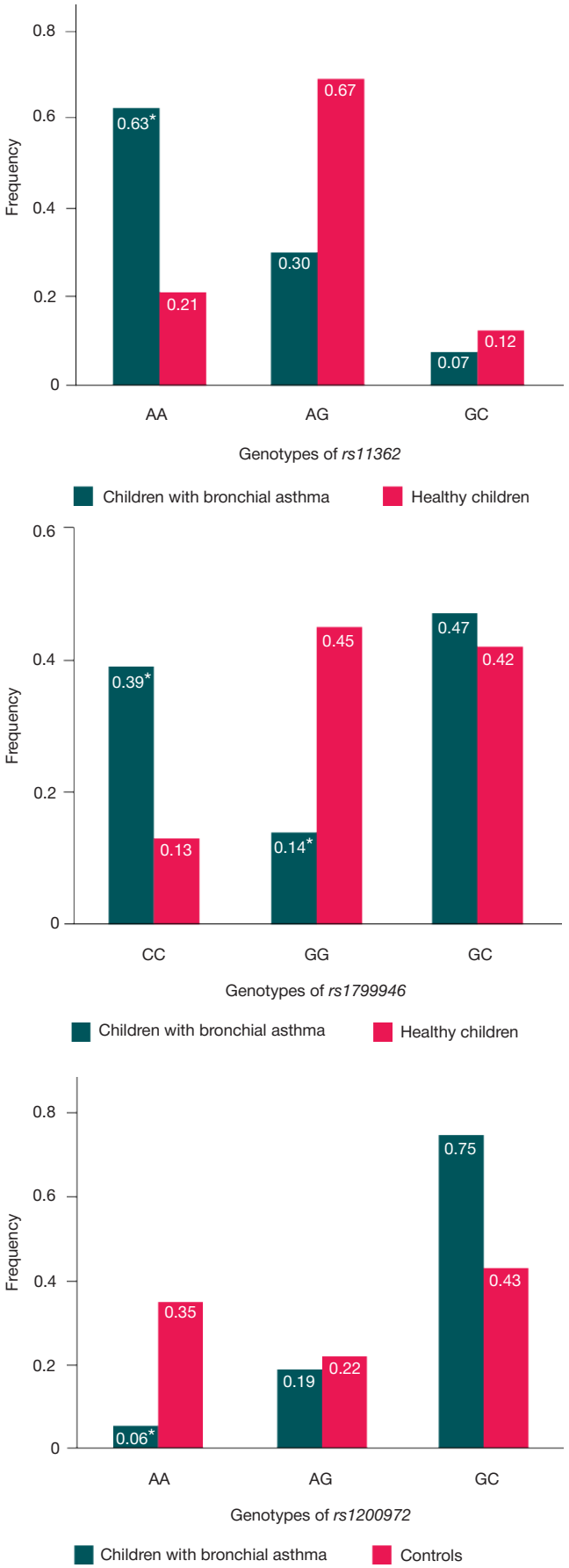

Fig.1. Genotype frequency distribution of single nucleotide polymorphisms *rs11362*, *rs1799946* and *rs1200972* in the *DEFB1* gene in asthmatic children (* — p <0.05, compared to the controls)

Care Unit for Children with Allergies and Respiratory Tract Diseases of the Scientific Center of Children's Health (Moscow). The study included 48 asthmatic children aged 3–7 years. The control group included 70 children without respiratory conditions, inflammatory and infectious diseases and allergies. Nasal scrapes were collected at the time of BA exacerbations that were accompanied by an acute respiratory infection.

For DNA extraction, the AmpliPRIME Ribo-sorb kit (InterLabService, Russia) was used. The real time PCR assay was conducted using SYBR Green I PCR Kit by Syntol, Russia. Data were statistically processed in MO Excel 2007 with Statistica 10.0 software (StatSoft, USA). Pearson's chi squared and Odd Ratio were computed (OR >1 indicated genotype association with BA, OR <1 indicated a genotype protective against BA) [10].

Expression of the *DEFB1*, *TLR2*, *TLR4* and *TLR9* genes was compared to β-actin gene expression. For RNA extraction, the AmpliPRIME Ribo-sorb kit was used. Reverse transcription was performed with the OT-1 kit by Syntol, real time PCR was carried out using the SYBR Green I PCR Kit. For statistical processing, Mann-Whitney test was applied ($p < 0.05$).

The study was approved by the Ethics Committee of Pirogov Russian National Research Medical University. Participants' parents gave their informed consent.

RESULTS

Genotype frequency distribution of *rs1799946*, *rs1800972* and *rs11362* polymorphisms of the *DEFB1* gene showed that the following genotypes are associated with the risk of asthma in children: AA of *rs11362* and CC of *rs1799946*, while genotypes GG and AA of *rs1799946* and *rs1200972* are protective against BA (fig. 1). Distribution of *DEFB1* alleles was alike in both groups.

Expression of the *DEFB1* gene was 3.5 times lower in children with bronchial asthma, compared to healthy children (fig. 2). A single nucleotide polymorphism in the promoter region can affect the level of gene expression and the amount of the produced protein. We divided patients of the experimental group into 3 subgroups based on the level of β-defensin-1 expression: low expression (>10,000 times higher than β-actin expression), moderate (10,000–30,000 times higher than β-actin expression) and high (>30, 000 times higher than β-actin expression). It was found that AG genotype of *rs11362* polymorphism is associated with the increased level of β-defensin-1 expression in epithelial cells. For example, the frequency of AG genotype in subgroups with high and low expression of *DEFB1* was 0.67 and 0.30, respectively. Genotype AA is associated with reduced expression of the β-defensin gene. Other genotypes of the studied polymorphisms are not associated with changes in the β-defensin gene expression. Patients with bronchial asthma showed a 19.5 times increased expression of the *TLR2* gene compared to the controls; *TLR9* expression was 9.5 times higher, *TLR4* expression was 8.3 times higher. Results are presented in the table below.

DISCUSSION

The obtained data can indicate that chronic inflammation of the bronchial mucosa in asthmatic children is partially associated with mutations in the 5'-untranslated region of *DEFB1*. Having assessed the expression of *DEFB1*, *TLR2*, *TLR4* and *TLR9*, we made a supposition that β-defensin-1 participates in BA pathogenesis. Antimicrobial peptides produced by epithelial cells of the respiratory tract mucosa prevent the invasion of pathogens into the mucosa. However, if antimicrobial peptide production is decreased and bacterial load is high, pathogens are recognized by TLRs of epithelial cells, which triggers a cascade of pro-inflammatory reactions, including synthesis of IL-1β, IL-6 and IL-12, INF-α, INF-β and chemokynes. Besides, through the activation of epithelial TLRs, production of thymic stromal lymphopoietin and IL-33 is induced. The latter interact with dendritic cells, boost activity of CD40 and CD80 costimulatory molecules, regulate Th0 and Th2 differentiation, and come into contact with mast cells inducing their degranulation [11, 12]. It facilitates the development of chronic inflammation (fig. 3).

CONCLUSIONS

Genotype AA of *rs11362* and genotype CC of *rs1799946* polymorphisms located in the 5'-untranslated region of the

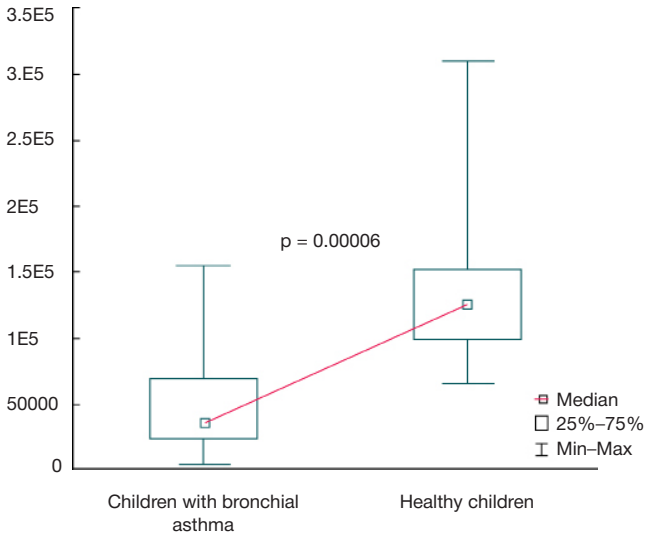

Fig.2. Expression of *DEFB1* in the epithelial cells of nasal mucosa in children with bronchial asthma and healthy children (compared to the expression of the β-actin gene).

Expression of *TLR2*, *TLR4*, *TLR9* and *DEFB1* in the epithelial cells of nasal mucosa in children with bronchial asthma and healthy children

Gene	Children with bronchial asthma	Healthy children
TLR2	130 000 (27 000; 270 000)	6 500 (5 000; 7 000)
TLR4	150 (30; 450)	18 (17; 120)
TLR9	30 000 (5 000; 50 000)	3 300 (3 000; 4 500)
DEFB1	35 000 (25 000; 68 000)	125 000 (100 000; 150 000)

Note: data are presented as a median (25 %; 75 %) of cDNA copies per 1 million copies of cDNA of β-actin, $p < 0.05$

DEFB1 gene are reliably associated with bronchial asthma in children. Genotype GG of *rs1799946* and genotype AA of *rs120097* polymorphisms are protective against asthma. Genotype AA of *rs11362* polymorphism is also associated with the reduced expression of the β-defensin-1 gene *DEFB1*. Thus, some mutations in *DEFB1* cause imbalances in the nasal mucosal innate immunity resulting in frequent exacerbations of BA in the setting of respiratory infections.

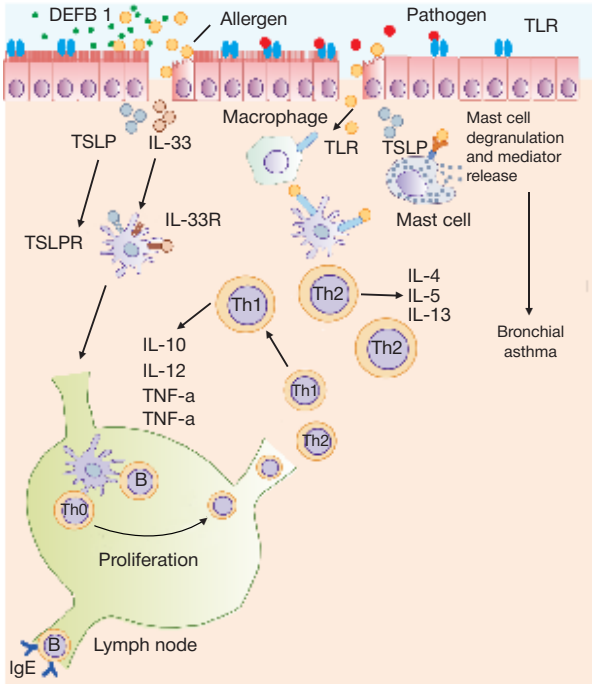

Fig. 3. Mechanism of chronic inflammatory response in bronchial asthma

When an allergen first comes in contact with the mucosa, it damages the epithelial barrier, which triggers cytokine secretion, including TSLP, IL-25 and IL-33. In the presence of cytokines, the secondary contact with the allergen induces maturation of dendritic cells (DCs) and their migration to lymph nodes, where DCs in collaboration with major histocompatibility complex molecules (MHC-II) "report" the allergen to Th0 cells (T-helpers) initiating their proliferation and differentiation into Th2 cells. Activated allergen-specific Th2s produce a wide range of cytokines: IL-4 (increases proliferation of B-lymphocytes and serves as their growth and differentiation factor, induces B-cell class switching to IgE), IL-5 (stimulates proliferation of eosinophils and facilitates release of the major basic protein) and IL-9 (activates mast cells). Allergen-specific IgE antibodies bind to high-affinity receptors (FceRI) of mast cells and basophils and to low-affinity receptors (FceRII) of eosinophils and macrophages. In case of a repeated allergen invasion, IgE of mast cell membranes binds to the allergen, thus ensuring its degranulation. Not all pathogens can be eliminated by antimicrobial peptides if bacterial load is high. Part of them is recognized by epithelial TLRs of the respiratory tract sustaining bronchial inflammation.

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DIAGNOSTIC ERRORS AND MANAGEMENT OF FOOT FRACTURES IN PATIENTS WITH MULTIPLE OR CONCOMITANT INJURIES

Korolev MA ✉, Yarmak DO, Miroschnikova CA, Moldakulov JM, Skoroglyadov AV, Korobushkin GV

Department of Traumatology, Orthopedics and Field Surgery, Faculty of Pediatrics,
Pirogov Russian National Research Medical University, Moscow, Russia

Delayed or missed diagnosis of foot fractures in patients with multiple or concomitant injuries often leads to the inadequate choice of treatment and causes serious long-term effects. This article reports the most common mistakes accompanying diagnostic procedures and therapy of this injury type. The study conducted in 2007–2015 enrolled 67 patients. Patients were divided into two groups: a prospective experimental group (n = 31) and a retrospective control group (n = 36). For both groups, diagnostic procedures and the range of therapeutic interventions applied were the same, but with the experimental group we used a stepped care approach, followed a specific sequence of activities and adjusted therapy considering the limb condition and the patient's overall state. In total, we identified 40 and 69 foot fractures in the prospective and retrospective groups, respectively. In the prospective group there were 5 delayed and 3 missed fracture diagnoses; in the second group those numbers were 7 and 9, respectively. The most common factors contributing to diagnostic errors were: excluding radiographic evaluation, severity of patient's overall condition, poor medical history. Missed fractures were often due to a combination of various factors. A one-step approach was prevalent in the controls (41 fractures); the experimental group underwent a multistep treatment (30 fractures). Therapy outcomes were assessed by Visual Analogue Scale. The results were statistically higher in the prospective group (Mann–Whitney U was 347), which indicates a better treatment applied in this group. The study also showed that using minimally invasive fixation for foot fractures improves treatment outcome.

Keywords: foot fractures, diagnostic error, concomitant injury, multiple injury

✉ **Correspondence should be addressed:** Maxim Korolev
Leninsky prospect, d. 10, korp. 7, Moscow, Russia, 117049; doctorkoroleff@mail.ru

Received: 30.03.2016 **Accepted: 22.04.2016**

ОШИБКИ ДИАГНОСТИКИ И ОСОБЕННОСТИ ЛЕЧЕНИЯ ПЕРЕЛОМОВ КОСТЕЙ СТОПЫ ПРИ СОЧЕТАННОЙ И МНОЖЕСТВЕННОЙ ТРАВМЕ

М. А. Королёв ✉, Д. О. Ярмак, Е. А. Мирошникова, Ж. М. Молдакулов, А. В. Скороглядов, Г. В. Коробушкин

Кафедра травматологии, ортопедии и военно-полевой хирургии, педиатрический факультет,
Российский национальный исследовательский медицинский университет имени Н. И. Пирогова, Москва

Переломы костей стопы при множественной и сочетанной травме часто диагностируются поздно или не диагностируются вовсе, что обусловливает ненадлежащее лечение и его неудовлетворительный отдаленный результат. В статье сообщается о наиболее распространенных ошибках диагностики и лечения этой группы переломов. В исследовании, проведенном в 2007–2015 гг., участвовали 67 пациентов. Их разделили на группу проспективного наблюдения (n = 31) — опытную и группу ретроспективного наблюдения (n = 36) — контрольную. Для обеих групп диагностические и лечебные мероприятия были одинаковыми, но для опытной группы соблюдали некоторые принципы лечения: этапность, последовательность всех действий, зависимость от состояния конечности и общего состояния пациента. Выявили 40 и 69 переломов костей стоп для группы проспективного и группы ретроспективного наблюдения соответственно, при этом в первой были поздно диагностированы или не диагностированы 5 и 3 переломов, а во второй — 7 и 9. Наиболее частыми ошибками диагностики стали: невыполнение рентгенологического исследования, тяжесть общего состояния пациента, скудный анамнез. Часто пропуск перелома был обусловлен влиянием сразу нескольких факторов. В контрольной группе преобладало одноэтапное лечение повреждений (41 перелом), а в опытной — многоэтапное (30 переломов). Оценка результатов лечения по шкале Visual Analogue Scale достоверно выше (критерий Манна–Уитни равен 347) в группе проспективного наблюдения, что свидетельствует о более высоком качестве лечения пациентов группы. Исследование также показало, что применение малоинвазивных способов фиксации переломов костей стопы улучшает результат лечения.

Ключевые слова: переломы костей стопы, ошибки диагностики, сочетанная травма, множественная травма

✉ **Для корреспонденции:** Королёв Максим Александрович
117049, г. Москва, Ленинский проспект, д. 10, корп. 7; doctorkoroleff@mail.ru

Статья поступила: 30.03.2016 **Статья принята в печать:** 22.04.2016

According to some estimates, 17–20 % of all bone fractures are foot fractures. [1]. Foot fractures are more frequent in patients with multiple and concomitant injuries. We have analyzed statistical data provided by the Trauma Unit of Pirogov City Clinical Hospital no.1, Moscow. In 2007– 2015 the hospital admitted 923 patients with multiple and concomitant injuries. We have found that 15 % of them were diagnosed with foot fractures; still, foot fractures accounted for only 7 % of all

fractures registered in the Unit during that period. It is probably because a large number of multiple and concomitant injuries are caused by car accidents, and the latter are often damaging for extremities [2–4].

Another important detail is a high rate of delayed diagnosis of foot fractures [5].Thus, in Guly's study fractures accounted for 79.7 % of delayed diagnoses of injuries; 11 % of them were injuries to the foot [6]. Delayed diagnosis affects the effectiveness and the duration of treatment [5, 7], patients develop persisting pain [8], and the quality of their life deteriorates [9]. Considering that, diagnostic and therapeutic approaches to this type of fractures should be improved.

At the first stage of providing medical service to the patient, the medical personnel should eliminate a life threatening condition, which is normally not caused by foot fractures, although the latter can negatively affect the treatment outcome [5]. After the patient has been resuscitated, urgently operated on and stabilized, the trauma specialist can perform a secondary survey to detect occult injuries to bones and soft tissues [10]. Rizoli et al. emphasize the importance of secondary physical examinations, since in their study about 30 % of injuries were diagnosed only because patients had repeatedly voiced their concerns [11].

Imaging is an important diagnostic tool. It includes ultrasonography (US), radiography, computed tomography (CT), and magnetic resonance imaging (MRI). Atilla et al. suggest performing US on the patients with injuries to the foot and ankle; they see it as helpful for diagnosing malleolar and fifth metatarsal fractures, but do not recommend it for other foot bone injuries [12]. Some researchers consider radiography ineffective [13], and many insist on a CT scan [14–17], the reason being its high accuracy and convenience. A CT scan is often ordered for patients with severe traumas. Given the indications, the foot can be scanned along with other body parts [17]. Magnetic resonance imaging is highly effective in detecting both soft tissue injuries, such as ruptured ligaments, tendons or muscles, and transchondrial foot fractures not visible on radiography [18].

Arthroscopy is a promising diagnostic and therapeutic technique. It is normally used to treat the talus due to the size of the talocrural joint and its relative accessibility [19], but there are reports on performing arthroscopy on other foot bones [20–22].

Poor diagnosis and/or treatment can result in a medical malpractice lawsuit. In 2010, 125 such lawsuits were filed in Moscow [23], while in 2013 their number increased to 325; in 58.5 % of cases the patient's claim was satisfied [24]. When treating foot fractures, orthopedic traumatologists face significant legal risks, because there are still no clinical guidelines for this type of fractures in Russia. There are guidelines for treating similar pathologies of different localization, but therapeutic methods they suggest cannot justify the doctor in case a lawsuit is filed.

Thus, diagnosis and treatment of foot fractures in patients with multiple injuries are a medical and a legal issue. The aim of our study was to analyze the most common errors in the diagnosis and treatment of foot fractures in patients with multiple and concomitant injuries and to elaborate guidelines for reducing the risk of missed fractures and improving their management.

METHODS

The study was conducted in 2007–2015 in the Trauma Unit of Pirogov City Clinical Hospital no.1 in Moscow. The study enrolled 67 patients with multiple and concomitant injuries, including foot bone fractures. The following exclusion criteria were applied: patient's early death, foot bone dislocations, or patient's refusal to participate. Two groups were formed: a group of prospective observation (n = 31; 22 men and 9 women, mean age of 38 years) and a control group of retrospective observation (n = 26; 29 men and 7 women, mean age of 41 years). In both groups injuries were caused by car accidents (81.2 % and 74.7 %, respectively), falls from height (8.3 % and 12.0 %, respectively) and other factors (11.5 % and 13.3 %, respectively).

Diagnostic and therapeutic procedures were the same for both groups; however, with the prospective observation group we were able to make adjustments in the course of treatment and adhere to some important principles of treatment tactics, such as using a stepped-care approach, following a specific sequence of diagnostic and therapeutic procedures, and considering how foot condition affected patient's geenral health. Diagnostic and therapeutic interventions in the retrospective observation group were assessed using medical histories, phone surveys and medical examinations in person.

During clinical examinations, the presence and the severity of edema, deformities and pain were assessed; foot mobility was tested with extra care. To detect acute neurocirculation disorders, dorsalis pedis pulse was palpated. Sensitivity was tested using external stimuli; local skin temperature was compared to body temperature. Biplanar radiography was performed in all cases; with the talus and calcaneus, a special projection was performed, when necessary. CT was performed to clarify the type of the fracture, to understand the need for therapeutic adjustments and to decide on postoperative procedures. In cases of capsular ligaments or damaged cartilages, MRI was ordered.

A diagnosis was classified as early if a fracture had been detected during the primary clinical examination or no later than within half of the time to bony union. A diagnosis was classified as delayed if a fracture had been detected at a different time prior to patient's discharge. A fracture was classified as missed if it had been detected in the course of outpatient treatment at a first aid facility.

A primary criterion for deciding on the surgical treatment of foot fractures in patients with multiple and concomitant injuries was patients' general condition. The surgery was under no circumstances to interfere with resuscitation and elimination of life-threatening conditions. It was also ruled out if the risk of anaesthetic complications was high.

Undisplaced closed foot bone fractures were fixed with plaster splints and bandages. If redisplacement of bone fragments was likely to occur, which is often the case with metatarsal and toe fractures, we used minimally invasive techniques, such as closed reduction and pin, screw or plate fixation.

Open foot fractures were an absolute indication for surgery. The extent of surgery was inversely proportional to the severity of patient's condition.

Displaced hindfoot fractures (involving the calcaneus or the talus) were treated surgically by open reduction or plate and/or screw osteosynthesis. If time elapsed after the injury exceeded 14 days, calcaneal and talar fractures were treated by open reduction and subsequent osteosynthesis; if time elapsed after the injury was less than 14 days, closed reduction with subsequent osteosynthesis was performed.

A one-step care approach implied only one type of treatment, while a multiple-step care approach implied primary atraumatic stabilization of the fracture (by casting, external fixation or adhesive tape fixation) followed by the introduction

of more complex and stable fixators (plates, screws and nails).

Treatment outcome was assessed using the following scales: SF-36 (Short Form 36), AOFAS (American Orthopaedic Foot and Ankle Society Score), FFI (Five-Factor Inventory), VAS (Visual Analogue Scale), MFTS (Moscow Foot Trauma Scale) and AQSA (Abbreviated Questionnaire of Subjective Assessment). The latter 2 scales had been developed at the department of Traumatology and Orthopedics of Pirogov Russian National Research Medical University [26]. The assessment was performed 1, 3, 6 and 12 months after the treatment had been completed, and once a year afterwards.

For all parameters the mean value and standard deviation were computed. To evaluate the significance of differences between the means and their correlations, Spearman's rank correlation coefficient (r) and Pearson's chi square were computed (considering Yates' correction, Tschuprow's T and Cramer's V). Since many samples were asymmetrical and distribution in those samples differed from normal, we used Mann-Whitney U test instead of Student's t-test.

The study was approved by the Ethics Committee of Piragov Russian National Research Medical University (Protocol no. 139 dated November 10, 2014). All patients gave written informed consent to participate.

RESULTS

Mean observation period was 4 years for each patient. In total, 109 foot fractures in 71 feet were detected; 40 of them, including 6 open, were detected in the prospective observation group; 69 fractures, including 7 open, were detected in the retrospective observation group (see table 1). In the control group, 53 fractures were diagnosed early, 7 diagnoses were delayed, and 9 were missed. For the prospective observation group, those numbers were 32, 5 and 3, respectively.

In both groups the majority of the fractures were diagnosed during the primary survey by the trauma surgeon, that is, 27 and 36 in the experimental and control groups, respectively, which accounted for 67.5 % and 52.1 % of the total number of fractures in both groups (see table 2). During the secondary survey in the resuscitation and intensive care unit, 8 fractures were detected in the experimental group, and 7 — in the controls (20.0 % and 10.1 %, respectively). After patients were transferred to the trauma unit, 3 fractures were diagnosed in the experimental group, and 11 — in the controls (7.5 % and 15.9 %, respectively). Of 16 delayed and missed diagnoses in the retrospective observation group, only 3 were radiographed (18.8 %); bipolar radiography was performed for 2 such fractures out of 8 in the prospective observation group (25.0 %). CT scans were performed on 6 patients in the experimental group and 3 controls, but in both groups patients with missed fractures did not have a CT scan. We should note that out of 67 patients 34 received a CT scan of other body regions, and 12 patients received multiple follow-up CT scans.

The most common reasons for missed foot fractures were as follows: no rfailure to perform radiography, severity of the patient's condition, scant medical records, and other (see table 3).

In the prospective observation group, 30 foot fractures were treated using a stepped-care approach; only 16 patients of the retrospective observation group were managed similarly. Plaster splints and bandages prevailed over surgical interventions: in the experimental group they were used for 10 fractures to which a one-step care approach was applied, and in 17 fractures to which a multiple-step care approach was applied. With the controls, those numbers were 41 and 4, respectively. In both groups, the most common surgical interventions were pin fixation and external fixation. Emergency osteosynthesis was not performed on any patient in both groups.

Arithmetic means of scores obtained from different scales indicate a better therapy outcome in the prospective observation group compared to the controls (see table 4). In the experimental group, standard deviations were lower than in the controls, which indicates a more stable treatment outcome. However, statistically significant differences were observed for VAS scale only, because Mann-Whitney U was 347, i.e., within the significance interval. For FFI scale, Mann-Whitney U was 420 and fell within the uncertainty range; it was insignificant for other scales. For all scales except VAS and FFI, Spearman's coefficient proves wrong the null hypothesis that early diagnosis does not affect the treatment outcome. For missed fractures, Pearson's coefficient was computed. Its value (2.517) shows a moderate association, which implies a possible correlation between the scores and indicates a need for earlier diagnosis and a therapy different from the one applied in the retrospective

Table 1. General description of the experimental and control groups

Criterion		Prospective observation group (n = 31)		Retrospective observation group (n = 36)	
		number	percentage, %	number	percentage, %
Number of patients with concomitant injuries (Injury Severity Score)	less than 16 points	5	16	2	5
	16 to 40 points	8	25	13	36
	over 40 points	0	0	0	0
Number of patients with multiple injuries	up to 2 fractures	4	12	5	13
	up to 3 fractures	5	16	8	22
	more than 3 fractures	9	29	8	22
Number of patients with foot fractures of various localization	on the right foot	15	48	16	44
	on the left foot	9	29	14	38
	bilateral	7	22	6	16
Number of foot fractures grouped according to the time to diagnosis	early diagnosis	32	80	53	74
	delayed diagnosis	5	12	7	11
	missed	3	8	9	15

observation group. Contingency coefficient, Tschuprow's T and Cramer's V indicate a weak association.

DISCUSSON

The most common reasons for delayed or missed diagnosis in our study were: failure to perform radiography, severity of patient's general condition and scant medical history. It is important to note that in many cases a fracture was not diagnosed early due to the combination of the factors mentioned above. Guly et al. mention poor analysis of radiological reports and poor radiography as the most common errors in foot fracture diagnosis [6]. Houshian et al. believe errors are due to the insufficient attention to detail exhibited by traumatologists and misinterpreted radiological data [27]. Brooks et al. report seven injuries visible on radiography (all images were good quality), but missed by trauma specialists [28]. Sharma et al. rank errors differently putting the severity of the patient's condition first, followed by the inaccurate assessment of his condition, misinterpretation of medical imaging data, and poor screening [29]. Alternative results provided by other authors are probably due to the fact that only lethal cases were studied.

We believe that severity of patient's general condition should not be seen as an obstacle to diagnosis of foot fractures. If a thorough medical examination is impossible in the resuscitation ward, it should be performed later by the trauma surgeon. With severe injuries, medical history is often scant, but signs of damage to the extremity are easy to discern, since it is usually characterized by conspicuous edema, deformities or pain. To improve radiographic image quality and thus reduce the number of missed fractures, digital equipment must be used; or data from an X-ray machine must be transmitted to a computer for better radiographic contrast control and stable image quality while scanning larger body regions. Unfortunately, not all hospitals in Russia are properly equipped.

Computed tomography is an important imaging tool; it is especially effective in identifying talar fractures [30–32]. The medical community is currently discussing a whole-body CT (WBCT) performed on patients with multiple injuries. Davies et al. report that WBCT helped them to diagnose a concomitant injury in 16 % of cases and some injury-related conditions in 42 % of cases; in the rest 42 % of cases it did not detect any injuries [17]. Based on the obtained results, the researchers recommend performing WBCT on patients with major trauma only after indications for this type of screening have been thoroughly considered. During WBCT a patient is exposed to a high dose of radiation (about 20 mSv), which can cause tissue malignization.

We also studied the effectiveness of various approaches to foot fracture management. Which is better: a one-step or a multiple-step approach? Minimally invasive or standard fracture fixation? Urgent or delayed intervention?

There are two main approaches to managing multiple traumas. The first is called Early Total Care (ETC) and implies urgent fixation of all fractures regardless of the patient's condition [33]. Pakhomov et al. believe that fixation of multiple fractures must be performed immediately and in one step. It is important, though, that their patients' condition was stable [14]. The second concept called Damage control orthopedics (DCO) implies that traumatologists must focus on the severe injuries first, while minor fractures can be treated later when patient's general condition improves [34]. This approach has some drawbacks. Nicola writes that DCO reduces the risk of complications caused by early medical intervention, but increases the need for a secondary surgery that can be less

Table 2. How foot fractures were diagnosed

How foot fractures were diagnosed	Group	Number of fractures	Percentage, %
On receiving a radiological report	R	33	47.8
	P	19	47.5
On receiving a CT report	R	3	4.3
	P	6	15.0
On receiving an MRI report	R	0	0.0
	P	2	5.0
During secondary examinations in the Resuscitation and Intensive Care Unit	R	7	10.1
	P	8	20.0
After patient's transfer to the Trauma Unit	R	11	15.9
	P	3	7.5
After radiographs were analyzed by the surgeon	R	2	2.9
	P	0	0.0
After radiographs were analyzed by the general practitioner	R	0	0.0
	P	0	0.0
After the first series of patient's complaints	R	3	4.3
	P	0	0.0
After the second series of patient's complaints	R	1	1.4
	P	0	0.0
After patient's transfer to the Surgical Unit	R	2	2.9
	P	0	0.0
After patient's transfer to the Medical Unit	R	0	0.0
	P	0	0.0
In a follow-up clinic	R	1	1.4
	P	0	0.0
Undocumented cases	R	6	8.7
	P	2	5.0
Total	R	69	100.0
	P	40	100.0

Note: R represents the retrospective observation group, P represents the prospective observation group.

Table 3. Reasons for delayed or missed foot fracture diagnosis

Reason for diagnostic error	Prospective observation group	Retrospective observation group
Failure to perform radiography	7	11
Severity of patient's general condition	7	10
Scant medical history	6	5
Absence of clinical signs of a fracture	2	6
Inaccurate assessment of trauma by the doctor	2	5
Poor quality of radiographs	2	4
Fractures detected on the other foot	1	3
Short stay in hospital	3	1
Other fractures detected on the same foot	0	3
Other	2	0

Note: in some cases there were several reasons contributing to the missed or delayed diagnosis of a foot fracture. Because of that, the absolute number of delayed or missed fractures does not coincide with the totals shown in table 1.

Table 4. Assessment of treatment outcome using standard scales and questionnaires

Parameter		SF-36		VAS	AOFAS	FFI	MFTS	AQSA
		PCS	MCS					
M	retrospective observation group	42.027	45.777	2.02	45.888	46.027	43.08	8.44
	prospective observation group	43.032	48.032	1.03	51.225	34.61	45.93	6.9
SD	retrospective observation group	9.78	8.45	1.66	18.89	21.88	19.54	8.23
	prospective observation group	9.63	8.31	1.04	19.121	17.45	19.98	7.06
Mann-Whitney U		526	461	347	459	420	514	488
Spearman's rank correlation coefficient		0.623	0.535	-0.05	0.494	0.138	0.641	0.698
Statistical analysis of missed fractures								
Pearson's chi square		2.517						
	Yates' correction	1.771						
	Contingency coefficient	0.19						
	Tschuprow's T	0.194						
	Cramer's V	0.194						

Note: M represents arithmetic mean, SD represents standard deviation.

effective, which, in turn, results in a longer hospital stay [35]. Our study convincingly demonstrates that surgical treatment of foot fractures should be postponed until the patient is stable, if possible.

There are many ways to fix a foot fracture: plaster splints, adhesive tape, pins, screws, plates and nails. Our study shows that patient's condition should be considered first when deciding on the fixation method. If a patient is stable, comminuted fractures of the calcaneus and metatarsal bones with displaced fragments should be fixed with plates, as plates ensure bone immobility. Phalanx fractures can be fixed with adhesive tapes, pins or miniplates. However, if a patient is hemodynamically unstable, hyperthermic or hypocoagulable, has a conspicuous edema, or the wound in the fracture area is contaminated, it is reasonable to use temporary fixation first, such as plaster splinting, skeletal extension or external fixation, and then proceed to surgery.

Some authors suggest using Ilizarov apparatus and external pin fixators, especially for calcaneal and talar fractures with displaced fragments [36–39]. Ilizarov apparatus was not used in this study, and there may be several reasons for that. First, some trauma surgeons in the emergency room had no experience using it. Second, there were no indications for its use. It is a complex and somewhat unwieldy system difficult to care for. We did use external pin fixators, though, mainly as a temporary solution. Those were later replaced by internal fixators.

Other authors report a beneficial effect of plates on fracture healing [14, 40]. We believe that plates are the most reliable fixation technique. They also allow the patient to manage almost on his own, given that the postoperative wound heals well and the patient gradually restores his physical activity. An alternative to plate fixation is minimally invasive screw fixation [41, 42].

CONCLUSIONS

In most cases, physical examination inadequacy and severity of patient's general condition are main reasons for delayed or missed diagnosis of foot fracture in patients with concomitant or multiple injuries. To improve diagnosis accuracy, a minimum of two projections should be performed during radiographic screening, digital equipment being a considerable advantage here.

If a trauma patient receives a CT scan of other body parts, and his medical history, nature of trauma and clinical symptoms indicate a foot injury, it is advisable to CT-scan the foot (or both feet) along with other body parts. When treating a patient with multiple or concomitant injuries, we recommend a stepped-care approach and minimally invasive fixation techniques, as those can improve treatment outcome, reduce the length of hospital stay and prevent complications.

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EVALUATION OF MICROCIRCULATION IN CHILDREN OF 8 AND 10 YEARS OF AGE USING INSPIRATORY BREATH HOLD

Baboshina NV ✉

Department of Medicine, Biology and Approaches to Teaching Biology, Faculty of Natural Sciences and Geography, Yaroslavl State Pedagogical University named after K. D. Ushinsky, Yaroslavl, Russia

Gas diffusion and transcapillary exchange take place in the microvasculature. Therefore, the evaluation of skin blood flow regulation and functional capacities of the microcirculatory system at various ontogenesis stages is of great importance. Using laser Doppler flowmetry in the group of boys (n = 15) and girls (n = 13) of 8 and 10 years of age, skin microcirculation and its regulatory mechanisms were evaluated. The study found an increase in the perfusion index in children between the age of 8 and 10 induced by the shifting roles of mechanisms of the microcirculatory regulation. The comparison of basal microcirculatory parameters did not display statistically significant differences related to sex in 8- and 10-year old participants. However, almost equal perfusion in boys and girls was maintained by different contributions of regulatory mechanisms. The breath holding test showed an increase in the initial microcirculation index and capillary blood flow reserve in the group of 10-year-old boys and girls. Our study revealed differences in various microcirculation parameters, in the intensity of active and passive rhythms of blood flow oscillations and response to inspiratory breath hold, which indicates age-related transformations of microcirculation system.

Keywords: laser Doppler flowmetry, microcirculatory regulation, inspiratory breath hold, capillary flow reserve

✉ **Correspondence should be addressed:** Natalia Baboshina
ul. Respublikanskaya, d. 108, Yaroslavl, Russia, 150000; pankrateva@bk.ru

Received: 01.06.2016 **Accepted:** 12.06.2016

ИССЛЕДОВАНИЕ МИКРОЦИРКУЛЯЦИИ КРОВИ У ДЕТЕЙ 8 И 10 ЛЕТ С ИСПОЛЬЗОВАНИЕМ ДЫХАТЕЛЬНОЙ ПРОБЫ

Н. В. Бабошина ✉

Кафедра медицины, биологии, теории и методики преподавания биологии, естественно-географический факультет, Ярославский государственный педагогический университет им. К. Д. Ушинского, Ярославль

В микроциркуляторном русле осуществляются процессы диффузии газов и транскапиллярный обмен. В связи с этим актуальной задачей является оценка состояния регуляции кровоснабжения кожи и функциональных возможностей системы микроциркуляции крови на отдельных этапах онтогенеза. С помощью метода лазерной допплеровской флоуметрии в группе мальчиков (n = 15) и девочек (n = 13) по достижении ими возрастов 8 и 10 лет оценивали состояние кожной микроциркуляции и функционирование механизмов ее регуляции. Обнаружено увеличение показателя микроциркуляции в возрастном периоде от 8 до 10 лет, вызванное перераспределением механизмов регуляции микрокровотока. При сравнении базальных показателей микроциркуляции достоверных половых различий в возрастах 8 и 10 лет не выявлено, однако поддержание примерно равного уровня перфузии у мальчиков и девочек достигается при разном соотношении регуляторных влияний на микрокровоток. При проведении дыхательной пробы выявлено увеличение исходного показателя микроциркуляции и резерва капиллярного кровотока в группе мальчиков и девочек в возрасте 10 лет. В ходе проведенного исследования между детьми 8- и 10-летнего возраста выявлены различия в показателях микроциркуляции, в степени выраженности активных и пассивных ритмов колебаний кровотока и реакции на дыхательную пробу, что свидетельствует о возрастных преобразованиях системы микроциркуляции.

Ключевые слова: лазерная допплеровская флоуметрия, механизмы регуляции микрокровотока, дыхательная функциональная проба, резерв капиллярного кровотока

✉ **Для корреспонденции:** Наталья Владимировна Бабошина
150000, г. Ярославль, ул. Республиканская, д. 108; pankrateva@bk.ru

Статья поступила: 01.06.2016 **Статья принята к печати:** 12.06.2016

According to present-day concepts, all functions of the organism undergo changes when the organism interacts with the environment. Therefore, the organism's adaptivity at various ages is determined by the morphological maturity of physiological systems and by how adequately the environment matches the organism's functional capacities [1].

The performance of the cardiovascular system, which is one of the most crucial life-sustaining systems, is often seen as an indicator of the functional status of the whole organism [2]. Still, current physiological studies focus more on the functional interactions of the circulatory and respiratory systems [3].

Such interest is dictated by the fact that the cardiovascular system that serves to deliver oxygen to the organism's cells and maintain homeostasis is one of the most important physiological systems. It determines both mental and physical performance capacities of the human and his adaptation to different activities [4]. Peripheral circulation provides adequate blood supply to separate organs and tissues in response to the constantly changing metabolism.

One of the high priority issues in the developmental physiology is assessment of separate elements and regulatory mechanisms of tissue perfusion that are ultimately responsible

for the normal performance of separate organs and the whole organism. It is also very important to study functional capacities of the microcirculatory system in ontogenesis [5], since it helps to reveal patterns of formation of the microvasculature and the specifics of its functioning, analyze its phenomenology and structure, assess its functional reserve and the conditions which contribute to the effective adaptation of the microcirculatory system at various stages of the child's development [6, 7].

Due to the extreme importance of the processes that take place in the terminal vessels, microcirculation is of particular interest for researchers. The functional contact of the microvasculature and the tissues plays a key role in maintaining homeostasis through a complex and subtle regulation of microcirculation in accordance with tissue metabolic needs. Because of that, the microvasculature is home for compensatory changes that largely determine the functional state of the organism [8]. A wide range of scientific works have described microvasculature in primary school children [7, 9–11]. Kutyreva et al. showed age-related differences in basal parameters of microcirculation and its regulatory mechanisms in children of 3-4 and 10-12 years of age; age-related changes in the microcirculatory functional reserve were detected.

The most common methods applied to study the microvasculature make use of Doppler ultrasound. Laser Doppler Flowmetry (LDF) combined with functional tests [12] is one of the major methods for studying microcirculation status and its regulatory mechanisms. The advantage of LDF is the ability to measure microcirculation *in vivo* and noninvasively, which is crucial for evaluating microhemodynamics in children.

The functional respiratory test (inspiratory breath-hold) allows obtaining a large amount of data that characterize microhemodynamics and its reserve capacities and to assess the functional contribution of various elements of microcirculatory modulation. Blood flow reduction during the vasoconstrictive respiratory test is indicative of the impact of both sympathetic innervation and vessel walls on microcirculation.

The inspiratory breath-hold test is highly informative and easy to use. In all healthy individuals, skin areas with high density of sympathetic nerve fibers respond to it positively [13].

The aim of this work was to study microcirculation in children of 8 and 10 years of age using the inspiratory breath-hold test.

METHODS

The study included virtually healthy children of both sexes (a group of boys, n = 15, and a group of girls, n = 13) after written informed consent had been obtained from their legal representatives (parents). The children were examined twice, in 2013 and 2015, when they reached the age of 8 and 10, respectively.

Microhemocirculation was evaluated by laser Doppler flowmetry using LAKK-02 computerized laser analyzer (LASMA Research and Production Enterprise, Russia).

Skin is a traditional and easily accessible object used to assess microcirculation in clinical practice [14]. For our tests, we chose a distal phalanx of the second finger of the right hand. This area is devoid of hair (glabrous skin). It is rich not only in arteriovenous anastomoses dependent on the sympathetic innervation, but also in autonomic and sensory nerve fibers [13].

The following values were computed: mean perfusion index M, mean square deviation σ (flux, or mean blood flow modulation), coefficient of variation K_V and amplitude-frequency features of the reflected signal.

Among the elements of microcirculatory regulation, passive and active mechanisms can be distinguished. They form 5 non-overlapping frequency bands in a spectrum of 0.005–3 Hz representative of endothelial activity (0.007–0.017 Hz), neurogenic (sympathetic adrenergic) activity (0.023–0.046 Hz), myogenic (smooth muscle) activity (0.05–0.145 Hz), respiratory rhythm (0.2–0.4 Hz), and cardiac rhythm (0.8–1.6 Hz) [15]. Superimposed oscillations recorded by LDF are induced by active and passive mechanisms of microcirculatory regulation. Active mechanisms (endothelial, neurogenic, and myogenic mechanisms of lumen regulation) generate transverse oscillations of the blood flow by cycles of muscle contractions and relaxations (vasoconstriction and vasodilatation episodes). Passive factors (respiratory and cardiac rhythms) are responsible for longitudinal blood flow oscillations expressed as recurrent changes of pressure and blood volume in a vessel [13].

The amplitude-frequency spectrum of oscillations was computed using wavelet transform; contribution of endothelial, neurogenic and myogenic components of microvascular tone and respiratory and cardiac rhythms was also evaluated [16]. We computed the neurogenic tone (NT) of precapillary resistance vessels and the myogenic tone (MT) of metarterioles and precapillary sphincters, as well as shunt index (SI), using the formulas below.

$$NT = (\sigma \times P_m) / (A_n \times M),$$
$$MT = (\sigma \times P_m) / (A_m \times M),$$
$$SI = A_n / A_m,$$

where σ is mean square deviation of perfusion index; P_m represents mean arterial pressure; M is mean perfusion index; A_n and A_m are maximum averaged oscillation amplitudes of sympathetic adrenergic and myogenic frequency bands, respectively [13].

Due to a large scatter in the results of measurements of oscillation amplitudes, it is difficult to assess performance of a regulatory mechanism using only amplitude values. Therefore, apart from A_{max} we analyzed the contribution of each component to the modulation of the microcirculatory flow calculated as $(A_{max} / 3\sigma) \times 100$ %, and a contribution to the total tissue perfusion calculated as $(A_{max} / M) \times 100$ %. Those normalized data were computed automatically after finding A_{max} in the respective frequency band [16, 17].

To study reserve capacities of the microcirculation, a respiratory vasoconstrictive test was used. The subjects were asked to take a deep breath and to hold it for 30 seconds, which led to the short-term reduction of perfusion index followed by the restoration of its initial level (see the picture below).

The following parameters were noted during the respiratory test: M_{init} — the initial value of perfusion index; P_{react} — the minimum perfusion value during the test; M_{rest} — perfusion index after normal breathing was restored; T3–T1 — time between the onset of breath-hold and the onset of microcirculatory flow reduction; T4–T3 — time between the onset of microcirculatory flow reduction and the minimal value of perfusion index; T5–T4 — time elapsed from the moment the minimum perfusion index value had been reached till breath recovery.

Using the inspiratory breath-hold test results, perfusion index shift (ΔM) was found and capillary flow reserve (CFR, %) was computed using the formulas:

$$\Delta M = [(M_{init} - M_{min}) / M_{init}] \times 100 \%,$$
$$CFR = (M_{min} / M_{init}) \times 100 \%,$$

where M_{min} and M_{init} are the minimum and the initial values of perfusion index [13].

The obtained values, including those of σ and K_v, are presented as arithmetic means and their standard deviations. After normality tests, the data were statistically processed in Microsoft Excel using a parametric Student's t-test. Because both parts of the study involved the same school children (measurements were performed in 2013 and 2015), a paired Student's t-test was used to evaluate statistical significance of age-related changes in perfusion index. The difference was considered statistically significant with p <0.05.

RESULTS

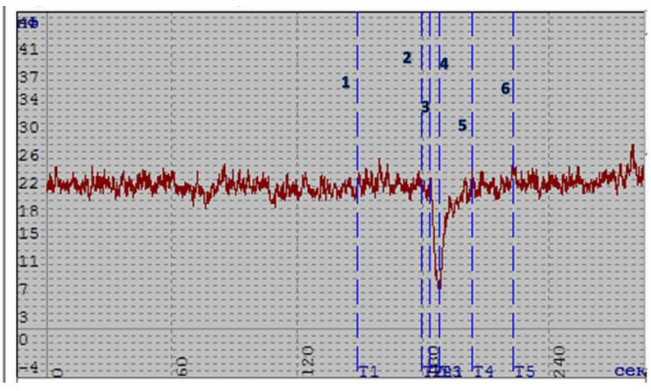
We observed age-related increase in perfusion index by 36.0 and 42.5 % (p <0.01) in the examined boys and girls, respectively. No statistically significant difference in flux (σ) was found in both groups. Blood flow variability (K_v) in the group of boys increased by 30.7 % (p <0.01), while girls displayed no statistically significant changes in K_v.

Parameters M, σ and K_v give a general idea of the microcirculatory system performance. A more detailed analysis was carried out at the second stage of the study when processing the amplitude-frequency spectrum of blood flow oscillations.

Boys showed age-related decrease in the normalized amplitude of endothelial rhythms by 19.7 % (p <0.05) the amplitude of myogenic oscillations increased by 24.4 % (p <0.01). In the group of boys aged 10 the values of maximum and normalized amplitudes in all frequency bands of passive factors of microcirculation regulation (respiratory and cardiac rhythms) were statistically higher compared to the data obtained from the same participants at the age of 8. The myogenic tone and shunt index decreased with age by 12.2 % (p <0.05) and 22.5 % (p <0.01), respectively (see table 1).

The functional contribution of endothelial rhythm and neurogenic oscillations to microcirculatory flow modulation decreased by 23.6 and 20.9 % (p <0.05) respectively in girls between the age of 8 and 10 years. The contribution of myogenic rhythms to total tissue perfusion decreased by 29.3 % (p <0.05). Maximum amplitudes of respiratory and cardiac rhythms increased by 84.2 (p <0.001) and 38.9 % (p <0.05). The neurogenic tone value increased by 31.4 % (p <0.05), shunt index decreased by 15.2 % (p <0.05) (see table 1).

Functional tests



Sample LDF data during a 30-second breath hold in a 8-year-old child

Legend: 1 — the initial value of perfusion index; 2 — the onset of breath hold; 3 — the onset of perfusion index reduction; 4 — the minimum value of perfusion index; 5 — the offset of breath hold; 6 —the value of perfusion index after normal breathing was restored.

Basal parameters of microcirculation were not statistically different in boys and girl between the ages of 8 and 10 years. In 8-year-old girls, the normalized amplitudes of respiratory and cardiac rhythms contributed to the blood flow modulation more than in boys of the same age (by 17.6 and 36.6 %, respectively, with p <0.05). In 10-year-old boys, the normalized amplitude of myogenic rhythms was 27.5 % (p <0.01) higher in comparison with the girls of the same age, the their neurogenic tone was 18 % (p <0.05) lower.

Results of the respiratory test showed that in 10-year-old children the initial level of perfusion was higher: by 36.0 % in boys and by 42.5 % in girls, compared with the data obtained from the same children at the age of 8 (p <0.01) (see table 2).

In boys, the interval between the onset of breath hold and the onset of microcirculatory flow reduction decreased by 18.3 % over two years; it increased by 23.3 % (p <0.05) in girls. As the children grew older, the time between the minimum value of perfusion index and breath recovery increased by 67.9 (p <0.01) and 135.0 % (p <0.01), respectively.

Reserve microvascular blood flow in boys did not change significantly, however, a tendency to its increase was observed. In girls, CFR increased by 31.5 % (p <0.01).

In 10-year-old girls the neurogenic tone was 31.4 % (p <0.05) higher and ΔM was 30.2 % (p <0.01) lower, compared to the corresponding values at the age of 8. 10-year-old boys showed no significant increase in neurogenic tone; the relative value of perfusion index reduction during the vasoconstrictive test decreased by 20.9 % (p <0.05).

DISCUSSION

It is known that the organism develops unevenly: long stable periods of development are followed by short unstable "critical" periods. It is those critical periods of development that are a basis for intensive formation of new properties and physiological systems, which is associated with the activation of energy metabolism [18].

The literature reports that mechanisms of microcirculatory regulation are formed at the age of 6, while specific patterns of the microcirculatory system performance are finally shaped in puberty followed by the formation of mature microcirculation [19].

In his work Litvin notes that tissue perfusion has a tendency to increase with age [1].

Our study demonstrated a conspicuous perfusion growth between the age of 8 and 10, which is probably associated with age-related changes in the microcirculatory system resulting from a more intensive metabolism. The increased amplitude of the pulse wave coupled with the increased perfusion index is indicative of a stronger arterial blood flow to the microvascular bed [16].

Decreased shunt index in the subjects indicates reduced muscle tone of precapillaries responsible for the regulation of nutritive blood flow. The reasons for it are different: reduced myogenic tone in boys and increased neurogenic tone in girls, which suggests a larger volume of blood coming into the nutritive capillaries.

When comparing basal parameters of microcirculation, we observed no significant differences in perfusion values in 10- and 8-year old boys and girls; however, in 10-year-old children an almost equal level of perfusion was maintained against various ratios of regulatory factors.

Respiratory oscillations originating from venular components penetrate into skin microvasculature; they are

Table 1. Perfusion index and mechanisms of perfusion regulation in children of 8 to 10 years of age

Parameter		Boys (n = 15)		Girls (n = 13)	
		8.13 ± 0.34 years	10.00 ± 0.33 years	8.00 ± 0.33 years	10.00 ± 0.33 years
Perfusion index	M, PU	19.65 ± 5.34	26.77 ± 6.00**	20.23 ± 4.03	28.89 ± 6.23**
	σ, PU	2.29 ± 0.85	2.22 ± 0.66	2.42 ± 0.92	2.70 ± 1.01
	KV, %	13.09 ± 4.91	9.07 ± 3.11**	12.22 ± 4.45	10.43 ± 4.65
Endothelial rhythms	A_{max}, PU	1.58 ± 0.83	1.28 ± 0.87	1.29 ± 0.45	1.40 ± 0.63
	(A_{max} / 3σ) × 100 %	20.84 ± 5.17	16.72 ± 5.23*	21.16 ± 4.77	16.16 ± 2.87*
	(A_{max} / M) × 100 %	8.23 ± 3.87	4.82 ± 2.88*	7.12 ± 2.68	5.35 ± 2.67
Neurogenic rhythms	A_{max}, PU	1.36 ± 0.49	1.35 ± 0.67	1.51 ± 0.68	1.43 ± 0.71
	(A_{max} / 3σ) × 100 %	18.02 ± 3.19	18.33 ± 3.90	20.52 ± 4.28	16.15 ± 4.65*
	(A_{max} / M) × 100 %	7.16 ± 2.67	5.13 ± 2.44*	7.53 ± 3.28	5.02 ± 2.49*
Myogenic rhythms	A_{max}, PU	1.00 ± 0.35	1.12 ± 0.41	1.09 ± 0.42	1.19 ± 0.49
	(A_{max} / 3σ) × 100 %	13.49 ± 2.92	16.84 ± 3.82**	15.26 ± 3.52	13.21 ± 3.38
	(A_{max} / M) × 100 %	5.19 ± 1.36	4.25 ± 1.47	5.49 ± 2.07	3.88 ± 1.45*
Respiratory rhythms	A_{max}, PU	0.31 ± 0.10	0.58 ± 0.19***	0.33 ± 0.11	0.61 ± 0.18***
	(A_{max} / 3σ) × 100 %	4.34 ± 1.08	8.41 ± 2.49***	5.28 ± 0.95	7.55 ± 2.48*
	(A_{max} / M) × 100 %	1.52 ± 0.36	2.16 ± 0.54**	1.79 ± 0.67	2.14 ± 0.68
Cardiac rhythms	A_{max}, PU	0.18 ± 0.04	0.35 ± 0.11***	0.28 ± 0.11	0.39 ± 0.14*
	(A_{max} / 3σ) × 100 %	2.67 ± 1.02	5.28 ± 2.11**	4.21 ± 2.16	5.08 ± 1.52
	(A_{max} / M) × 100 %	0.99 ± 0.24	1.32 ± 0.34**	1.33 ± 0.81	1.47 ± 0.52
NT, AU		1.84 ± 0.38	1.82 ± 0.32	1.69 ± 0.38	2.22 ± 0.62*
MT, AU		2.37 ± 0.48	2.08 ± 0.51*	2.29 ± 0.51	2.41 ± 0.61
SI, AU		1.38 ± 0.36	1.07 ± 0.15**	1.38 ± 0.32	1.17 ± 0.20*

Note: here and in table 2 data are presented as arithmetic mean ± standard deviation. M represents mean perfusion index; σ represents mean square deviation (mean blood flow modulation); K_v represents the coefficient of variation of perfusion index (blood flow variability); NT — neurogenic tone; MT — myogenic tone; SI — shunt index; A_{max}, (A_{max} / 3σ) × 100 % and (A_{max} / M) × 100 % — maximum and normalized amplitudes.

* — p <0.5; ** — p <0.01; *** — p <0.001 in comparison with the same parameters in children of an earlier age; PU— perfusion units; AU-— arbitrary units.

Table 2. Perfusion parameters in 8- and 10-year-old children during the inspiratory breath hold

Parameter	Boys (n = 15)		Girls (n = 13)	
	8.13 ± 0.34 years	10.00 ± 0.33 years	8.00 ± 0.33 years	10.00 ± 0.33 years
M_{init}, PU	20.26 ± 5.49	26.91 ± 6.49**	19.73 ± 5.91	29.54 ± 6.36**
P_{react}, PU	10.12 ± 4.06	16.17 ± 7.90*	9.38 ± 2.58	18.68 ± 7.69**
M_{rest}, PU	18.87 ± 4.40	26.90 ± 6.65**	19.93 ± 5.22	29.68 ± 6.94**
T3–T1, s	11.28 ± 3.95	9.23 ± 2.28*	10.34 ± 1.66	12.76 ± 3.03*
T4–T3, s	8.01 ± 4.10	4.74 ± 1.88*	9.65 ± 4.22	6.58 ± 2.45*
T5–T4, s	10.64 ± 4.64	17.81 ± 6.75**	9.35 ± 3.06	22.02 ± 6.00***
CFR, %	54.09 ± 17.81	59.57 ± 24.35	50.88 ± 13.32	66.78 ± 16.48**
ΔM, %	50.20 ± 26.10	39.70 ± 23.10*	52.40 ± 22.50	36.96 ± 20.77**

Note: M_{init} is the initial value of perfusion index before the inspiratory breath hold; P_{react} is minimal perfusion during the respiratory test; M_{rest} is the value of perfusion index after normal breathing was restored; T3–T1 is a time interval between the onset of breath hold and the onset of microcirculatory flow reduction; T4–T3 is a time interval between the onset of microcirculatory flow reduction and the minimum value of perfusion index; T5–T4 is a time interval between the minimum value of perfusion index and breath recovery; CFR is capillary flow reserve; ΔM is perfusion index shift.

— p <0.05; ** — p <0.01; *** — p <0.001 when compared to the corresponding values in children of an earlier age.

registered mainly in venules. Formation of those oscillations in human skin microvasculature is affected by at least two mechanisms: a mechanic transmission of respiratory variations of intrathoracic pressure mediated by the venous system (suction effect of the thorax during inspiration as veins get filled with blood) and an autonomic interaction of cardiovascular and respiratory centers. A passive hydrostatic nature of the former mechanism means that respiration-related oscillations originate from pressure wave propagation, while active mechanisms of vascular tone regulation are not involved. In the latter case, blood flow oscillations form active vasoconstrictive mechanisms of neurogenic nature, one of which is a well-known vasomotor reflex that is expressed as short-term reduction in tissue perfusion in response to the inspiratory gasp. The inspiratory vasomotor reflex is implemented through sympathetic peripheral innervation [20].

The vasomotor reflex triggered by the inspiratory gasp induces constriction of arterioles and short-term reduction in skin blood flow. Reduced perfusion during the respiratory test is a result of sympathetic regulation limited mainly by the neurovascular synapse [13].

Levin's work [21] showed that between between the age of

7 and 20, the functional reserve of the microcirculatory system grows. The respiratory test we used demonstrated the increase in the microcirculatory reserve capacity accompanying short-term hypoxia in girls; boys showed a conspicuous tendency to increased CFR.

The vascular response to the activation of adrenergic fibers is affected by sympathetic innervation and vessel wall reactivity. Therefore, the degree of blood flow reduction during the respiratory test reflects a combination of both factors that cannot be assessed separately. Because of that, for adequate sympathetic perivascular innervation assessment, LDF data should be interpreted carefully taking into account the initial neurogenic tone at rest and a relative value of perfusion index reduction during the respiratory test [13]. Changes in NT and ΔM in 10-year-old girls lead us to conclude that sympathetic activity increases with vasoconstrictive stimulation, while in 10-year-old boys changes in the perfusion index shift are induced by a weaker reactivity of preganglionic neurons against the functional load [16].

The perfusion index shift measured during the respiratory test is a result of sympathetic regulation limited mainly by the neurovascular synapse. Therefore, the age-related decrease of ΔM in boys and girls indicates a less conspicuous response of the vascular wall to the inspiratory breath hold. With age, perfusion index reduction becomes weaker in response to the respiratory test.

Physiologically, the time interval between 8 and 10 years of age is crucial because it lies between two critical periods: a growth spurt (at the age of 5–6) and puberty. The former is associated with significant morphological and functional changes in the nervous system [22]. Puberty is characterized by hormonal and muscle changes.

In 8-10-year-old children the intensity of oxidative processes remains pretty high, though metabolism remains quite stable. However, at this age the majority of physiological systems, including the cardiorespiratory system, enhance their capacities. Tissues and organs demand more oxygen, which leads to a specific performance pattern of the cardiovascular and respiratory systems. Though the circulatory and respiratory systems are not that resource conserving in children as in adults, they are very co-operative [22]. Close functional interconnection of the respiratory and cardiovascular systems implies their interdependence. Changes in the respiratory system performance lead to adaptive changes in the circulation and oxygen delivery to blood tissues.

In the ontogenesis, the cardiorespiratory system develops heterochronically in close interaction with the physical development of the organism, morphological changes in the lungs, heart, thorax, age-related metabolism dynamics and the development of regulatory mechanisms. Because of that, the cardiorespiratory system of a primary school child at various ages has different qualitative and quantitative characteristics based on the continuous development of morphological structures and functional changes [23].

CONCLUSIONS

Our study has revealed changes in the peripheral blood flow characterized by the age-related increase in tissue perfusion in the group of boys and girls. Those changes are most likely to be associated with the shifting roles of various regulatory mechanisms; oscillation amplitudes of active modulation components contribute less to the microcirculatory regulation, while passive elements of microhemodynamics modulation contribute more. A considerable growth of the pulse wave indicates a bigger arterial blood flow to the microvasculature. The increased amplitude of the respiratory wave resulting from venous pressure indicates decreased microcirculatory pressure.

The study demonstrated that basal microcirculatory parameters in boys of girls of 8 and 10 years of age did not differ significantly. However, active and passive factors of microcirculatory regulation made different contribution to maintaining equal levels of perfusion in boys and girls.

While assessing reserve capacities of microcirculation using the inspiratory breath hold, we found that improvement of autonomic regulation of the microvasculature between the age of 8 and 10 was expressed as decrease in the vessel wall reactivity in response to sympathetic adrenergic stimuli.

Further research is necessary to study the microvasculature and its regulatory mechanisms at various ontogenesis stages, including puberty which gives birth to sex-related differences in hemomicrocirculation performance. Such studies will help to understand human microcirculation dynamics better and to explore age-related specifics of peripheral circulation.

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IMPACT OF LEARNING ENVIRONMENTS ON THE PHYSICAL DEVELOPMENT OF MOSCOW SCHOOLCHILDREN: HYGIENE ASPECTS

Bokareva NA ✉, Milushkina OYu, Ovchinnikova ZA, Pivovarov YuP, Sheina NI

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

Physical development and health of today's schoolchildren are influenced by various factors, including the way the learning process is organized. This article presents data on some aspects of physical development of 1585 teenagers (15 to 17 years old) from 35 Moscow schools, including general education schools, specialized schools, schools with optional advanced courses in biomedical sciences, and health promoting schools. We studied basic anthropometric characteristics of the participants (body height and weight), assessed their psychoemotional status using the Children's Form of Manifest Anxiety Scale and their lifestyle using questionnaire surveys. The control group included teenagers from general education schools. The study showed that the number of teenagers with no abnormalities in their physical development was significantly higher in health promoting school while the number of overweight students there was significantly lower, compared to the controls ($p < 0.05$). For other groups of participants, the results were statistically insignificant. We established statistically significant correlations between well-proportioned physical development and the impacts of night sleep deficit ($r = -0.44$, $p < 0.05$), time spent working on the computer ($r = -0.44$, $p < 0.05$), psychological climate in the family ($r = -0.20$, $p < 0.05$), and meal frequency (Pearson's contingency coefficient was 0.41, with $p < 0.01$, Cramer's contingency coefficient was 0.32, with $p < 0.03$).

Keywords: physical development of schoolchildren, learning environment, health promoting schools, advanced courses in biomedical sciences, classroom AV equipment, physical activity

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

Received: 23.05.2016 **Accepted:** 12.06.2016

ГИГИЕНИЧЕСКАЯ ОЦЕНКА ВЛИЯНИЯ ОРГАНИЗАЦИИ ОБРАЗОВАТЕЛЬНОГО ПРОЦЕССА НА ФИЗИЧЕСКОЕ РАЗВИТИЕ ШКОЛЬНИКОВ Г. МОСКВЫ

Н. А. Бокарева ✉, О. Ю. Милушкина, З. А. Овчинникова, Ю. П. Пивоваров, Н. И. Шеина

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

На здоровье и физическое развитие современных школьников влияют различные факторы, в том числе организация образовательного процесса. В статье представлены данные об особенностях физического развития 1585 подростков в возрасте 15–17 лет из 35 школ г. Москвы: общеобразовательных школ, школ с углубленным изучением отдельных предметов, школ с медико-биологическими классами, школ здоровья. Изучали основные антропометрические показатели — длину и массу тела, а также психоэмоциональный статус по шкале явной тревожности для детей (The Children's Form of Manifest Anxiety Scale) и образ жизни с помощью анкетирования. За контрольную приняли группу подростков из общеобразовательных школ. Исследование показало, что среди учащихся школ здоровья статистически значимо больше подростков с нормальным физическим развитием и меньше — с избытком массы тела, чем среди учащихся группы сравнения ($p < 0,05$), в то время как для других групп результаты были статистически незначимы. Были установлены статистически значимые корреляционные связи между гармоничностью физического развития и несколькими факторами влияния: дефицитом ночного сна ($r = -0,44$, $p < 0,05$), продолжительностью работы за компьютером ($r = -0,44$, $p < 0,05$), психологическим микроклиматом в семье ($r = -0,20$, $p < 0,05$), кратностью приема пищи (коэффициент сопряженности Пирсона — 0,41, $p < 0,01$, коэффициент сопряженности Крамера — 0,32, $p < 0,03$).

Ключевые слова: физическое развитие школьников, организация образовательного процесса, школы здоровья, медико-биологические классы, технические средства обучения, двигательная активность

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

Статья поступила: 23.05.2016 **Статья принята к печати:** 12.06.2016

Physical development in childhood and adolescence still remains an important area of research, because factors that influence it and the intensity of these factors are constantly changing. Modern literature reports the increase in the overall body size of young Russians as compared to their age-mates of previous generations, more rapid biological maturation, earlier menarche, thicker layers of subcutaneous fat, higher body mass index, and reduced functional abilities of the body [1–9].

The way the learning process is organized heavily affects health and physical development of modern schoolchildren [10]. Education quality standards are very strict and course load is increased, especially in specialized schools or schools that offer industry-specific training.

Reasonable adjustments in learning and teaching help to improve the quality of knowledge acquired at school, to motivate the child to voluntarily choose a future profession, to help him/her adapt to the learning environment in a higher

education institution in the future. Some researchers have shown that innovative practices introduced at schools are often accompanied by health problems in children and teenagers, including functional deficits and chronic diseases [11–13].

The aim of this study was to investigate how new learning and teaching practices influence physical development of modern children in Moscow schools.

METHODS

We have summarized and analyzed the data obtained during three scientific experiments in 2011–2015 [14–16].

We have described learning environments in 35 Moscow schools, including general education schools, specialized schools, schools with optional advanced courses in biomedical sciences, and health promoting schools. The following aspects were evaluated: sanitation and hygiene, technical equipment, medical service, food service, and learning arrangements according to the recommendations of the "Complex assessment of education of children and teenagers at educational institutions" guide [17]. The study included 1585 schoolchildren aged 15–17 (732 boys and 853 girls). Table 1 shows how children were distributed into groups depending on their school type. Teenagers from general education schools formed the control group.

Physical development of schoolchildren was assessed by the unified anthropometric method using standard tools [18]. Major anthropometric parameters of physical development were studied, namely, body weight and height. To assess how well-proportioned the child's physical development was, we used a regional modified weight-on-height regression scale [19]. Psychoemotional status of teenagers was assessed by the Children's Form of Manifest Anxiety Scale (CMAS) adapted by Prikhozhan AM [20, 21]. To identify risk factors responsible for developing health disorders and to describe children's lifestyle, schoolchildren and their parents were surveyed using standard questionnaires [20].

Data were statistically processed using Statistica 6.0 software (StatSoft, USA). Statistical significance was assessed using Student's t-test. Correlation and contingency coefficients were used to assess how studied factors affected body proportions. The study was approved by the Ethics Committee of Pirogov Russian National Research Medical University (Protocol 130 dated December 9, 2013). Headmasters and parent boards gave consent to include teenagers into the experiment.

RESULTS

Learning environments are generally favorable for Moscow schoolchildren: 83.3 % of educational institutions were assigned to category 1 of sanitary and epidemiological welfare; the rest 16.7 % were assigned to category 2.

Table 1. Participants' distribution into groups according to their school type

School type	Number of schools	Number of students	
		Boys	Girls
General education schools	5	152	135
Specialized schools	5	185	204
Schools with optional advanced courses in biomedical sciences	23	347	476
Health promoting schools	2	48	38

Among the most common violations of sanitation regulations were the following: no public transport available in the vicinity of a school, small school grounds and lack of vegetation on them, sports grounds failing to meet sanitation requirements. School maintenance was also an issue, as well as small territories for medical facilities or the lack of the latter. Natural and artificial lighting checks revealed dirty windows and light fitting, unreplaced light bulbs and broken lamps. Besides, in some schools air and temperature conditions failed to meet the standards, technical equipment was arranged improperly, and the furniture did not match children's height. Weekly study load met the requirements almost in every school [22], except for schools with optional advanced courses in biomedical sciences, where the number of hours was by 2.1–2.4 h higher and did not match the physiological performance curve based on the weekly performance of high school students.

The comparative analysis of physical development of schoolchildren showed that the number of teenagers with no abnormalities in their physical development was significantly higher in health promoting school, while the number of overweight students there was significantly lower, compared to the controls ($p < 0.05$) (see table 2). The numbers of children with no abnormalities in their physical development and body weight deficit in schools with advanced courses in biomedical sciences and in general education schools (the controls) were comparable; the number of overweight children was 1.3 times lower. In specialized school, the number of pupils with normal physical development was the lowest. Excess weight in this group was observed 1.4 times less often, and weight deficit — 1.4 times more often, compared to the controls. Still, those observations are statistically insignificant.

A more challenging curriculum and a wider range of information that children have to process lead to the increased volume of homework. In average, high school students (84.6 % girls and 71.6 % boys) spend 2 hours on it. We have not found any significant differences between the studied groups in relation to this parameter. Questionnaire results in all the groups were similar; summarized data are presented in the table below.

Normal sleep duration (8 hours or more) was observed in only a quarter of teenagers participating in the study, 32.9 % of them being boys and 26.7 % being girls. The majority of schoolchildren (51.3 % boys and 50.0 % girls) slept 6–7 hours a day. Another 15.8 % boys and 23.3 % girls slept less than 6 hours. We have found a statistically significant correlation between night sleep deficit and physical development ($r = -0.59$, $p < 0.05$): the excess weight was 1.9 times higher in teenagers who suffered from night sleep deficit, compared to those who slept more than 8 hours a day (fig. 1).

All the girls who participated in the study and 99.1 % of the boys had a computer at home and used it on a regular basis; 70 % of schoolchildren started using the computer at the age of 8–11 (at primary school). 74.3 % of high school students used their computer every day. In average, boys spent 12.36 ± 1.73 hours a week working on the computer, while girls spent 12.24 ± 1.95 hours.

Table 3 shows how schoolchildren are subgrouped depending on the weekly hours spent working on the computer. We found a statistically significant correlation between the hours spent working on the computer and the physical development of teenagers ($r = -0.44$, $p < 0.05$). Among those who use a computer 7 hours a week or more, prevalence of weight deficit is 1.5 times higher (24.6 %) than among those who use it less than 7 hours (16.9 %) ($p < 0.05$). The survey showed that 65–80 % teenagers knew about health problems associated

Table 2. Physical development of Moscow schoolchildren of 15-17 years of age from schools of various types

School type	Percentage of teenagers with no abnormalities in their physical development, %	Percentage of overweight teenagers, %	Percentage of teenagers with body weight deficit, %
General education schools	69.7	15.1	15.1
Specialized schools	66.1	10.7	21.4
Schools with optional advanced courses in biomedical sciences	68.4	11.1	17.7
Health promoting schools	80.8*	3.8*	15.4

Note: * — p <0.05 (compared to the controls).

with using a computer but were still unwilling to give it up as a homework support tool.

A considerable number of schoolchildren (29.3 %) emphasized that they were permanently stressed throughout the learning process. Among them, the number of those overweight was 2.3 higher than among children who were under no stress (13.0 and 5.6 %, respectively), (p <0.05). Only 29.3 % of boys and 20.9 % of girls said that there were no conflicts in their families. Children from families where frequent conflicts were typical had no abnormalities in their physical development 1.5 times less often than children from zero-conflict families; they also suffered from body weight deficit 3 times more often (p <0.05) (r = –0,20, with p<0.05).

In addition to the compulsory classes at school, 63.2 % of boys and 69.9 % of girls attended extra curricular classes where static postures prevailed 2–3 times a week in average. A total duration of such classes was 7–8 hours a week in average. 53.9 % of boys and 55.9 % of girls did sports or attended dance classes spending an average of 5–6 hours a week on these activities. Among the teenagers whose physical activity was limited to PE lessons at school, the number of those with no abnormalities in their development was significantly lower and the number of those with excess body weight was higher, with p <0.05 (fig. 3).

There were some deviations from school hygiene standards regarding meals. 30.1 % of high school students never had breakfast and 35.7 % never had lunch at school. Only 76.3 % of boys and 60.0 % of girls had meals three or more times a day. Only 61.8 % of boys and 48.8 % of girls received hot meals two or more times a day. Long intervals between the meals that exceeded 5-6 hours were reported by 67.1 % of boys and 85.9 % of girls. Among the teenagers who did not have regular meals, body weight deficit was observed 1.7 times more often than in the group of children whose meals were regular (fig. 4). We have found a statistically significant correlation between physical development and meal frequency (Pearson's contingency coefficient of 0.41, p <0.01; Kramer's contingency coefficient of 0.32, p <0.03). In the group of schoolchildren who had hot meals once a day or did not have hot meals at all, the number of overweight teenagers was twice as higher than in the group of schoolchildren who had hot meals twice a day or more frequently (p<0.05; see fig. 5).

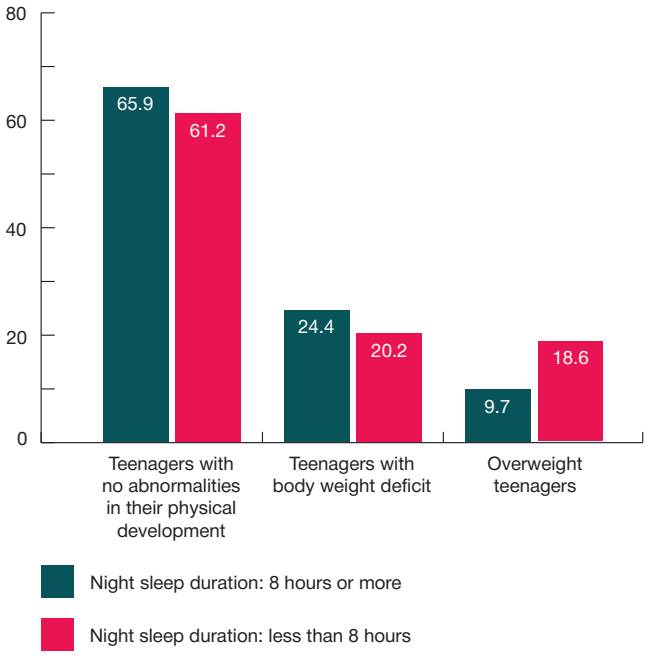

Fig. 1. The impact of sleep duration on the physical development of Moscow schoolchildren of 15–17 years of age

DISCUSSION

The data describing the impact of various forms of the learning process, including industry-specific training, on the physical development of children and teenagers are scanty and controversial [12, 23, 24]. Some studies report positive impact of industry-specific training on the functional and psychoemotional state of the students; others describe its negative impact. The majority of the results obtained in our study are statistically insignificant, but the analysis of various factors, such as night sleep deficit, long hours spent working on the computer, hypodynamia, irregular meals, that influence the physical development of teenagers indicates that the most negative impact is associated with those learning practices that increase academic loads.

The obtained data in support of the negative influence of night sleep deficit on the physical development of schoolchildren correlate with the results of other studies. Excess body weight is more likely to be found in children who suffer from sleep deficit than in those who get enough sleep [25, 26].

Hypodynamia is a distinct feature of the lifestyle of modern children and teenagers. Deficit of physical activity amounts to 30 % in preschool educational institutions [27]; in primary

Table 3. The use of the computer and TV by Moscow schoolchildren of 15–17 years of age

Questionnaire question	Answer	Boys	Girls	Total
Computer use, times a week	1–2	8.4	7.6	7.9
	3–4	16.8	18.5	17.7
	Every day	74.8	73.9	74.3
Computer use, hours a week	1–7	34.4	47.3	41.3
	8–14	22.9	25.4	24.3
	15–21	21.9	16.4	18.9
	22 and over	20.8*	10.9	15.5
Watching TV, hours a week	I do not watch TV	10.1	8.2	9.1
	1–7	54.5	52.7	53.6
	8–14	20.2	29.1	24.8
	15–21	8.1	6.4	7.2
	22 and over	7.1	3.6	5.3

school this number drops by 2.0–2.5 times [28, 29]. According to some researchers, the deficit of physical activity among primary school children amounts to 35–40 % it amounts to 75-85 % among high school students [30]. The data on physical activity deficit obtained in our study correlate with the data provided by other researchers.

Poor and unbalanced diet negatively affects physical development of children and teenagers and their academic progress and increases the risk of diseases [3, 6, 31]. The results of our research confirm it.

Health promoting schools are the most beneficial for teenagers from a health perspective. Such schools provide two hot meals during the day (breakfast and lunch); third courses are enriched with vitamin C November through May; students have unlimited access to water. Physical education is an important trend in such schools. To increase physical activity of students, an extra 20 minute break for active games was introduced; there are PT breaks during the lessons. 1st to 4th year students have 5 lessons in their weekly schedule that involve physical activity; 5th year students have 4 such lessons, 6th–11th year students — 3 lessons. Once a trimester health days are celebrated by playing outdoor games and holding competitions. In February children have extra holidays, namely, a sports week during which 2nd to 11th grade children participate in various team competitions in different kinds of sports, mainly outdoor sports. To preserve and improve neurological and mental health of schoolchildren, a weekly physical load is strictly regulated, the schedule is drawn considering physiological curves of daily and weekly performance of the students; a lesson's duration is reduced to 40 minutes; an individual approach to students

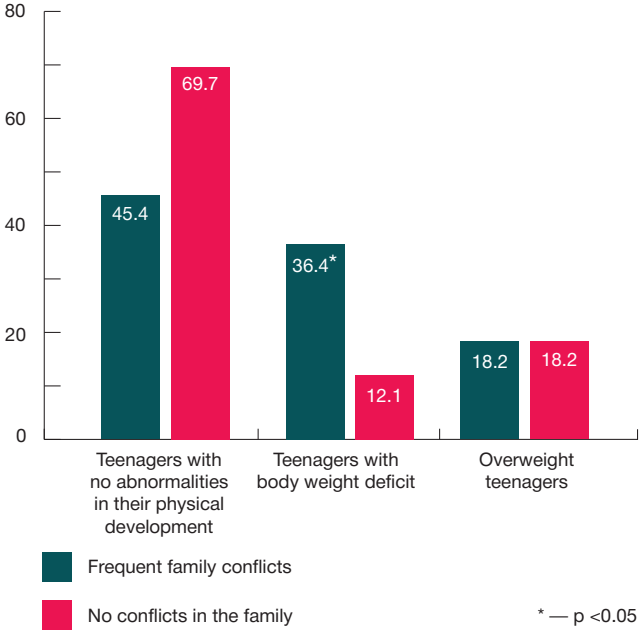

Fig. 2. The impact of family climate on the physical development of Moscow schoolchildren of 15–17 years of age

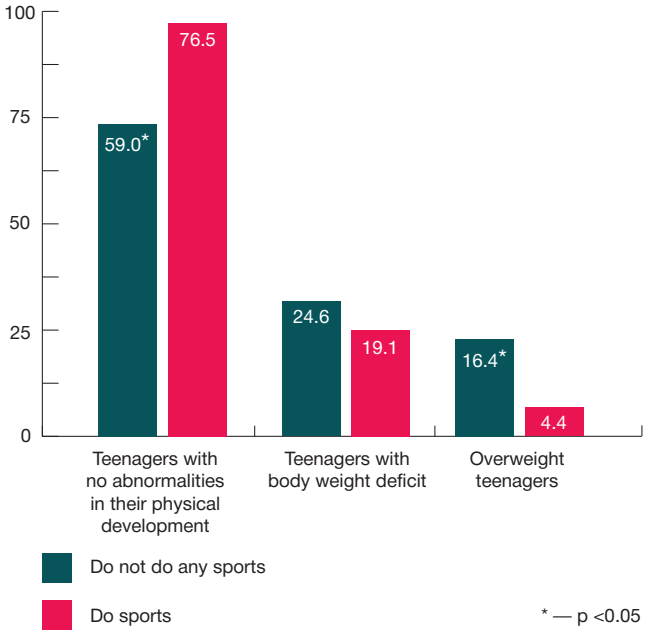

Fig. 3. The impact of extra sports activities on the physical development of Moscow schoolchildren of 15–17 years of age

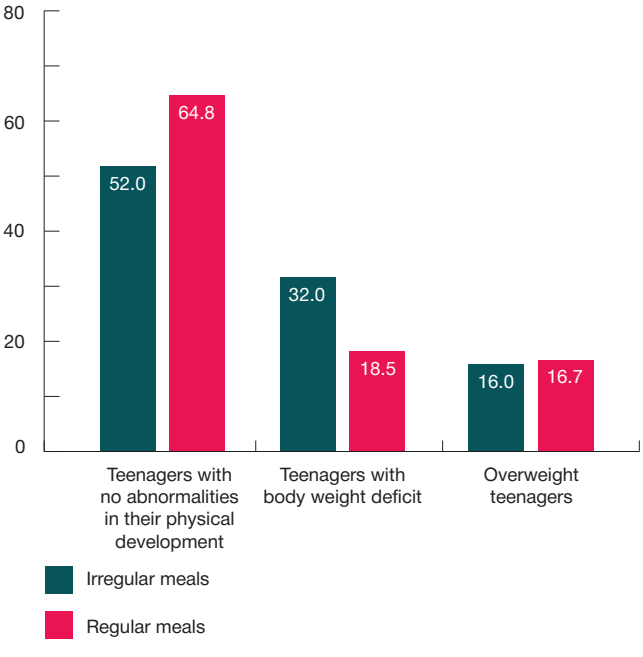

Fig. 4. The impact of meals regularity on the physical development of Moscow schoolchildren of 15–17 years of age

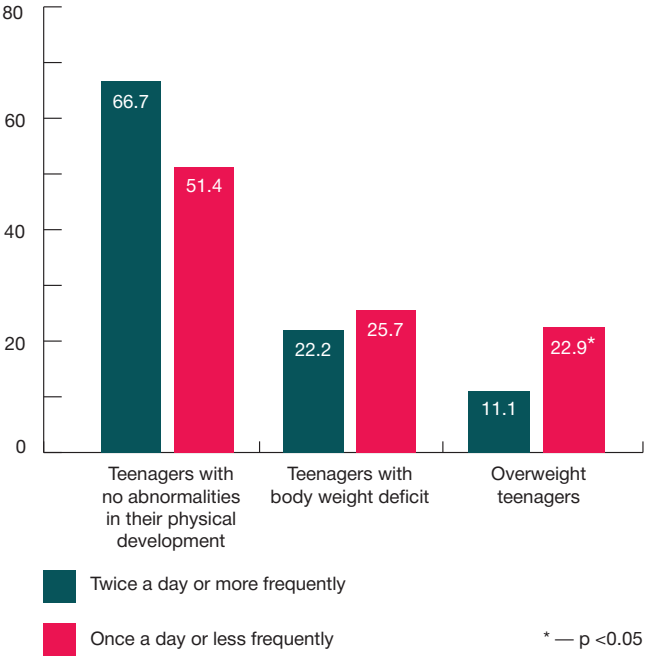

Fig. 5. The impact of hot meals frequency on the physical development of Moscow schoolchildren of 15–17 years of age

became possible after reducing the maximum number of pupils per class to 22; teachers are taught to identify the signs of intellectual exhaustion and to help alleviate psychoemotional tension; at school, children are supported by psychologists. Here, a particular focus is on teaching children, their parents and educators the basics of healthy lifestyle and nutrition, and on motivating them to be more active and do sports.

CONCLUSIONS

This study revealed a number of factors that have a negative impact on the physical development of schoolchildren: increased academic load, unregulated use of technical equipment during the learning process, conspicuous hypodynamia, psychoemotional tension and stress associated with the learning process. Among the factors that negatively affect the physical state of the teenagers are night sleep deficit, family conflicts and irregular meals. The results of this work helped the authors to elaborate practical recommendations for preventing abnormalities in the physical development of schoolchildren that can be used by medical personnel, teachers, parents and schoolchildren themselves.

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HOW AWARE ARE MEMBERS OF THE MEDICAL UNIVERSITY COMMUNITY OF THE RISKS AND CONSEQUENCES OF SKIN TATTOOING? RESULTS OF THE ONLINE SURVEY

Trushina EV[1]✉, Minkina OV[1,2], Dvornikov AS[1], Skripkina PA[1], fon Zimfer EI[1], Konyshev YaI[1]

[1] Department of Dermatology and Venerology, Faculty of General Medicine, Pirogov Russian National Research University, Moscow, Russia

[2] Family Medical Center, OOO, Moscow, Russia

This article presents the results of an anonymous online survey on skin tattooing conducted in the community of medical students and professors. The article covers issues of tattoing from medical and sociocultural perspectives. The survey was carried out in Pirogov Russian National Research University and included 210 participants of different ages. The results demonstrate the insufficient knowledge of medical indications, contraindications and potential complications associated with tattooing. The obtained data can be a good starting point for developing programs aimed at raising awareness among young people, especially in higher medical institutions. The opinion expressed in this article is not necessarily shared by students and professors from other institutions for higher medical education and does not necessarily reflect the level of expertise in other medical communities. This work is an example of how socially oriented practical training can be organized for the 3rd- and 4th-year students of the Faculty of General Medicine at Pirogov Russian National Research University.

Keywords: tattoo, tattooing, online survey, medical students, medical contraindications, dermatologic complications

✉ **Correspondence should be addressed:** Evgeniya Trushina
ul. Ostrovityanova, d. 1, Moscow, Russia, 117997; trushina.evgeniia@gmail.com

Received: 23.05.2016 **Accepted:** 06.06.2016

КОМПЕТЕНТНОСТЬ СООБЩЕСТВА МЕДИЦИНСКОГО ВУЗА В ВОПРОСАХ РИСКОВ И ПОСЛЕДСТВИЙ ТАТУИРОВАНИЯ КОЖИ ПО РЕЗУЛЬТАТАМ ОНЛАЙН-АНКЕТИРОВАНИЯ

Е. В. Трушина[1]✉, О. В. Минкина[1,2], А. С. Дворников[1], П. А. Скрипкина[1], Е. И. фон Цимфер[1], Я. И. Конышев[1]

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

[2] ООО «Семейный медицинский центр», Москва

В статье представлены результаты анонимного онлайн-анкетирования студенческого и преподавательского сообществ медицинского вуза по вопросам, связанным с татуированием кожи. Освещены проблемы нанесения тату-изображений с медицинской и социокультурной точек зрения. В опросе, проходившем в РНИМУ им. Н. И. Пирогова, приняли участие 210 респондентов различных возрастных групп. Анкетирование выявило недостаточный уровень знаний участников о медицинских показаниях/противопоказаниях и потенциальных осложнениях при проведении тату-процедур. Полученные данные являются достаточным основанием для начала формирования программ повышения специализированной грамотности в молодежной среде, особенно в медицинских учреждениях высшего профессионального образования. Мнение, выраженное в публикации, не обязательно отражает точку зрения и уровень знаний студенческих и преподавательских сообществ других вузов. Работа представляет собой вариант организации и проведения социальной практики для обучающихся 3-го и 4-го курсов лечебного факультета РНИМУ им. Н. И. Пирогова.

Ключевые слова: тату, татуирование, татуировка, онлайн-анкетирование, студенты медицинского вуза, медицинские противопоказания, дерматологические осложнения

✉ **Для корреспонденции:** Евгения Валерьевна Трушина
117997, г. Москва, ул. Островитянова, д. 1; trushina.evgeniia@gmail.com

Статья поступила: 23.05.2016 **Статья принята к печати:** 06.06.2016

Adorning the body with images dates back centuries. Such images vary by geographical location or between ethnic groups, are used for identification and as a decoration, provide information about the wearer, or have religious meaning. Tattooing is a kind of the avant-garde art and a way to change one's appearance. Tattooing is performed by various piercing tools that break skin integrity and inject ink into deeper skin layers thus creating an indelible (permanent) image.

We do not know exactly when the first tattooing procedure was performed. Polynesian tattoos originated in the 3rd-2nd millennium BC in the Lapita archaeological culture on Santa Cruz Island (Solomon Islands, Melanesia) [1]. The earliest evidence of tattooing among Europeans dates back to the early XVIII century when tattooed people demonstrated their skin adornments at fairs [2]. The history of mankind has numerous examples of using tattoos to mark offenders. Prisoners

of concentration camps were branded with identification numbers. Tattoos often indicate a rank in the criminal hierarchy [3-6].

We live in the era of tattoo renaissance, when skin adornment, once bizarre, has become quite common, especially among young people of 18 to 30 years of age. Tattooing has lost its rudimentary pagan flavor and its shady criminal reputation.

Considering a lasting interest for tattooing, we should elucidate the questions of contraindications, consequences and safety for future generations of doctors [7]. On the one hand, to run the procedure smoothly, one should clearly understand its sanitation requirements; on the other hand, medical personnel should be aware of the legal liability that ensues from performing the procedure in spite of contraindications. Unfortunately, this fact is often ignored by both the customer and the tattoo service provider.

Contraindications for tattooing are diabetes mellitus, blood clotting disorders, diseases of blood-forming organs, anamnestic immune response to metals or their chemical compounds, blood thinning therapies, epilepsy, systemic autoimmune disorders, cancer, acute bacterial and viral infections. Other contraindications include alcohol or narcotic intoxication, pregnancy and lactation. Up to now, in the Russian Federation whole blood and component donors must specify in the blood donor screening questionnaire whether they have a tattoo. Having a tattoo is also a temporary contraindication to being a donor (Addenda 1 and 2 to Order 364 of the Ministry of Health of the Russian Federation dated September 14, 2001 "On approval of rules for medical examination of whole blood and component donors"). Worldwide, potential donors can be deferred or rejected if they have a tattoo, due to a supposedly high association between tattooing and some transfusion-transmitted infectious diseases [8–10].

Recently doctors, especially dermatologists, have started to pay closer attention to body tattoos because of a huge number of complications related to tattooing. Dermatologic complications of tattooing vary from acute short-term superficial inflammatory reactions that patients do not usually report to the doctor to infectious, persistent allergic or phototoxic reactions, granulomatous and lichenoid rashes [11–14].

Modern literature classifies tattoo-related medical complications as pyogenic infections (impetigo, furunculosis, cellulitis); non- pyogenic infections (syphilis, lepra, viral hepatitis); dermatologic manifestations of systemic diseases of unclear etiology in the tattoo area (psoriasis, lichen ruber planus, discoid lupus erythematosus); acquired hypersensitivity to tattoo ink (pigments); complex pathological states (keloids, erythema multiforme, lymphadenopathy) [15].

Inflammatory reactions are usually manifested by a localized edema, itching, hyperemia of various intensity, and papular rash in the tattoo area. According to the literature, the most common trigger for developing such complications is a red pigment that contains mercury and its sulfides [16, 17] (fig. 1). However, there is a growing number of reports on acute and subacute inflammatory response and even cancers associated with the use of modern organic pigments, such as Pigment Red 170 and 181 [18–21].

We distinguish between superficial and deep tissue infectious complications depending on the zone they affect, and between bacterial, viral and fungal complications depending on the etiological agent that causes them (fig. 2).

The number of scientific works that describe complications caused by various infectious pathogens during tattooing is growing. Along with such pathogens as *Saksenaea vasiformis* [22] and *Molluscum contagiosum* [23], some others have been reported, such as *Mycobacterium fortuitum* [24, 25], *Mycobacterium chelonae* [26, 27], *Mycobacterium haemophilum* [28], *Aspergillus fumigatus* [29], and *Human pappillomavirus* [30, 31].

No comprehensive and effective reorganization is possible in any management structure without proper personnel training. Medical students can be seen as a potential resource for health system; thus, they should be ready to convincingly promote the basics and principles of healthy and safe lifestyle in the future.

The aim of this work was to evaluate personal involvement of students and professors of a medical university into the problem of tattooing (the presence and nature of body tattoos and the reasons for having them) and to understand the general level of awareness of tattoo-related medical issues using an anonymous online survey.

METHODS

Using an anonymous online survey developed by the authors of this work, we collected and analyzed responses of students, medical residents, postgraduate students and professors of Pirogov Russian National Research Medical University who agreed to take part in the survey, submitted information on having a tattoo and shared their knowledge of medical indications/contraindications and possible complications following the tattooing procedure.

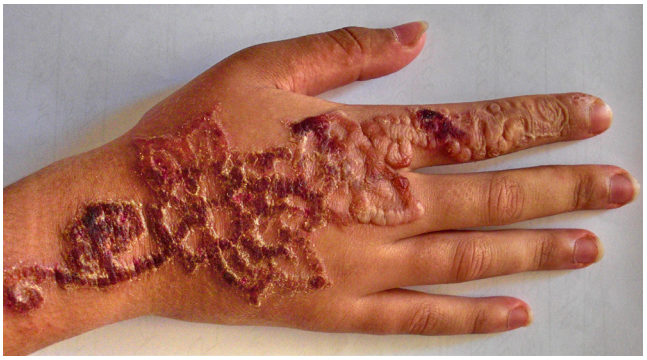

Fig. 1. Inflammatory response in the tattoo area (the dorsum of the right hand), still persistent 5 days after the procedure

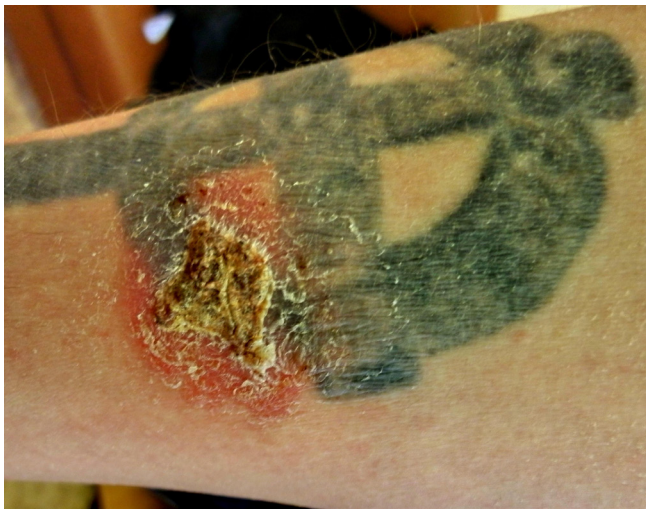

Fig. 2. Mixed bacterial and fungal infection in the tattoo area (the inner forearm)

For our study, we decided on a one-off group online questionnaire and unselected participants. Such study design allows for the quickest collection of the initial data, its results are easy to mathematically process; it also allows to survey a large number of respondents within a short period of time, not to mention that this method is very cost-effective. The link to the online questionnaire was published on the web page of the dean's office of the Faculty of General Medicine, Pirogov Russian National Research Medical University, since it is the biggest faculty here, which would allow us to collect and analyze as much data as possible from target respondents within a short time period.

The data were collected within 30 days. An invitation to participate in the survey was sent over to the personnel and students of the Faculty of the General Medicine. The questionnaire consisted of 16 close-ended questions that limited possible answers to a number of choices (see the table below). Answers to key questions determined what question would be offered to the respondent next, as shown in fig.3. Anonymity of the participants was a prerequisite. To prevent respondents from filling out the survey form more than once, an IP filter for blocking repeated access attempts to the questionnaire was applied. The survey was approved by the university administration.

RESULTS

210 individuals took part in the survey. The majority of the respondents (140, or 66.7 %) were female. 198 (94.3 %) respondents were 19–24 years of age, 8 (3.8 %) respondents were 25–30 years of age, 1 (0.5 %) was 16-18 years of age, 3 respondents (1.4 %) were 30-40 years of age. According to the obtained data, 188 (89.5 %) respondents were undergraduate students; 7 participants (3.3 %) were postgraduates, 15 (7.1 %) participants had completed their secondary vocational education by the time of the survey.

As suggested by the results of our survey, only 16 (7.6 %) individuals were not aware of the complications that may occur during or after tattooing procedures. Three times as many respondents — 45(21.4 %) did not know about contraindications that must be considered before tattooing. About one third of the respondents (68 individuals, or 32.4 %) did not even have basic knowledge about ink ingredients used for tattooing.

Only 15 participants (7.1 %) disclosed that they had a tattoo. Interestingly, most of them (9 participants) were female. 10 (67 %) respondents with tattoos had some understanding of what chemical components the ink is made of; all of those respondents had searched for the relevant information before they got a tattoo.

10 (67 %) of the respondents had only one anatomical region covered with tattoos. 5 (33 %) respondents had two or more anatomical regions covered with tattoos. 9 (60 %) of those respondents had a monochrome image.

Respondents were asked to specify the reasons that had motivated them to get a tattoo. 9 (60 %) participants saw their tattoos as a modern body adornment; 4 (47 %) respondents said their tattoos reminded them of some important event in their life or were a way to celebrate romantic relationship; few respondents — 1 (7 %) for each answer option — viewed tattoos as a symbol of being a member of a specific social group, or used them to mask their birth- or acquired defects and to cover up the previous body image.

Only 3 (20 %) respondents with tattoos thought about removing them: 2 were unsatisfied with the way the tattoo looked (the image looked faded and distorted), 1 respondent had personal reasons and either was bored with it or found it inconsistent with his current social status.

Only 38 (18 %) respondents gave a positive answer to the question "Are you planning to get a tattoo?"; none of them had had a tattoo before. For the majority of the respondents (20 individuals), the key motivating factor for getting a body image was aesthetics; they saw a tattoo as a modern body adornment. Among other motives were: cosmetic corrections (masking skin defects, old tattoo improvement, covering up old images) specified by 3 participants; personal reasons (tattoos being a reminder of romantic relationship or important event) specified by 11 individuals; social reasons (being a member of a certain social group or a subculture) specified by 3 individuals; ethnicity-related or religious reasons specified by 3 people.

DISCUSSION

According to the literature, up to 15 % of the world population have at least one tattoo [32]; 10 to 30 % of young people have at least one body image; half of those who still have not received a tattoo, are planning to have it done [33, 34]. Unfortunately, there are no accurate statistical data on the number of people with tattoos in the Russian Federation, on their age and social status.

Results obtained in this study do not differ drastically from the data provided by various research works. Only 7.1 % of the respondents had body tattoos at the time of the survey. Interestingly, there were only few participants (5) who had more than one tattoo, and there were no respondents who considered getting another tattoo. These data allow for a supposition that in nonverbal communication, ideographic body marks do not have any value as an adaptation tool or as a means of establishing authority in the student community of a higher education institution (which is the social group we studied)

The survey showed that 18 % of the respondents do not exclude the possibility of getting a tattoo in the future, and only 9.5 % see the esthetical aspect of tattooing as attractive. Such result indicates the absence of growing interest in the decorative meaning of tattooing among the educated youth.

Although most of the respondents (92.4 %) were aware of the complications that can occur during or after the tattooing procedure, 21.4 % of participants (1/5 of all respondents) did not know about medical contraindications to getting a permanent body image, and 32.4 % of the respondents did not even have a general idea of what ingredients the tattoo ink consists of. Considering that the survey was conducted in the higher education institution, where the primary goals are to give and receive medical education, the authors of this work expected that respondents' awareness, that is, basic theoretical knowledge of indications, contraindications, complications, and safety arrangements during the procedure, should be higher than it actually was.

Changing one's appearance by permanent skin images is challenging. Still, thinking that tattoo will always remain an element of skin adornment is misleading. People often feel the need to get rid of the tattoo to conform to the company's image, for example, to observe a dress code when applying for a position in a bank, a law enforcement agency, state media. According to the scanty data available at the moment, almost

Questions and sets of answers used in the online survey

№	Question	Answer code	Sets of possible answers
1	Please, specify your sex		– Male – Female
2	How old are you?		– 16–18 years – 19–20 years – 21–24 years – 25–30 years – 31–40 years – 41 years or older
3	Please, specify your level of education		– Secondary vocational education – Undergraduate student – Higher education
4	Are you aware of the risks related to tattooing?		– Yes – No
5	Do you know about contraindications to tattooing?		– Yes – No
6	Do you know the ingredients of ink used for tattooing?	6.1 6.2	– Yes – No
7	Do you have tattoos?	7.1 7.2	– Yes – No
8	When did you decide to find out about the ingredients of ink used for tattooing?		– Before I had my tattoo done – During the tattooing procedure – After I had my tattoo done
9	Your tattoo covers	9.1 9.2	– One anatomical region – Two or more anatomical regions
10	Your tattoo is		– Monochrome – Color
11	You have		– Only monochrome tattoos – Only color tattoos – A combination of both
12	What reason did you have to get a tattoo? Several answers are possible		– Cosmetic (to mask skin defects, to cover up an old tattoo) – Esthetic (body adornment) – Personal (to celebrate a romantic relationship, to remind myself of an important person or event in my life) – Social (I'm a member of some social group or a subculture) – Ethnicity-related or religious (I'm a member of some ethnic or religious community) – It was a spontaneous decision
13	Have you ever thought of removing your tattoo?	13.1 13.2	– Yes – No
14	What are the reasons that made you consider removing your tattoo? Several answers are possible		– Cosmetic (the tattoo faded, the image got distorted) – Esthetic (I do not see my tattoo as body adornment; it does not match my current look) – personal reasons (I'm bored of it; it is not in accord with my new relationship) – Social (I'm reluctant to show that I belong to a specific social group or subculture) – Ethnicity-related or religious (I'm reluctant to show that I'm a member of some ethnic or religious community)
15	Are you planning to have a tattoo done?	15.1 15.2	– Yes – No
16	What reasons do you have to get a tattoo? Several answers are possible		– Cosmetic (masking skin defects, improvement of a previous image, covering up my old tattoo) – Esthetic (I see my tattoo as a modern adorning element) – Personal (I want to celebrate my romantic relationship or remember someone or some important event) – Social (I belong to a specific social group or subculture) – Ethnicity-related or religious (I'm a member of some ethnic or religious community)

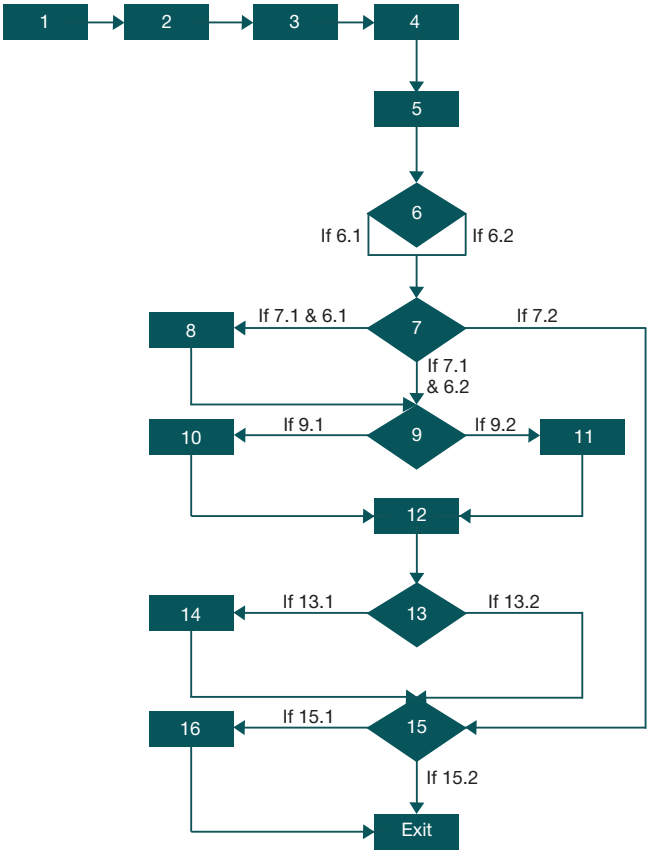

Fig. 3. Questionnaire flowchart.

half of tattoo studios' customers undertake an attempt to remove the tattoo within the first 10–12 years after receiving it [35]. Currently tattoos can be removed using various methods, such as dermabrasion, laser techniques, exposure to acids or liquid nitrogen, and surgical excision followed by suturing. However, a complete removal is not always possible, because there is still no ideal method or technology to extract the artificial pigment from deeper skin layers. Such methods are often painful, very expensive, lead to hypo- or hyperpigmentation of skin or scarring. It is necessary to emphasize that medical students or their teachers should be qualified enough to understand the issues of tattooing or tattoo removal, and to widely promote their knowledge.

CONCLUSIONS

The results of this study demonstrate that in general, the interest in skin tattooing among medical students is quite low. However, we should also acknowledge that although they will provide medical services in the future, their awareness of tattoo-related issues is also low.

We believe it sensible to introduce optional interdisciplinary (fundamental and clinical) courses into the curriculum starting from the first year of education that will engage experts from the esthetic medicine industry. Immersed into this kind of practical training, students will be able to form a holistic system of universal knowledge, to exploit "knowledge asymmetry" unlike students from other higher education institutions, and to adapt successfully to the society, considering real changes and needs of the medical service market.

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