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Multiple drug resistance of nosocomial bacterial strains provoked by the unwise and uncontrolled use of antibiotics in medicine and agriculture seriously threatens modern healthcare: minor and trivial infections are about to kill again. A solution may lie in the development of new alternatives to antibiotics. This review highlights the most interesting approaches to the development of antibacterial drugs focusing on the most promising ones, such as phage therapy and phage lytic enzymes (lysins).

Keywords: antibiotic, bacterophage, phage lysin, phage therapy, antibiotic treatment, multiple drug resistance

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АЛЬТЕРНАТИВЫ АНТИБИОТИКАМ: ЛИТИЧЕСКИЕ ФЕРМЕНТЫ БАКТЕРИОФАГОВ И ФАГОВАЯ ТЕРАПИЯ

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Множественная лекарственная устойчивость госпитальных штаммов бактерий, возникшая в результате неразумного неконтролируемого применения антибиотиков в медицине и сельском хозяйстве, представляет одну из серьезнейших угроз для современного здравохранения: привычные инфекции вновь могут убивать, как раньше. Решение проблемы может быть найдено в процессе разработки альтернатив антибиотикам. В обзоре приведены краткие сведения о наиболее интересных подходах к созданию новых антибактериальных средств и подробно проанализированы наиболее преспективные из них, такие как фаговая терапия и литические ферменты бактериофагов (фаголизины).

Ключевые слова: антибиотик, бактериофаг, фаголизин, фаговая терапия, антибиотикотерапия, множественная лекарственная устойчивость

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The antibiotic era: from victory to defeat

Back in the early 19th century an opinion prevailed that disease is caused by imbalances in the body. It was only in the mid-century that Louis Pasteur linked infections to their causative agents, germs. The day in 1928 when penicillin was discovered by the British bacteriologist Alexander Fleming became a milestone in the history of medicine, marking the advent of the antibiotic era. Antibiotics were initially seen as a universal remedy, a super weapon capable of turning the tide in the war against infectious diseases. But the euphoria did not last long. As our knowledge accumulated, more new antibiotics were discovered with various mechanisms of action, different properties and spectra of activity; but bacteria struck back by developing resistance to those drugs, frustrating the efforts of researchers and doctors [1]. So hopes were pinned on last-resort antibiotics, such as colistin and daptomycin.

In May 2015 the World Health Organization (WHO) admitted that bacterial resistance to antibiotics was the underlying cause of the ongoing healthcare crisis and proposed the Global Action Plan on Antimicrobial Resistance [2]. However, it was only a few months after that a mobile colistin-resistance gene was discovered in November 2015 [3]. A similar report followed in October 2016 describing a daptomycin-resistant *Staphilococcus aureus* strain capable of inactivating the antibiotic by releasing membrane phospholipids into the surrounding environment once they have bound daptomycin [4]. In September 2016, a month before that report, an

American patient died of sepsis caused by the gram-negative "superbacteria" *Klebsiella pneumoniae* resistant to all 26 antibiotics approved in the US [5]. The analysis of all known antibiotics and their therapeutic combinations published by WHO in 2017 [6] uncovered a sad truth: there is currently no cure for infections caused by gram-negative multidrug-resistant bacteria. Last-resort antibiotics, the defense we thought reliable, no longer work, and trivial infections can kill once again. This urges us to develop novel antibiotics or start searching for an alternative that will be just as effective.

Sources for new antibiotics

Antibiotics are natural, synthetic or semisynthetic chemicals whose small concentrations are capable of inhibiting microbial growth. The primary source of clinically important antibiotics is actinomycetes and sometimes non-mycelial bacteria, so these microorganisms may still have something new to offer. Another way to discover a novel antibiotic is a screening for a candidate chemical structure and predicting its orientation and position in the active site of a target protein.

That said, the search for novel antibiotics has almost stopped, and for a few understandable reasons. First, most antibiotics target one of three key prokaryotic metabolic pathways and processes, including protein biosynthesis, DNA replication and bacterial cell wall synthesis [7]. The majority of possible approaches to these targets have already been proposed and scrutinized. Besides, there is always a risk of spontaneous mutations in bacteria that can ruin years of efforts spent in a scientific lab and incur additional costs. Second, to discover a single antibiotic, one has to analyze about a million new actinomycetes [8], which is a very costly procedure.

Over 90 % of all bacterial species found in the environment cannot be cultured in a lab under standard conditions [9]. However, recently it has become possible to create specific conditions favorable for such bacteria in order to identify producers of yet unknown antibiotics among those species. This gave rise to two conceptually different search strategies: screening for a source of new antibiotics among unculturable bacteria and production of novel synthetic antibiotics. Both strategies are starting to pay off. For example, screening of uncultured soil bacteria has revealed a previously unknown antibiotic (teixobactin) that kills gram-positive bacteria [10]. Another antibiotic PEG-2S that inhibits Na^+ translocating NADH:ubiquinone oxidoreductase has been synthesized in the lab [11].

Of particular interest are synthetic antibiotics targeting bacterial bioenergetics [12]. In 2012, the Food and Drug Administration (USFDA) approved bedaquiline synthesized after 4 decades of research for the treatment of tuberculosis. This drug targets mycobacterial ATP synthase suppressing bacterial bioenergetics, which is causing death for the affected cells [13].

Based on the symbiotic theory a supposition was made that antioxidants that induce collapse of mitochondrial membrane potential can be effective against bacteria. Lately it has been found that the antioxidant SkQ1, a synthetic triphenyl phosphonium-based compound, can kill bacteria by decreasing their membrane potential [14, 15].

Alternatives to antibiotics

In spite of the astonishing variety of approaches to the discovery of effective alternatives to antibiotics and more than ten years of painstaking research, there have been no serious breakthroughs in the therapy of infections. Few candidate substitutes seem to work.

Among the most interesting alternatives to antibiotics are vaccines, antibodies, probiotics, immunostimulants, photosensitizers, natural bacteriophages, phage lytic enzymes, synthetic bacteriophages, antimicrobial peptides, host-defense peptides, antibiofilm compounds, multidrug efflux pump inhibitors, immunosuppressants, liposome entrapment of toxins, metal chelators, antibacterial nucleic acids, anti-resistance nucleic acids, and antibacterial peptides. This list is not exhaustive, though.

Based on the evaluation of clinical potential and relative simplicity of use, a conclusion can be drawn that phage lysins and multidrug efflux pump inhibitors are the most promising therapeutic alternatives to antibiotics, while vaccines and antibodies seem to have a good potential as prevention tools, and probiotics can be used for both treatment and disease prevention. Bacteriophages, antibiofilm compounds, antimicrobial peptides and photosensitizers also hold promise; however, it is not clear yet whether they can enter the market as finished pharmaceutical products to replace existing antibiotics. There are also doubts about immunostimulants that are sometimes used for disease prevention or as complementary drugs: their clinical significance is yet to be elucidated; therefore, they do not seem to be an adequate substitute for antibiotics.

Many of the approaches listed above are either undergoing experimental trials or exist as theories. More information about them can be found in the review [16] ordered by the Wellcome Trust foundation (UK) and prepared by the researchers from academic circles and the pharmaceutical industry. In this article we focus on the most promising and interesting alternatives to antibiotics, including vaccines, antibodies, multidrug efflux pump inhibitors, photosensitizers, bacteriophages and phage lysins.

Vaccines

Vaccines are a well-established and effective method of disease prevention. In 2015 the novel multicomponent vaccine Bexsero (GlaxoSmithKline Biologicals, UK) against *Neisseria meningitidis* was introduced into the national infant immunization program in the UK. *Neisseria meningitidis* is the causative agent of pediatric meningitis and bacteriaemia. Because its capsular polysaccharide MenB, which is a virulence factor, resembles human cell adhesion molecules, effective induction of antibodies against meningococcal infection is a difficult task. A group of British researchers managed to solve it by conducting a bioinformatic search for a candidate antigen. The antigen contained in the outer membrane vesicles was later shown to elicit sustained immune response upon vaccination. The approach used by the British researchers is referred to as reverse vaccinology. Bexsero is effective against 73 % to 88 % of group B meningococcal strains (MenB) [17]. That said, it is still very unlikely that antibiotics will be fully replaced by such vaccines in the nearest future.

Antibodies

A wide range of unique properties makes antibodies a keystone of contemporary medicine. Pathogen-specific monoclonal antibodies are either used independently for disease prevention or in combination with antibiotics to treat bacterial infections [18]. Antibodies neutralize the effects of bacterial toxins [19–22]; they can also be directed against bacterial antigens [23–25] and quorum-sensing signals [26, 27]. Together, traditional antibiotics and antibodies open new possibilities for

inhibiting biofilm formation enhancing the effect of antibiotic–based treatment and can be employed for combating persister cells insensitive to antibiotics [28, 29].

Multidrug efflux pump inhibitors

Although these compounds were known before, they are now receiving closer attention [30, 31] owing to the peculiar interactions between the substrates of multidrug efflux pumps and their inhibitors [15, 32]. Efflux pump inhibitors are a conceptually new approach to fighting bacteria not mentioned in the review [16], helping to reduce therapeutic concentrations of antibiotics administered to a patient by an order of magnitude or two. This phenomenon was first demonstrated using a novel antibiotic SkQ1, with pump inactivation leading to a 50-fold reduction in its minimum inhibitory concentrations. The bad news is that normally several pumps are involved in pumping antibiotics out, and the inhibitors often affect a multitude of different pumps simultaneously. Importantly, SkQ1 is recognized only by the main multidrug efflux pump of *Escherichia coli*, AcrAB-TolC [15]. Indeed, the use of efflux pump inhibitors provides a solution to the problem of antibiotic resistance.

Photosensitizers

Antimicrobial photodynamic therapy or inactivation (aPDT or aPDI) is new effective method of killing gram-negative and gram-positive bacteria and yeast [33–36]. aPDI employs a non-thermal reaction induced by the interactions between visible light photons and a photosensitizer, such as methylene blue, chlorin, porphyrin, chlorophyll or their derivatives, in the presence of oxygen. Reactive oxygen species produced as shown in Fig. 1 effectively kill bacterial cells, including *Pseudomonas aeruginosa*. This therapy is in particular demand in dentistry and dermatology [36] where it can replace local antibiotic treatment.

Phage therapy

Bacterial viruses or bacteriophages constitute the largest group of viruses with double-stranded genomic DNA, although there are phages with single stranded DNA and single/double-stranded RNA [37]. In total, the number of phages is estimated to be as high as 10^{31}–10^{32} [38]. They play an important role in the regulation of the world's bacterial population. Phages kill 20–40 % of sea bacteria per day [39]. The use of phages as therapeutic agents was proposed and successfully tested by Twort [40], D'Herelle [41], Bruynoghe and Maisin [42] in the early 20th century. However, phages did not become a popular treatment option at that time largely because antibiotics kept gaining ground and there was a lack of theoretical knowledge to explain phages' failure in clinical trials [43].

According to the contemporary views, phages used for treating bacterial infections must meet a few requirements: 1) they have to be lytic; 2) their therapeutic concentrations must be calculated for each particular infection; 3) a phage receptor involved must be well studied; 4) the final formulation must be free of bacteria and 5) contain viable bacteriophage particles [44–46]. The positive effect of phage therapy, i.e. reduction in the pathogen count down to the level at which the organism can handle the infection on its own [47], varies between individuals depending at the same time on a few other factors, which prevents phages from entering American and European markets [46].

Both bacteriophages and antibiotics directly attack bacteria; therefore, their effectiveness can be compared. Advantageously, bacteriophages are more specific for certain bacterial strains, such as *Clostridium difficile* that causes intestinal infections or diarrhea following antibiotic treatment [48]. Also, phage therapy is more sparing than antibiotic-based therapies [49, 50]. However, if infection is caused by multiple different bacteria, as is the case with wounds, bacteriophages are far less effective than antibiotics [51].

In the experiment [52] *E. coli* were infected with bacteriophages collected in two geographically distant regions: Mexica and Bangladesh. The tested phages turned to be highly specific for bacteria from their home region [52]. However, no significant differences were observed in the outcome of two different treatments (with a Russian bacteriophage cocktail and a placebo) applied to the cohort of 160 Bangladeshi children with *E. coli*-associated diarrhea [53]. Bacteriophages directed against antibiotic-resistant bacterial strains are easier to find in those regions where these bacteria are indigenous [54], which

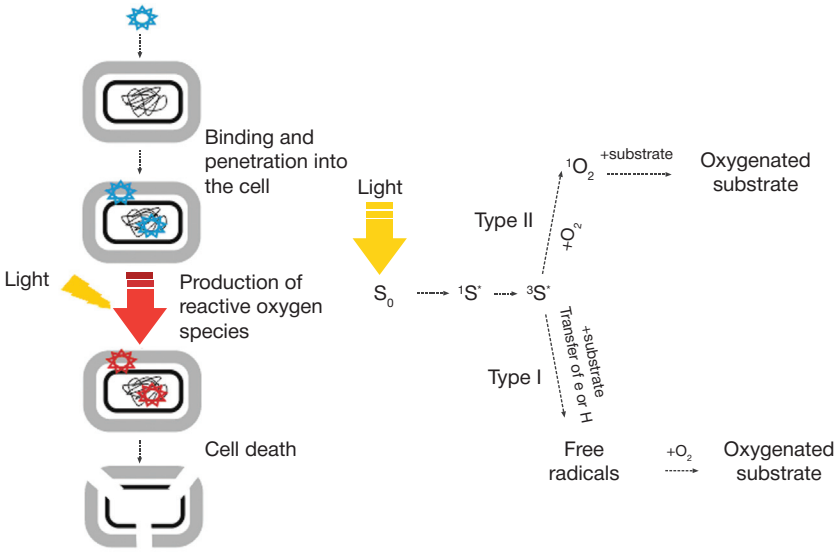

Fig. 1. Schematic illustration of antibacterial photodynamic therapy (left). Photodynamic effects explained on the molecular level (right). Upon absorbing a photon, a photosensitizer molecule (S_0) enters a short-lived excited singlet state ($^1S^*$), moving then to a triplet state ($^3S^*$). After that, two scenarios are possible: either $^3S^*$ reacts with the substrate and intermittent radicals are produced that damage cell structures and macromolecules inside the cell (type I), or energy from $^3S^*$ is transferred to oxygen, and reactive singlet oxygen $^1O_2$ is produced that also damages cell structures or its macromolecules

provides a new insight into the problem of antibiotic resistance and the use of phage therapy.

An idea to use bacteriophages for treating infections caused by biofilm-forming bacteria seems quite attractive. Antibiotics are not the best option here: they inhibit biofilm formation only when administered in high toxic doses [55]. Experiments *in vitro* have demonstrated that bacteriophages prevent formation of biofilms and even destroy them, especially in the case of *Listeria monocytogenes*, *P. aeruginosa* and *Staphylococcus epidermidis* [56, 57].

To predict possible complications or adverse effects of phage therapy, one should be aware of all nuances of phage biology and genome since many phages contain genes coding for virulence factors or toxins [58] or conferring resistance to antibiotics [59–62]. Phage therapy is difficult and requires more caution than antibiotic treatment because of the phenomena of phage-associated botulinum toxins [59], diphtheria toxins [58], cholera toxins [63], and phage-triggered conversion of non-toxic bacteria into toxic [64]. It is believed that life cycles of bacteriophages (Fig. 2) can vary from lytic to lysogenic, including pseudo lysogenic and defective [65, 66]. The genomic analysis of the gigantic bacteriophage *P. aeruginosa* [67–69] has demonstrated that its gene products very much resemble proteins with yet uncharacterized function produced by other organisms.

To sum up, complicated life cycles, a risk of conversion and resistance gene transfer and the variability of phages, as well as mutations in the bacterial population, render phage therapy somewhat unreliable and unpopular, in spite of its good clinical potential.

Phage lytic enzymes (phage lysins)

A bacterial cell envelope is a barrier in the way of bacteriophage DNA that enters the cell in order to infect it or is released back into the surrounding environment as a viral particle. The cell envelope is a complex organized system of lipid and peptidoglycan layers protecting the cell from the invasion of foreign agents. The cell envelope of gram-negative bacteria has three components, including the inner plasma membrane, peptidoglycans and the outer membrane [70, 71], while the cell envelope of gram-positive microorganisms consists of only two components and misses the outer membrane.

The hardest to penetrate is the peptidoglycan layer built from alternating residues of N-acetylglucosamine and N-acetylmuramic acid linked by β-1,4-glycosidic bonds and short peptide chains. Linked together, peptidoglycan blocks (referred to as murein) form a gigantic macromolecule that ensures mechanical stability of the layer and its impermeability to viruses and toxic factors with big molecular weight [37, 70, 71]. Therefore, to get inside the cell, the phage has to locally disrupt the integrity of cell membranes and the peptidoglycan layer. This is done by lytic enzymes called bacteriophage lysins (phage lysins, endolysins or virolysins).

Importantly, the peptidoglycan layer of an infected cell is lysed in a two-step process [72] which includes “lysis from without” followed by phage DNA passage into the cell and “lysis from within” facilitating release of new phage particles into the environment. Normally, the first type of lysis is performed by a capsid-associated phage lysin, such as the gp5 baseplate protein produced by the T4 bacteriophage; this lysin has a functional domain with lytic properties [73, 74]. “Lysis from without” is limited to a particular site on the membrane and lasts just enough time for phage DNA to enter the cell and not be killed by the phage. “Lysis from within” is usually performed by a soluble phage lysin and is not site-restricted but very limited in time. It follows the massive synthesis of phage lysins in the cell and is activated in parallel with holin integration into the cell membrane. Holin is a regulatory protein that forms nonselective pores in the bacterial membrane. These pores disrupt bacterial metabolism and make the peptidoglycan layer vulnerable to phage lysins, which results in the total lysis of the layer, cell damage and release of phage particles into the environment [75–77].

Soluble phage lysins triggering massive “lysis from within” are interesting candidate alternatives to antibiotics: they can effectively lyse peptidoglycan and, unlike phages, do not depend on holin presence.

There are a few classifications of phage lysins [78–85], including the one based on the mechanism of their action: 1) lysozymes, which hydrolyze β-1,4-bonds between the residues of N-acetylmuramic acid and N-acetylglucosamine in the peptidoglycan molecule; they are subdivided into muramidases and lytic transglycosylases; 2) N-acetylmuramoyl-L-alanine amidases, which hydrolyze the amide bond between N-acetylmuramic acid and L-alanine; 3) peptidases, which hydrolyze the peptide bond between peptidoglycan amino acids, and 4) esterases (Fig. 3).

A study of autolytic enzymes of *S. pneumoniae* revealed that phage lysins have a modular structure, with one domain recognizing the site of lysis and the other cleaving peptidoglycan [86]. The modular structure appeared to be typical for both previously discovered and new phage lysins [87–89], which was later confirmed by genetic engineering experiments involving rearrangement and combination of functional domains [90].

Of course, not all phage lysins have equal potential to become therapeutic agents but some of their properties, such as the ability of PlySs2 to retain its function after 10 hours of freezing and thawing cycles [91] and the ability of PlyG to attack endospores of *Bacillus anthracis* [92], make phage lysins an interesting object for research. They are also species-specific, i. e. they kill only those bacterial (sub)species that are targeted by their phages [93, 94].

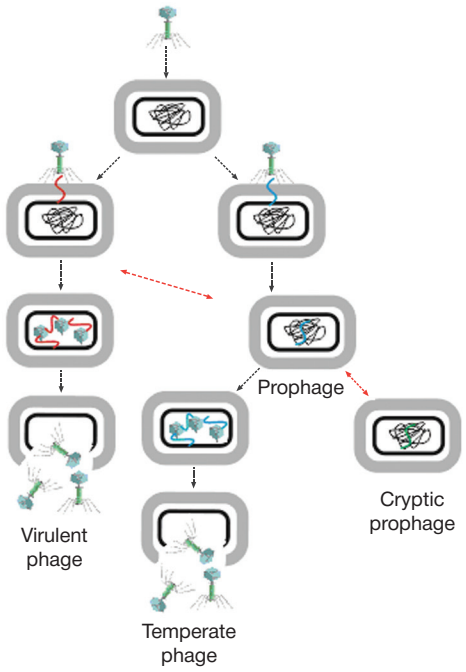

Fig. 2. Possible scenarios of phage infection in the bacterial population. Reversible stages are shown in red (mutations, premature sequence termination in prophages, deletions, insertions, etc.)

Considering the modular structure of natural phage lysins, through simple genetic modifications researchers managed to design chimeric phage lysins for treating complex bacterial infections caused by methicillin-resistant *S. aureus* (MRSA) [95]. Also, combined with low molecular weight antibiotics, such as penicillin or gentamycin, phage lysin Cpl-1 can totally eliminate penicillin–resistant pneumococci [96].

However, the absence of phage machinery facilitating passage through the bacterial cell wall restricts the use of phage lysins. The outer membrane of gram-negative bacteria reduces the therapeutic effect of phage lysins almost to nothing [94]. However, they do work in gram-positive bacteria. In addition, there is a risk of antibody production in response to phage lysins, which may interfere with phage lysin "mission". Both *in vitro* and *in vivo* experiments demonstrate that antibodies impede but not totally block lysis of bacterial cells by phage lysins [97], which may be explained by the fact that the affinity of the latter to their substrates is possibly higher than the affinity of a generated antibody to the enzyme.

CONCLUSION

Although there are a lot of promising alternatives to antibiotics, none of them seems to be a perfect substitute for antimicrobial drugs. All of them are not so safe, predictable, controllable, easy to use or effective. Still, it is obvious that development of alternative approaches to treating bacterial infections is a vital necessity for today's healthcare. Combination therapies will probably win over others due to the synergistic effect of their components: antibiotics and their alternatives working together.

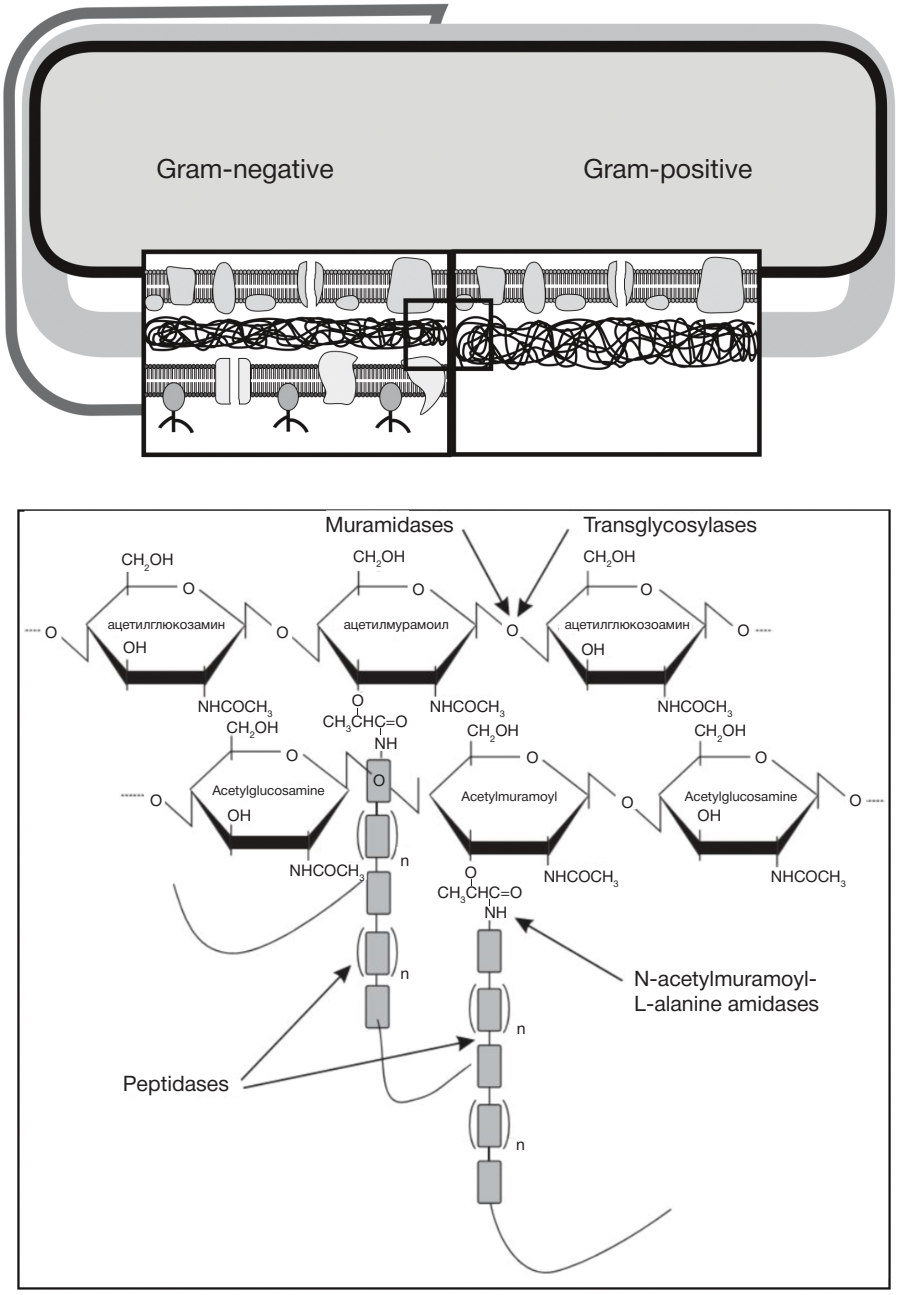

Fig. 3. Schematic of bacterial cell envelopes and phage lysin classes that degrade the peptidoglycan layer

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TRIPHENYL PHOSPHONIUM-BASED SUBSTANCES ARE ALTERNATIVES TO COMMON ANTIBIOTICS

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There is an urgent need for new antimicrobial and therapeutic strategies to deal with the ever evolving antimicrobial resistance among the most prevalent bacterial pathogens. Infections due to virulent bacteria remain significant causes of morbidity and mortality despite progress in antimicrobial therapy, primarily because of the increasing of antimicrobial resistance levels among such group of bacteria. Despite significant advances in the understanding of the pathogenesis of infection due to these organisms, there are only limited strategies to prevent infection. Recently it was reported that SkQ1, triphenyl phosphonium-based mitochondria-targeted antioxidant and antibiotic, effectively kills all tested Gram-positive laboratory strains including of *Bacillus subtilis*, *Staphylococcus aureus* and *Mycobacterium sp*. Moreover, SkQ1 demonstrated effectiveness towards Gram-negative strains too, except *Escherichia coli*. The mechanism of the bactericidal action of TPP-based antibiotics could be also described by its ability to suppress bacterial bioenergetics by collapsing membrane potential through activation of protonophorous uncoupling. To this date, there are no reports of resistance to SkQ1 among Gram-positive strains; therefore, triphenyl phosphonium-based antibacterial agents would be effective towards planktonic and sessile cells of clinical resistant strains.

Keywords: triphenyl phosphonium, protonophore, bacteria, antibiotic resistance, clinical strains, membrane potential, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pneumoniae*

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ПРОИЗВОДНЫЕ ТРИФЕНИЛФОСФОНИЯ КАК АЛЬТЕРНАТИВА ОБЫЧНЫМ АНТИБИОТИКАМ

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В связи с тем, что наиболее распространенные патогены постоянно эволюционируют, приобретая устойчивость ко все большему числу антибиотиков, в настоящее время существует острая потребность в новых антимикробных препаратах и стратегиях лечения инфекций, вызываемых антибиотикорезистентными бактериями. Хотя в понимании патогенеза подобных инфекций удалось значительно продвинуться, стратегий для борьбы с ними не так много. Недавно было показано, что SkQ1, антиоксидант и антибиотик на основе трифенилфосфония, воздействующий на митохондрии, эффективен в отношении грамположительных лабораторных штаммов, включая *Bacillus subtilis*, *Staphylococcus aureus* и *Mycobacterium sp*. Более того, SkQ1 также показал эффективность в отношении грамотрицательных штаммов за исключением *Escherichia coli*. Механизм бактерицидного действия трифенилфосфониевых соединений можно объяснить их способностью вызывать нарушения энергетического обмена у бактерий за счет резкого снижения мембранного потенциала путем стимуляции протонофорного разобщения. На текущий момент случаи устойчивости к SkQ1 среди грамположительных бактерий не зафиксированы, поэтому антимикробные препараты на основе трифенилфосфония могут быть эффективно использованы для борьбы с планктонными и сессильными клиническими штаммами, нечувствительными к обычным антибиотикам.

Ключевые слова: трифенилфосфоний, протонофор, бактерии, устойчивость к антибиотикам, клинические штаммы, потенциал мембраны, *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pneumoniae*

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Antimicrobial resistance threatens the very core of modern medicine. Systematic misuse and overuse of antibiotics in human medicine and food production have put every nation at risk. Without significant effort and immediate global action, the world is heading towards post-antibiotic era in which common and trivial infections could once again kill [1].

The resistance to antimicrobial agents is happening around the world, and leading to a significant decrease of therapeutic options for bacterial diseases. Antimicrobial resistance affects all areas of health, and direct consequences of infection with resistant microorganisms can be severe.

In 2017, the World Health Organization issued a global priority pathogen list [2] to guide efforts to find new antibiotics against the current public health bacterial threats. Gram-positive bacterial pathogens, such as *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecium*, are responsible for community-acquired and hospital-associated infections and are an increasing public health threat. Despite causing infections, such bacterial species are also normal inhabitants of human and animal microbiota. To establish infection, these pathogens possess a wide array of virulence factors, which are responsible for successful colonization in human hosts as well as evasion of the immune system. One of the virulence aspects that contributes to the successful infection by these bacteria is their ability to form biofilm, both on biotic (bones and heart valves) and abiotic surfaces (catheters, prostheses and other medical devices) [3–5]. Biofilms can be defined as a sessile microbial community in which cells are attached to a surface and/or other cells and incorporated into the protective extracellular polymer matrix. The process of biofilm formation induces many phenotypic alterations, including loss of motility, reduced growth rate, increased surface adhesion and higher tolerance to antibiotics and host immune response [6]. Thus, biofilm can be associated with chronic and recurrent infections caused by these bacterial species. Due to ability to produce biofilm and high antimicrobial resistance rates found in certain variants of *S. pneumoniae*, *S. aureus* and *E. faecium* strains, therapeutic options are limited. Therefore, the search for new therapeutic approaches is necessary.

Staphylococcus aureus

S. aureus is an important human pathogen and is a leading cause of soft tissue, blood-borne and device related infections in adults and children. Currently, *S. aureus* is the most common bacterial species isolated in intensive care units in the United States [7]. In addition, *S. aureus* is the second most common bacterial pathogen that causes infections in outpatients [8]. At the same time, it can also be found colonizing asymptomatically the skin and nares of approximately 20 % of the population [9]. Risk factors for infection include disruption of mucosal or cutaneous surfaces, introduction of a foreign body or medical device, surgery, hemodialysis or host immunosuppression. Antimicrobial resistance, especially methicillin-resistant *S. aureus* (MRSA), is an important public health problem among *S. aureus* clinical isolates, complicating treatment and prevention of staphylococcal infections. Since its first report in the 1960's, the incidence of nosocomial infections caused by MRSA has been increasing steadily. MRSA can be responsible for nearly 50 % of all reported *S. aureus* hospital-associated infections, being highlighted in surgical site infections and catheter-associated urinary tract infections. Resistance to methicillin, and other beta lactams, in MRSA strains results from the acquisition of the *mecA* gene cassette, which modifies the penicillin binding proteins in the cell wall [10]. More recently, the emergence of vancomycin-intermediate (VISA) and vancomycin-resistant *S. aureus* strains (VRSA) has further increased the concerns regarding antimicrobial-resistant *S. aureus* infections. The proportion of MRSA strains with reduced susceptibility to vancomycin nearly doubled between 2004 and 2009 [11].

Enterococcus sp.

Enterococci have become important nosocomial pathogens in the last decades, being the third most common opportunistic pathogen in the hospital environment and causing more than 12 % of all healthcare-associated infections [12]. In addition, *Enterococcus* are normal inhabitants of the intestinal microbiota of humans and animals. *Enterococcus faecalis* and *Enterococcus faecium* account for the majority of enterococcal strains recovered from colonization and infections in humans. Enterococcal isolates are frequently associated with resistance to multiple antimicrobials. The occurrence of vancomycin-resistant enterococci (VRE) has peculiar importance due to the high ability for dissemination and association with therapeutic challenges [13]. Resistance to vancomycin is more common among *E. faecium* strains, and is mediated through the acquisition of a group of genes collectively known as the *van* gene complex. These genes are harbored by a transposon and encode an alteration in the cell wall that leads to reduced affinity for vancomycin. VRE isolates can also be highlighted as reservoirs of antimicrobial resistance genes, which can be transferred to other bacterial species, such as *S. aureus*. VRSA strains have acquired the van genotype from enterococcal isolates [14]. Approximately 8 % of colonized patients develop a VRE infection either during or shortly after hospital admission [15], and the associated mortality for these infections can be high (13–46 %) [16]. VRE strains are usually associated with intra-abdominal, skin and soft tissue, urinary tract, bloodstream infections and endocarditis. VRE transmission often occurs via healthcare workers and once acquired may be life-long. Approximately one-third of the reported enterococcal infections were due to vancomycin-resistant strains. Therefore, we cannot rely on the availability of effective antimicrobial agents for treatment of VRE infections and highlight the importance of preventing transmission of these microorganisms.

Streptococcus pneumoniae

S. pneumoniae, also known as pneumococcus, is a significant human pathogen, being the leading cause of community-acquired pneumonia and one of the major agents of bacterial meningitis. This microorganism can be part of the nasopharynx microbiota of healthy individuals, but asymptomatic colonization can evolve to disease once pneumococcus is capable to migrate from the nasopharynx to sterile sites such as the brain, lungs and bloodstream, especially among immunocompromised individuals such as the elderly and young children. Penicillin nonsusceptible pneumococci (PNSP) are currently listed among the most important antimicrobial resistant threats worldwide [2, 17]. Increasing and alarming numbers of PNSP isolates have been detected since the first report in the 1960's. These isolates are usually resistant to different beta-lactam antibiotics, and appear as consequence of the genetic modification of penicillin-binding protein (PBP) genes, leading to the production of PBPs with reduced affinity to beta-lactams. In addition to resistance to beta-lactams, pneumococcal isolates resistant to macrolides have emerged worldwide, representing up to 40 % of pneumococcal isolates

recovered in Europe [18] and more than 70 % of the strains from Asian countries. Resistance to macrolides among pneumococci is mainly attributed to ribosomal target site alteration, but can also be due to alteration in antibiotic transport and modification of the antibiotic [19]. Antimicrobial resistance in *S. pneumoniae* has been modulated by the widespread use of antibiotics and also by the introduction of pneumococcal conjugate vaccines (PCV).

Bacterial energetics is a target for new antimicrobials

The keystone of the bacterial bioenergetics is cellular membrane, which acts as a barrier to allow the energy of electrochemical gradients to transform into pure chemical energy, in accordance with cellular demands. All bacteria require an electrochemical gradient of a proton motive force (PMF) to be consumed by for a variety of processes, such as the synthesis of ATP and active transport of nutrients from the environment for their growth and metabolic activity. Moreover, the generation of the PMF is evolutionarily conserved and existed in the last universal common ancestor (LUCA) [20, 21].

Some chemical compounds, such as gramicidin, can disrupt membranes by forming physical pores, therefore decreasing electrochemical gradients. Other chemical compounds, named protonophores, can decrease electrochemical gradients by specifically binding to protons, characterizing the protonophoric cycling on membranes (Figure, left panel). Still, there is an additional group of chemical compounds able to decrease electrochemical gradients, named protonophore-like compounds (protonophorous uncouplers). Compared to protonophores, protonophorous uncouplers carry another proton-binder across membranes (Figure, right panel), such as fatty acids [22].

Therefore, targeting bacterial bioenergetics by new antimicrobial agents brings new effective possibilities to subvert bacterial infections, especially those caused by antimicrobial resistant variants, by initiating an idle running of bacterial bioenergetics, which is presumably without the threat of the acquisition of resistance.

Triphenyl phosphonium-based substances

Quaternary ammonium and phosphonium compounds have been used as antiseptics and disinfectants for many decades [23–28]. Recently developed mitochondria-targeted antioxidants, a wide range of compounds having an antioxidant group linked to a mitochondria-targeted moiety, are exemplified by triphenyl phosphonium-conjugated chemical groups, such as ubiquinone (MitoQ) [29], plastoquinone (SkQ1) [30]. Such compounds have been reported as effective in killing reference strains of *Bacillus subtilis* [31, 32]. Moreover, SkQ1 demonstrated effectiveness also against certain Gram-negative bacterial species, except *Escherichia coli*, and different Gram-positive species, including *S. aureus*, and *Mycobacterium sp*.

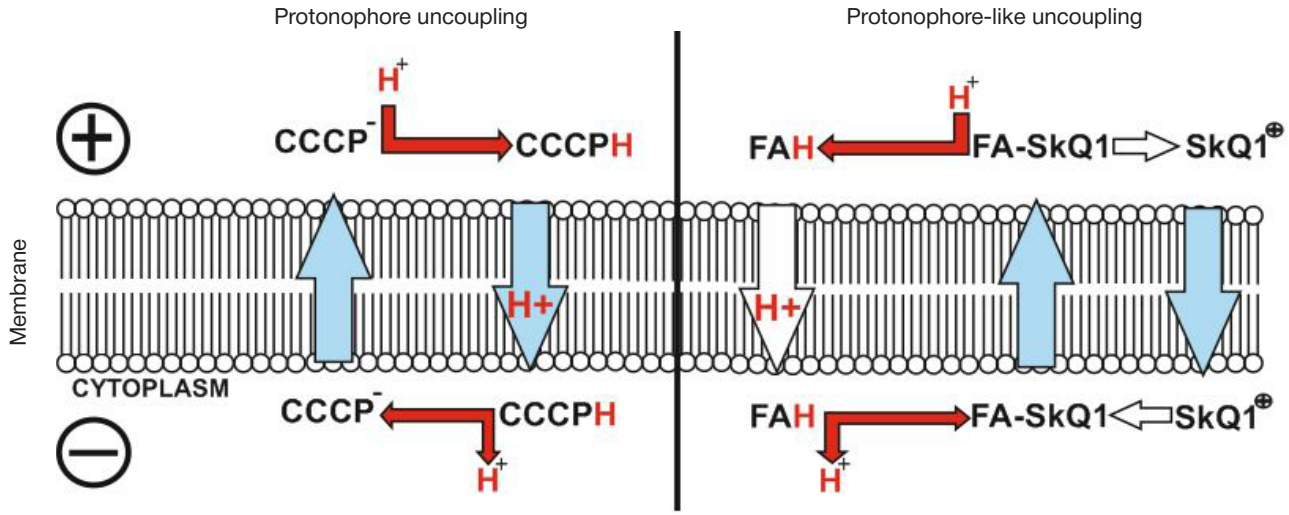
The mechanism of the bactericidal action of triphenyl phosphonium-based antibiotic SkQ1 could be also ascribed to its ability to suppress bacterial bioenergetics by collapsing membrane potential through activation of protonophorous uncoupling. Using *in silico* screening it has been found that antiinfective compounds for treating some parasitic infections are uncouplers for bacteria bioenergetics [33]. Moreover, it was found recently that widely used broad-spectrum biocide triclosan induced collapse of membrane potential in bacterial cells together with well-known inhibition of enoyl-acyl carrier protein reductase, one of the key enzymes in bacterial fatty acid synthesis [34].

SkQ1 contains not only a strong antimicrobial moiety but also an antioxidant moiety targeting mitochondrial reactive oxygen species; therefore, SkQ1 can be considered as a new type of dual-acting “hybrid” antibiotic. The absence of cytotoxicity of 1–2 μM SkQ1 for human and animal cells means that SkQ1 might be safely used to treat many bacterial infections by killing invading bacteria on the one hand, and curing damaged host cells on the other hand.

Such compounds also present potential to be used in bacterial infections where biofilms can be present. To this date, there are no reports of resistance to SkQ1 and alkyl-triphenyl phosphonium among Gram-positive bacteria.

CONCLUSIONS

Relying on the current level of knowledge of mechanisms of bacterial antibiotic resistance and bioenergetic processes in bacterial cells, it is possible to suggest that TPP-based compounds could be potential alternative antimicrobial agents for certain antibiotic-resistant pathogenic bacteria, representing a novel path through which antimicrobial resistance could be subverted and mortality rates associated with such infections could decrease.



Protonophoric cycling performed by protonophore uncoupler CCCP and protonophore-like triphenyl phosphonium-based coupler SkQ1 with help of fatty acids (FA)

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PHYSICAL AND CHEMICAL PROPERTIES OF RECOMBINANT KPP10 PHAGE LYSINS AND THEIR ANTIMICROBIAL ACTIVITY AGAINST *PSEUDOMONAS AERUGINOSA*

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One of the most promising approaches to combatting multiple drug resistance is based on the use of recombinant endolysins. In this work we study physical, chemical and lytic properties of phage KPP10, a recombinant endolysin effective against *Pseudomonas aeruginosa* at concentrations of 3 μg/ml. Fused with a fragment of positively charged SMAP-29 peptide into one reading frame, artilysin KPP10 is effective against laboratory strains and clinical isolates of *Pseudomonas aeruginosa* in the absence of permeabilizers. Our findings encourage preclinical trials of this artilysin in infectious models.

Keywords: nosocomial infections, antimicrobial resistance, bacteriophages, lysins of bacteriophages, phage KPP10, artilysin KPP10

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ФИЗИКО-ХИМИЧЕСКИЕ СВОЙСТВА И ПРОТИВОМИКРОБНАЯ АКТИВНОСТЬ РЕКОМБИНАНТНОГО ФАГОЛИЗИНА БАКТЕРИОФАГА KPP10, ДЕЙСТВУЮЩЕГО НА *PSEUDOMONAS AERUGINOSA*

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Одним из наиболее перспективных современных подходов для преодоления множественной лекарственной устойчивости можно назвать применение рекомбинантных эндолизинов. Нами получены данные о физико-химических и литических свойствах бактериолизина фага KPP10 — рекомбинантного эндолизина, который эффективно действовал на штаммы *Pseudomonas aeruginosa* в концентрации от 3 мкг/мл. Слитый в одной рамке трансляции с фрагментом положительно заряженного пептида SMAP-29, артилизин KPP10 действует на лабораторные штаммы и клинические изоляты *Pseudomonas aeruginosa* без использования пермеабилизаторов. Полученные результаты позволяют рассчитывать на успешное проведение доклинических испытаний эффективности артилизина AL-KPP10 с использованием инфекционных моделей.

Ключевые слова: внутрибольничные инфекции, антибиотикорезистентность, бактериофаги, эндолизины бактериофагов, фаг KPP10, артилизин AL-KPP10

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The alarming rate of antibiotic resistance is one of the major concerns of contemporary healthcare. The number of bacterial strains with multiple or extensive resistance keeps growing. Some of them do not respond to treatment with otherwise highly effective medications, including drugs of last resort; some become totally resistant “superbugs” causing persistent infections to any used antibiotic. Current approaches to the therapy of bacterial infections cannot handle the spreading variety of rapidly emerging mechanisms of antibiotic resistance. A temporary solution lies in the use of improved approaches to

standard therapies based on known antibiotics. However, in the long run we need novel antibacterial agents with a reduced resistance potential.

A possible alternative to antibiotics and synthetic antibacterial agents comes in the form of bacteriophages that have been long used to combat infections [1, 2]. Bacteriophages offer a few advantages, including the ability to auto-dose depending on a bacterial titer, very little impact on the normal microbiota, low resistance potential, compatibility with antibiotics, the ability to destroy biofilms, rapid discovery, and low cost; a therapeutic effect of a bacteriophage can be achieved with a single or low dose, and phages are relatively environmentally friendly [3]. Still there are downsides to their clinical application. A pure phage isolate is difficult to obtain. Its pharmacokinetic properties need to be carefully controlled and normalized throughout the entire storage period. Besides, to produce a target phage host strains/species closely related to the pathogen of interest often have to be cultured in the absence of effective standard protocols under strict safety requirements. Also, bacteriophages can carry genes of bacterial toxins and harbor certain resistance potential. Lastly, phages are highly immunogenic, meaning that they will have a weaker therapeutic effect if used repeatedly [3–5].

Phage lysins (endolysins) constitute a new class of antibacterial agents free of the abovementioned limitations. Phage lysins are enzymes encoded by bacteriophages that are capable of degrading the peptidoglycan layer of the bacterial cell wall. Over the course of their lifecycle, phages secret these enzymes to facilitate phage DNA passage into the bacterial cell and to aid release of new virions from the infected bacterium into the surrounding environment. The lytic activity of their catalytic domains allows phage lysins to cleave peptidoglycan bonds in the bacterial cell wall, which eventually leads to hypotonic lysis of the infected bacterium caused by the difference of the osmotic pressures inside and outside the cell [6].

Phage lysins have a few indisputable advantages over other antimicrobial agents. First, they are selective and attack only certain bacterial species while sparing the normal microbiota. Second, phage lysins ensure rapid lysis as they do not rely on slow metabolic reactions. Therefore, phage lysin-based therapies may not take as much time as standard antibacterial treatments. Third, the risk of developing resistance to phage lysins is low. Phage lysins target specific molecules crucial for the normal life cycle of the host, rendering emergence of a resistant isolate highly improbable as it would have to be accompanied by the massive rearrangement of the bacterial cell wall. Fourth, phage lysins can kill antibiotic-resistant bacterial strains providing a solution to one of the most pressing problems of contemporary healthcare. Fifth, due to their capacity to destabilize the peptidoglycan layer of the cell wall, phage lysins can both kill metabolically active or latently rested cells and access bacterial cells hidden by biofilms [7].

One of the most dangerous causative agents of nosocomial infections is *Pseudomonas aeruginosa*. It causes a variety of different infections which are hard to cure, because it rapidly develops resistance to different antibiotics and is capable of forming biofilms [8]. Discovery of new drugs against *Pseudomonas aeruginosa* is a matter of a nearly paramount importance.

Bacteriophage KPP10 has a broad spectrum of activity directed against *Pseudomonas aeruginosa* strains [9]. A study of physical and chemical properties and the spectrum of lytic activity of its lysin could yield interesting results and reveal its potential as a basis for a novel antibacterial drug against *Pseudomonas aeruginosa*. Below we describe a synthetic recombinant artilysin of the KPP10 bacteriophage, its physical and chemical properties and bactericidal activity.

METHODS

Synthesis of proteins AL-KPP10 and L-KPP10

Sequences coding for endolysin L-KPP10 and artilysin AL-KPP10 were cloned into the commercial vector pAL2-T (Evrogen, Russia) and checked for errors using Sanger sequencing. The L-KPP10-encoding gene inserted in the pAL2-T vector was cleaved by restriction nucleases at sites BamH — SacI and BamHI — PstI. The same procedure was performed on the pQE-30 expression vector. The obtained restriction fragments were separated by agarose gel electrophoresis, viewed under UV light and extracted from the gel using the Silica Bead DNA Extraction Kit (Thermo, USA). The restriction fragments of the vector and the inserts were then ligated together in the standard T4 ligase reaction buffer (Thermo, USA).

For recombinant protein production, two obtained expression vectors pQE-30 carrying genes AL-KPP10 and L-KPP10, respectively, were introduced into the competent *Escherichia coli* cells (strain M15) using the heat shock transformation protocol. Briefly, the competent cells were added to the ligase mixtures, incubated on ice for 25 min and then kept at 42 °C for 45 s, chilled on ice for 2 min and combined with plain LB. The cells were then grown at 37 °C for 1 hour, seeded on Petri dishes and left there overnight at 37 °C. Upon the overnight incubation the colonies were picked and grown in LB containing appropriate antibiotics.

Once the cultures reached OD_{600} = 0.6, they were ready for induction (3 hours in 1 mM IPTG) followed by purification in the Ni-NTA agarose column. Briefly, the induced cells were lysed in a buffer containing 6 M guanidine hydrochloride, 10 mM Tris and 100 mM $NaH_2PO_4 \bullet H_2O$ (pH 8.0) for 1 hour. The obtained lysate containing the protein of interest was run through a gravity flow column. The protein was eluted from the sorbent by lowering the pH of the gradient buffer (8 M urea, 10 mM Tris, 01.M $NaH_2PO_4 \bullet H_2O$, pH range from 8.0 to 5.5).

The obtained fractions of proteins AL-KPP10 and L-KPP10 were purified by affinity chromatography in the HisTrap HP column (GE Healthcare, UK). Before the protein solution was applied to the column, the column was equilibrated with a buffer consisting of 8 M urea, 10 mM Tris, 0.1 M $NaH_2PO_4 \bullet H_2O$, and 10 mM imidazole. The elution buffer contained 8 M urea, 10 mM Tris, 100 mM Na_2HPO_4 and 500 mM imidazole (pH 5.5). The obtained protein fractions were dialyzed from the buffer containing 5 mM HEPES and 1 mM DTT (pH 5.5) at 4 °C. Protein concentrations were measured spectrophotometrically on HITACHI U-2900 (Hitachi, Japan) in cuvettes with a 1 cm long optical path. The optical density of the solutions was measured at 280 nm wavelength. Protein concentrations were calculated given that the molar extinction coefficient $E^{0.1\%}_{280}$ nm computed in ProtParam [10] was 1.455.

Physical and chemical measurements

1. Circular dichroism spectroscopy

Circular dichroism spectra of the recombinant proteins obtained under denaturing conditions and dialyzed in 0.1 mM phosphate buffer were measured on the modified Jobin-Yvon Mark V dichrograph (Horiba, Japan) at room temperature in cuvettes with a 1mm long optical path at protein concentrations of about 200 μg/ml. The spectra were measured over the range of 190 to 260 nm.

2. Dynamic laser light scattering

The hydrodynamic size of the particles in the solution was measured on ZetaSizer Nano-ZS (Malvern Instruments LTD, USA) at 25 °C in polystyrene cuvettes with an optical path of 1 cm. The laser wavelength was 633 nm. The dialyzed protein samples studied at this stage of the experiment were obtained under denaturing conditions using the technique described above. Protein concentrations were measured spectrophotometrically in advance. The data were processed in Dispersion Technology Software, version 5.10 (Malvern, USA).

3. Fluorescence spectrometry

Tryptophan fluorescence emission from 40 μg/ml protein samples obtained under denaturing conditions and purified chromatographically was recorded by FluoroMax-3 (HORIBA Jobin Yvon GmbH, Horiba, USA) at room temperature in cuvettes with an optical path of 1 cm at the excitation wavelength of 280 nm, at which tryptophan absorbs actively. The spectra were measured over the range of 300 to 400 nm.

Investigating antibacterial properties of the lysins

1. Bactericidal activity of L-KPP10 and AL-KPP10

The samples of L-KPP10 and AL-KPP10 preparations were purified in the chromatography column and dialyzed in the buffer containing 5 mM HEPES and 1 mM DTT (pH 5.5). Protein concentrations were adjusted using the same buffer.

Two ml of the bacterial culture left to sit in LB overnight were diluted and grown until the cells reached the optical density OD_{600} of 0.6. The resulting culture (2 ml) was pelleted by centrifugation at 3000 g for 10 min; then the cells were resuspended in 2ml of 5 mM HEPES (pH 5.5); turbidity of the obtained suspension was 0.5 McFarland standards. The culture was then diluted 100-fold using the same buffer (the final cell density was 10^6 cells per ml). The following mixtures were prepared in a 96-well plate:

1) 100 μl bacterial suspension, 50 μl protein preparation at the required concentration and 50 μl EDTA (the final concentration was 0.5 mM);

2) 100 μl bacterial suspension and 100 μl protein preparation at the required concentration;

3) 100 μl bacterial suspension bacterial suspension and 100 μl buffer (the control mixture);

4) 100 μl bacterial suspension, 50 μl buffer and 50 μl EDTA (the control mixture).

Those mixtures were incubated at room temperature for 30 min and diluted tenfold in the phosphate buffer (pH 7.2); 100 μl of each dilution were applied on Petri dishes containing agarised LB. The colonies were picked after the overnight incubation at 37 °C.

In our study we used the PA103 strain of *Pseudomonas aeruginosa* and clinical bacterial isolates Ts 38-16, Ts 43-16, Ts 44-16, Ts 47-16, Ts 48-16 and Ts 49-16 from the collection of Gamaleya Federal Research Center for Epidemiology and Microbiology.

2. Electron microscopy

AL-KPP10 at 50 μg/ml concentrations was added to 10^6 CFU of the sensitive Ts 43-16 isolate combined with the HEPES buffer and incubated for 10, 20 or 30 min at room temperature.

Ten μl of the mixture were applied on a grid mesh, dried at room temperature, stained with 1% uranyl acetate solution by submerging the mesh in a drop of the stain for 30 seconds. The residual uranyl acetate was removed by washing the mesh in a drop of water for 30 seconds, blotted with filter paper and dried at room temperature. The obtained protein preparations were inspected under the electron microscope JEOL JEM-101 (FEI Phenom World, Netherlands).

RESULTS

Synthesis of the recombinant endolysin and artilysin and their physical and chemical properties

To obtain recombinant endolysin L-KPP10 and its modified variant artilysin AL-KPP10 with a fragment of the myeloid antimicrobial peptide SMAP-29 ([K2,7,13]-SMAP-29(1–17)) at its C-terminus, we used custom-synthesized gene sequences. The genes were inserted into the vector pQE-30 for further expression. Both expression vectors carrying AL-KPP10- and L-KPP10-encoding genes, respectively, were introduced into the competent *Esherichia coli* cells (strain M15) using a heat shock transformation protocol. Protein production was induced in the cultured cells by isopropyl β-D-1-thiogalactopyranoside (IPTG), the synthetic lactose analog. Upon induction, the cells were lysed under denaturing conditions. The resulting fractions of AL-KPP10 and L-KPP10 proteins His-tagged at the N-terminus were purified and refolded by dialysis using affinity chromatography.

In the next step we assessed physical and chemical properties of the obtained proteins. Structures of L-KPP10 and its modified variant AL-KPP10 were studied under denaturing conditions and after renaturation. Among the methods used were circular dichroism spectroscopy (to study the secondary structure of the proteins), fluorescence spectrometry (to measure tryptophan spectra in order to assess the presence of a tertiary structure) and dynamic light scattering (to study the ability of the proteins to aggregate or oligomerize).

Circular dichroism spectroscopy revealed that AL-KPP10 and L-KPP10 had a typical αβ-protein spectrum, with a small contribution of disorganized structures (Fig. 1A). The presence of the peptide component [K2,7,13]-SMAP-29(1–17) did not affect the secondary structure of AL-KPP10 (protein and peptide spectra almost entirely overlapped).

Tryptophan fluorescence measurements were necessary to assess whether the proteins could fold into a tertiary structure (Fig.1B). Unlike denatured proteins, renatured proteins demonstrated a shift of emission peaks to shorter wavelengths. This indicates that tryptophan located inside the protein core started to lose its contacts with the solvent as a result of tertiary structure recovery, suggesting that renatured AL-KPP10 and L-KPP10 could retain their enzymic activity.

Stability of protein solutions was assessed by dynamic laser light scattering. This method is used to measure the hydrodynamic size of a protein complex in a solution and assess the ability of the molecule to aggregate. Small (3 to 7 nm) sizes of the complex suggest that the protein is monomeric, implying its stability in a solution. We discovered that the majority of native proteins were aggregates of 60 nm in size, which is normal for recombinant proteins obtained through renaturation (Fig. 1C, 1D).

Antimicrobial activity of the recombinant endolysin and artilysin against *Pseudomonas aeruginosa*

Antimicrobial activity of the obtained recombinant endolysin and artilysin was tested on the laboratory strains and clinical isolates from the collection of the Laboratory for Translational Biomedicine (Gamaleya Federal Research Center for Epidemiology and Microbiology). Prepared *Pseudomonas*

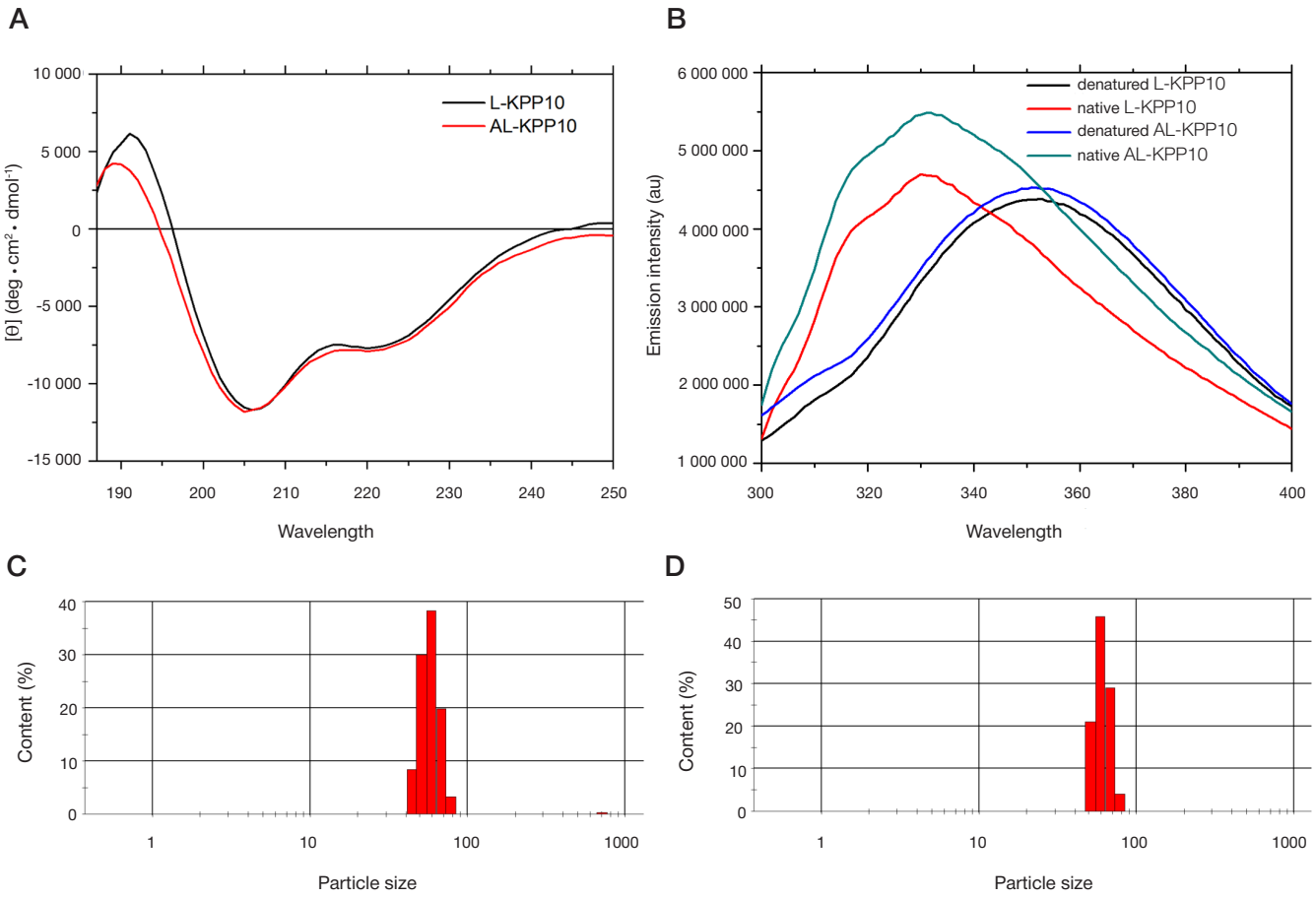

Fig. 1. Physical and chemical properties of endolysin L-KPP10 and artilysin AL-KPP10. Circular dichroism spectra of AL-KPP10 and L-KPP10 (**A**). Spectra of tryptophan fluorescence of proteins under denaturing and native conditions (**B**). Dynamic laser light scattering in the solutions of native AL-KPP10 (**C**) and L-KPP10 (**D**)

aeruginosa cells were treated with different concentrations of the recombinant endolysin and artilysin, and then seeded onto solid agarized LB.

The studied concentrations of endolysin L-KPP10 did not have a bactericidal effect on strain PA103 (Fig. 2A). This was not unexpected: the outer membrane protects the cell wall of gram-negative bacteria from the attacks of the protein. Treatment of bacterial cells with a combination of L-KPP10 and 0.5 mM EDTA increased membrane permeability and stimulated lysis at concentrations as low as 3 μg/ml (Fig. 2B).

In contrast, AL-KPP10 exhibited antimicrobial activity against PA103 and 4 of 6 clinical *Pseudomonas aeruginosa* isolates (Table 1 and Fig.3) due to the presence of a positively charged peptide component. Upon incubation of 10^6 bacterial cells with 25 μg/ml or 50 μg/ml AL-KPP10 for 30 min, the population of *Pseudomonas aeruginosa* CFU decreased by 10^5 cells (Fig. 3A).

Lysis was visualized by electron microscopy. Briefly, the clinical isolate Ts 43-16 of *Pseudomonas aeruginosa* sensitive to artilysin was treated with 50 μg/ml AL-KPP10 and incubated at room temperature for 10, 20 or 30 minutes. The cells were stained with uranyl acetate and inspected under the electron microscope (Fig. 3B). As shown in the picture, the phage lysin causes the cell wall of *Pseudomonas aeruginosa* to degrade (in the pictures the cell wall appears as an electron-dense colored flake-resembling mass).

To sum up, incubation of AL-KPP10-treated *Pseudomonas aeruginosa* strains sensitive to this protein results in the massive death of cultured bacteria, as exemplified by the clinical isolate Ts 49-16 (Fig. 3C).

DISCUSSION

There is an ongoing search for phage lysins that could be used to effectively combat gram-positive and gram-negative bacteria. Gram-negative pathogens are harder to kill because of their outer membrane that protects the peptidoglycan layer from phage attacks. One of the possible solutions to this problem lies in the use of permeabilizers, such as polymyxins or their derivatives, aminoglycosides, EDTA, citric acid, etc. [11]. For example, endolysin KZ144 obtained from the antipseudomonal phage phiKZ has been shown to exhibit bactericidal activity against Pseudomonas strains in the presence of permeabilizers [12]. Another example is endolysin OBPgpLYS. It has a broad spectrum of activity against gram-negative bacteria and can reduce their population by an order of magnitude. Its combinations with small quantities of EDTA have been shown to enhance the antimicrobial effect against multidrug-resistant *Pseudomonas aeruginosa* by 2 to 3 orders of magnitude [13].

However, it is possible to do without permeabilizing agents and use a new class of phage lysins called artilysin instead [14, 15, 16]. An artilysin consists of an endolysin and a positively charged peptide fused into one reading frame. The role of the peptide is to facilitate the passage of a protein through the outer membrane of gram-negative bacteria. One of the most promising peptides here is the sheep myeloid antimicrobial peptide SMAP-29. This amphiphilic molecule binds to a negatively charged membrane phospholipids incorporating its hydrophobic moiety into the membrane and thus creates pores [17]. One of the examples of SMAP-29-containing artilysin is called Art-175, a modified endolysin of phage KZ144. Unlike

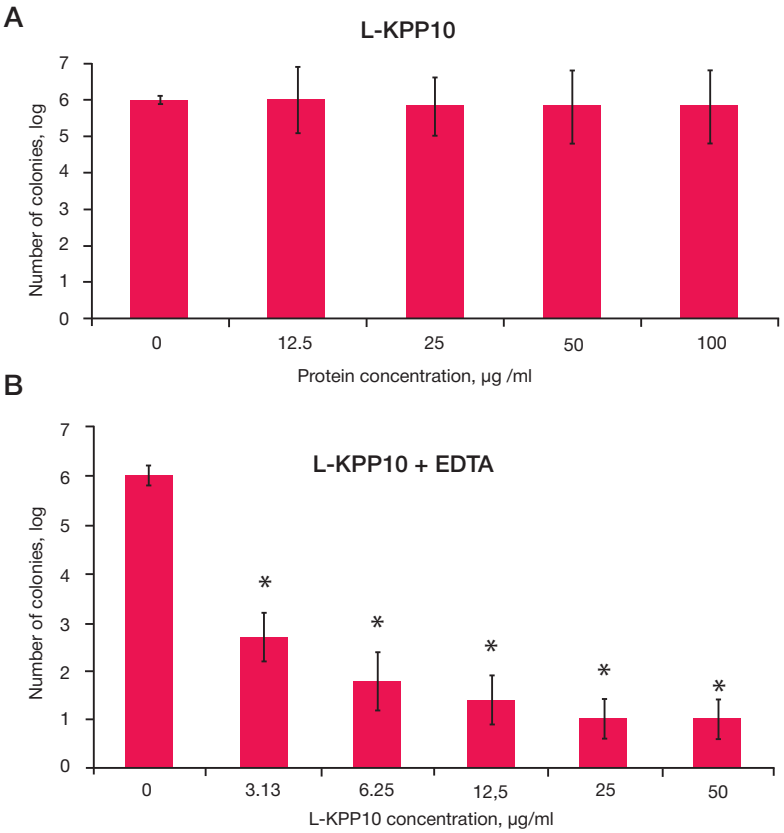

Fig. 2. Antimicrobial activity of the recombinant endolysin against strain PA103 of *Pseudomonas aeruginosa*. Treatment with different concentrations of the recombinant L-KPP10 in the absence of permeabilizing agents (**A**). 0.5 mM EDTA added to the bacteria combined with L-KPP10 (**B**). * — Difference is significant at p = 0.05 (Mann-Whitney U)

the original KZ144, Art-175 can pass through the outer membrane of *Pseudomonas aeruginosa* and kill multidrug resistant bacteria reducing their population by more than 10^4 [16]. The bactericidal effect of phage lysins is not limited to intraspecies attacks [18]. Thus, Art-175 has been shown to be effective against *Acinetobacter baumannii*, including persistent strains and strains with multiple drug resistance, [19].

Unfortunately, the original SMAP-29 is cytotoxic for human red cells. Its antibacterial and hemolytic activity has been shown to depend on its length and the original sequence. The non-toxic but still optimally bactericidal variant of SMAP-29 was obtained by removing and substituting some its original amino acids and had the following structure: [K2,7,13]-SMAP-29(1–17) [20, 21].

In the course of this work we obtained two recombinant phage lysins: endolysin L-KPP10 and its modified variant artilysin AL-KPP10. These proteins have a tertiary structure that can recover after renaturation, suggesting that they can retain their enzymic activity. Both proteins are highly stable in a solution. Importantly, the presence of the positively charged peptide SMAP-29(1–17) in KPP10 does not change the structure and properties of the protein. Those findings allowed us to continue our investigation and explore the antimicrobial activity of the obtained recombinant molecules.

We have established that the studied concentrations of endolysin L-KPP10 are effective against Pseudomonas only in the presence of EDTA. Being a permeabilizing agent, EDTA aided the passage of the endolysin into the cell through the outer membrane of the cell wall. To exert a bactericidal effect against a wide range of *Pseudomonas aeruginosa* strains, artilysin AL-KPP10 did not need permeabilizers, such as EDTA, since it contained a fragment of the myeloid peptide SMAP-29 ([K2,7,13]-SMAP-29(1–17)). Five of seven studied clinical isolates of *Pseudomonas aeruginosa* turned out to be sensitive to the recombinant artilysin. The best antimicrobial effect was seen at concentrations of 25 μg/ml and 50 μg/ml, when the bacterial populations shrunk by 10^5 cells within 30 min.

The closes functional analog of AL-KPP10 is artilysin Art-175. It consists of endolysin KZ144 linked to the original SMAP-29. Just like AL-KPP10, Art-175 can pass through the outer membrane of *Pseudomonas aeruginosa* and kill the cell. These two artilysins are different in their structure: AL-KPP10 retains the original KPP10 sequence and contains a modified shortened peptide SMAP-29 with three amino acid substitutions at positions K2,7,13, which makes the protein less toxic to human cells [20, 21]. We also expect AL-KPP10 to have a better antibacterial effect than Art-175, because the bactericidal activity of AL-KPP10 exhibited in our experiments was higher than that of Art-175 [16].

CONCLUSIONS

Our findings confirm that lytic properties of phage lysins targeting gram-negative bacteria from both inside and outside the cell can be modified and enhanced by the use of

Table 1. Antimicrobial activity of artilysin AL-KPP10 against clinical isolates of *Pseudomonas aeruginosa*

Strain/Clinical isolate of *P. aeruginosa*	Effect
PA103	+
Ts 38-16	–
Ts 43-16	+
Ts 44-16	+
Ts 47-16	–
Ts 48-16	+
Ts 49-16	+

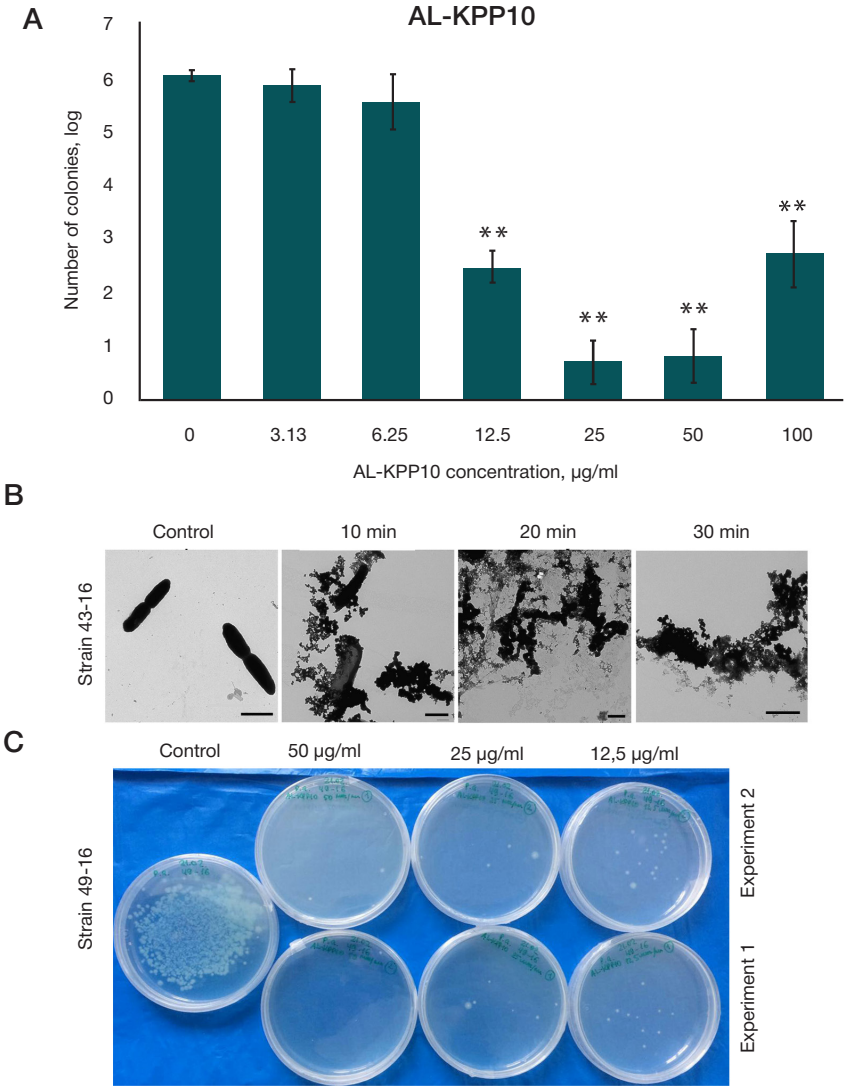

Fig. 3. Antimicrobial activity of the recombinant artilysin AL-KPP10 against sensitive strains of *Pseudomonas aeruginosa*. Treatment of *Pseudomonas aeruginosa* sensitive strains with different concentrations of recombinant AL-KPP10 in the absence of permeabilizing agents (**A**), $p < 0.01$ (Mann-Whitney U). Electron microscopy of Ts 43-16 (clinical isolate of *Pseudomonas aeruginosa*) following incubation with 50 μg/ml AL-KPP10 for 10 min, 20 min and 30 min (**B**). A Petri dish with artilysin and clinical isolate Ts 49-16 of *Pseudomonas aeruginosa* upon incubation for 30 min (**C**)

permeabilizing agents and positively charged peptides. The results of our work are consistent with previously published data on endolysin KZ144 isolated from antipseudomonal bacteriophage phiKZ and endolysin OBPgpLYS that exhibited antimicrobial activity in the presence of permeabilizers. This gives hope for the discovery of therapeutic agents based on recombinant phage lysins that could become a real alternative to antibiotics.

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THE USE OF ANTIMICROBIAL PHOTODYNAMIC THERAPY MEDIATED BY MC540 IN THE INFECTED WOUND MODEL

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Photodynamic therapy (PDT) is an alternative to conventional therapies of infections. It can kill drug-resistant bacteria without damaging host tissues. In the present work we use the multiresistant strain *PA21* of *Pseudomonas aeruginosa* to model a wound infection in mice and study the effect of PTD mediated by aqueous solutions of the anionic photosensitizer merocyanine 540 (solubilized in water and in 0.25 M sodium chloride) on bacterial decontamination and wound healing. To assess a therapeutic effect of PDT, we monitored bacterial contamination of the wound, measured the wound size in two planes using a caliper and carried out a histopathological examination of infected tissue sections. Our study reveals that PDT mediated by MC540 in the sodium chloride solution can induce bacterial death, inhibit bacterial re-growth and accelerate wound healing.

Keywords: antimicrobial photodynamic therapy, multiresistant clinical strain, *Pseudomonas aeruginosa*, skin infection, soft tissue infection, wound healing, merocyanine 540, wound infection

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ПРИМЕНЕНИЕ АНТИМИКРОБНОЙ ФОТОДИНАМИЧЕСКОЙ ТЕРАПИИ НА ОСНОВЕ МЦ540 К МОДЕЛИ РАНЕВОЙ ИНФЕКЦИИ

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Фотодинамическая терапия (ФДТ) является альтернативным методом лечения инфекций, позволяющим убивать лекарственно-устойчивые бактерии без повреждения ткани хозяина. В настоящем исследовании использован полирезистентный клинический штамм *Pseudomonas aeruginosa PA21* (*P. aeruginosa*) в модели раневой инфекции на мышах для изучения влияния ФДТ (в водных растворах анионного фотосенсибилизатора Мероцианина 540 (МЦ540): в воде и 0,25 М NaCl) на бактериальную инактивацию и заживление ран. После проведения ФДТ гибель бактерий оценивали путем определения бактериологической нагрузки в ранах, процесс заживления ран контролировали прямым измерением штангенциркулем в двух проекциях, а также проведением патоморфологических исследований послойных срезов инфицированных ран. Полученные результаты показали, что ФДТ в присутствии МЦ540 в растворе хлорида натрия (но не МЦ540 в воде) способна вызывать гибель бактерии, препятствовать их восстановлению и значительно ускорять процесс заживления ран.

Ключевые слова: антимикробная фотодинамическая терапия, полирезистентный клинический штамм *Pseudomonas aeruginosa*, инфекция кожи и мягких тканей, заживление ран, мероцианин 540, раневая инфекция

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Skin is the body's natural physical barrier and the first line of defense against pathogens. Damaged skin can end up getting infected. In turn, infection can delay wound healing, compromise a person's general health and even increase the risk of death. Antibiotics once revolutionized the therapy of infections, but today the growing incidence of resistant bacteria compels the search for new alternatives to the conventional management of localized infections. One of the promising solutions here is photodynamic therapy (PDT) that is normally used in cancer treatment [1–3] but has been recently proposed to combat bacterial infections [4–7]. Antimicrobial PDT relies on the use of photosensitizers (PS), non-toxic dyes that can be activated

by a specific wavelength of light. The PS is introduced into the infected wound that is subsequently exposed to a source of monochromatic light with a required wavelength [8–9]. Once activated, PS starts producing free radicals and/or singlet oxygen that are lethal for pathogens.

The aim of this study was to investigate the effect of MC540-mediated PDT on bacterial growth in the wound and wound healing in a murine model. BALB/c mice were infected with the multidrug resistant clinical strain *PA21* of *Pseudomonas aeruginosa*. To assess a therapeutic effect of PDT, we monitored bacterial contamination of the wound, measured the wound size in two planes using a caliper and carried out a histopathological examination of infected tissue sections.

METHODS

Photosensitizer

The stock solution of 1×10^{-3} M anionic dye merocyanine 540 (Sigma-Aldrich, Switzerland) was prepared in 96% ethanol on the day of the experiment. MC540 concentrations were determined spectrophotometrically based on the extinction coefficient of MC540 in water ε_{500} = 63 000 $M^{-1}cm^{-1}$. The stock solution was diluted with water or 0.25 M sodium chloride solution (ACROS, USA) to reach working MC540 concentrations of 25 μM.

Bacterial culture

The multiresistant strain PA21 of *Pseudomonas aeruginosa* used in our study was obtained from a collection of Gamaleya Federal Research Center for Epidemiology and Microbiology.

P. aeruginosa cells were incubated overnight at 37 °C in the Brain Heart Infusion broth (BHI Difco, USA), diluted 100-fold in the fresh medium, and grown until they reached the optical density OD_{600} of 1, which corresponds to the concentration of 10^9 CFU/ml. The culture was washed twice in PBS (pH = 7.4) (Ecoservice, Russia) and applied onto the wound in 50 μl aliquots.

Bacterial burden in the wounds

Swabs were taken from wound surfaces using cotton balls. Then the balls were placed into the test tube containing 0.9 ml normal saline, which was subsequently used to prepare a series of dilutions: 1:10, 1:100, and 1:1000. Each dilution was seeded onto two dishes treated with agarized media (BHI Difco, USA). Twenty-four hours later, the colonies were harvested and counted; the counts were converted to log CFU/ml.

Antimicrobial photodynamic therapy

All manipulations were performed using aseptic techniques and gentle anesthesia. The experiments were carried out in compliance with the principles of bioethics, international guidelines of the European Convention for the Protection of Vertebrate Animals (Strasbourg, March 1986) and the Declaration of Helsinki.

The experiments were conducted in 6–8 weeks old BALB/c mice weighing 17 to 21 g. Twenty-four hours before PDT we shaved the fur from a 2-cm^2 area on animals' backs and outlined the wound contours with a permanent marker. Before the surgery, the animals were anesthetized with inhaled isoflurane. The surgical site was cleansed with an antiseptic agent twice. The excised wound was 1.5 cm in diameter and extended through the skin, subcutaneous tissue and fascia down to the muscle. The wound was inoculated with a 50 μl aliquot of 10^7 CFU/ml *P. aeruginosa suspension*. Six hours after wound modeling the mice received photodynamic therapy.

The animals were divided into 4 groups (Table 1). Mice from group A (n = 18) were used as absolute control (no treatment); mice from group B (n = 18) were used as light control (irradiation with 530 nm monochromatic light for 5 min); mice from group C (n = 18) received PDT mediated by 25 μM aqueous solution of MC540 (irradiation with 530 nm monochromatic light for 5 min); mice from group D (n = 18) received PDT mediated by 25 μM aqueous solution of MC540 containing 0.25 M sodium chloride (irradiation with 530 nm monochromatic light for 5 min). Three animals were selected from each group to study the long-term effects of treatment (36 days).

The wounds were treated with 50 μl of 25 μM aqueous solution of MC540 containing 0.25 M NaCl and then exposed for 5 min to a source of monochromatic light with a 530 nm wavelength and a power density of 2 mW/cm^2, which ensured the total dose of 6 J/cm^2.

Histological analysis

The samples were fixed in 10% buffered formalin and embedded in paraffin. The paraffin-embedded 2–7 μm-thick sections were stained with hematoxylin-eosin and subjected to microstructural and morphometric analyses. Morphometric evaluation was performed using the AxioimagerA-2 microscope (Carl Zeiss, Germany).

Wound healing

While monitoring the condition of the wounds, we noted the signs of inflammation, type and quantity of wound drainage, etc. Wound sizes were measured with a ruler and a caliper in two planes 1 hour before PDT on days 2, 4, 7, and 14. To understand how wound healing progressed, the following parameters were calculated:

1. A change in the wound surface area on different days of the experiment (ΔS, %):

$$\frac{(S_0 - S_n)\times 100}{S_0},$$

where S_0 is the initial wound area, S_n is the wound area on day n.

Table 1. Study design

Group	Number of animals	Bacteria	Treatment
Group A: absolute control	18	*P. aeruginosa*	No treatment
Group B: light control	18	*P. aeruginosa*	Light (6 J/cm^2; λ = 530 nm)
Group C: PDT + MC540	18	*P. aeruginosa*	MC540 + light (6 J/cm^2; λ = 530 nm)
Group D: PDT + MC540+ 0.25 M NaCl	18	*P. aeruginosa*	MC540_0.25 M NaCl + light (6 J/cm^2; λ = 530 nm)

2. Relative rate of wound area reduction ($\nu_{heal.}$, %/day):

$$\frac{(S_0 - S_n)\times 100\%}{S_0 \times n},$$

where S_0 is the initial wound area, S_n is the wound area on day n, n is the day of the experiment.

3. Epithelialization rate ($\nu_{epith.}$, mm^2 / day):

$$\frac{S_0 - S_n}{T},$$

where S_0 is the initial wound area, S_n is wound area on day n, T is the number of days between the measurements.

All data were processed in Universlab DeskTer River V3.3.3269

Statistical processing

We calculated the mean values of the parameters mentioned above and the mean error SEM = $\frac{s}{\sqrt{n}}$ (*s* is a mean square deviation).

RESULTS

A day after the animals received PDT, their condition was satisfactory. The animals were active, mucous membranes looked normal and coats were smooth. All wounds were starting to suppurate; the most pronounced suppuration was observed in the controls.

Bacterial contamination

One of the most important tools used to estimate a therapeutic effect of treatment in patients with soft tissue infection is a microbiological test. We measured the degree of bacterial contamination prior to treatment, and 48, 96 and 168 hours after PDT. The results are presented in Table 2.

Six hours after inoculation but prior to PDT, bacterial burden was the same in all groups: $5.03 \pm 0.04\times10^6$ CFU/ml.

Forty-eight hours after PDT the number of *P. aeruginosa* decreased by 10^2 and 10^3 CFU/ml in groups C (PDT mediated by the aqueous solution of MC540) and D (PDT mediated by the aqueous solution of MC540 containing NaCl), respectively.

Healing progress

Over the course of monitoring, we established a few differences in the healing dynamics with regard to ΔS between the animal groups (Table 3).

The ΔS values in both control groups A and B were significantly lower ($p < 0.05$) than in groups C and D at all stages of the experiment (the only exception was groups B and C on day 7 when no significant differences were noticed).

Healing rates (ν_{heal}) turned to be very different within the groups (Table 3). The maximum rate was observed in group D, while in other groups ν_{heal} remained quite stable for 14 days ($p > 0.05$). Of note, the healing rates in all groups had slowed by day 14 and almost leveled off.

Epithelization rates (ν_{epith}) turned out to be significantly different between some groups and within the groups (Table 4).

The highest epithelization rate (ν_{epith}) between days 4 and 7 was observed in group D ($p < 0.05$ in all cases), by day 14 epithelization rates had decreased in all groups.

In the first 48 h after wound modeling a blood clot was formed in the wound. The major component of the clot was fibrin, which eventually hardened into a scab. The wound produced exudate dominated by neutrophils. Blood cells underwent lysis, manifesting suppurative inflammation (Fig. 1 A).

Table 2. Bacterial contamination of the wound measured on different days of the experiment, CFU/ml

	Time after PDT			
Group	0 h	48 h	96 h	168 h
Group A	5.00×10^6	1.5×10^5	1.7×10^5	1.4×10^4
Group B	4.95×10^6	1.2×10^5	1.6×10^5	1.0×10^4
Group C	5.01×10^6	2.0×10^4	0.7×10^2	negative
Group D	5.15×10^6	1.5×10^3	negative	negative

Table 3. Morphometry of wound matrix remodeling in BALB/c mice

Changes in the wound surface area throughout the course of the experiment, ΔS				
	Time after PDT			
Group	Day 2	Day 4	Day 7	Day 14
Group A	11.11 ± 0.37	25.00 ± 0.82	41.11 ± 1.36	84.43 ± 3.78
Group B	13.55 ± 0.45	32.22 ± 1.39	50.01 ± 1.65	85.30 ± 3.75
Group C	16.66 ± 0.53	40.02 ± 2.17	48.33 ± 1.76	93.31 ± 1.89
Group D	30.01 ± 2.33	55.55 ± 3.61	67.77 ± 4.75	94.77 ± 4.17
Relative rate of wound reduction, ν_{heal}				
	Time after PDT			
Group	Day 2	Day 4	Day 7	Day 14
Group A	5.55 ± 0.18	6.25 ± 0.21	5.87 ± 0.19	6.03 ± 0.12
Group B	7.50 ± 0.25	10.51 ± 0.35	7.14 ± 0.24	5.95 ± 0.13
Group C	8.33 ± 0.29	10.00 ± 0.87	6.90 ± 0.77	5.92 ± 0.28
Group D	15.01 ± 0.16	13.88 ± 1.91	9.68 ± 1.01	7.33 ± 0.45

Beneath the scab immature connective tissue started to grow actively with plenty of thin-walled capillaries, sinuses and lacunae filled with blood (Fig. 1B). On days 4 through 7 the connective tissue underwent fibrosis, transforming in some cases into hyalinized or even calcified scar tissue (Fig 1E). Although such infiltrates are not always deeply seated, they cause contractures and immobilize the skin.

Histological analysis revealed that in the first 24–48 h basal epidermal cells proliferated slowly, and then rapid proliferation took over and keratinization increased. Growing and differentiating, the epidermis gradually closed the wound (Table 4, Fig. E). In general, the wound underwent different stages of healing, which are usually affected by a number of factors, including those set in our experiment (Table 3 and 4, Fig. 1).

Good blood supply, increased oxygenation and a variety of enzymic processes occurring in the wound stimulated tissue substitution and even triggered intracellular regeneration of myosatellite cells (Tables 3 and 4, Fig. 1D).

To sum up, results of the microstructural analysis are consistent with the data obtained in the experiment (Tables 2–4, Fig. 1). Wound healing was exuberant owing to the active growth, differentiation and maturation of the microvasculature, formation of granulation tissue and vigorous epithelization.

DISCUSSION

In our previous study *in vitro* we have demonstrated that inactivation of *P. Aeruginosa* occurs ten times more effectively after PDT mediated by the MC540 solution containing 0.25 M sodium chloride than after PDT mediated by the aqueous solution of MC540 [10], which could be explained by the different degree of MC540 aggregation in water and in the presence of salt. In water MC540 exists both as a monomer and a dimer [11], but only monomers can generate reactive oxygen species, specifically singlet oxygen [12, 13]. In saline solutions MC540 forms large crystal-like aggregates capable of producing free radicals [14–17].

The results of the present study conducted in animals convincingly confirm the data obtained in our previous *in vitro*

Table 4. Rates of epithelial repair of infected wounds in BALB/c mice

Epithelization rates, v_{epith}				
	Time after PDT			
Group	Day 2	Day 4	Day 7	Day 14
Group A	1.07 ± 0.04	5.17 ± 0.23	15.69 ± 1.18	6.33 ± 1.05
Group B	2.24 ± 0.07	6.19 ± 0.27	16.07 ± 0.82	6.41 ± 1.16
Group C	2.63 ± 0.09	5.41 ± 0.24	14.31 ± 0.45	6.09 ± 1.24
Group D	3.52 ± 0.11	8.01 ± 0.35	16.94 ± 0.86	8.11 ± 1.53

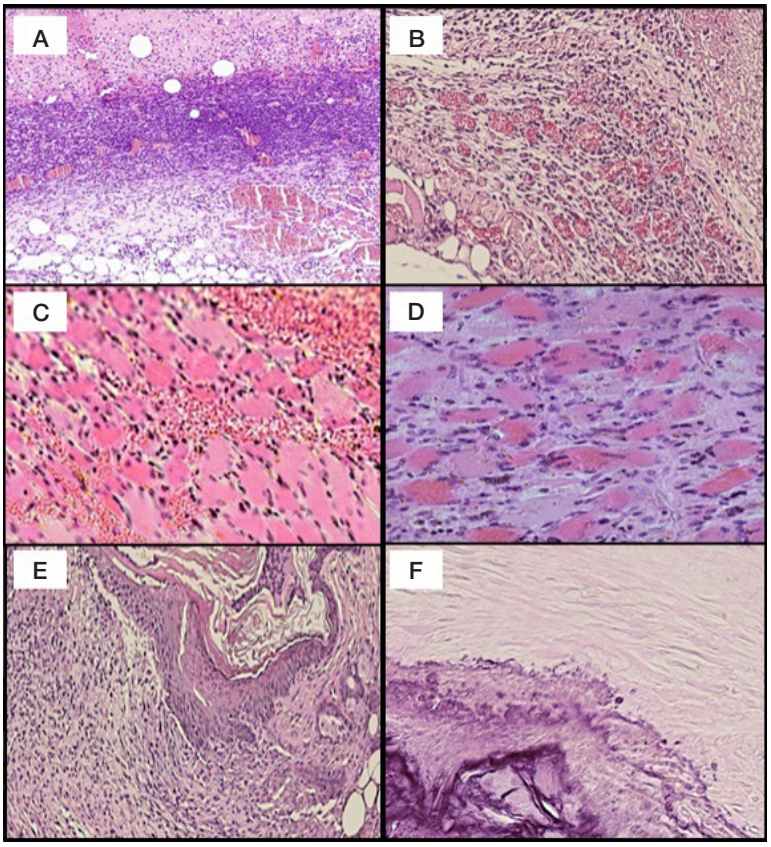

Fig. 1. Microscopy of infected wounds of mice after PDT. Stain used: hematoxylin and eosin (**A**) Immature granulation tissue on day 4, ×150. (**B**) Vascular hyperemia and fluid accumulation around the capillary bed on day 4, ×350. (**C**) Blood in the muscle tissue on day 4, ×250. (**D**) Fragments of muscle tissue showing intracellular cell regeneration of day 7, ×450. (**E**) Epithelialization and scarring on day 14, ×150. (**F**) Hyalinized and calcified scar tissue on day 36, ×150

work with regard to bacterial death, inhibition and rapid wound healing.

Table 2 shows that after PDT mediated by the MC540 solution containing sodium chloride, full decontamination of the wound infected with multiresistant *P. aeruginosa* is observed as early as on day 4, while after PDT mediated by the aqueous solution of MC540 it happens on day 7 only.

Morphometry (Table 3) confirms the better effect of MC540 aggregates in comparison with its monomers or dimers with regard to the rate of healing and wound area reduction. By day 4 the wound area in group D shrunk by 45% whereas in group C it shrunk by 60%, which is close to the results demonstrated by group B (68%).

The relative rate of wound healing (Table 3) in group A was linear (mean square deviation considered). For groups B and C it was parabolic with a peak on day 4, for group G-exponential. The healing process slowed on day 14 in all groups. These data correlate with the rate of wound area reduction (Table 3).

Regeneration of the epithelium was faster in group D where it became very pronounced on day 4 (Table 4), in comparison with the controls and the group that received PDT mediated by the aqueous solution of MC540.

Results of the microstructural analysis correlate well with other obtained data (Tables 2–4, Fig.1) indicating rapid wound healing owing to the active growth, differentiation and maturation of the microvascular bed, formation of granulation tissue and vigorous epithelization. But although wound healing and decontamination were faster when PDT was mediated by MC540 in the sodium chloride solution, the healing process was rough, accompanied by fibrin breakdown and fibrinoid formation. PDT mediated by the aqueous solution of MC540 resulted in a slower and not so harsh healing without active fibrinoid formation.

CONCLUSIONS

The results of our experiment carried out in mice suggest that PDT mediated by the aqueous solution of MC540 containing sodium chloride is more effective against bacterial infection of the modeled wound, inhibits bacterial re-growth and accelerates healing in comparison with the controls and the group that received PDT mediated by the plain aqueous solution of MC540.

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ASSESSMENT OF PERIOPERATIVE PROPHYLAXIS OF INFECTIOUS COMPLICATIONS IN POST-OP PATIENTS

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Perioperative antimicrobial prophylaxis (PAP) involves administration of antimicrobial agents (AMA) to patients undergoing a surgical intervention and aims to reduce the risk of postoperative infectious complications, especially at surgical sites. In the present work we assess efficiency and safety of AMA used for prevention of postoperative infectious complications. In the course of our study we pre-analyzed 576 medical histories of post-op patients aged 18 to 87 years (mean age M ± SD was 57.4 ± 14.5 years), of which 347 (60.2%) were male and 229 (39.8%) female. Only 481 histories were selected for final analysis. We assessed the choice of antibacterial therapy, the frequency of adverse reactions (AR) and infectious complications and the type of the latter. PAP regimens were consistent with the official guidelines in 207 (43.04%) cases. PAP recommendations were ignored in 274 cases (56.96%), and the timing was wrong in 364 cases (75.7%). Incorrect dosages were administered in 225 cases (46.8%). We also discovered an association between irrational PAP regimens and 1) the length of patient's stay in the intensive care unit ($p = 0.003$ and $p < 0.005$), 2) the frequency of reoperations associated with infection ($p = 0.001$), 3) mortality rates ($p = 0.002$), and 4) isolation of strains with multidrug resistance ($p = 0.016$). We conclude that PAP regimens for the inpatients of surgical wards are often compromised by failure to comply with the official guidelines, wrong timing and incorrect dosage, which negatively affects hospital statistics.

Keywords: antibacterial agents, perioperative antimicrobial prophylaxis, infectious complications, surgery, efficiency, adverse reactions

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ОЦЕНКА РАЦИОНАЛЬНОСТИ ПРОВЕДЕНИЯ ПЕРИОПЕРАЦИОННОЙ АНТИМИКРОБНОЙ ПРОФИЛАКТИКИ ИНФЕКЦИОННЫХ ОСЛОЖНЕНИЙ У ПАЦИЕНТОВ ПОСЛЕ ХИРУРГИЧЕСКИХ ВМЕШАТЕЛЬСТВ

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Назначение антибактериальных препаратов (АБП) для проведения периоперационной антимикробной профилактики (ПАП) пациентам хирургического профиля необходимо для снижения частоты возникновения инфекционных осложнений в послеоперационном периоде, в том числе инфекций области хирургических вмешательств. Целью работы была оценка рациональности и безопасности выбора АБП для проведения ПАП инфекционных осложнений у пациентов после хирургических вмешательств. Проведен фармакоэпидемиологический анализ 576 историй болезней пациентов в возрасте от 18 до 87 лет после хирургических вмешательств, средний возраст (M ± SD) составил 57,4 ± 14,5 года, мужчин — 347 (60,2%), женщин — 229 (39,8%). В финальный анализ рациональности схем ПАП вошли 481 история болезни. Оценивали рациональность выбора антибактериальной терапии, частоту развития неблагоприятных побочных реакций (НПР), частоту и характер инфекционных осложнений. Показано, что выбор схем ПАП соответствовал рекомендациям в 207 (43,04%) случаях. Выявлена высокая частота несоблюдения рекомендаций по проведению ПАП (274; 56,96%) и нарушения сроков проведения ПАП (364; 75,7%). Общее число случаев нарушения режимов дозирования составило 225 (46,8%). Обнаружена взаимосвязь нерациональных схем ПАП с длительностью пребывания в ОРИТ ($p = 0,003$ и $p < 0,005$), частотой повторных оперативных вмешательств, ассоциированных с инфекцией ($p = 0,001$), уровнем летальности ($p = 0,002$) и выделением полирезистентных штаммов ($p = 0,016$). Таким образом, у пациентов хирургического профиля в условиях реальной клинической практики сохраняется высокая частота несоблюдения рекомендаций по проведению ПАП, нарушения сроков проведения ПАП и режимов дозирования АБП, что негативно сказывается на госпитальных показателях.

Ключевые слова: антибактериальные препараты, периоперационная антимикробная профилактика инфекционных осложнений хирургического вмешательства, рациональность применения, неблагоприятные побочные реакции

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Perioperative antimicrobial prophylaxis (PAP) is an internationally accepted standard of care for surgical patients. It involves the use of antibacterial agents (AA) and aims at lowering the risk of infectious complications in general and surgical site infections (SSI) in particular. Any postoperative infectious complication negatively affects the outcome of surgery, extends a patient's stay in hospital, incurs high costs, increases the risk of re-surgeries, contributes to hospital death rates and requires additional drug-based therapy [1].

According to the European Center for Disease Prevention and Control (ECDC) and the World Health Organization (WHO), infectious complications associated with multi-drug resistant and pan-resistant strains have recently become alarmingly incident [2, 3].

Among the factors promoting antibiotic resistance are overuse and misuse of AA [4]. In this light, a wise approach to the choice of antibacterial therapy becomes particularly important [5]. Inappropriate dosing, including administration of subtherapeutic doses of AA both for treatment and prevention, and prolonged post-operative prophylaxis (>24 h) reduce PAP efficacy and contribute to antimicrobial resistance [6, 7].

The Russian Federation actively supports measures for curbing antibiotic resistance. Revised clinical recommendations proposed in the National Strategy for Antimicrobial Treatment Control highlight the necessity of control over the spread of nosocomial infections [8]. Systemic monitoring of antibiotic resistance and trade regulations are essential components of this strategy [9].

Therefore, the rational use of AA in the clinical setting becomes an important tool for reducing the risk of SSI and adverse reactions (AR) and curbing antibiotic resistance.

The aim of this study was to assess the choice of AA with regard to its adequacy and safety in patients undergoing PAP.

METHODS

We have analyzed the regimens of antimicrobial PAP given to the inpatients of surgical units with regard to their adequacy and compliance with national and international clinical guidelines [1, 10]. We set up a database containing details of patients' clinical and demographic profiles (sex, age, diagnosis, comorbidities, creatinine levels, creatinine clearance rates before and after surgery) and surgical interventions, including blood loss volume, wound contamination, complications, adverse reactions, and AA doses).

A total of 576 medical histories were selected for the analysis. Of all patients included in the preliminary analysis, 347 (60.2%) were men and 229 (39.8%) — women. Their age ranged from 18 to 87 years (mean age M ± SD was 7.4 ± 14.5 years). All of the patients had undergone a surgical intervention between June 2016 and December 2016. Details are presented in Table 1.

All surgical interventions performed on the analyzed patients were divided in three groups: general surgeries (356; 61.8%), cardiac surgeries (177; 30.7%), and cancer surgeries (21; 3.6%). The majority of the surgical interventions were elective (468; 81.3%). Most of the surgical wounds were clean (310; 53.8%). Infected wounds were observed in 113 (19.6%) patients who had septic suppurative inflammation at various locations. Clean-contaminated and contaminated wounds were observed in 70 (12.2%) and 84 (14.6%) patients, respectively.

Only 481 medical histories were selected for the final analysis. The rest 95 (16.5%) were excluded due to the lack of reliable data about PAP.

PAP adequacy and safety were assessed based on:

– AA regimens;

– adequacy of regimens, timing and duration of PAP;

– frequency of adverse reactions.

The total frequency of post-operative infectious complications and SSI were also estimated.

In addition, we have analyzed the impact of different factors, such as PAP regimens, clinical and demographic characteristics of patients (age, sex, BMI, underlying medical condition, renal function, type of surgery, wound contamination, blood loss volume) on the risk of infectious complications, the length of hospital stay, the length of stay in intensive care and death.

Statistical analysis was performed in STATISTICA 10.0 (StatSoft Inc., USA). The data were presented as means (M) and the standard deviation (SD). Normality of sample distribution was evaluated by the Shapiro-Wilk W test; homogeneity of variances across two samples was tested using Fisher's T-test. The differences were considered significant at $p < 0.05$. Apart from the correlation analysis, we also used the Mann-Whitney U and Kolmogorov-Smirnov tests to compare two independent

Table 1. Clinical characteristics of patients included in the analysis

Parameter	Patients n = 576	Male n = 347		Female n = 229		p
	M ± SD	M	SD	M	SD	
Age. years	57.4 ± 14.5	57.8	13.6	56.9	15.6	0.468
BMI. kg/m^2	28.20 ± 5.67	27.70	4.80	29.01	6.80	0.005
Hospital stay. days	18.10 ± 22.05	17.20	10.70	19.60	32.40	0.197
Onset of infectious complications. days after surgery	1.5 ± 3.6	1.1	3.6	1.3	3.7	0.596
Onset of non-infectious complications. days after surgery	0.89 ± 4.39	0.88	2.70	0.90	6.20	0.942
Re-operation. days after initial surgery	1.18 ± 3.96	1.40	4.30	0.85	3.30	0.114
Length of stay in intensive care. days	3.10 ± 7.60	2.76	6.20	3.63	9.20	0.180
Duration of mechanical ventilation. days	0.61 ± 4.12	0.30	2.67	1.04	5.60	0.041
Blood loss. ml	214.1 ± 483.1	169.9	426.7	243.3	515.5	0.074
Creatinine^{0*}. mg/dl	0.95 ± 0.49	1.05	0.56	0.83	0.32	< 0.0001
Creatinine^{1**}. mg/dl	1.07 ± 0.94	1.13	0.76	0.98	1.16	0.058
Creatinine^{0*} clearance rate. ml/min	96.80 ± 42.50	100.61	44.91	91.17	38.01	0.009
Creatinine^{1**} clearance rate. ml/min	74.79 ± 52.20	76.32	48.79	72.48	57.03	0.401

Note: * — creatinine levels and clearance rates (Cockcroft-Gault equation) before surgery; ** — creatinine levels and clearance rates (Cockcroft-Gault equation) 24–48 h after surgery

continuous variables not complying with normal distribution. Two qualitative independent variables were compared using the two-tailed Fisher's test or χ^2 with Yates' correction.

RESULTS

The retrospective analysis of medical histories of 481 patients has revealed that in 297 (43.04%) cases the choice of antibiotics was rational and consistent with existing clinical guidelines. In 274 (56.96%) cases the choice of AA was not rational because it did not account for wound contamination and the specifics of surgery. On the whole, PAP regimens were characterized by the frequent use of 3rd generation cephalosporins (CPh) and cycling of 1st, 2nd, 3rd and 4th generations of CPh in pre- and postoperative management; the regimens also included carbapenems and inhibitor-protected aminopenicillins in combination with aminoglycosides (amikacin), metronidazole, and fluoroquinolones (ciprofloxacin), which were administered to the patients with clean and clean-contaminated wounds. In the studied patients' sample wrong PAP timing was observed in 364 (75.7%) cases. Inappropriate dosing was noticed in 225 (46.8%) cases.

Good choice of PAP regimens (207; 43.04%) was spoiled by inappropriate AA doses in 64 (30.9%) cases (Table 2).

In 364 (75.7%) cases, the inadequate choice of AA (274; 56.96 %) was accompanied by prolonged PAP (regimens were extended beyond 24–48 hours); subtherapeutic doses were prescribed in 161 (58.8%) cases (Tables 2, 3).

Analysis of safety of antibacterial agents used for perioperative prophylaxis

The retrospective analysis of medical records revealed that the total number of adverse reactions was 23 (3.99%); all of them were observed in the group of patients who received prolonged PAP. No adverse reactions were observed in the group of patients who received PAP before surgery and in the group where PAP regimens were limited to 48 hours. Thus, the risk of adverse reactions increases with PAP duration (Table 4).

Retrospectively, the following AR were observed:

– antibiotic-associated colitis — 9 cases (39.1%);

– psychomotor agitation — 6 cases (26.1%);

– pseudoallergies — 3 cases (13.0%);

– elevated transaminases — 3 cases (13.0%);

– antibiotic-induced nephropathy (vancomycin) — 2 cases (8.6%);

– prolonged QT interval — 2 cases (8.6%).

Further analysis revealed positive correlations between the risk of adverse reactions and: age ($r = 0.109$; $p = 0.009$), the length of hospital stay ($r = 0.291$; $p < 0.0001$), the length of stay in the intensive care unit ($r = 0.374$; $p < 0.0001$), death ($r = 0.269$; $p < 0.0001$), incidence of non-infectious postoperative complications ($r = 0.340$; $p < 0.0001$), postoperative creatinine levels and creatinine clearance rates ($r = 0.256$; $p < 0.0001$). No correlations were found between AR and: allergies ($r = 0.039$; $p = 0.348$), the choice of PAP regimens ($r = 0.340$; $p = 0.387$), dosing ($r = 0.028$; $p = 0.504$), PAP duration ($r = 0.017$; $p = 0.687$) and infectious complications ($r = 0.032$; $p = 0.443$).

The Mann-Whitney and Kolmogorov-Smirnov tests confirmed the presence of reliable associations between AR and age, the length of hospital stay, the length of stay in the intensive care unit, duration of mechanical ventilation and postoperative complications. However, the Kolmogorov-Smirnov test did not yield significant values for death ($p = 0.121$, Table 5). Importantly, low creatinine clearance rates in postoperative patients receiving antibiotics were a significant predictor of AR development.

Table 2. Antibacterial agents used in perioperative prophylaxis and inappropriate dosing

PAP regimen	Number of regimens, n	Inappropriate dosing, n
1. Adequate PAP regimens	**207**	**64**
1st and 2nd generation cephalosporins	93	29
1st generation cephalosporin + metronidazole	27	24
Inhibitor-protected aminopenicillins	87	11
2. Inadequate PAP regimens	**274**	**161**
3rd and 4th generation cephalosporins	141	68
3rd and 4th generation cephalosporins + metronidazole	72	56
Cycling of 1st, 2nd and 3rd generation cephalosporins	39	23
Cycling of cephalosporins and vancomycin	11	8
Carbapenems	7	2
Inhibitor-protected aminopenicillins in combination with aminoglycosides or fluoroquinolones	4	4

Table 3. Duration of perioperative prophylaxis

PAP timing	Number of patients	
	abs.	%
Administration of a single AA dose before surgery	117	24.3
Inadequate duration of PAP:	364	75.6
PAP extended to 24 h	92	25.3
PAP extended to 48 h	71	19.5
3–4 days	100	27.5
5–7 days	63	17.3
8–10 days	26	7.1
11–14 days	12	3.3

Frequency of infectious complications in the postoperative period

Postoperative infectious complications were observed in 90 (15.6%) cases, dominated by SSI (45; 50%) and infections of the lower respiratory tract (31; 34.4%), including nosocomial pneumonia in 24 patients (77.4%) and nosocomial tracheobronchitis in 7 patients (22.6%); sepsis (7; 7,8%); intrabdominal infections (6; 6.7%); infections of the urinary tract (1; 1.1%), and infective endocarditis (1; 1.1%). The frequency of infectious complications in patients with different types of wound contamination is shown in Table 6.

PAP was administered to the majority of patients with clean (91.3%) and clean-contaminated (91.6%) wounds. The frequency of infectious complications in such patients was 14.1% and 19.5%, respectively (Table 6). All patients with contaminated wounds underwent PAP; the frequency of infectious complications in this group was 27.1% (n = 19). There were no reliable records about the administration of AA before surgery (usual timing is 30–60 min before the operation) to the patients with infected wounds although those patients did receive AA in the postoperative period (61; 54.5%). In these patients the frequency of infectious complications was significantly higher than in the patients who did not receive PAP (11; 18.03%) than in the patients who received adequate PAP in compliance with clinical guidelines (6; 11.8%). Re-operations were necessary in 86 (14.9%) cases, of which 32 (37.2%) were associated with infectious complications and the rest 54 (62.8%) were not.

Additionally, we have analyzed the associations between a few different factors, such as the bad choice of PAP, patients' clinical and demographic characteristics (age, sex, diagnosis, renal function, type of surgery, wound contamination, blood loss), the frequency of infectious complications, a need for a re-operation, the length of hospital stay, the length of stay in the intensive care unit, bacterial growth, and mortality (Table 7).

Statistically significant were the associations between the bad choice of PAP and mortality ($p = 0.002$), between prolonged PAP /inappropriate dosing and the length of stay in intensive care ($p = 0.003$ and $p < 0.005$, respectively). Inappropriate doses were shown to increase the risk of re-operations associated with post-op infection ($p = 0.001$).

Importantly, elevated creatinine levels measured 24 h after the surgical intervention are a marker of renal function and demonstrate strong associations with the frequency of infectious complications ($p = 0.006$), the length of stay in intensive care ($p = 0.049$), the length of stay in hospital ($p = 0.001$), and mortality ($p = 0.003$).

DISCUSSION

The rational choice of PAP is one of the major tools for regulating the spread of nosocomial infections in surgical patients. PAP aims at reducing the risk of postoperative complications, the length of stay in intensive care and hospital in general, and mortality from septic or suppurative complications. Surgeons, anesthesiologists, clinical pharmacologists, epidemiologists and hospital administration should be encouraged to actively participate in the studies of compliance with international clinical standards for PAP.

Our study demonstrates that in 52.4% cases PAP regimens are consistent with international and national clinical guidelines. Failure to comply with clinical guidelines was observed in 47.6% cases when the choice of antibiotics was not rational, the regimens were extended beyond necessity (85.4%) and the administered doses were inappropriate (66.4%).

Our findings are consistent with those of Khan et al. [11], Vessal et al. [12] and El Hassan et al. [13] who also discovered the lack of compliance with the clinical guidelines for PAP in surgical patients; in those research works compliance varied

Table 4. Frequency of adverse reactions depending on PAP duration

PAP duration	AR	
	абс.	%
Administration of a single AA dose before surgery	0	–
PAP extended to 24 h	0	–
PAP extended to 48 h	5	21.7
3–4 days	3	13.0
5–7 days	3	13.0
8–10 days	5	21.7
11–14 days	7	30.4
Total	23	100.0

Table 5. Associations between clinical and laboratory parameters and the risk of adverse reactions

Parameter	Adverse reactions	
	p1 (Mann-Whitney U test)	p2 (Kolmogorov-Smirnov test)
Age	0.025	0.315
Allergies	p = 0.308	p > 1
Duration of mechanical ventilation	< 0.0001	0.017
Length of stay in intensive care	< 0.0001	< 0.0001
Length of hospital stay	< 0.0001	< 0.0001
Death	< 0.0001	0.121
Postoperative non-infectious complications	< 0.0001	< 0.0001
Postoperative creatinine levels and clearance rate	< 0.0001	< 0.0001
Infectious complications	p = 0.165	p > 1

from 1.7% to 82%. The majority of those studies were focused on the timing of preoperative prophylaxis.

According to Gouvêa et al., who have analyzed a few research works on the issue, PAP regimens are observed in 70.3% to 95% cases, the rational choice of PAP varies between 22% and 95 %, bad — between 2.3% and 100%, wrong timing occurs in 73% to 100% cases, and total compliance with clinical standards for PAP — in 5.8%–91.4% cases [14].

A retrospective study by Prospero et al. has demonstrated that over the course of 6 years covered by the study PAP standards were observed in 58% cases. The frequency of postoperative infectious complications was mostly affected by the length of surgery (OR 1.68; 95% CI: 1.56–1.82) and emergency (OR 2.16; 95% CI: 1.96–2.37). The authors note that in spite of poor adherence to PAP guidelines in general, the group where PAP protocols were observed had a low frequency of infectious complications [15].

To encourage medical personnel to adhere to PAP standards and improve clinical care, WHO experts recommend the use of checklists in the perioperative period [16].

In their works, some researchers focus on the choice of antibiotics used for PAP. According to our data, third generation cephalosporins, including those against Pseudomonas, are extensively used for perioperative prophylaxis. International studies by Lautenbach et al. [17] and Rodríguez-Baño et al. [18] demonstrate a high correlation between the use of 3rd generation cephalosporins and the spread of strains producing broad-spectrum β-lactamases. Our microbiological monitoring (n = 84) indirectly confirms a high incidence of such strains isolated from patients' samples (22; 26.2%). There is a concern about the emergence of strains resistant to carbapenems Carb+ (23; 27.4%) because the choice of AA for treating infectious complications caused by such strains is very limited.

In our study, adverse reactions were registered in 23 patients (3.99%). There was a relatively high incidence of antibiotic-associated colitis in patients receiving cephalosporins (9; 1.6%) and episodes of psychomotor agitation in elderly patients receiving cephalosporins in combination with metronidazole (6; 1.04%). According to one of the epidemiological studies, antibiotic-associated diarrhea develops in 8% of inpatients, 1–3% of them have a fulminating course. The researchers conclude that apart from antibiotics, among the risk factors contributing to this condition are anticholinergics and medications inhibiting intestinal motility [19].

Grill et al. report a high incidence of neurological disorders following administration of fluoroquinolones to surgical patients. Among the conditions observed are episodes of psychomotor agitation, seizures, myoclonus, delirium, dysarthria, and ataxia. Severe neurologic responses are particularly frequent in elderly patients and patients with a history of neurological disorders. The researchers note that such adverse reactions are equally frequent for all types of fluoroquinolones [20].

The increased risk of adverse reactions following prolonged PAP supports the importance of adherence to clinical protocols. Our correlation analysis shows that adverse reactions correlate with the length of hospital stay ($r = 0.291$, $p < 0.0001$), the length of stay in the intensive care unit ($r = 0.374$; $p < 0.0001$), mortality ($r = 0{,}269$; $p < 0{,}0001$), and the duration of mechanical ventilation ($r = 0.249$; $p < 0.0001$).

Importantly, in patients receiving antibiotics the frequency of adverse reactions correlates with creatinine levels and creatinine clearance rates ($p < 0.0001$), which are used as markers of renal damage. The study [21, 22] has demonstrated that deteriorating renal function affects pharmacokinetics of antibiotics and increases the risk of adverse reactions to the point of life threatening. These data need to be prospectively studied in more detail.

CONCLUSIONS

Compliance with the guidelines for perioperative prophylaxis in the clinical setting remains poor.

Our study has revealed associations between the inadequate choice of antibiotics and mortality, prolonged PAP and the length of patient's stay in intensive care, inadequate dosing and the frequency of re-operations associated with post-op

Table 6. Frequency and type of infectious complications in patients with different types of wound contamination undergoing perioperative prophylaxis

Infectious complications n = 90 (15,6%)	Wound contamination, n (%)			
	clean n = 310	clean-contaminated n = 84	contaminated n = 70	infected n = 112
SSI	15 (5.3)	6 (7.8)	12 (7.1)	4 (7.8)
Nosocomial pneumonia	15 (5.3)	3 (3.9)	4 (5.7)	0
Sepsis	5 (1.8)	1 (1.3)	0	0
Other*	5 (1.8)	5 (6.5)	3 (4.3)	2 (3.9)
Total	40 (14.1)	15 (19.5)	18 (27.1)	6 (11.8)
PAP administered	283 (91.3)	77 (91.6)	70 (100)	51 (45.7)

Note: * — infective endocarditis, urinary tract infection, нnosocomial tracheobronchitis, intraabdominal infection

Table 7. Impact of inadequate PAP regimens on hospital statistics

Inadequate PAP	p (Mann-Whitney U)					
	Infectious complications	Length of stay in intensive care	Length of hospital stay	Re-operations associated with post-op infection	Presence of multidrug resistant bacteria in patients' samples	Death
Prolonged PAP	> 1	0.003	0.530	0.934	0.290	0.465
Inappropriate dosing	0.603	<0.005	0.500	0.001	0.016	0.980
Elevated creatinine levels 24 h after surgery	0.006	0.049	0.001	0.567	0.899	0.003
All regimens	0.900	0.116	0.206	0.103	0.610	0.002

infections, as well as the length of stay in the intensive care unit.

Adverse reactions are a risk factor for extended mechanical ventilation and extended stay in the intensive care unit or hospital in general.

Considering the reliable association between creatinine levels and the risk of infectious complications and death, we believe that renal function tests has a high prognostic value, which can be elucidated by further prospective studies of patients receiving PAP.

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ANALYSIS OF THE ASSOCIATION BETWEEN THE RS767455 T>C *TNFRSF1A* AND RS1061622 T>G *TNFRSF1B* POLYMORPHISMS AND NONALCOHOLIC STEATOHEPATITIS

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Poor diet, sedentary behavior and genetic background are major factors contributing to the etiology and pathogenesis of non-alcoholic fatty liver disease (NAFLD). It is hypothesized that polymorphisms of the *TNFRI* and *TNFRII* genes coding for the receptors that bind the proinflammatory cytokine tumor necrosis factor alpha (TNFα) can be implicated in the susceptibility to NAFLD, but not much data is available in the literature. In the present work we aimed to investigate a possible association between the rs767455 T>C *TNFRSF1A* and rs1061622 T>G *TNFRSF1B* polymorphisms and one of NAFLD forms, nonalcoholic steatohepatitis (NASH), and to assess their effect on blood biochemistry. Samples of DNA isolated from the venous blood of 151 healthy donors and 242 patients with NASH were genotyped using PCR-RFLP. TNFα concentrations were measured by ELISA. We have not found any association between the rs767455 T>C *TNFRSF1A* polymorphism and the development of NASH in the residents of Karelia. However, we have discovered an association between NASH and the T>G *TNFRSF1B* rs1061622 polymorphism. Carriers of the G allele have a higher risk of developing NASH (OR = 4.83; 95% CI: 2.72–8.57). The rs1061622 T>G genotype of the *TNFRSF1B* gene appears to have no effect on TNFα concentrations and the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Our findings suggest a possible association between the rs1061622 T>G *TNFRSF1B* polymorphism and a risk of developing NASH in the residents of Karelia.

Keywords: non-alcoholic steatohepatitis, tumor necrosis factor alpha, tumor necrosis factor alpha receptors, mbTNFRI, sTNFR, *TNFRSF1A* gene, *TNFRSF1B* gene, gene polymorphism, alanine aminotransferase, aspartate aminotransferase

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АНАЛИЗ АССОЦИАЦИИ ПОЛИМОРФНЫХ ВАРИАНТОВ T>C RS767455 ГЕНА *TNFRSF1A* И T>G RS1061622 ГЕНА *TNFRSF1B* С РАЗВИТИЕМ НЕАЛКОГОЛЬНОГО СТЕАТОГЕПАТИТА

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В этиологии и патогенезе неалкогольной жировой болезни печени (НАЖБП) важны особенности питания, малоподвижный образ жизни и наследственность. Предполагают, что полиморфные варианты генов, кодирующих рецепторы к провоспалительному цитокину фактору некроза опухоли альфа (TNFα) (*TNFRI* и *TNFRII*), влияют на предрасположенность людей к развитию НАЖБП. Однако сведения о связи данного заболевания с носительством полиморфных вариантов генов *TNFRI* и *TNFRII* почти отсутствуют в литературе. Целью исследования было изучить связь полиморфных вариантов генов *TNFRSF1A* (T>C rs767455) и *TNFRSF1B* (T>G rs1061622) с развитием одной из форм НАЖБП — неалкогольного стеатогепатита (НАСГ) и их влияние на биохимические показатели крови. Методом ПЦР-ПДРФ генотипировали ДНК, выделенную из венозной крови 151 здорового донора и 242 пациентов с диагнозом НАСГ. Содержание TNFα оценивали с помощью иммуноферментного анализа (ИФА). По результатам, связь полиморфного маркера T>C rs767455 гена *TNFRSF1A* с развитием НАСГ у жителей Карелии отсутствует. Обнаружена ассоциация с НАСГ полиморфного варианта T>G rs1061622 гена *TNFRSF1B*. У носителей аллеля G повышен риск развития данного заболевания ОШ = 4,83 (95% ДИ: 2,72–8,57). Влияние генотипа по T>G rs1061622 маркеру гена *TNFRSF1B* на содержание TNFα и активность аланинаминотрансферазы (АлАТ), аспартатаминотрансферазы (АсАТ) и щелочной фосфатазы (ЩФ) не выявлено. Сделано заключение, что полиморфный вариант T>G rs1061622 гена *TNFRSF1B* может быть вовлечен в предрасположенность населения Карелии к НАСГ.

Ключевые слова: неалкогольный стеатогепатит, наследственность, фактор некроза опухоли альфа, рецепторы к фактору некроза опухоли альфа, mbTNFRI, sTNFR, ген *TNFRSF1A*, ген *TNFRSF1B*, полиморфизм генов, аланинаминотрасфераза, аспартатаминотрансфераза

Финансирование: финансирование осуществлялось из федерального бюджета на выполнение государственного задания КарНЦ РАН (тема № 0221-2017-0049) на оборудовании Центра коллективного пользования ФИЦ «Карельский научный центр Российской академии наук», при поддержке стипендии Президента РФ для молодых ученых и аспирантов, осуществляющих перспективные научные исследования и разработки по приоритетным направлениям модернизации российской экономики на 2015–2017 гг.; в рамках «Программы стратегического развития Петрозаводского государственного университета в 2013–2017 г.», (НИОКР № 115070110006, ИКРБС № 216022450003), проекта «Разработка технологии скрининговой диагностики неалкогольной жировой болезни печени у лиц с избыточным весом и метаболическим синдромом» № 9173ГУ/2015 от 15.12.2015 г. программы «У.М.Н.И.К.».

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Progression of nonalcoholic fatty liver disease (NAFLD) and more specifically one of its serious forms, nonalcoholic steatohepatitis (NASH), is accompanied by elevated concentrations of proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα), in the blood plasma and liver [1, 2]. Abnormally high TNFα promotes hepatic inflammation, lipid deposition and peroxidation, stimulates activation of Kupffer cells and hepatocyte apoptosis, and leads to insulin resistance [3]. As plasma TNFα levels go back to normal, the liver function recovers [4, 5].

Proteins belonging to the TNF family exert their biological effects by interacting with TNFR superfamily receptors [6]. TNFα-binding receptors (mbTNFR) are represented by two types of transmembrane proteins: mbTNFRI and mbTNFRII. The intracellular region of mbTNFRI carries a death domain absent in mbTNFRII. Once activated, the death domain triggers either apoptosis or necroptosis [7]. Another type of TNFα receptors are soluble sTNFRs, a product of mbTNFR ectodomain shedding mediated by ADAM metalloproteinases [8]. sTNFRs bind to TNFα and act as mbTNFR antagonists preventing activation of TNFα-signaling pathways. Low concentrations of soluble TNFα receptors can be found in the blood serum and urine of healthy individuals. Patients with chronic viral hepatitis [9], cirrhosis [10], or NAFLD [11, 12] have elevated levels of circulating TNFR, which indicates inflammation and activation of T-cell immunity, in particular $CD8^+$ T-cells that express metalloproteinase ADAM-17 [8]. It is hypothesized that both levels and ratio of soluble to membrane-bound TNFα receptors play a significant role not only in inducing hepatocyte death and damage to the liver, but also in the regeneration and homeostasis of this organ [13, 14, 15, 16]. It appears that the ratio of soluble to membrane-bound TNFRI and TNFRII largely determines the intensity of immune response and inflammatory reactions. It has been shown that mutations in the *TNFRSF1A* and *TNFRSF1B* genes affect sTNFRI and sTNFRII concentrations in the blood plasma and the number of mbTNFRI and mbTNFRII proteins on the surface of innate immunity cells [17]. Therefore, we can hypothesize that polymorphisms of genes coding for TNFα receptors may substantially contribute to the etiology and pathogenesis of liver diseases, including NAFLD. At present, attempts are made to establish an association between polymorphic variants of TNFR-encoding genes and NAFLD. The data is still scarce, describing mostly a link between *TNFRSF1A* or *TNFRSF1B* polymorphisms and biliary cirrhosis, alcoholic liver disease and hepatocellular carcinoma [18, 19, 20]. Associations between polymorphisms of TNFα receptor genes and NAFLD are hardly reported in the literature. That said, we decided to investigate how *TNFRSF1A* and *TNFRSF1B* polymorphisms contribute to the development of NAFDL in Karelian residents.

METHODS

Venous blood sample collection was aided by the Department of Propedeutics of Internal Diseases and Hygiene (Institute of Medicine, Petrozavodsk State University) and the Laboratory for Clinical Diagnostics of the Clinical Hospital at Petrozavodsk Station (Russian Railways JSC). The study recruited 110 male and 132 female patients with NASH (242 patients in total) and 151 healthy individuals (64 males and 87 females). The healthy donors also underwent a medical checkup by the doctors of the Clinical Hospital at Petrozavodsk Station (Russian Railways JSC). All participants were divided into 2 groups: healthy controls with no clinical symptoms of NAFLD (mean age of 48.04 ± 2.26 years) and patients with NASH (mean age of 50.14 ± 2.46 years). The age did not differ significantly between the groups (U = 132.5; p = 0.637). The study included individuals of both sexes who gave informed consent to participate. Among other general criteria for inclusion were: Karelian residency, negative HBsAg and hepatitis C antibody tests (no chronic viral hepatitis), the absence of alcoholic, drug-induced or autoimmune liver diseases confirmed by medical history and clinical or laboratory tests. The main group included patients with a first-time diagnosis of mild to moderate NASH (prior to treatment). Exclusion criteria for both groups were: infectious or inflammatory diseases within a month before the study, pregnancy or lactation, smoking, diabetes mellitus, body mass index ≥ 30 kg/m^2, drug therapy, intake of hepatotropic drugs. The diagnosis was established based on standard clinical, laboratory, instrumental and histological tests. The following blood parameters were evaluated: ALT, AST, and ALP (measured on the RandomAccessF-15 analyzer by BioSystems, Spain). Ultrasound scans revealed enlarged liver and increased parenchymal echogenicity in all patients with NASH. In some cases, the diagnosis was confirmed by liver biopsy.

Prior to drug therapy, 10 ml of venous blood were collected into EDTA-containing vacuum test tubes, of which 250 μL were used for DNA extraction. Some venous blood was used to obtain 200 μL plasma samples for measuring TNFα concentrations. The remaining blood volume was used for biochemistry tests.

The study was approved by the Committee on Medical Ethics of Petrozavodsk State University and Ministry of Health and Social Development of the Republic of Karelia (Protocol 39 dated November 15, 2017).

TNFα concentrations were measured in randomly selected blood plasma samples by ELISA using the Human TNFα Platinum ELISA kit (eBioscience, Austria). It total, 30 plasma samples of healthy donors (mean age of 49.11 ± 1.81 years) and 60 samples of patients with NASH (mean age of 49.95 ± 2.74 years) were tested; male and female samples were equally represented. The age did not differ significantly between the groups (U = 181.5; p = 0.535). Optical density of the solution was measured on the microplate reader Sunrise (Tecan, Austria) at 450 nm wavelength and 620 nm reference wavelength.

DNA was extracted from the peripheral blood on microcolumns using the K-Sorb kit (Syntol, Russia). Quality and quantity of the obtained DNA were evaluated on the SmartSpec spectrophotometer (Bio-Rad, USA).

To amplify the region of the *TNFRSF1A* gene harboring position 339 (rs767455), the following primers were used: forward 5'agtggctgaggttaggac3' and reverse 5'ctatgcccgagt ctcaac3' described in [21]. To amplify the region of the *TNFRSF1B* gene harboring position 587 (rs1061622), the following primers were used: forward 5'gcacacatcgtcactctc3' and reverse 5'aaggagtgaatgaatgagac3'described in [21]. Polymerase chain reaction (PCR) was carried out in the iCycler iQ5 (Bio-Rad, USA) using a reaction mix by Evrogen, Russia. PCR products containing rs767455 were incubated with 1 unit Bse1 I restriction endonuclease (SibEnzyme, Russia) for 3 hours at 65 °C. PCR products containing rs1061622 were incubated with 1 unit Fat I restriction endonuclease (SibEnzyme, Russia) for 1 hour at 55 °C. Then DNA fragments were separated in 1.5% agarose gel using the tris-acetate buffer.

The obtained data were processed in Statgraphics 2.1. Differences in allele and genotype frequencies between the two groups were assessed using the χ^2 test; differences in biochemical parameters were assessed using the nonparametric Mann–Whitney–Wilcoxon U test. The latter was employed because distribution in the groups was not normal.

To assess how different genotypes affected blood biochemistry, the Kruskal–Wallis test was used. To estimate the risk of developing NASH, we calculated the odds ratio (OR) and the 95% confidence interval (CI) [22]. Differences were considered significant at $p < 0.05$.

RESULTS

Figures 1 and 2 show electrophoresis of rs767455-and rs1061622- containing PCR products after restriction digest.

TNFRSF1AT>C (rs767455) allele and genotype frequencies have been analyzed in patients with NASH and healthy controls.

The datasets were tested for deviations from the Hardy-Weinberg equilibrium. Both healthy controls and patients with NASH demonstrated deviations for allele and genotype frequencies ($\chi^2 = 8.25$ (df = 2, $p < 0.05$), $\chi^2 = 21.64$ (df = 2, $p < 0.05$), respectively).

Table 1 shows that frequencies of T>C (rs767455) alleles and genotypes did not differ between patients with NASH and healthy donors.

We have also analyzed the frequencies of *TNFRSF1B* 587T>G alleles and genotypes in patients with NASH and healthy controls.

The two studied groups did deviate from the Hardy-Weinberg equilibrium ($\chi^2 = 0.30$ (df = 2, $p > 0.05$), $\chi^2 = 4.16$ (df = 2, $p > 0.05$) for healthy donors and patients with NASH, respectively).

Table 2 shows that *TNFRSF1B* 587T>G allele and genotype frequencies differed between the healthy donors and patients with NASH. The G allele was far more frequent in patients with NASH than in healthy individuals. Carriers of the G allele are at a higher risk of developing NASH (OR = 4.83; 95% CI: 2.72–8.57).

We have also assessed the effect of the *TNFRSF1B* polymorphism (rs1061622) on liver function tests and plasma TNFαlevels (Table 3). No significant differences were observed regarding the studied parameters between carriers of two different genotypes in the compared groups. The genotype did not have any effect on blood biochemistry both in patients with NASH and healthy controls ($p > 0.05$).

DISCUSSION

We have attempted to establish an association between two polymorphisms rs767455 and rs1061622 of genes *TNFRSF1A* and *TNFRSF1B*, respectively, and susceptibility to NASH. According to the literature, these polymorphisms are associated with a few inflammatory diseases and abnormal levels of TNFα in the blood plasma [23]. The rs767455 polymorphism of gene *TNFRSF1A* is a synonymous mutation at position 36 of exon 1. Synonymous mutations are known to disrupt mRNA splicing, alter mRNA structure and affect protein folding [24]. It has been shown that adenine to guanine substitution at position 36 of *TNFRSF1A* leads to a CCA to CCG codon change, disrupting translation [25]. In combination with other *TNFRSF1A* mutations (haplotype T-A-T at rs4149570-rs767455-rs1800692), it leads to the reduced abundance of exon 2-skipping products [26]. We have not established an association between the rs76 7455polymorphismandsusceptibilityto NASH in the study participants. However, we have discovered an association between the G allele carriership (rs1061622, *TNFRSF1B*) and the risk of this disease.

Thers1061622 polymorphism of the *TNFRSF1B* gene is a thymine to guanine substitution at position 587 of exon 6 that leads to a methionine to arginine amino acid substitution at position 196 of the protein's transmembrane domain, near the site of proteolytic cleavage by ADAM metalloproteases. This mutation affects ectodomain shedding (cleavage of the intracellular fragment of the transmembrane protein and its release into the extracellular matrix). Some researchers have shown that TT (Met196) genotype carriers have lower levels of sTNFRII than those with the Arg196 receptor variant [27]. Other authors report that carriers of TT+TG genotypes at this locus have higher levels of sTNFRII in the blood plasma than donors with the GG genotype [28].

Thus, the rs1061622 polymorphism can alter the ratio of membrane-bound to soluble TNFRII both in health and inflammation. Patients with liver diseases have elevated levels of sTNFRI and sTNFRII in the blood plasma and liver that positively correlate with disease severity [10, 12, 29, 30]. However, the role of increased ectodomain shedding of TNFα receptors in inflammation is not absolutely clear. Elevated concentrations of sTNFRII accompanied by reduced number of mbTNFRII on cell surface can trigger mbTNFRI-mediated signaling pathways leading to apoptosis [7]. Besides, soluble TNFR can act as physiological attenuators of TNFα activity, competing for the ligand with membrane-bound receptors. However, it appears that soluble receptors are capable of stabilizing and preserving circulating TNFα and thus act as its agonists [31].

The Met196 andArg196 variants of TNFRII differ in their ability to mediate TNF signaling and trigger apoptosis or necroptosis. Epithelial HeLaS3 cells transfected with the pcDNA3.1 plasmid containing the Arg196 allele of *TNFRII* demonstrated reduced activity of the nuclear factor kB and poor recruitment of TRAF2 upon stimulation with recombinant TNFα [32]. Subsequent activation of TNFRI signaling pathway in these cells induced apoptosis while in the cells transfected with the plasmid containing the wild type Met196, survival rates were better. Importantly, NASH is accompanied by the

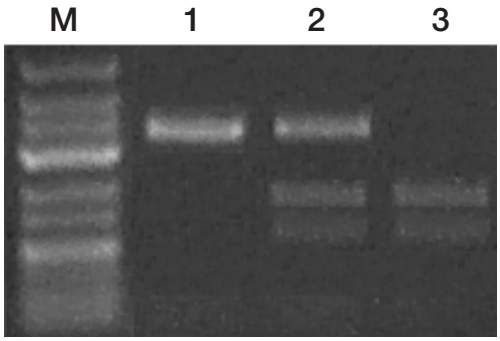

Fig. 1. Electrophoresis of rs767455-containing PCR products after restriction digest: M — Thermo Scientific GeneRuler Low range DNA Ladder, 1 — genotype CC (330 bp), 2 — genotype TC (330, 184 and 146 bp), 3 — genotype TT (184 and 146 bp)

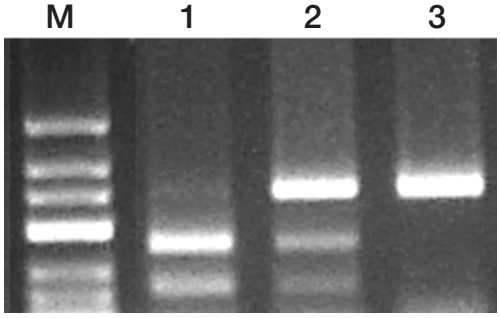

Fig. 2. Electrophoresis of rs1061622-containing PCR products after restriction digest: M — Thermo Scientific GeneRuler Low range DNA Ladder, 1 — genotype TT (235 and 144 bp),2 — genotype TG (379, 235 and 144 bp), 3 — genotype GG (379 bp)

Table 1. Distribution of *TNFRSF1AT*>C (rs767455) alleles and genotypes in patients with NASH and healthy control

Alleles and genotypes	Controls (n = 131)	Patients with NASH (n = 242)	χ^2
T	119 (0.45)	229 (0.47)	0.24 (df = 1, p > 0.05)
C	143 (0.55)	255 (0.53)	
TT	35 (0.26)	72 (0.30)	0.61 (df = 2, p > 0.05)
TC	48 (0.37)	85 (0.35)	
CC	48 (0.37)	85 (0.35)	

Table 2. Distribution of *TNFRSF1BT*>G (rs1061622) alleles and genotypes in patients with NASH and healthy controls

Alleles and genotypes	Controls (n = 151)	Patients with NASH (n = 133)	χ^2
T	206 (0.68)	116 (0.44)	16.60 (df = 1, p < 0.05)
G	96 (0.32)	150 (0.56)	
TT	69 (0.46)	20 (0.15)	37.07 (df = 2, p < 0.01)
TG	68 (0.45)	77 (0.58)	
GG	14 (0.09)	36 (0.27)	

Table 3. Liver function parameters in healthy and diseased *TNFRSF1B587T*>G (rs1061622) carriers

Parameter	Controls		Patients with NASH	
Genotype	TT (n = 14)	TG + GG (n = 16)	TT (n = 20)	TG + GG (n = 40)
ALT, un/l	17.29 ± 2.05 (17.05)	18.31 ± 2.34 (18.27)	59 36 ± 8.53 (45.90)	65.56 ± 8.68 (48.30)
AST, un/l	24.36 ± 2.64 (19.50)	22.08 ± 2.72 (19.09)	51.22 ± 9.65 (41.05)	51.51 ± 6.27 (35.60)
ALP, un/l	118.42 ± 10.82 (117.46)	123.51 ± 17.14 (125.48)	218.00 ± 18.70 (210.00)	214.26 ± 11.28 (197.00)
TNFα, pg/ml	5.53 ± 1.38 (4.69)	4.82 ± 0.39 (5.08)	6.09 ± 0.43 (5.83)	6.36 ± 0.27 (6.27)

Note: data are represented as mean and error mean (M ± m). The median is shown in brackets.

death of hepatocytes [33]. We hypothesize that the rs1061622 polymorphism of the *TNFRSF1B* gene contributes to the development and progression of NASH through activation of signaling pathways that induce hepatic cell death.

The rs1061622 polymorphism affects the levels of proinflammatory cytokines, which provides another explanation of its involvement into the etiology and pathogenesis of NASH[34]. We have studied plasma concentrations of TNFα in healthy and diseased carriers of different alleles and genotypes to reveal no significant differences between the groups. However, we cannot claim the absence of any effect of the *TNFRSF1B* polymorphism rs1061622 on TNFα levels because of a small sample size (especially true for the controls) and some other factors that may affect this parameter. Therefore, the role of this polymorphism in the development of NASH needs to be further investigated.

CONCLUSIONS

No association has been found between the rs767455 T>C *TNFRSF1A* polymorphism and the development of NASH in Karelian residents. We have however discovered an association between the rs1061622 T>G *TNFRSF1B* polymorphism and the disease. This polymorphic marker can be implicated in the genetic predisposition to NASH among the residents of Karelia.

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PREVALENCE OF ICHTHYOSIS VULGARIS AND FREQUENCY OF *FLG* R501X AND 2282DEL4 MUTATIONS IN THE POPULATION OF THE ROSTOV REGION

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Ichthyosis vulgaris (IV), a serious skin condition that runs in families, is actively studied worldwide. In this work we aimed to evaluate the prevalence of IV and frequency of two *FLG* mutations R501X and 2282del4 in the population of the Rostov region. Our genetic epidemiology study of hereditary monogenic disorders covered a total of 497,460 residents of 12 districts to identify 230 separate nosological entities. In the course of the analysis, we calculated the prevalence of IV per district and in the entire region and compared our findings with the results of earlier studies. The average prevalence of IV in the Rostov region was 1:5,025, which is consistent with the average prevalence of the disease across Russia (1:5,151). Tselinsky and Millerovsky districts demonstrated the highest prevalence rates (1:1,942 and 1:2,032, respectively). To evaluate the frequency of two *FLG* mutations R501X and 2282del4, we assayed the samples of 58 patients with IV and 127 healthy unelated controls by PCR followed by the restriction fragment length polymorphism analysis. In patients with IV, the frequency of the 2282del4 mutation was 48.28%, which is in line with European figures and also 30 times higher than in the controls (1.58%), suggesting the pathogenicity of the mutation. The R501X mutation was not identified both in patients with IV and healthy controls.

Keywords: ichthyosis vulgaris, mutations R501X and 2282del4, *FLG* gene, prevalence rates, Rostov region

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РАСПРОСТРАНЕННОСТЬ ВУЛЬГАРНОГО ИХТИОЗА И ЧАСТОТА МУТАЦИЙ R501X И 2282DEL4 В ГЕНЕ *FLG* В РОСТОВСКОЙ ОБЛАСТИ

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Вульгарный ихтиоз (ВИ) — серьезное поражение кожного покрова, передающееся по наследству и активно изучающееся специалистами всего мира. Целью работы стала оценка распространенности ВИ и частот мутаций R501X и 2282del4 в гене *FLG* у населения Ростовской области. Проведено генетико-эпидемиологическое исследование широкого круга моногенной наследственной патологии у населения в 12 районах. Изучена частота мутаций R501X и 2282del4 в гене *FLG* у больных ВИ и в контрольной группе (здоровой популяции). Суммарная численность обследованных составила 497 460 чел. Выявлено 230 нозологических форм. Рассчитана распространенность ВИ в каждом районе и по области в целом, проведен сравнительный анализ с ранее обследованными популяциями. Средняя распространенность ВИ в области составила 1:5025 и соответствует среднему значению по России, равному 1:5151. Определено накопление ВИ в Целинском (1:1942) и Миллеровском районах (1:2032). Методом полимеразной цепной реакции (ПЦР) с последующим анализом полиморфизма длин рестрикционных фрагментов изучена частота мутаций R501X и 2282del4 в гене *FLG* у больных ВИ (58 пациентов) и в контрольной группе (127 здоровых неродственных индивидов). Частота мутации 2282del4 в гене *FLG* среди пациентов с ВИ составила 48,28% (соответствует данным по Европе), в контрольной группе 1,58%. Сравнительный анализ частоты мутации 2282del4 в гене *FLG* в двух группах показал, что среди пациентов с ВИ частота мутации (48,28%) 2282del4 в гене *FLG* в 30 раз превышает частоту в контрольной группе (1,58%), что косвенно подтверждает патогенное действие мутации в группе больных ВИ. Мутация R501X не выявлена у больных ВИ и в контрольной группе.

Ключевые слова: вульгарный ихтиоз, мутации R501X и 2282del4, ген *FLG*, генетико-эпидемиологическое исследование, Ростовская область

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Ichthyosis vulgaris (IV, OMIM #146700, also known as ichthyosis simplex) is the most common and relatively mild type of hereditary nonsyndromic ichthyosis. Usually, its clinical symptoms start to show as early as one month after birth and include dry and flaky skin, plate-like or branny greyish scaling especially prominent on the abdomen, chest and extensor surfaces, follicular keratosis and hyperlinear palms and soles [1, 2].

The disease is caused by mutations in the filaggrin gene (*FLG*, OMIM *135940). This gene is part of the epidermal differentiation complex, a gene cluster on the short arm of chromosome 1q21. It consists of three exons and two introns; exon 3 is responsible for protein synthesis [2].

According to the classic research study conducted in Great Britain, the different forms of ichthyosis affect 1 in every 3,665 individuals: ichthyosis vulgaris strikes 1 in 5,300 individuals, X-linked ichthyosis occurs in 1 per 6,190 male population and autosomal recessive forms – in 1 per 300,000 population. However, the actual frequency of the disease was estimated to be even higher: of 6,051 schoolchildren surveyed by the researchers 24 had the autosomal recessive form, which brings the frequency to 1:250 [3].

European DNA studies conducted in patients with IV have revealed that the most common *FLG* mutations associated with the disease are R510X and 2282del4. According to different estimates, their frequencies vary from 30% to 67.3% [4]. In a study conducted in 2006, slight differences were observed in the frequencies of these mutations between the Irish (4.1% and 0.5%, respectively), Scottish (2.1% and 1.2%, respectively) and American (2.4% and 1.1%, respectively) populations [5]. Another study reported a higher frequency of R501X and 2282del4 genetic variants (2.9% and 1.9%, respectively) in Scottish schoolchildren [6]. In Western Austria, though, these two mutations were found to be identically frequent (1.4%) [7]. Perhaps, such discrepancy is to be blamed on the different approaches to patient screening used by the researchers.

Based on molecular genetic screening of patients with IV carried out in South-East Asia a few other *FLG* mutations were described, including 321delA, S2554X, 441delA, 1249insG, 7945delA, Q2147X, E2422X, and R4307X. The frequency of the 3321delA mutation is estimated to be as high as 52.31% in Asian patents with IV and only 4% in healthy controls. Mutations found in the European populations are rare in Asia [8]. So far, allelic heterogeneity associated with various hereditary disorders has been described for many different populations.

In the Russian city of Novosibirsk, the frequency of the *FLG* 2282del4 mutation is 3.8% [9]. In the Republic of Bashkortostan heterozygous carriers of this mutation account for 3.86% of the control group [10]. The frequencies of the R510X and 2282del4 mutations in the Ukrainian population are 2.1% and 1.0%, respectively [11].

To date, ichthyosis vulgaris is believed to be a semi-dominant disease [1, 5]. Smith et al. have shown that in patients with IV heterozygous for either R501X or 2282del4 genetic variants disease manifestations are mild, while patients homozygous for the R501X mutation and compound-heterozygous carriers of R501X/2282del4 develop severe clinical symptoms [5]. Also, a few authors have reported a dosage-dependent effect of *FLG* mutations [6].

The present study aimed to evaluate the prevalence of IV and frequency of the *FLG* R501X and 2282del4 mutations in the population of the Rostov region.

METHODS

Our genetic epidemiology study was carried out in 12 districts of the Rostov region, including Volgodonskoy, Dubovsky, Yegorlyksky, Zimovnikovsky, Krasnosulinsky, Matveevo-Kurganinsky, Millerovsky, Miasnikovsky, Rodionovo-Nesvetaysky, Tarasovsky, Tselinsky, and Tsimliansky. In total, 497,460 individuals were surveyed [12]. In our work, we relied on the guidelines for genetic epidemiology studies conducted over the period between 2000 and 2017 [13, 14]. Those guidelines were developed at the Research Center for Medical Genetics; they have been used as a basis for Russian epidemiologic research studies for over 30 years. The protocol includes a complete health assessment by different specialists, including a geneticist, pediatrician, dermatologist, ophthalmologist, neurologist, psychiatrist, etc. Using this protocol, up to 4,000 or 5,000 separate monogenic nosological entities can be identified in the course of field research.

Prevalence of ichthyosis vulgaris was calculated based on the ratio of the surveyed individuals to the individuals with IV. The obtained results were compared to the data from the genetic epidemiology studies conducted by the Research Center for Medical Genetics [13, 14] because the approaches to data acquisition described in the available literature vary considerably.

A tendency towards increasing incidence of IV in a few districts of the Rostov region was inferred using the F-distribution test ($\alpha < 0.001$) [15].

Table 1. Prevalence of ichthyosis vulgaris in the population of 12 districts of the Rostov region

District	Total size of population	Number of affected individuals	Prevalence
Volgodonskoy	23,542	5	1:4,708
Dubovsky	23,185	4	1:5,796
Yegorlyksky	36,098	13	1:2,777
Zimovnikovsky	38,071	2	1:19,036
Krasnosulinsky	77,847	0	0
Matveevo-Kurgansky	37,600	11	1:3,418
Millerovsky	75,201	37	1:2,032
Miasnikovsky	37,432	5	1:7,486
Rodionovo-Nesvetaisky	30,760	0	0
Tarasovsky	45,575	0	0
Tselinsky	38,830	20	1:1,942
Tsimliansky	33,319	2	1:16,660
Total	497,460	99	1:5,025

Molecular genetic screening of samples for the R501X and 2282del4 mutations in the *FLG* gene was performed in the Laboratory for Genetic Epidemiology, Research Center for Medical Genetics.

To determine the frequencies of 2282del4 and R501X mutations, we collected blood samples from two groups of participants. The first group included 58 affected individuals with IV identified in the course of our epidemiology study conducted in 12 districts of the Rostov region. The second group included 127 unrelated healthy controls who had been living in Millerovsky or Volgodonskoy districts for at least three generations (71 and 56 individuals, respectively). The participants gave written informed consent covering such aspects of the study as voluntary participation, medical examination, blood collection and publication of the results. In the case of underaged children, informed consent was obtained from their parents. A few families insisted that some of their diseased members should not be examined. The study was approved by the Ethics Committee of the Research Center for Medical Genetics (Protocol No. 5 dated December 20, 2010).

Genomic DNA was isolated from peripheral blood leukocytes using the DIAtom DNAPrep100 kit according to the manufacturer's protocol. Screening for c.2282del4 and R501X mutations was done by PCR followed by the analysis of restriction fragment length polymorphisms. Mutation frequencies were compared between healthy and diseased individuals using Fisher's exact test [15].

RESULTS

In the course of our genetic epidemiology study conducted in 12 districts of the Rostov region, we identified 230 separate hereditary monogenic nosological entities. In the present work, we focus on the prevalence and molecular basis of ichthyosis vulgaris, one of ichthyosis form. In total, we have identified 99 patients with IV coming from 49 nuclear families.

IV prevalence was calculated for each district and the Rostov region in general (Table 1). The analysis demonstrates that most patients with IV reside in Millerovsky (37) and Tselinsky (20) districts, while Tarasovsky, Rodionovo-Nesvetaysky, and Krasnosulinsjy districts are free of IV.

IV is the most prevalent in Tselinsky (1:1,942) and Millerovsky (1:2,032) districts. Figures showing IV prevalence in the European part of Russia are provided in Table 2.

Our study shows that prevalence of the disease in the Rostov region corresponds to the average prevalence of IV (1:5,151) in the European part of Russia. The lowest prevalence (1:88,210) was registered in the Bryansk region, while the highest prevalence (1:2,130) was observed in the Republic of Mari El [13, 14]. In our study, a tendency towards increasing incidence was observed in Millerovsky and Tselinsky districts ($\alpha < 0.001$) as compared with average figures on the Rostov region and Russia in general.

Of all surveyed individuals with IV, 58 patients from 38 families agreed to take a DNA test, which confirmed that 28 individuals were heterozygous for the 2282del4 mutation, making 48.28% of all examined unrelated participants with IV. All tests came negative for the R501X mutation. In the group of healthy controls, two (1.58%) were heterozygous for the 2282del4 mutation. Healthy residents of Millerovsky district also tested positive for this mutation, but negative for R501X.

DISCUSSION

In the course of our epidemiology study, we discovered that clinical manifestations of the disease vary markedly from mild to severe both between and within families. All patients started showing symptoms shortly after birth. The symptoms included mucosal dryness, dry scalp and hair, striate patterns on palms and soles, mildly to severely flaky skin, with small or large greyish plate-like scales localized on the abdomen and lower legs. Some patients had scaly skin on the chest and extensor surfaces; follicular keratosis was also observed. Scaling was present on the scalp and along the vermilion zone.

About half of all patients with IV reside in Millerovsky district or were born there (37 patients from 16 families). They come from the Russian families who have been living in the area for at least three generations. We have identified 50 affected individuals in those families (4 died, 7 moved to other areas), which suggests the founder effect. Using the pedigree analysis, we were able to establish kinship between some of the surveyed families. For example, 5 of 16 families (16 patients with IV) living in the khutor of Gray-Voronets share the same ancestor who was born in the late 19th century, in 1896. Their pedigree is presented in Fig. 1.

The analysis demonstrated that the frequency of the *FLG* 2282del4 mutation is lower than in the European population but consistent with the results obtained in Novosibirsk [7, 9].

Table 2. Prevalence of ichthyosis vulgaris in the surveyed regions of the European part of Russia

Regions of the Russian Federation	Total size of population	Prevalence
Rostov region	497,460	1:5,025
Arkhangelsk region	40,000	1:6,667
Bryansk region	88,210	1:88,210
Kirov region	286,616	1:3,675
Kostroma region	444,476	1:8,386
Tver region	76,000	1:12,667
Krasnodar region	426,700	1:8,534
Republic of Adygea	101,800	1:5,358
Republic of Bashkortostan	250,000	1:6,944
Republic of Mari El	276,900	1:2,130
Republic of Tatarstan	268,894	1:7,469
Udmurt Republic	267,655	1:6,225
Chuvash Republic	264,490	1:3,149
Russia (average)	2791,741	1:5,151

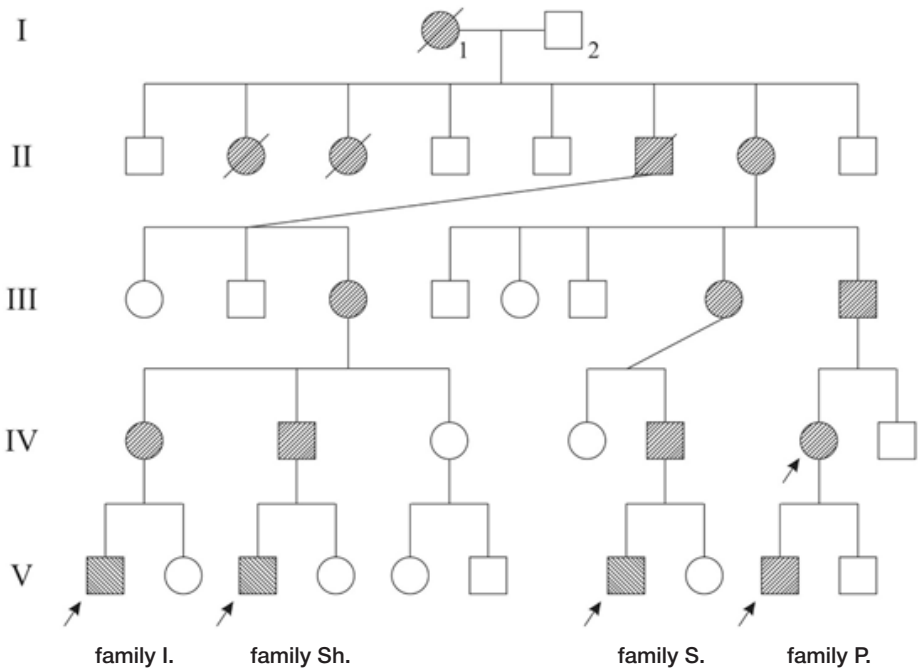

Fig. 1. Pedigree of the family with ichthyosis vulgaris

Our study has revealed significant differences (Fisher's $p < 0.00001$) in the frequencies of *FLG* 2282del4 between individuals with IV (0.483, 28/58) and healthy controls (0.016, 2/127).

CONCLUSIONS

On average, ichthyosis vulgaris occurs in 1 of 5,025 surveyed residents of the Rostov region. Its prevalence varies from 0 in Tarasovsky, Rodionovo-Nesvetaysky and Krasnosulinsky districts to 1:1,942 in Tselinsky and 1:2,032 in Millerovsky districts. The prevalence of the disease in the Rostov region is consistent with the average figures across Russia (1:5,151) [13, 14]. The founder effect is observed for the majority of the surveyed families. Among patients with IV the 2282del4 mutation is 30 times more frequent (48.28%) than among the controls (1.58%), which suggests its pathogenicity. In our study, the R501X mutation was absent in both diseased individuals and healthy controls.

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CLINICAL MANIFESTATIONS AND IMMUNOLOGY OF NUMMULAR ECZEMA

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Being a relatively common chronic skin condition with understudied pathogenesis, nummular eczema captures attention of medical researchers. The aim of this wok was to describe clinical manifestations of the disease, revise criteria for its accurate diagnosis and understand the role of malfunctioning components of the adaptive and innate immunities in triggering the inflammatory response. Using high-sensitivity ELISA assays, we assessed the cytokine profiles, determined the levels of adhesion molecules and the affinity of serum antibodies in 51 patients with nummular eczema. The immune profiles of the patients were dominated by proinflammatory interleukins, being deficient in regulatory cytokines. The relative abundance of mononuclear $CD50^+$ and $CD54^+$ cells was increased. Natural antibacterial immunity was weakened by the production of low-affinity serum antibodies. Based on our findings, we have established criteria for the differential diagnosis of nummular eczema and described a contribution of both regulatory and effector immunity components to the abnormal immune homeostasis. We conclude that the discovered defects of the humoral regulation and non-specific resistance in patients with nummular eczema are pathogenic and determine the course of the disease.

Keywords: nummular eczema, clinical manifestations, interleukins, IL-10, IL-12, IL-17, proinflammatory cytokines, adhesion molecules, antibody affinity, diagnostic criteria, indirect immunofluorescence

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КЛИНИКО-ИММУНОЛОГИЧЕСКИЕ ХАРАКТЕРИСТИКИ НУММУЛЯРНОЙ ЭКЗЕМЫ

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Широкая распространенность, длительное хроническое течение заболевания и недостаточно изученный патогенез определяют интерес к проблеме нуммулярной экземы. Целью настоящего исследования было изучение особенностей клинического течения экземы, клинических критериев для постановки диагноза и определение роли нарушений основных систем врожденного и адаптивного иммунитета в развитии воспалительного процесса. С использованием высокочувствительного ИФА для определения цитокинового статуса, молекул адгезии и аффинности сывороточных антител обследован 51 больной нуммулярной экземой. Установлено, что у больных нуммулярной экземой наблюдались изменение соотношения выработки интерлейкинов с преобладанием провоспалительных вариантов, а также дисбаланс в системе иммунорегуляторных цитокинов. Обнаружен значительный рост относительного количества мононуклеарных клеток $CD50^+$ и $CD54^+$. Выявлены нарушения естественного антибактериального иммунитета, которые обусловлены продукцией низкоаффинных сывороточных антител. Проведенная работа позволила установить критерии дифференциальной диагностики нуммулярной экземы. Определены особенности нарушений со стороны иммунного гомеостаза, касающиеся как иммунорегуляторных, так и иммуноэффекторных звеньев. Обнаруженные нарушения в системе гуморальной регуляции иммунного ответа и состоянии неспецифической резистентности у больных нуммулярной экземой имеют патогенетическое значение и определяют особенности клинического течения данного заболевания.

Ключевые слова: нуммулярная экзема, клинические проявления, интерлейкины IL-10, IL-12 и IL-17, молекулы адгезии, аффинность антител, диагностические критерии, непрямая иммунофлюоресценция

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Eczema, a common skin condition, remains a therapeutic challenge due to its chronicity, frequent and continuous relapses, understudied pathogenesis and complicated treatment [1–3]. One of eczema types, nummular dermatitis, has been recently reported to become increasingly incident and resistant to standard therapies. To date, eczema is believed to be a polyetiologic disease caused by a variety of overlapping exogenous and endogenous factors [4–6]. Among the major contributors that trigger eczema development and maintain chronic inflammation are endocrine, metabolic and neurohumoral factors and genetic predisposition [7–9]. Some authors point to the suppressed immunoregulatory function, deficit of regulatory T cells and increased activity of humoral immunity that accompany nummular eczema, suggesting that abnormalities in both cellular and humoral components of the immune system may have a role in the development of this disease and its relapses [10–13].

It has been established that non-specific resistance and functional activity of neutrophils are often compromised in patients with nummular eczema; their complement system is dysfunctional, lipid peroxidation is aggravated and the compensatory antioxidant system is hyperactive [14, 15]. On

the whole, though, cytokine profiles of such patients have hardly been investigated. The literature is scarce [10] and does not give a full picture of how cytokines are involved in this pathology. With that in mind, we decided to study how proinflammatory IL-17 and regulatory IL-10 and IL-12 behave in patients with nummular eczema.

Our aim was to describe clinical manifestations specific for nummular eczema and understand the roles of defective interleukin-based modulation of the immune response and non-specific resistance in the development of this condition.

METHODS

The study was carried out at the facilities of Cheryomushinsky Moscow Research and Clinical Center for Dermatology, Venereology and Cosmetology of Moscow Department of Healthcare. The study was approved by the local Ethics Committee of Pirogov Russian National Research Medical University, Protocol № 164 dated April 17, 2017. All patients gave informed consent to participate. The study was conducted in patients over 18 years of age who had developed clinical symptoms of nummular eczema more than 6 months before the study and had not been receiving immunosuppressive drugs in the last 6 months before the study. The exclusion criteria were severe chronic conditions, TB, syphilis, cancer, and refusal to participate.

The participants included 23 males and 28 females aged 18 to 64 years. The onset of the disease varied considerably, occurring on average 4.8 ± 0.4 years before the study.

In most cases, the first clinical symptoms appeared between 18 to 28 years of age. The most common triggers were household irritants (10 patients, or 19.6%), stress (9 patients, or 17.6%), and diet (6 patients, or 11.8%). Microbial contamination was observed in all participants and was chronic and recurrent in 44 patients (86.3%). The number of relapses varied from 2 to 8 per year, lasting from 2–3 weeks to 3 months. The symptoms were very pronounced, indicating acute inflammation. Twenty-one patients (41.2%) had lesions on upper and lower extremities (the back of the hands, soles, forearms and lower legs). Other patients (58.8%) had extensive cutaneous lesions on the back, shoulders, lateral body surfaces and abdomen. The disease started with itchy round-shaped erythematous spots with clear boundaries, miliary or vesicular papules, pustules, crusting and scaling. In rare cases patients developed microvesicles, followed by the formation of serous wells and oozing.

Using ELISA assays, we measured the levels of IL-10, IL-12 and IL-17 in the patients' blood serum. The tests were performed using the Proplan microplate washer (Picon, Russia), the SkyLine shaker (ELMI, Latvia) and the Uniplan spectrophotometer (Picon, Russia). Reagent kits were by Vector-Best, Russia.

Peripheral blood mononuclear cells were immunotyped by indirect immunofluorescence using ICO monoclonal antibodies. The relative antibody affinity was determined by solid phase immunoassays with different molar concentrations of sodium thiocyanate, which breaks the bonds in the antigen-antibody complex. The levels of soluble antigens sCD50 and sCD54 were measured by ELISA using monoclonal antibodies ICO-60 and ICO-184. The obtained concentrations were presented in arbitrary units per ml (U/ml) and compared to the average results of laboratory tests conducted in healthy individuals. The data were processed by variance analysis in Microsoft Excel. Mean arithmetic M and mean error m were computed. Significance of differences was assessed using Student's t-test. The differences were significant at $p < 0.05$ (95% probability).

RESULTS

The tests revealed elevated concentrations of IL-12 in the peripheral blood of patients with nummular eczema, which were as high as 159.8 ± 5.9 pg/ml in comparison with the reference values (32.1 ± 2.6 pg/ml). The IL-17 levels were elevated in all patients, reaching 6.1 ± 1.0 pg/ml, which was well above the reference range (0.22 ± 0.1 pg/ml). The IL-10 levels were as low as 4.1 ± 0.18 pg/ml, which is substantially below the reference values (14.1 ± 0.2 pg/ml; see the Table). The levels of sCD50 and sCD54 (196.5 ± 4.6 U/ml and 31.5 ± 2.8 U/ml, respectively) fell outside the reference range, but the difference was minor. Still, the relative quantities of mononuclear cells $CD50^+$ and $CD54^+$ (79.8 ± 9.9% and 81.8 ± 10.8%, respectively) in the blood serum of the participants were significantly above the norm (59.3 ± 7.8% and 60.2 ± 7.9%, respectively). The analysis of the immunoassay data showed reduced affinity of serum antibodies to the common antigenic determinant in 26 (51.1%) patients with nummular eczema. Normal affinity was observed in 25 (49.9%) patients. Reduction to 500–1000 units was observed in 16 (31.4%), reduction below 500 units – in 10 (19.7%) patients.

DISCUSSION

The symptoms of nummular eczema have changed significantly over the years complicating differential diagnosis and affecting diagnostic accuracy. The typical location of lesions is now different, their pattern tending to be infiltrative, with rare exudating vesicles or microerosions.

The conducted immunoassays have demonstrated that the onset and development of nummular eczema are accompanied by immunological events typical for acute inflammation. The literature [2] mostly reports abnormal production of IL-1, IL-2 and IL-6. Our study, however, provides a more objective picture of cytokine behavior in patients with nummular eczema. Specifically, we have established a multifold increase in IL-12 and IL-17, indicating a tendency of lymphocytes to differentiate more into Th1 than into Th0 cells, which probably results in intense production of proinflammatory cytokines and triggers acute inflammatory response in the dermis and epidermis. In turn, the lack of IL-10 leads to the immunoregulatory dysfunction and promotes pathological changes that determine the clinical symptoms of the disease. A huge increase in low-affinity antibodies downregulates the activity of the complement system and facilitates microbial opsonization, eliminating the bacterial threat. At the same time, low-affinity antibodies bind to the membrane receptors of regulatory T cells making them more sensitive to microbial antigens. The number of soluble adhesion molecules falls, while their membrane-bound forms

Table1. Cytokine concentrations in the blood serum of patients with nummular eczema

	IL-10 (pg/ml)	IL-12 (pg/ml)	IL-17 (pg/ml)
Patients with nummular eczema (n = 38)	4.1 ± 0.18*	159.8 ± 5.9*	6.1 ± 1.0*
Reference values	14.1 ± 0.2	32.1 ± 2.6	0.22 ± 01

Note: *significant at $p < 0.01$, as compared to the reference values.

become abundant, contributing to the increased migration of cells to the inflammation sites and promoting chronicity.

CONCLUSIONS

Our study reveals that these days the symptoms of nummular eczema start to show at a young age, the typical location of lesions is different, and exudating microvesicles, vesicular papules, microerosions, and oozing are rare.

We have demonstrated the role of imbalanced levels of cytokines and adhesion molecules in the formation of the pathologic immune response in patients with nummular eczema. Increased production of low-affinity antibodies suggests compromised natural antibacterial immunity.

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EXPRESSION OF STEROID HORMONE RECEPTORS IN THE TISSUE OF ENDOMETRIOMAS IN FIRST-TIME AND RELAPSING PATIENTS

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A molecular marker for the risk of endometriosis recurrence can help to customize a post-operative treatment plan for an individual patient. The aim of this study was to compare the expression of genes coding for estradiol (mER, ER_{α}, ER_{β}) and progesterone ($PGRmC_1$, mPR, PR-A, PR-B) receptors in first-time and relapsing patients with ovarian endometriosis. Our study included 94 women of reproductive age with ovarian endometriosis: 82 first-time and 12 relapsing patients. The expression of genes coding for steroid receptors was measured using reverse transcription polymerase chain reaction. Recurrent conditions were characterized by a change in the expression of estrogen receptors and unchanged expression of progesterone receptors. Expression of mER in the tissue of patients with first-time endometriosis was 15.09 ± 1.18. Patients undergoing recurrence demonstrated a 3-fold increase in mER expression (from 15.09 ± 1.18 to 44.45 ± 9.1). Also, in such patients ER_{β} expression was 5 times higher increasing from 11.71 ± 0.22, which is an average value for first-time patients, to 10.02 ± 3.81, while ER_{α} expression surged 7-fold from 10.47 ± 1.05 to 1.68 ± 0.55 ($p < 0.05$). Transcription of the studied receptors in the pathological tissue depended on the stage of the disease: in relapsing patients expression of estradiol receptors underwent some changes, while expression profile of progesterone receptors remained unchanged. Sensitivity of endometrial tissue to gestogens is clinically important and serves as a basis for a successful hormone-based relapse prevention.

Keywords: ovarian endometriosis, PCR, estradiol receptors, progesterone receptors

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ОСОБЕННОСТИ ЭКСПРЕССИИ ГЕНОВ РЕЦЕПТОРОВ СТЕРОИДНЫХ ГОРМОНОВ В ТКАНЯХ ПЕРВИЧНЫХ И РЕЦИДИВИРУЮЩИХ ЭНДОМЕТРИОДНЫХ ОБРАЗОВАНИЙ ЯИЧНИКОВ

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Для разработки индивидуального плана послеоперационного ведения пациенток необходим поиск молекулярно-фармакологического маркера принадлежности пациенток к группе риска рецидивирования эндометриоза яичников. Целью исследования было провести сравнительный анализ экспрессии генов рецепторов эстрадиола (mER, ER_{α}, ER_{β}) и прогестерона ($PGRmC_1$, mPR, PR-A, PR-B) в ткани первичного эндометриоидного образования яичников и при рецидиве заболевания. В исследование вошли 94 пациентки репродуктивного возраста с эндометриоидными образованиями яичников: 82 пациентки с первичным эндометриозом и 12 с рецидивом заболевания. Для определения экспрессии генов стероидных рецепторов использовали метод полимеразной цепной реакции с обратной транскрипцией (ОТ-ПЦР). При рецидиве заболевания в эндометриоидной ткани яичника выявлен сдвиг спектра эстрогеновых рецепторов, а уровень рецепторов прогестерона оставался неизмененным. Уровень экспрессии mER в ткани первичного эндометриоза составил 15,09 ± 1,18. Обнаружено увеличение экспрессии mER в 3 раза (с 15,09 ± 1,18 в первичной эндометриоидной ткани до 44,45 ± 9,1 в ткани с рецидивом эндометриоза), ER_{β} в 5 раз (с 11,71 ± 0,22 до 10,02 ± 3,81), а также снижение экспрессии рецептора ER_{α} в 7 раз (с 10,47 ± 1,05 до 1,68 ± 0,55) по сравнению с эндометриоидной тканью яичника при первичном заболевании ($p < 0,05$). Транскрипция рецепторов эндометриоидной ткани яичника зависит от стадии заболевания: при рецидивировании наблюдается изменение спектра рецепторов эстрадиола на фоне интактного рецепторного аппарата для прогестерона. Клиническое значение имеет сохранность чувствительности ткани к гестагенам как основа успешной противорецидивной гормональной терапии.

Ключевые слова: эндометриоз яичников, ПЦР, рецепторы эстрадиола, рецепторы прогестерона

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Endometriosis is a chronic benign estrogen-dependent condition [1, 2]. A tendency to recur and a negative impact on a woman's general health, quality of life and ability to work make endometriosis a clinically and socially significant disease. In patients receiving surgical treatment for endometriosis, cumulative recurrence rates are 6.4%, 10%, 19.9%, and 30.9% at 2, 3, 5, and 6 years after the surgery, respectively [3]. There is no consensus in the medical community on what triggers the recurrence of the disease, which complicates the search for biochemical markers that could be used to monitor the effect of treatment in first-time and relapsing patients [4].

Among the possible causes of the recurrence proposed in the literature is incomplete excision of endometriotic lesions during the surgery; it is still debatable, though, whether it is a true recurrence or a new primary disease. Hormone prevention therapy can alter the status of steroid receptors in the endometrium and affect the sensitivity of lesions to further treatment. Therefore, transcription profiles of steroid receptors can differ in first-time and relapsing patients. The aim of this work was to compare the expression levels of genes coding for membrane and nuclear estrogen (mER, ER_α, ER_β,) and progesterone (PGRMC1, mPR, PR-A, PR-B) receptors in the endometriotic tissue of first-time patients with ovarian endometriomas and patients with recurrences. This will help us understand whether progestins are a rational treatment choice for recurrent endometriosis.

METHODS

The study included 94 women of reproductive age diagnosed with endometriosis: 82 were first-time patients and 12 had a relapse. All patients gave informed consent to participate.

The patients diagnosed with endometriosis for the first time were included in the study if they were of reproductive age, their ultrasound scans suggested ovarian endometriomas, and the diagnosis was confirmed laparoscopically and histologically.

For relapsing patients, inclusion criteria were as follows: reproductive age, prior surgery for endometriosis, ultrasound scans suggestive of endometriomas that were later confirmed laparoscopically and histologically.

Patients with extragenital and genital comorbidities (uterine myoma, adenomyosis, ovarian tumors) and those who had undergone a hormone therapy before were excluded from the study.

All tests were carried out in the proliferative phase of the menstrual cycle.

Based on the histologically verified diagnosis, the women were divided into 2 groups: group I included 82 patients with ovarian endometriomas, group II consisted of 12 relapsing patients. The age of the participants varied from 18 to 44 years, averaging 31.4 ± 5.2 years. The age of the patients included in group I varied from 22 to 41 years, the mean age was 34.2 ± 5.3 years. In group II the mean age was 33.4 ± 6.9 years, implying that the groups were comparable in terms of age. Disease duration ranged from 1.5 to 5 years in group I and from 2 months to 1.5 years in group II. The most common complaint in both groups was pelvic pain (35 cases or 53% in group I and 6 cases or 67.4% in group II).

The frequency of menstrual disorders was significantly lower in group I than in group II: 25.6% vs. 14.7%, respectively. In group I, 35 (52.4%) women had a history of pregnancy; in group II such women made 45.5% (4 individuals). Infertility was diagnosed in 8 (65.7%) relapsing and 32 (25.9%) first-time patients. The percentage of women with somatic pathology in both groups was almost equal: 35.5% and 34.9% respectively.

Apart from general and pelvic examinations, the patients underwent a 2D Doppler ultrasound scan on VOLUSON-730 Expert (GEKretz, Zipf, Austria). The procedure was performed using the standard technique and an endovaginal probe (3.3–10.0 MHz). All patients underwent a laparoscopic surgery (equipment by Karl Storz, Germany). The excised tissue was subjected to the histological analysis. Expression of steroid receptors was also analyzed.

The endometriotic tissue samples were tested for the expression of genes coding for membrane (mER) and nuclear (ER_α и ER_β) estrogen receptors and membrane (mPR и PGRMC-1) and nuclear (PR-A и PR-B) progesterone receptors. Briefly, mRNA was extracted from the tissue samples using the RIBO-prep kit (AmpliSens, Russia) according to the manufacturer's protocol. cDNA was synthesized from an mRNA template through reverse transcription using the set of reagents REVERTA-L (AmpliSens, Russia). Real-time PCR was performed in iCycler iQ5 (BioRad, Germany) using the reagent kit 2.5x for RT-PCR with SYBR Green I detection. Genes coding for GAPDH (glyceraldehyde-3- phosphate-dehydrogenase) were used as control (see the Table).

Gene expression was measured using the values of $0.5^{-\Delta Ct}$ (to assess the differences between the groups) and $2^{-\Delta\Delta Ct}$ (to calculate relative quantities), where ΔCt = Ct (target gene) — Ct (GAPDH) and $\Delta\Delta Ct = \Delta Ct$ (first-time patients) – ΔCt (relapsing patients).

All data were processed in GraphPadPrism 5.0. Normality of distribution was tested using the Kolmogorov-Smirnov test. Independent variables were compared using the nonparametric Mann-Whitney U test. Differences were considered significant at $p \le 0.05$.

RESULTS

Estrogen and progesterone are major regulators of proliferation of the endometrium or tissues related to it in the reproductive female tract. Each steroid hormone regulates expression of hundreds of genes at different stages of the menstrual cycle [5]. Relative mRNA quantities for estrogen (mER, ER_α, ER_β) and progesterone (PGRmC1, mPR, PR-A, PR-B) receptors expressed in the endometriotic ovarian tissue obtained from first-time and relapsing patients are shown in Fig. 1.

The comparative analysis demonstrates that in relapsing patients estrogen expression levels in the endometriotic tissue are different from those in first-time patients: a 3-fold increase is observed for mER ($p = 0.0016$), a 5-fold increase — for ER_β ($p = 0.0024$), and a 7-fold drop — for ER_α ($p = 0.0001$).

Table. The sequence of synthetic oligonucleotides used to study expression of steroid receptors and GAPDH by RT-PCR [http://www.ncbi.nlm.nih.gov/protein/].

	Up	Low
GAPDH	gaa-ggt-gaa-ggt-cgg-agt	gaa-gat-ggt-gat-ggg-att-tcc
mER	agg-gac-aag-ctg-agg-ctg-ta	gtc-tac-acg-gca-ctg-ctg-aa
ER_α	tgc-caa-gga-gac-tcg-cta-ct	ctg-gcg-ctt-gtg-ttt-caa-c
ER_β	tca-gct-tgt-gac-ctc-tgt-gg	tgt-atg-acc-tgc-tgc-tgg-ac
PR-A	aaa-tca-ttg-cca-ggt-ttt-cg	tac-agc-atc-tgc-cca-ctg-ac
PR-B	gac-tga-gct-gaa-ggc-aaa-gg	cga-aac-tcc-agg-caa-ggt-gt
mPR	tgc-cct-gct-gtg-tga-tct-ta	gat-agc-tga-ggc-tcc-tgg-at
PGRmC1	tgc-cct-gct-gtg-tga-tct-ta	gat-agc-tga-ggc-tcc-tgg-at

Note: GAPDH — glyceraldehyde-3-phosphate-dehydrogenase, mER — membrane estrogen receptor, ER_α, ER_β — nuclear estrogen receptors, PR-A, PR-B — nuclear progesterone receptors, mPR, PGRmC1 — membrane progesterone receptors.

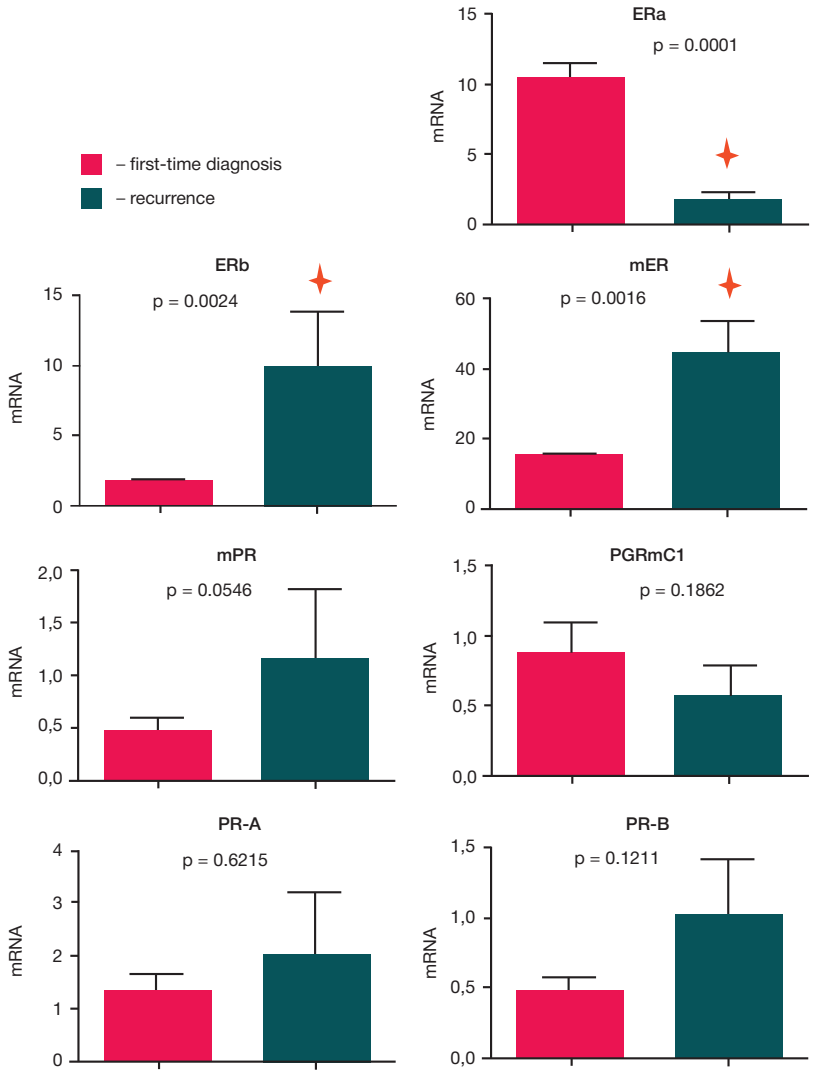

Fig 1. Expression of sex steroid receptors in the endometriotic tissue of patients with first-time diagnosed and recurrent endometriomas

Note: p — significance of differences (Mann-Whitney U); [mRNA] — expression of the target gene relative to GAPDH (0.5^ΔCt•100)

DISCUSSION

Estrogen and progesterone effects are largely mediated by the nuclear receptors of these hormones (ER and PR). Estrogen and progesterone interact with their receptors and activate them. Unlike ER and PR, membrane receptors of steroid hormones are understudied but still known to have an auxiliary role in modulating the expression of nuclear receptors [6].

Estradiol is a key regulator of endometrial tissue growth and survival; it also has a crucial role in inflammation and endometrial pain. Estradiol exerts its steroid growth-promoting effect on the endometrium when produced locally or transported to the endometrial tissue with blood. ER subtypes (ER_α и ER_β) are proteins with high affinity to estradiol and are coded for by separate genes. Although ER_α and ER_β are both present in the endometrium, ERα seems to be the major mediator of estrogen effect [7]. Elevated levels of ER_α are associated with the activation of target tissue proliferation, whereas ER_β limits transcriptional activity of ER_α, exerting an antiproliferative effect [8, 9].

Endometriotic tissue differs from the eutopic endometrium in terms of expression of steroid receptors. The literature reports (though scarcely) higher levels of ER_β, lower levels of ER_α, and very low levels of both PR isoforms, in particular PR-B, in the endometriotic tissue in comparison with the eutopic endometrium [10, 11]. In this study we employed RT-PC to quantify mRNA of nuclear receptors in primary endometrial and endometriotic stromal cells. For ER_α, mRNA levels were 7 times lower in the endometriotic stroma than in the endometrial stromal cells. For ER_β, mRNA levels were dramatically higher (~34 times) in the endometriotic stromal cells in comparison with the normal endometrial stroma where ER_β demonstrated a very low expression or the absence of it. For PR and PR-B, total mRNA levels were significantly lower in the endometriotic stroma than in the endometrial stromal cells [12]. Recently, there have been reports of elevated PR-A in patients with endometriosis regardless of the menstrual cycle phase [13]. It is known that ER_β activation leads to the transcription of a number of genes in the stromal cells of endometriotic lesions, such as the SGK1 kinase gene which maintains viability of stromal cells by inhibiting proapoptotic factors and through phosphorylation and inactivation of FOXO3a [14].

It our previous study we have demonstrated that endometriotic lesions are characterized by the increased expression of nuclear ER_β, in comparison with healthy ovarian tissue. Those findings are consistent with the literature [14]. Elevated ER_β levels inhibit ER_α expression in the endometriotic lesions, and the increased ER_β to ER_α ratio observed in

endometriotic stromal cells is proportionate to the degree of inhibition of progesterone receptor expression and the increase in cyclooxygenase-2 mRNA levels.

In the present study, we do not discriminate between different histological subtypes of the endometriotic tissue in relapsing patients. These conditions can lead to local inflammation and resistance to progestins [15]. We have not found any changes in the expression of genes coding for membrane and nuclear progesterone receptors that could accompany changes in the expression profiles of estrogen receptors.

Our study demonstrates that ER_β mRNA levels (perhaps owing to the abnormal hypomethylation of its promoter) are significantly higher in the endometriotic tissue in relapsing than in first-time patients. At the same time, ER_α mRNA concentrations are lower in relapsing patients. No reliable reduction in the expression of progesterone receptor genes has been observed in our study accompanying the changing estrogen receptor profiles, therefore, sensitivity of the endometriotic tissue to exogenous progestins is retained in relapsing patients.

Considering the above, changes in the expression profiles of estrogen receptors in patients with recurrent endometriosis can be reflective of the aggravating estrogen-dependent processes in the endometrium. It is known that the endometriotic stromal cell contains a full pack of genes involved in a steroidogenic cascade sufficient for converting cholesterol to estradiol [16]. Besides, the endometriotic tissue is characterized by the aberrant expression of aromatase, which stimulates local synthesis of estrogens, some cytokines and metalloproteinases. Moreover, deficiency of type 2 17β-hydroxysteroid dehydrogenases converting 17β-estradiol to a not so active estrone contributes to the accumulation of active estrogens in the tissue [17].

In our previous work we have demonstrated the differences in the expression of steroid receptors between the healthy ovarian tissue and the endometriotic tissue. In endometriosis, ER_β expression (2.3 ± 0.6) was 2.4 times higher ($p = 0.022$) than the healthy tissue (0.8 ± 0.3); PR-A expression in the endometriotic tissue (5.5 ± 2.0) was 9.7 times higher ($p = 0.008$) than in the healthy tissue (1.3 ± 1.3); PR-B expression in the endometriotic tissue (0.7 ± 0.2) was 3.5 times higher ($p = 0.005$) than in the controls (0.08 ± 0.03) [18].

We conclude that in patients with recurrent endometriosis the expression of progesterone receptors remains unchanged against the background of changing estrogen expression profiles, providing a theoretical support for a progestin-based prevention therapy for patients with endometriosis in the post-operative period.

CONCLUSIONS

Our findings demonstrate that in relapsing patients estrogen expression levels in the endometriotic tissue are different from those in patients with first-time diagnosis: a 3-fold increase is observed for ($p = 0.0016$), a 5-fold increase — for ER_β ($p = 0.0024$), and a 7-fold drop — for ER_α ($p = 0.0001$). In other words, the receptor status of the endometriotic tissue is different between first-time and relapsing patients in terms of mRNA levels of estrogen receptors. This confirms the involvement of estrogen receptors in promoting proliferation of hormone-dependent tissues of the female reproductive tract. The absence of differences in the expression of progesterone receptors indicates retained sensitivity of the tissue to progestins in recurrent endometriosis.

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A STUDY OF THE REPERTOIRE OF ACTIVATED T-CELL CLONES OBTAINED FROM A PATIENT WITH ANKYLOSING SPONDYLITIS

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Recent studies of T-cell clonal repertoires of patients with ankylosing spondylitis (AS) have led to the discovery of AS-associated T-cell clones with a highly homologous T-cell receptor structure. The role of T-lymphocytes in the disease progression cannot be elucidated without analyzing the diversity and abundance of functionally different T-cell clones found in patient with AS. Using a state-of-the-art technique for T-cell repertoire profiling based on massively parallel sequencing, we, for the first time, studied the T-cell repertoire of activated T-cells from the peripheral blood of a patient with AS. We have demonstrated that a subpopulation of $CD38^{+}HLA\text{-}DR^{+}$ T-lymphocytes is highly diverse both in terms of clonal diversity and abundance of the identified clonotypes, suggesting diverse antigen specificity of the activated peripheral blood T-cells. Most of the activated T-cell clonotypes had low abundance in total population of peripheral blood T-cells. In the repertoire of activated T-cells we have found the clonotype TRBV9_CASSVGVYSTDTQYF_TRBJ2-3, previously discovered in AS and reactive arthritis, and a few other clonotypes of cytotoxic and helper T-cells that may have a role in promoting inflammation in AS patients. Presence of the AS-associated clonotypes in activated T-cell subset suggests that the T-cells might play an active role in ongoing inflammation during the disease progression. This provides rationale for further research of their antigen specificity and role in triggering or maintaining AS.

Keywords: T-cell repertoire, ankylosing spondylitis, activated T lymphocytes, Bekhterev's disease, T-cell subpopulation, clonal repertoire

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ИССЛЕДОВАНИЕ КЛОНАЛЬНОГО РЕПЕРТУАРА ФРАКЦИИ АКТИВИРОВАННЫХ Т-ЛИМФОЦИТОВ У ПАЦИЕНТА С АНКИЛОЗИРУЮЩИМ СПОНДИЛИТОМ

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Недавние исследования клонального репертуара Т-клеток при анкилозирующем спондилите (АС) позволили идентифицировать группу клонов Т-лимфоцитов с высокогомологичной структурой Т-клеточного рецептора, ассоциированных с развитием АС. Определение роли Т-лимфоцитов в развитии заболевания требует исследований клонального состава функционально различных субпопуляций Т-клеток больных АС. С использованием современной технологии реконструкции клонального репертуара Т-клеток на базе массированного параллельного секвенирования нами был впервые исследован клональный репертуар активированных Т-лимфоцитов периферической крови пациента с АС. Мы обнаружили высокое разнообразие клонального состава фракции Т-лимфоцитов, экспрессирующих маркеры активации CD38 и HLA-DR, как по численности клеток, представляющих идентифицированные клонотипы, так и по структуре Т-клеточного рецептора клонотипов, что свидетельствует о разнообразной антигенной специфичности активированных Т-лимфоцитов периферической крови пациента. Основу клонального разнообразия составили малопредставленные в общем репертуаре клонотипы Т-клеток. В составе репертуара фракции был обнаружен ранее ассоциированный с АС и реактивным артритом клонотип TRBV9_CASSVGVYSTDTQYF_TRBJ2-3, а также ряд других клонотипов субпопуляций цитотоксических и хелперных Т-клеток, вероятно, связанных с воспалительным процессом при АС. Экспрессия маркеров активации клетками ассоциированных с АС клонотипов демонстрирует активное участие таких клонов в воспалительной реакции при заболевании, определяя актуальность исследования их антигенной специфичности и роли в возникновении и/или развитии АС.

Ключевые слова: Т-клеточный репертуар, анкилозирующий спондилит, активированные Т-лимфоциты, болезнь Бехтерева, субпопуляция Т-лимфоцитов, клональный репертуар

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Ankylosing spondylitis (AS, also known as Bekhterev's disease) is a chronic rheumatic, presumably autoimmune disorder. Genome-wide association studies link the risk of AS development to certain allelic variants of genes of antigen-presenting system (HLA and ERAP1/2) and cytokine receptors involved in proinflammatory response [1]. Strong association between risk of the disease and certain alleles of *HLA-B* and protective effect of some other *HLA-B* variants (HLA-B is involved in antigen presentation) have inspired a hypothesis suggesting that T-cell recognition of self-antigens in complex with HLA-B*27 triggers AS [2]. Recent studies of T-cell clonal repertoires in patients with AS have allowed to discover a group of cytotoxic T-lymphocyte clones with a highly homologous antigen-recognizing part of T-cell receptor (TCR); these clones are specific to AS and found in patients' peripheral blood and synovial fluid obtained from the inflamed joints [3, 4]. Previously, clones of T-lymphocytes with a similar or identical TCRs were found in the synovial fluid of patients with reactive arthritis, another member of the spondylarthropathies family [5–7].

Of particular interest are functional characteristics of the T-cell clones hypothesized to trigger and/or maintain inflammation. This work was aimed to study the clonal repertoire of subpopulation of activated T-lymphocytes isolated from the peripheral blood of a patient with active ankylosing spondylitis. For T-cell repertoire profiling, we applied a state-of-the-art technology based on the next-generation sequencing (NGS) of TCRβ cDNA libraries that were prepared using molecular barcoding technique [8, 9]; molecular barcoding is essential for correction of sequencing errors and estimation of the abundance of T-cell clones in the sample. After reconstruction of the repertoire and investigation of it's structure, we searched for the published AS-associated clones among the clonotypes of the studied T-cell subset. To estimate the abundance of the clonotypes identified in activated T-cell subset and attribute them to one of the two major functional T-cell subpopulations, we profiled the repertoire of the entire T-cell population and $CD4^+$ and $CD8^+$ T-cell subsets obtained from peripheral blood of the patient.

METHODS

Patient

A 28-year old man with axial ankylosing spondylitis (Bekhterev's disease) was diagnosed 7 years before our study. The patient was *HLA-B*27* positive. The treatment he was receiving included nonsteroidal anti-inflammatory drugs (arcoxia), methotrexate, and TNF-α inhibitors (Humira). Three years before blood collection, the patient underwent high-dose immunosuppression therapy with 200 μg/kg cyclophosphamide+anti-thymocyte globulin followed by autologous hematopoietic cell transplantation without $CD34^+$ enrichment. Six months later the patient had relapse of the disease following a respiratory infection. At the time of blood collection the patient had active AS manifested as severe pain and limited mobility of joints.

Isolation of PBMC and T-cell subsets

Eight milliliters of patient's peripheral blood were collected into K3-EDTA vacutainers (BD Biosciences, USA). Mononuclear cells were isolated by standard Ficoll density gradient centrifugation (1.077 g/cm^3, PanEco, Russia). Two peripheral blood samples of identical volume (F1 and F2) were obtained simultaneously and later used as two independent replicates for whole T-lymphocyte repertoire profiling. Subsets of $CD4^+$ and $CD8^+$ cells were isolated from separate blood samples using Dynabeads cell isolation kit (Invitrogen, USA).

Subpopulation of the activated T-lymphocytes expressing CD38 and HLA-DR markers was isolated from peripheral blood mononuclear cells by flow cytometry (FACSVantage, BD Biosciences). At the time point 1 we isolated a subpopulation of $CD3^+CD38^+HLA\text{-}DR^+$ lymphocytes. Two months later (time point 2) subpopulations of $CD3^+CD8^+CD38^+HLA\text{-}DR^+$ and $CD3^+CD8^-CD38^+HLA\text{-}DR^+$ cells were isolated from the patient's peripheral blood sample. The panel of antibodies for flow cytometry included CD3-APC (Invitrogen), CD8-FITC (Invitrogen), HLA-DR-PerCP (Invitrogen) and CD38-PE (IQProducts, Netherlands). Staining with antibodies was performed according to the protocols of manufacturers. The cells were lysed in 350 μl RLT buffer (Qiagen, Netherlands), and total RNA was then isolated for subsequent TCR repertoire profiling.

Preparation of cDNA libraries

RNA from PBMC, and $CD4^+$ and $CD8^+$ cell subsets, was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA of T-lymphocytes obtained by flow cytometry was isolated using the RNAeasy Mini reagent kit (Qiagen) according to the manufacturer's protocol. TCRβ cDNA libraries were prepared as described in [10]. The libraries were sequenced on HiSeq 2000/2500 (Illumina, USA) in the paired-end mode with a 100 bp read length.

Sequencing data processing

To correct sequencing errors and count unique TCR cDNA molecules in the library, we used MiGEC (a software pipeline utilizing the principle of molecular barcoding) [8, 11]. V-, D- and J-segments and CDR3 sequence were determined using MiXCR [12], which was also employed to quantify clonotypes and form a list of the identified clonotypes for each sample. For clonal sequence assembly only TCR cDNA sequences covered by at least two reads were used (based on analysis of unique molecular identifiers (UMI)), the restriction allowed to eliminate the majority of sequencing errors and to reduce the artificial clonal diversity of the repertoire [13]. Further bioinformatic analysis was performed using R programming language [14].

The identified clonotypes were associated with a particular T-cell phenotype based on detection of each particular clonotype (combination of CDR3 nucleotide sequences with TCR V-segment) in repertoires of the T-cell subpopulations.

The use of published TCRβ cDNA sequencing data

In this work, we also used data on TCRβ cDNA repertoire profiling peripheral blood and synovial fluid samples from *HLA-B*27* positive patients with AS. These data have been already published and are currently available in NCBI SRA database (SRP111372) [4]. The published sequencing data and data obtained for the present study were processed identically.

RESULTS

Characteristics of the studied cell samples and technology used for clonal repertoire profiling

For clonal T-cell repertoire analysis 50,000 cells expressing CD3, CD38 and HLA-DR were isolated using flow cytometry

from peripheral blood sample of the patient with AS. At the time of blood sampling the patient had joint pain and limited mobility of joints and was not receiving any anti-cytokine therapy. Flow cytometry revealed that the subpopulations of CD3+HLA-DR+ and CD3+CD38+HLA-DR+ cells accounted for 2.6 % and 1,5 % of patient's T-lymphocytes, respectively. These results are consistent with the literature reporting similar proportions for peripheral blood lymphocytes of healthy donors of the same age [15]. In our case, increased frequency of $CD3^+CD38^+$ T-cells was observed: 39.2 % for the AS patient vs. 5.85 % to 24.6 % in healthy donors of the same age.

At that time point, samples of cytotoxic ($CD4^+$) and helper ($CD8^+$) T-lymphocytes and two samples of unfractionated mononuclear cells (F1 and F2) were obtained from peripheral blood of the donor.

For TCRβ clonal repertoire profiling, we applied an original technology, which includes the following features:

1) template-switch-based cDNA synthesis, that allowed us to use universal primers for TCR cDNA amplification to minimize amplification bias associated with differences in the structure of a variable V-segment (part of a mature TCR gene);

2) molecular barcoding [8], that allows to estimate the depth of T-cell repertoire analysis and abundance of the identified clonotypes [9]; during preprocessing of sequencing data, molecular barcodes are used for correction of sequencing errors that helps to reduce the artificial diversity of the clonal repertoire originating from such errors [11];

3) introduction of sample-specific barcodes on both ends of cDNA library molecules. One of the barcode is introduced during cDNA synthesis step [10] allowing to exclude cross-contamination of samples both at the stage of sample preparation and during sequencing. Low probability of cross-contamination, in turn, allows to associate of the identified clonotypes with a particular T-cell phenotype. Such association is based on the identification of a unique nucleotide sequence of a variable TCR region in repertoires of different T-cell subsets ($CD4^+$, $CD8^+$, F1 and F2, $CD3^+CD38^+$HLA-DR^+).

Sequencing of TCRβ cDNA libraries yielded a total of 20.25 million reads: 6.23 and 7.53 million reads for F1 and F2 samples, respectively; 2.78 million reads for the $CD4^+$ subset; 2.96 million reads for the $CD8^+$ subset and 0.75 million reads for the $CD3^+CD38^+$HLA-DR^+ cell subpopulation. For further analysis and reconstruction of a variable TCRβ region (recovery of a CDR3 nucleotide sequence and a V-segment, i.e. identification of a T-cell clonotype) we used cDNA sequences covered by at least two reads. This allowed us to further reduce the artificial diversity of the repertoire originating from erroneous clonotypes [13, 16].

The total T-cell repertoire profiling depth reached about 1.6 million unique clonotypes. For samples F1, F2, CD4 and CD8, 3.3 million TCRβ cDNA molecules were analyzed (969,000, 1,260,000, 601,000 and 652,000, respectively). According to the previously published efficiency of the TCR repertoire profiling technology used, the total depth of analysis was about 3.3 million T-cells [13, 16]. Sequencing yielded 17,296 TCRβ cDNA sequences for $CD3^+CD38^+$HLA-DR^+ cells, covered by at least two reads, while the total number of cells in this sample was 50,000. Thus, on average every TCRβ cDNA sequence corresponded to a TCR mRNA of a distinct T-cell, and the total depth of TCR repertoire analysis of the subpopulation was about 17,000 unique T-cells.

Abundance of activated T-cell clonotypes in the total repertoire of T-cells

On the first step of TCR repertoire analysis of the activated T-cell subpopulation, we studied the distribution of the identified clonotypes in the total repertoire of peripheral blood T-cells. Over 85 % (10,212 of 11,927) of the identified activated T-cell clonotypes were not found in repertoires of any other samples: unfractionated T-cells (F1 and F2) or cytotoxic/helper T-cell subsets (Table 1). The probability to observe a specific clonotype in the sample is roughly proportional to the number of T-lymphocytes bearing the same TCR in the total T-cell population (i. e. to the abundance of a clonotype in the repertoire). High repertoire diversity and different abundance of T-cell clones combined with a relatively small volume of the sample can explain the substantial part of unique T-cell clonotypes between two independent blood samples obtained at the same time point.

Current estimate of the potential diversity of TCRβ repertoire is about 10^8 variants [17]; in our study the abundance of clonotypes in the total T-cell population varied between 1.5 % to 8.6 x 10^{-6} % T cells. To determine whether the observed high number of unique clonotypes was specific to the $CD3^+CD38^+$HLA-DR^+ subset, we estimated the repertoire overlap between total T-cell sample F1 and T-cell subsets expressing CD4 and CD8 surface markers with the sample F2. To obtain comparable repertoire analysis depth with fraction of activated T-lymphocytes, the repertoires of F1, CD4 and CD8 samples were reconstructed based on the corresponding number (17,296) of randomly selected *in silico* TCR cDNA molecules (repertoires *F1, *CD4 and *CD8). After 100 independent rounds of analysis, about 30% of clonotypes in each repertoire were shown to overlap with the clonotypes in sample F2, representing 30% to 50 % of the total number of analyzed T-cells from *F1, *CD4 and *CD8 repertoires. For the $CD3^+CD38^+$HLA-DR^+ subset these proportions were twice as low (Fig. 1). This fact indicates a considerable enrichment of the $CD3^+CD38^+$HLA-DR^+ subset with T-cell clones which have too low abundance in the total repertoire to be detected in the independent sample of the same volume.

Over 1,500 clonotypes in the repertoire of activated T-lymphocytes accounted for 13.1 % of the clonal diversity and for 20 % of the total number of analyzed T-cells, and were present in at least one of the two independent samples of unfractionated peripheral blood lymphocytes (F1 and F2). The depth of analysis was 2 million T-cells for the total T-cell repertoire. The proportion of peripheral blood T-cells represented by such clonotypes was 8.5 %. Of those clonotypes, only a few

Table 1. Comparison of $CD3^+CD38^+$HLA-DR^+ clonal composition with the total repertoire of peripheral blood T-cells

Associated phenotype*	Found in both samples (F1 and F2)**	Found either in F1 or F2**	Absent in both samples***
CD4	505 (4.2 %)	148 (1,2 %)	119 (1 %)
CD8	282 (2.4 %)	29 (0,2 %)	32 (0.3 %)
Unknown	175 (1.5 %)	425 (3,6 %)	10212 (85.6 %)

Note. * — association with the subset of cytotoxic of helper T-cells based on the analysis of $CD8^+$ and $CD4^+$ samples' repertoires; ** — percent of the total clonal diversity of the studied subset; *** — the number of clonotypes detected only in the $CD3^+CD38^+DR^+$ subset.

were relatively abundant in the total repertoire of the patient's T-cells (Fig. 2). Thus, of 100 most abundant clonotypes in total repertoire only 45 were discovered in the CD3⁺CD38⁺HLA-DR⁺ subset (39 and 6 clonotypes from cytotoxic and helper T-cell subsets, respectively).

The analysis of clonal overlap revealed that the majority of CD3⁺CD38⁺HLA-DR⁺ T-lymphocytes in terms of clonal diversity and clonal abundance were represented by the clones with low abundance in the total T-cell pool. Low abundance of 90 % of clonotypes results in the inability to detect them in the samples of unfractionated peripheral T-cells at a standard depth of analysis (a few millions of separate peripheral blood T-cells).

The structure of clonal repertoire of CD3⁺CD38⁺HLA-DR⁺ T-lymphocytes

Variable domain of TCRβ largely or fully determines the specificity of a T-lymphocyte clone to an antigen complexed with an MHC molecule (main histocompatibility complex, MHC) [18–20]. In our study, for analysis of clonal repertoire of activated T-cells each clonotype was defined as a combination of an amino acid sequence of a hypervariable CDR3 region and a V-segment identifier, which is a component of a mature TCR gene and determines the structure of the rest part of the beta-chain variable domain.

The clonal repertoire of activated T-cells from peripheral blood of the AS patient was relatively diverse; this diversity was comparable to that of the total T-cell repertoire. Analysis of the sequencing data demonstrated that majority of T-cells in the subpopulation has a unique variant of a TCRβ variable domain: we reconstructed as many as 12,000 different clonotypes from 17,000 TCR cDNA molecules. Note that for the samples F1 and F2 representing the total T-cell population, we identified about 550,000 unique clonotypes from 1 million sequences of TCR cDNA molecules.

The distribution of clonal abundance of CD3⁺CD38⁺HLA-DR⁺ fraction was the same as in the total T-cell repertoire (sample F1). The most abundant clonotype in the fraction accounted for 0.27 % of the total T-cell population. Note that the most abundant clonotype in the total T-cell repertoire (sample F1) accounted for 1.6 % of all T-cells in the population. Top 100 most abundant clonotypes of activated T-cells or total repertoire accounted for 5.6 % of the CD3⁺CD38⁺HLA-DR⁺ subset or 9 % of the total repertoire, respectively. No significant differences were observed between the sample F1 and the CD3⁺CD38⁺HLA-DR⁺ subset in terms of the abundance of clonotypes having a specific V-segment in their TCRβ gene (those are clonotypes that presumably recognize peptides in complex with same MHC molecule). Compared to the total T-cell repertoire, there was a lack of enrichment of the activated T-cell population in clonotypes with a specific structure of a variable TCRβ region.

A search for AS-associated clonotypes

T-cells expressing activation markers are directly involved in the immune response. This encourages us to hypothesize that the repertoire of activated T-cell subpopulations might be enriched with clonotypes associated with inflammation in patients with active spondylitis. To profile the CD3⁺CD38⁺HLA-DR⁺ subset, we performed a search for the AS-associated clonotypes that had been previously discovered in the peripheral blood and synovial fluid of *HLA-B*27* positive AS patients [3, 4]. The search list included clonotypes observed in the synovial fluid of at least two of three *HLA-B*27* positive patients whose repertoires had been characterized in our previous work [4]. This was necessary to minimize accidental and irrelevant clonotype matches between the repertoires of two donors and to focus on similar clonotypes found in inflamed joints of different individuals. The search list included 1,913 clonotypes: 11 CDR3 sequences constituting a motif associated with AS (as reported by the two studies mentioned above) and 1,902 clonotypes found in the synovial fluid of patients with AS.

We found 32 identical amino acid sequence of a TCRβ variable domain between 11,927 clonotypes coming from the subset of activated T-lymphocytes and the clonotypes from our list. The majority of shared clonotypes had low abundance in the CD3⁺CD38⁺HLA-DR⁺ T-cell subset (Fig. 3).

Eight of these 32 clonotypes were not found in the T-cell repertoires of peripheral blood samples from *HLA-B*27* positive healthy donors (data from [4]) (Table 2), suggesting the lack of expansion of these clonotypes in healthy individuals [4, 21, 22]. One of those 8 clonotypes, TRBV9_CASSVGVYSTDTQYF_TRBJ2-3, belongs to the group of 11 variations of the variable TCR sequence that constitute the CDR3 motif associated with AS. The motif was also previously found in the synovial fluid of *HLA-B*27* positive patients with reactive arthritis [5, 23]. In our study this clonotype was associated with the CD8⁺ T-cell subset just like in three previously published works reporting its presence in the samples of patients with spondyloarthropathies (AS and reactive arthritis).

For two months after the first sampling the disease status of the patient remained unchanged, and we profiled the repertoire of his activated T-cell clones once again (time point 2). Using flow cytometry, we obtained 16,000 CD3⁺CD8⁺CD38⁺HLA-DR⁺ and 10,500 CD3⁺CD8⁻CD38⁺HLA-DR⁺ lymphocytes and reconstructed 3,900 and 8,300 TCRβ clonotypes, respectively. The depth of analysis was comparable to that performed two months before in terms of quantity of the identified clonotypes. The subpopulations of the activated T-cell clones isolated at time points 1 and 2 shared 3 % of clonotypes coming from the cytotoxic and helper subsets in equal proportions: of 323 clonotypes of activated T-cells covered by the analysis at both time points, 154 were associated with the CD8⁺ phenotype, 136 — with the CD4⁺ phenotype; the remaining 33 clonotypes

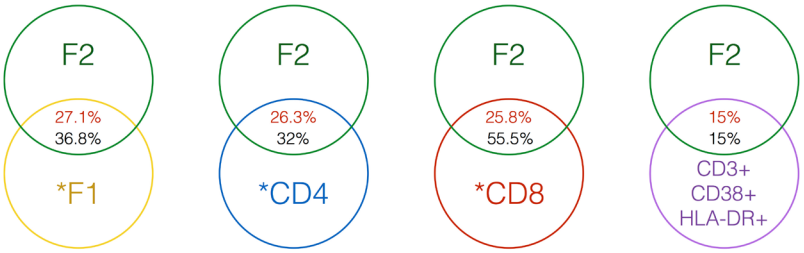

Fig. 1. Comparison of clonal composition of different T-cell subpopulations. Percent of the clonal diversity (shown in red) and percent of the T-cell repertoire (shown in black) represented by clonotypes shared by two compared samples. *F1, *CD4, *CD8 are repertoires of F1, CD4, and CD8 samples, respectively, reconstructed on the basis of 17, 523 randomly *in silico* selected TCR cDNA molecules (that equals to the number of TCR cDNA molecules covered by the analysis of the CD3⁺CD38⁺HLA-DR⁺ subset)

were not attributed to any phenotype during the analysis at time point 1.

In repertoire of CD8⁺ activated T-lymphocytes in time point 2, we also found the clonotype TRBV9_CASSVGVYSTDTQYF_TRBJ2-3 along with the three other clonotypes that had been previously discovered in the synovial fluid of patients with AS but not in the peripheral blood of healthy *HLA-B*27* positive donors (Table 2). The amino acid sequence of the variable TCRβ region of another clonotype discovered at time point 2 was highly homologous to the published clonotypes associated with AS due to the identical V-segment in the TCR gene sequence and a similar sequence of a hypervariable CDR3 region. This clonotype was observed in the repertoire of peripheral blood lymphocytes in 40 % of patients with AS (n = 25) [4] and in two of the three studied T- cell repertoires of the synovial fluid; it was, however, absent in all blood samples of *HLA-B*27* positive donors (n = 7) (Table 2).

DISCUSSION

Surface molecules CD38 and HLA-DR are markers that discriminate activated T-cells, a subpopulation of T-lymphocytes exerting their effector function during the immune response, such as the response to viral invasion [24–27]. Increased frequency of T-cells with the CD38⁺HLA-DR⁺ phenotype was shown in the peripheral blood of patients with autoimmune inflammatory bowel diseases that often accompany spondyloarthropathies and ankylosing spondylitis, in particular [28]. According to the recent study of anti-cytokine treatment effectiveness, unlike healthy donors, patients with AS have higher frequency of cytotoxic HLA-DR⁺ T-cells in their peripheral blood; the study also demonstrates that the frequency of HLA-DR⁺ helper T-cells differ significantly between patients who respond to treatment and those who do not [29]. Discovery of AS-associated T-cell clones with a highly homologous TCR structure [3, 4] encourages research of clonal composition of functionally different T-cell subsets in patients with AS that could elucidate the role of T-cells in the disease. In our study we used a state-of-the-art technique for cDNA library preparation and sequencing data analysis, and pioneered the exploration of the structure and clonal composition of the repertoire of activated peripheral blood T-cells from patients with AS. Low probability of clonal composition bias and quantitative assessment of the abundance of each clonotype in a sample were important features of the TCR repertoire profiling technology used for the present study.

The analysis of clonotype abundance in the activated T-cell subset and the total T-cell repertoire has revealed a unique composition of the activated T-cell subpopulation: it is enriched in clonotypes that cannot be detected in unfractionated blood samples or in the subpopulations of CD8⁺- and CD4⁺ T-lymphocytes owing to their low abundance. A low degree of overlap between the repertoires of activated T-cells obtained at two different time points is probably the result of low abundance of these clonotypes in peripheral blood.

The fact that the majority of clonotypes found in activated T-cell subset had low abundance could be explained by the enrichment of the fraction with clones participating in inflammation and normally localized at inflammation sites. Komech et al. [4] report higher abundance in synovial fluid compared to peripheral blood of patients with acute synovitis for AS-associated clonotypes. However, at the given depth of analysis achieved in our study, clonal diversity of the activated T-cell subpopulation almost mirrors that of the total T-cell repertoire in peripheral blood. We have not observed prominent clonal expansions or enrichment in clonotypes with a specific structure of a variable TCRβ domain in the activated T-cell subset. Using a recently created database of clonotypes with well-characterized specificity to different MHC/peptide

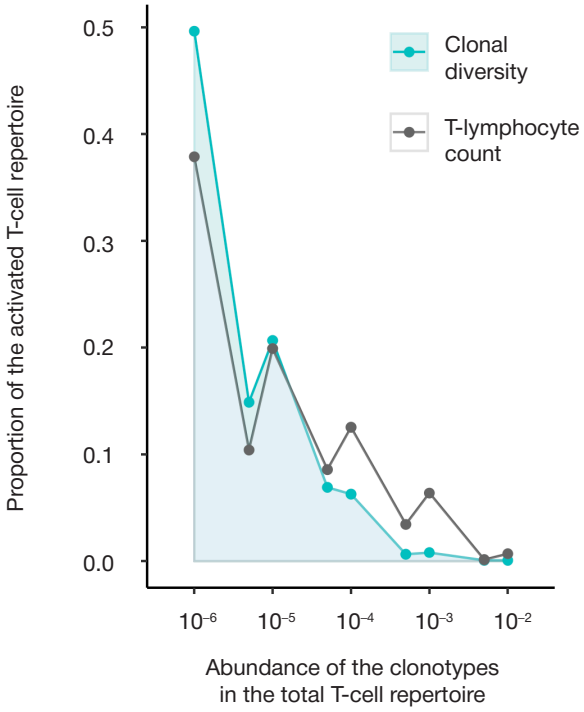

Fig. 2. Distribution of the clonotypes identified in the CD3⁺CD38⁺HLA-DR⁺ subset depending on their abundance in the total repertoire (F1). The Y axis represents the proportion of clonotypes in the studied subset relative to the total number of clonotypes in the subset; the X axis represents the abundance of clonotypes in the total repertoire of T-cells

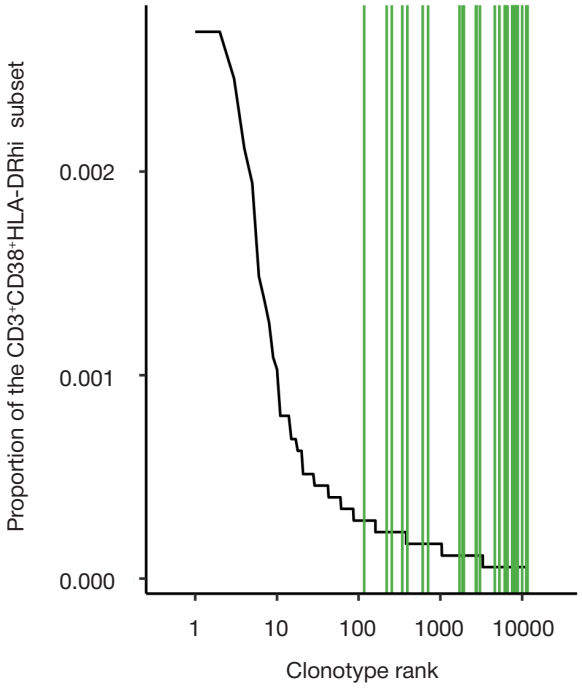

Fig. 3. Abundance of the clonotypes associated with AS and found in the synovial fluid of patients with AS in the repertoire of activated T-cells. Rank (reference number) in the repertoire of activated T-cells for each clonotype from the list (see explanation in the text), is represented by green vertical line

Table 2. Analysis of frequency in the peripheral blood and synovial fluid samples of patients with AS for the clonotypes identified in $CD3^+CD38^+HLA\text{-}DR^+$ subset

Amino acid sequence	V-segment	Frequency in the samples obtained from patients with AS** (n = 25), %	Number of synovial fluid samples (n = 3)	Associated phenotype***	Sample
CASSLGPGSYEQYF	TRBV5-1	32.0 (8)	3	CD4	Time point 1
CASSVGVYSTDTQYF	TRBV9	24.0 (6)	1	CD8	Time points 1 and 2 ($CD8^+$)
CASSSRGPYEQYF	TRBV7-2	16.0 (4)	2	–	Time point 1
CASSDYNEQFF	TRBV2	16.0 (4)	2	CD4	Time point 1
CASSQEGQESDTQYF	TRBV4-2	12.0 (3)	2	–	Time point 1
CASSLGGRNNEQFF	TRBV5-1	4.0 (1)	2	–	Time point 1
CAWSLGVNQPQHF	TRBV30	4.0 (1)	2	–	Time point 1
CASSYSGGSGYTF	TRBV6-5	0 (0)	2	CD4	Time point 1
CASSVGGDYGYTF	TRBV9	40.0 (10)	2	CD8	Time point 2 ($CD8^+$)
CASSLGLSGANVLTF	TRBV5-6	8.0 (2)	2	–	Time point 2 ($CD8^+$)
CASSQAGAYQETQYF	TRBV4-2	0 (0)	2	–	Time point 2 ($CD8^-$)

Note. * — The list includes clonotypes of the $CD3^+CD38^+HLA\text{-}DR^+$ cell subset isolated from the peripheral blood of the patient that were not found in the repertoires of healthy *HLA-B*27* positive donors; ** — the number of peripheral blood samples of AS patients, where the clonotype was identified, is specified in brackets; *** — association with the subset of cytotoxic of helper T-cells based on the analysis of the repertoire of $CD8^+$ and $CD4^+$ samples.

complexes [30], we have found a few matches of a TCRβ amino acid sequence between clonotypes in the repertoire of activated T-cell subset and clonotypes in the database, which are specific to some immunogenic of influenza virus, CMV and EBV. This suggests that T-lymphocytes found in the patient's peripheral blood expressing HLA-DR and CD38 activation markers represent a subset of T-cells that actively participate in the immune response against a broad range of antigens.

To search for the clonotypes associated with AS we used T-cell repertoire data for the peripheral blood and synovial fluid samples of healthy *HLA-B*27*-positive donors obtained earlier using with the same technology for T-cell repertoire reconstruction. After analysis of two repertoires of activated T-cells obtained at different time points using different flow cytometry gating strategies, we found 11 clonotypes that were absent in the repertoires of healthy donors but observed in the synovial fluid of at least two patients with AS. None of those 11 clonotypes was found in the repertoires of patients' peripheral blood. The presence of these clonotypes in the repertoires of *HLA-B*27* positive patients with AS and their absence in healthy *HLA-B*27* positive patients demonstrates their ability to undergo selection in the thymus in the presence of HLA-B*27, followed by the clonal expansion in patients but not in healthy *HLA-B*27* positive individuals [21].

Among the aforementioned 11 sequences of the variable TCRβ domain there was the TRBV9_CASSVGVYSTDTQYF_TRBJ2-3 clonotype previously associated with AS based on the recent studies of the T-cell clonal repertoire of the peripheral blood [3] and synovial fluid [4] of patients with AS. An identical clonotype was previously discovered in the synovial fluid of *HLA-B*27* positive patients with reactive arthritis [5, 23]. This clonotype was associated with the subpopulation of cytotoxic T-lymphocytes in the mentioned research works and in our present study as well. Upon analyzing the clonal repertoire of two independent samples of activated T-cells isolated from the peripheral blood using different gating strategies at two different time points, we managed to prove the long-term presence of this clonotype in the activated T-cell subpopulation of peripheral blood of the patient.

The activated cytotoxic T-cell subset contained the clonotype TRBV9_CASSVGGDYGYTF_TRBJ1-2 found in the peripheral blood samples of 40 % of AS patients (n = 25) and in two of three samples of synovial fluid from patients. A high frequency of this clonotype in the synovial fluid and peripheral blood of patients with AS, a certain degree of homology of its variable TCRβ domain to other clonotypes previously discovered in patients with reactive arthritis and AS and its absence in the peripheral blood of healthy *HLA-B*27* positive donors allow to suggest a possible role of this clonotype in inflammation in ankylosing spondylitis. Besides, some clonotypes from the helper subpopulation might also be linked to AS, given the presence of a few $CD4^+$ clonotypes in the repertoire of the activated T-cells that share the same structure of the variable TCRβ domain with the clonotypes of the $CD4^+$ T-cell subset isolated from the synovial fluid of patients with AS and considering the absence of these $CD4^+$ clonotypes the peripheral blood of healthy *HLA-B*27* positive donors.

CONCLUSIONS

We have performed for the first time a study of clonal repertoire of a T-cell subset expressing activation markers HLA-DR and CD38 from the peripheral blood of a patient with active ankylosing spondylitis. Clonal repertoire of the subpopulation was not highly oligoclonal and was enriched in clonotypes poorly represented in the total T-cell repertoire. We have discovered a number of clonotypes of cytotoxic and helper T-cells possibly implicated in inflammation process in AS. Most importantly, in the subpopulation of cytotoxic activated T-cells we have identified a clonotype, which was previously associated with AS in other recent studies. Expression of activation markers by T-cells of the AS-associated clones suggests their active role in inflammation. Further research is necessary to investigate the clonal repertoire of functionally different T-cell populations and to determine antigen specificity of the identified clonotypes, which will facilitate our understanding of mechanisms underlying AS initiation and development and help to design specific treatment strategies for the disease.

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OBSERVING THE DYNAMICS OF THE NAD+ TO NADH RATIO IN *DANIO RERIO* EMBRYO TISSUES USING A GENETICALLY ENCODED BIOSENSOR

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The zebrafish *Danio rerio* is a popular model species for biomedical research focusing on modeling human diseases and screening of therapeutic drugs. Genetically encoded biosensors based on fluorescent proteins are widely used in many laboratories worldwide to study the biology of living systems of various complexity *in vivo*. The main advantage of these molecular tools is that they allow observing biological processes in intact systems in real time. In the present work we study the dynamics of the intracellular ratio of NAD+ to NADP in the yolk sac and tissues of *D. rerio* larvae using the SoNar biosensor following the injection of glycolisis end products, lactate and pyruvate.

Keywords: genetically encoded biosensors, *Danio rerio*, pyruvate, lactate

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РЕГИСТРАЦИЯ ДИНАМИКИ СООТНОШЕНИЯ НАД+/НАДН В ТКАНЯХ ЭМБРИОНОВ РЫБ *DANIO RERIO* С ПОМОЩЬЮ ГЕНЕТИЧЕСКИ КОДИРУЕМОГО БИОСЕНСОРА

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Рыба *Danio rerio* является популярным модельным объектом во многих медико-биологических исследованиях, в том числе для моделирования человеческих заболеваний и скрининга лекарственных препаратов. Генетически кодируемые биосенсоры на базе флуоресцентных белков широко используются во всем мире для исследований сложных биологических процессов в живых системах любого уровня сложности *in vivo*. Главное преимущество таких молекулярных инструментов в том, что генетически кодируемые индикаторы позволяют исследователям наблюдать за биологическими процессами в интактных системах в режиме реального времени. В данной работе мы впервые протестировали биосенсор SoNar для регистрации такого важного внутриклеточного параметра, как соотношение НАД+/НАДН, в тканях эмбрионов рыбы *D. rerio*. Мы исследовали, как изменяется динамика соотношения НАД+/НАДН в желточном мешке и тканях тела малька рыбы при инъекции продуктов гликолиза лактата и пирувата.

Ключевые слова: генетически кодируемые биосенсоры, *Danio rerio*, пируват, лактат

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The zebrafish *Danio rerio* is a popular model species for *in vivo* biomedical research. It has a few advantages over other lab animals: fish are prolific breeders that require little upkeep and develop rapidly. Due to their optical clarity, *Danio rerio* embryos are a good object for microscopy. Besides, this fish's genome is very well studied and hence easy to manipulate. Therefore, *D. rerio* are often used to study gene functions and developmental biology of vertebrates. Because cell biochemistry and functional activity of proteins in *D. rerio* resemble those of mammals, this species is used for modeling human diseases, testing toxicity of chemical compounds in preclinical trials, and in therapeutic drug screening.

In the last few years *D. rerio* has been successfully exploited as a model organism to study acute lymphoblastic leukemia

[1–3], melanoma [4, 5], muscular dystrophy [6], diabetes mellitus [7], pathological conditions of the heart [8–10], kidneys [11, 12], and central nervous system [13, 14], including brain ischemia [15, 16]. Such vertebrate models are handy when it comes to investigating effects of various chemical compounds on the organism in health and pathology. The easiest way to administer a compound of interest to the fish is to add it into the water in the tank: the chemical will enter the fish's bloodstream through the gills. The majority of experiments are carried out in larval fish, though, meaning that drugs are normally injected straight into the yolk sac. This method of drug delivery can be employed to assess cardiotoxicity of aspirin, clomipramine, nimodipine, varapamil and some others medications [17, 18]. Larval fish are also used to study metabolites that are part of normal mammalian biochemistry. For example, in one of the experiments excess amounts of glucose were administered to fish embryos via injections to describe expression profiles of genes involved in carbohydrate metabolism [19]. It is also possible to infect *D. rerio* larvae with injections of bacterial cells to observe how infection progresses [20–22] or even mammalian cells to study tumor growth [23, 24].

Genetically encoded biosensors based on fluorescent proteins have taken *in vivo* studies of physiology and pathology to a new level. Each biosensor is a chimeric protein molecule consisting, as a rule, of a sensing moiety and a fluorescent domain. The sensor domain detects changes in the surrounding environment, such as fluctuations in the intracellular concentrations of a studied compound. The fluorescent component "reports" interactions between the sensing domain and the compound of interest, and the obtained signal is then interpreted. Such biosensors are encoded by a gene that can be incorporated into any living organism. What is important is that this approach can be used in real-time imaging of biochemical processes in a living organism [25]. Oftentimes genetically encoded biosensors are the only available tool for looking into complex biological events. Fluorescent biosensors have been extensively used in model *D. rerio* species to study embryogenesis [26], inflammation [27], and organ regeneration [28].

In this work we have pioneered the use of the genetically encoded biosensor SoNar [29] as a tool for monitoring the redox state of nicotinamide adenine dinucleotide (NAD) in the cytoplasm of *D. rerio* tissues. The ratio of its oxidized to reduced forms (NAD^+/NADH) is critical not only for cell metabolism but also for the regulation of many signaling cascades [30–32]. We have found out that the NAD^+/NADH ratio changes both in the yolk sac and body of a *D. rerio* embryo following lactate and pyruvate injections.

The SoNar biosensor [29] is based on the bacterial protein T-Rex of *Thermus aquaticus* that regulates transcription of a few enzymes in response to fluctuations in the intracellular NAD^+/NADH ratio. One of the biosensor's components is the fluorescent protein cpYFP integrated into the mobile region of T-Rex. Conformational changes accompanying the binding of T-Rex to NAD^+ or NADH are propagated to the fluorescent protein affecting its spectral properties (Fig.1). SoNar fluorescence excitation spectrum typically has two peaks at 420 and 490 nm; the sensor emission peak is at 518 nm. The signal is calculated as a ratio of fluorescence intensities excited at 420 nm and 490 nm (F_{420}/F_{490}) [29]. Thus, the signal is ratiometric, which is particularly important for *in vivo* research, as it helps to avoid artifacts related to different expression levels of the sensor in different cells, motion, changes of the object's shape or tissue thickness.

METHODS

AB/TL *Danio rerio* fish were kept in a specially equipped room at 26.5 °C under 12:12 light conditions. The experiments were conducted in compliance with the Declaration of Helsinki. To obtain progeny, parent fish were paired in separate tanks containing 500 ml of E3 medium 4 hours before the lights went out. Eggs were collected when the lights were turned back on.

In our experiment we used SoNar [29] and SypHer-2 [33] biosensors. To obtain larval *D.rerio* fish expressing these biosensors in their tissues, we synthesized biosensor mRNA *in vitro* using the commercial mMessage mMachine SP6 Transcription kit (Ambion, USA). Then 1 nl of 0.1 μg/μl mRNA was injected into the yolk sac of single-cell fish embryos using Eppendorf Microinjector 5242. The treated fish were kept in Petri dishes at 26.5 °C under standard 12:12 light conditions. Twenty four hours after the injection, the embryos were examined under the fluorescence microscope to select those that were fluorescent. Before microscopy the embryos were manually stripped of chorions, immobilized in 0.02 % tricaine solution and mounted in a drop of 1.5 % low-melting point agarose. One nl of 200 mM sodium lactate and sodium pyruvate solutions was injected in the yolk sac of each immobilized embryo. Prior to the injections of metabolites, biosensor signals were recorded in the yolk sac and body of each larval fish.

Microscopy was performed using the wide field fluorescence microscope Leica DM6000 (Leica, Germany), equipped with the HC PL FLUOTAR 10.0*0.30 DRY lens. Fluorescence was excited using excitation filters CFP BP436/20 and GFP BP470/40. Frame rate was 1 frame per minute. Images were processed in ImageJ (NIH). Graphs showing the dynamics of sensor signals were constructed using OriginPro 8.6 (OriginLab, USA). The same software was used to process data obtained at each time point.

RESULTS

We have tested the use of the genetically encoded sensor SoNar [29] in monitoring the NAD^+/NADH ratio in *D. rerio* tissues. Briefly, we synthesized biosensor mRNA and injected it into the yolk sac of single-cell fish embryos. Twenty four

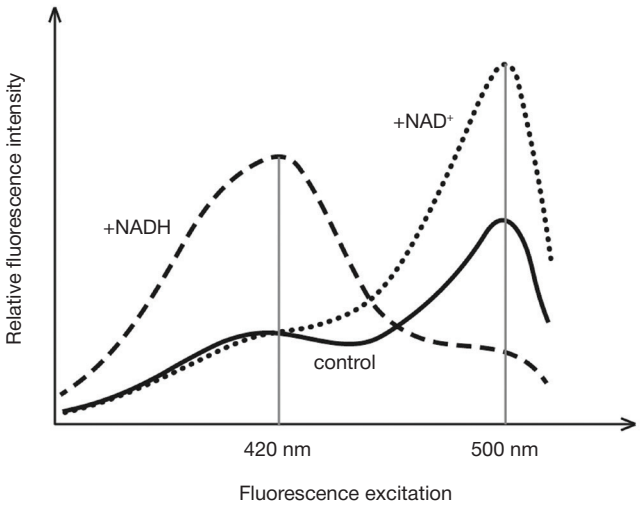

Fig. 1. Fluorescence excitation spectrum of the SoNar protein-based biosensor with two typical peaks at 420 nm and 490 nm. When NADH is added, fluorescence intensity increases at 420 nm and weakens at 490 nm. When NAD^+ is added, signal intensity increases at 490 nm. The SoNar biosensor has one emission peak with emission maximum at 518 nm

hours after the injection we selected fluorescent embryos for further microscopy. SoNar fluorescence was recorded in two independent channels; fluorescence excitation wavelengths in each channel matched the two peaks of SoNar's fluorescence excitation spectrum (Fig. 1). We obtained two images of each fish embryo at 420 nm (F_{420}) and 490 nm (F_{490}) excitation. Using ImageJ we divided the images by one another; the resulting image was then painted in pseudocolors corresponding to the values of the F_{420}/F_{490} ratio (Fig. 2, A, D). Again, using ImageJ we selected a few zones on the embryo's body and made further calculations. Graphs showing the dynamics of the F_{420}/F_{490} ratio were constructed in OriginPro 8.6.

To monitor the dynamics of the $NAD^+/NADH$ ratio in the embryo tissues of *D. rerio* using the SoNar biosensor, we injected sodium lactate and pyruvate into the embryos. It is well known that the lactate/pyruvate pair is in equilibrium with free cytoplasmic $NAD^+/NADH$ owing to the reaction catalyzed by lactate dehydrogenase [34, 35]. Bearing that in mind, we injected 1 nl of 200 mM sodium lactate solution in the yolk sac of 10 embryos and 1 nl of 200 mM sodium pyruvate solution in the yolk sac of another 11 embryos. Prior to the injections, we recorded the signal emitted by the biosensor in the untreated tissues. This initial value was taken as 1. After the injection, the fish were quickly put under the microscope and imaging was resumed with the same settings. Embryos injected with lactate demonstrated an increase in the F_{420}/F_{490} ratio, indicating reduction of the NAD pool (Fig. 2, B). Similar signal dynamics observed in different parts of the embryo's body can be explained by rapid delivery of the metabolites from the yolk sac to other organs. The signal reached its maximum 5 min after the injection, gradually losing its intensity for the next 30 min until it finally reached its initial value, while lactate was being metabolized by cellular organelles. We expected to see an opposite effect with pyruvate. But the F_{420}/F_{490} ratio decreased only in the yolk sac and even slightly increased in the embryo's body (Fig. 2, E). It means that small doses of pyruvate trigger different redox events in the intracellular NAD pool: oxidation in the yolk sac and reduction in the embryo's body.

Lactate or pyruvate transport into the cells can happen against the background of changing pH because it occurs in symport with H^+ [36, 37]. The specific chromophore structure makes fluorescent proteins sensitive to pH fluctuations [38]. To assess a possible effect of pH on the SoNar signal intensity, we used SypHer2 previously developed in our lab as a control

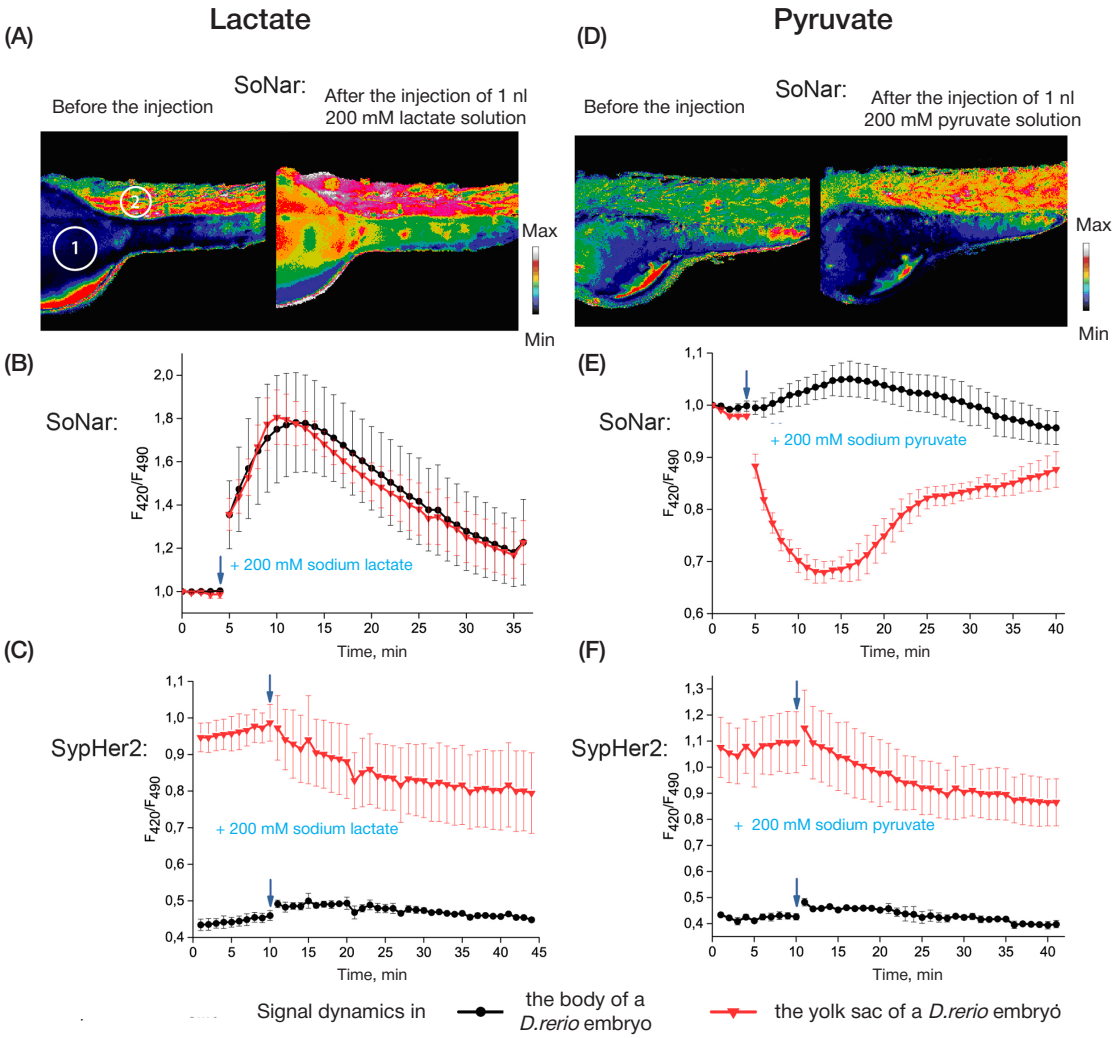

Fig. 2. Changes in the NAD+/NADH ratio and pH in the tissues of one-day old *D. rerio* embryos before and after pyruvate and lactate injections into the yolk sac. **(A and D)** Images of a *D. rerio* embryo body before and after injections of 1 nl 200 mM lactate **(A)** and pyruvate **(D)** solutions. Images are shown in pseudo-colors corresponding to the SoNar signal values (F_{420}/F_{490}). Zones 1 and 2 circled in white in the first picture indicate regions on the embryo's body where F_{420}/F_{490} fluctuations were registered. These regions were the same for all inspected fish. **(B and E)** Changes in the $NAD^+/NADH$ ratio measured using the SoNar biosensor in the yolk sac (red line) and body (black line) of the *D. rerio* embryo before and after injections of lactate **(B)** and pyruvate **(E)** solutions. **(C and F)** Changes in pH registered using the SypHer2 in the yolk sac (red line) and body (black line) of a *D. rerio* embryo before and after injections of lactate **(C)** and pyruvate **(F)**. For all graphs **(B, C, E, F)** the error bar corresponds to the standard deviation. Graph **(C)** was constructed based on the imaging of 10 fish. Graph **(E)** — based on the imaging of 11 fish. Graphs **(C)** and **(F)** — based on the imaging of 6 animals (per graph)

biosensor [33]. We synthesized SypHer2 mRNA and conducted the same series of experiments as with SoNar. Lactate and pyruvate injections did not induce any changes in pH in the embryos' bodies. Injected into the yolk sac, both metabolites caused a slight and slow increase in pH in this tissue which lasted for 20 min following the injection (Fig. 2, C, F).

DISCUSSION

Our findings lead us to conclude that the genetically encoded biosensor SoNar developed to record NAD^+/NADH fluctuations can be successfully used in the experiments involving *D. rerio* model species. The NAD^+/NADH ratio is an important biological parameter that can be significantly affected by pathology. A fish that expresses the SoNar biosensor in its tissues or cells can be effectively used as a model organism to assess the impact of different compounds on the NAD^+/NADH ratio and therefore on disease progression.

We have found out that lactate injections in the yolk sac cause significant simultaneous reduction of the NAD pool in all tissues of the fish. However, pyruvate injections induce NAD oxidation only in the yolk sac; moreover, they cause slight reduction in the embryo's body. One of the recent works studied the role of redox processes in embryogenesis using the *D. rerio* model to reveal that massive oxidation starts at gastrulation and goes on to subside when the fish is three days old. That work demonstrates that oxidative stress plays a key role in the development of some organs in the course of embryogenesis [26]. Perhaps, we have not observed significant oxidation in embryo tissues following the pyruvate injection because at this developmental stage redox homeostasis is shifted towards oxidation. Notably, in the article cited above the minimum level of reactive oxygen species (hydrogen peroxide) was observed in the yolk sac – the organ in which (in our experiment) NAD tended to be oxidized after the injection of pyruvate. Insignificant reduction of the NAD pool in the embryo's body is probably compensation.

Another possible explanation is that in the yolk sac pyruvate is reduced to lactate, which can be confirmed by decreased NADH concentrations, but it is mainly lactate that is transported to the embryo's body. Perhaps, this is why we observed a signal change indicative of a slight elevation of NADH concentrations. This hypothesis needs to be tested.

CONCLUSIONS

We have demonstrated that the genetically encoded biosensor SoNar developed to measure the NAD^+/NADH ratio can be used in *D. rerio* tissues. Lactate injections into the yolk sac of a one-day old fish embryo lead to rapid reduction of the NAD pool in all tissues of the fish, showing that substances present in the yolk sac can be easily transported to other tissues. Pyruvate injections induce oxidative reactions in the yolk sac only, accompanied by slight reduction in the embryo's body. Using the control SypHer2, we have established that pyruvate and lactate cause slight pH fluctuations only in the yolk sac.

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A BIOLUMINESCENT SYSTEM OF FUNGI: PROSPECTS FOR APPLICATION IN MEDICAL RESEARCH

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Bioluminescence is chemical oxidation of a small luciferin molecule by air catalyzed by luciferase and accompanied by the emission of photons in the visible spectrum. This reaction is used in bioluminescent bioimaging, the method for the visualization of organism's interior. Bioimaging is a popular tool used in medical research. However, it has an unfortunate drawback: it requires introduction of external luciferin to the system before every experiment. In this work we discuss a possibility of developing an autonomous luminescent system in eukaryotes based on the bioluminescent system of higher fungi.

Keywords: bioluminescence, bioimaging, tumor models *in vivo*, photodynamic therapy

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НОВАЯ БИОЛЮМИНЕСЦЕНТНАЯ СИСТЕМА ГРИБОВ: ПЕРСПЕКТИВЫ ИСПОЛЬЗОВАНИЯ В МЕДИЦИНСКИХ ИССЛЕДОВАНИЯХ

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Биолюминесценция — это химическое окисление небольшой молекулы люциферина кислородом воздуха под действием белка люциферазы, которое сопровождается эмиссией кванта света в видимой области спектра. На основе этой реакции были разработаны различные методы биолюминесцентного имиджинга — получения изображения в живом организме. Они активно используются в медицинских исследованиях. Однако недостатком существующих люминесцентных систем является необходимость добавления люциферина извне перед каждым экспериментом. В настоящей работе обсуждается возможность разработки автономной люминесцентной системы эукариот на основе биолюминесцентной системы высших грибов.

Ключевые слова: биолюминесценция, биоимиджинг, опухолевые модели *in vivo*, фотодинамическая терапия

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There are thousands of species capable of emitting light. They are referred to as bioluminescent. The majority of them inhabits the depths of the world's seas and oceans, but some glowing species such as insects, worms and fungi can also be found above the water surface. Bioluminescence is a result of luciferase-catalyzed oxidation of a small luciferin molecule by air, accompanied by the emission of a photon in the visible spectrum. This reaction has inspired the development of bioluminescence imaging aimed to visualize processes happening inside the living organism. Bioluminescence imaging has found its way into medical research, including cancer research and development of anticancer drugs. In the last few years this method has become increasingly popular due to its unsurpassed sensitivity in producing accurate images of the interior of living organisms, down to the level of a single cell.

The number of unique luciferin-luciferase pairs occurring in nature is estimated to be as high as 40 [1]. However, in spite of such variety, only a few well-studied bioluminescent systems are currently applied in medical science, including firefly D-luciferin, bacterial and coelenterazine systems. For each type of a luciferin there are a few "complementary" luciferases found in different organisms. For example, for D-luciferin as many as 30 natural luciferases are known, while coelenterazine is "complementary" to 15 luciferases and 8 photoproteins, i.e. stable substrate/protein complexes [2]. Once we know the exact luciferase amino acid sequence, we can insert the gene for this enzyme into another organism's genome and stimulate its expression in it. Introduction of external luciferin into this system will cause the cells to glow. In turn, the emitted light can be registered and subsequently analyzed. Bioimaging is possible not only with cells and tissues but also with cell organelles and for cell-cell/protein-protein interactions. Because different types of luciferins, their functional analogs and luciferases (both natural and synthetic) can be used in

parallel, bioluminescence becomes an amazingly powerful tool laying the basis for multicolor imaging [3–6].

Bioluminescence in medicine

Firefly D-luciferin is the most popular substrate used in medical research. To react with luciferase, it needs adenosine triphosphate (ATP). Therefore, a bioluminescence signal can be obtained only in the presence of ATP. Obviously, this means that bioluminescence can be employed to accurately measure ATP concentrations. Because ATP is a universal energy carrier, its levels characterize cell's metabolic potential and may be used to assess cytotoxicity of various drugs or their impact on cell proliferation [7]. In turn, bacterial luminescent systems strongly rely on the presence of such cofactors as flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide hydride (NADH). This property is exploited by bioluminescence-based analytical methods developed to assay NAD(P)H, dehydrogenases and various metabolites, like malate, sorbitol and ethanol, whose oxidation is accompanied by the reduction of NAD^+ to NADH. Used in combination, the firefly and bacterial luminescent systems become a tool for constructing metabolic maps of tumor tissues facilitating discovery of novel approaches to cancer therapy [8–10].

Indeed, the most accurate picture of cancer progression can be obtained using *in vivo* models. MRI, PET, radiography and similar imaging methods afford to investigate metastasis and angiogenesis of tumors as well as their respond to treatment, especially when it comes to deep tissues. But bioluminescent imaging is preferable to standard methods due to its high sensitivity; 3D-imaging is also possible [11].

Non-invasive imaging techniques normally employ luciferases of *Photinus pyralis* and *Pyrophorus plagiophtalamus* (for D-luciferin) and luciferases of *Renilla reniformis* and *Gaussia princeps* (for coelenterazine). Recently, the small NanoLuc luciferase has become increasingly popular; it is applied with synthetic luciferin called furimazine [12]. Luciferases are sometimes conjugated to fluorescent proteins and quantum dots to shift the signal to longer wavelenghthusing bioluminescence resonance energy transfer (BRET); this technique is applied to obtain images of deep tissues [13, 14].

The phenomenon of bioluminescence is also used in quantum dot-based photodynamic therapy of tumors. A photosensitizer with a powerful cytotoxic effect on cancer cells is delivered to tumors residing in deep tissues where it can be activated using BRET by photons emitted from a luciferase [15, 16].

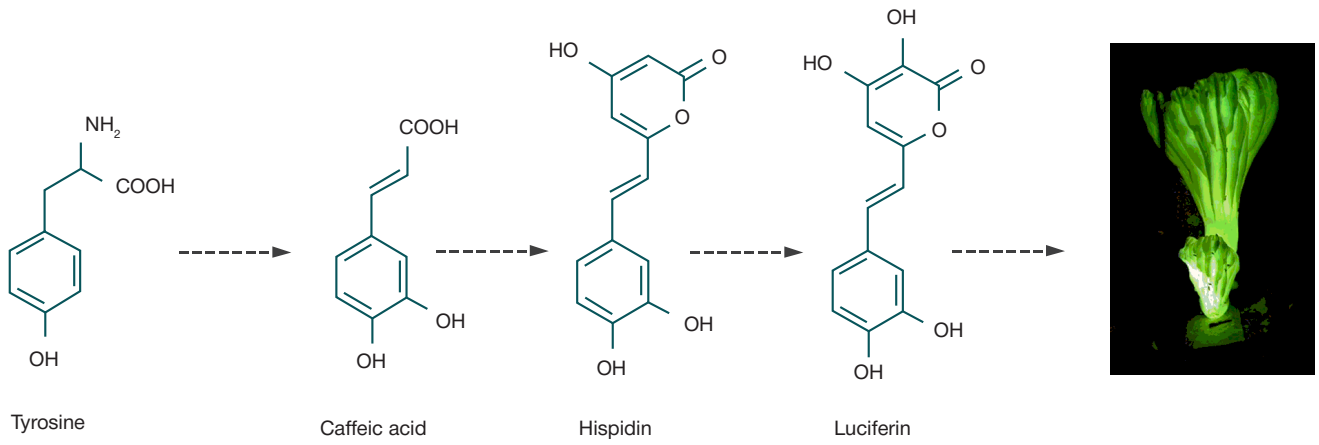
Luminescent system of higher fungi is a new alternative

An unfortunate disadvantage of luminescent systems used for bioimaging in cancer research is their dependence on luciferin from external sources that needs to be added before every experiment. The only autonomous bacterial luminescent system available nowadays is toxic for eukaryotes. Its key element is the *luxCDABE* operon. Initially, every attempt to express a bacterial luminescent system in eukaryotic cells resulted in failure. But after a massive structural rearrangement of the operon, bioluminescence in yeast and human cells became finally possible under the condition that luciferin should be added into the system externally [17]. In 2010 after the additional rearrangement of genes in the operon, human cells capable of autonomous luminescence were obtained [18].

At the moment, bacterial luminescent systems are not so readily implemented in eukaryotic cells. To incorporate such systems into a different organism, the same time-consuming manipulations with the operon will probably be needed, including rearrangement of regulatory and operon sequences and introduction of additional linker regions. That said, creating an autonomous luminescent system is still a problem.

For luciferins used in contemporary research, such as D-luciferin and coelenterazine, the problem seems to have no quick solution, because no clear picture of how these molecules are synthesized in the living organism is available. Recently a structure of luciferin of higher fungi (3-hydroxyhispidin) has been described [19] and its bioluminescence studied in depth [20]. A biosynthetic precursor of luciferin in fungi is caffeic acid. Luciferin is produced from it in two steps. First, hispidin is made from two caffeic acid derivatives, namely caffeoyl-CoA and malonyl-CoA, in the presence of polyketide synthase [21]; then, hispidin is converted to luciferin in the presence of hydroxylase (see the Figure).

Caffeic acid is a common secondary metabolite i plants. Transfer of genes coding the proteins involved in synthesis and bioluminescence of luciferin in fungi to plant cells seems to be more realistic solution than manipulations with prokaryotic bacterial genes. But we still believe that autonomously luminescent cells can be obtained not only from fungi but also from other organisms, such as yeast and mammals. Enzymes involved in a two-step process of caffeic acid biosynthesis from L-tyrosine are already known; they are phenylalanine ammonia lyase and cinnamate 4-hydroxylase. Also the genes encoding these enzymes are known (sam8 and sam5, respectively) [22]. Insertion of them into the cluster of genes responsible for fungal luminescence will help to create the first fully autonomous



A schematic of luciferin biosynthesis in luminescent higher fungi

bioluminescent system that can be used for bioimaging in eukaryotic organisms.

CONCLUSIONS

Bioluminescence imaging applicated in studiesof mechanisms of disease progression or response to treatment has a few advantages over other methods, one of them being incredibly high sensitivity. However, all bioluminescence systems share a common downside: to function, they require a luciferin substrate from external sources. Development of an autonomous luminescent system based on the gene cluster responsible for bioluminescence in higher fungi may offer a potential solution to this problem.

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REHABILITATION OF PATIENTS WITH INFERIOR ALVEOLAR NERVE INJURIES

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Mandibular fractures are a common type of injuries of the facial bones. Most of them affect the angle and body of the mandible in the areas innervated by the inferior alveolar nerve (IAN). Inpatients of maxillofacial units often lack health literacy; therefore, the aim of our study was to improve the effect of treatment in patients with mandibular fractures and IAN injuries by motivating patients toward a healthy lifestyle and by pioneering the use of therapeutic agents Mexicor and Combilipen in the standard regimen.

Keywords: mandibular fracture, inferior alveolar nerve injury, rehabilitation, healthy lifestyle, Mexicor, Combilipen

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РАЗРАБОТКА МЕРОПРИЯТИЙ ПО РЕАБИЛИТАЦИИ ПАЦИЕНТОВ С ПОВРЕЖДЕНИЕМ НИЖНЕГО АЛЬВЕОЛЯРНОГО НЕРВА

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Среди повреждений костей лицевого скелета переломы нижней челюсти встречаются часто. Наиболее распространенной локализацией переломов нижней челюсти при которых происходит травма нижнего альвеолярного нерва (НАН), являются угол и тело, в толще которых проходит НАН. Пациенты челюстно-лицевых стационаров имеют низкую медицинскую и социальную грамотность, поэтому целью исследования явилось повышение эффективности лечения больных с переломами нижней челюсти, сопровождающимися травмой НАН, путем внедрения нового комплекса фармакологических препаратов — Мексикора и Комбилипена, ранее не использовавшихся в челюстно-лицевой хирургии, и медико-социальных мероприятий, направленных на формирование мотивации к ведению здорового образа жизни у пациентов.

Ключевые слова: перелом нижней челюсти, повреждение нижнего альвеолярного нерва, социальная реабилитация, здоровый образ жизни, Мексикор, Комбилипен

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In recent years, severe traumatic injuries of facial bones have become increasingly incident, including multiple fractures and associated injuries received in road accidents, assaults, sports competitions, etc. [1]. According to foreign and domestic reports, mandibular fractures make up to 87% of such injuries; most of them involve damage to the inferior alveolar nerve (IAN) [2, 3] caused by bruising, overstretching or pressure from the fragments of the injured mandible. Strains are observed in 70.1% of cases, contusion in 16.8%, incomplete rupture in 12.5%, and complete rupture in 0.6% of cases [4]. IAN injuries are subdivided into subclinical, mild, moderate, moderate-to-severe, and severe [5, 6]. Regardless of their type, they trigger neurotrophic changes in all tissues innervated by IAN, resulting in poor bone healing and sensory deficit. One of the major symptoms of IAN damage suffered by every patient is pain [7, 8]. Inpatient care is normally provided at maxillofacial units; outpatients are referred to special rehabilitation units upon discharge from hospital. Treatment includes surgical interventions, such as immobilization of fractured bones or external fixation if bones are severely displaced, and conservative drug therapies [9, 10, 11, 12, 13].

Management of mandibular fractures should also include psychological counselling to motivate patients towards a healthy lifestyle and health literacy [14, 15].

Delayed diagnosis, immobilization or surgical intervention, ineffective therapy, failure to comply with doctor's recommendations and unwillingness to give up bad habits lead to the irreversible damage to IAN, which ultimately impedes fracture healing, promotes inflammation, and causes the loss of sensation and muscle dysfunction in the affected zone. Therefore, there is a need for an improved rehabilitation plan for patients with mandibular fractures.

METHODS

The study recruited 212 men aged 19 to 63 with unilateral mandibular fractures and no severe comorbidities who presented to Pirogov City Clinical Hospital No. 1 (Moscow) in 2011–2016 within 24 hours after the injury. Patients with severe comorbidities, inflammation, and multiple fractures of the mandible were excluded from the study. All the participants gave their informed consent. The study was approved by the Ethics Committee of Pirogov Russian National Research Medical University (Protocol No. 107 dated April 18, 2011). The participants were surveyed to obtain information about their medical history and social background. Upon discharge from the hospital the patients were referred to the rehabilitation unit. The patients also received psychological counseling.

Generally, treatment included fracture immobilization and extraction of teeth in the fracture line, if indicated. If brain injury was suspected, the patients were referred to a neurologist or a neurosurgeon. Possible comorbidities were ruled out by a primary care physician, trauma surgeon, and otolaryngologist. If maxillomandibular fixation turned out to be ineffective or the patients developed posttraumatic malocclusion, titanium plate osteosynthesis was performed. Conservative therapy included antibiotics to prevent inflammatory complications (IM injections of 2 ml lincomycin and 1 g cefazolin, both twice a day), analgesics (2 ml diclofenac, IM, twice a day), oral irrigations, and medications prescribed by other specialists.

The patients were divided into 2 groups. The control group (86 patients) received 1 ml 0.5% neostigmine methyl sulfate solution (marketed as Proserin by Dalhimfarm, Russia) for one month, 500–1,000 μg cyanocobalamin alternated with 3 ml 5% thiamine chloride (15 to 20 injections), and 0.005 g dibazol per os for one month. The main group consisted of 126 patients who received Combilipen by Pharmstandard-UfaVITA, Russia (pyridoxine + thiamine + cyanocobalamin + lidocaine) and ethyl methyl hydroxypyridine succinate (marketed as Mexicor, EcoPharmInvest, Russia).

The dose of ethyl methyl hydroxypyridine succinate recommended by the manufacturer was 150 mg; it was administered to the patients intramuscularly twice a day for 14 days. After that the patients received the medication in capsules (100 mg twice a day for 21 day).

Combilipen was administered to the patients intramuscularly at the dose of 2 ml once a day for 10 days, followed by a 3-week regimen of 3 times a week.

Based on the classification of IAN injuries proposed by A.Korzh in 1989, the patients in both groups were subdivided into three subgroups depending on whether their IAN injuries were mild, moderate or moderate-to severe/severe.

Treatment effect was evaluated based on:

1. The electrical excitability of the lower lip and chin on the affected side. To measure it, we needed to determine the actual site of nerve damage and estimate its size.

To ensure maximum accuracy of both measurements and result interpretation, we selected three landmarks on the patient's face two of which were located midway between the lower lip and the most prominent point of the chin (A) and midway between the mouth corner and the lower border of the mandible (B). Thus, the line connecting A and B was horizontal. Point C was located halfway between the middle of the lower lip and the mouth corner. The vertical line going downwards from point C crossed the horizontal line and thus formed 4 test zones [16].

The sensory exam included light stimulation of the chin skin with a brush or a cotton pad. To assess patient's deep sensations, the chin was palpated.

Electrical excitability of the skin on the affected side was measured in the 4 zones mentioned above within 24 hours after admission, on day 10, on days 32–40, after the splints were removed, and 6 months after the injury.

The skin of the examined zones was cleansed with 70% ethyl alcohol (ethyl alcohol also ensured better electrical conductivity). The electrode of PARKELL Digitest 2 (Parkell, USA) was placed on the sites innervated by IAN in the 4 studied zones. We determined the minimum current that evoked a sensation in the patient (tingling, prickling, etc.). The patient reported their sensation to the doctor. The current was supplied in pulses increasing from 0 to 160 μA [17].

Skin excitability on the affected side corresponded with the severity of injuries to IAN aiding the distribution of patients into the groups. For uniformity, skin excitability was measured in zone 3 in all patients. On the intact side it varied from 25 to 35 μA in all patients. In patients with mild injuries excitability on the affected side ranged from 35 to 69 μA; patients with moderate injuries demonstrated a range between 70 and 129 μA; in patients with severe injuries skin excitability was 130 μA and higher.

2. Social background and hygiene of the patients.

More than half of the patients with mandibular fractures were under the influence of alcohol at the time of admission. Of those patients who were behind the wheel during the accident, 29.8% were drunk. Our survey revealed that 38.4% of the participants had a drinking problem. There were 2.3 times more alcohol abusers among the unemployed patients than among those who had a job (56.6% vs 24.6%). Among the patients with a university degree the number of alcohol abusers was significantly lower ($p < 0.05$) than among those who did not have a degree. The coefficient r of correlation between alcohol abuse and education was -0.889 ($m = \pm 0.114$, $p < 0.05$). Of all surveyed patients, 70.4% were regular smokers; half of them (50.8%) admitted that smoking was bad for their health but could not give it up. Only 3.4% did not believe smoking could have a negative effect on their health.

Ninety percent of the respondents reported that they had never tried narcotic drugs; 7.1% had tried them once, and 1.9% still used them occasionally. One in 100 patients was a regular user. All of those patients were below 40 years of age.

Among the patients with mandibular fractures 44.2% were overweight. Only 13.4% of the participants watched their weight. Overweight and its metabolic consequences can delay bone healing. A very thick subcutaneous tissue layer complicates palpatory and visual examination of fractures and facial deformities. Sometimes blood vessels leak fluid into the subcutaneous tissue, causing hematomas that can get infected and start festering. In our study the coefficient r of correlation between these parameters was + 0.749 ($n = \pm 0.114$, $p < 0.05$). Only 9.8% of patients followed doctor's advice and watched their diet. Among them the majority were over 50 years of age.

Body weight may be indicative of a particular diet a patient follows and his/her attitude to physical exercise. More than

half of the study participants (59.8%) tended not to engage in physical activity. Among them the number of patients over 50 years of age was 2.3 times higher than among their younger counterparts (74.4% vs 31.1%, p<0.05). It total, three quarters of the patients rarely visited a doctor when they got ill, did not undergo regular checkups, did not do any therapeutic exercise or follow doctor's advice, etc.

RESULTS

Using PARKELL Digitest 2 (Parkell, USA) we obtained data about the electrical excitability of the skin in IAN-innervated areas located on the chin and the lower lip. Dental pulp tests were not performed because patients were wearing fixation splints at that moment.

Our study was conducted in two groups of patients: the first consisted of patients with angle fractures of the mandible who received Mexicor and Combilipen as part of their complex therapy, and the second group included patients who did not receive those medications. Measurements were taken on the day of admission, on day 10 following the admission, days 32–40 and 6 months after the injury.

Skin excitability was higher in zones 2 and 3 than in zones 2 and 4.

In patients with mild injuries to IAN mean excitability values were 55 ± 1.5 μA and 58 ± 1.5 μA (zones 2 and 3, respectively). The patients reported a tingling sensation in the lower lip on the affected side and a reduced sense of touch. In patients with moderate injuries skin excitability varied from 109.6 ± 3.1 μA to 121.9 ± 3.5 μA. In patients with severe injuries it ranged from 159.5 ± 4.5 μA to 168.1 ± 4.8 μA, suggesting a more serious loss of sensation in the studied zones. Patients with moderate and severe damage to IAN reported a total loss of sensation in these zones, complained of pain, dysfunction of facial muscles, impaired mucosal and dental sensation on the affected side. Because IAN branches supply sensation to the front teeth, small contralateral zones of paresthesia were observed in the lower lip and chin of 18.6% of patients.

Over the course of treatment, the main group patients with mild injuries demonstrated reduction in skin excitability in zones 1 and 4 (29.4 ± 1.4 μA and 27.4 ± 1.5 μA, respectively). These values correlated with the electric excitability of the skin on the intact side. In zones 2 and 3 excitability decreased to 40.1 ± 4.2 μA and 42.3 ± 4.0 μA, respectively, in the main group. This tendency was less pronounced in the controls in which skin excitability decreased to 35.1 ± 1.4 μA in zone 1, 49.6 ± 4.6 μA in zone 2, 50.8 ± 4.6 μA in zone 3, and 36.2 ± 1.6 μA in zone 4.

In the main group the patients with moderate and severe injuries also demonstrated reduction in the electric excitability of the skin on the affected side. The values were 1.2-1.3 times lower than in the controls.

Our analysis reveals that after completing their treatment, the patients with mild injuries both in the main and control groups had the same level of skin excitability on the affected and intact sides. Only 15% of the controls retained a tingling sensation, while others no longer showed any clinical symptoms of IAN damage.

In the patients with moderate injuries skin excitability was the highest in zones 2 and 3, equaling on average 40.5 ± 3.9 μA and 59.7 ± 3.7 μA, respectively, in the main group, and 65.1 ± 5.4 μA and 85.7 ± 5.1 μA, respectively, in the controls. In the main group the paresthetic area grew smaller, but in 25% of the controls its size did not change.

In the patients with severe damage to IAN, skin excitability in the studied zones was 1.3–1.4. times lower than in the controls. At the end of the treatment course 73% of the controls still had pronounced clinical symptoms indicating IAN damage, whereas the patients in the main group were gradually improving (smaller areas of paresthesia, less severe pain). As seen from the measurements taken on days 32–40 of treatment, skin excitability in all 4 zones was decreasing in the patients who had been receiving Combilipen and Mexicor. The choice of those therapeutic drugs was not random: Order 1497n of the Ministry of Health of the Russian Federation on the Treatment of patients with damage to the facial nerve dated December 24, 2012, recommends that such patients should be prescribed vitamins B1, B6 and B12 as part of their complex treatment. The beneficial effect of antioxidants in the complex therapy of peripheral nerve injuries has also been demonstrated by a number of foreign and Russian researchers [18, 19].

Rehabilitation of patients with mandibular fractures included psychological counselling, drug therapy and surgical interventions. Only half (47.7%) of the patients complied with medical recommendations, 32.7% ignored those recommendations and 19.6% followed them selectively.

According to the patients, among the factors interfering with their medical rehabilitation were the absence of positive effect of treatment (49.3 cases), queues in rehab units (41.2), pain or unpleasant sensations during medical procedures (38.7), lack of money (37.6), lack of motivation (28.4), lack of trust in doctors (23) (cases are specified per 100 surveyed patients). Almost half of the surveyed patients who only partially complied with the medical recommendations named 2 to 3 reasons that prevented them from completing the full course of treatment.

DISCUSSION

The electrical excitability of the skin in the areas innervated by IAN measured at the time of admission was 55.5 ± 1.5 μA in zone 2 and 58 ± 1.5 μA in zone 3 in the patients with mild injuries; in the patients with moderate injuries the figures were 109.6 ± 3.1 μA and 121.9 ± 3.5 μA, respectively; in the patients with severe injuries skin excitability in zones 2 and 3 was as high as 159.5 ± 4.5 μA and 168.1 ± 4.8 μA, respectively. Ten days after the injury skin excitability decreased 1.2–1.3-fold in the main group in comparison with the controls. On days 32–40 excitability in zones 2 and 3 was 40.5 ± 3.9 μA and 59.7 ± 3.7 μA, respectively, in the main group patients with moderate injuries, and 65.1 ± 5.4 μA and 85.7 ± 5.1 μA, respectively, in the controls. In the patients with severe injuries it was 1.3–1.4 times lower than in the controls. 73% of the controls retained clinical symptoms at the same level, while the dynamics in the main group was positive (smaller areas of paresthesia, alleviated pain).

Table 1. Electrical excitability of the skin of the lower lip in zones 2 and 3 at the time of admission, corresponding with the severity of damage to IAN

Zone	Severity of IAN damage		
	mild	moderate	severe
2	55 ± 1.5 μA	109.6 ± 3.1 μA	159.5 ± 4.5 μA
3	58 ± 1.5 μA	121.9 ± 3.5 μA	168.1 ± 4.8 μA

By the end of treatment, the patients with mild and moderate injuries to IAN had regained normal electrical excitability of the skin. Clinical symptoms of IAN damage such as numbness in the lower lip, pain during palpation of the lower lip, and the loss of sensitivity on the affected side, were gone.

In the patients with severe injuries (the main group) who completed the treatment course electrical excitability in all studied zones fell within the normal reference range. But in the control group 12 patients still had increased excitability in zones 2 and 3 (45.1 ± 3.2 μA and 43.2 ± 3.1 μA, respectively). Increased skin excitability was accompanied by such symptoms as numbness and tingling.

By days 32–40, the area of paresthesia in the lower lip, mouth corner, chin skin, and gums on the affected side had shrunk in 103 patients from the main group (as compared to the controls) and covered only zones 2 and 3. Because Mexicor had a positive effect on microcirculation that was visible already on day 3 of treatment: soft tissue edema on the affected side diminished in size in 74 patients.

Motivating the patients toward a healthy lifestyle was one of the most important components of the treatment plan. Our study shows that low health literacy is one of the major factors negatively affecting person's general health. The patients were encouraged to change their attitude to health care in general and diet and exercise in particular. As a result, the proportion of patients following a healthy diet increased from 13.6% to 36.2%; 30% of patients improved their sleeping habits (vs 16.1% before the study), and 23.2% (vs 12.6% before the study) became more physically active. Eleven percent of the patients gave up smoking.

CONCLUSIONS

The measurements taken on days 32–40 of treatment demonstrated that the electrical excitability of the skin had decreased in the 4 studied zones in the patients with moderate and severe injuries who had been receiving Combilipen and Mexicor.

The paresthetic area in the patients from the main group had also shrunk and covered only zones 2 and 3 at the time of measurements, while in the controls it still covered zones 1, 2 and 3. Therefore, we conclude that Combilipen and Mexicor stimulate regeneration of the damaged nerve and alleviate clinical symptoms of IAN injuries.

Rehabilitation also included psychologic counselling aimed to motivate the patients toward a healthier lifestyle and change their attitude to medical care in general. As a result, the number of patients who started to trust medical recommendations increased by 31.2%. This, in turn, led to a 2.9-fold increase in the number of patients with IAN injuries who enjoyed a complete recovery.

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REPAIR OF FRESH INJURIES TO THE ACROMIOCLAVICULAR JOINT BY DOUBLE-BINDLE RECONSTRUCTION

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Dislocation of the lateral end of the clavicle (LEC) constitutes over 26% of all dislocations, 11% of sports injuries and over 10% of acute injuries to the shoulder girdle, ranking 3rd after elbow and wrist joints dislocations. The majority of surgical techniques used to repair fresh injuries to the acromioclavicular joint (ACJ) do not account for its anatomy and biomechanics, resulting in postoperative instability of the joint in both vertical and horizontal planes. The aim of this study was to propose a highly effective technique for the surgical treatment of acute injuries to ACJ ensuring a better recovery of its function. Below we present the results of 112 patients who underwent minimally invasive acromioclavicular joint reconstruction. The outcome was very good in 111 patients (99.1%). The proposed technique helps to avoid damage to the biomechanics of the joint and to fully restore its anatomy within short time.

Keywords: dislocation of the clavicle, acromioclavicular joint, double-bundle reconstruction, fresh injury

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ВОССТАНОВЛЕНИЕ НЕДАВНО ПОЛУЧЕННЫХ ПОВРЕЖДЕНИЙ АКРОМИАЛЬНО-КЛЮЧИЧНОГО СОЧЛЕНЕНИЯ МЕТОДОМ ДИНАМИЧЕСКОЙ ДВУХПУЧКОВОЙ РЕКОНСТРУКЦИИ

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Вывихи акромиального конца ключицы (АКК) составляют более 26% всех вывихов, 11% всех случаев спортивных травм, более 10% всех случаев острых травм плечевого пояса, занимая третье место после вывихов в локтевом и лучезапястном суставах. Большинство техник оперативного лечения недавно полученных (свежих) повреждений акромиально-ключичного сочленения (АКС) не учитывают анатомию и биомеханику сочленения, в связи с чем в послеоперационном периоде у пациентов сохраняется один из возможных в этом сочленении видов нестабильности (горизонтальная или вертикальная нестабильность). Целью нашего исследования было разработать высокоэффективную методику оперативного лечения недавно полученных повреждений, позволяющую восстановить функцию АКС в ранние сроки. Представлены результаты оперативного лечения методом малоинвазивной динамической реконструкции АКС у 112 пациентов со свежими вывихами АКК. В 111 (99,1%) случаях получен хороший результат. Использование предложенного метода позволяет не нарушать биомеханику сочленения, полностью восстановить анатомию и в кратчайшие сроки получить хороший функциональный результат.

Ключевые слова: вывих ключицы, акромиально-ключичное сочленение, АКС, двухпучковая фиксация, недавно полученные повреждения

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Over 26% of all dislocations are those of the lateral end of the clavicle (LEC) [1]. LEC is especially important in the world of sports, since the overwhelming majority of patients with such a dislocation are sportsmen, amateurs and professionals, aged from 25 to 45 years.

Incomplete and late diagnostics is one of the main reasons behind unsatisfactory outcomes of LEC dislocation treatment. However, even when the dislocation is found early, it does not mean there is no discussion as to how to treat it [2]. Treatment recommendations are often not just conflicting but mutually exclusive: they range from restorative surgery during acuity to refusal to do any surgery even when LEC dislocation is a complete one.

There are over 200 conservative and aggressive (surgery) approaches to LEC dislocation treatment described in the published papers [3]. This diversity results from the anatomy and biomechanics of ACS, which is a "suspension" of the upper limb [4]. Surgery also leads to a failure quite often: practitioners tend to choose outdated techniques, which do not take biomechanics of the joint into account [5].

Many studies confirm that it is the coracoclavicular ligament that takes the load born by ACJ [6]. Coracoclavicular ligament is double bundle; it includes trapezoidal and conical ligaments. These ligaments are angled relative to each other and form the unique biomechanics of ACJ [7, 8]. Based on this, we believe that restoration of both coracoclavicular ligament's bundles is a successful LEC dislocation treatment technique.

Recently, there appeared new approaches to LEC dislocation treatment. These approaches make use of external fixation, shape memory tighteners, minimally invasive techniques etc.

However, it is still the surgeon who decides upon the LEC dislocation treatment method in each case, and often the great variety of available techniques is not taken into account.

Fixation hardware is a source of serious inconvenience for patients. Tensioned needles often cause uncontrolled LEC hypercorrection, which contributes to the relapse of dislocation. Their use is limited by the complexity of application.

LEC fixation with thread and a wire loop (Weber technique) often led to a relapse of dislocation. Typical complications accompanying this technique are migration and deformation of threads and wire breakage [9].

The hook plate technique, which is widely used nowadays to keep LEC fixed, is rigid, highly traumatic, and implies consequent implant removal surgery. The registered LEC dislocation recurrence rate is high, as are migration and fixator fracture [10]. Chronic traumatization of acromion caused by the plate combines with severe pain and restriction of shoulder movements, which often leads to ACJ arthrosis (18.1%), subacromial impingement (8.76–37.5%), acromion osteolysis (2.56–30.3%), ACJ osteoarthritis (18.1%) [11, 12, 13].

LEC fixation with thread is not an optimal technique, either: cerclage in the coracoid's can lead to an incomplete dislocation of the clavicle, while its rotation leads to wires cutting through the bone. Generally, putting wires in means trauma.

Despite the advantages of arthroscopic operations, many traumatologists still choose long-established clavicle stabilization techniques over them because they require special skills and equipment. Arthroscopic approach involves extensive debridement of the coracoid's lower surface, which can result in extra damage to the remaining coracoclavicular ligament and the neurovascular structures found there. On the other hand, such techniques allow precision in positioning tunnel in the coracoid.

Currently, anatomical reconstruction of the ACJ ligaments is the primary target of LEC dislocation treatment [14, 15, 16, 17].

The choice is often made in favor of minimally invasive ACJ operation techniques [18, 19, 20]; one of the most promising of them is MINAR, Minimally Invasive Acromioclavicular Joint Reconstruction [21]. This technique was developed by professor Wolf Petersen et al at the Martin Luther Hospital in Berlin. Gear used in MINAR: Flipptack (KARL STORZ GmbH & Co, Germany) fixators and Ethibond 2.0 (Johnson & Johnson, USA) cord. The technique involves immobilizing LEC with one bundle to stabilize and hold the treated distal part of the clavicle in position, which allows the coracoclavicular ligament to heal on its own. This technique has been used by medical doctors practicing at the Department of Traumatology, Orthopedics and Military Field Surgery of the Russian National Research Medical University named after N.I. Pirogov since 2009. The results of treatment of 156 patients were analyzed. 78% of patients with IV and V type dislocations (Rockwood classification [22]) suffered from persisting horizontal instability, which gave us the idea to develop a minimally invasive dynamic double bundle ACJ reconstruction technique. This technique allows complete reconstruction of ACJ anatomy by replacing both portions of the coracoclavicular ligament. The goal of this research was to develop a highly effective surgical treatment method to address fresh LEC dislocations through minimally invasive ACJ reconstruction aimed at rapid restoration of the shoulder joint's function.

PATIENTS AND METHODS

From 2011 to 2017, the minimally invasive dynamic double bundle ACJ reconstruction technique (patent RU 2017112434 of 19.10.2017) was applied in 112 cases, all of which were fresh injuries. The patients were treated in Hospitals #1 and #64 operating under the Russian National Research Medical University named after N.I. Pirogov. The criteria for inclusion into this research were as follows: men and women aged 18 to 70 years (Table 1); injury freshness — 3 to 7 days, sports related and not (Table 2); clinical and radiological diagnosis, injury class determined by Rockwood classification (1984), which allows the most reliable assessment of damage to ligamentous stabilizers and degree of displacement of the clavicle's acromial end (Figure 1). Exclusion criteria: age under 18 and over 70; injury freshness over 7 days; other discrepancies with the inclusion criteria. Ultrasound, MRI, CT and other examination methods applied in combination allowed realistic pre-surgery evaluation of the injury and post-surgery assessment of the healing process (figure 2).

Statistica 10 software enabled statistical analysis of the research data.

Surgery technique

The technique revolves around fixing clavicle to scapula with two "fastener – thread" systems; their location and direction mimic portions of the coracoclavicular ligament (conical and trapezoidal).

1. Skin incision and surgical access preparation. A "saber" vertical incision 3 cm long (or less) 2–3 cm from ACJ, projection — coracoclavicular ligament. The incision line should cover 2/3 of clavicle and run 1 cm anteriad to the clavicle towards the apex of the coracoid process.

2. Deltoid muscle splitting with blunt and sharp instruments; coracoid process palpation in the projection of coracoclavicular ligament attachment. Important: deltoid muscle dissection was started at the very edge of the clavicle and along the muscle fibers. Such an approach simplified further surgical manipulations. The special canal drilling guide (canal in the coracoid process) was introduced from the lateral side and under the coracoid process. The design of this guide ensures protection of neurovascular structures during canal drilling.

The goal is to make the canal at the base of the coracoid process in the projection of coracoclavicular ligaments

Table 1. Patients by sex and age

Age/Sex	Male	Female	Total
Under 18	2	0	2 (1.8%)
19–30 y.o.	58	3	61 (54.5%)
31–40 y.o.	40	2	42 (37.5%)
41–50 y.o.	4	1	5 (4.5%)
51–60 y.o.	1	0	1 (0.9%)
61–70 y.o.	1	0	1 (0.9%)
TOTAL:	112		112 (100%)

Table 2. Patients by type and mechanism of injury

Type of injury	Injury mechanism	Number of patients
Domestic	direct	19
	indirect	18
Sports	direct	44
	indirect	27
Traffic accident	direct	4
	indirect	0
TOTAL:		112

attachment. Topography of the canal defines if there develops an anteriad subluxation of the clavicle afterwards.

3. A special sleeve was inserted into the guide; this sleeve guided the needle, diameter of which was 2.4 mm. Cannulated drill with the diameter of 4.3 mm went along the needle and made a through canal in the coracoid process.

4. Two dynamic block "fastener – thread" were modeled. The fasteners used were 4-hole titanium Flipptack, 12 mm long and 4 mm wide. Titanium alloy (TiAl6V4) is an inert material, i.e. the surrounding soft tissues do not react to its presence actively.

Central holes of both fasteners received non-resorbable woven braided polyester suture (diameter — 1 mm). Block system modeling implied joining the two fasteners and leading one end of the thread through their central holes. Next, that end went through the nearby hole, then again into the same holes. As a result, the fasteners were interconnected and the thread ran through the central holes twice, its ends remaining on one side. The fasteners were then driven 7–8 cm apart, which resulted in appearance of two loops, one of them closed. Pulling at the ends of the thread allowed bringing the fasteners closer to each other; tying the ends resulted in fixation of the fasteners while keeping the required distance between them.

5. Next, fasteners of each block system were driven into the drilled canal with a special pusher. 4.3 mm drill was used to make two through tunnels in the clavicle, projections of lig. conoideum and lig. trapezoidum attachments. With the help of a Dechamp's needle the loop was made through the canals; forceps were used to bring it out of the wound. One of the outer holes of the upper fastener received a thread, the ends of which were lead through the exposed loop. By tightening the ends of the loop the thread that ran through the fastener was brought outside (upwards) through the the canal in the clavicle. Subsequent pulls at this thread allowed leading the upper fastener upwards, through the canal in the clavicle. Thus were the fasteners brought upwards onto the clavicle. Alternating tensioning of ends of both block systems' threads enabled pulling the fasteners together and fixation of the adjusted LEC. The ends of the thread were tied together.

Clinical case. Patient U. 35 y.o., injured during a hockey game, hit the rink's board. Clinical and radiological examination at admission. Diagnosis: dislocation of the right clavicle's acromial end, Rockwood classification type V.

Pre-surgery preparation included laboratory and instrumental examination (Figure 3).

The patient went into surgery on the 3rd day after receiving the injury; the technique applied was the minimally invasive dynamic double bundle ACJ reconstruction. Figure 4 is the the postsurgery image.

The postoperative period saw no complications; primary tension healed the wound. Immobilization (bandage) — 3 days, up to the disappearance of pain. The patient strictly followed the rehabilitation recommendations and started restoring the movement abilities the next day. The sutures were removed in the office. Strength exercises were added to the recommendation after 3–5 weeks. Follow-ups — 6 weeks and 6 months after the operation (Figure 5).

The result is considered to be good. Unrestricted limb loading was allowed 6 weeks after. 2.5 months later, after the rehabilitation, the patient started practicing sports on the professional level again. The radiograph taken 6 months later showed no migration of the fixator and no subluxation of the clavicle. Full functional recovery was acknowledged.

RESULTS

We studied long-term results in all 112 patients. They were examined and questioned 3–4 times a year, the interval between such sessions was at least 3 months. The treatment outcomes were evaluated on the basis of the latest examination.

Clinical tests helped determine the stability of ACJ. All patients had their ACJ radiographed in standard projections while loaded and in Zanca projection. When necessary, the patients went through MRI, CT. DASH (Disability of the Arm, Shoulder and Hand) scale [23] was used to register and systematize subjective feelings of patients, degree of functional recovery of the shoulder joint, daily activities limitations imposed by the upper extremity. The same scale was applied to objective examination results.

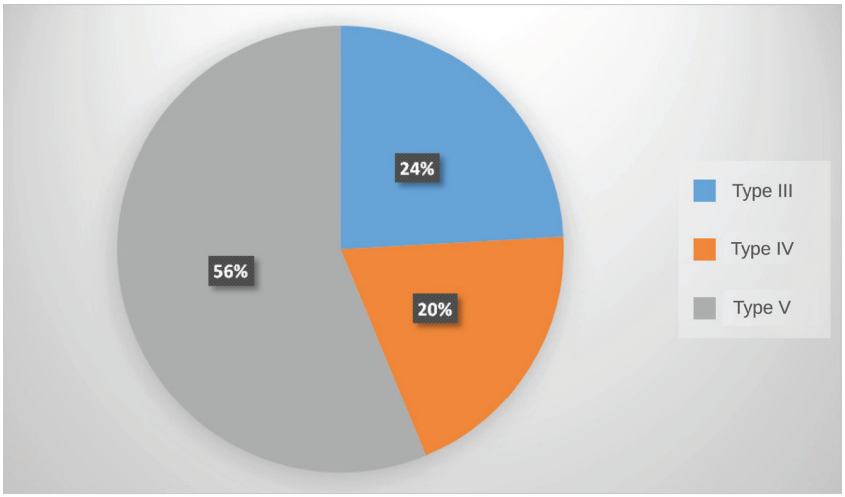

Fig 1. Patients by types of damage to ACJ

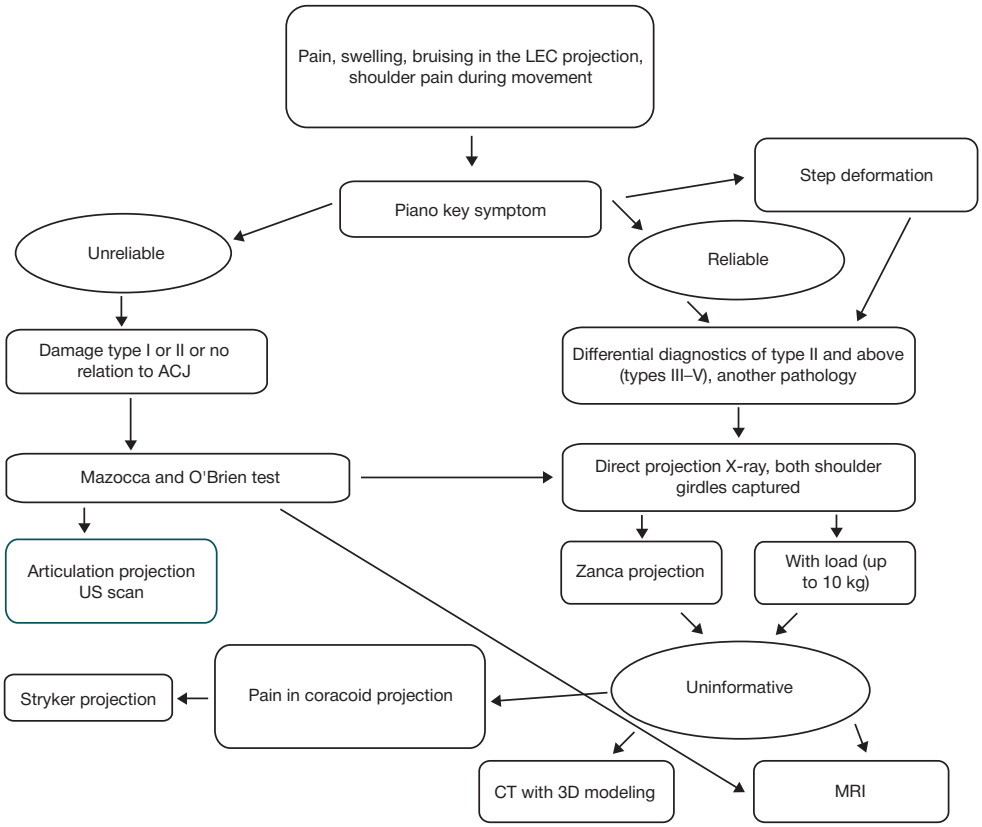

Fig 2. ACJ injury patients diagnostics diagram

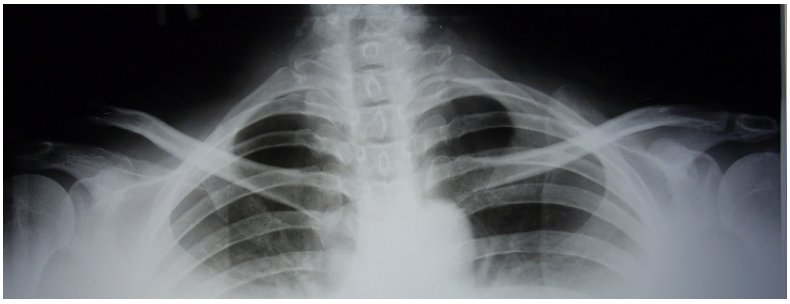

Fig 3. Results of the X-ray examination. The radiogram reveals a dislocation of the acromial end of right clavicle

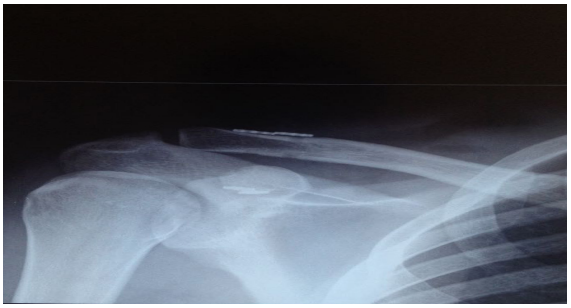

Fig 4. Dislocation reduced, joint fixed. State after double bundle ACJ reconstruction

Patients complaints were taken into account when assessing results of the treatment; VAS (visual analog scale) [24], a psychological test revealing subjective assessment, was used to register pain and learn its type. Other factors considered: residual deformation in the LEC projection; shoulder joint movements amplitude; functional capabilities of the upper limb; clinical tests results (piano key symptom) and X-ray tests (joint congruence) results.

In 111 (99.1%) cases the results of the treatment were good. One patient suffered from an operative wound infection that was limited to epidermis and required no further surgery. 8 days later that patient was discharged from the hospital.

DISCUSSION

The length of period between receiving the injury and seeking medical assistance is of great importance for surgery outcomes [25]. The advantages offered by the dynamic double bundle ACJ reconstruction technique when applied to fresh injuries are obvious: it is minimally invasive (the incision is 3 cm max); special

guides minimize the risk of damaging neurovascular structures; surgery does not imply exposing the ACJ and traumatizing its soft tissues; the technique is simple and fast (20 to 40 min average operation time); there is no need to remove an implant. Functional results shown by the technique surpass those offered by other treatment methods: anatomical connection and its dynamics do not violate anatomy and biomechanics of ACJ and allows restoring the limb's function within the shortest possible time, which is extremely important for professional athletes. Moreover, neither vertical nor horizontal instability never occur post-surgery.

CONCLUSIONS

We have developed and introduced into practice the minimally invasive dynamic double bundle ACJ reconstruction technique (patent RU 2017112434 from 19.10.2017) applicable to fresh injuries. Reconstruction of the joint does not hinder its biomechanics and allows complete restoration of the coracoclavicular ligament's anatomy through building two thread cerclages between coracoid process and clavicle where natural ligaments run. These aspects are fundamentally important about the developed technique. Vertical and horizontal stability prevents recurrence while maintaining physiological mobility of the limb. The developed technique can be introduced into daily practice of traumatology departments.

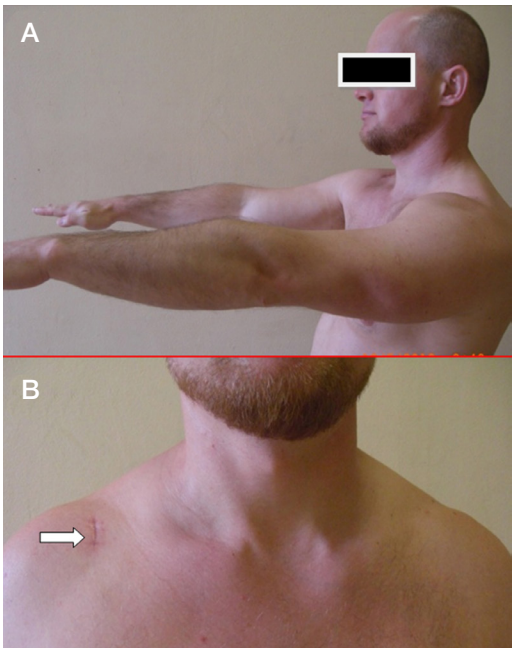

Fig 5. Surgery results after 6 months. **A**. Movement capabilities fully restored. **B**. Type of postoperative scar (*indicated by the arrow*)

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