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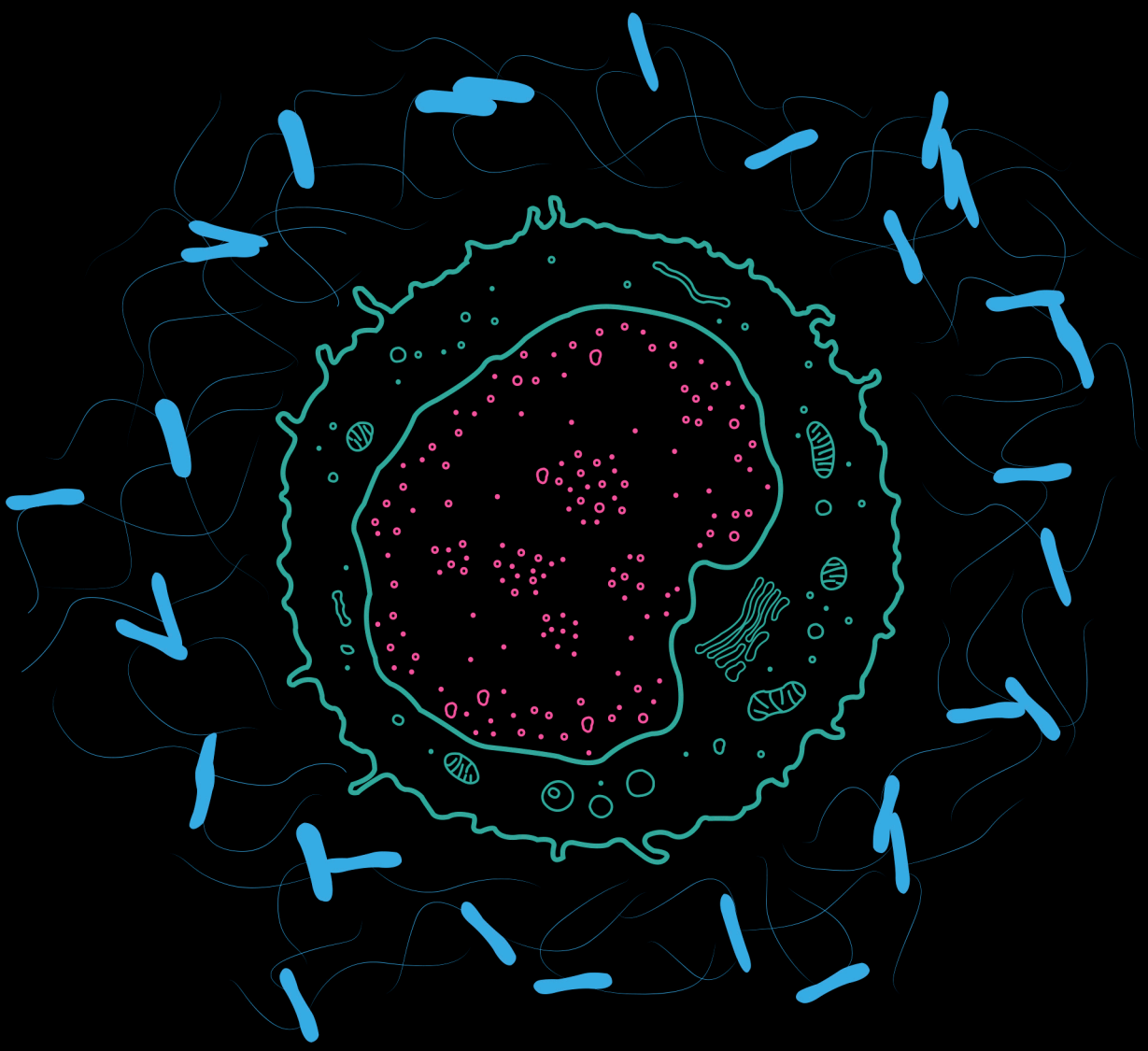

AMYLOIDS OF MICROORGANISMS REVIEW

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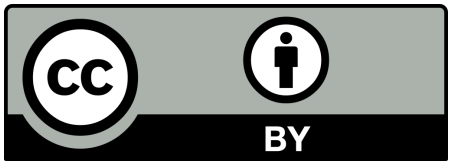

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CELL SURFACE AMYLOID PROTEINS OF MICROORGANISMS: STRUCTURE, PROPERTIES AND SIGNIFICANCE IN MEDICINE

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This review summarizes data which describe properties of microbial cell surface amyloids proteins. Definitions of amyloids and microbial functional amyloids are given. The review provides numerous examples of research in which the presence of amyloid-like properties in microbial cell surface proteins is demonstrated convincingly. Studies of the important role of pili, curli, tafi and some other bacterial fibrillar proteins in host colonization are reviewed. Data on amyloid proteins of yeast cell surface, their properties and potential association with candidiasis development are summarized. This review also appeals to experts in biology and medicine in an attempt to draw their attention to the issue which is increasingly discussed in scientific work at present, namely to a possible role of bacterial extracellular matrix amyloids and amyloid proteins of eukaryotic microorganism surface, yeast in the first place, in the development of amyloidosis in animals and humans.

Keywords: microbial cell surface, microbial amyloid, functional amyloid, pili, curli, tafi, phenol soluble modulin, adhesin, class I hydrophobin, amyloidosis

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АМИЛОИДНЫЕ БЕЛКИ ПОВЕРХНОСТИ МИКРООРГАНИЗМОВ: СТРУКТУРА, СВОЙСТВА И ЗНАЧЕНИЕ ДЛЯ МЕДИЦИНЫ

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В обзоре суммированы данные, посвященные описанию свойств амилоидных белков поверхности клеток микроорганизмов. Определены понятия «амилоид» и «функциональный амилоид» микроорганизмов. Приведены многочисленные примеры исследований, в которых убедительно показано наличие амилоидных свойств у белков клеточной поверхности микроорганизмов. Рассмотрены работы, демонстрирующие важную роль пилей, курлей, тафи и некоторых других фибриллярных белков бактерий в колонизации организма хозяина. Обобщены данные об амилоидных белках поверхности клеток дрожжей, их свойствах и возможной роли в развитии кандидозов. Обзор также призван привлечь внимание специалистов в области медицины и биологии ко все более активно обсуждаемому в литературе вопросу о возможном участии амилоидов внеклеточного матрикса бактерий, а также амилоидных белков поверхности эукариотических микроорганизмов, в первую очередь дрожжей, в развитии амилоидозов животных и человека.

Ключевые слова: клеточная поверхность микроорганизмов, амилоид микроорганизмов, функциональный амилоид, пили, курли, тафи, фенол-растворимый модулин, адгезин, гидрофобин класса I, амилоидоз

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The human microbiome is composed of an average of 10^{14} microbial cells [1], many of which have amyloid proteins on their surfaces. Some recent studies have lead us to hypothesize that the presence of those amyloids can contribute to the onset and development of many diseases such as systemic amyloidoses in higher animals and humans, tuberculosis and Alzheimer's disease [2–6].

From a medical perspective, the analysis and deep understanding of processes and molecular mechanisms underlying the assembly of amyloid structures in pro- and

eukaryote microorganisms offer broad opportunities. In the first place, the above-said refers to the elaboration of protection strategies against the negative impact of amyloids on humans and animals. It is important to understand how to most effectively prevent the formation of bacterial biofilms by pathogenic microorganisms or destroy those already formed, and to mitigate the effect of amyloid formation in animals and humans caused by exposure to microbial amyloids.

Amyloids are protein fibrils with cross-β-structure. Composed of monomers, they are β-sheets in which parallel or antiparallel β-strands run perpendicular to the fiber axis. The distance between the neighboring strands inside a β-sheet is 0.47 nm; the one between the neighboring β-sheets is 0.8 to 1.2 nm [7, 8]. Hydrogen bonding between peptide backbones of neighboring strands has an important role in stabilizing the structure of amyloid fibrils. Interactions between lateral groups of amino acid residues of neighboring polypeptides, such as hydrogen bonding, ionic and hydrophobic interactions, and stacking interactions, also contribute to the stabilization of the amyloid structure. High resistance to the fluctuations of such environmental parameters as hydrophobicity, salt concentration, pH, temperature, pressure, exposure to denaturing agents and proteinases is characteristic of amyloids, which is determined by a large number of interactions involved in stabilizing their structure [2, 9–12].

Because amyloids cause many widely spread incurable diseases (the amyloidoses), they have long been actively explored in humans and animals. Pili (from Latin *pilus* — a hair) were described in the middle of the 20th century in gram-negative and gram-positive bacteria [13]. However, it has been discovered recently that many structures on microbial surfaces are amyloid fibrils. By now, curli (from English *a curl*) or tafi (thin aggregative fimbriae) have been described in such bacteria genera as *Escherichia, Neisseria, Yersinia, Shigella* and *Salmonella* [2, 3, 10, 11, 14–17]. Pili have been described in *Streptococcus* genus, specifically, in *Streptococcus agalactiae, Streptococcus pyogenes* and *Streptococcus pneumoniae*, in *Mycobacterium tuberculosis* and other gram-positive bacteria. The assembly mechanisms of these structures and their role in host colonization have been described in sufficient detail [13].

It is well known that amyloids, specifically the so-called class I hydrophobins, are present on the surface of filamentous fungi, such as *Aspergillus fumigatus* [18]. Amyloids are found in microorganisms among structural molecules, adhesins and toxins. Along with the structures mentioned above, a growing list of already described amyloids includes phenol-soluble modulins of *Staphylococcus aureus* [12, 19], adhesins of *Candida albicans* [20, 21], and amyloids formed by TasA protein in *Bacillus subtilis* [22–24].

In the course of study of microbial surface amyloid proteins, the term “functional amyloids” was coined [25]; functional amyloids are amyloid-forming proteins that are not associated with pathologies in microorganisms and perform functions useful for microbial cells. A number of published works have demonstrated that the formation of functional amyloids is possible not just in microorganisms; a supposition has been made that they exist in all domains of the living world and participate in various processes, from biofilm formation in microbial communities to long-term memory regulation in animals [7]. This review will look at some examples of how amyloid proteins of microbial surfaces contribute to the development of diseases in animals and humans, and present some data characterizing the structure of these amyloids and the conditions under which they are formed.

Amyloids participating in the formation of bacterial extracellular matrix

Curli and tafi are the main protein components necessary for the extracellular matrix formation. They are present on the surface of many gram-negative bacteria, including a number of strains of *Escherichia coli, Salmonella spp*. and other *Enterobacteriaceae* [10, 11, 14–17]. *E. coli* curli bind to many human proteins, including fibronectin, laminin, type I collagen, major histocompatibility complex class I molecules, plasminogen and some others [26–29], and contribute to pathogenesis facilitating further microbial invasion of the host. [14, 30–32]. Curli are fibrillar structures attached to the bacterial outer membrane at one end. They can be up to several micrometers long and 3 to 4 nm wide. Curli tend to aggregate laterally by forming clusters up to 60 nm in diameter [33]. Curli fibrils are highly resistant to denaturing agents and proteinases but can be depolymerized after the short-term treatment with concentrated formic acid [10, 11, 34]. The data from circular dichroism spectroscopy indicate that the secondary structure of curli fibrils is rich in β-sheets [11]; curli fibrils also interact with amyloid-specific dyes, namely, congo red (CR) and thioflavin T (TT) [11, 27]. This information makes it possible to classify curli as amyloid fibrils [11].

Curli are necessary for bacterial biofilm formation and are the major protein component of the extracellular matrix formed along [33, 35]. It has been shown that curli genes are best expressed at temperatures below 30 °C, low concentration of nutrients, low osmolality and at the stationary growth phase, i.e. under the conditions that *E. coli* and other *Enterobacteriaceae* encounter outside the host. Under such conditions biofilm formation can contribute to bacteria survival [33]. Curli mediate the attachment of bacteria to various surfaces, including plant cells [36, 37], stainless steel [38], glass and plastic [33], and can considerably enhance microbial cell resistance to chlorine [38] and mercuric compounds [39].

Curli assembly is a process strictly regulated by the cell [14, 40]; it involves proteins encoded by at least two operons: *csgABC* and *csgDEFG* in *E. coli* [41]. Curli consist of two homologous proteins, namely, CsgA and CsgB, the main structural component of fibrils being CsgA protein [27]. Purified CsgA forms amyloid fibrils *in vitro* in the absence of other proteins. However, their β-strands are arranged into β-spirals instead of β-sheets. *In vivo* the presence of CsgB is necessary for CsgA amyloid fibril assembly [11, 27, 42]. CsgA secreted by a *csgB* deletion mutant of *E.coli* can polymerize on the surface of CsgB producing cells [11, 14, 27]. This phenomenon is called interbacterial complementation and is widely used in mutation studies aimed at detecting protein genes participating in curli formation [11, 43]. Interbacterial complementation proves that CsgB is a nucleating agent for CsgA polymerization [11].

The majority of CsgBs are localized on the bacterial surface, which indicates that the supposition of CsgB nucleating function is accurate [44]. CsgF provides the proper folding and localization of CsgB nucleator protein and is probably a chaperon-like protein [43]. CsgE periplasmatic protein is likely to participate in CsgA secretion and inhibit CsgA polymerization *in vitro* [45] due to the unmediated interaction between CsgE and CsgA molecules [46]. Thus, CsgE can be seen as a CsgA-specific chaperon. The evolving concept of the nucleation properties exhibited by microbial cell surface proteins in the course of amyloid formation allowed some authors to consider microbial-derived amyloid proteins as a real risk factor for amyloidoses and Alzheimer’s disease development [47].

The majority of experiments on curli biogenesis and functions were carried out on *E.coli* and *Salmonella spp.* Curli homologues were discovered among the representatives of *Bacteroidetes, Firmicutes* and *Thermodesulfobacteria* genera by bioinformatic analysis [48]. *CsgEFG* operons were found in the majority of the bacteria mentioned above with potential CsgA and CsgB homologues, while CsgC and CsgD proteins were often absent. In spite of the fact that many bioinformatic assays are awaiting the experimental confirmation, there are grounds to suppose that structures similar to curli can be more widely spread in biofilm-forming bacteria than it was thought before [49].

Adhesin P1 located on the cell surface of *Streptococcus mutans* that causes dental caries is an amyloid protein [50]. This adhesin induced a shift in the CR dye absorption spectrum, green birefringence in the CR stained sample and a specific TT fluorescence. Using microscopic methods, fibrils were detected in the sample of this adhesin; this, coupled with spectrophotometric assay results, confirmed its amyloid nature [50]. The obtained data indicate that P1 is not the only protein of *S. mutans* cell surface capable of forming amyloids, because the colonies of the bacteria deprived of this adhesin still induced green birefringence after CR staining [50].

Mycobacterium tuberculosis pili are another example of how amyloids of microbial extracellular matrix can possibly contribute to pathology. This microorganism causes tuberculosis that leads to 3 million deaths every year worldwide [2]. Pili on gram-positive *M. tuberculosis* surfaces are not soluble in the chloroform/methanol mix (2:1) and in the sodium dodecyl sulfate-containing buffer (SDS); they also interact with amyloid-specific CR dye, which suggests their amyloid nature [2]. Pilus protein deletion mutants of *M. tuberculosis* exhibited reduced virulence [2]. The researchers explain that pili are capable of binding to laminin, the extracellular matrix protein, thus contributing to the firm adhesion of a microorganism to host tissues. Thus, M.tuberculosis uses these amyloid proteins to successfully colonize the host [2]. In the serum of patients with tuberculosis, high titers of antibodies interacting with M.tuberculosis pili are found. [2].

Other gram-negative microorganisms that can colonize different human organs and tissues, such as cocci *Staphylococcus aureus*, cause various diseases, from minor skin infections to bacteremia and sepsis. Many of these diseases are associated with biofilm formation in the host [20]. Extracellular amyloid fibrils have been identified in S.aureus biofilms. They consist of short peptides called phenol-soluble modulins (PSM) [12].

S. aureus or *S. epidermidis* PSMs have many functions [51–54]. It has been shown that in their fibrillar form PSMs are necessary for *S. aureus* to provide biofilm stability against various dispersing (biofilm degrading) agents and physical impact [12]. The authors of that work believe that the inhibition of phenol-soluble modulins export is a promising research area that can contribute to preventing diseases induced by pathogenic staphylococci. The search for minor molecules – amyloid polymerization inhibitors – is one of the ways that can lead to the development of drugs for staphylococci elimination on the stage of biofilm formation [49].

Bacillus subtilis pili are an important component of biofilm extracellular matrix formed by the bacteria on hard surfaces and at water–air interface [55]. This microorganism is not pathogenic, however, it is widely spread and can be found in soil, air, water and food. The main protein subunit of *B. subtilis* pili is TasA protein [22, 56]. Fibrils formed by TasA *in vitro* are very similar to B. subtilis pili morphologically [22]; at the same time they interact with amyloid-specific dyes such as CR and TT, are rich in β-sheets, as suggested by CD-spectroscopy, and can be depolymerized only after the incubation in the presence of formic acid [22]. It should be noted that TasA was first identified as a secreted protein and a protein of *B. subtilis* spore surfaces with distinct antibacterial properties [57, 58]. Antibodies used in the diagnosis of neurodegenerative diseases recognize both metastable intermediates generated in the course of amyloid fibril formation and TasA oligomers, which suggests a possible structural similarity of these two oligomer types [22, 59, 60]. Antibodies used in the diagnosis of neurodegenerative diseases in humans recognize TasA oligomers [22, 59, 60], which suggests their immunological similarity.

Amyloids forming amphypathic membranes on microbial cell surfaces

Hyphae, spores and fruiting bodies of many fungi are covered with amphypathic (i.e., having both hydrophilic and hydrophobic areas) rodlet layers that form a mosaic of parallel fibrils 5 to 12 nm wide [18]. Those amphypathic layers do not dissolve when boiled in the presence of 2 % SDS and 1 M NaOH, and dissociate into monomers only when treated with formic or trifluoroacetic acids [9]. The main and probably the only component of fungal rodlet layers is class I hydrophobins [61, 62]. The polymerization of hydrophobins is most effective at interfaces with high surface tension, such as liquid-air interface; agents reducing surface tension also reduce the rate of hydrophobin polymerization *in vitro* [63].

Hydrophobins are a large family of low molecular weight proteins (7–9 kDa) found in fungi [61]. This family got its name due to being rich in hydrophobic amino acid residues [9]. Hydrophobin encoding genes are present in many fungi. Class I hydrophobins are typical functional amyloids because they have a role in spore and fruiting body formation; they are also important for adhesion to the host cell surface and protection against the host immune system [18, 64]. Thus, in the infection caused by filamentous fungi *Arthroderma benhamiae* (dermatophytes, i.e., surface mycosis pathogens in humans and animals), hydrophobin HypA has a masking function and protects the microorganism from the host immune system. Deletion of the hydrophobin gene leads to a rapid wetting of fungal filaments and conidia, which induces increased activation of granulocytes, neutrophils and dendritic cells and is accompanied by elevated titers of interleukins IL-6, -8, -10 and tumor necrosis factor TNF-α [65]. RodA hydrophobin, a component of the rodlet layer that covers pathogen spores, contributes to the development of the infection induced by another filament fungus *Aspergillus fumigatus* that can lead to invasive aspergillosis. In the experiments on animals the spores of the mutant strain with deleted RodA or Δ*laeA* mutant containing 60 % less hydrophobins, were susceptible to macrophage phagocytosis [66].

Amyloids as a part of yeast cell walls: adhesins and glucantranspherase Bgl2p

The development of systemic amyloidosis in mice injected with *Candida sp.* lyophilized cells is well known, but is not widely discussed [67]. The authors of that article emphasized that amyloid depositions could occur in experimental animals as a response to casein, albumin, bacteria or *E. coli* endotoxin administration [68–71]; but after the injections had been cancelled, amyloid depositions started to reduce gradually or

disappeared. [72, 73]. Laboratory mice injected with *Candida sp.* lyophilized cells died of systemic amyloidosis within 400 days after the last injection [67]. Separate experiments showed that injecting mice with *Candida sp*. intracellular matter did not cause amyloidosis. The authors concluded that amyloidosis development was stimulated by cell walls components [67].

Bioinformatic analysis of *Saccharomyces cerevisiae* yeast proteome detected the abundance of amyloidogenic proteins in cell walls [74]. Als proteins (from *agglutinin-like sequence*) are the example of well described proteins of yeast cell surfaces with amyloid properties [20, 21, 75]. In *Candida albicans* genome eight ALS genes were detected, each of them encoding the protein that consists of a signal sequence necessary for the protein secretion, three tandem immunoglobulin (Ig)-like domains, a T-domain rich in threonine, a various number of 36-amino-acid-long glycosylated tandem repeats (TR), a highly glycosylated stem domain and a signal sequence of glycosylphosphatidylinositol anchor attachment protein that ensures protein covalent attachment to the cell wall glucan [76]. Ig-like domain ensures binding to a substrate; T-domain is necessary for proper folding of Ig-like domain and secretion TR increases affinity of Ig-like region to ligands and can promote yeast aggregation independent of Ig-like region. Due to the presence of stem domain, active regions are at a considerable distance from the stem wall [76].

In spite of the intense glycosylation, Als family proteins are low soluble and form amyloid fibrils even at low concentrations when purified [20]. The conformation of N-terminal regions of Als1p (Ig-fragment) and Als5p (Ig-T-fragment) proteins in a solution has been studied [76]. The obtained data indicated that in both cases β-sheets were prevailing elements of a secondary structure of the polypeptide of interest [76]. It was also shown that Als5p, Als1p and Als3p had a highly conserved potentially amyloidogenic region (PAR) in T-domain [20].

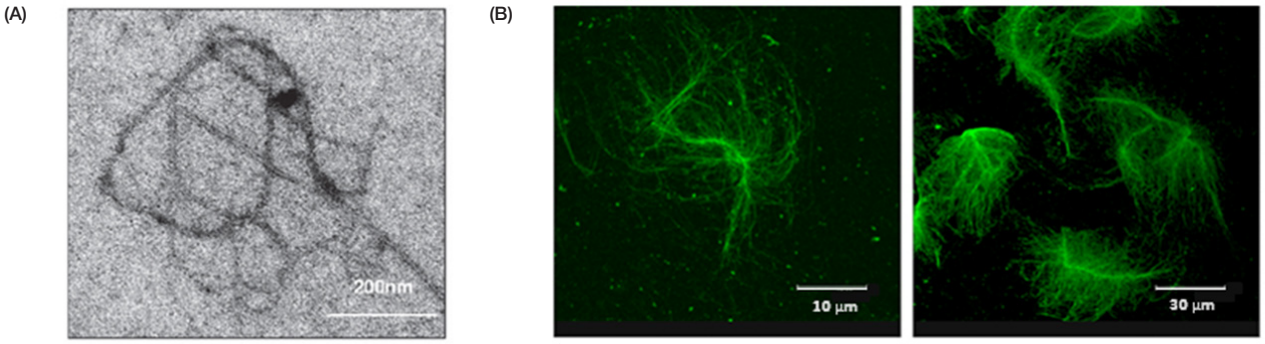
Interestingly, PARs were detected in amino acid sequencies of both Als proteins and yeast adhesins of different families [75]. Peptides containing those PARs formed fibrils that interacted with amyloid-specific dyes, and according to the CD-spectroscopy assay had a secondary structure rich in β-sheets [75]. Amyloid formation is likely to be a very common phenomenon [75].

The opportunistic yeast pathogen *C. albicans* forms biofilms facilitating colonization of host tissues and making *C. albicans* cells extremely resistant to antimicrobial treatment [77, 78]. An important role in the pathogenesis and biofilm formation is played by Als-adhesins described above, along with many other adhesins produced by *C. albicans* [78, 79]. Some Als-adhesins form amyloid structures [20, 21, 75], which probably contributes to *C. albicans* cell autoaggregation and *C. albicans* interaction with extracellular matrix proteins (fibronectin, laminin, type IV collagen) and other mammalian peptide ligands, cells of other yeast species and bacterial cells [76, 78]. The ability of *Candida sp.* to attach to the mucosal surfaces of different organs and to synthetic materials surfaces by means of surface adhesins is an important factor in the pathogenicity of these fungi that contributes to the development of the infection. This property is most conspicuous of *C. albicans* yeast [80, 81].

Glucantransferase Bgl2p is another protein of yeast cell wall (CW) exhibiting amyloid properties. It is a small (31.5-34 kDa depending on the yeast species) conserved major noncovalently bound protein. Its presence in the CW has been detected in many yeast species, such as *S. cerevisiae*, *C. albicans, A. fumigatus* [82–84]. Bgl2p of *S. cerevisiae* is highly homologous to Bgl2p of *C. albicans*. Antibodies against *S. cerevisiae* Bgl2p react with C. albicans Bgl2p [82, 85]. Bgl2p of the CW is resistant to trypsin and proteinase K and cannot be extracted from it when treated with 1% SDS solution in water at 37 °C, in contrast to other noncovalently bound polysaccharide backbone proteins of the CW [86].

Bgl2p extracted from *S. cerevisiae* CW can form structures with fibrillar morphology [86, 87] clearly seen in microscopic assays (see the figure below). Bgl2p protein extracted from the CW induced specific fluorescence of TT and exhibited a circular dichroism spectrum characteristic of a protein rich in β-structure [86, 88], which also indicated the amyloid nature of the structures formed by Bgl2p. The ability of Bgl2p to fibrilize at different pH values was also studied using isolated proteins and synthetic peptides with potential amyloidogenic determinants predicted in the Bgl2p sequence by a bioinformatic assay [87]. It was shown that Bgl2p extracted from the cell wall formed fibrils at neutral and mildly acidic pH values, while in mildly alkaline media it lost its ability to form amyloid fibrils [87]. The mechanism of Bgl2p formation in the cell wall and its physiological role in the functioning of yeast are yet to be discerned [89].

Presumably, Bgl2p has a crucial role in pathogenic yeast virulence, since BGL2 gene deletion reduces the infecting ability of those microorganisms [82]. Jang et al. found that *C. albicans* Bgl2p also functions as an adhesin and ensures cell attachment to the immobilized saliva components [85]. It was shown that antibodies to *C. albicans* Bgl2p are a diagnostic biomarker of systemic candidiasis, and their high levels correlate with the reduced death probability, which may be related to the protective function of these antibodies [90].



Photomicrograph of glucantransferase Bgl2p samples extracted from Saccharomyces cerevisiae yeast cells. **(A)** — electronic microscopy. Negative staining[86]. **(B)** — fluorescent microscopy. Staining with antibodies against Bgl2p [87]

CONCLUSIONS

When describing amyloid proteins of microbial surfaces, we did not review the articles dedicated to such amyloids as chaplins, microcins and harpins, because their role in human and animal pathogenesis has not yet been identified or studied. Still, the studies of the amyloid structures and formation mechanisms, which are actively carried out in a number of big research centers and laboratories in Russia and abroad, hold promise for important discoveries in this field. We think it necessary to pay close attention to the analysis of a possible role of amyloids and other microbial cell surface components in the development of diseases with vague etiology. Microorganisms are abundant in the bodies of higher eukaryotes including humans. For many animals microorganisms are essential. The number of microbial cells can be significantly higher than the number of host cells [1]. Components of microbial cell surfaces including amyloid proteins are in permanent contact with host cells and liquids. One should not underestimate the potential role of these molecules, localized on the surface of both pro- and eukaryotic microorganisms, in the metabolism of animals and humans including pathogenic mechanisms.

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NEURODEGENERATIVE CHANGES INDUCED BY INJECTION OF β-AMYLOID PEPTIDE FRAGMENT (25-35) IN HIPPOCAMPUS ARE ASSOCIATED WITH NGF-SIGNALLING ACTIVATION

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β-amyloid peptide (Aβ) is an important component of the neurodegeneration mechanism in Alzheimer's disease. This work investigates the effect of intrahippocampal injection of Aβ(25–35) fragment on nerve growth factor (NGF) signalling. Aggregated Aβ(25–35) was injected into rat dorsal hippocampus. Rats in the control group received injections of the peptide with an inverted amino acid sequence and a solvent. It was shown that Aβ(25–35) induces neuron death in rat hippocampus. Neurodegeneration was accompanied by a statistically significant increase ($p < 0.05$) in p75NTR neurotrophin receptor expression in all animals who had received exogenous peptides, and by an increased level of NGF in the hippocampus of those rats who had been injected with Aβ(25–35). The study results demonstrate that changes in the hippocampus induced by Aβ(25–35) are accompanied by increased NGF signalling, which, to some extent, supports the current clinical data obtained from patients with Alzheimer's. The changes mentioned above are compensatory. However, both damage reparation and further degenerative processes can be the ultimate outcome.

Keywords: β-amyloid peptide, hippocampus, nerve growth factor, p75NTR receptor, neurodegeneration, Alzheimer's disease

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НЕЙРОДЕГЕНЕРАТИВНЫЕ ИЗМЕНЕНИЯ, ВЫЗВАННЫЕ ВВЕДЕНИЕМ ФРАГМЕНТА (25–35) β-АМИЛОИДНОГО ПЕПТИДА В ГИППОКАМП, СВЯЗАНЫ С АКТИВАЦИЕЙ NGF-СИГНАЛИНГА

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В механизме нейродегенерации при болезни Альцгеймера важную роль играет β-амилоидный пептид (Aβ). В работе исследовали влияние интрагиппокампальной инъекции фрагмента Aβ(25–35) на систему сигналинга фактора роста нервов (NGF). Крысам вводили агрегированный Aβ(25–35) в область дорзального гиппокампа. Контрольной группе проводили инъекции пептида с обратной аминокислотной последовательностью и растворителя. Показано, что Aβ(25–35) вызывал гибель нейронов в гиппокампе крыс. Нейродегенеративные процессы сопровождались достоверным ($p < 0,05$) увеличением экспрессии рецептора нейротрофинов p75NTR у всех животных, получавших экзогенные пептиды, и повышением уровня NGF в гиппокампе только тех крыс, которым делали инъекцию Aβ(25–35). Результаты исследования демонстрируют, что вызванные Aβ(25–35) изменения в гиппокампе сопровождаются усилением NGF-сигналинга. Данное усиление в определенной степени подтверждает имеющиеся данные клинических наблюдений у пациентов с болезнью Альцгеймера. Указанные изменения носят компенсаторный характер, однако конечным результатом может быть как репарация повреждения, так и дальнейшее усиление дегенеративного процесса.

Ключевые слова: β-амилоидный пептид, гиппокамп, фактор роста нервов, рецептор p75NTR, нейродегенерация, болезнь Альцгеймера

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One of the key components of Alzheimer's disease pathogenesis is a β-amyloid peptide (Aβ). It consists of 40 to 42 amino acids and is an intramembrane fragment of a large transmembrane protein precursor. Aβ is a product of its precursor proteolytic processing in the amyloidogenic pathway. Though the ultimate role of Aβ is still unclear, its accumulation in patient's brain in the form of soluble aggregates and insoluble deposits is the most important marker of Alzheimer's disease. Because Aβ exhibits toxicity to neurons, intracerebral injections of this peptide in animals can help to model some aspects of a complicated pattern of Alzheimer's disease. Toxicity is characteristic of both a full-length Aβ peptide and some of its shortened fragments,

in particular Aβ(25–35) undecapeptide that is often seen as a functional domain of Aβ and is responsible for its aggregating properties [1, 2].

Aβ(25–35) neurotoxicity following its injection into the hippocampus was demonstrated by D. R. Rush et al [3]. Aβ(25–35) injection induced the adjacent tissue loss and neuronal degeneration [3]. However, other authors [4] found no neurotoxic effect of Aβ(25–35) following its administration into the ventral pallidum and substantia innominata . They observed the formation of cavities containing protein aggregates that were positively stained with congo red. Aggregated Aβ(25–35) caused more conspicuous damage of the CA1 pyramidal layer in the hippocampus compared to the peptide synthesized from the same amino acids in the reversed sequence, Aβ(35–25) [5–8]. Degenerating neurons were also detected in the temporal cortex following the Aβ(25–35) injection into the nucleus basalis magnocellularis of rats [9]. It is important to note that undecapeptide injection into some brain structures can induce transsynaptic cytoskeletal damage and astroglial activation that are observed in the actual injection area and also spread to more distant brain areas. Such changes were detected in the hippocampus following the Aβ(25–35) injection into the amygdala [10]. Our previous work showed that Aβ(25–35) also induced the activation of astrocytes and microglia in the hippocampus after being injected into this structure [8].

Neuroglial activation in the lesion is a controversial phenomenon. On the one hand, being the actual components of neuroinflammation, activated astrocytes and microgliocytes contribute to the degeneration. When triggered, the mechanisms of neuroinflammation can lead to the dysfunction and death of neurons, which exacerbates further inflammation. Thus, the vicious circle is established in which neuroinflammation causes neurodegeneration [11]. On the other hand, glial activation is a distinct compensatory tissue response, with microglia actively phagocyting a pathogen that caused tissue damage, e.g., injected Aβ or amyloid plaque components, and astrocytes contributing to a better supply of neurons with substances necessary for their repair, such as neutrophins.

One of the most important neutrophins of the mammalian brain is a nerve growth factor (NGF). NGF is the main neutrophin that ensures support and functioning of cholinergic neurons in the brain of adult mammals [12, 13]. It is synthesized and released into the extracellular medium by hippocampal and neocortical cells, targets for cholinergic neurons of basal nuclei. In turn, cholinergic neurons in the brain of young, adult and senescing animals express a high affinity NGF receptor (TrkA) and a low affinity NGF receptor (p75NTR) [14], which demonstrates the dependence of the metabolism of those cells on the levels of neutrophins in both the developing and the mature brain. A signal cascade triggered by NGF becomes particularly important in Alzheimer's development. On early stages of the disease (mild cognitive decline) NGF levels are reduced [15], while later stages (severe dementia) are associated with their increase [15, 16]. Considering a specific role of Aβ at different stages of the disease, it is likely to be involved in NGF metabolism regulation in Alzheimer's.

The aim of this work was to study the changes in NGF signaling system in the hippocampus of rats following the administration of aggregated Aβ(25–35).

METHODS

Experiments on animals were conducted in compliance with the Directive of the European Parliament and of European Council, dated September 22, 2010, and the Order № 267 of the Ministry of Healthcare of the Russian Federation, dated June 19, 2003, on the protection of animals used for scientific experiments. The protocol of the experiment was approved by the Ethics Committee of the Institute of Higher Nervous Activity and Neurophysiology, RAS.

The study was carried out in male Wistar rats from Stolbovaya breeding nursery of the Medical Center for Biomedical Technologies, FMBA (Moscow oblast, Russia), with weights ranging from 290 to 350 g. The rats were kept in plastic cells in fives under vivarium housing conditions with 12h artificial lighting (8:00 — 20:00) and free access to water and food.

The rats were anaesthetized by an intraperitoneal injection of chloral hydrate (350 mg/kg). Aqueous solutions of Aβ(25–35), a control peptide synthesized from the same amino acids in the reversed sequence Aβ(35–25), and a vehicle (sterile water) were administered bilaterally in the hippocampus at AP –3.8 mm; L ± 2.0 mm; DV +3.8 mm from bregma using Model 900 stereotaxic instrument (David Kopf Instruments, USA) [17]. The rats were injected with 3 nmol aggregated Aβ(25–35) or Aβ(35–25) (Bachem, Switzerland) in a total volume of 2 μL (1.5 nmol/μL), the control group received the equal volume of sterile water. Injections were performed at a rate of 1 μL/min. The needle was left in the injection site for 5 minutes for proper substance distribution and for preventing its leakage. Peptide aggregation was performed as described in [18].

7 days after the surgery the rats were decapitated, their brains removed and washed in ice-cold 0.9 % NaCl solution; hippocampus and cerebral cortex were isolated on ice. Those brain structures were frozen and stored at –85 °C for analysis. To measure the NGF level, the tissue was homogenized at the ratio of 1:10 (mass/volume) in a buffer consisting of 100 mM Tris-HCL (pH 7.0), 2 % bovine serum albumin, 1 M NaCl, 4 mM Na2EDTA, 2 % Triton X-100, 0.1 % NaN3 and protease inhibitors, namely, 157 μg/mL benzamidine, 0.1 μg/mL pepstatin A and 17 μg/mL PMSF.

The total amount of NGF was measured using ChemiKine Nerve Growth Factor Sandwich ELISA Kit, a reagents kit for the enzyme-linked immunosorbent assay (Merck Millipore, USA), according to the manufacturer's guide. Measurements were performed using Wallac VICTOR 1420 multitasking reader (PerkinElmer, Finland). Protein concentration in the tissue was measured using Coomassie Brilliant Blue G-250 dye. The NGF content was presented in pg/mg protein.

For the histological and enzyme immunoassay, the rats were re-anaesthetized with chloral hydrate (450 mg/kg). Then the brains were fixed by intracardiac perfusion of 4 % paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4) and stored in the same fixative for 24 hours. 50 μm thick frontal sections were prepared using VT1200 S vibrating microtome (Leica Biosystems, Germany) and stored at –20 °C in a cryoprotectant. The sections were Nissl-stained with cresyl violet (Merck, Germany). The expression of p75NTR receptor was evaluated on free-floating sections by immunohystological assay using polyclonal rabbit antibodies (Sigma-Aldrich, USA) and diluted 1:100. Antibody binding was detected using goat-anti-rabbit IgG conjugated with biotin (Sigma-Aldrich, USA) diluted 1: 800, and VECTASTAIN Elite ABC Kit (Vector Laboratories, USA), an avidin-biotin complex with horseradish peroxidase. Diaminobenzidine (SIGMA Fast kit; Sigma-Aldrich, USA) was used as a chromogen.

A quantitative evaluation of damage degree was performed using the images of Nissl-stained sections taken with Camedia C-4000 (Olympus, Japan). The length of lesions in the dentate gyrus and CA1 hippocampal field was measured using Image-J

(NIH, USA) software. Based on the length and thickness of the sections, the total CA1 damaged area was measured as described in our earlier work [19]. Evaluation of p75NTR expression was based on the total area exhibiting positive staining in three sections with the most severe hippocampal damage located 500 μm from each other. To estimate the level of expression in an individual animal, the results were averaged and presented in pixels (pxl).

Reagents by Sigma-Aldrich (USA) were used in the study if not specified otherwise

Data were presented as a group arithmetic mean (M) and a standard error mean (SEM). The impact of peptides on the lesion size was evaluated by Kruskal–Wallis test. Differences between the groups were calculated using Mann - Whitney test.

RESULTS

The study of structural changes was carried out in animals (n = 5) that received 3 nmol aggregated Aβ(25–35) injection in the dorsal hippocampus of the left hemisphere 7 days after the peptide had been administered. 3 nmol Aβ(35–25) were injected in the dorsal hippocampus of the right hemisphere of the same animals. To assess the effect of the vehicle, the controls (n = 5) were injected with the equal volume of sterile water in the hippocampus of the left hemisphere and sterile 0.9% NaCl solution into the hippocampus of the right hemisphere. The majority of neurons in the assayed brain sections of the controls had normal morphology. Chromatophilic neurons in neocortex and primary olfactory cortex were rarely observed. Injections of isotonic solution did not induce a considerable damage in rat hippocampus. Small lesions associated with the needle penetration were found in the vicinity of the injection site after the vehicle had been introduced to the CA1 hippocampal field. Single chromatophilic cells were found in the CA3 field. At the same time, distinct structural changes of dentate gyrus (DG) were observed. In the first place, those changes were reflected in the considerable cell death of the DG upper blade. It should be mentioned that lesions were most conspicuous in the injection area and decreased in size further from the injection site. On the whole, these data correlate with the results of our previous works [8, 20].

Intrahippocampial administration of non-toxic Aβ(35–25) resulted in the conspicuous cavitation in the studied brain area. Along with it, a substantial damage of the DG upper and sometimes lower blades was observed. The degree of CA1 hippocampal field damage was comparable to the one in the brains of the controls who had received sterile water. In contrast to Aβ(35–25), administration of toxic aggregated peptide Aβ(25–35) induced a more statistically significant ($p < 0.05$) damage in the CA1 field (Fig. 1). Variance analysis showed the dependence of the CA1 field lesion size on Aβ(25–35) activity [$H(2.15) = 8.9$; $p < 0.02$]. The CA1 field lesion size was significantly ($p < 0.05$) bigger compared to the hippocampus of the control rats that had been injected with water and to the hemisphere where a non-toxic peptide had been injected. No correlation was observed between the DG lesion size and the peptide administration [$H(2.15) = 4.0$; $p = 0.1$] (Fig. 2). Thus, a higher sensitivity of CA1 neurons to a toxic effect of Aβ(25–35) was shown compared to DG neurons.

The development of neurodegenerative processes induced by Aβ(25–35) administration in the hippocampus is accompanied by significant changes in the system of neurotrophin supply. Thus, in the hippocampus of rats a statistically significant ($p < 0.05$) change in the expression of p75NTR receptor was observed. A statistically significant expansion of the area stained with specific antibodies to p75NTR protein was observed after both Aβ(35–25) and toxic Aβ(25–35) injections (Fig. 3). No specific effect of Aβ(25–35) on this value was detected. Peptide injections in the hippocampus resulted in the increased levels of NGF in this brain region (Fig. 4). At the same time, the injection of toxic Aβ(25–35) produced a more prominent effect on NGF levels compared to Aβ(35–25).

DISCUSSION

This work has demonstrated that Aβ(25–35) injection in the hippocampus leads to neurodegeneration that is most conspicuously expressed in the pyramidal layer of CA1 field cells. Cell damage and death were localized mainly in the injection area; the lesion size in the pyramidal layer was significantly bigger in the rats that had received the injection of Aβ(25–35), in contrast to the injections of the control peptide with a reversed amino acid sequence or the vehicle (sterile water). We should note that DG damage was observed in the hippocampus of animals in all groups except for those that had been injected with sterile isotonic NaCl solution. The damage of this structure is likely to have been caused by the syringe point being at the edge of the dentate gyrus lateral blade in accordance with the stereotaxic atlas coordinates, and granule cells were subjected to osmotic shock.

Neurodegenerative processes were accompanied by the increased p75NTR neurotrophin receptor expression that was observed in the hippocampus of the rats that had been administered to both Aβ(25–35) and Aβ(35–25). The functions of this receptor in the brain are diverse [21]. It can contribute to the survival of damaged neurons by enhancing the effective functioning of Trk receptors; it can also induce apoptosis of

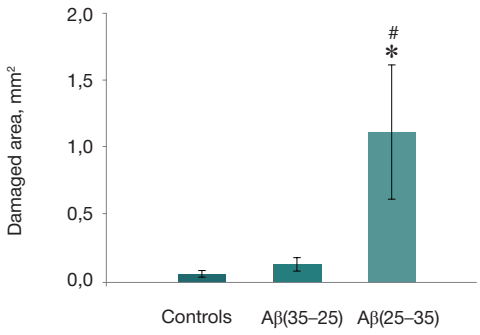

Fig. 1. Effect of amyloid peptide injection on the lesion size in CA1 hippocampal field of rats. * — $p < 0.05$ compared to the controls (vehicle injection), # — $p < 0.05$ compared to the group injected with Aβ(35–25); Mann–Whitney test

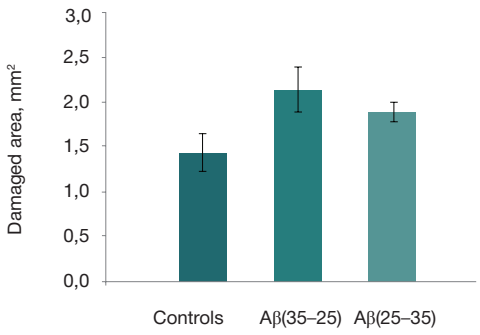

Fig. 2. Effect of amyloid peptide injection on the lesion size in dentate gyrus of rats

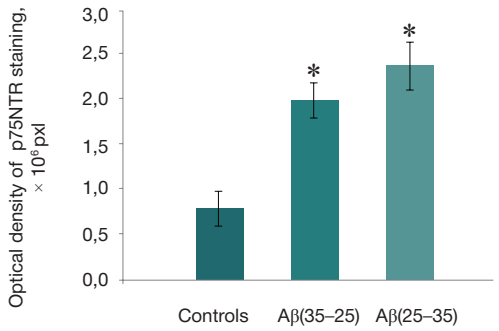

Fig. 3. Effect of amyloid peptide injection on p75NTR receptor expression in the hippocampus of rats

∗ — p <0.05 compared to the controls (vehicle injection); Mann–Whitney test.

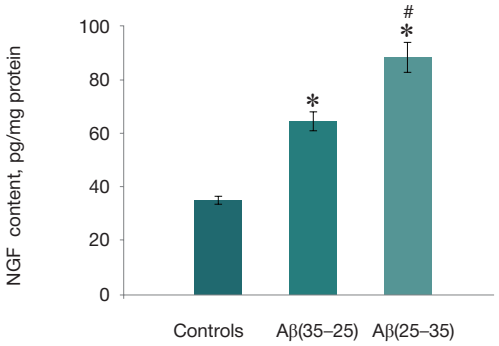

Fig. 4. Effect of amyloid peptide injection on the amount of NGF in the hippocampus of rats

∗ — p <0.05 compared to the controls (vehicle injection),
— p <0.05 compared to the group injected with Aβ(35–25); Mann–Whitney test.

damaged cells to reduce the inflammatory response, maintain the microenvironment for regeneration purposes, and control neuroinflammation. It is known that the expression of this receptor increases considerably in the hippocampus of patients with Alzheimer's, where Aβ can interact with p75NTR, contributing to cell death [22]. It was shown that in SH-SY5Y neuroblastoma cell culture Aβ(25–35) can bind to this receptor just as the full length peptide Aβ(1–42) [23]. At the same time, a peptide with a reversed amino acid sequence Aβ(42–1) did not display such properties. It should be noted that the mechanisms that help Aβ trigger the expression of p75NTR have not been fully understood. Moreover, it is a common belief that in the hippocampus p75NTR is expressed only on the afferent endings of basal nuclei cholinergic neurons [22].

Some authors indicate that p75NTR can be present in the membranes of subgranular zone neuroblasts [24] and in the dendritic spines and afferent terminals of CA1 pyramidal cells [25]. Besides, hippocampal astrocytes can actively express p75NTR, for example, in response to NMDA-receptor antagonists [26]. Microglial cells also express this receptor [27]. Administration of exogenous peptides into the hippocampus of rats similar to the one described in this work led to the significant activation of astrocytes and microglia [8, 19]. No significant difference in p75NTR levels can be explained by the increased expression of astrocytes and microglial cells in response to Aβ(25–35) and Aβ(35–25) injections.

After Aβ(25–35) injections the accumulation of NGF in the hippocampus was more conspicuous than after the injection of a peptide with a reversed amino acid sequence. Alzheimers's pathogenesis is associated with the fluctuations in NGF synthesis. Later disease stages, at which patients are diagnosed with dementia and neurodegeneration, are characterized by the increased NGF levels in brain structures [15, 16]. In contrast to earlier ontogenesis stages, the accumulation of NGF in the brain in pathology can be a controversial phenomenon. On the one hand, NGF is the main neurotrophin that ensures the survival of cholinergic neurons of basal nuclei due to the interaction of its mature form with TrkA and p75NTR receptors [28]. On the other hand, the binding of NGF pro-form to p75NTR receptor can trigger neuronal death [29]. The enzyme immunoassay used in this work for assessing the levels of NGF in tissue did not allow us to separately estimate the levels of NGF pro-form and mature form. Considering the works of other researchers, we can hypothesize that after interaction with Aβ(25–35), NGF pro-form will be a prevailing NGF molecule in the hippocampus [30]. Thus, it is possible that more intense interaction of NGF pro-form with a large number of p75NTR receptors will contribute to further neuronal death in the hippocampus and the lesion expansion.

CONCLUSIONS

The data obtained in this study show that aggregated Aβ(25–35) administration into the hippocampus of rats leads to neuronal degeneration in the CA1 field accompanied by the increased levels of NGF. The expression of p75NTR receptor increases in all animals that received Aβ(25–35) or Aβ(35–25) exogenous peptides. We hypothesize that Aβ(25–35) induces NGF signaling activation that contributes to the lesion expansion in the pyramidal cell layer of the hippocampus. Further research is necessary to clarify the molecular mechanisms of the developing neurodegeneration.

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CLARIFICATION OF THE STATUS OF SOME MUTATIONS CONSIDERED PATHOGENIC, BY HARMLESS MUTATIONS ATTRIBUTES

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Prediction of mutation pathogenicity and its effect on the phenotype is an important task of modern bioinformatics. This task is particularly difficult in regard to single nucleotide polymorphisms, as their effect is very hard to predict. Information on pathogenic mutations is provided by curated databases such as Online Mendelian Inheritance in Man (OMIM) and The Human Gene Mutation Database (HGMD) which include data from experimental works. However, as different authors interpret the term "mutation pathogenicity" differently, it is necessary to double-check data before using them. We have assessed HGMD database quality using the most common bioinformatic tools, namely, snpEff, polyphen2 and SIFT. Our study relied on the characteristics specific for harmless mutations: high frequency in a population, weak effect on amino acid sequence of a protein, low pathogenicity as computed by the utilities used in the study. As a result, we have identified clearly harmless variants among those in the mutation database, as well as ambiguous ones in which a mutation type depends on characteristics and tools used for the analysis.

Keywords: human genetics, high-throughput sequencing, pathogenicity, population analysis, search for mutations

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УТОЧНЕНИЕ СТАТУСА НЕКОТОРЫХ МУТАЦИЙ, СЧИТАЮЩИХСЯ ПАТОГЕННЫМИ, С ПОМОЩЬЮ ПРИЗНАКОВ БЕЗВРЕДНЫХ МУТАЦИЙ

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Важной задачей современной биоинформатики является предсказание патогенности мутации и ее влияния на фенотип. Она особенно трудна для однонуклеотидных полиморфизмов, чей эффект сложнее всего предсказать. Патогенные мутации берут из курируемых баз данных, таких как Online Mendelian Inheritance in Man (OMIM) и The Human Gene Mutation Database (HGMD), куда включают данные из экспериментальных статей. Однако поскольку различные авторы вкладывают разный смысл в понятие «патогенность мутации», необходимо контролировать данные баз перед их использованием. Мы проанализировали качество данных базы HGMD с помощью наиболее часто используемых биоинформатических инструментов: snpEff, polyphen2 и SIFT. В исследовании мы опирались на признаки, характерные для безвредных мутаций: высокую частоту в популяции, слабое влияние на аминокислотную последовательность белка, низкую патогенность по оценке вычислительных методов. В результате среди мутаций базы нами выявлены однозначно безвредные варианты, а также варианты со спорным значением, для которых тип мутации зависит от используемых для анализа признаков и инструментов.

Ключевые слова: генетика человека, высокопроизводительное секвенирование, патогенность, популяционный анализ, поиск мутаций

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The impact of single nucleotide polymorphisms (SNP) on the phenotype is hard to predict. Currently existing tools for predicting mutation pathogenicity have a number of flaws, such as low sensitivity and specificity of no more than 75–80 % for SNP. Besides, they often do not annotate insertions and deletions [1–3].

Pathogenic mutations described in experimental articles are collected into databases, such as the Online Mendelian Inheritance in Man database (OMIM, [4]) and the Human Gene Mutation Database (HGMD [5]). However, the term *pathogenicity* can be interpreted widely; there is no unanimous opinion on what it implies. As a result, different approaches are applied while selecting mutations for their inclusion in a database; thus, the data in different databases are not the same and need rectification.

To identify non-pathogenic mutations, their indirect indicators are often used, such as allele frequency in a population and the effect on the amino acid sequence of a protein. With new data coming into sight, these indicators can help us understand how the existing databases can be improved. Knowing that mutations described as pathogenic meet the criteria for non-pathogenic variants is important for the practical usage of the data derived from these databases. This knowledge can help us understand why certain genetic variants affect the phenotype while others do not.

For scientists who rely on HGMD in their research it may not be obvious that apart from clearly deleterious mutations, it currently includes harmless ones assessed as pathogenic. Within the framework of this study, the pathogenicity of mutations included in HGMD was evaluated using bioinformatic tools. Allele frequencies annotated in HGMD were compared to those from Exome Aggregation Consortium 0.3 [6]; the effect of HGMD mutations on the amino acid sequence of proteins was analyzed, and their pathogenicity was predicted using the most common bioinformatic tools: snpEff, PolyPhen-2 and SIFT.

METHODS

A public version of HGMD (of the fourth quarter of 2014) was used as a source of pathogenic mutations. It contained 73,208 mutations. Their allele frequencies were calculated using snpEff 4.0. The obtained data were compared to the allele frequencies from Exome Aggregation Consortium 0.3 that included whole exome and whole genome sequencing data from 60,706 samples of unrelated patients. ExAC provides allele frequency data on six populations: African, Latino, East Asian, South Asian, Finnish and European (non-Finnish). All unidentified samples are grouped as “Other”. When we used the database, the number of genotyped samples for each annotated mutation varied in different populations, from about 500 for “Other” to 30,000 for Europeans. Allele frequencies were compared using bcftools [7].

HGMD mutations affecting the amino acid sequence of proteins were identified using snpEff 4.0 [8]. A possible level of pathogenicity was predicted using PolyPhen-2 and SIFT utilities. These utilities are standard tools for predicting mutation pathogenicity; neither of them used HGMD data as a training set.

RESULTS

snpEff annotation

Mutations obtained from HGMD were annotated by snpEff, frequencies of each mutation type were established according to snpEff classification. We have found that in many cases mutations have more than one prediction, meaning they can refer to various types at the same time. It usually happens when a mutation is located within the gene and the adjacent genes are used for its annotation. We have filtered variants belonging to more than one type and selected those with the most conspicuous impact according to the algorithm suggested by snpEff developers (see the table below) [8].

Annotation with ExAC

18,159 (25 %) mutations present in HGMD are described in ExAC.

Results obtained by PolyPhen-2 and SIFT

We have predicted mutation pathogenicity using PolyPhen-2 and SIFT utilities. PolyPhen-2 uses two models for pathogenicity prediction: HumDiv and HumVar. According to the developers’ description, HumVar predicts Mendelian diseases better, while HumDiv is more efficient with complex phenotypes and mildly deleterious alleles [9]. We have chosen HumDiv model to use a wider pathogenicity definition. Threshold for cutting off pathogenic and possibly pathogenic variants was set by default.

PolyPhen-2 annotated 52,248 mutations, 39,032 (72 %) of them were identified as pathogenic and 6,220 (11 %) as possibly pathogenic. SIFT utility analyzed 53,097 mutations with 34,638 (65 %) identified as pathogenic and 4,358 (8 %) as possibly pathogenic (with low probability). Both utilities recognized the variants submitted to the database as pathogenic in 70–80 % cases, which corresponds to their expected performance [2, 3].

DISCUSSION

Using ExAC database as a resource containing data on allele frequency

Technical description of ExAC has not been released yet, but the database is known to include data from both population genetic studies and sequencing projects describing the samples of patients with various diseases. We believe that such projects use less samples compared to population genetic research works, and their effect on the resulting frequency must be negligible, especially if samples of a large number of individuals have been analyzed in population genetic studies. That is why our analysis did not cover mutations that had been genotyped in a few individuals only. That being said, we believe that ExAC can certainly be used to estimate the frequencies in such studies as ours. The developers of this database claim that it can be used as a reference set of allele frequencies for disease studies.

Presence of synonymous mutations in HGMD

95 % of all mutations obtained from HGMD were distributed by snpEff in two groups: missense mutations and nonsense mutations. However, about 2.5 % of mutations were identified as synonymous (see the table). Although the pathogenicity of synonymous variants has been described in literature, in most cases synonymous mutations are considered harmless. We focused on this group as a group of variants with the most disputable pathogenicity. PolyPhen-2 utility does not perform the pathogenicity assessment of synonymous mutations because it relies on the effect of a mutation on the protein amino acid sequence. SIFT utility allows for the assessment of the synonymous mutation pathogenicity; it identified only 4 out of 1,793 synonymous mutations as pathogenic. It is highly probable that the rest of 1,789 mutations (~2.5 % of all mutations in HGMD) are not pathogenic because they do not have any other signs of pathogenicity.

Number of the most important mutations obtained from HGMD and predicted by snpEff

Type*	Number of mutations	Type *	Number of mutations
missense_variant	56136	sequence_feature	66
stop_gained	13513	initiator_codon_variant	61
synonymous_variant	1793	intron_variant	54
start_lost	465	non_coding_exon_variant	44
3_prime_UTR_variant	363	splice_donor_variant	39
downstream_gene_variant	245	splice_acceptor_variant	23
upstream_gene_variant	162	stop_retained_variant	4
stop_lost	136	5_prime_UTR_variant	3
splice_region_variant	99	intergenic_region	2

*Names are given as they appear in snpEff. missense_variant – missense mutations; stop_gained – nonsense mutations; synonymous_variant - synonymous mutations; start_lost – a codon variant that changes at least one base of the canonical start codon; 3_prime_UTR_variant – a UTR variant of the 3' UTR; downstream_gene_variant – a sequence variant located 3' of a gene. upstream_gene_variant - a sequence variant located 5' of a gene; stop_lost – a sequence variant where at least one base of the terminator codon (stop) is changed resulting in an elongated transcript; splice_region_variant – a sequence variant in which a change has occurred within the region; of the splice site, either within 1-3 bases of the exon or 3-8 bases of the intron; sequence_feature – a sequence variant within any region initiator_codon_variant – a codon variant that changes at least one base of the first codon of a transcript; intron_variant – a transcript variant occurring within an intron non_coding_exon_variant – a sequence variant that changes non-coding exon sequence of a noncoding transcript; splice_donor_variant – a splice variant that changes the 2 base pair region at the 5' end of an intron; splice_acceptor_variant - a splice variant that changes the 2 base region at the 3' end of an intron; stop_retained_variant – a sequence variant where at least one base in the terminator codon is changed, but the terminator remains; 5_prime_UTR_variant - a UTR variant of the 5' UTR; intergenic_region – a region containing or overlapping no genes that is bounded on either side by a gene, or bounded by a gene and the end of the chromosome.

Analysis of synonymous pathogenic mutations in HGMD

Only one of the four synonymous mutations in HGMD identified as pathogenic by SIFT utility is described in dbSNP [10]. It is NM_005228.3:c.2361G>A (NP_005219.2:p.Gln787=) mutation with rsid *rs1050171*. According to Zhang et al. [11], this mutation is associated with lung cancer; its molecular mechanism of action has not been identified yet. The frequency of the alternative ("mutant") allele A is about 43 %, according to the "1000 genomes" project data presented in dbSNP. The ClinVar database [12] defines this SNP as benign [12]. The reasons for SIFT classifying this mutation as pathogenic are probably related to the conservative position where the mutation occurred. It is located at codon position 3 that is usually less conservative than positions 1 and 2, and gets a lower score. However, for this mutation the PhyloP Vertebrate evolutionary conservation score obtained from UCSC Genome Browser [14], combined with the scores of positions 1 and 2 of adjacent codons, is much higher than the score of other third codon position nucleotides, which is indicative of high conservation of the nucleotide of interest.

After all, the true nature of this mutation is hard to identify. On the one hand, there is evidence that this mutation is non-pathogenic, such as the data from ClinVar database, its synonymous type, the high frequency of the allele variants in the population. On the other hand, the results of prediction using SIFT utility in HGMD and the high evolutionary conservation suggest the pathogenicity of this variant. This example illustrates the difficulty of mutation pathogenicity prediction: even manual analysis cannot provide the unambiguous interpretation of the results, because the mutation type depends on the choice of a tool for analysis.

Variants with a mutation present in a heterozygote only

To analyze the mutations absent in the samples in the homozygous state, we have chosen four mutations, each being present in a heterozygote in more than 75 % of samples and in a homozygote in less than 5 % of samples (according to the ExAC data):

1. chr1:1650845G>A (*rs1059831*, gene *CDK11A*, HGMD phenotype: associated with type 2 diabetes) [14],
2. chr2:112614429G>A (*rs72936240*, gene *ANAPC1*, HGMD phenotype: protein deficit associated with the risk of cancer) [15],
3. chr7:142458451A>T (*rs111033566*, gene *PRSS1*, HGMD phenotype: hereditary pancreatitis) [16],
4. chr17:7197581G>T (*rs189257850*, gene *YBX2*, HGMD phenotype: associated with male infertility) [17].

Homozygous variants 2 and 3 have never been present in any population, homozygous variant 1 has been found in only one out of 8,209 samples in the South Asian population. Strangely, for variant 4 only 203 samples have been genotyped, while for variants 1–3 about 60,000 samples have been genotyped. For variant 4 only one individual out of 52 in the East Asian population has been described as homozygous and 13 individuals out of 62 have been described as homozygous in the Latin American population.

These mutations are mainly found in heterozygotes, which can be explained by the fact that they cause death or at least cannot be inherited. Based on the phenotype analysis, variants 2 and 4 can be excluded as heterozygous because of early death or infertility of their carriers. Variant 4 is the most interesting one, but it is the only variant that has not been genotyped widely. It is difficult to understand why this mutation is highly frequent in one of the populations and why the number of individuals analyzed in this population is so low. Because the number of the individuals analyzed is low, those data have been possibly obtained by analyzing diseased individuals (see the description of ExAC specifics above), so no predictions for this variant are possible. Variant 2 can be described as lethal in the homozygous state. We make a supposition that although it is not obvious that variants 1 and 3 are lethal, the existent data prove that these mutations cause death or infertility in homozygotes.

CONCLUSIONS

Assessing mutation pathogenicity is a difficult task. Sometimes neither automatic nor manual analysis can classify it as clearly pathogenic or harmless. However, in the absence of

experimental data on transgenic organisms with a mutation of interest, the existing databases can still be used for pathogenicity analysis, but one should use them carefully. Automatic use of those databases is restricted by the quality of data presented there. It is important to manually check if the mutations described in experimental works are pathogenic, especially if the claims of their pathogenicity do not correspond to the database prediction.

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T-CADHERIN GENE POLYMORPHISM IS ASSOCIATED WITH CORONARY HEART DISEASE MANIFESTATIONS

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A number of studies have shown that a *CDH13*-encoded T-cadherin protein, which is a receptor for low density lipoproteins and adiponectin, an adipocyte hormone, is associated with atherosclerosis and coronary heart disease (CHD) development. Some single nucleotide polymorphisms in *CDH13* gene affect the expression of T-cadherin and the levels of adiponectin and blood plasma lipids, but the connection between these polymorphisms and CHD development has not been studied yet. In this work the role of *rs12051272, rs4783244*, *rs12444338* and *rs11646213* single nucleotide polymorphisms in CHD development and its manifestations was investigated. The study enrolled men under 55 years of age: 79 patients with stable effort angina with no prior myocardial infarction, 107 patients with prior myocardial infarction being the first manifestation of CHD, and 99 healthy subjects. All subjects were clinically examined; laboratory tests and genotyping were conducted. The results of genotyping were evaluated using SNPStats on-line software. This study has not found a connection between *CDH13* gene polymorphisms and CHD development. However, it was shown that *rs12051272* polymorphism is associated with the specifics of the disease onset: GT genotype was detected in 13 (16.5 %) patients with stabile effort angina and only in 3 (2.8 %) patients with myocardial infarction (odd ratio of 7.54; 95 % confidence interval of 2.01–28.35). Thus, the study demonstrates that *CDH13* gene polymorphism can affect atherogenesis and CHD manifestations.

Keywords: T-cadherin, *CDH13*, gene polymorphism, low density lipoproteins, adiponectin, coronary heart disease, myocardial infarction

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ПОЛИМОРФИЗМ ГЕНА Т-КАДГЕРИНА (*CDH13*) АССОЦИИРОВАН С ХАРАКТЕРОМ МАНИФЕСТАЦИИ ИШЕМИЧЕСКОЙ БОЛЕЗНИ СЕРДЦА

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Ряд исследований показал, что белок Т-кадгерин, кодируемый геном *CDH13* и являющийся одновременно рецептором липопротеидов низкой плотности и адипоцитарного гормона адипонектина, играет роль в развитии атеросклероза и ишемической болезни сердца (ИБС). Некоторые однонуклеотидные замены в гене *CDH13* влияют на экспрессию Т-кадгерина, уровни адипонектина и липидов плазмы крови, однако связь между данными заменами и развитием ИБС не исследована. В настоящей работе изучали роль однонуклеотидных замен *rs12051272*, *rs4783244*, *rs12444338* и *rs11646213* в развитии ИБС и характере ее манифестации. В исследование включили мужчин в возрасте до 55 лет: 79 пациентов со стабильной стенокардией напряжения без инфаркта миокарда, 107 человек, перенесших инфаркт миокарда как дебют ИБС, и 99 здоровых лиц. Всем исследуемым проводили клинико-лабораторное обследование и генотипирование. Результаты генотипирования оценивали с помощью онлайн-программы SNPStats. В настоящей работе взаимосвязи полиморфизма гена *CDH13* с развитием ИБС не выявлено, однако показано, что замена *rs12051272* ассоциирована с характером дебюта заболевания: генотип GT выявили у 13 (16,5 %) пациентов со стабильной стенокардией напряжения и только у 3 (2,8 %) человек с инфарктом миокарда (отношение шансов — 7,54; 95 % доверительный интервал — 2,01–28,35). Таким образом, показано, что полиморфизм гена *CDH13* может влиять на процессы атерогенеза и характер манифестации ИБС.

Ключевые слова: Т-кадгерин, *CDH13*, генетический полиморфизм, липопротеиды низкой плотности, адипонектин, ишемическая болезнь сердца, инфаркт миокарда

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Coronary heart disease (CHD) is an extremely important medical and social issue. This disease is currently one of the leading causes of death and disability worldwide [1].

Acute coronary syndrome is often the first symptom of CHD. Intravascular thrombi are formed on the surface of a damaged atherosclerotic plaque, which leads to the development of myocardial infarction (MI) [2]. The median percent stenosis of the infarct-related artery is 48% [3]. Thus, patients with unstable plaques can be spared angina and other myocardial ischemia symptoms, but are very likely to develop acute MI. In case the atherosclerotic plaque and CHD develop gradually, stable effort angina (SEA) often becomes the first manifestation of the disease.

The mechanism of unstable atherosclerotic plaque formation has not been fully studied. Some studies have shown that T-cadherin has an important role in the development of atherosclerosis and CHD [4-7]. T-cadherin is a glycosylphosphatidylinositol-anchored protein; it belongs to the cadherin superfamily and is a receptor for low density lipoproteins (LDL) [8] and high molecular weight adiponectin, a hormone secreted by adipose tissue [9]. Many works describe the antiatherosclerotic effects of adiponectin resulting from the increased synthesis of high density lipoproteins in the liver, the reduction of cholesterol concentration in the atherosclerotic plaque [10-12] and the suppression of macrophage-to-foam-cell transformation [13]. M.M.Joosten et al. showed that low adiponectin was associated with atherosclerosis development: reduced adiponectin levels in blood serum correlated with the presence of multiple atherosclerotic vascular lesions [14, 15]. By contrast, X.J.Cai et al. demonstrated that adiponectin suppresses proliferation, migration and transformation of adventitial fibroblasts [16], which possibly causes cap thinning and increases the risk of MI. T-cadherin also functions as an LDL receptor [8,17] and thus contributes to the build-up of an unstable atherosclerotic plaque independently of adiponectin.

Some studies showed that single nucleotide polymorphisms in T-cadherin gene (*CDH13*) can affect a diponectin concentrations in blood and thus be a part of the mechanism of cardiovascular disorder development. However, there are almost zero data on the *CDH13* polymorphism association with CHD and MI. For this work we have selected four single nucleotide polymorphisms in *CDH13* gene and investigated the connection between them and both CHD development and symptoms indicative of the disease onset. It was established that *rs12051272* (G→T) and *rs4783244* (G→T) polymorphisms [18, 19] are associated with the adiponectin level changes in blood serum. The single nucleotide polymorphism *rs12444338* (G→T) is related to the changes in T-cadherin gene promoter activity [20] and to the carotid intima-media thickness [21], which indicates its possible impact on the atherogenesis. No similar data were obtained in relation to *rs11646213* (A→T) polymorphism [19, 22]; however, allele A is associated with the reduced risk of arterial hypertension (AH) and the increased risk of metabolic syndrome development [22, 23]. All polymorphisms studied in this work are associated with blood serum lipid levels [22, 24-26].

METHODS

The study enrolled 285 men aged 26 to 55. Blood samples and clinical data were obtained from the biobank of the Faculty of Fundamental Medicine of Lomonosov Moscow State University. All patients signed the informed consent as required by the Declaration of Helsinki. The control group consisted of 99 individuals: military air forces pilots without arterial hypertension, dyslipidemia and CHD signs according to cardiac stress test results. The group of patients with CHD included 186 individuals, with the age of onset being below 55. Based on the symptoms accompanying the disease onset, two subgroups were formed. For the first subgroup (n=79), the inclusion criteria was SEA without MI confirmed by cardiac stress test or coronary angiography. The second subgroup (n=107) included men in whom CHD first manifested itself as a clinically, laboratorially (elevated levels of myocardial necrosis markers) and instrumentally (electrocardiography, echocardiography, radionuclide diagnostics) confirmed MI without prior effort angina. Coronary angiography data were not used as a criterion for MI diagnosis; however, coronary angiography was performed on the patients with MI for deciding on the further treatment when the connection between MI and coronary atherosclerosis was not certain. Glucose tolerance defects and diabetes mellitus were exclusion criteria for all groups.

Patients were diagnosed with AH if their systolic blood pressure was higher than 140 mmHg and diastolic blood pressure was higher than 90 mmHg, or if they were undergoing the antihypertensive therapy. Patients were diagnosed with dyslipidemia if total blood cholesterol was over 5.3 mmol/l, LDL was over 3.0 mmol/l, or if patients were undergoing the antihyperlipidemic therapy at the time of CHD onset. Patients with body mass index over 30 were considered obese. For this study we used the data obtained from the first medical examination at the time of CHD diagnosis.

Genomic DNA was extracted using QIAamp DNA Blood Mini Kit (QIAGEN, Germany) and QIAcube robotic workstation (QIAGEN, Germany) for sample preparation of venous blood stabilized by EDTA. Genotyping was performed using TaqMan SNP Genotyping Assays (Applied Biosystems, USA).

For qualitative characteristics, the significance of differences between the groups was assessed by Yates chi-squared test. Distribution of qualitative characteristics was evaluated by Shapiro-Wilk test. Characteristics with near normal distribution were assessed using Student's t-test; other qualitative characteristics were assessed by Mann-Whitney U-test. In all cases the difference was interpreted as statistically significant with $p<0.05$. Genotyping data were analyzed using SNPStats software. To assess the probability of disease development with different genotypes, odds ratio (OR) and the corresponding 95% confidence interval (CI) were calculated. Akaike information criterion (AIC) was used to detect the inheritance pattern (codominant, dominant, recessive, superdominant and log-additive) that best matched the obtained results [27].

RESULTS

Major risk factors and their prevalence in the individuals enrolled in the study are presented in tables 1 and 2. Differences between the group of patients with CHD and the controls based on the prevalence of major risk factors and age were accounted for in the mathematical models describing the obtained results. At the same time, no significant differences were observed in the prevalence of major risk factors of cardiovascular diseases between the subgroups of patients.

No statistically significant difference was found in the frequencies of different genotypes while comparing the controls with the group of patients who had CHD, and while comparing the controls with each of subgroups of patients who had stable effort angina and prior myocardial infarction.

However, while comparing the controls with the subgroup of patients with SEA disregarding such traditional risk factors as age, obesity, smoking, dyslipidemia and AH, differences in the

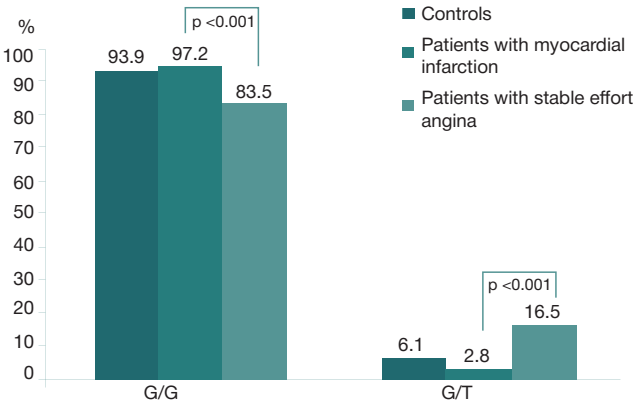
frequencies of *rs12051272* and *rs11646213* polymorphisms were detected (see tables 3, 4).

To clarify the role of these polymorphic markers, subgroups of patients with MI and SEA were compared. When introducing traditional risk factors to the model, statistically significant differences were obtained for *rs12051272* polymorphism only; for G/T genotype the OR (95% CI) of stable effort angina development was 7.54 (2.01-28.35) (see table 5). For *rs11646213* polymorphism no statistically significant difference was found.

Thus, no statistically significant differences were found between the controls and each of the studied subgroups; however, the association of *rs12051272* polymorphism with CHD manifestation pattern (MI or SEA) was shown . *rs12051272* genotype frequency data are presented in the chart below.

DISCUSSION

Reduced T-cadherin level in blood plasma is associated with the severity of atherosclerotic damage of coronary arteries and acute coronary syndrome development [7], which indicates a possible connection between *CDH13* gene polymorphism that affects protein levels and the development of CHD and its manifestation patterns. It is known that *rs12444338* (G/T) polymorphism is associated with both adiponectin level and *CDH13* promoter activity [20], that is why we expected that this marker would be associated with CHD development.



Frequency of *rs12051272* polymorphism genotypes of *CDH13* gene in the studied groups

However, no data indicated the correlation of *rs12444338*, *rs4783244* and *rs11646213* polymorphisms with CHD development and its manifestation patterns. Similar results were presented by H. Morisaki et al. for *rs12444338*; they did not find any effect of that polymorphism on MI development and the levels of LDP and adiponectin [19].

Mathematical models applied in this study accounted for the traditional factors of cardiovascular risk (age, AH, smoking, obesity and dyslipidemia), but the specifics of the controls did not allow for the demonstration of *CDH13* polymorphism association with CHD development. Still, the association of *CDH13* polymorphism with the disease manifestation pattern was shown: the frequency of G/T genotype of *rs12051272* polymorphism was significantly higher in the group of patients with SEA and without MI (16.5 and 2.8 %, respectively); OR was 7.54; 95 % CI was 2.01–28.35. The obtained data can indicate the possible protective role of T allele, which is a paradox, because this allele is associated with a lower level of adiponectin in blood plasma [19].

There are a number of possible explanations for the association discovered in this work. First, it should be noted that detecting the level of circulating adiponectin in patients with MI is hindered: it binds to T-cadherin and accumulates in the zone of myocardial damage [28], thus the reduced adeponectin level in patients with MI can be merely a result of this process [29]. Besides, adiponectin is likely to induce a number of various effects on the build-up of atherosclerotic plaques and MI development. On the one hand, high levels of adiponectin prevent the development of MI by normalizing the lipid profile [10] and suppressing macrophage transformation to foam cells [13]. On the other hand, some works have shown that adiponectin suppresses the migration of fibroblasts and their transformation to miofibroblasts [16]. As a part of this

Table 1. Prevalence of risk factors in the studied groups

Risk factors	Controls, n=99	Patients with CHD, n=186
Age, years*	36.0 (32.0; 39.0)	47.0 (44,0; 51.0)#
Dyslipidemia	0 (0)	52 (27.96 %)#
Obesity	7 (7.07 %)	57 (30.65 %)#
Smoking	27 (27.27 %)	93 (50.00 %)#
AH	0 (0)	116 (62.37 %)#

For patients with CHD, the age of the disease onset is shown; the median (interquartile range) # is presented; # — p<0.001 when compared with the corresponding figure in the controls.

Table 2. Prevalence of risk factors in the controls and the subgroups of patients

Risk factors	Controls, n=99	Patients with SEA, n=79	Patients with MI, n=107	p^{1-2}	p^{1-3}	p^{2-3}
Age, years*	36.0 (32.0; 39.0)	48.0 (43.0; 51.0)	47.0 (44.0; 52.0)	<0.001	<0.001	0.971
Dyslipidemia	0 (0)	24 (30.38 %)	28 (26.17 %)	<0.001	<0.001	0.64
Obesity	7 (7.07 %)	29 (36.71 %)	28 (26.17 %)	<0.001	<0.001	0.167
Smoking	27 (27.27 %)	35 (44.30 %)	58 (54.21 %)	<0.05	<0.001	0.235
AH	0 (0)	53 (67.09 %)	63 (58.88 %)	<0.001	<0.001	0.322

* — for the subgroups of patients, the age at the time of the disease onset is shown; the median (interquartile range) is presented; p^{1-2} —statistically significant differences between the controls and the subgroup of patients with SEA; p^{1-3} — statistically significant differences between the controls and the subgroup of patients with MI; p^{2-3} — statistically significant differences between the subgroups of patients.

Table 3. Frequency of *rs12051272* polymorphism genotypes of *CDH13* in healthy subjects and patients with stable effort angina without consideration of risk factors

Genotype	Controls, n (%)	Patients with SEA, n (%)	OR (95% CI)	p	AIC
G/G	93 (93.9)	66 (83.5)	1	< 0.05	243.5
G/T	6 (6.1)	13 (16.5)	**3.05 (1.10–8.45)**		

Table 4. Frequency of *rs11646213* polymorphism genotypes of *CDH13* in healthy subjects and patients with stable effort angina without consideration of risk factors

Inheritance pattern	Genotype	Controls, n (%)	Patients with SEA, n (%)	OR (95% CI)	p	AIC
Codominant	T/T	36 (36.4)	35 (44.3)	1	0,07	245.2
	A/T	44 (44.4)	38 (48.1)	0.89 (0,47–1.68)		
	A/A	19 (19.2)	6 (7.6)	**0.32 (0.12–0.91)**		
Dominant	T/T	36 (36.4)	35 (44.3)	1	0,28	247.4
	A/T-A/A	63 (63.6)	44 (55.7)	0.72 (0.39–1.31)		
Recessive	T/T-A/T	80 (80.8)	73 (92.4)	1	0,023	243.3
	A/A	19 (19.2)	6 (7.6)	**0.35 (0.13–0.91)**		
Superdominant	T/T-A/A	55 (55.6)	41 (51.9)	1	0,63	248.3
	A/T	44 (44.4)	38 (48.1)	1.16 (0.64–2.10)		
Log-additive	–	–	–	0.66 (0.42–1.02)	0,058	244.9

Table 5. Frequency of *rs12051272* polymorphism genotypes of *CDH13* in patients with stable effort angina and myocardial infarction with the consideration of all risk factors of interest

Genotype	Patients with MI, n (%)	Patients with SEA, n (%)	OR (95% CI)	p	AIC
G/G	104 (97.2)	66 (83.5)	1	< 0.001	251.1
G/T	3 (2.8)	13 (16.5)	**7.54 (2.01–28.35)**		

mechanism, adiponectin can cause thinning of fibrous cap of the already formed atherosclerotic plaque, which eventually causes its rupture, atherothrombosis and MI. It should be noted that changes in T-cadherin level can affect sensitivity to insulin, activity of endothelial nitric oxide synthase, endothelial cells migration and angiogenesis [30], contractile activity of vascular smooth muscle cells and organization of extracellular matrix [31]. All these factors can change the pattern of atherosclerosis development.

There is some evidence that T-cadherin level (at least in blood plasma) negatively correlates with the level of adiponectin in young males, while in females this correlation is positive [32]. The inclusion of only male individuals was a limitation of this study.

CONSLUSIONS

It has been shown that genetically determined variations in T-cadherin expression are associated with the pattern of CHD onset: myocardial infarction or stable effort angina. This indicates T-cadherin participation in atherogenesis and its effect on the stability of atherosclerotic lesions. The mechanism of this effect can be associated with adiponectin or LDP activity and requires further examination. The obtained results can be useful for the assessment of the myocardial infarction risk and for the prediction of how the initial atherosclerotic changes will progress.

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DIAGNOSTIC ADVANTAGES OF A LONG-TERM HOLTER ECG MONITORING COMPARED TO A STANDARD 24-HOUR MONITORING

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Longer observation periods have been proven to increase the diagnostic value of Holter ECG monitoring for paroxysmal atrial fibrillation mainly. The aim of this work was to study the diagnostic efficacy of a long-term ECG monitoring in detecting various types of arrhythmias and to assess the uneven distribution of arrhythmias over different observation days. In this study 27 patients were examined, including 13 men and 14 women with a mean age of 42.8 ± 10.8 years, their complaints suggesting various types of tachy- and bradyarrhythmias. Compact recorders and original software were used. ECG recording time was 5-7 days. Using quantity criteria characterizing the level of uneven inter-day distribution of arrhythmias, advantages of long-term observations over a 24-hour monitoring were confirmed for such arrhythmias as sinus pauses over 2.5 seconds, a second degree AV block, paroxysmal supraventricular tachycardia, single and paired ventricular extrasystoles, ventricular salvos, ventricular tachycardia.

Keywords: Holter monitoring, long-term ECG monitoring, arrhythmia

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ДИАГНОСТИЧЕСКИЕ ПРЕИМУЩЕСТВА МНОГОСУТОЧНОГО ХОЛТЕРОВСКОГО МОНИТОРИРОВАНИЯ ЭЛЕКТРОКАРДИОГРАММЫ ПЕРЕД СТАНДАРТНЫМ 24-ЧАСОВЫМ ИССЛЕДОВАНИЕМ

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Повышение диагностической ценности холтеровского мониторирования ЭКГ при увеличении длительности исследования убедительно доказано в основном в отношении пароксизмальной фибрилляции предсердий. Цель исследования — изучение диагностической эффективности метода многосуточного мониторирования ЭКГ в выявлении различных видов аритмий, оценка неравномерности распределения аритмий между различными сутками наблюдения. Обследованы 27 пациентов (13 мужчин, 14 женщин, средний возраст — 42,8 ± 10,8 года) с жалобами, вызывающими подозрение на наличие различных видов тахи- и брадиаритмии. В исследовании использованы малогабаритные кардиорегистраторы и оригинальное программное обеспечение. Длительность регистрации ЭКГ составила 5–7 сут. С использованием предложенных количественных критериев, характеризующих степень неравномерности межсуточного распределения аритмий, подтверждены преимущества многосуточных исследований перед 24-часовым наблюдением в отношении таких аритмий, как паузы более 2,5 с, АВ-блокада II степени, пароксизмальная суправентрикулярная тахикардия, одиночные желудочковые экстрасистолы, парные и групповые желудочковые экстрасистолы, желудочковая тахикардия.

Ключевые слова: холтеровское мониторирование, многосуточное мониторирование ЭКГ, аритмия

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By now, various authors have proved that longer observation periods increase the informative value of ECG monitoring. For example, G. Senatore at al. [1] compared the incidence of asymptomatic recurrences of atrial fibrillation (AF) in patients who had undergone radiofrequency catheter ablation of AF by a 90-day continuous transtelephonic ECG monitoring, standard ECG recorded 12, 24 and 36 hours after ablation and on the 14th, 30th and 120th days after ablation, and 24-hour Holter recording on the 30th and 120th days after ablation. It was demonstrated that long-term transtelephonic ECG monitoring was of a higher diagnostic value and decreased the success of ablation from 86 %, as detected by two other methods, to 72 %.

N. Dagres et al. [3] studied the influence of Holter duration on the detection of AF recurrences after ablation for this arrhythmia and established that a 24-hour Holter would have detected 59 % of patients with recurrences, a 48-hour Holter — 67%, a 72-hour Holter— 80 %, a 4-day recording — 91 % of all recurrences identified upon completing the 7-day observation. T. Hanke et al. [4] compared the effectiveness and accuracy of cardiac rhythm assessment in patients with prior standard radiofrequency catheter ablation by standard ECG Holter monitoring and long-term monitoring with an implantable medical device (IMD) (Reveal XT 9525, Medtronic Inc., USA) over a 3-month period. During the 24-hour surveillance, sinus rhythm was documented in 53 readings, but confirmed by the IMD in 34 cases only. D. Jabaudon et al. [5] showed that 7-day ambulatory ECG monitoring using an event-loop recorder enables to detect AF episodes more effectively than standard ECG and standard Holter monitoring. The effectiveness of standard ECG was 2.7 %; Holter monitoring identified another 5 % among those patients whose standard ECG records were of no diagnostic value; event-loop recording identified another 5.7 % of AF in patients with a normal ECG and a normal 24-hour Holter. D. Andresen et al [6] demonstrated the advantages of long-term ECG monitoring in the detection of complex ventricular tachyarrhythmias.

A positive diagnostic experience in using continuous long-term ECG monitoring for the detection of various arrhythmias has been described by a number of Russian researchers [7–10]. Long-term ECG monitoring is successfully applied when deciding on the appropriate antiarrhythmic therapy [11, 12].

It is important to note that the majority of previous studies focused on such arrhythmias as paroxysmal atrial fibrillation. Other arrhythmias, including those of clinical significance, were studied less. Little attention is paid to studying the uneven inter-day distribution of arrhythmias. Qualitative criteria showing the advantages of long-term studies have been insufficiently elaborated.

The aim of this study is to investigate the diagnostic advantages of long-term Holter monitoring over a standard 24-hour observation in detecting different types of arrhythmias, excluding AF, and to analyze the uneven inter-day distribution of arrhythmias.

METHODS

We examined 27 patients (13 male and 14 female) with a mean age of 42.8 ± 10.8. The inclusion criteria were as follows: complaints of infrequent subjective symptoms of arrhythmia (1-4 times a week), such as sudden palpitations, an acute sensation of abnormal heart activity, blackouts and sudden intense dizzy spells. Written informed consent was obtained from all patients.

For long-term ECG monitoring portable 3-channel “Machaon-03” recorders (Altonika, Russia) were used. These recorders allow for 7-day recording without battery replacement. The records were processed by the original software designed by the authors of this work. It enables to process and analyze a 7-day long 3-channel record without splitting it into separate 24-hour long segments, in one pass. Previously, the software was tested on the ECG MIT-BIH database [13–15] using a traditional method [16]. The accuracy of R-peak automatic detection was as follows: sensitivity (SE) of 99.56 %, positive predictive value (+P) of 98.67 %. A test was conducted using the Russian Society of Holter Monitoring and Non-invasive Electrophysiology database [17] with the following R-peak detection accuracy: sensitivity (SE) of 99.8 % and positive predictive value (+P) of 99.5 %.

Patients were distributed into groups based on the ECG recording duration: 21 patient had a 7-day recording, 5 patients — a 6-day recording, 1 patient — a 5-day recording. The mean duration of observation was 6 days 17 hours (161 hour).

We analyzed 7 types of arrhythmias: sinus pauses over 2.5 seconds, second degree AV block, supraventricular extrasystoles, including paired and salvos, paroxysmal supraventricular tachycardia, single and paired ventricular extrasystoles, ventricular salvos, ventricular tachycardia.

In each case the observation duration was nominally divided into 24-hour intervals. The number of various rhythm and conduction disorders in each interval was calculated. Then the uneven distribution of arrhythmia episodes over the observation period was analyzed based on the difference in their number in every 24-hour interval.

For qualitative evaluation of the results, we suggest the following parameters:

– PN1 positive number of patients — number of patients with the arrhythmia of interest detected in only one of all 24-hour intervals, whereas in the rest of 24-hour intervals this arrhythmia was not detected at all;

– PN1 positive number of patients, % — a proportion of patients with only one diagnostically significant 24-hour interval to the total number of patients with detected arrhythmia of interest, expressed as a percentage For example, the studied type of arrhythmia was detected in 7 patients; 2 of them recorded arrhythmia only during one 24-hour interval of the total observation period. Thus, the percentage of PN1-positive number of patients will be 28.5 %;

– PN1 negative number of patients — number of patients who did not have the arrhythmia of interest registered within at least one of 24-hour intervals, but had it documented on other days;

– PN1 negative number of patients, % — a proportion of patients with at least one 24-hour interval free of the arrhythmia of interest, to the total number of patients in whom this arrhythmia type was detected, expressed as a percentage. For example, a given type of arrhythmia was detected in 8 patients, 5 of them had a day when this type of arrhythmia was not registered. The percentage of PN1-negative number of patients will be 62.5 %;

– PD24 — a probability of detection of the arrhythmia of interest in case the study would have covered a 24-hour interval only. For each patient, this value was calculated as a proportion of the number days when arrhythmia was detected, to the total number of days in the observation period, and expressed as a percentage. For example, if arrhythmia is identified in 3 out of 6 24-hour intervals (a 6-day monitoring), PD24 will be 50 %;

– VC — a variation coefficient, a non-uniformity index, calculated as a ratio of standard deviation of arrhythmias number in each observation day to their daily average in a given patient.

RESULTS

Results of data analysis obtained during the continuous long-term ECG Holter monitoring, are presented in the table below.

Using the quantity criteria listed above the advantages of long-term ECG recording over a standard 24-hour Holter were demonstrated. As the table suggests, the standard 24-hour ECG monitoring can fail to detect potentially dangerous arrhythmias.

When summarizing the results of all arrhythmia cases analyzed in this work, the following mean values were obtained. The probability of detecting an arrhythmia within a 24-hour surveillance was 51.4%, compared to long-term observations.

Parameters of detection various arrhythmia types in a long-term surveillance

Parameters	Supraventricular extrasystoles	Single ventricular extrasystoles	Paroxysmal supraventricular tachycardia	Sinus pause over 2.5 sec	Second degree AV block	Paired and group ventricular extrasystoles	Ventricular tachycardi	Mean values for all arrhythmia types
Number of patients with a given type of arrhythmia	27	26	15	3	3	13	5	-
PN1 positive number of patients	0	2	7	2	1	8	3	-
PN1 positive number of patients, %	0	7.7	46.7	66.7	33.3	61.5	60	39.4
PN1 negative number of patients	3	15	13	2	3	12	5	-
PN1 negative number of patients, %	11.1	57.7	86.7	66.7	100.0	92.3	100.0	73.5
PD24, %, mean valu	98.4	71.2	39.8	29.4	55.6	33,2	31.9	51.4
VC, mean value	61.4	106.7	181.8	203.3	147.5	198.5	199	156.9

Arrhythmia was detected in only one 24-hour interval of the total observation period in 39.4% of cases. In 73.5% of cases there was at least one 24-hour interval when arrhythmia was not present.

A mean value of arrhythmia variation coefficient was 156.9, which is 5 times higher than a standard threshold value for a uniform distribution.

Examples illustrating the uneven distribution of arrhythmia episodes between different days are presented below.

Patient S., 46 years of age, male, sought medical advice with a cardiologist at Regional Clinical Hospital no.1. The patient complained of sudden palpitations which lasted from several seconds up to several minutes and occurred once or twice a week, mainly in the evening or at night. The patient had had those symptoms for about a year. In spite of the fact that except palpitations no other symptoms were present, those episodes caused a considerable psychological discomfort for the patient. The cardiologist suspected ventricular dysrhythmia. Shortly before that the patient had undergone a 24-hour Holter that only registered singular supraventricular extrasystoles. We conducted a 7-day Holter monitoring. During the analysis of a 4th day record, a single episode of paroxysmal superventricular tachycardia was detected with the heart rate of up to 145 beats per minute and the duration of 5 seconds (Fig. 1), which matched the patient's subjective sensations. It was the only episode within a 7-day observation. As a result, it was proved that arrhythmia episodes in this patient were of low risk, which made it possible to cancel the aggressive antiarrhythmic treatment planned before.

Patient L., 32 years of age, male, sought medical advice with a cardiologist at Regional Clinical Hospital no.1. The patient complained of periodic short dizzy spills and presyncopes that occurred approximately once a week. On the 6th day of a 7-day monitoring the patient suddenly felt intense dizziness, which coincided with the episode on the tape consisting of two consecutive sinus pauses of 2.8 and 3.1 seconds long (Fig. 2). This episode of sinus node suppression was a single indication of bradycardia over the whole 7-day recording. The patient was referred to surgeons to decide on the pacemaker implantation.

Patient P., 62 years of age, female, sought medical advice with a cardiologist at Regional Clinical Hospital No 1. The patient complained of periodic short sudden anxiety episodes, palpitations, chest discomfort and pressure unrelated to physical exercise. Those episodes had been present for several months

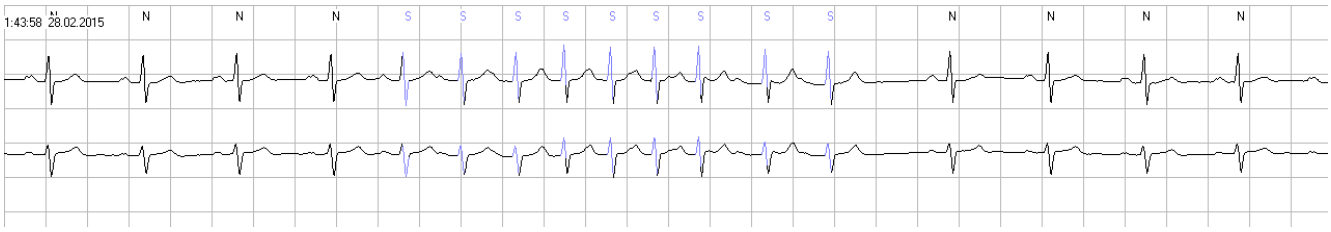

Fig. 1. A single episode of paroxysmal supraventricular tachycardia on the 4th day of the 7-day observation

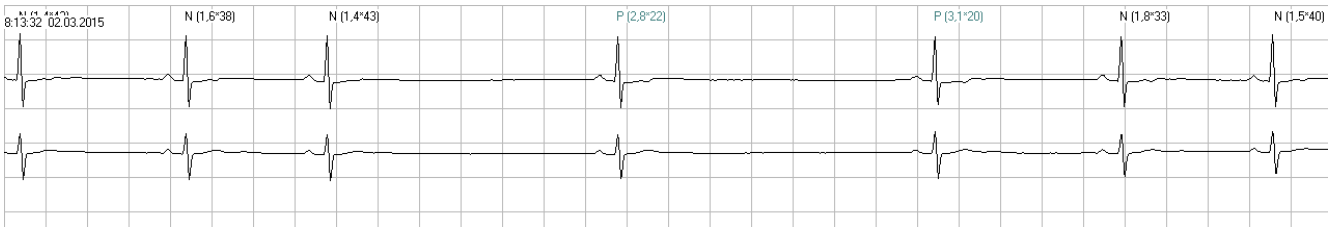

Fig. 2. Subsequent sinus pauses of 2.8 and 3.1 sec resulting from sinus node suppression

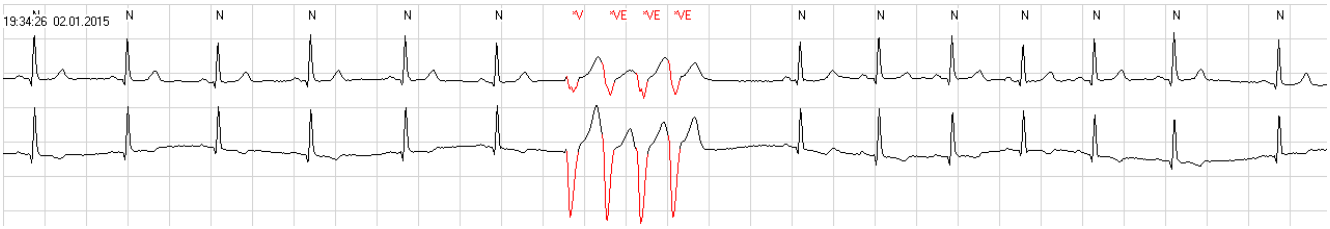

Fig. 3. A single episode of paroxysmal ventricular tachycardia on the 4th day of the 7-day study

and occurred once or twice a month, but became more frequent (2-3 times a week) by the time of the consultation. To exclude a cardiovascular disease, a 7-day monitoring was conducted. A single episode of ventricular tachycardia was documented on the 4th day of the observation (fig. 3), confirmed by the patient's complaints. The patient was referred to a cardiologist to decide on the appropriate antiarrhythmic therapy.

DISCUSSION

Different authors have shown that longer observation periods increase the informative value of ECG monitoring. Increased observation duration was achieved by different means and in all cases resulted in a higher informative value of the examination. However, less attention was paid to investigating of how uneven the inter-day arrhythmias distribution is. We believe that the data we obtained prove that in patients with infrequent clinical symptoms of heart dysrhythmias, the inter-day distribution of arrhythmias is significantly uneven. Cases of total absence of the studied arrhythmias over a period of several observation days and cases of dysrhythmias recorded only in one 24-hour interval of the whole observation period make up quite a big proportion. Such distinct unevenness points to a very high probability of false positive diagnostic results within this group of patients in case if only a one-time 24-hour Holter monitoring is performed.

Longer (up to 7 days) observation increases the probability of detecting various arrhythmias. The results of this study suggest that the above-said is true not only for paroxysmal AF, but for other arrhythmias as well. Still, a longer surveillance is more expensive as compared to a 24-hour observation, which is the reason why a 7-day monitoring cannot be recommended as a routine procedure instead of a standard 24-hour Holter in every case.

Longer observation (up to 7 days) can be recommended if a 24-hour monitoring has proved to be of no informative value in patients with typical and distinct complaints and infrequent (1–4 times a day) rhythm and conduction defects.

Noninvasive 7-day observation is definitely not as long as an observation based on the usage of an implantable medical device, but it has a number of advantages based on other parameters: no implantation surgery is required, it is cheaper, the recorder can be used many times, it offers a possibility for continuous ECG recording.

CONCLUSIONS

The quality criteria we suggest enable to objectively assess the inter-day distribution of arrhythmias and clearly show their irregular occurrence.

In patients with infrequent arrhythmia symptoms, a considerable uneven inter-day distribution of arrhythmias is observed. This conclusion applies not only to paroxysmal atrial fibrillation, but to other arrhythmias as well.

During the superlong 5-, 6-, 7-day Holter observations there is a possibility to extract more valuable diagnostic information than during standard ECG monitoring. The probability of detecting arrhythmias in case of their infrequent occurrence (1–4 times a week) increases considerably. This is particularly true for clinically significant arrhythmias, such as sinus pauses over 2.5 sec, second degree AV block, paroxysmal supraventricular tachycardia, paired ventricular extrasystoles, ventricular salvos, ventricular tachycardia. However, the advantages of using this method for supraventricular extrasystole diagnosis are disputable.

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ECG-BASED BIOMETRIC IDENTIFICATION: SOME MODERN APPROACHES

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The uniqueness of electrical activity of every human heart prompts us to use the ECG as a biometric parameter in various security and authentication systems as it is easy and cheap to extract the signal and difficult to fake it or obtain nonconsensually. At the moment various approaches to researching a possibility of human identification by ECG are used. Identification mode includes the following stages: data collection, procession, feature extraction, classification. Researchers use different mathematical algorithms at every stage: principal component analysis, wavelets, neural networks, etc. This article reviews the most significant studies of ECG based human identification and compares their results and accuracy of conceptual approaches.

Keywords: ECG, identification, classification, biometrics

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БИОМЕТРИЧЕСКАЯ ИДЕНТИФИКАЦИЯ, ОСНОВАННАЯ НА ЭКГ: НЕКОТОРЫЕ СОВРЕМЕННЫЕ ПОДХОДЫ

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Уникальность электрической активности сердца каждого человека побуждает использовать электрокардиограмму в качестве биометрического параметра в различных системах безопасности и аутентификации в связи с легкостью и дешевизной извлечения сигнала, а также сложностью его подделки и недобровольного извлечения. На данный момент применяют различные подходы к исследованию возможности идентификации человека по ЭКГ. Режим идентификации включает в себя следующие стадии: сбор данных, обработка, извлечение характерных признаков, классификация. На каждом из этих этапов группы исследователей используют различные математические алгоритмы: метод главных компонент, вейвлеты, нейронные сети и т. п. В статье рассмотрены наиболее значимые исследования в области идентификации человека по ЭКГ. Проведено сравнение результатов и точности концептуальных подходов.

Ключевые слова: ЭКГ, идентификация, классификация, биометрия

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In this era of technological infrastructure, security issues are particularly important. Growing industries, network integration, a rapid development of information technologies urge us to search for new identity-based means of data protection.

Applications often need to identify a person: to match un unknown individual to a known identity from a database, to perform a “one-to-many” comparison, to verify an individual, i.e. to check if he is the person he claims to be, to perform a “one-to-one” comparison against a specific template. Such tasks can be found everywhere: from computer systems to systems that grant access to closed or corporate facilities. The identification of family members in their daily life also presents a particular interest.

Traditional password-based and identification systems have a number of flaws. A password can be forgotten or elicited, and such systems are easy to hack. One needs to always carry the identification key around, which is inconvenient. There are chances that the intruder can get hold of your password or a physical identifier. Besides, a person cannot be identified without any specific physical carrier. Together, all those factors prompt us to look for new approaches to the problem.

Biometrics (life measurement in Greek) refers to a system of human identification based on one or more than one physiological or behavioral traits [1]. Various physiological or behavioral characteristics can serve as biometrics if they more or less satisfy the following criteria: universality; uniqueness; permanence; measurability; performance; acceptability; circumvention (ease of use of a substitute) [2].

Currently the following biometric characteristics are used: fingerprints, face, iris, hand geometry, voice, DNA,

facial thermogram, signature, gait, labial form, etc. [3–12]. The advantages and disadvantages of these characteristics are related to the criteria listed above [13]. For example, it is almost impossible to make a mistake with a DNA-based identification or verification, and the samples can be used in forensics; however, this method requires special laboratory equipment. The same is true for fingerprints: though the reader can be quite miniature in comparison to a device used for a DNA-based detection, fingerprints can change with time or be affected by other factors. Thus, the use of various biometric identifiers is dependent on the goals, limitations and resources within a specific task.

Recently, scientists have focused their attention on the development of a new type of biometric recognition, namely on the electrical activity of the heart, a human physiological trait. Specifically, the ECG is becoming an adequate mean for a medium level protection in applications since it is easy and cheap to extract the signal and difficult to fake it or obtain nonconsensually. It is worth noting that the uniqueness of the ECG is a sum of various physiological factors such as heart anatomy, weight, gender, chest size, age, health, etc. Thus, with age or affected by a disease, the heart electrical activity changes, and it is not reasonable to use the ECG as a long-term biometric parameter. For example, Bionym, a Canadian company, has announced the development of the Nymi band, an electronic device that will record a user's ECG every day, verify him and grant him access to certain infrastructure objects (a mobile phone, a computer, a hotel room, a car, etc.). For identification purposes, the ECG is most likely to be used while working with databases, as the advancement of telemedicine technologies allows storing huge data arrays, including patients' ECG records. If an operator or a doctor fills in patient data incorrectly (mistypes a family name, date of birth, etc.), the identification of such records can contribute to a more accurate observation of the course of a disease.

Another possible field of application can be found with a small fixed number of users of certain ECG recorders: for example, in various medical institutions for the sake of convenience patients will only need to record an ECG, and the identification system will determine whose record it is. With identification, using ECG recorders at home will be easier; gadgets in the form of mobile telephone cases have already appeared on the market. They can record patient's heart electrical activity and send it over to a doctor via Internet.

The main principles of building a biometric identification system and different approaches to the ECG-based human identification are reviewed below. The diversity of mathematical tools is described. The results of basic research works are presented.

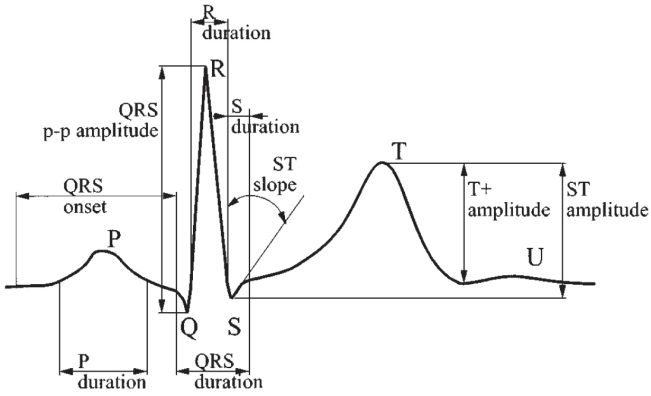

Fig. 1. ECG feature combination: (Biel et al., 2001)

ECG signal formation

Electrocardiogram is a time curve of a total electrical potential occurring in a heart muscle due to the flowing of ions through a muscle membrane [3]. IECG recording is one of the most common tools used for the diagnostics of cardiovascular disorders due to its high informative value and accuracy.

In cardiology, the ECG is often measured in several leads that carry information about the potential difference between the two points of the heart electric field, using electrodes. Each of the leads reflects the condition of a certain region of the heart muscle.

Basic principles of building EGG-based human identification system

The identification process includes the following stages:

– initial data collection;
– signal pre-processing (filtration etc.);
– extraction of typical features, their procession and template creation;
– comparison of a submitted template with previously enrolled templates in a database.

After that, an identification decision is made using various classification algorithms.

The most difficult task with identification is to select those features that are truly characteristic of a studied object. This particular area offers broad opportunities for experimenting with various approaches. The main idea is that a plurality of such features (descriptors) forms a vector that can be compared to other vectors using various mathematical methods.

There are approaches based on the extraction of such features as amplitudes, angles, vertical and horizontal constituents of ECG signal segments [15, 16].

Another approach is related to the extraction of analytical properties presented by signal decomposition coefficients in various bases, such as Fourier coefficients [17], wavelets, linear prediction coefficients [18], etc.

On this stage of the identification process, standard methods of classification are used. The simplest is the "nearest centroid" method. It labels a new input feature vector as the class that gives a minimal distance to the class centre. Another common approach is the "k-nearest neighbours" algorithm; it is based on assigning an object to the most common class among its neighbours. For recognition, support vector machines and neural networks are also used [19].

Comparison and results

One of the first scientific works that demonstrated the possibility of using the ECG for identification purposes, was an article by Lena Biel et al. [15] In the experiments with 20 healthy subjects it was shown that for a quality ECG-based identification 1 lead instead of 12 standard leads is sufficient.

As a basis for the ECG signal analysis, 30 signal features characterizing its form were chosen. These features are normally used for medical diagnosis. Their correlation with each other was analyzed, which helped to reduce the total number of features and to select those most specific for each individual. A set (vector) of 8 features (variables) characterizing (classifying) each individual was considered the most successful combination (Fig. 1). To account for the variability of feature changes, the sample data were obtained from each participant at different times.

For identification, the so-called SIMCA method (Soft Independent Modeling of Class Analogy) was applied. It is widely used in chemometrics for spectroscopic data classification. It also allows working with a large number of features [20]. Classification tasks and algorithms and identification tasks often overlap, if we treat an object chosen for identification as a class.

The first step in SIMCA is a more common PCA (Principal Component Analysis), which in its essence is a mathematical tool for reducing data dimensionality or data compression [21]. Transforming a large number of variables to a new representation with considerably lower dimensions makes it possible to simplify data by orders of magnitude, for example, to reduce 1000 variables to 100, with no data loss and no variables being ignored. At the same time, the data which are irrelevant for the analysis are detected and removed as noise. Being discovered, principal components give an indication of hidden variables controlling data structure. Thus, an ECG feature space distinguishing an individual is projected on the principal components direction, which in that particular work was a plane, where each point is related to an individual, or a class in mathematical terms. In this space classification can be performed.

After building a PCA-decomposition, SIMCA is used to calculate the distance between classes as well as the distance from each class to a new object. Two values are used as such metrics. The distance between an object and a class is calculated as a root mean squared residual, occurring when projecting the object onto the class. The other value defines the distance from an object to a class centre and is calculated as the range (squared Mahalanobis distance). In this space a classification rule is set up and identification becomes possible.

In Biel's work, the results of human ECG-based identification depended on a number of ECG features selected for the research. In average, the scientists accomplished 49 correct identifications out of 50.

Another study was performed by Steven A. Israel et al. [16]. They established that the psychological state of the subjects did not affect the outcome of the identification process. Interestingly, that the authors used LDA (Linear Discriminant Analysis) as a method for reducing the space of the studied parameters. The efficiency of LDA compared to Principal Component Analysis, as well as the combination of both, was studied by Y. Wang et al [22].

In his work Y. Wang also used coordinate ECG parameters (amplitudes, angles and distances) as a basis for classification. However, the alignment of each complex by the R-peak was this work's distinctive feature (Fig. 2).

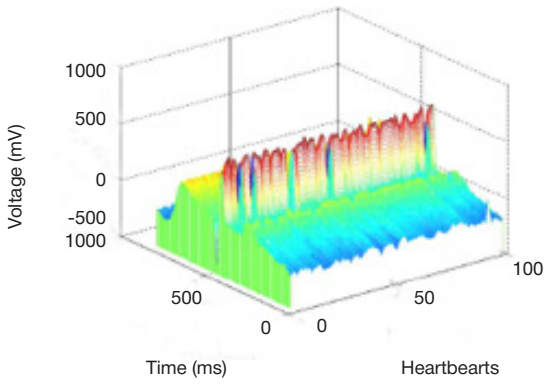

Fig. 2. A signal aligned by R-peak: (Wang et al., 2006)

LDA is one of the oldest statistical methods [23] and is used for finding linear combinations of features that best discriminate two or more objects. Like PCA, it is commonly used as a classifier or for dimensionality reduction. Initially, Y. Wang investigated, which of the two following simple classification methods was more efficient as an algorithm for reducing the number of ECG signal properties: the k-nearest neighbours (a class of a classified object is the most common class among k-nearest neighbours) and the nearest centroid method (the closer a classified object is to a "gravity centre" of a group of objects belonging to a known class, the higher is the probability that it belongs to this class). It was shown that the best result can be achieved by using Principal Component Analysis with the k-nearest neighbours. Using a hierarchical combination of LDA and PCA, Y.Wang achieved a 98.9 % accuracy in recognition. 13 volunteers participated in the experiments; identification was performed more than once at different times and under different conditions.

Methods based on the extraction of analytical properties and neural network classification present a particular interest. In 2010 a study was conducted by a group of scientists led by Justin Leo Cheang Loong [18]. ECGs with one chest lead were recorded in 15 subjects. Two bases were chosen as algorithms for the analytical ECG signal representation and for comparison of their performance with each other, namely wavelets and coefficients of linear prediction.

A basis for the wavelet packet decomposition algorithm (WDP) is a wavelet, a term introduced by A.Grossman and J.Morlet in the mid 1980s in the context of feature analysis of seismic and acoustic signals [24]. Wavelet transform based algorithms are also used for electrocardiogram analysis. A wavelet transform is a tool that splits data into different frequency components. Each frequency is then studied with a required resolution. Thus, a wavelet transform is a tool for time-frequency localization of signal features. Among the advantages of WPD are a high decomposition rate, universality and a possibility to alter the decomposition level. However, this method cannot be automated. For best decomposition it is necessary to manually analyze several WPD levels. Another drawback is related to the core of wavelet analysis: a necessity to choose a basis wavelet depending on the character of initial time series.

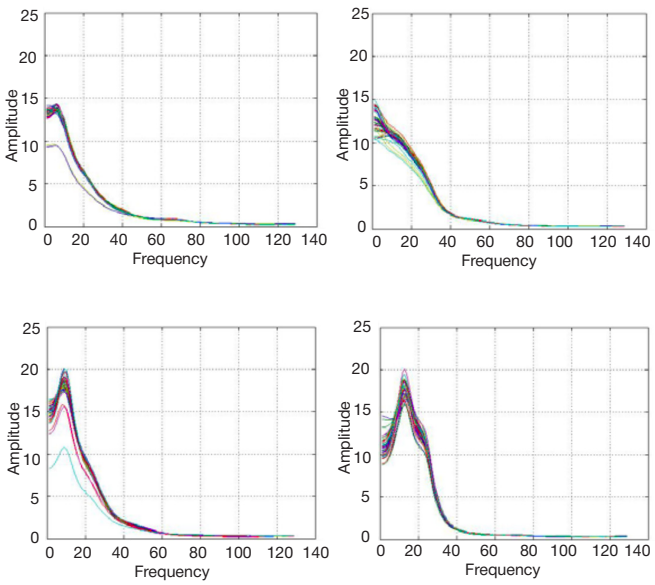

Fig. 3. The LPC spectrum of different subjects (Loong et al., 2010).

J. L. Ch. Loong et al. subjected a signal to a 5-level wavelet packet decomposition using the db2 (Daubechies wavelet) and obtained overall 50 parameters that were used as a feature set for identification.

The Linear Predictive Coding (LPC) is normally used to model different parameters of human speech transmitted instead of samples or sample differences that require a larger bandwidth [14]. LPC algorithms are traditionally used for studying the vocal tract signal, i.e. for the analysis, recognition and procession of human speech. LPC coefficients allow predicting signal feature values as a linear function of previous segments. For ECG-based identification, the signal was processed using LPC algorithms. The first 40 points of the LPC spectrum were taken as a feature set for further research. Figure 3 shows ECG LPC spectrum differences in 4 subjects.

As a classifier, an artificial neural network (ANN) was used as a classifier after applying the error back propagation algorithm. The idea of ANN originated from an attempt to describe the processes of information perception in human brain. Like human brain, the ANN consists of neurons, multiple elements that are connected to each other and imitate brain neurons. A basic structure of this network is shown in figure 4.

Each neuron in a neural network transforms input signals into output signals and is connected to other neurons. Input neurons form the so-called neural network interface. Data input to a neural network is performed through the input layer that receives signals. All neural network layers process signals until they reach the output layer that generates output signals.

The ANN task is to transform data in a required way. For that, the network needs to be trained. During the training process, ideal (reference) values of input-output pairs or "teachers" are used. The "teacher" evaluates the behaviour of the neural network. For training the so-called training algorithm is used. The untrained neural network cannot imitate the anticipated behaviour. The training algorithm modifies individual neurons of the network and its connection weights in such a way, that its behaviour matches the expected performance. The main idea of the method applied in that study is that error signals propagate reversely from the network output to its input while in the standard operation mode the signals propagate from the input to the output. The LPC algorithm showed better results against the WPD method with the recognition accuracy of 99.5 % and 91.5 % respectively [18].

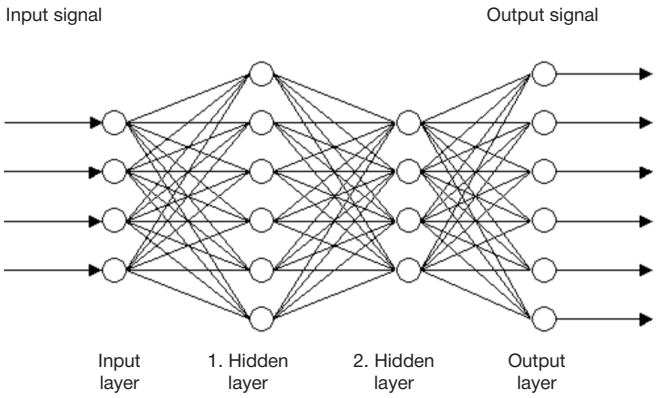

Fig. 4. Neural network structure

CONCLUSIONS

The possibilities of human ECG-based identification have not been sufficiently studied so far. However, research in this area is actively pursued at the moment. The increasing number of such experiments all over the world gives us the reason to consider the electrical activity of the heart a very prospective research object. Different approaches to extracting individual parameters of ECG receive the most attention; uniform standards and effective methods are yet to be developed. However, the inspiring results obtained in previous studies hold promise for future development in this area.

Summary of ECG based human identification results reviewed in this work

Study	Number of subjects	ECG features type (number)	Data procession algorithm	Classifier	Result %
L. Biel, et al. ECG Analysis: A New Approach in Human Identification [15]	20	signal form features (8)	PCA (Principal Component Analysis)	SIMCA (Soft Independent Modeling of Class Analogy)	98,00 (49 correect identifications of 50)
Y. Wang, et al. Integrating Analytic and Appearance Attributes for Human Identification from ECG Signal [22]	13	signal form features (15)	PCA (Principal Component Analysis)	K-NN («k-nearest neighbours»)	95.55
			LDA (Linear Discriminant Analysis)	K-NN («k-nearest neighbours»)	93.01
			LDA/PCA hierarchial algorithm	K-NN («k-nearest neighbours»)	98.9
J. L. Ch. Loong, et al. A New Approach to ECG Biometric Systems: A Comparitive Study between LPC and WPD Systems [18]	15	analytical features (50)	WPD (Wavelet Packet Decomposition)	neural network	91.52
		analytical features (40)	LPC (Linear Predictive Coding)	neural network	99.52

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SURFACE PHENOTYPE OF BLOOD LYMPHOCYTES IN CHILDREN WITH MEDIUM AXIAL MYOPIA IN THE PRESENCE OR ABSENCE OF SECONDARY IMMUNODEFICIENCY

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Investigating the role of secondary immunodeficiency in the development of myopia in children is a promising research area. We studied the surface phenotype of blood lymphocytes in healthy children and children with medium axial myopia in the presence or absence of secondary immunodeficiency clinical manifestations. The mean age of study participants was 16 ± 0.25 years. The control group and each of the two experimental subgroups included 8 children. Using indirect immunofluorescence, the expression of CD3, CD4, CD8, CD16, CD56, CD20, CD72, CD38, CD25, CD71, HLA-DR, CD95, CD54, mIgM, mIgG, ICAM-1 antigens was studied. For children with myopia and secondary immunodeficiency, only one statistically significant ($p < 0.05$) difference from the control group was detected, namely, a reduced expression of CD4 antigen. For children with myopia and without secondary immunodeficiency, a statistically significant ($p < 0.05$) increase in CD20 antigen expression and a reduced ICAM-1 antigen expression were observed.

Keywords: nearsightedness, myopia, medium axial myopia, secondary immunodeficiency, lymphocytes, lymphocyte surface phenotype, antigens

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ПОВЕРХНОСТНЫЙ ФЕНОТИП ЛИМФОЦИТОВ КРОВИ У ДЕТЕЙ С ОСЕВОЙ МИОПИЕЙ СРЕДНЕЙ СТЕПЕНИ ПРИ НАЛИЧИИ И ОТСУТСТВИИ ВТОРИЧНОГО ИММУНОДЕФИЦИТА

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Изучение влияния вторичного иммунодефицита на развитие близорукости у детей — перспективное направление исследований. Нами был изучен поверхностный фенотип лимфоцитов крови у здоровых детей и детей с осевой миопией средней степени при наличии и отсутствии клинических признаков вторичного иммунодефицита. Средний возраст участников исследования составил 16 ± 0,25 года. В контрольную группу и в каждую из двух опытных подгрупп включили по 8 детей. Изучали экспрессию антигенов CD3, CD4, CD8, CD16, CD56, CD20, CD72, CD38, CD25, CD71, HLA-DR, CD95, mIgM, mIgG, ICAM-1 методом непрямой иммунофлюоресценции. Для детей с близорукостью и вторичным иммунодефицитом выявили лишь одно достоверное ($p < 0,05$) отличие от показателей контрольной группы — сниженную экспрессию антигена CD4. Для детей с близорукостью и без вторичного иммунодефицита отметили достоверное ($p < 0,05$) усиление экспрессии антигена CD20 и снижение экспрессии антигена ICAM-1.

Ключевые слова: близорукость, миопия, осевая миопия средней степени, вторичный иммунодефицит, лимфоциты, поверхностный фенотип лимфоцитов, антигены

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Myopia is not only the most common type of refractive error, but is also ranked first in the general structure of ocular pathology [1]. The prevalence of myopia is growing. According to available data, myopia of varying degrees affected approximately 1.6 billion people worldwide in 2000. This figure is expected to in crease to 2.5 billion by 2020 [2]. It is important to note that prevalence of the disease among children [3, 4] is growing, and progression of the disease is often observed in school children [5, 6].

Myopia is considered a polyetiological disease, but some of its causes are not yet fully studied. Studying the role of immune disorders in the development of myopia is a promising research area [5–9]. Children with myopia have clinical signs of secondary immunodeficiency more often than their peers with other types of clinical refractive errors [5, 7–9].

Our study aims at investigating the surface phenotype of blood lymphocytes in children with emmetropia and medium axial myopia, with or without clinical signs of secondary immunodeficiency.

METHODS

The study was conducted in 2013–2015 and featured 24 school children in Moscow aged between 10 and 18 years (mean age of 16 ± 0.25 years): 16 boys and 8 girls. The control group included 8 children (16 eyes) with emmetropia without chronic diseases but with incidence of acute respiratory infections (ARIs) for less than five times a year. The experimental group consisted of 16 children (32 eyes) and was divided into two subgroups. The first subgroup (Group I) included 8 children diagnosed with medium axial myopia and clinical signs of secondary immune deficiency (SID). These children were observed with increased incidence of ARIs (more than 7 times a year). The exclusion criterion was the presence of autoimmune diseases. The second subgroup (Group II) included 8 children with medium axial myopia, but without clinical signs of SID and with incidence of ARIs for less than five times a year and absence of ARIs for two months prior to the study. The diagnosis was verified at the Diagnostic and Consultative Unit, Children's Medical Centre of the Administrative Directorate of the President of the Russian Federation, Moscow, Russia. The presence or absence of SID was determined based on dispensary data.

All the children examined underwent standard ophthalmic examination, including visometry, (digital chart OAP-250, Carl Zeiss, Germany), autorefractometry (auto kerato-refractometer KR-8900, Topcon, Japan), biomicroscopy (SL 120 slit lamp, Carl Zeiss, Germany), identification of relative accommodation reserves by Avetisova method [10], and ophthalmoscopy and echo biometry (HiScan scanner, OPTICON, Italy).

Lymphocytes were separated from peripheral blood using a one-step Boyum density gradient technique [11]. The expression of CD3, CD4, CD8, CD16, CD56, CD20, CD72, CD38, CD25, CD71, HLA-DR, CD95, CD54, mIgM, mIgG, ICAM-1 antigens was studied using monoclonal antibodies ICO and LT by indirect immunofluorescence under the Luman I-3 microscope (LOMO, Russia).

The results were statistically processed using software package Statistica. The statistical significance was estimated using Student's test.

RESULTS

The research results are presented in the table. Group I children (with SID signs) showed reduced expression of CD3, CD4 and CD8 antigens in comparison with the control group. However, the difference was significant only for lymphocytes with surface phenotype $CD4^+$ ($p < 0.05$). In Group II children (with no SID signs), on the contrary, the number of lymphocytes expressing CD3, CD4 and CD8 antigens was higher than that of the control group.

But for all of them, the difference was insignificant. Differences in expression of CD16 and CD56 antigens identified for both groups in comparison with the control group was also not statistically confirmed.

The content of lymphocytes with surface phenotype CD20+ in Group I children was 14.54 ± 2.36 %, which is lower than similar indicator in the group of healthy children (19.87 ± 2.15 %). However, the difference was insignificant. A significant increase in the expression of this antigen in Group II children was observed. The identified differences on lymphocytes with

Lymphocyte count with different surface phenotype in the peripheral blood of children in the experimental and control groups (% of the total lymphocyte count)

Surface markers	Group I	Group II	Control group
$CD3^+$	39.97 ± 2.01	58.47 ± 1.96	56.33 ± 3.35
$CD4^+$	29.81 ± 4.38*	44.73 ± 4.76	38.03 ± 0.87
$CD8^+$	22.66 ± 2.49	32.02 ± 2.63	26.48 ± 0.98
$CD16^+$	19.01 ± 3.12	25.74 ± 4.51	23.01 ± 3.07
$CD56^+$	18.28 ± 4.62	19.88 ± 3.52	18.06 ± 1.65
$CD20^+$	14.54 ± 2.36	29.21 ± 2.84*	19.87 ± 2.15
$CD72^+$	15.08 ± 1.63	24.805 ± 4.07	19.23 ± 2.27
$CD38^+$	16.17 ± 3.16	25.93 ± 5.41	22.89 ± 2.08
$CD25^+$	17.11 ± 2.13	19.52 ± 2.22	17.92 ± 4.23
$CD71^+$	18.73 ± 4.14	21.17 ± 2.99	17.8 ± 2.84
$HLA\text{-}DR^+$	22.27 ± 2.06	24.63 ± 3.88	21.45 ± 2.20
$CD95^+$	15.88 ± 2.68	20.97 ± 2.46	15.73 ± 1.87
IgM^+	10.69 ± 1.73	27.19 ± 5.79	15.84 ± 1.07
IgG^+	16.29 ± 3.67	20.49 ± 3.23	20.49 ± 3.23
$ICAM\text{-}1^+$	6.69 ± 0.70	22.905 ± 6.42*	11.93 ± 1.40

* — $p < 0.05$ compared with the control group.

surface phenotypes CD72, CD38, CD25, CD71, HLA-DR, CD95, mIgM and mIgC were not statistically confirmed.

A significant ($p < 0.05$) decrease in the level of expression of ICAM-1 antigen was observed in Group II: number of lymphocytes corresponding to the phenotype was 6.69 ± 0.70 % against 11.93 ± 1.40 % in the control group.

Thus, children with medium axial myopia and clinical signs of SID showed reduced expression of CD4 antigen when compared with healthy children. In children with the same diagnosis, but with no clinical signs of SID, there was a significant increase in the expression of CD20 antigen and reduced expression of ICAM-1 antigen.

DISCUSSION

Despite the fact that most of the differences revealed were statistically insignificant, it is undeniable that children with medium axial myopia and clinical signs of SID tend to have decreased immunity. Examining a larger number of patients is most probably required. However, there is reduced number of major subpopulations of T-lymphocytes and NK-cells in sickly children compared with healthy children.

Increased expression of adhesion molecules in children with myopia can be associated with the action of peroxy compounds on lymphocytes [12, 13]. It is known that transretinal — the product of isomerization of cis-retinal in the light-dependent visual cycle process – activates lipid peroxidation [14]. Also, myopia is associated with the toxic effect of peroxide compounds on the sclera and increase in the longitudinal dimensions of the eyeball [15].

CONCLUSIONS

The trend towards inhibition of expression of the antigens characterizing lymphocyte subpopulations in myopia in children with clinical signs of SID shows that the immune system is involved in the pathological process, and is apparently not associated with myopia.

Children with medium axial myopia without clinical signs of SID exhibit elevated blood lymphocytes expressing ICAM-1 antigens in the leukocyte membrane. This may be associated with production of free radicals, which are generated during visual act by one of the active metabolites of vitamin A.

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VITAMIN STATUS OF URBAN AND RURAL SCHOOL CHILDREN AND SPECIFICS OF FREE RADICAL REACTIONS IN THEIR BLOOD SERUM

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The rate of free radical reactions is one of stress markers. The ability of the organism to resist oxidation is determined by various factors, including vitamin supply. Vitamins A, E, C and group B vitamins directly or indirectly affect the degree of antioxidant protection. We have studied vitamin supply in school children aged 12 to 17 in urban (n = 250) and rural areas (n = 200) and the rate of free radical reactions in their blood serum by induced chemiluminescence. Deficiency of vitamins A and E, which have antioxidant properties, was detected in both urban and rural school children; however, the former had a higher deficiency level. This corresponds to the chemiluminiscence assay data: all chemiluminescence assay values in urban school children were 2.2-7.6 times higher than in rural school children, which indicates a higher intensity oxidation in their bodies. A deficiency of group B vitamins was also detected in rural school children, riboflavin being an exception in a subgroup of 15 to17 year old subjects.

Keywords: vitamin supply, retinol, tocopherol, ascorbic acid, thiamine, riboflavin, pyridoxine, antioxidants, oxidative stress, free radical reactions, school children

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СОДЕРЖАНИЕ ВИТАМИНОВ В ОРГАНИЗМЕ ГОРОДСКИХ И СЕЛЬСКИХ ШКОЛЬНИКОВ И ОСОБЕННОСТИ СВОБОДНОРАДИКАЛЬНЫХ РЕАКЦИЙ В ИХ СЫВОРОТКЕ КРОВИ

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Уровень свободнорадикальных реакций в организме является одним из маркеров стресса. Способность организма противостоять окислительным процессам определяется различными факторами, в том числе витаминной обеспеченностью. Витамины А, Е, С и витамины группы В прямо или косвенно влияют на степень антиоксидантной защиты. Нами была изучена витаминная обеспеченность городских (n = 250) и сельских (n = 200) школьников в возрасте 12–17 лет и уровень свободнорадикальных реакций в их сыворотке крови (методом индуцированной хемилюминесценции). Дефицит витаминов А и Е, обладающих антиоксидантными свойствами, был выявлен у учащихся как городских, так и сельских школ, однако у первых он был выше. Это согласуется с данными хемилюминесцентного анализа: значения всех показателей хемилюминесценции у городских школьников были в 2,2–7,6 раза выше, чем у сельских, что свидетельствует о более высокой интенсивности окислительных процессов в их организме. Также был выявлен недостаток витаминов группы В в организме сельских школьников за исключением рибофлавина в возрастной подгруппе 15–17 лет.

Ключевые слова: витаминная обеспеченность, ретинол, токоферол, аскорбиновая кислота, тиамин, рибофлавин, пиридоксин, антиоксиданты, окислительный стресс, свободнорадикальные реакции, школьники

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Change in parameters of homeostasis, particularly the level of free radical reactions in the body, may indicate stress in modern school children studying in urban and rural schools [1–5]. It has been established that in stress, neurohumoral regulation systems are activated under the influence of a "primary mediator" [6]. Free radicals and lipid peroxidation products [7] are act as primary mediators.

Change in redox balance triggers protective and adaptive reactions in the body, which involve antioxidant vitamins [8]. Vitamin A (retinol), E (tocopherol) and C (ascorbic acid) are the classical antioxidants [9–12].

In particular, tocopherol is the universal protector of cell membranes, preventing oxygen from contacting with unsaturated fatty acids of membrane lipids and lipid peroxidation.

Due to the presence of double bonds in the molecule, retinol can react with various reactive oxygen species. The antioxidant function of ascorbic acid is due to its ability to easily give two hydrogen atoms required for neutralization of free radicals. This vitamin is very effective in high concentrations [13]. B vitamins may have indirect influence on free radical oxidation and antioxidant system. This assumption is based on the fact that the body's antioxidant system consists of various protective mechanisms [14]. These include superoxide dismutase, catalase, peroxidase, and other enzymes. Proper amino acid metabolism, including metabolism regulated by thiamine (vitamin B_1), riboflavin (vitamin B_2) and pyridoxine (vitamin B_6) is important for these enzymes.

The study aims at conducting a comparative analysis of the vitamin status of urban and rural school children with levels of free radical reactions in their blood serum.

METHODS

Two experimental groups were created. The first group included urban school children living in the city of Orenburg (n = 250), while the second group consisted of school children living in villages located in Orenburg region (n = 200). Subgroups — 12–14 year olds (n = 150 and n = 100 among urban and rural school children respectively) and 15–17 year olds (n = 100 in both groups) – were marked out from each of the two experimental groups. The study included school children aged 12–17 years, who have no chronic diseases, have not been sick for a month before the study and residing in an area with the same anthropogenic load (within the group). The exclusion criterion here was unwillingness by the children or their parents to participate in the study. The Ethics Committee of Orenburg State Medical University approved the study. The parents of the school children gave a written consent for the participation of their children in the study. The samples for the study in the two groups were obtained simultaneously in three months after the children have commenced classes at the school.

Venous blood and urine were the biological material for the study. Blood was collected in the morning before the children could take their breakfast. 10 ml of blood was collected in Vacuette (BD, USA) vacuum tubes without an anticoagulant. It took 2 hours to deliver the blood samples to the lab. Transportation and storage was carried out at a temperature of 18–25 °C. Urine was collected in the morning after sleep or no earlier than after 2-3 hours after the previous urination. The urine samples (not less than 20 ml in volume) were collected in dry sterile vials. Transportation and storage took 3 hours. The biomaterial was transported and stored at a temperature of 18–25 °C.

Iron-initiated chemiluminescence was used to assess the level of free radical reactions in the blood serum. After collecting blood, it was held for 30 minutes and then centrifuged at 1500 rpm for 15 minutes. The resulting serum was diluted with phosphate buffer (2.72 g KH_2PO_4 and 7.82 g KCl in 1 liter of distilled water, pH 7.4). The resulting solution was titrated with saturated KOH solution until pH 7.45 was achieved. Chemiluminescence registration was performed according to Farkhutdinov technique [15] on Chemiluminometer-3 device (Lumex, Russia). The sensitivity of the device is about 200 photons/sec. The device was calibrated before commencement of work on reference light source (luminescent uranium glass JS-19). The maximal intensity of the fast signal, lightsum of the slow signal (S) and the tangent of the backside angle of the chemiluminescence signal were determined. Indicator values were given in relative units, having calculated their ratio for experimental and blank control samples.

The level of excretion of B vitamins and vitamin C in the urine and the content of vitamins A and E in the blood serum were examined.

Analysis of specimens for the presence of retinol and tocopherol was performed using bioliquid analyzer Fluorat 02-ABLF (Lumex, Russia). In preparing the samples, 1 ml of blood serum and 1 ml of ethanol were placed in one tube for centrifugation, while 1 ml of distilled water and 1 ml of ethanol (calibration pattern) were placed in the other tube. Both tubes were shaken in Vortex apparatus for 30 seconds after which 5 ml of hexane was added and again shaken for 1 minute. After shaking the tubes, they were centrifuged for 10 minutes at 1500 rpm. The separated hexane layer (extract) was used for fluorimetric analysis. Content of vitamin A or E (X, μg/ml) in the blood serum was calculated using the formula:

$$X = \frac{C_{изм} \times V_э \times Q}{V_c},$$

where Cvit is vitamin concentration in the extract (measured in μg/ml); VE is the extract volume (measured in ml); Vc is the blood serum volume taken for analysis (measured in ml); Q is the coefficient reflecting extract dilution.

Content of ascorbic acid and B vitamins was determined by the level of their excretion in urine. Visual titration with Tillman's reagent (2,6-dichlorophenol sodium) was used for vitamin C. 10 ml of urine, 10 ml of distilled water and 1 ml of 10 % HCl solution were measured in two conical flasks. The contents of each flask was stirred and titrated with 0.001 N solution of Tileman's reagent until pink color appeared and remained for 30 seconds. Vitamin C content (X, mg/h) was calculated by the formula:

$$X = \frac{0{,}088 \times A \times B}{Б},$$

where 0.088 is the coefficient reflecting the amount of ascorbic acid, which is equivalent to 1 ml of 0.001 N solution of 2,6-dichlorophenol sodium (measured in mg); A is the arithmetic average of results of titration of 0.001 N solution of 2,6-dichlorophenol sodium of two urine samples (measured in ml); B is the amount of urine taken for titration (in ml); C is the average daily amount of urine (1500 ml for boys and 1200 ml for girls).

Thiamine excretion in the urine was determined by Wang & Harris technique. Riboflavin levels in urine were estimated by Maslennikova & Gvozdeva method, while those of pyridoxine were calculated by fluorimetric method [16].

The actual nutrition by the school children was estimated based on the meal taken by them in the last 24 hours [17]. This was done using a questionnaire. After that, the biological values of the diets were estimated using tables of chemical composition of food products proposed by Skurikhin & Tutelian [18]. The data obtained were compared with physiological norms for each age group [19].

The sample size was calculated using Sanetliyev's formula (1968):

$$n = \frac{t^2 \times p \times q}{\Delta^2},$$

where n is the number of observations, t is the confidence coefficient, p is the prevalence indicator, q = 100 % – p, Δ is the confidence interval. Considering that in medical research, 95% is the minimum confidence level, which corresponds to a confidence factor t = 1.96, we took p equal to q, i.e. 50 %, in order to maximize the product of p and q, while 100 % was taken as the confidence interval. The amount of minimum sample calculated in this way (ensuring representativeness) was n = 100. Student's test was applied with subsequent calculation of significance (p). This was to identify statistically significant differences in the experimental groups. The data were statistically processed using software package Statistica 5.0, which automatically calculated the mean values, standard deviation and standard error of the mean.

RESULTS

The retinol content and tocopherol content in the blood serum of urban schoolchildren aged 12–14 was less than the physiological norm (hereinafter compared with a lower bound) by 26.7 % and 12.8 % respectively, and 20.0 % and 9.8 % respectively in children aged 15–17 (Table 1). The content of retinol and tocopherol in the serum of rural schoolchildren was slightly higher, but also was less than the physiological norm: 6.7 % and 6.8 % respectively for children aged 12–14, and 10.0 % and 9.8% respectively for children aged 15–17. Vitamin C excretion in the urine of urban schoolchildren of both age subgroups corresponded with the lower bound of the physiological norm. In rural children, the figure was 14.8 % and 12.1 % below the norm for the students aged 12–14 and 15–17 respectively.

The low urinary excretion levels of thiamine compared with the norm were established only for the rural schoolchildren in both age subgroups (Table 2). Insufficient urinary excretion of riboflavin was observed in all the schoolchildren, except for rural children aged 15–17 (14.2 ± 0.24 mkg/h – matches the lower bound of the physiological norm). Low urinary excretion of pyridoxine was observed only in rural children: in a group of children aged 12–14, vitamin B6 excretion was by 5.2 % below the norm, 15-17 years – by 6.5 %.

Analysis of the daily diets of the schoolchildren showed that vitamins A and E were seriously lacking in the diets of urban and rural school children (Table. 3). In the 12–14-year age sub group, the content of retinol in their diet was by 88.3 % and 66.7 % below the norm in urban and rural school children respectively. In the 15–17-year age subgroup, the figures were 78.0 % and 83.0 % respectively. Tocopherol deficiency was 59.2 % and 71.7 % in urban and rural school children respectively, aged 12–14, and 66.0 % and 70.7 % for those aged 15–17 years. Ascorbic acid content in the diets of all groups was within the norm. Thiamine deficiency was detected in urban and rural school children aged 15–17 years, riboflavin — in all groups, except the urban group, aged 12–14 years, pyridoxine — in all groups

The values of all chemiluminescence parameters were significantly higher for both age subgroups of urban schoolchildren ($p < 0.05$). For instance, the value of the maximal intensity of the fast signal of blood serum in the urban children aged 12-14 and 15-17 were respectively 4.3 and 5.9 times higher than in the rural children. This indicates a higher content of lipid hydroperoxides in the blood serum of the urban children; the value of the lightsum (lipid peroxidation) was 2.2 and 4.2 times higher; the value of the tangent of the backside angle of the chemiluminescence signal (speed of lipid oxidation) was 3.8 and 7.6 times higher (Table 4).

DISCUSSION

The low vitamin status of modern schoolchildren has been also confirmed by other researchers [21–23]. The identified

Table 1. Serum levels of vitamins A and E, and urinary excretion of vitamin C in urban and rural schoolchildren, M ± m

Schoolchildren		Vitamins		
		A	E	C
		Physiological norm		
		0.3–0.7 μg/ml	8.0–12.0 μg/ml	0.7–1.0 mg/h
12–14 years	urban	0.22 ± 0.01	6.98 ± 0.16	0.72 ± 0.01
	rural	0.28 ± 0.01*	7.46 ± 0.09*	0.60 ± 0.02*
15–17 years	urban	0.24 ± 0.01	7.22 ± 0.17	0.71 ± 0.01
	rural	0.27 ± 0.01*	7.22 ± 0.08	0.62 ± 0.01*

Physiological norms are given according to Standards of Biochemical Analysis [20]. * — $p<0.05$ when comparing the indicators of urban and rural schoolchildren in each age group.

Table 2. Urinary excretion of B vitamins in urban and rural schoolchildren, M ± m

Schoolchildren		Vitamins		
		B_1	B_2	B_6
		Physiological norm		
		15–35 μg/ml	14–30 μg/ml	40–60 mg/h
12–14 years	urban	20.42 ± 0.49	12.34 ± 0.37	41.11 ± 0.80
	rural	12.58 ± 0.25*	13.68 ± 0.32*	37.92 ± 0.57*
15–17 years	urban	21.3 ± 0.39	12.76 ± 0.41	40.33 ± 0.64
	rural	13.19 ± 0.20*	14.2 ± 0.24*	37.40 ± 0.37*

Table 3. Vitamin content in the daily diet of the schoolchildren, M ± m

Vitamin	Schoolchildren					
	12–14 years			15–17 years		
	urban	rural	PN	urban	rural	PN
Vitamin A, μg	100.0 ± 2.0	200.0 ± 4.0*	600	220.0 ± 5.0	170.0 ± 3.0*	1000
Vitamin E, μg	4.9 ± 0.7	3.4 ± 0.6*	12	5.1 ± 2.3	4.4 ± 1.1	15
Vitamin C, μg	59.1 ± 13.8	63.1 ± 12.3*	60.0–70.0	99.4 ± 24.8	89.6 ± 21.0	70.0–90.0
Vitamin B_1, μg	2.7 ± 0.3	1.4 ± 0.1*	1.3	0.9 ± 0.2	0.81 ± 0.1	1.3–1.5
Vitamin B_2, μg	2.1 ± 0.1	1.1 ± 0.1*	1.5	0.4 ± 0.08	0.65 ± 0.09*	1.5–1.8
Vitamin B_6, μg	1.2 ± 0.1	0.9 ± 0.1*	1.6–1.7	0.9 ± 0.14	0.99 ± 0.15	1.6–2.0

PN — Physiological norm of vitamin consumption for this age group. [19] * — p <0.05 when comparing the indicators of urban and rural schoolchildren in each age group.

Table 4. Indicators of blood serum chemiluminescence in urban and rural schoolchildren, M ± m

Indicators	Schoolchildren			
	12–14 years		15–17 years	
	urban	rural	urban	rural
Maximal intensity of the fast signal(relative unit).	2.82 ± 0.67	0.65 ± 0.10*	3.83 ± 0.84	0.44 ± 0.04*
Lightsum (relative unit).	4.36 ± 0.60	1.95 ± 0.19*	6.35 ± 0.78	1.51 ± 0.19*
Tangent of the backside angle of the chemiluminescence signal (relative unit).	1.14 ± 0.22	0.30 ± 0.07*	1.51 ± 0.25	0.20 ± 0.03*

* — p <0.05 when comparing the indicators of urban and rural schoolchildren in each age group.

difference in the level of free radical reactions in the blood serum of urban and rural school children is consistent with the findings about their vitamin status: the antioxidant vitamin A and E status of urban school children is slightly lower than that of rural children. The level of free radical reactions in the blood serum of urban children is higher than rural children.

The diet of urban children is typically more balanced than the diet of children in rural areas. According to Ushakov & Sokolova [5], about 70 % of urban schoolchildren in winter take butter, cheese, meat, fowl, fish, raw vegetables and fruits almost every day or 2-3 times a week. On the other hand, less than 40 % of rural schoolchildren take similar diet. Predominance of potatoes, pork and milk in the diet of the schoolchildren has a positive correlation with ownership of a kitchen garden and part-time farms by the rural population. This explains why the serum levels of vitamin C in urban school children corresponds with the physiological norm but are deficient in the body of rural children. At the same time, according to Leshchenko, 53.5 % of urban schoolchildren and 35.5 % of rural schoolchildren eat only 1-2 times a day [24]. This eating frequency cannot provide the body with adequate amount of vitamins. This could explain the fact why even at eating more diverse meals, the vitamin A and E status of the urban school children still remained lower.

CONCLUSIONS

The blood serum of urban and rural schoolchildren was found to have vitamins A and E deficiency. However, the deficiency is more pronounced in urban schoolchildren. In all likelihood, this is due to higher content of oxidation products in their blood serum, as evidenced by the chemiluminescence indicator values that are by 2.2–7.6 times higher than in the rural children. Analysis of diets confirmed the findings: it was established that there is deficiency of vitamins A, E and pyridoxine in the diets of all the groups of students and that there is deficiency of thiamine in the diets of older students. The foregoing necessitates adjustment of nutrition in schools.

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TISSUE CHEMILUMINESCENCE AS A METHOD OF EVALUATION OF SUPEROXIDE RADICAL PRODUCING ABILITY OF MITOCHONDRIA

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Mitochondrial dysfunctions are an underlying cause of many human diseases including degenerative diseases. One of the consequences of mitochondrial dysfunctions is apoptosis of functionally active cells. During the initial stage of apoptosis, increased production of superoxide anion-radical (SAR) is observed. A promising method of SAR detection in cells and tissues is chemiluminescence (CL), primarily, in the presence of lucigenin, a SAR specific amplifier of CL. In this study a means of improving CL was proposed, and its effectiveness in detecting SAR level in living tissues of laboratory animals in hypoxia and parkinsonism models was evaluated. Aerobic (O_2 — 15 %, CO_2 — 5 %, N_2 — 80 %) and anaerobic (CO_2 — 5 %, N_2 — 95 %) gas mixtures proposed for samples aeration, maintained a constant pH of 7.4, necessary for accurate recording of CL. Using the studied method, a statistically significant increase (1.8 and 2.0 times) in SAR production level in rat heart tissue was detected with hypoxia duration of 150 to 240 minutes. In the parkinsonian model SAR production in mouse brain tissue samples of striatum and substantia nigra was 1.7 and 1.3 times higher after administration of the final dose of proneurotoxin, as compared to the control group.

Keywords: mitochondrial disorders, apoptosis, superoxide anion-radical, superoxide radical producing ability, hypoxia, parkinsonism, tissue chemiluminescence, lucigenin

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ТКАНЕВАЯ ХЕМИЛЮМИНЕСЦЕНЦИЯ КАК МЕТОД ОЦЕНКИ СУПЕРОКСИД РАДИКАЛ-ПРОДУЦИРУЮЩЕЙ СПОСОБНОСТИ МИТОХОНДРИЙ

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Дисфункция митохондрий лежит в основе развития многих заболеваний человека, включая дегенеративные. Одно из следствий митохондриальной дисфункции — апоптоз функционально-активных клеток. На начальной стадии апоптоза отмечается усиление продукции супероксид анион-радикала (САР). Перспективным методом обнаружения САР в клетках и тканях является метод хемилюминесценции (ХЛ), прежде всего — в присутствии люцигенина, специфичного для САР химического активатора ХЛ. В исследовании был предложен способ усовершенствования метода, а также оценена его эффективность при определении уровня образования САР в переживающих тканях лабораторных животных при моделировании гипоксии и паркинсонизма. Предложенные для аэрации опытных образцов кислородсодержащая (O_2 — 15 %, CO_2 — 5 %, N_2 — 80 %) и бескислородная (CO_2 — 5 %, N_2 — 95 %) газовые смеси обеспечивали постоянство pH 7,4, необходимое для корректной регистрации ХЛ. С помощью изучаемого метода было показано достоверное увеличение уровня образования САР в ткани сердца крыс при циклах гипоксии длительностью 150 и 240 мин — в 1,8 и 2,0 раза. При паркинсонизме уровень образования САР в ткани мозга мышей, содержащей стриатум и черную субстанцию, через 12 ч после введения последней дозы пронейротоксина оказался выше в 1,7 и 1,3 раза соответственно в сравнении с контролем.

Ключевые слова: митохондриальные заболевания, апоптоз, супероксид анион-радикал, супероксид радикал-продуцирующая способность, гипоксия, паркинсонизм, тканевая хемилюминесценция, люцигенин

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Human mitochondria, apoptosis and diseases

Mitochondria are important organelles found in cells. They are vital because they perform a variety of functions. But above all, they generate energy through oxidative phosphorylation and regulate growth, aging and cell apoptosis. There are about 400 mitochondrial diseases recognized. Also, mitochondrial medicine has developed as an independent scientific direction.

Cellular energy dysfunction may lead to neuromuscular abnormalities [1]. Nerve tissue is particularly sensitive to reduced energy metabolism [2]. However, energy dysfunction may not be the most dangerous mitochondrial dysfunction. Mitochondria are the major source of formation of intracellular free radicals [3], first of all superoxide anion radical (SAR). Accumulation of SAR and its derivatives – reactive oxygen species – leads to mitochondrial oxidative stress. This is extremely dangerous for cells because it can trigger programmed cell death called apoptosis [4]. There are two basic pathways to triggering apoptosis: external (receptor) and internal (mitochondrial) pathways [5]. Most forms of apoptosis in vertebrates come through the second pathway [6].

Oxidative stress in mitochondria is known to be the causative factor or pathogenesis link of many diseases: neurodegenerative diseases (Parkinson's disease [7], Alzheimer's disease [8], multiple sclerosis [9], amyotrophic lateral sclerosis, and others), neuro-ophthalmopathy, glomerulonephritis [10], insulin resistance, as well as aging. Higher risk of a number of diseases (cancer, diabetes [11], cardiovascular diseases) is associated with polymorphisms of antioxidant enzymes, manganese-dependent superoxide dismutase (MnSOD) and glutathione peroxidase, which arrest the consequences of mitochondrial oxidative stress. The essential role of mitochondrial dysfunction in the development of cancer was also identified [5].

Mitochondrial dysfunction and accumulation of free radicals in the cell are influenced by adverse factors. One of such factors is gene mutation. Unlike other organelles, mitochondria have deoxyribonucleic acid (mtDNA), which encodes a subunit of some complexes of oxidative phosphorylation. Mutations in mtDNA, as well as the genes of nuclear DNA that encodes mitochondrial proteins cause Leber's hereditary optic neuropathy (or Leber optic atrophy) [12], NARP (Neuropathy, Ataxia, and Retinitis Pigmentosa) syndrome [13], MERRF (Myoclonus Epilepsy with Ragged-Red Fibers in skeletal muscle) syndrome, MELAS (Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) syndrome [14], Kearns-Sayre syndrome (retinitis pigmentosa, external ophthalmoplegia, and complete heart block, ptosis, cerebellar syndrome) [15], Pearson syndrome (abnormal functioning of the bone marrow, liver, and pancreas) [16], and others.

Mitochondria as sources of free radicals in apoptosis

During aerobic respiration, there is leak of 1.2 % of electrons from the mitochondrial electron transport chain, which restores oxygen with production of superoxide anion radical [17]. Complex I and complex III of the respiratory chain are the main centers of production of reactive oxygen species. Normally, the body's antioxidant system neutralizes free radicals formed. However, under the influence of adverse factors, the level of free radicals increases by many times.

First, electron transfer is blocked in the mitochondria [18] resulting in respiratory depression, reduced synthesis of adenosine triphosphate (ATP) and, most importantly, increased formation of mitochondrial superoxide anion radical [19]. Then, under the influence of mitochondrial superoxide, hydrogen peroxide is formed in the matrix. After that, the complex of mitochondria-specific phospholipid - cardiolipin with cytochrome c is formed [20]. As demonstrated in model experiments, this complex has peroxidase activity [4]. In the presence of hydrogen peroxide, it oxidizes organic substrates, including polyunsaturated fatty acids. This leads to production of lipid free radicals [21] and triggers a chain reaction of lipid peroxidation [22]. Lipid peroxidation in mitochondrial membranes leads to swelling of the matrix [23], rupture of the outer membrane or at least formation of large pores in it through which cytochrome c leaves from the mitochondria. Appearance of cytochrome c in the cytoplasm triggers apoptosis reaction [24].

Methods of assessing the radical-producing ability of mitochondria in a living tissue

Various methods, including chemiluminescence (CL) [25], are used to assess the radical-producing ability of mitochondria in a living tissue. CL allows to register the concentration of radicals (which is extremely low in living systems) and the rate of reaction in which the radicals participate. A method for registering tissue CL using a refrigerated photomultiplier [26] has been recently developed. This method has several advantages over the labor-consuming and costly method by Japanese researchers using a refrigerated photomatrix [27]. The essence of the proposed method for assessing the radical-producing ability of a tissue [26] is that under regulated temperature (37 °C), weak air flow is fed from a peristaltic pump, through a capillary, to a system comprising of the test samples of the tissue and lucigenin activator (Fig. 1, A). The selected position level of the capillary and the aeration rate allow to observe formation of superoxide anion radicals in tissues affected. However, a study [26] showed CL to rise over time, which complicates analysis of curves obtained. Possibly, additional CL amplification is associated increased pH value of the medium solution over time, and not with additional production of radicals in the tissue. It is known that the intensity of lucigenin luminescence depends on the pH of the medium [28]. Increased pH of the medium enhances production of lucigenin cation, which interacts with SAR, followed by emission of quantum of light.

Our study included two experiments. The first experiment was aimed at determining the gas mixture composition optimal for maintenance of a constant pH level at aeration of prototypes using the method studied. The aim of the second experiment was to assess the level of production of superoxide anion radicals in hypoxia and parkinsonism in the tissue samples of rat brain and mouse heart using the studied method.

METHODS

Installation scheme

SmartLum-I100 chemiluminometer (DISoft, Russia) additionally equipped with peristaltic pump Pumps 323 (Watson Marlow, UK) was used in the experiments. Tanks containing different gas mixtures were connected to the pump. The installation scheme is shown in Figure 1, A.

The distance (*l*) from the bottom of the cuvette to the tip of the capillary supplying gas to the working solution was 1.5 cm (Fig. 1, B). This contributed to rapid diffusion of gas to the test sample without disturbing its position in space. Slices were placed at the bottom of the cuvette making the sample side with the largest area to face the detector (Fig. 1, B).

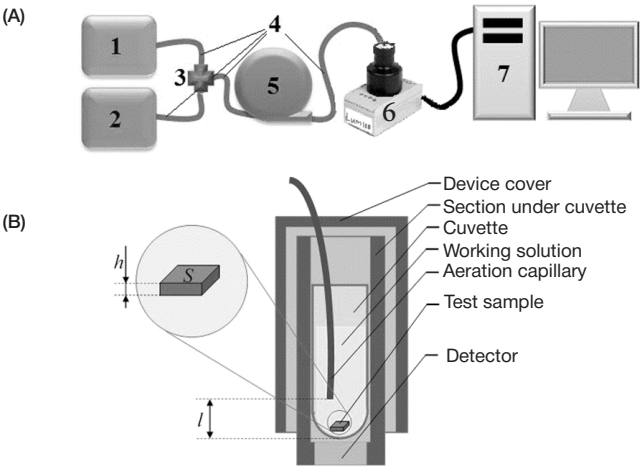

Fig. 1. (A) Experimental setup scheme. 1, 2 — tanks for two different gas mixtures (oxygen and oxygen-free mixtures); 3 — gas mixture switch; 4 — tubes supplying gas mixture to the chemiluminometer cuvette; 5 — peristaltic pump feeding gas mixture to the system; 6 — SmartLum-100 chemiluminometer; 7 — computer. **(B)** Position of the test sample in the chemiluminometer

Animals and manipulations

The study used the heart of white male Wistar rats aged 2–3 months and the brain of C57BL/6 mice aged 2.5–3 months. While working with the animals, regulations (No 755, Order of August 12, 1977) established by the Russian Ministry of Health were complied with. The Bioethics Commission of Lomonosov Moscow State University approved the experiments. The animals were kept in a vivarium, 6 animals each in standard T4 cells with controlled lighting (12 hours in the night and 12 hours in the day), with free access to feed and water.

Rats. All organ-harvesting manipulations were performed after the animals were deeply anesthetized with chloral hydrate (400 mg/kg). After harvesting the organs, they were washed in saline solution (0.9% NaCl). A sharp blade was used to cut out a small rectangular piece of the left ventricle, not more than 5 x 5 x 5 mm in size and then washed.

Mice. To model the early symptomatic stage of Parkinsonism, a 12 mg/kg dose of proneurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Sigma, USA) was administered subcutaneously to the mice four times, at two-hour intervals between injections [29]. The control animals were administered with a saline solution (0.9 % NaCl) in the same way. 12 hours after administering the last proneurotoxin dose, the mice were decapitated (without anesthesia) and the brain isolated. Thin frontal slices, 300 microns thick, were obtained using vibratome (Vibratome 1000 Plus, USA). Tissue blocks containing the substantia nigra (place of localization of the bodies of dopaminergic neurons) and striatum (area of axonal projections of dopaminergic neurons) were isolated in the slices.

To preserve tissue viability, all manipulations were performed in ice-cold Krebs-Ringer solution, comprising of NaCl 6.96 g, KCl 0.36 g, CaCl2 0.22 g, $MgSO_4 \cdot H_2O$ 0.33 g, NaHCO3 2.1 g, D-glucose 1.82 g, HEPES 4.8 g, distilled water 1.0 l. After that, the pH level was brought to 7.4. The experiments used only a freshly prepared solution.

Determining the optimal composition of the aeration gas mixture

The optimal composition of aeration gas mixture was determined using the heart tissue samples of rats. The tissue sample was placed in a cuvette containing 90 μm of lucigenin in Krebs-Ringer solution. Lucigenin was used as a selective probe for SAR. The chemiluminescence of tissue samples were registered for 125 minutes under controlled temperature (37 °C) and aeration at pump rotor speed of 6 rpm. Such a rate of gas mixture supply allowed to maintain sufficient rate of saturation of the solution with gas, mix the solution and wash the sample, keeping it fixed at the bottom of the cuvette. The pH of the solution was measured every 20 minutes.

Three gas mixtures of the following composition were examined:

– atmospheric air: 21 % O2, 0.03 % CO2, 78 % N_2,
– carbogen: 95 % O_2, 5 % CO_2,
– human exhaled air: 15% O2, 4 % CO_2, 74 % N_2,
others – 7 %.

Registration of tissue chemiluminescence in hypoxia simulation and in Parkinsonism

Based on the results of the experiment carried out to determine the optimal composition of the aeration gas mixture, technical mixtures of new composition were prepared and were used for chemiluminescence registration in modeling of hypoxia and parkinsonism:

– oxygen-containing gas mixture (OCGM): 15 % O_2, 5 % CO_2, 80 % N_2,
– oxygen-free gas mixture (OFGM): 5 % CO_2, 95 % N_2.

Hypoxia modeling. The study was conducted on samples of rat heart tissue using three hypoxia models. The CL of one sample was registered for each model. To create hypoxic conditions, anoxic gas mixture was passed through a solution containing a piece of the tissue. To create reoxygenation conditions, oxygen-containing gas mixture was passed. Three hypoxia models were investigated.

Model 1 — hypoxia for 15 minutes. For the first 30 minutes, CL was registered at OCGM aeration, followed by 15-minute CL registration at OFGM (hypoxia) aeration. After that, OCGM aeration was resumed for 30 minutes (reoxygenation). This hypoxia cycle was repeated six times. The total CL registration time was 400 minutes.

Model 2 — hypoxia for 150 minutes. For the first 30 minutes, CL was registered at OCGM aeration, followed by 150-minute CL registration at OFGM (hypoxia) aeration. After that, OCGM aeration was resumed for 30 minutes (reoxygenation). This hypoxia cycle was repeated twice. The total CL registration time was 325 minutes.

Model 3 — hypoxia for 240 minutes. For the first 30 minutes, CL was registered at OCGM aeration, followed by 240-minute CL registration at OFGM (hypoxia) aeration. After that, OCGM aeration was resumed for 30 minutes (reoxygenation). The total CL registration time was 350 minutes.

Change in radical formation was assessed by S/S0 parameter, where S is the area under the CL curve within 30 minutes of reoxygenation at the end of the experiment with the model. Parameter S reflects the amount of radicals formed. S_0 is the area under the CL curve over the first 30 minutes of luminescence registration. S0 reflects the initial amount of radicals. Unit of measure S — (imp/s) × min.

Modeling of Parkinsonism. The study was performed on mouse brain slices. The CL of three tissue slices of the area of the substantia nigra and the three sections of the striatum area of the brain (of both experimental and control animals) was registered. Chemiluminescence was registered over 25 minutes under aeration with oxygen-containing gas mixture. SAR formation was assessed by S/S_0 parameter (after 20

minutes of aeration), where S is the area under the CL curve of the tissue of the experimental animals, while S_0 is the area under the CL curve of the tissue of the control animals.

Data were statistically processed using software packages Statistica 7.0 and MS Office Excel 2010. The results were presented as mean value and standard deviation. The significance of differences between the groups was determined using the Mann–Whitney U test. The differences were acknowledged to be statistically significant at a significance level of $p < 0.05$.

RESULTS

Dependence of pH on the composition of the aeration gas mixture

The influence of the composition of three different gas mixtures on the pH level of a solution containing a sample of the heart tissue was investigated. With atmospheric air aeration, pH rose from 7.4 to 9.0 (Fig. 2, A). A change in the pH value influenced the intensity of lucigenin-dependent chemiluminescence. The registered changes in the kinetics of the CL curve are a direct consequence of the alkalinity of the medium: the growth dynamics of CL and increase in pH coincided over time (Fig. 2).

On the contrary, aeration with gas mixtures with high content of CO_2 (carbogen and exhaled air) did not result in a significant change in pH of the medium during the experiment. CL luminescence remained at a constant level (Fig. 2, B).

Thus, CO_2 content in gaseous mixtures at a 4-5 % level is sufficient to maintain the pH at physiological norm (7.4). Therefore, for further CL registration of tissue samples, technical gas mixture was prepared in which the percentage content of the main components was similar to the exhaled air. However, there were no impurity gases (OCGM). Oxygen-free gas mixture (OFGM) with high content of CO2 was used to create hypoxic conditions.

Formation of free radicals in the heart tissues of rats in hypoxia

Under repeated cycles of hypoxia lasting for 15 minutes, a significant increase in the formation of superoxide anion radical was observed only at the time of reoxygenation at the 300th minute of the experiment: the amount of SAR increased by 1.5 times in comparison with the baseline. Under longer cycles of hypoxia (150 and 240 minutes), statistically significant increase in CL was also observed at the end of the experiment. Here, formation of SAR increased by 1.8 and 2.0 times respectively (Fig. 3, Table 1).

Formation of free radicals in the brain tissues of mice in parkinsonism

The modeled stage of parkinsonism in mice corresponds to early symptomatic stage in people. At this stage, increased formation of superoxide anion radical was observed in the animals. In the case of brain tissue slices containing the striatum, a significant increase (1.7 times) in SAR production was detected. For brain tissue slices containing the substantia nigra, there were no significant differences between the experimental and control animals. However, a tendency towards 1.3 times increase in production of radicals was detected (Fig. 4, Table 2).

DISCUSSION

Lucigenin-enhanced chemiluminescence is a promising method for measuring the level of SAR production and assessing disorders that occur in individual cells and the tissue in general. However, applying his method requires maintenance of a constant pH of the medium at 7.4. When aerating samples with atmospheric air, the pH index changes, while the volume of the Krebs–Ringer buffer system (2 ml per 1 mg tissue) is not enough to stabilize the pH at 7.4. On one hand, the use of a continuous-flow system can help solve the identified problem by constantly renewing the solution used to wash the tissue. However, the necessary technical retrofit measures and increased reagent consumption make this approach difficult to implement. On the other hand, the possibility of changing the composition of the gas mixture for aeration of the solution – increasing the CO_2 content – is a simpler and more affordable method. Saturating the washing solution with carbon dioxide allows to maintain the pH at a constant level by dissolving the gas in water and establishing the balance state:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3 \leftrightarrow 2H^+ + CO_3^{2-}$$

We showed that aeration with a gas mixture containing 4–5 % of carbon dioxide is optimal for the method.

The hypoxia/reoxygenation cycle is known to play a key role in human infarction. Moreover, the tissue suffers the most severe damage at sharp increase in production of reactive oxygen species and dies after resumption in blood supply. Assessment of the level of formation of superoxide radical

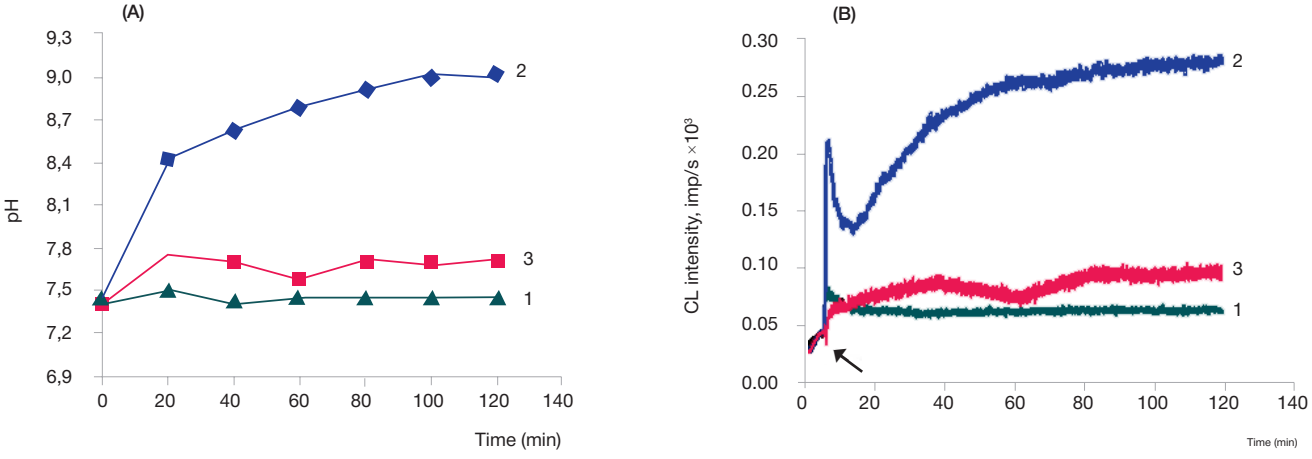

Fig. 2. (A) Change in pH and **(B)** development of lucigenin-enhanced chemiluminescence in one of the rat heart samples with aeration of samples with gas mixtures of different composition

1 — curve for exhaled air (O_2 — 5 %, CO_2 — 4,3 %, N_2 — 74,0 %); 2 — curve for atmospheric air (O_2 — 21,0 %, CO_2 — 0,03 %, N_2 — 78,0 %); 3 — curve for carbogen (O_2 — 95,0 %, CO_2 — 5,0 %). Arrow indicates start of aeration.

anion at different periods of hypoxia showed that the cyclical effects of short periods of hypoxia led to lower production of free radicals than longer periods of hypoxia.

Registration of lucigenin-enhanced CL under aeration of tissue sample with oxygen-containing gas mixture showed an increase in formation of SAR in the brain tissue 12 hours after the last dose of proneurotoxin was administered. This suggests that increased production of free radicals, leading to degeneration of nerve cells, occurs long before the first clinical symptoms of the pathology appear.

CONCLUSIONS

Conditions for the use of lucigenin-enhanced chemiluminescence to evaluate the radical-producing ability of biological tissues were optimized. Compositions of oxygen-containing and oxygen-free gas mixtures for aeration of the test sample to maintain at 7.4 the pH of the solution used to wash the sample were selected.

The possibility of using the method for estimation of the level of production of radicals in hypoxia and parkinsonism was demonstrated. There was significant increase in the level of production of radicals in heart tissue for hypoxia cycles – 1.8 times increase for 150-minute hypoxia cycle, and 2.0 times increase for 240-cycle. The level of SAR production in Parkinsonism in areas of the striatum and substantia nigra 12 hours after the last dose of proneurotoxin was administered was 1.7 and 1.3 times higher respectively than in the control sample.

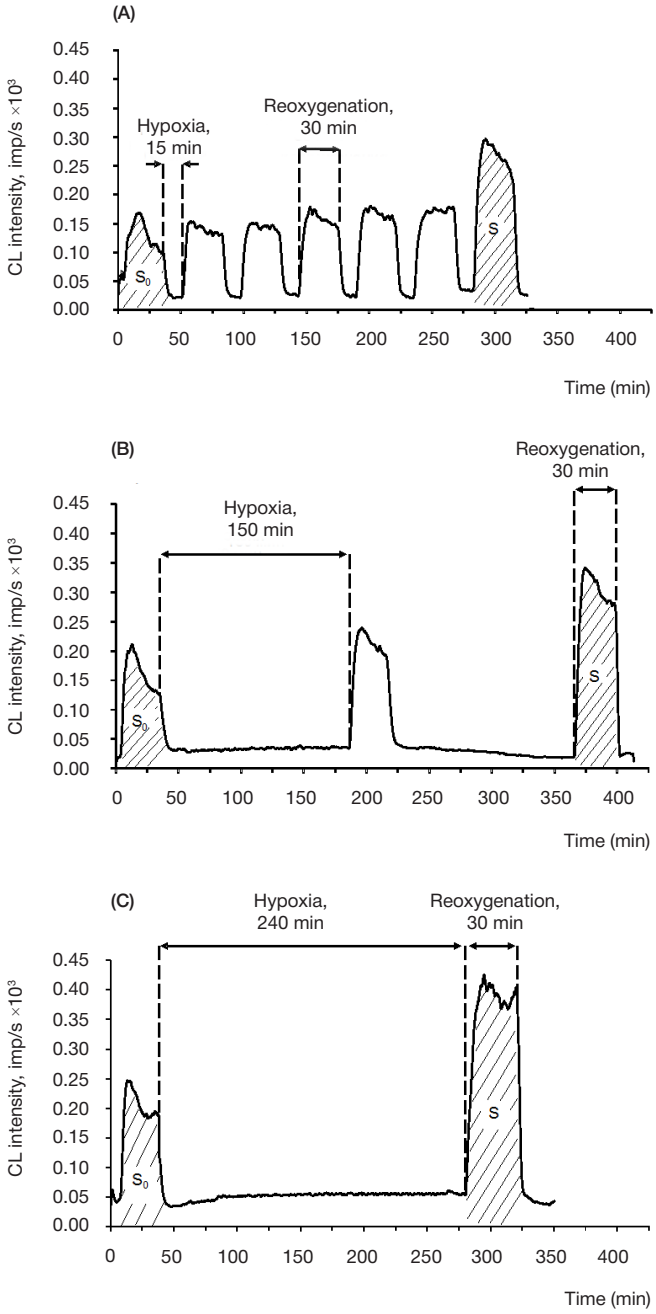

Fig. 3. Curves of lucigenin-enhanced chemiluminescence in one of the rat heart samples for different hypoxia models. **(A)** Model 1 (hypoxia cycle — 15 min). **(B)** Model 2 (hypoxia cycle - 150 min). **(C)** Model 3 (hypoxia cycle — 240 min)

Table 1. Influence of hypoxia duration on formation of superoxide anion radical in rat heart tissue, M ± m (n = 5, p <0.05)

Indicator	Hypoxia duration (min)		
	15	150	240
S/S_0	1.49 ± 0,10	1.76 ± 0.20	2.04 ± 0.15

Table 2. Formation of superoxide anion radical in mouse brain tissue when modeling parkinsonism, M ± m (n = 5, * — p <0.05)

Tissue	Indicator		
	S (parkinsonism)	S_0 (control)	S/S_0
Striatum	71.0 ± 5.0*	121.0 ± 15.0	1.7 ± 0.4
Substantia nigra	30.0 ± 10.0	40.0 ± 6.0	1.3 ± 0.5

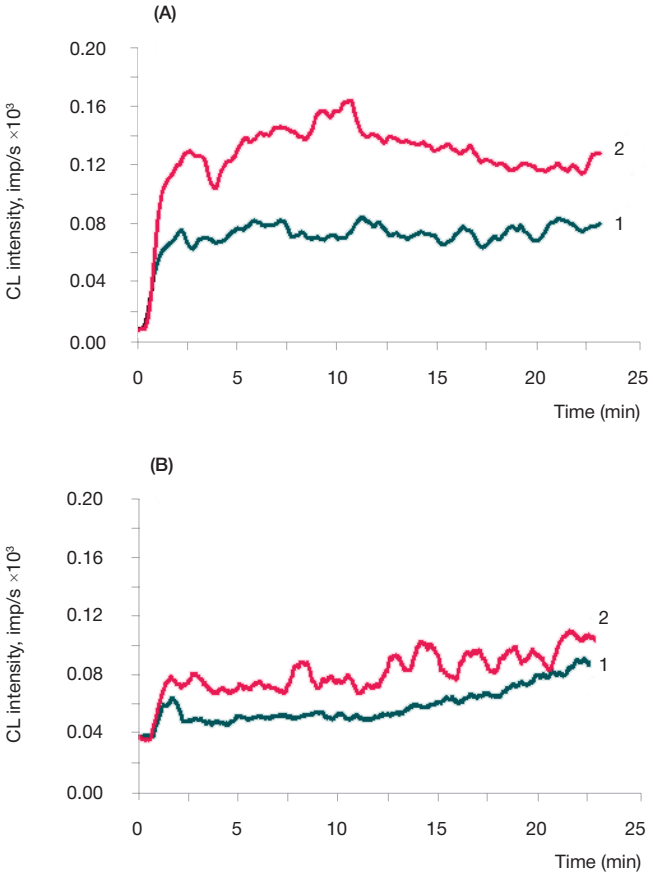

Fig. 4. Development of lucigenin-enhanced chemiluminescence in mouse brain tissue sections containing the striatum **(A)** and substantia nigra **(B)**

1 — curve for control sample, 2 — curve for test sample.

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SERUM ALBUMIN AS A SOURCE OF AND A TARGET FOR FREE RADICALS IN PATHOLOGY

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Oxidative stress caused by excessive accumulation of pro-oxidants and/or depletion of antioxidants, is an important pathogenic factor. Oxidative stress leads to oxidative modification of macromolecules. Proteins are a target for oxidizing agents. Of other antioxidants in human blood plasma, serum albumin is particularly interesting as a target for reactive oxygen species. In this brief review albumin is looked upon as a target for free radicals, an antioxidant, and a source of free radicals in its complexes with copper ions. Possible targets for free radicals in protein structure and the consequences of their exposure to free radicals attacks have been analyzed. The role of glycosylation in contributing to protein oxidative modification has been studied. The original experimental data on albumin structure changes in various models of oxidative stress obtained by a spectrofluorimetric method are pesented. Increased antioxidant properties of albumin modified in a physical model of oxidative stress (UV-irradiation) have been described.

Keywords: oxidative stress, free radicals, human serum albumin

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СЫВОРОТОЧНЫЙ АЛЬБУМИН КАК ИСТОЧНИК И МИШЕНЬ СВОБОДНЫХ РАДИКАЛОВ В ПАТОЛОГИИ

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Окислительный стресс, вызванный избыточным накоплением прооксидантов и/или истощением антиоксидантов, является важным патогенетическим фактором. Он вызывает окислительную модификацию макромолекул, и одной из мишеней окислителей являются белки. Среди антиоксидантов в плазме крови человека особый интерес в качестве мишени для активных форм кислорода представляет сывороточный альбумин. В нашем кратком обзоре он рассмотрен как мишень для свободных радикалов и антиоксидант, а также как источник свободных радикалов в комплексе с ионами меди. Проанализированы возможные мишени свободных радикалов в структуре белка и последствия воздействия радикалов на них. Уделено внимание роли гликозилирования как одного из факторов, способствующих окислительной модификации белков. Приведены собственные экспериментальные данные об изменениях в структуре альбумина при разных моделях окислительного стресса, полученные спектрофлуориметрическим методом, проиллюстрировано усиление антиоксидантных свойств альбумина при физической модели окислительного стресса (ультрафиолетовое облучение).

Ключевые слова: окислительный стресс, свободные радикалы, сывороточный альбумин человека

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Free radicals are an important component of cellular metabolism. They induce a number of negative effects if accumulated in excess, including the structural and functional damage of the cell and even its death through necrosis or apoptosis. A shift in balance between free radicals (pro-oxidants) and antioxidants in favor of the former is called oxidative stress (OS). Various factors cause OS, but all of them eventually lead to the oxidative modifications of macromolecules, such as DNA or proteins, and to lipid peroxidation. A new research area has emerged, namely, research of protein oxidative modification (POM) [1]. The knowledge accumulated in this area is not only of fundamental significance, but also is widely applied in actual practice. Recently, tests detecting oxidized proteins in blood cells and tissues have been introduced, making it possible to collect extensive factual material. POM has been found to induce formation of tyrosine and tryptophan oxidation products,

including *o*- and *m*-tyrosines, 3,4-dihydroxyphenylalanine (DOPA), carbonyls and other oxidized derivatives; dimers (dityrosines) are formed; auto-oxidative glycosylation of proteins occurs [2].

A complex antioxidant system of the organism resists free radical oxidation. Blood plasma proteins with antioxidant properties are components of this system [3]. Among them, a key role is assigned to human serum albumin (HSA). This protein interacts with free radicals, undergoes oxidative modification and protects the body. Albumin oxidative modification causes complete or partial loss of its diverse functional activity, which in itself can produce a variety of effects. However, during this process HSA acquires new properties and, possibly, new functions. Besides, albumin modified by oxidation can be an effective marker of OS.

Considering the increased interest in HSA role in free radical oxidation, we thought it important to summarize all the data collected and to examine this protein from two perspectives: as a source of and as a target for free radicals.

Serum albumin as a source of free radicals

In blood plasma there are two main proteins responsible for copper binding and transportation that at the same time prevent copper damaging effect on other plasma proteins, blood cells and surrounding tissues [4]. Ceruloplasmin contains copper in its active site. Reactions involving this protein are not accompanied by the formation of any radicals. The second largest copper pool in blood plasma is associated with human serum albumin that contains a high affinity site for copper, namely, the N-terminal tripeptide Asp-Ala-His (Cu^{2+}/Ni^{2+}-binding motif) [5]. Under normal conditions less than 1% of total albumin is copper-bound, but this amount is enough to generate a big number of radicals in blood. In some pathological states, for example, Wilson's disease or arthritis, the level of albumin bound copper can be considerably higher (2 to 5 times)[6–8].

Y.A. Gryzunov et al. [9] thoroughly investigated the conditions and reasons related to the change in the radical producing (pro-oxidant) activity of HSA-copper ions complex. Those authors studied, firstly, the effect of cystein-34 amino acid residue (Cys-34) modification on catalytic activity of the complex, and, secondly, the result of non-esterified fatty acid binding to albumin. To monitor the pro-oxidant activity of the complex, the rate of ascorbate radical formation was measured by the electron paramagnetic resonance assay (EPR), since ascorbate is one of the main interceptors of free radicals in blood plasma. At copper-albumin ratios below 1:1, the bound copper was almost redox-inactive, as long as Cys-34 was in a reduced state. We will term the inactive complex Cu/HSA-SH. Alkylation, nitrosylation and oxidation of thiol groups induced the catalytic radical producing activity of the Cu/HSA complex. This activity was more than an order of magnitude lower than the activity of free copper ions not bound to albumin. However, the evidence of such activity itself is important. Using ultrafiltration, it was shown that it is the complex with copper:protein stoichiometry of 1:1 and not free copper ions, generated accidentally during Cu/HSA-SH processing, that exhibits such activity.

In that work [9] it was also established that being catalytically inactive, Cu/HSA-SH complex displayed radical producing activity as a result of protein conformational changes when bound to free fatty acids, given that albumin did not contain any fatty acid impurities. Both conformational changes measured by probe fluorescence (fig.1, probes I and II) and catalytic activity reached their maximum at a fatty acid to protein molar ratio of 3:1 for oleic acid and 2:1 for linoleic acid. Parallel to fatty acids binding and profound conformational changes caused by this process, oxidation of Cys-34 SH-groups and a simultaneous increase in redox activity of copper-albumin complex were observed. The authors concluded that fatty acids regulate anti- and pro-oxidant properties of Cu/HSA complex by changing Cys-34 redox status.

The process described above includes the following stages (fig.1):

1) binding of fatty acids in protein domains I, II and III;

2) albumin conformational changes (measured by the fluorescence of probes I and II);

3) activation of catalytic (redox) activity of the Cu complex in the binding site;

4) Cys-34 SH-group oxidation;

5) oxidation of other molecules by dissolved oxygen facilitated by Cu/HSA and accompanied by free radicals formation (oxidative stress).

Thus, pro-oxidant properties of HSA complex with copper ions are implemented only after protein SH-group has been oxidized or a thiol group has interacted with nitrogen monoxide (NO).

Serum albumin as a target for free radicals

A lot of data confirm that HSA antioxidant activity is determined by at least three factors: 1) its binding of variable valency metals, such as copper; 2) its reactions with free radicals (a radical trap); 3) the formation of products with antioxidant properties during its oxidative modification.

If HSA is added to blood lipoproteins that are quickly oxidized in the presence of copper ions, then lipid peroxidation in lipoproteins is inhibited [10], but not blocked completely, because copper retains its catalytic activity in the complex with albumin. It means that HSA is an antioxidant because it forms a complex with copper ions. However, this complex alone cannot be a pro-oxidant, which depends on the amount of albumin-bound copper in blood plasma and this complex activity. As stated previously, binding of NO and fatty acids, as well as a chemical modification of Cys-34 thiol group, makes Cu/HSA complex catalytically active. In contrast, native HSA completely inhibits catalytic activity of copper ions.

In serum albumin, Cys-34 SH-group is a primary radical interceptor; because of this group HSA constitutes the majority of reactive thiols in blood plasma [11, 12]. Cys-34 oxidation results in the formation of sulfenic acid (RSOH) that is later oxidized to sulfinic (RSO_2H) or sulfonic (RSO_3H) acids [13]. As mentioned before, SH-groups serve as a defense mechanism against free radical oxidation [14, 15], their concentration in blood plasma lowers considerably when OS increases, which occurs in various diseases [16–18] and aging [19]. Using high performance liquid chromatography (HPLC) with fluorescence detection, K. Oettl et al. studied HSA redox state as a potential systemic marker of OS in patients with various diseases (cataract, glaucoma, age related macular degeneration, diabetes mellitus, diabetic retinopathy and hypertension), with or without complications and with consideration of possible effects on age [20].

Another amino acid sensitive to free radical attacks is methionine. HSA contains 6 methionine residues. Its oxidation by various oxidizing agents leads to the formation of methionine sulfoxide (MetSO). However, changes in HSA properties induced by free radicals are tricky to interpret. To look at enzymes from this perspective, one can refer to the work of R. Levine et al. [21], who found that preferential oxidation of unprotected methionine residues of enzymes had little effect

on the biological functions of glutamine synthetase. At the same time a supposition was made that methionine residues redox cycle in biological systems can be a factor of defense against reactive oxygen species and prevent other functionally important changes in protein structure.

In serum albumin, aromatic amino acids are a third target for free radicals; they can be susceptible to oxidative modification under oxidative stress. HSA consists of 18 tyrosine residues and 1 tryptophan residue. The result of oxidative modification of free tyrosine, tryptophan and albumin is the augmentation of protein protective properties due to the formation of oxidation products that are antioxidants [22]. One of such compounds is DOPA [22].

Thus, attacked by free radicals, HSA loses its free cysteine thiol group, some of tyrosine groups and a tryptophane residue. The higher is the level of OS in human blood (systemic OS in other terms), the higher is the degree of thiol and aromatic amino acid loss. Both of these criteria are currently used for evaluation of OS levels in clinical practice. It is important to note that aromatic amino acids, which are constituents of HSA, are natural fluorophores and their oxidative damage can be measured by a simple and sensitive method of ultraviolet fluorescence registration. This subject was looked upon in a number of studies. Reduced HSA fluorescence was observed when studying the effect of glycosylation [23] and free radicals [24] in a diabetes model. Those authors established a clear correlation between protein molecule conformational changes and protein antioxidant properties; a key role of copper ions in implementing albumin pro-oxidant properties was confirmed. Likewise, reduced analytical signal intensity, which is a useful index of amino acid degradation and displays a clear dependence between oxidation and protein conformational restructuring, was observed when studying the effect of an individual hydroxyl radical (•OH) and its combinations with superoxide anion radical (•OH + $•O^{2-}$) on proteins [25]; when studying thiol groups oxidation and elevated fructosamine levels in patients with obstructive sleep apnea [26]; when assessing protein structural changes mediated by peroxynitrite (by tryptophan and cystein oxidation, tyrosine nitration, dityrosine formation, production of 2,4-dinitrophenilhydrazine, carbonyls and molecule fragmentation) [27]; when modeling a "soft" OS, induced by ascorbate, oxygen and trace amounts of metals [28]; finally, when studying a correlation between HSA oxidative modification growth and the severity of hepatic failure characterized by increased carbonyls and Cys-34 oxidation [29].

It has been shown that protein glycosylation leads to its more intense oxidative modification [30]. In the work of J. V. Hunt and S. P. Wolff [30] this fact was illustrated by the example of tryptophan. Moreover, many observations showed that glycosylation and oxidation are closely related to each other: glycosylation both boosts oxidation and is boosted by it. To describe this property, a new term has been introduced, namely, *"glycoxidation"* — glycooxidation, derived from glycosylation + oxidation [31]. F. Monacelli et al. [31] used fluorescence spectroscopy and circular dichroism analysis to study the end products of oxidation and glycosilation and HSA conformational changes after its incubation with ribose, ascorbic acid (AA) and diethylenetriaminepentaacetic acid (DTPA) in various combinations. Ribose was found to induce a considerable increase of pentosidine (a glycosilation marker), with AA and DTPA preventing its accumulation, especially at later incubation stages. Ribose increased oxidation protein products level moderately, while AA inhibited their formation. Besides, in combination with AA ribose contributed to further formation of oxidation products, while DTPA inhibited oxidation protein products formation induced by AA. Using a circular dichroism analysis, F. Monacelli et al. obtained the results proving that AA and DTPA are strong modifiers of α-spiral part of HSA structure while ribose affects protein structure at late incubation stages only.

After studying the relevant literature, we carried out a series of experiments in our laboratory on using HSA as a marker in various OS models. Albumin structural changes were evaluated using spectrofluorometry; albumin antioxidant properties were evaluated by luminol-enhanced chemiluminescence assay (with some modifications) [32]. Solutions of luminol $C_8H_7N_3O_2$ (Sigma-Aldrich, USA, molecular weight of 177.16), HSA (Sigma-Aldrich, molecular weight of 69,000), AAPH (2,2′-Azobis(2-methylpropionamidine) dihydrochloride, Fluka, Germany) were prepared by dissolving weighted amounts of corresponding substances in phosphate buffer solution (KH_2PO_4, reagent grade). A working concentration of N-formyl-methionine-leucine-phenylalanine (a substance used for neutrophil stimulation) by FMLP,Sigma-Aldrich, was obtained by diluting the initial solution with a medium (Hank's solution containing glucose / HEPES). For irradiation, samples with optical density of no less than 0.2 were used to provide the uniform UV absorption throughout the solution volume and to avoid nonlinearity of fluorescence spectra registration. Neutrophils were extracted from the blood of patients with Wegener's granulomatosis (Tareev Clinic of Nephrology, Internal and Occupational diseases). Measurements were done using RF-5301 PC spectrofluorophotometer (SHIMADZU, Japan) and Lum-5773 chemiluminometer (DISoft, Russia) with PowerGraph software; absorption spectra were registered by Specord 200 spectrophotometer (Jena Eng., Germany). Samples were irradiated in Bio-Link crosslinker (Vilber Lourmat, France), which allows irradiation dose control, with effective short wavelength of 254 nm. The following OS models were used: physiochemical (thermally induced decomposition of AAPH), chemical (albumin exposure to superoxide and hydroxyl radicals produced in Co^{2+}/H_2O_2), physical (exposure to different dozes of UV-irradiation), and biological (radical production after phagocytes activation). Data obtained in the experiments are presented in fig. 2. In all our experiments a 100 Mm phosphate bugger solution with pH of 7.4 was used as a medium. Excitation wavelength for fluorescence spectrum registration was 260 nm.

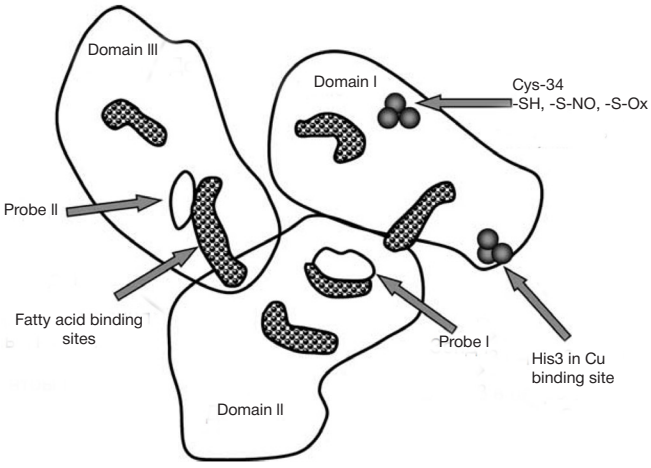

Fig. 1. Location of fatty acid- and Cu-binding sites; location of probes I and II in domains I, II and III in HSA structure

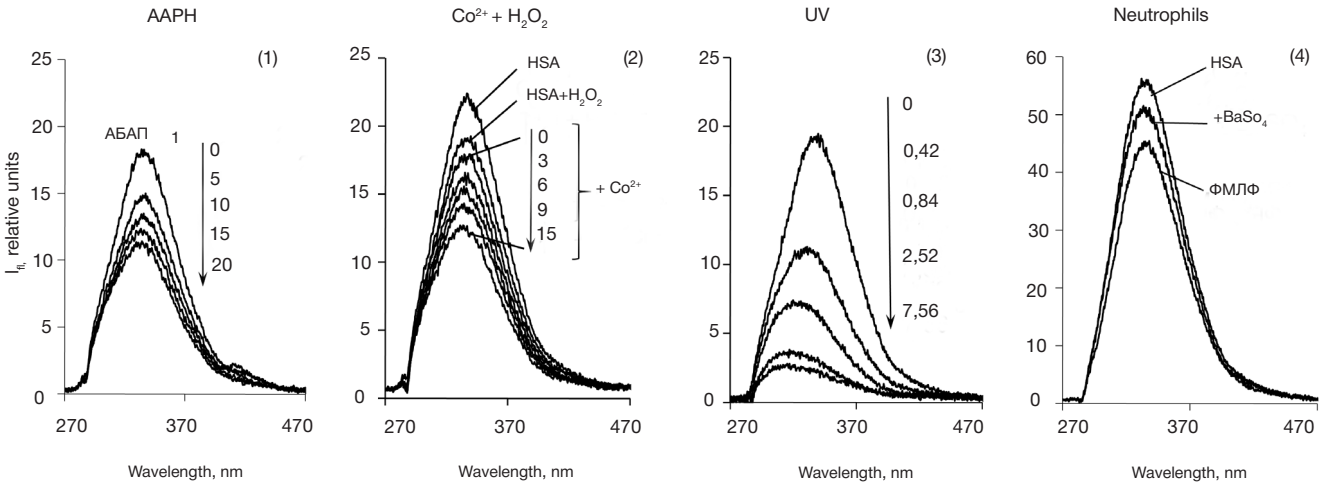

Fig. 2. Fluorescence spectra obtained in the experiments. (1) HSA (0.66 μm) and AAPH (2.5 mM), figures next to curves show incubation time. (2) HSA (0.6 μm), H_2O_2 (3 mM) and Co^{2+} (0.3mM), figures next to HSA + H_2O_2 + Co^{2+} curves show time after introducing Co2+ to the system: 0, 3, 6, 9 and 15 minutes. (3) HSA (0.6 μm), figures next to curves show UV-irradiation dosage, kJ/cm². (4) HSA (fraction isolated from blood plasma and diluted 1:100) combined with neutrophils stimulated by barium sulfate and FMLP

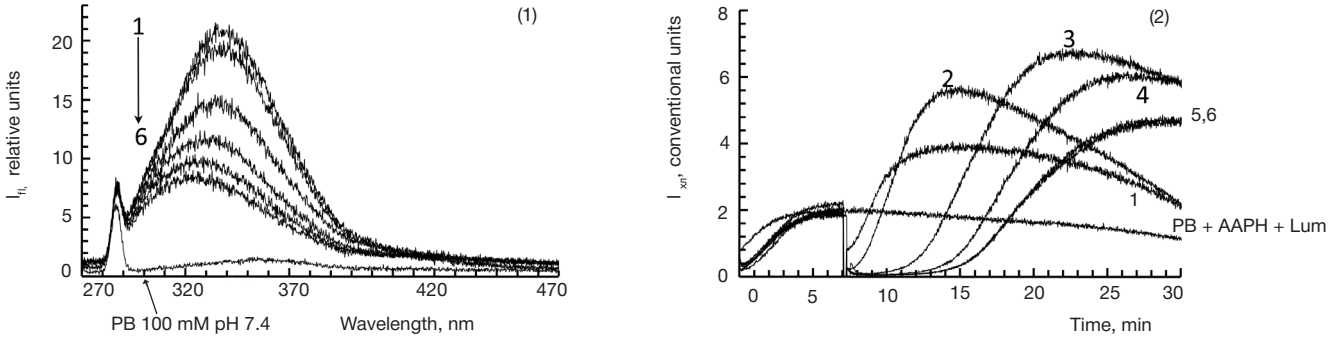

Fig. 3. Changes in fluorescent and antioxidant properties of HSA exposed to different doses of UV-irradiation. (1) HSA fluorescence spectra (0.66 μm); the protein was exposed to different doses of UV-irradiation (figures show the dosage, J/ cm²: 0 — native protein, 1 — 0.050, 2 — 0.200, 3 — 0.400, 4 — 0.600, 5 — 0.800, 6 — 1.000). (2) Chemiluminescence curves of HSA (0.66 μm) exposed to different doses of UV-irradiation (figures next to curves show the dosage, kJ/cm²) in the system containing phosphate buffer solution (PB) (37 °C), AAPH (2.5 mM), luminol (Lum) (2 μm), system total volume 1.000 ml

Study results show that in all OS models protein oxidative modification is observed, which is demonstrated by reduced fluorescence intensity. A physical model of OS (UV radiation) was investigated in greater detail. Experimental data are presented in fig.3. In all experiments 100 Мм phosphate buffer solution with pH of 7.4 was used as a medium; excitation wavelength for registering fluorescence spectra was 260 nm. To register chemiluminescence, the following steps were taken: AAPH and luminal solutions were mixed in a cuvette, the resulting mixture was incubated for 20 minutes at room temperature in the dark, then a phosphate buffer heated up to 37°C in a thermostat was added to the AAPH-luminol mixture. The cuvette was placed in the device, and chemiluminescence was registered until the curve reached the plateau. After the curve displayed a steady level of radical generation, an aliquot of the antioxidant (HSA) was introduced to the system.

Fig. 3 shows a dosage-dependent reduction of analytical signal intensity of the sample exposed to UV, and a simultaneous increase in antioxidant properties of HSA: a “dip” area is growing (latent period, t_{lat} — time during which fluorescence decay is observed below the curve). It can be explained by the fact that products with antioxidant properties are a result of aromatic amino acids oxidation [22]. Fig. 4 shows a correlation between reduced fluorescence intensity and antioxidant activity growth (t_{lat}, min).

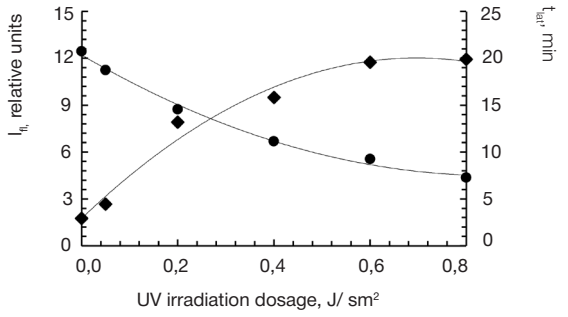

Fig. 4. Comparison of HSA fluorescence changes (0.66 μm) (λ^{340}_{max} = 337 nm) and the increase in the total antioxidant activity (t_{lat}, min) with the increased dosage of short wavelength radiation, J/ cm²

CONCLUSIONS

We have analyzed and summarized known experimental data on albumin being a source of and a target for free radicals. The second largest copper pool in blood plasma is associated with albumin. In different pathological conditions the level of albumin-bound copper increases. The mechanisms and conditions under which pro-oxidant properties of the complex

are implemented were studied. At the same time, albumin itself is the main blood plasma protective protein; it becomes possible because of albumin ability to intercept free radicals. Albumin reveals its protective properties due to the presence of cystein-34 SH-groups. Some contribution is made by 6 residues of another amino acid, namely, methionine, sensitive to oxidation. Finally, aromatic amino acids are responsible for production of substances with prominent antioxidant properties. This fact is supported by our own experimental data. Oxidative modification of serum albumin in various models of oxidative stress was assessed by spectrofluorometry. A physical model (ultraviolet radiation) was studied in more detail: dosage-dependent reduction of analytical signal intensity was demonstrated with the simultaneous increase in antioxidant protein properties that were detected using luminol enhanced chemiluminescence. One of the products of tyrosine oxidation, 3,4-dihydroxyphenylalanine (DOPA), was found to exhibit aintioxidant properties.

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TWO HMG DOMAINS OF YEAST MITOCHONDRIAL PROTEIN ABF2P HAVE DIFFERENT AFFINITY TO DNA

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Maintaining mitochondrial genome integrity is essential for the viability of the whole organism. Mitochondrial genome mutations lead to muscular dystrophies, neurodegenerative diseases, and are associated with aging. In this work a baker's yeast (*Saccharomyces cerevisiae*) mitochondria model was used to investigate DNA-binding abilities of different domains of a mitochondrial Abf2p protein which participates in homologous recombination and reparation. A weak non-specific HMG1 binding to linear DNA and a specific HMG1 binding to a branched DNA with a dissociation constant of 510 nM have been discovered. The HMG2 domain itself does not bind to any DNA and either has other functions or demonstrates its DNA-binding activity in a full-length protein only.

Keywords: mitochondria, mitochondrial genome, Abf2p, recombination

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ДВА HMG-ДОМЕНА МИТОХОНДРИАЛЬНОГО БЕЛКА ДРОЖЖЕЙ ABF2P ОБЛАДАЮТ РАЗЛИЧНЫМ СРОДСТВОМ К ДНК

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Поддержание постоянства митохондриального генома имеет большое значение в обеспечении функционирования организма в целом. Мутации в геноме митохондрий могут быть причиной развития мышечных дистрофий и нейродегенеративных заболеваний, установлена также их связь с процессом старения организма. В данной работе исследована ДНК-связывающая способность отдельных доменов митохондриального белка пекарских дрожжей *Saccharomyces cerevisiae* Abf2p, участвующего в процессах гомологичной рекомбинации и репарации. Выявлено, что домен HMG1 неспецифично и слабо связывает линейную ДНК и при этом специфично взаимодействует с разветвленной структурой ДНК с константой диссоциации комплекса 510 нМ. Домен HMG2 сам по себе не обладает способностью связываться с ДНК и, вероятно, предназначен для осуществления других функций либо же проявляет ДНК-связывающую активность в составе полноразмерного белка.

Ключевые слова: митохондрия, митохондриальный геном, Abf2p, рекомбинация

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Mitochondria are intracellular organelles that play a key role in providing most eukaryotic cells with energy through adenosine triphosphate (ATP) synthesis. Mitochondria obtains from the cytoplasm most of the compounds needed to function. However, they have their own genetic information storage and transfer unit — namely their DNA and protein biosynthesis system. Mitochondrial DNA (mtDNA) is typically represented as a closed circular DNA molecule encoding mitochondrial rRNA, mitochondrial tRNA and protein involved in oxidative phosphorylation.

Mitochondrial DNA repair is key to maintaining the normal functioning of the organelle. This is due to the high incidence of mtDNA damage compared with nuclear DNA [1], caused by high concentration of reactive oxygen species in the mitochondria. Homologous recombination is one of the most important ways of repairing double-stranded breaks both in nuclear DNA and mtDNA. Indeed, mitochondrial homologous recombination processes are found in almost all major groups of eukaryotic organisms (plants [2], fungi [3] and invertebrates [4]). It is assumed that DNA recombination mechanisms in the

nucleus and mitochondria are generally similar [5]. The study of homologous recombination in mitochondrial genome became particularly essential after obtaining evidence that this process exists in mammals [5] and that there is link between accumulation of mediated recombination of deletions and several human diseases (muscular dystrophy, neurodegenerative diseases), aging and tumorigenesis process [6].

Brewer's/baker's yeasts (*Saccharomyces cerevisiae*) are extremely convenient object for the study of mitochondrial processes. In this organism, mitochondria have considerable functional similarity with human mitochondria. Moreover, yeast can perform vital functions in the absence of functionally full-fledged mitochondria, allowing for deletion of the genes of certain mitochondrial proteins and study of the phenotypic manifestations of such mutations. The mitochondrial genome of *S. cerevisiae* is a structure (nucleoid) organized in space. The nucleoid contains up to 10 mtDNA copies that interact with different proteins. Mitochondrial DNA located in the nucleoid is composed of three-dimensional organization, closely connected with the mechanisms of replication, transcription, and inheritance. Laying in the nucleoid also protects the DNA from the attack of reactive oxygen species, appearing during oxidative phosphorylation [7].

Abf2p is one of the most widely represented proteins interacting with mtDNA in the mitochondrial nucleoids in yeast. It was extracted for the first time in its pure form in 1979 [8], and the name 'Abf2p' was given to it in 1991 [9]. This protein contains two domains — HMG1 (High-Mobility Group 1) and HMG2 (HighMobility Group 2). Abf2p is presently the most studied among other proteins of yeast mitochondrial nucleoid.

Abf2p is a unique protein with the highest basicity among nucleoid proteins [10]. It generates negative DNA supercoiling when in contact with a circular plasmid in the presence of topoisomerase 1 [10]. Abf2 mutant yeast can support mtDNA growth in YPG medium containing glycerol as a carbon source. However, when culturing in media with fermentable carbon sources such as glucose, there is gradual loss of mtDNA [10]. It is also proved that in Abf2p gene mutation, the number of mitochondrial recombination events when paired with wild-type strain significantly reduces [11]. Besides, Abf2p stabilizes Holliday recombination junction intermediates, which also points to the importance of this protein in recombination [12].

Despite the large number of studies on Abf2p, the molecular mechanisms of its involvement in mtDNA recombination processes remain unexplored. The presence of two HMG domains in mitochondrial DNA-binding protein Abf2p is typical of most HMGB proteins: they are known to bind DNA minor groove with limited specificity or completely non-specific [13]. Besides, it is assumed that HMG domains that make up these proteins bind DNA independently of each other with similar efficiency [13]. However, mitochondrial HMG proteins apparently have much broader functionality than their nuclear homologues. For example, human mitochondrial DNA-binding protein, TFAm, apart from compaction of nucleoids, is a transcription factor and is probably involved in the processes of mtDNA recombination and repair [14]. TFAm is similar to bacterial HU proteins when it comes to its multi-functionality and DNA binding characteristics [14]. It is assumed that Abf2p also possesses multifunctional properties, as it is a structural analog of TFAm in yeast mitochondria.

In this paper, recombinant proteins corresponding to two Abf2p domains — HMG1 and HMG2 — were obtained. The DNA-binding ability of these domains with respect to linear DNA duplex and structure imitating late homologous recombination intermediates (Holliday junction) was studied.

METHODS

Gene cloning and expression

ABF2 gene sites corresponding to domains HMG1 (amino acid residues 27-115) and HMG2 (amino acid residues 112-183) were amplified using primer pairs abfhmg1F (GATACATATGGGTCCTAAAAGGCCC ACATC) / abfhmg1R (CGTCCTCGAGAGGAAGTTTTTCGTCA AACTCC) and abfhmg2F (GGCGCATATGGAGTTTGACGAAAAA CTTCC) / abfhmg2R (GAGGCTCGAGAGCATTATATTCTTGG ATAGC) respectively. Yeast genomic DNA strain BW303 was used as a template. The obtained amplification products were treated with restriction endonucleases *NdeI* and *XhoI* (Thermo Fisher Scientific, USA) and cloned into expression vector pET32a (Novagen, USA). In this way, we obtained the pET32a_HMG1 and pET32a_HMG2 vectors. Conformity of cloned sequences with the reference sequence was verified through Sanger sequencing in a post-genomic lab belonging to the Institute of Physico-Chemical Medicine of Russia, a Federal Medical & Biological Agency. The plasmids obtained were transformed into expression strain Escherichia coli B834 (DE3), single colonies of transformants were transferred to a 2xYT liquid medium with 100 μg/ml of carbenicillin, cultured at 37 °C under vigorous agitation to OD_{600} ~ 0.6–0.8. After that, expression of cloned genes was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 0.25 mM. Culturing was continued at 30 °C for 3 hours. The cells were then pooled by centrifugation (3000 g, 10 min) and re-suspended in the starting buffer (25 mM sodium phosphate buffer with pH 7.4; NaCl 500 mM; imidazole 20 mM). The re-suspended cells were sonicated in 4 pulses by 15 seconds each at 20 % amplitude. The cell lysates obtained were centrifuged at 17,000 g for 20 min. Supernatants containing recombinant proteins were pooled and further purification of the target product was carried out by metal-chelate affinity chromatography in 1 ml HisTrap column filled with Ni-NTA sepharose (GE Healthcare, USA), using high-performance protein chromatography system AKTA Purifier (GE Healthcare System, US) according to manufacturer's recommendations. Elution of target proteins was monitored by absorbance at 280 nm wavelength. A fraction with the target protein was immediately transferred into the storage buffer (25 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA) using a 5 ml HiTrap Desalting column that contained Sephadex G-25. Efficient protein transfer to the buffer for storing was monitored through the absorption peak ratio at 280 nm wavelength to the conductivity of the solution.

Protein concentrations were determined by absorbance at 280 nm wavelength on NanoPhotometr spectrophotometer (Implen, Germany) according to known molar extinction coefficient (12090 $M^{-1} \times cm^{-1}$ for both proteins) and taking into account exact molecular weights: 12632.39 12 Da for HMG1 and 10177.58 Da for HMG2.

Assembling DNA structures

The structures used (DNA duplex and Holliday structure) were collected from the following oligonucleotides - x-FAM (FAM-AGTCTAGACTGCAGTTGAGTCCTTGCTAGGAC GGATCCCT), x-com (AGGGATCCGTCCTAGCAAGGACTCAA CTGCAGTCTAGACT), b (AGGGATCCGTCCTAGCAAGGGGC TGCTACCGGAAGCTTCT), r (AGGAATTCAACCACCGCTCAA CTCAACTGCAGTCTAGACT), h (AGAAGCTTCCGGTAGCAGC CTGAGCGGTGGTTGAATTCCT) – which are similar to those in the study by Duckett & Lilley [15]. Linear double-stranded

DNA was pooled from x-FAM and x-com oligonucleotide, while Holliday structure was collected from x-FAM, b, r and h oligonucleotides. The pooling was performed as follows. 5l of 1 μm of solution of labeled oligonucleotide (x-FAM) and 5l 2 mM of solutions of other oligonucleotides were added to 20 mcl of double buffer (Tris-HCl 25 mM, pH 7.5; NaCl 150 mM), and if necessary, adjusting the volume of the reaction mixture to 40 μm of deionized water. The reaction mixture was heated in a water bath to a temperature of 95 °C, and then passively cooled over 3 hours to 25 °C.

Electrophoretic mobility shift assay (EMSA)

Each of the reaction mixtures consisted of 2 mcL of 5-fold EMSA buffer (100 mM Tris-HCl, pH 8.0; 1 M NaCl; 1 mg/ml BSA; 35 % glycerol), 1 mcL of 100 nM of solution of DNA structures and various concentrations of the recombinant protein. The total volume of each reaction mixture was 10 mcL and if necessary, brought to the final volume using deionized water.

The reaction mixtures were incubated for 15 minutes in the dark at room temperature. Thereafter, they were applied to 6% polyacrylamide gel (20 × 20 cm) prepared on TBE buffer (90 mM Tris-borate, 2 mM EDTA). Before applying the samples, the gel was subjected to preliminary electrophoresis at 400 V for 40 minutes with active cooling to a temperature of 10°C. Electrophoretic separation of samples was performed for 120 minutes under the same conditions. After electrophoresis, the gel was scanned using Storm 860 scanner (GE Healthcare, USA) with blue fluorescence excitation. The resulting images were analyzed using the ImageJ program, determining the area for each track and the fluorescence band intensities of the bound and free DNA. By ratio of integral values of the fluorescence band intensities to the sum of the band areas in the track, free and bound DNA concentrations were calculated. Dissociation constants were calculated using the formula:

$$K_D = [Df] \times [P_0 - Db] / [Db],$$

where [Df] is the free DNA concentration, [Db] is bound DNA concentration and [P0] is protein concentration. All concentrations were expressed in nM. At least three independent experiments were carried out for each DNA/recombinant protein pair.

RESULTS

Obtaining recombinant proteins

In order to clarify the role played by each of the HMG domains of mitochondrial protein Abf2p in performing its functions, recombinant proteins corresponding to both domains were obtained. After isolation and purification of these proteins, their degree of purity was assessed using denaturing electrophoresis (Fig. 1). The resulting protein preparations had sufficient purity for further research. The recombinant proteins yielded 8 mg/l culture for HMG1 and 4.2 mg/l culture for HMG2.

Analysis of interaction of individual HMG domains with linear and cruciform DNA

As already noted, the HMG domains of HMGB proteins are presently believed to bind DNA independently of each other with similar efficiency. To verify whether this is true of Abf2p, we analyzed the binding of linear DNA duplex of 40 base pairs (bp) and synthetic cruciform structure that mimics the Holliday structure, with the obtained recombinant proteins through EMSA.

HMG1 domain in the conditions used by us almost did not interact with the linear DNA (Fig. 2, A). It was only at high protein concentration (500 nM) that lower band intensity corresponding to free DNA was observed. However, there was no formation of complex (which would have been visible as a clear band at the top of the gel). From this, it can be concluded that HMG1 weakly and nonspecifically interacts with linear DNA. However, it effectively bound with cruciform structure. DNA/protein complexes were detected even at a protein concentration of 100 nM (Fig. 2, A, C1). Through three independent experiments, we calculated the apparent dissociation constant of the HMG1 complex with cruciform DNA. The constant was equal to 510 ±11.78 nM. At the same time, the HMG2 domain did not form complexes with linear DNA duplex nor with cruciform structure. Even at concentrations of recombinant protein equal to 1 μm, there was no reduction in the intensity of the band corresponding to free DNA (Fig. 2, B).

According to various literature sources, full-length protein Abf2p forms a complex with linear DNA with a constant from 40 to 150 nM, that is, considerably smaller than HMG1 should [11, 15]. It was assumed that increased affinity of the full-length protein to DNA with respect to HMG1 is due to interaction of the two domains. To verify this assumption, we conducted an EMSA experiment after incubation with a mixture of two recombinant proteins. The results showed that addition of HMG2 does not

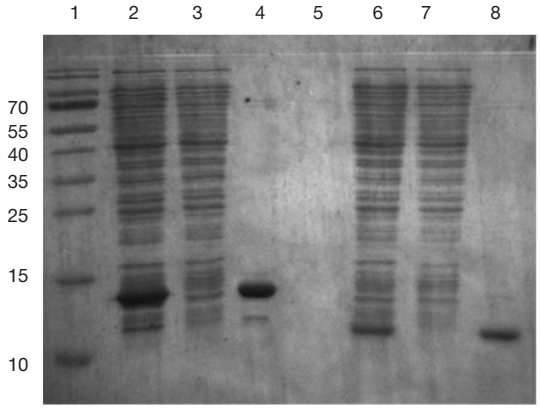

Fig. 1. Isolation and purification of recombinant proteins corresponding to HMG1 and HMG2 domains of the Abf2p protein

Recombinant proteins were purified by metal affinity chromatography on Ni sepharose. 1 — molecular weight markers (the molecular weights of marker proteins are shown on the left); 2, 6 — damaged cell lysates; 3, 7 — factions that didn't interact with affinity column; 4, 8 — purified preparations of recombinant proteins HMG1 and HMG2 respectively.

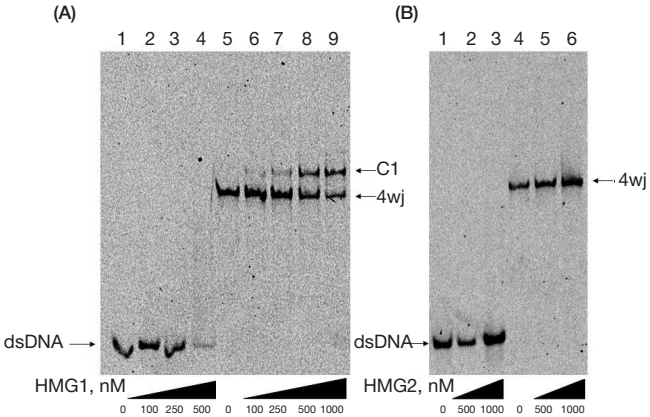

Fig. 2. Analysis of the binding of recombinant proteins corresponding to HMG1 and HMG2 domains with DNA using EMSA

Linear DNA duplex (double-stranded DNA - dsDNA) and cruciform DNA (4-way junction — 4wj) at a concentration of 10 nM were incubated with increasing concentrations of recombinant proteins, after which the reaction mixtures were separated in 6% polyacrylamide gel. **(A)** Binding with DNA of HMG1 domain; 1–4 — interaction with DNA duplex, 5–9 — binding with cruciform DNA; C1 — resulting complex. **(B)** Binding with DNA of HMG2 domain; 1–3 — interaction with DNA duplex, 4–6 — binding with cruciform DNA. The lower part of the figure shows the used concentration of recombinant proteins (in nM).

affect the characteristics of DNA binding with HMG1 (data not shown).

DISCUSSION

Based on data obtained, the following can be concluded. HMG1 domain makes a major contribution to the DNA binding activity of Abf2p. Here, the HMG2 domain does not possess direct DNA-binding activity in vitro. However, it is important for performance of this function by the full-length protein since the dissociation constant of the HMG1/linear DNA complex, according to our results, is more than 1 μm, while the dissociation constant of the same complex with Abf2p is lower by 1–2 orders of magnitude. It should be highlighted that HMG1 can, similarly to full-length protein, specifically bind cruciform DNA structure. This feature is characteristic of many HMGB proteins involved in maintaining DNA integrity of the process, namely repairing by recombination [14]. There are reports that Abf2p is important for homologous recombination of yeast mitochondrial DNA, and apparently, the specificity of binding of full-length protein is achieved directly by HMG1 [11, 12]. The role of HMG2 in the various functions of Abf2p is less obvious. As our data show, HMG2 by itself does not bind to a linear or branched DNA in the selected experimental conditions. This is quite unusual for HMG domain. Nevertheless, relying on the results obtained, it is not clear whether HMG2 can exhibit DNA-binding activity inside a full-length protein. Apparently, apart from strengthening the DNA-binding properties of HMG1, the second domain may be involved in Abf2p interaction with other proteins, for example, in attracting enzymes needed for repair and recombination.

CONCLUSIONS

The study conducted allowed to establish that the HMG1 domain of yeast mitochondrial protein Abf2p is not specific and weakly binds linear DNA, at the same time forming a specific complex with cruciform DNA with a dissociation constant of 510 nM. HMG2 cannot bind with DNA by itself *in vitro.*

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STABILITY OF GADOLINIUM-BASED CONTRAST AGENTS IN THE PRESENCE OF ZINC AND CALCIUM IONS IN DIFFERENT MEDIA

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To improve the safety of Gd^{3+}-based contrast agents (GBCA) in clinical practice, it is recommended to use the most stable substances and to consider conditions determining their stability. The aim of this study was to compare the stability of GBCAs for magnetic resonance imaging in the presence of zinc and calcium ions and polyvinylpyrrolidone (PVP) in water, phosphate buffer solution and blood serum using proton NMR relaxometry. The study demonstrated that macrocyclic gadobutrol is more stable than all linear contrast agents. The addition of PVP (10 mg/ml) improved the stability of linear GBCAs in phosphate buffer solution and blood serum. Calcium ions have a much weaker destabilizing effect on GBCAs than zinc ions.

Keywords: gadolinium-based magnetic resonance contrast agents, polyvinylpyrrolidone, calcium ions, zinc ions, NMR relaxometry

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СТАБИЛЬНОСТЬ ГАДОЛИНИЙСОДЕРЖАЩИХ МАГНИТНО-РЕЗОНАНСНЫХ КОНТРАСТНЫХ СРЕДСТВ В ПРИСУТСТВИИ ИОНОВ ЦИНКА И КАЛЬЦИЯ В РАЗЛИЧНЫХ СРЕДАХ

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Для повышения безопасности клинического использования гадолинийсодержащих магнитно-резонансных контрастных средств (МРКС) рекомендовано применять наиболее стабильные препараты и учитывать условия, определяющие их стабильность. Целью исследования был сравнительный анализ стабильности Gd^{3+}-содержащих МРКС в присутствии ионов цинка, кальция и поливинилпирролидона в воде, фосфатном буфере и сыворотке крови с использованием метода протонной ЯМР-релаксометрии. Было показано, что макроциклический гадобутрол обладает большей стабильностью, чем все линейные МРКС. Поливинилпирролидон (10 мг/мл) способен улучшить стабильность линейных МРКС в фосфатном буфере и сыворотке крови. Ионы кальция обладают значительно менее выраженным дестабилизирующим действием на МРКС, чем ионы цинка.

Ключевые слова: гадолинийсодержащие магнитно-резонансные контрастные средства, поливинилпирролидон, ионы кальция, ионы цинка, ЯМР-релаксометрия

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It is known that gadolinium-based contrast agents have found their widest application in MRI studies [1]. Although gadolinium is present in them as a chelate, one should bear in mind that the toxicity of this rare earth element in its free form can be compared to that of mercury and lead [2] and that the stability of gadolinium-based magnetic resonance contrast agents (MRCAs) varies and is determined by two major factors: 1) a chemical structure of a chelator; 2) a presence of some organic and non-organic ligands in the medium that can compete for binding to Gd^{3+} ions or a chelating compound thus facilitating Gd^{3+} release.

Using an unstable contrast agent can be life threatening for patients with impaired renal function since free gadolinium retains in tissues and can cause nephrogenic systemic fibrosis [3–5].

Recent studies demonstrated an increased intensity signal in such brain structures as globus pallidus and dentate nucleus on unenhanced T_1-weighted MR images in patients [6] or laboratory animals [7] who had received low stability linear MRCAs before, which is possibly related to Gd^{3+} depositing. After administration of high stability macrocyclic MRCAs, no such "residual" increased signal was observed. It is also known that gadolinium release from MRCAs depends on the presence of various ions in the surrounding medium [8]. Therefore, a complex study on how the above mentioned factors interact can shed some light on the dynamics of Gd^{3+} release from a chelate complex in various media, as well as estimate the risk of administering certain contrast agents to patients with renal insufficiency or conditions accompanied by increased zinc or calcium ions concentration in blood. Improving the stability of these contrast agents, as by means of adding a substance with strong chelating properties, is also important. Polyvinylpyrrolidone (PVP) with its chelating and detoxifying properties can be regarded as such a substance [9].

The aim of this study is to conduct the comparative analysis of the stability of Gd^{3+}-based MRCAs in the presence of zinc ions, calcium ions and PVP in water, phosphate buffer solution and human serum.

METHODS

The following linear Gd^{3+}-based MRCAs were studied: gadopentetate dimeglumine (Magnevist 0.5 M, Bayer, Germany); gadobenate dimeglumine (MultiHance 0.5 M, Bracco, Italy); sodium gadopentetate + PVP (Dipentast 0.125 M, Epidbiomed Group of Companies, OOO, Russia); gadopentetate-β-cyclodextrin (Cyclogadopentetate 0.125 M, Epidbiomed Group of Companies, OOO, Russia), and gadobutrol, a macrocyclic MRCA (Gadovist 1 M, Bayer, Germany)

Contrast agents stability was assessed by proton NMR relaxometry (Minispec mq 20, Bruker, Germany). Gadolinuim release from a chelate affects proton relaxation times in the medium [10]. T_1 relaxation time was measured since MR signal intensity depends on this parameter. Stability assays of the substances listed above were performed in distilled water (pH 6.0), phosphate buffer and blood serum (pH 7.4). In the experiments with zinc, stability of five MRCAs was assessed, i.e. gadopentetate dimeglumine, sodium gadopenetate with PVP, gadopentetate-β-cyclodextrin, gadobutrol and gadobenic acid, whereas in the experiments with calcium only gadopentate dimeglumine was involved.

To obtain 0.2 M phosphate buffer (pH 7.4), aqueous solutions of NaH_2PO_4 and Na_2HPO_4 were prepared [11]. Blood serum was obtained from the patients of A.N.Ryzhikh State Scientific Centre for Coloproctology. All donors signed the informed consent to their biological material being used in the scientific research under the conditions of respecting their privacy and confidentiality. Blood was collected in sterile tubes with a clot activator and a barrier gel. Serum was obtained by centrifuging blood at 1200 g for 10 minutes and stored frozen at –20 °C for no more than 10 days. Prior to freezing, serum samples were tested for albumin concentration on Spotchem EZ SP-4430 clinical chemistry analyzer (Arkray Inc., Japan). Then the samples were diluted in phosphate buffer until albumin concentration of 10^{-4} M (close to physiological) was obtained.

A 200 mM $ZnCl_2$ aqueous solution (Komponent-reaktiv, Russia) was prepared by dissolving the weighted amount of 2.7 g in 100 ml distilled water. The final concentration of $ZnCl_2$ in the sample was 2 mM. While adjusting $ZnCl_2$ final concentration, we drew on the study by M. Taupitz et al. [12] that demonstrated the most illustrative results at this particular $ZnCl_2$ concentration. The concentration of the initial $CaCl_2$ aqueous solution (Komponent-reaktiv, Russia) was also 200 mM (2.2 g $CaCl_2$ in 100 ml distilled water), the final concentration in the sample was 2 mM. The initial aqueous solution of PVP (Kollidon® 17 PF, BASF) was prepared by dissolving 500 mg PVP powder in 1 ml distilled water.

To assess the stability of the studied MRCAs, two samples were prepared simultaneously. The first sample was a 0.2 mM MRCA solution. T_1 relaxation time of the 0.2 mM MRCA solution was measured at 40 °C (temperature value in the sample chamber of the MR relaxometer). Then a zinc chloride or calcium chloride solution was added to the sample until the final concentration of 2 mM was reached; then relaxation time was measured again. After that the sample was incubated in the thermostat at 40 °C; T_1 measurements were repeated in 1, 2 and 24 hours. The second sample was similar to the first one, the difference being a PVP solution with a final concentration of 10mg/ml added to it after adding zinc chloride or calcium chloride. In the second sample relaxation time was measured at the same time points.

Within the framework of this study all experiments were repeated sixfold to improve the reliability of the results. Using Statistica 10 software, mean values and standard deviations were computed. Because of the normal distribution of the obtained data (in all cases of sample checks using the Kolmogorov-Smirnov test, the p-value was substantially higher than 0.05), a statistical significance of differences between the means was determined by Student's t-test, the difference being significant with $p < 0,05$.

RESULTS

Effect of zinc ions on MRCAs stability

In distilled water T_1 longitudinal relaxation time of all linear MRCAs shortened by an average of 23-28% (Fig. 1) in the absence of PVP 24 hours after the addition of zinc chloride. In the gadopentetate dimeglumine sample T_1 value lowered by 25.7 ± 0.6 %, in the sodium gadopentetate sample — by 28.1 ± 0.7 %, in the Cyclogadopentetate sample (CGP)— by 22.0 ± 0.5 %, in the gadobenate dimeglumine sample— by 24.8 ± 0.4 %, respectively. For macrocyclic gadobutrol T_1 did not change significantly.

In phosphate buffer without PVP, T_1 of all linear MRCAs lowered by an average of 13–19 % 24 hours after the addition of zinc chloride. We observed a reduction in T_1 by 18.1 ± 0.7 % in the gadopentetate dimeglumine sample, a reduction by 19.3 ± 0.8 % in the sodium gadopentetate and PVP sample, a reduction by 12.8 ± 0.6 % in the CGP sample, a reduction by 15.9 ± 0.5 % in the gadobenic acid sample. In the gadobutrol

sample T_1 did not undergo any significant alterations (Fig. 2). The lowest T_1 value was observed 1 and 24 hours after the addition of zinc chloride as opposed to its immediate reduction in the previous series of experiments with distilled water being a medium.

After adding PVP to gadopentetate dimeglumine, T_1 decreased by 7.9 ± 0.7 %, in the sodium gadopentetate sample it decreased by 12.3 ± 0.7 % (Fig. 3). Thus these MRCAs showed a statistically significant improvement in stability in the presence of PVP by an average of 10% and 7 %, respectively. PVP improved the stability of CGP by 13 %, with T1 displaying no significant changes 24 hours after its addition. In the gadobenic acid sample T_1 final values in the presence and in the absence of PVP did not show a significant difference. No effect of zinc ions on gadobutrol relaxation time was observed in phosphate buffer in the absence or presence of PVP.

In blood serum in the absence of PVP T_1 of MRCAs decreased by an average of 31–61 %, the most significant reduction was observed 1 and 24 hours after the addition of zinc chloride to the solution (Fig. 4). Of all linear MRCAs the best stability figures were observed in CGP— T_1 decreased by an average of 31.2 ± 0.3 %; the worst results were observed in gadopentetic acid salts: in the dimeglumine salt sample T_1 decreased by 61.2 ± 0.6 %, in the sodium salt sample — by 56.1 ± 0.1 %. In the gadobenic acid samples T_1 lowered by 50.2 ± 0.1 %, in the gadobutrol samples no significant decrease in T_1 was observed. Stability improvement of gadobenic acid by PVP was slight, but statistically significant (by 5%). PVP did not have any effect on the stability of other MRCAs (Fig. 5).

Effect of calcium ions on MRCAs

Exposed to calcium and zinc ions, gadopentate dimeglumine showed no significant variation in T_1 in the absence of PVP in water, and a T_1 reduction by 7.8 ± 0.7 % and 9.1 ± 1.1 % in phosphate buffer and blood serum respectively. The addition of PVP resulted in a statistically significant improvement in gadopentetate dimeglumine stability in phosphate buffer and blood serum. In the tests with calcium ions gadopentetate dimeglumine stability did not change in the presence of PVP in water, which a constant T_1 value is indicative of.

DISCUSSION

According to the obtained results T_1 longitudinal relaxation time shortens in all linear MRCAs samples after the addition of zinc regardless of PVP presence. A shortened T_1 relaxation time can be explained by a transmetalation reaction between zinc and a MRCA molecule: zinc ions replace gadolinium ions in a chelate, while in its free form gadolinium can shorten the proton relaxation time in the medium. Zinc ions did not have any effect on macrocyclic gadobutrol.

The results of our work demonstrate a higher stability of macrocyclic MRCAs and confirm literature data on gadolinium dissociation in vivo when linear MRCAs are used as opposed macrocyclic [13]. They also confirm that zinc facilitates gadolinium release from linear but not macrocyclic chelates as a result of transmetalation [12].

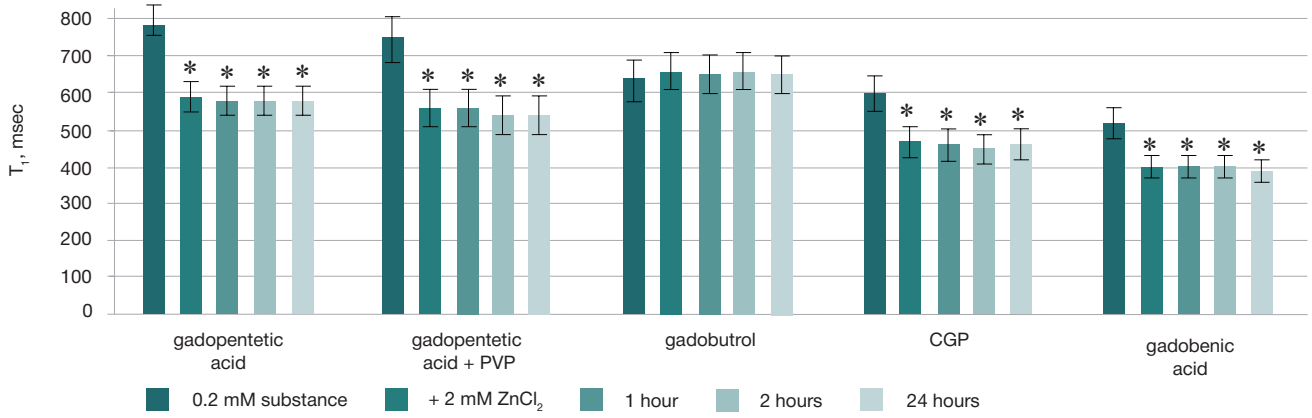

Fig. 1. Effect of zinc ions on T_1 water proton relaxation time in the studied MRCAs solutions in water (pH 6.0) Here and in fig. 2-5 below: CGP — gadopentetate-β-cyclodextrin .* — statistically significant difference from the control. Procedures and conditions of the experiment are described in "Materials and Methods"* — statistically significant difference from the control (p <0.05).

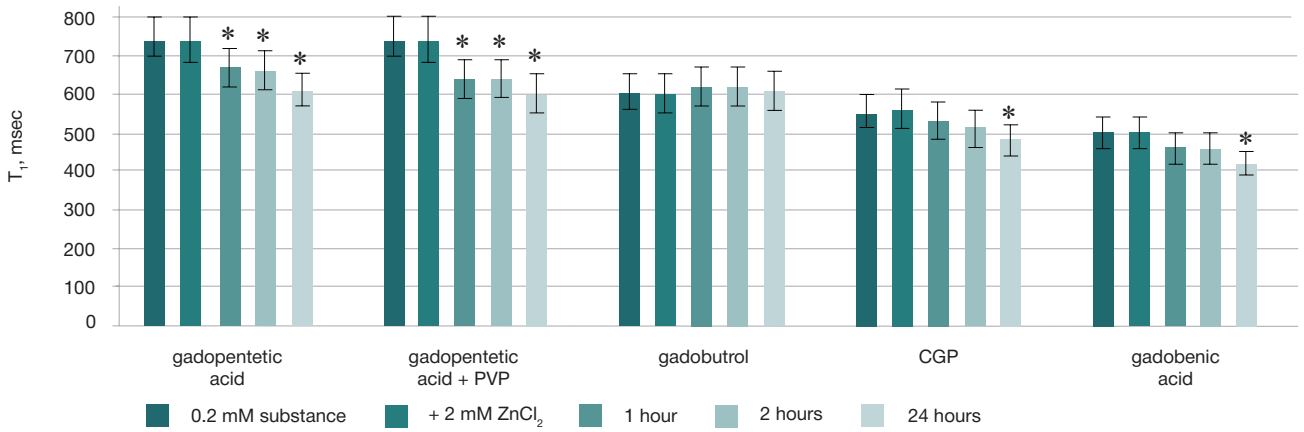

Fig. 2. Effect of zinc ions on T_1 water proton relaxation time in MRCAs solutions in phosphate buffer (pH 7.4)

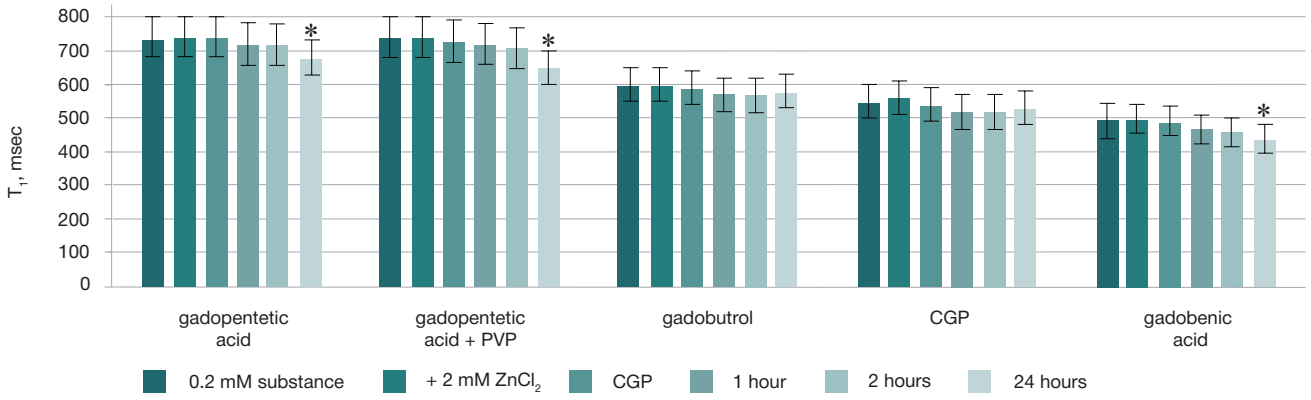

Fig. 3. Effect of zinc ions and PVP on T_1 water proton relaxation time in MRCAs solutions in phosphate buffer (pH 7.4)

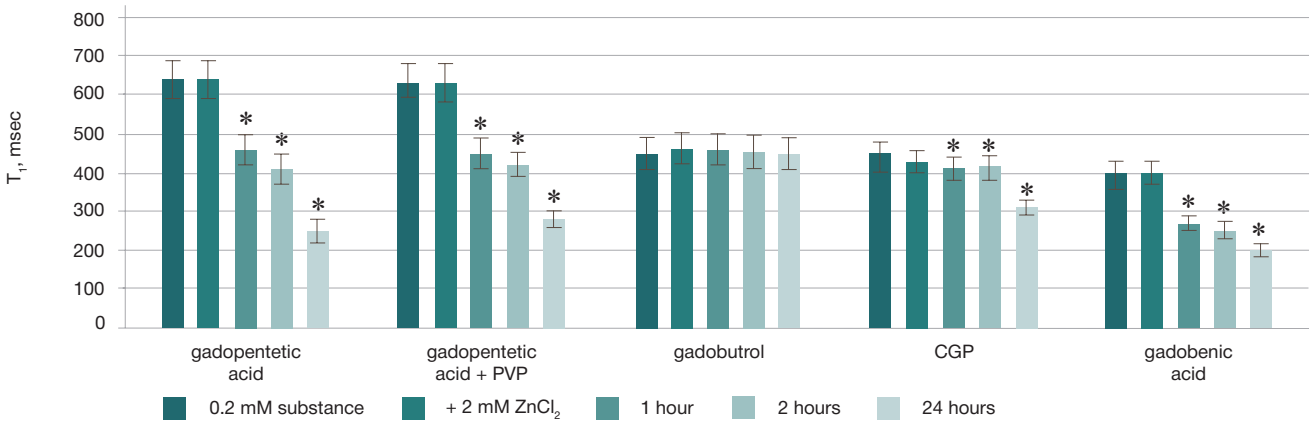

Fig. 4. Effect of zinc ions on T_1 water proton relaxation time in MRCAs solutions in blood serum diluted in phosphate buffer (pH 7.4)

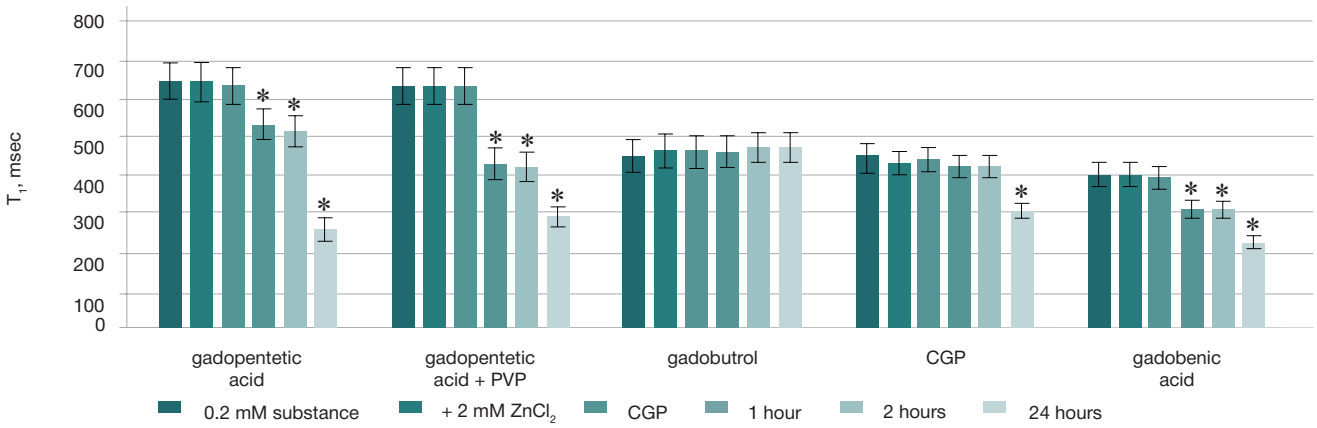

Fig. 5. Effect of zinc ions and PVP on T_1 water proton relaxation time in MRCAs solutions in blood serum diluted in phosphate buffer (pH 7.4)

In serum diluted down in phosphate buffer to albumin concentration of 10^{-4} M, T_1 decreased more than in two other media within 24 hours. It is probably the result of a larger number of compounds in the serum that can interact with both positively charged Gd^{3+} ions (phosphate, citrate, carbonate, heparin and others) and negatively charged chelates (metal cations), which leads to the destabilization of a large number of MRCAs molecules and creates a higher concentration of free gadolinium compared to other media. As a result, T_1 reduction in blood serum tests is the most considerable. The results of tests with zinc and calcium ions showed that calcium ions effect on gadopentetate dimeglumine stability is weaker.

PVP significantly improved stability of three studied linear MRCAs in phosphate buffer, as opposed to water solution. Thereby a question of adding PVP as an auxiliary component to the pharmaceutical forms of linear MRCAs should be raised.

In patients with renal insufficiency MRCAs half-life is prolonged. Administering linear MRCAs, specifically non-ionic that are less stable than macrocyclic, to such patients should be avoided [14]. This recommendation is also relevant for patients with conditions accompanied by increased zinc and phosphate levels in blood.

CONCLUSIONS

Macrocyclic gadobutrol is more stable than other studied linear magnetic resonance contrast agents. Zinc ions do not have any effect on its relaxation properties. Linear MRCAs show the highest stability in the presence of zinc ions in phosphate buffer, and the lowest stability in blood serum. Polyvinylpyrrolidone demonstrates a statistically significant although slight improvement of linear MRCAs stability in phosphate buffer (by 10% in gadopentetate dimeglumine, by 7% in sodium gadopentetate, by 9 % in CGP) and in blood serum (by 5% in gadobenic acid) and not in water. Calcium ions have a much less destabilizing effect on gadopentetate dimeglumine than zinc ions.

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APPROACHES TO IMPROVING TUBERCULOSIS CARE IN HIV-INFECTED PATIENTS AND CRITERIA FOR ITS EVALUATION

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Despite various anti-tuberculosis measures in the setting of HIV infection, the epidemiological situation of tuberculosis in Russia is deteriorating. We have analyzed the data of statistical report form no.61 for years 2004-2014, surveillance data on individual TB cases with HIV coinfection for years 2004-2014 (personal data) and TB care arrangements for patients with HIV in 20 regions. The main causes of the deteriorating epidemiological situation are the growing immunodeficiency in patients with TB coinfection, unseparated epidemiologically dangerous patient flows (patients with tuberculosis and HIV-infected patients) and low quality preventative measures in special care medical facilities. Chemoprophylaxis can be an effective method of controlling the spread of tuberculosis among HIV-infected patients if it is recommended by a qualified tuberculosis therapist to patients adhering to regular drug intake under supervision of medical personnel. Otherwise a large scale chemoprophylaxis can result in an increased proportion of patients with drug-resistant tuberculosis. This works suggests criteria for the evaluation of tuberculosis care effectiveness considering the pathogenesis of the disease during late stages of HIV.

Keywords: tuberculosis, HIV-infection, tuberculosis care, tuberculosis chemoprophylaxis

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ПОДХОДЫ К УСОВЕРШЕНСТВОВАНИЮ ПРОТИВОТУБЕРКУЛЕЗНОЙ ПОМОЩИ БОЛЬНЫМ ВИЧ-ИНФЕКЦИЕЙ И КРИТЕРИИ ЕЕ ОЦЕНКИ

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Несмотря на различные меры по борьбе с туберкулезом, сочетанным с ВИЧ-инфекцией, эпидемиологическая ситуация по заболеванию в России продолжает ухудшаться. Нами были проанализированы данные отчетной формы № 61 за 1999-2014 гг., данные персонифицированного мониторинга больных туберкулезом, сочетанным с ВИЧ-инфекцией, за 2004-2014 гг. (личные данные) и организация противотуберкулезной помощи больным ВИЧ-инфекцией в 20 регионах. Основными причинами ухудшения эпидемиологической ситуации являются нарастание иммунодефицита среди пациентов с сочетанным туберкулезом, отсутствие разделения эпидемиологически опасных потоков пациентов (больных туберкулезом и больных ВИЧ-инфекцией) и невысокий уровень профилактической работы в специализированных медицинских учреждениях. Химиопрофилактика может быть эффективным средством борьбы с распространением туберкулеза среди больных ВИЧ-инфекцией, если она будет назначаться подготовленным врачом-фтизиатром пациентам, готовым принимать лекарства под наблюдением медицинского персонала. Иначе масштабная химиопрофилактика может вызвать рост доли пациентов с туберкулезом с лекарственной устойчивостью. Предложены критерии оценки эффективности противотуберкулезной помощи с учетом патогенеза заболевания на поздних стадиях ВИЧ-инфекции.

Ключевые слова: туберкулез, ВИЧ-инфекция, противотуберкулезная помощь, химиопрофилактика туберкулеза

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The World Health Organization defines the fight against tuberculosis among HIV-infected patients as one of the priorities in health care, while high-quality epidemiological surveillance is an important aspect here [1]. Russia is also developing a strategy for combating tuberculosis among persons with HIV infection [2]. Discussions on the epidemiological situation and approaches to tuberculosis care delivery to HIV-infected TB patients began in the late 1990s [3, 4].

Russia began in 1999 to keep records of TB patients with HIV infection according to report form No 61 of the Federal statistical surveillance "Information About HIV Patient Population". In 2001, the first results of its analysis were published [5], while in 2002, the first doctor's TB care manual for HIV patients was published [6]. Later, approaches developed by Russian researchers were approved by WHO experts and issued by joint recommendations [7, 8]. Russia became the first and still the only country that introduced a personalized monitoring system for TB patients with HIV infection [9]. In 2008, doctors in the regions started entering a number of information about cases of tuberculosis in HIV-infected patients into report form No 61 based on personalized monitoring data. In 2004, under the auspices of the Russian Ministry of Health, the country launched a training program for TB specialists and infectious disease experts working with HIV-infected patients. In 2014, Russian Society of Phthisiologists issued the Federal Guidelines for the Diagnosis and Treatment of Tuberculosis in HIV-Infected Patients. The Guidelines expanded and modernized the approaches to tuberculosis chemoprophylaxis [10]. A draft instruction on TB chemoprophylaxis in HIV-infected patients is currently being discussed. It has been sent to regional centers for prevention and control of AIDS. The authors of this article have a copy. However, despite the measures taken, the epidemiological situation of the disease continues to worsen.

We analyzed the data from report form No 61 collected between 1999 and 2014 and data obtained from personalized monitoring of TB patients with HIV infection for 2004-2014 (personal data). We also examined how provision of TB care to HIV patients is organized, as well as information materials about the disease provided to patients in 20 regions. The aim was to identify possible causes of inefficiency of the existing TB care approaches for HIV patients. Based on the analysis, some measures on how to improve TB care to HIV patients were proposed.

Analysis of the epidemiological situation

According to report form No 61, there were 0.2 TB cases per 100,000 persons with HIV infection in 1999. The figure rose to 9.8 in 2014. Prevalence of the disease has also risen from 0.35 to 25.8 cases per 100,000 persons. Tuberculosis is increasingly becoming a cause of death in HIV-related severe immunodeficiency: in 2008 (the beginning of record keeping in form No 61), tuberculosis accounted for 75.7 % of deaths in patients with advanced HIV infection. By 2014, the figure has risen to 86.9 %. Considering the pathogenesis of tuberculosis in the late stages of HIV infection, it can be assumed that the main cause of a rise in tuberculosis in Russia is precisely an increase in immune deficiency among HIV patients.

Another reason for the deterioration of the epidemiological situation is the low level of preventive measures among TB patients with HIV infection. For example, in 2014, 7.8 % of such patients had HIV-infected family members. However, in the information materials for them, we found no information about tuberculosis preventive measures in the family and about TB peculiarities in the late stages of HIV infection. This led to a rise in the incidence of tuberculosis in children with early stages of HIV infection: 143 children with TB infection in 2014 and 47.5 % of them had early-stage HIV infection. This means that tuberculosis in these children was not caused as a result of immunodeficiency.

Survey of HIV patients for tuberculosis in different regions showed that it is often at the stage of examination that conditions for the spread of tuberculosis are formed. For instance, for the exclusion of TB, persons with severe immune deficiency were hospitalized for diagnosis in the TB unit, where there may be patients with yet undiagnosed tuberculosis with bacterial excretion. Sometimes, all HIV-infected patients are hospitalized in one unit regardless of whether there is bacterial excretion or indications for hospitalization (diagnosis or treatment). This procedure aggravates the epidemiological situation of tuberculosis among HIV-infected patients. For example, among patients with mycobacteriosis (with HIV infection) who were treated in TB facilities, 9 % were infected and fell sick of tuberculosis [12].

Efficacy of tuberculosis chemoprophylaxis

Tuberculosis chemoprophylaxis in HIV-infected persons is undoubtedly one of the most effective means of preventing the spread of the disease if drug administration is controlled by a doctor. However, ensuring such a control is extremely difficult because most of the patients with HIV infection are socially disadvantaged individuals. For instance, in 2014, among HIV patients with tuberculosis, 75.5 % were of working age who were not working for a long time, 66.2 % were infected through drug injection, 42.1 % were currently or previously in prisons. In this connection, an indication to provide coverage of tuberculosis chemoprophylaxis for at least 50 % of HIV-infected patients [13] is worrying because it can trigger increase in drug resistance in mycobacterium tuberculosis.

This assumption is supported by data obtained from personalized monitoring conducted by us: in 2011, primary multidrug-resistant tuberculosis (caused by an organism that is resistant to at least isoniazid and rifampin) was detected in 41 % of patients, while in 2014, the figure increased to 42.1 %. Besides, primary drug-resistant tuberculosis (caused by an organism that is resistant to other two or more drugs) was observed in 15.4 % of patients in 2011 and 15.9 % in 2014. The situation is worse in prisons: in 2014, primary multidrug-resistant TB (MDR-TB) was identified in 55.9 % of patients, while primary multidrug-polyresistant TB in 16 % (obviously due to the fact that patients in such places are extremely socially disadvantaged, and that the infection source is a person at the same place). It is important to note that these parameters are not decreasing.

The question now is whether uncontrolled TB chemoprophylaxis of HIV patients will be an additional reason for emergence and spread of mycobacterium strains that are resistant to anti-TB drugs. Will this uncontrolled TB chemoprophylaxis be effective even when drugs are taken regularly, if the draft instruction on TB chemoprophylaxis encourages HIV patients to take rifampicin to which there is primary drug resistance in many of the patients and which is not combined with antiretroviral drugs included in the basic HIV treatment scheme? In addition, there are doubts over whether it is possible to cover such a number of HIV-infected patients (at least 50 %) since, according to our data, about 30 % of them are not included in records at HIV/AIDS prevention and control centers (HAPCC). Moreover, over 12 % of patients on record do not undergo medical examination. At the same

time, requiring HIV-infected patients to visit a clinic without their consent has been prohibited by law since 1995 [14].

In our opinion, only a TB doctor can prescribe tuberculosis chemoprophylaxis to HIV patients. Such doctor must be trained on this problem and the patients must be only those who are committed to regular use of drugs. Drug administration itself should be under the supervision of an HAPCC medical staff or personnel of a unit providing such functions at the municipal level. Tuberculosis chemoprophylaxis of HIV-infected patients at TB facilities, that is, at the infection source, is unacceptable.

Ways of improving the quality of TB care to HIV-infected patients

It is necessary to deploy a procedure for TB care to HIV patients that would minimize the likelihood of contact with severely immunocompromised persons and TB patients.

Medical care to TB patients with HIV infection should be provided at different TB facilities, depending on whether the patient has bacterial excretion and there is drug resistance in mycobacterium tuberculosis. For their treatment at a TB clinic, the salaries of TB doctors and infectious disease physicians must be provided for. Doctors may be taken into these positions only after occupational retraining. Their number should be determined by load (number of patients). Both specialists must be staff of the entire clinic and not just of a single ward, and manage HIV-infected patients distributed in the wards.

To minimize the likelihood of contact with patients with advanced HIV infection and tuberculosis patients with bacterial excretion, only TB specialists should terminate a TB treatment in the continuation phase at persistent absence of bacterial excretion. But this should be done at facilities providing specialized care to HIV patients. The same is true of follow-up of patients from the third record group. Treatment of HIV-infected patients with chronic forms of tuberculosis with bacterial excretion should be done only at a TB clinic.

Thus, most of the work on prevention, detection and diagnosis of tuberculosis, as well as differential diagnosis of tuberculosis and other secondary diseases in HIV infection should be conducted by HIV care facilities.

Criteria for assessing the efficiency of TB care in HIV-infected patients

Some standard criteria in TB are not relevant in the later stages of HIV infection and may compromise the work of TB facilities. For example, it is not proper in cases where TB detection in patient at the later stage is considered as not satisfactory. This is so because in a person with severe immune deficiency, clinical manifestations of the disease often develop before the radiographic changes appear. Therefore, it is typically not possible to identify the disease through scheduled X-ray diagnostics. Deaths from TB can occur in persons without chest radiography changes. Even with a small delay by the patient in seeking medical care, death occurs as a result of acute progression of the disease. It is impossible to consider death from tuberculosis in these cases as a result of poor diagnosis. For detection of tuberculosis in the late stages of HIV infection, HIV awareness programs on the need to seek immediate treatment at a specialists hospital when the first symptoms of the disease appear are particularly important [15].

In connection with the above, criteria for evaluating the efficiency of TB care in HIV patients should be formed taking into account peculiarities of the pathogenesis of tuberculosis in the late stages of HIV infection. Perhaps to this end, it is advisable to consider the following: the proportion of TB focal points examined by medical staff during the first three days (of the number of detected focal points of tuberculosis which are home to patients with HIV infection); the proportion of children born to women with HIV infection, who are isolated from the focal points of tuberculosis (among the patients in the focal points); the proportion of discrepancies between clinical and pathologic diagnosis (if the patient was in the clinic for a month or more) taking into account the frequency of autopsies in cases of death with combined infection; the proportion of cases in which autopsy in patients with tuberculosis detected no other secondary diseases characteristic of HIV infection (if the patient was in the clinic for a month or more).

CONCLUSIONS

The spread of tuberculosis among HIV-infected patients is often caused by a breach in anti-epidemic requirements for assistance of such patients, in particular: lack of separation of epidemiologically dangerous patients (TB patients and those with immunodeficiency). Prevention, detection and diagnosis of tuberculosis in patients with advanced HIV infection should be performed at HIV/AIDS prevention and control centers or specialist institutions.

Uncontrolled TB chemoprophylaxis among socially disadvantaged patients may contribute to the spread of mycobacterium tuberculosis strains that are resistant to anti-TB drugs. Chemoprophylaxis should be done on those who are willing to regularly take medication under medical supervision. Chemoprophylaxis should be prescribed by specially trained phthisiologists only.

Standard TB criteria for evaluating the efficiency of TB care in patients with late-stage HIV infection are biased. Criteria factoring in the peculiarities of tuberculosis pathogenesis in severe immunodeficiency should be used.

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COMPARATIVE ANALYSIS OF MODERN APPROACHES TO THE PERFORMANCE ASSESSMENT OF SCIENTIFIC MEDICAL ORGANIZATIONS IN RUSSIA AND ABROAD

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The article reviews basic methods of performance assessment of scientific medical organizations in Russia. Qualitative and quantitative effectiveness criteria are provided. International practices are described; a comparative analysis of assessment methods used in Russia and abroad is carried out. Global trends in the development of approaches to analyzing the effectiveness of scientific organizations are reviewed. Based on our analysis, a compelling rationale for developing more up-to-date criteria for evaluating the performance of scientific medical organizations is given.

Keywords: performance assessment of scientific medical organizations, effectiveness and relevance of scientific research, human resources, integration into world science, dissemination of scientific knowledge, enhancing the prestige of science, resource maintenance of the scientific organization

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СРАВНИТЕЛЬНЫЙ АНАЛИЗ СОВРЕМЕННОГО РОССИЙСКОГО И ЗАРУБЕЖНОГО ПОДХОДОВ К ОЦЕНКЕ ДЕЯТЕЛЬНОСТИ НАУЧНЫХ ОРГАНИЗАЦИЙ В СФЕРЕ ЗДРАВООХРАНЕНИЯ

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В статье приведены основные методы оценки деятельности российских медицинских научных организаций. Описаны количественные и качественные показатели эффективности. Представлен мировой опыт, проведен сравнительный анализ методов оценки, используемых в России и за рубежом. Рассмотрены мировые тенденции развития подходов к изучению эффективности научных учреждений. На основании проведенного анализа продемонстрированы убедительные данные о необходимости разработки более совершенных критериев оценки деятельности научных медицинских организаций.

Ключевые слова: оценка деятельности медицинских научных организаций, результативность и востребованность научных исследований, кадровый потенциал, интеграция в мировое научное пространство, распространение научных знаний, повышение престижа науки, ресурсное обеспечение деятельности научной организации

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A modern approach to assessing the performance of medical research institutions in Russia's health sector is mostly quantitative in nature and features a large number of indicators. On one hand, such comprehensive analysis facilitates collection of miscellaneous data about organizations and certain areas of their activities. On the other hand, however, it complicates regular monitoring and receipt of objective results. Use of numerical scales involves calculation of the total average indicator based on which further comparative analysis is to be conducted. Combination of diverse factors, inability to identify the most important ones and complexity in clear interpretation of values increase the likelihood of errors in assessing research institutions while applying a quantitative approach. The assessment results may be incomplete or not sufficiently reliable to address the issue of further prospects of the research institution [1].

According to information on the activities of research institutions carrying out research, developmental and technological activities for the purpose of monitoring and evaluation, approved by Order No 162 of the Russian Ministry of Education and Science on March 5, 2014, the following are the key performance indicators:

– effectiveness and relevance of scientific research;
– human resource development;
– integration into the global scientific space, dissemination of scientific knowledge and enhancement of the prestige of science;
– resourcing the activities of research institution.

Effectiveness and relevance of research institutions

The effectiveness and relevance of the activities of medical research institutions are enough for evaluation of the quantitative and qualitative characteristics of research activities of the institutions. For a comprehensive evaluation of this sector, resource indicators — funding, personnel and logistics — are also factored in.

Application of mainly quantitative methods for performance analysis of research institutions has led to active development of scientometric systems [2–4]. Presently, Russia is ranked 15–18th in the world in terms of number of scientific publications. However, in terms of number of cited published works, the country does not make it in the list of 20 leading countries [5].

Scientometrics are actively used in Russia and abroad as a reliable tool for evaluating scientific organizations and communities. Domestic scientometrics has been used by Russian Science Citation Index since 2005 [2, 6]. The main indicators used to measure the impact of research include publication activity of scientists in Russian and foreign scientific journals [6–8]. The number of publications and number of cited works included in global databases Web of Science and Scopus, as well as national information-analytical system Russian Science Citation Index are mainly analyzed [9]. Quantitative analysis of publication activity is a simple, yet reliable and intuitive method for determining the effectiveness of a research organization. Qualitative evaluation methods are characterized by accuracy, timeliness, representativeness and accessibility [3, 4].

Most industrialized nations, including Russia, use quantitative assessment of indicators [10]. For example, the UK uses three main criteria for analysis. The first of these involves the study of the newness, importance and degree of development of scientific subjects. The second examines the magnitude of the research results [11]. The third investigates the competitiveness of a research institution [12]. Examination procedure uses double-blind method [13]. The effectiveness of the activities of national universities in the UK is assessed via the Research Assessment Exercise program once every 4 years. The final rating data serve as the basis for further financing. A similar analysis in the preparation of economic decisions is also applied in the United States [14, 15].

In the US, the main method for evaluation of the effectiveness and safety of the technologies created are systematic literature reviews, whose analysis is carried out by organizations specially created for these purposes. The second evaluation method is cost-effectiveness analysis in which the costs of achieving additional year of life adjusted for quality is calculated. Based on the values obtained, decisions on further financing are taken. Clinical and economic analysis method is less widely used [16].

Medical science is the most cited in the global scientific community. The most famous scientometric database — Web of Science and Scopus, high-demand library fund — the U.S. National Library of Medicine [16, 17].

National databases are widely used by non-English speaking scientific communities. For example, the Chinese Social Sciences Citation Index covers most of the country's journals. At the same time, they try to increase the share of publications in European and American journals. Similar projects have been implemented in Taiwan (Taiwan Humanities Citation Index) and in Japan (Citation Database for Japanese Papers) [18].

Among scientometric indicators in foreign countries, the impact factor of publications, which is annually calculated at the Institute for Scientific Information (ISI) and published in the Journal Citation Reports is used. Since this criterion has been used in the West since the 70s of the 20th century, this impact factor is quite high for many European and American journals, unlike Russian journals [19]. For example, the 2015 impact factor of one of the most prestigious medical periodicals The New England Journal of Medicine is 55.87 [20]. European and American researchers try to publish in journals with very high impact factors to increase prestige and promote career development [16].

The resource estimate of the effectiveness and relevance of scientific research is important, but today it is a vulnerable area for many Russian medical and scientific institutions. Often, research institutions operate a poor assessment system for evaluating the prospects of patents and intellectual right management [21, 22]. Only in recent years that effective measures aimed at creating small innovative enterprises that can make productive use of patented innovative products and services were applied. To upgrade this direction, a mechanism for state guarantee of procurement of innovative products and formation of technology transfer centers was created [5].

In most economically developed countries in the world, intellectual property is commercialized [23]. The US is characterized by transfer of property rights to intellectual products or services created through state support to the private sector due to the fact that the state by law could not be the owners of such products or services. In the EU, UK and Japan, the government owns certain rights to intellectual activity and is actively involved in commercialization of research products [24].

The financial impact of scientific research in Russia is evaluated using two indicators: sources of income of the research institution and type of activity. From the side of the government, financial support is provided from federal budget as part of Russian government's programs "Healthcare Development" for 2013-2020 and "Development of Science and Technology" for 2013–2020, and in accordance with government directives and support grants [16]. At the same time, government financial support for research institutions decreases and the share of private capital increases as the final result comes nearer. Russia is ranked 9th by level of research funding in the world [5, 25]. Improving public funding with increase in the cost of medical science is necessary, but it is not enough for modernization of this sector.

Most foreign countries actively search for the most effective mechanisms for funding of medical research institutions. The most common are grants and state support, as well as sponsorship from research funds, councils and business organizations [26]. The importance of innovation for the public sector is growing. The US is characterized by funding of both fundamental and applied research. In most EU countries,

the government provides effective resourcing; private sector support is weak and irregular [27]. At the same time, there is joint performance assessment of both research institutions and agencies responsible for research development and funding [28]. Quantitative analysis approaches should be complemented with qualitative analysis approaches, particularly the criteria of efficiency and safety of innovative medical technologies [16].

Human resource development

Among all the performance criteria of institutions, evaluation of personnel potential in Russian medical research institutions receives the least number of indicators (only four). Data obtained in the end is insufficient for system analysis. However, the majority of performance indicators of any research institution and its competitiveness depend precisely on the human resource capacity of that institution.

By number of scientists, Russia is ranked 4th behind China, USA and Japan [5]. In recent years, a new theory accounts for a shift in approach relating staff to costs. This theory considers the staff as the most important resource of the effectiveness of any institution.

Qualitative and quantitative assessment indicators of human resource capacity reflect the degree of implementation of research programs, the effectiveness of the institution's structure and use of human resources, increase in productivity and quality of research. Using unrelated criteria with different analysis significance makes it difficult to obtain an objective final assessment of the entire institution [29]. Lack of strictly formal assessment of personnel potential of research medical institutions and databases, as well as qualitative factors affecting the result of activities pose challenges for the objective assessment of the sector [30]. Human potential assessment in foreign countries depends primarily on the status and quality of research activities of the scientist and research institution in the international community [31].

The US National Science Foundation applies scientific management to modernization of the staffing sector. They use lifelong learning, professional development motivation, manifestation of leadership skills and creative potential of employees [5, 32].

Enhancing the prestige of Russian science

Evaluation of indicators of integration into the global scientific space, dissemination of scientific knowledge and enhancement of the prestige of science for Russia is extremely urgent thanks to the obvious need to modernize this sector. Russians have recently been coming up with the idea of prestige of scientific work and academic status [33]. Socio-economic instability has led to sharp decline in the reputation of scientists. Owing to low wages, only about 9 % of Russians regard research profession as prestigious [5]. The ongoing loss of staff by many research institutions due to economic reasons reduces the efficiency of research and the overall level of institutions. This, of course, is reflected in the assessment of this sector. Translational barriers [34], unattractiveness of investment in medical science and lack of competitiveness of intellectual production when compared with economically developed countries are only a small list of problems to be addressed.

In most economically developed countries worldwide, public opinion polls clearly demonstrate the opposite results. According to statistics, about 51 % of respondents in the US believe that a career as a scientist is highly prestigious, 25 % said it is very prestigious, and 20 % consider it as prestigious [5]. International experience shows that high social status of the research elite reflects the socio-economic level of the country and the pursued information policy [33].

In the European Union, issues of protection of rights of not only those involved in clinical trials, but also the animals that take part in the experiments, as well as the problem of changes in the legislation are traditionally important for the medical research community. Some European and American scientific communities are in favor of public access to information on patients who participated in clinical trials with the aim of further analysis and receipt of reliable data for further diagnosis and treatment of a wide range of diseases, especially cancer. The European scientific elite, with support from one of the leading research organizations in Germany — Max Planck Society — is in favor of open access to scientific publications for any person in accordance with the Berlin Declaration on Open Access to Knowledge in the Sciences and Humanities. To draw attention to these issues, research societies and institutions, which are widely represented on the Internet and receive enough response from a non-indifferent population, are being created [35–37].

Resourcing the activities of a research institution

To ensure effective monitoring of Russian research and medical institutions, indicators responsible for resourcing of the activities of research institutions are among the most important [33]. In recent years, Russia has taken a number of measures to modernize her research institutions, which, in contrast to Western countries, has led to a reduction in the number of researchers in the research sector [38].

Mainly quantitative indicators, which are apparently formal in nature, are used to analyze the sector. The number of young scientists under the age of 39 is one of the consistently low indicators in many research institutions. Sociological studies showed that there is little interest among young people in research careers due to low pay, poor prestige of the profession, lack of research funding, poor social conditions and increasing bureaucracy [39]. The average number of young researchers does not exceed 25 % of the entire number of researchers [5].

Intangible assets in the modern Russian scientific institution are becoming an important criterion of the effectiveness of the organization. They are accounted for in the balance sheet as non-current assets. Exclusive intellectual rights of an institution contribute to monopolization of the right to use such rights, including to receive income from transfer of the rights to the industrial sector. Presence of innovative intangible assets allows to pay royalties, while adding the cost into the cost of the assets [40]. However, the actual use of these assets is small compared with other Russian science sectors. Patented technologies are often not used in production.

The salary of researchers is also an important factor in the effectiveness of the activities of scientific institutions and its final evaluation [41]. In most economically developed countries of the world, there is increased research funding, thereby attracting more researchers [33].

According to the US National Science Foundation, 60–65 % of funds are spent on salaries of researchers, graduate and undergraduate students, 12 % goes to the purchase of new equipment, 11 % is spent on education, about 6 % on technology transfer and only 1–3 % on administration [16, 42].

FINDINGS

In order to effectively implement the Strategy Of Development Of Medical Science In The Russian Federation For The Period Till 2025, approved by Order No 2580-r of the Government of the Russian Federation on 28 December 2012 [5], there is need to develop better performance criteria for evaluation of research medical institutions.

According to some researchers [43], mainly qualitative indicators resulting from examination should be used to evaluate the effectiveness of research institutions. However, most experts recommend building a model that factors in constant monitoring, assessment algorithms, creation and maintenance of a personalized database of employees' register [20]. Such an analytical model will form the final assessment of research institutions and will contribute to decision-making with regards to prospects. Therefore, it should include a systematic approach and have data accumulation, processing and storage functions [44]. Based on the above, at this stage of developing methods for research evaluation, the task of developing an objective approach that would obtain final performance indicators for Russian research health institutions is extremely important.

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