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Review

ASPECTS OF LYOPHILIZATION OF CARDIAC BIOIMPLANT

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The use of implants of biological origin in clinical practice has led to the search for methods of long-term storage of tissues without damaging their functional and structural characteristics. Xenografts (extracted from pericardium of pigs, horses, bulls) are drawing more and more interest. The bovine pericardium is exposed to chemical and physical factors providing complete purification of tissue from cells and their components. Such scaffolds are protein (collagen) complexes that fully replicate the microstructure of the pericardial tissue. Lyophilisation ensures long-term preservation of the extracellular matrix properties. The principle of the method is in drying pre-frozen tissue, in which water is sublimated. The method is intended for storage, transportation, and the subsequent use of the bioimplant in clinical practice. However, the lyophilization process may be accompanied by various undesirable factors that can lead to denaturation of the matrix protein or loss of its functionality and structure. To preserve the natural microstructure, stabilizers or various modifications (slow/fast freezing, reducing the degree of supercooling, etc.) of the lyophilization process are applied to biological prostheses. In this review, the main processes of lyophilization of biological tissue are described, which can affect the operation of a cardiac implant. A deep understanding of the parameters of the lyophilization process is crucial for creation of stable tissue grafts and their subsequent long-term storage.

Keywords: scaffold; lyophilization; tissue engineering; bovine pericardium; extracellular matrix.

Introduction

Modern treatments for heart failure are aimed at correcting (masking) symptoms and while the latest research strategies are aimed at eliminating the causes of their occurrence – repairing damaged myocardium or regenerating it, often regenerative medicine approaches are used. Despite recent advances in this field, few patients fully restore heart function. Today, the most effective treatment for patients with end-stage heart failure is cardiac allotransplantation [1, 2]. However, the number of patients requiring transplantation significantly exceeds the number of donor hearts [3]. Therefore, the development of alternative treatments for heart failure remains a top priority. One of the possible methods is the use of xenograft-derived scaffolds which is a decellularized matrix, these scaffolds are used to replace or support damaged heart tissue. The decellularized scaffold can be applied to the surface of the heart to prevent, or even regress, the spread of heart damage. Also, grafts that are to a certain extent populated with cells and delivered to the site of injury can help restore lost heart cells and further recovery. An ideal scaffold should be compatible with all types of heart cells, provide mechanical strength in the right place, properly order cells and transmit biochemical signals for the proper functioning of cells in the heart [4]. Sources of such scaffolds can be biological or synthetic materials, each of which has its own advantages and disadvantages. Synthetic materials are not always biodegradable and often do not meet the requirements for the attachment and infiltration of cells in the vessels and parenchyma; however, it is convenient to make grafts of almost any size and shape from them [5].

Unlike synthetic scaffolds, biological ones, which usually originate from the extracellular matrix (ECM), support the transmission of biochemical signals necessary for cell migration, proper positioning, and differentiation. However, they can be mechanically unstable when exposed to physical conditions (sterilization, lyophilization, etc.).

One of the widely used methods for the preservation of donor tissues is lyophilization – a method of drying pre-frozen tissue intended for long-term storage and its further use (after rehydration) in clinical practice. The peculiarity of this method lies in the fact that drying occurs during the transition of water from a solid state to steam, bypassing the liquid phase [6]. Drying of tissues during lyophilization leads to dehydration of the amorphous matrix surrounding the collagen fibers. As a result, certain structural transformations of the biotechnologically transformed tissue matrix occur [7]. For the first time, a systematic study of the properties and qualities of lyophilized grafts

took place in the 1950s of the last century. Some of the first researches devoted to tissue lyophilisation belong to E.W. Flosdorf [8]. To date, a fairly large number of studies have been devoted to comprehensive research of lyophilized graft architectonics and biomechanical properties [9, 10]. Scientists have proven that during lyophilisation tissues acquire resistance to environmental factors and the ability to maintain a complex of structural and biochemical properties that are important for the transplantation [11, 12]. In addition, lyophilisation of xenograft is necessary prior to gamma-ray sterilization to avoid substantial changes in the tissues without dehydration that make impossible their clinical use. The lyophilisation process does not significantly affect the structure of tissues such as bone, cartilage and pericardium [13].

Lyophilized bovine pericardial transplants treated with glutaraldehyde are used in surgery for heart valve replacement. The use of such scaffolds often leads to implant calcification. However, some authors report that lyophilisation before chemical treatment reduces inflammation and prevents calcification, as well as not changing the mechanical characteristics of the biomaterial; it does improve its immune properties, and allows the graft to be preserved for future use [14].

Scientific advances in tissue engineering make it possible to create grafts using various chemical and physical methods. At the same time, the influence of the lyophilisation process on the properties of new grafts requires further study, which determines the relevance of the selection of the optimal parameters and reagents of the method (Figure). The structure of the lyophilisation process can have a significant impact on tissue stability. To reduce structural damage and preserve the natural properties of the fabric, it is necessary to analyse the freezing rate and temperature, drying rate and moisture content in stages. Therefore, the purpose of this review was to consider the main processes of lyophilization and its effect on biological tissues.

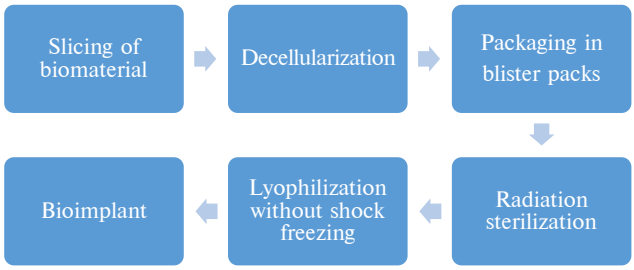

Figure: Scheme of creating a lyophilized bioimplant

Modern approaches to long-term storage of biological tissues

Lyophilisation is one of the most effective techniques for the long-term storage of tissues for surgical use. The peculiarity of this method is that drying takes place during the transition of water from a solid state to vapour without a liquid phase [15]. This phenomenon of evaporation of a solid without melting is called freeze drying. The displacement of moisture from a frozen state of biological tissue is due to the property of water to pass from the state of ice to the vapour state at temperatures below zero, under the influence of vacuum and at a pressure of less than 4.6 mm Hg, that is, the solid passes into vapour without the formation of an intermediate liquid state. It is important that during lyophilisation to create conditions that regulate tissue temperature and water vapour pressure to ensure a continuous transition of ice to steam. The crystallization temperature is usually determined by measuring the conductivity of an electric current [16, 17]. Dehydration is carried out by maintaining the equilibrium of the concentration of water vapour in the tissue and the environment. If the space around the tissue is released from water vapour, their loss is compensated by the sublimation of an appropriate amount of water vapour from the frozen tissue [18].

Freeze drying of biological tissue (homo- or heterograft) aims to create an implant bank for use in modern cardiac surgery. Lyophilisation ensures optimal preservation of all functional properties and architectonics of the extracellular matrix (ECM) of the cardiac implant. This method avoids the problem of storing frozen tissues due to the transition of the tissue into a dry form. Lyophilisation is used for storage of bio-prostheses and in tissue engineering [19, 20].

The lyophilisation process includes the stages of freezing, primary and secondary drying, taking into consideration stability of the protein, the characteristics of the inert filler, and the critical temperatures of the structure [21].

Freezing is the initial stage using several such freezing agents as liquid nitrogen, liquid oxygen and dry ice. When freezing biological materials, the cooling rate plays an important role, on which the size of the ice crystals depends. With fast freezing, a fine-crystalline structure of the frozen tissue is formed, with slow freezing, a coarse-crystalline one is formed. The formation of ice crystals and their

size depends to a large extent on the degree of hypothermia, the start point of crystallization temperature and of cooling rate [22–24]. In order to obtain frozen tissue without microscopically visible crystals, the temperature must be reduced every second by 10–20 °C, since slow freezing allows water to crystallize [25]. The cooling rate of biological tissue for conservation depends mainly on the cooling temperature, the nature of the environment and the size of the tissue being processed [26]. Under optimal conditions, freezing forms large ice crystals with minimal surface area.

Annealing is a stage of the freezing process during which the product temperature is kept higher than the final freezing temperature for a sufficiently long time. Annealing promotes the crystallization of inert fillers (mannitol and glycine), which in turn prevents the breakdown of matrix proteins and strengthens their storage stability [27–29].

Primary dehumidification. The next stage is the evaporation of the solution from the ice, which occurs at an increasing product temperature, but within the limits below the critical temperature (glass transition temperature and collapse temperature). This process can be controlled by the pressure in the lyophilisation chamber providing that the pressure in the chamber is lower than the pressure of saturated water vapour at the required temperature [30]. The difference between the pressure in the chamber and the vapour pressure serves as the driving force for the sublimation reaction. Parameters of the primary drying process, such as temperature and duration, heating intensity, pressure in the chamber, can affect the quality of the final product.

Secondary dehumidification. Residual moisture is removed by secondary dehumidification using desorption. Compared to the primary process, the product temperature is higher and the chamber pressure is lower [31]. When heating during primary drying to the temperature at the beginning of secondary drying, excessive increase heating intensity should be avoided to prevent both protein denaturation and product polymerization [32]. In general, it is recommended to set a high temperature for a short period in order to reduce the lyophilisation duration. The temperature, the pressure in the chamber and the duration of secondary drying must be optimized taking into account the required moisture content of the dried product, which is necessary to preserve the protein structure [33].

Unfavourable aspects during lyophilisation of biological tissues

Biomaterials for implants in cardiac surgery are an extracellular matrix (collagen protein) purified from cells and their components. The structure of water around collagen was investigated by various methods [18]. It has been shown that water plays an important role in maintaining the microstructure of the collagen matrix and provides the mechanical properties of collagen fibrils [34]. Water can interact with collagen in three states: free, bound, or structural. Free water fills the space between the microfibrils and the protein fibrils. At the same time, the bound water stabilizes the collagen double helix. Structural water is responsible for the stabilization of the protein triple helix by forming hydrogen bonds between the helix [26]. These differences lead to a significant effect on the dynamics of water removal and the composition of residual moisture in dry preparations. The amount of bound water, as well as the strength of its binding to the substrate that is measured by the binding energy in different preparations, can vary widely depending on the type of tissue that is subjected to conservation [18].

Water molecules coming out of tissue can behave in different ways. Very often, water molecules from the inside of the tissue turn back into ice on the surface of the tissue. In other cases, water molecules freely leave the tissue and are retained by the condenser of the lyophilizer. The resistance that water molecules meet on their way is due to the shape and size of the tissue and the thickness of the dried layer. The thinner the tissue, the smaller its size and the faster it cleans from the water. An important factor in tissue dehydration is its residual moisture, which affects the preservation of the morphological structures of the tissue that is being preserved. Residual moisture is defined as the percentage between the weight of the native and lyophilized biological tissue. The acceptable degree of dehydration for vessels is a threshold within 5% of residual free water [35].

At the end of the first drying period, when the free water is completely removed, the temperature rises to about 0 °C. The residual moisture of the tissue, due to the presence of the bound water, remains at 5–10% of the dry mass. This moisture could not be removed under the physical conditions that are characteristic of the first drying period. In the second period, when the temperature of the material rises to +30 – +40 °C, conditions

are created for the water removal, which is bound to the substrate by stronger bonds. Until the end of the second period, the residual tissue moisture usually decreases to 1-2% [36].

Thus, the lyophilisation process can significantly damage the microstructure of the ECM, which is important for the effective operation of the biological prosthesis. At this time, various chemical and physical methods are used to preserve the structure of the matrix after freeze drying. One of the widely used methods is the use of glutaraldehyde (GA) as a crosslinking agent. GA stabilizes the collagen structure, prevents the destruction of tissues by enzymes or bacteria, and also reduces the antigenicity of the material [37]. However, the use of GA may result in uneven collagen linking (surface linking), which may result in the formation of multilayer material and, as a consequence, a limitation of the mechanical properties of the valve leaflets under cyclic loads. M. Lopez-Moya *et al.* mention that the main controversial aspect of this method is the appearance of a progressive calcification process leading to deterioration in the structure of the bio-prosthesis and loss of its function [38]. To eliminate the process of calcification of bio-prostheses, several methods have been proposed, such as: treatment with heparin, hyaluronic acid coatings and photochemical crosslinking [39].

Complete rejection of GA is possible with cross-linking using soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and an organic substance derived from proline (N-hydroxysuccinimide). Stabilization of the "cross-linking" process is carried out in the MES buffer (2-(N-morpholino) ethanesulfonic acid). The effectiveness of this technique was positively assessed according to the results of the biomechanical properties of the implant [40].

However, the successful course of the lyophilisation process depends not only on the presence of a stabilizing agent, but also on factors such as crystallization, pH shift, formation of ice crystals and so on. A change in the chemical equilibrium in the system during lyophilisation can lead to protein denaturation and loss of its biological activity.

Freezing can cause structural and conformational changes in the protein, which are usually reversible. During lyophilisation, a two-phase matrix is formed, consisting of ice and a frozen matrix, containing solvent molecules and a fraction of water that is not frozen. The solidification of the frozen matrix leads to separation into a phase without ice and a phase with ice [41]. Protein products may become unstable during freezing and crystallization. The cause of instability during lyophilisation is incomplete crystallization of cryoprotectants.

Crystalline inert fillers such as mannitol and glycine must be completely crystallized upon freeze-drying because complete crystallization of mannitol can result in further crystallization during storage, which, in turn, can lead to loss of protein stability [42]. Taking into account the properties of crystallization and characteristics of metastable forms during transformation is a prerequisite for the development of stable protein products [43]. A review of previous publications suggests that crystallization with cryoprotectants is one of the main protein destabilizing factors. Thus, the choice of cryoprotectants with a low tendency to crystallization can be a solution to ensure stability and preserve the biological activity of the protein in the frozen state [44, 45].

Protein products are stable at isoelectric pH values between 6 and 7. At higher values, the repulsion of the same charges of the protein molecules occurs, which causes its denaturation or unfolding. The change in pH in a frozen product can be observed during freezing of components which contain less soluble buffer components. During freezing of a protein product with such buffer components as sodium phosphate and succinate, their possible crystallization results in a decrease in pH by 3 units, which significantly destabilizes proteins [46, 47]. When such salts as sodium or potassium phosphates are used as buffering components, the difference between the freezing point of mono-ionized (salt) and non-ionized (free acid or base) samples causes one component to freeze earlier than the other, leading to destabilization of proteins (denaturation or conformational changes) [48].

Thus, biological tissues dried by the freeze-drying method differ significantly in their properties from similar tissues dried from a liquid state. A characteristic feature of lyophilized tissues is their almost complete keeping the original volume turning into a dry sponge. Before use, lyophilized tissues are placed in sterile saline solution, which may contain antibiotics (penicillin, streptomycin, gentamicin) for further rehydration. The essence of rehydration is that the lyophilized tissue absorbs moisture and again acquires the properties characteristic of the original state. With the correct rehydration regimen, the physicochemical, plastic and structural features of the pericardium can be restored.

Lyophilized tissue when packed under vacuum, can be stored indefinitely both in a refrige-

rated state and at room temperature. However, today, according to the standards, freeze-dried xeno- and allografts are recommended to be stored for no more than 5 years. Transportation and storage of such tissues are also carried out without any special conditions and reservations [35].

Conclusions

According to the analytical review, it was determined that today a large number of biocompatible materials are used to create implants, but none of them are ideal, therefore, the search for new materials for plastic surgery of cardiovascular tissue is relevant at the current stage of tissue engineering development. Increasing preference is given to natural matrices, in particular an extracellular matrix based on the bovine pericardium, which has advantages over synthetic analogues. The production of xenograft-derived cardiac prostheses requires guaranteed shelf life and transportation conditions. Therefore, methods of long-term storage of bio-prostheses are being actively developed, which can provide storage without functional and structural changes in the implant. The most promising method for today is drying a bio-prosthesis by lyophilisation, which creates conditions under which biological tissues undergo minimal chemical changes. A lyophilized cardiac implant under vacuum packing can be stored indefinitely and does not require special transportation conditions. The possibility of long-term storage of lyophilized bio-prostheses for cardiac surgery, ease of transportation provides the opportunity to create a large supply of biomaterial. This makes it possible to create an almost unlimited supply of valuable material that can be transported at any time and over unlimited distances. Therefore, this method of preserving biomaterials in our time is considered to be one of the most promising and convenient for practical purposes and tasks.

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ПРИНЦИПИ ЛІОФІЛІЗАЦІЇ БІОЛОГІЧНИХ КАРДІОІМПЛАНТІВ

Використання в клінічній практиці імплантів біологічного походження обумовило пошук методів довготривалого зберігання тканин без пошкодження їх функціональних і структурних особливостей. Усе більший інтерес викликають імпланти, виготовлені з ксенотканин (перикарда свиней, коней, биків). Бичачий перикард піддають дії хімічних і фізичних чинників, що забезпечують повне очищення тканини від клітин та їх компонентів. Такі скафолди являють собою білкові (колагенові) комплекси, що повністю відтворюють мікроструктуру перикардіальної тканини. Ліофілізація забезпечує збереження властивостей екстрацелюлярного матриксу протягом тривалого часу. Принцип методу полягає у висушуванні попередньо замороженої тканини, за якого вода повністю сублімується. Метод призначений для зберігання, транспортування та подальшого використання біоімпланта в клінічній практиці. Однак процес ліофілізації може супроводжуватися різними несприятливими чинниками, що можуть призводити до денатурації білка матриксу або втрати його функціональності та структурності. Для збереження природної мікростурктури біопротеза використовують стабілізатори або різні модифікації (повільне/швидке заморожування, зменшення ступеня переохолодження тощо) процесу ліофілізації. В нашому огляді розглянуто основні процеси ліофілізації біологічної тканини, що можуть впливати на роботу кардіоімпланта. Глибоке розуміння параметрів процесу ліофілізації має вирішальне значення для створення стабільних тканинних трансплантатів із подальшим їх довготривалим зберіганням.

Ключові слова: скафолд; ліофілізація; тканинна інженерія; бичачий перикард; позаклітинний матрикс.

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ПРИНЦИПЫ ЛИОФИЛИЗАЦИИ БИОЛОГИЧЕСКИХ КАРДИОИМПЛАНТОВ

Использование в клинической практике имплантов биологического происхождения привело к началу поиска методов длительного хранения ткани без повреждения структурных и функциональных особенностей. Большой интерес вызывают импланты, изготовленные из ксенотканей (перикарда свиней, лошадей, быков). Бычий перикард подвергают воздействию химических и физических факторов, обеспечивающих полную очистку ткани от клеток и их компонентов. Такие скаффолды представляют собой белковые (коллагеновые) комплексы, которые полностью воспроизводят микроструктуру перикардиальной ткани. Лиофилизация обеспечивает сохранение свойств экстрацеллюлярного матрикса в течение длительного времени. Принцип метода заключается в высушивании предварительно замороженной ткани, при котором вода полностью сублимируется. Метод предназначен для хранения, транспортировки и последующего использования биоимпланта в клинической практике. Однако процесс лиофилизации может сопровождаться различными неблагоприятными факторами, которые могут приводить к денатурации белка матрикса или потере его функциональности и структурности. Для сохранения природной микростурктуры биопротеза используют стабилизаторы или различные модификации (медленное/быстрое замораживание, уменьшение степени переохлаждения и т.д.) процесса лиофилизации. В данном обзоре рассмотрены принципы процесса лиофилизации биологической ткани, которые могут влиять на работу кардиоимпланта. Глубокое понимание параметров процесса лиофилизации имеет решающее значение для создания стабильных тканевых трансплантатов с последующим их длительным хранением.

Ключевые слова: скаффолд; лиофилизация; тканевая инженерия; бычий перикард; внеклеточный матрикс.

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Review

HUMAN GUT MICROBIOME AS AN INDICATOR OF HUMAN HEALTH

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The undeniable achievement in the study of the gut microbiome as an association of different microorganisms, including viruses, that colonize various organs and systems of the body, is the establishment of the fact that some diseases that were consmicrobiotaidered as non-infectious can also be transmitted through microorganisms. This resulted in the gut microbiome being called a forgotten organ that could serve as an additional and kind of missing link for a more objective and better diagnosis and treatment of many diseases that were not considered infectious. The rapid development of gut microbiome research in recent years not only is connected with better understanding of the functioning of the microbiome by the scientific community, but also inseparable from the strategic support of each country. Global investment in researches, related to the human microbiome, has exceeded $1.7 billion over the past decade. These researches contribute to the development of new diagnostic methods and therapeutic interventions. Our review is dedicated to the analysis of the possibilities of application of the human gut microbiome for the diagnosis of diseases, and the role of the intestines in the provocation and causing of certain diseases. Significant differences in the composition and diversity of the human microbiome are shown depending on geographical location and the change of socio-economic formations towards a gradual decrease in the diversity of the gut microbiome due to three stages of human population's existence: food production, agriculture and industrial urban life. We analyze the influence of dietary patterns, various diseases (including malignant neoplasms) and viral infections (in particular, coronavirus) on the gut microbiome. And vice versa – the influence of the gut microbiome on the drugs effect and their metabolism, which affects the host's immune response and course of the disease.

Keywords: human intestine; gut microbiome; coronavirus; immune response; drug metabolism.

Introduction

The human microbiome is an association of different species and types of microorganisms, including viruses, that colonize various organs and systems of the body, ranging from the skin and oral cavity to internal organs: respiratory tract, gastrointestinal tract, urinary tract, reproductive tract, etc. [1].

Over the past century, the world's scientific databases have accumulated enormous factual experimental material on research in various fields of microbiology, including (over the past few decades) research on the gut microbiome. If we imagine the volume of information on the gut microbiome, we can state the following: according to database Web of Science Core Collection, by 2005 the number of relevant publications was just over 500 per year while in 2019 alone over 9,500 publications have been published (over 2,000 are highly cited ones) 449 journals. The top 15 journals with the most cited articles on this topic are: Nature, Gut, Science, PNAS, Cell Host&Microbe, Gastroenterology, Cell, PloS One, ISME Journal, Nature Communications, Nature Reviews Microbiology and Nature Reviews Gastroenteric Medicine [2].

Major countries that have made contributions to gut microbiota researches are the USA, China, the UK, Germany, France, Canada, Italy, Japan, Spain, the Netherlands and Australia.

An undoubted achievement in the study of the gut microbiota is the establishment of the fact that some diseases, which earlier were considered to be non-infectious, can also be transmitted through microorganisms. This led to the gut microbiome being called a forgotten organ that could serve as an additional and even a missing link for more objective and better diagnosis and treatment of many diseases that were not considered infectious [3]. As a result, in recent years more than $3 billion has been invested in scientific research, related to the study of the gut microbiome [2].

In this artivle, we are aimed to present theoretical analysis of scientific information on the gut microbiome, which was published for the last 5 years. Acquisition and forming of knowledge in this field of medical microbiology will enable to use the obtained information more quickly and professionally for the purposes of detection and effective treatment of some diseases considered to be non-infectious. To achieve the set goal, it is necessary to perform the following objectives:

– to analyze and establish the relevance of research basing on key financial indicators and the participation of different countries in research and publications on the topic of human gut microbiome;

– to highlight the general characteristic of the human microbiome and give its species composition and structure;

– to describe the external and internal influences on human gut microbial diversity;

– to analyze the impact of microorganisms from the environment on the human body and establish their role in the pathophysiology of diseases of different spectrum, as well as to outline methods of correction of dysbiotic conditions;

– to create a holistic view of practical advances in human gut microbiome research.

Identification of patterns of existence, development and interaction of certain types of microorganisms of the gut microbiome, that can affect the physiological state of the host in dependence to certain diseases and various pathological conditions, depends on the composition and structure of the human microbiome. The systemization and summarizing of the results, obtained in this study of the gut microbiome, makes it possible to identify its role in biological processes occurring in the human body.

General characteristics and composition of the gut microbiome

The composition and density of the human microbiota differs significantly from organ to organ and in different parts of the organ system. For example, the upper respiratory tract is more densely populated than the lower. The gastrointestinal tract (GIT), the stomach, duodenum and ileum (the lower part of the small intestine) are characterized by low density of microbial population, while the small intestine, cecum and large intestine are quite densely populated [4].

There is an outdated information that the ratio of "own microorganisms" (normal microbiota of human organs, existing pathogenic and conditionally pathogenic microorganisms in the body) and the human cells is 10:1. However, a refined estimation of the quantitative ratio of "human microorganisms" to the total number of macroorganism cells actually showed that there is 1 human cell for every 1.3 microorganism cells [5]. We should note that this approach to quantitative counting of human microbiome does not take into account fungi, viruses and phages present in various biotopes and, in the case of viruses and phages, may be equal to the number of bacteria or, according to [6], may exceed the number of the latter by at least an order of magnitude. Despite the fact that more accurate counts somewhat reduce the degree to which the number of microbial cells exceeds the number of human cells, the results of counts do not reduce the level of influence of human microbiome associated with the diversity of microbial life on the organism.

The species composition of the human microbiome is very diverse. An approximate estimate (and comparison) of 1,000 species of intestinal microorganisms with 2,000 genes per species (microorganism) was made. And it made possible to estimate 2,000,000 genes. This figure is 100 times higher than the usually assumed number of about 20,000 human genes.

As already noted, the intestines of a healthy person contain a number of bacterial cells roughly comparable to the number of cells that make up his body. It has been experimentally shown that an average fecal sample contains up to 700,000 bacterial genes, which is about 38 times more than the genes expressed by the human genome. Based on these data, it can be assumed that the metabolic capacity of the entire gut microbiota may exceed the metabolic capacity of the host organism. However, there is no numerical evidence in this sense yet [7].

If we consider the microbiome of an individual, it is estimated that 150 to 400 species live in the intestines of each individual [8]. Typically, most of these species belong to the following phyla: *Bacteroidetes* (consist of three large classes whose representatives are widely distributed in the environment, including soil, marine sediments, seawater and animal intestines), *Firmicutes* (a division of bacteria, most of which are Gram-positive, some have no cell wall at all and are not Gram-stained, but also have no outer membrane, found in other Gram-negative forms), *Actinobacteria* (gram-positive bacteria that are high in guanine and cytosine DNA and have a fungal-like mycelial structure, the largest subgroup is Actinomycetes) and *Proteobacteria* (a group of bacteria identified by their ribosomal RNA (16S rRNA) sequence, the most numerous group of bacteria, comprising 1,534 species, or about one-third of all known bacterial species).

The relative proportions of each of these taxa vary greatly between individuals [9] and even within an individual during his or her lifetime [10]. It is known that the microbiome of each individual is unique; however, when studying the microbiome of different human populations, several trends have been identified, as shown in Table 1 [11].

Table 1: Prevailing types and classes of human microbiome bacteria. Adopted from [11]

The bacterial type	Class	Example	Localization	Characteristics
Actinobacteria	Acidimicrobia, Actinobacteria, Coriobacteriia, Rubrobacteria, Thermoleophilia, Nitriliruptoria	*Corynebacterium, Mycobacterium, Nocardia, Bifidobacterium, Streptomyces*	Intestine, oralcavity, skin	Gram-positive, filamentous, physiologically aerobic, can be heterotrophic or chemoautotrophic, but most are chemoheterotrophic and can use a wide range of food sources.
Bacteroi detes	Bacteroidia, Flavobacteria Sphingobacteria	*Bacteroides, Prevotella*	Intestine, oralcavity	Aerobic and anaerobic, non-sporulating, Gram-negative bacilli
Firmicutes	Bacilli,Clostridia, Erysipaelotrichia, Thermolithobacteria, Negativicutes	*Clostridium, Staphylococcus, Enterococcus, Lactobacillus* spp	Bowel, skin,stomach	Gram-positive, bacilli,cocci, spiral-shaped,anaerobic, aerobic, include comensal and beneficial bacteria
Proteobacteria	Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria	*Escherichia, Salmonella, Vibrio, Helicobacter, Yersinia, Legionellales*	Colon, skin	Gram-negative bacteria

The human digestive tract is 6.5 m long and consists of three organs: the stomach, small intestine and large intestine; however, most studies of the human microbiome have focused on the microbial association of the large intestine. Each milliliter of the large intestine (chyme) contains approximately 10^{11} microbial cells compared to 10^{8} cells in the small intestine [12].

Most of the information about the human gut microbiome was obtained through the following projects: the Human Microbiome Project (HMP) and the Human Gut Metagenomics Project (MetaHIT), funded by the US National Institutes of Health and the European Commission, respectively. However, it should be noted that the results obtained for the quantitative and qualitative composition of the gut microbiomes of the different countries populations, within the framework of the above projects, differ from each other in several parameters [13].

When comparing the quantitative and qualitative indicators of microbiomes, it was shown that their composition is more similar to each other within the same segment (e.g., oral cavity, small or large intestine) of different people than microbiomes of different segments of the same person. As for the individual representatives of microorganisms in different segments of the human body, it was shown that the oral cavities are inhabited by various representatives of the microbiome and, as a rule, they are dominated by *Streptococcus* spp. Skin areas are distinguished primarily by local skin properties (dry or wet) and are mainly inhabited by *Corynebacterium*, *Propionibacterium* and *Staphylococcus* species. A healthy vagina contains representatives of the genus *Lactobacillus* (a genus of gram-positive facultatively anaerobic bacteria that convert lactose and other sugars into lactic acid – *L. crispatus*, *L. iners*, *L. jensenii* or *L. gasser*) [14]. A significant indicator of the state of female microbiota is their belonging to different races and ethnic groups, although even in this structured ecosystem intra-organismal variations are significant and to date have no fully explained causes. During human ontogenesis, the gut microbiome is formed under the influence of various factors. The most interesting and indicative is the variation of microbiome representatives depending on gestational age, method of newborn birth and method of feeding and human age [15]. The data of these studies are shown in Table 2.

Some important human segments usually have a particularly low microbial biomass in healthy individuals and therefore are more difficult to characterize. The lungs, for example, are almost sterile in the absence of infection or chronic disease, which leads not only to much interest in identifying their residents, but also includes considerable technical difficulties in sampling from these segments, so large-scale carefully controlled studies are needed to establish the functionality of these complex habitats of low-density microorganisms [16].

Table 2: Variation of microbiota depending on some exposure factors. Adopted from [15]

		Actinobacteria	Bacteroidetes	Firmicutes	Proteobacteria
Gestational age	Preterm birth (<37weeks gestation)	*Bifidobacterium* spp.↓ *Atopobium* spp.↓	*Bacteroides**↓	*Firmicutes**↓ *Lactobacillus*↑ *Ruminococcus* spp. *Lachnospiraceae** *Peptostreptococcaceae** *Clostridiaceae**	*Enterobacteriaceae**↑ *Enterococcus* spp.↑
	Premature babies	*Bifidobacterium* spp.↑	*Bacteroidetes**↑	*Ruminococcus spp.* *Lachnospiraceae** *Peptostreptococcaceae** *Clostridiaceae**	*Enterobacteriaceae**
	Vaginal birth	*Bifidobacterium* spp.↑, *Bifidobacterium catenulatum*↑ *Bifidobacteriumlongum*↑	*Prevotella*↑ *Bacteroidesfragilis*↑	*Lactobacillus*↑ *Staphylococcus*↑ *Streptococcus*↑	*Escherichia*↑
Method of delivery	Caesarean section	*Corynebacterium*↑ *Propionibacterium*↑	*Bacteroides**↓	*Staphylococcus* ↑	*Escherichia*↓ *Shigella*↓
Feeding methods	Breast milk	*Bifidobacterium*↑↑		*Lactobacillus*↑ *Staphylococcus*↑	*Enterococcus*↑
	Milk substitutes	*Bifidobacterium*↑	*Bacteroides*↑	*Clostridium*↑ *Clostridium difficile*↑ *Lactobacillus*↑	*Escherichia*↑
	Solid food	*Bifidobacterium*↑	*Bacteroidetes**↑ *Bacteroides*↑	*Firmicutes**↑ *Lactobacilli*↑ *Clostridiumcoccoides*↑	
Human age	The first year of life	*Bifidobacterium*	*Bacteroides*	*Veillonella,* *C. coccoides,* *C. botulinum*	
	From 2-3 years old to adult	*Bifidobacteriaceae** *Coriobacteriaceae**	*Bacteroidaceae** *Prevotellaceae** *Rikenellaceae**	*Lachnospiraceae* *Ruminococcaceae*	*Proteobacteria**
	Over 70 years old.	*Bifidobacteriaceae*↓		*Clostridium**↓	*Proteobacteria**↑

Notes. *Unknown genera, ↑ increasing, ↓ decreasing.

Differences in the composition and diversity of the human microbiome depending on the geographical location

An important component of the study of the human microbiome is a comprehensive characterization of the microbiota of a healthy person, It is necessary for comparison and for the establishment of deviations from the norm during a disease. No less important is the establishment of indigenous (normal) microbiota of practically healthy people depending on their race and ethnicity. And, as recent studies have shown, there are significant differences in the structure of the microbiome of such population groups [17]. However, one should be aware that these data cannot be completely accurate because of the huge diversity of situations that can affect the final outcome of studies. But to draw an overall picture of the microbiomes of healthy humans of different origins, this information is certainly useful.

It is also indisputable that the change of socio-economic formations gradually influences the qualitative and quantitative composition of both the general human microbiome and (to a greater extent) the gut microbiome. These changes were accompanied by a gradual decreasing in the diversity of the microbiome, especially the gut microbiome. These phenomena can be explained by the fact that human populations have passed through three stages of existence, such as food extraction, agriculture and industrially developed urban life (if we count only purely industrial urban life; if we add to these figures the microbiomes of people engaged in agriculture, the overall picture changes somewhat).

Researchers compared the diversity and composition of the gut microbiota of people of three different modes of existence:

– remote hunter-gatherer populations such as the Hadza (an indigenous people living in northern Tanzania – Arusha, Singida, Shinyanga regions, around Lake Eyasi); Pygmies (a group of stunted – average height of adult males is less than 150 centimeters – negroid peoples living mainly in equatorial forests of Africa) and Indians (common name for the indigenous population of America from Venezuela, except for Eskimos and Aleuts, that was given to them by the navigator Christopher Columbus who discovered the continent India) [18, 19];

– traditional Bantu farming or fishing populations (a group of Central and Southern African peoples, the largest among them Rwanda, Makua, Shona, Congo, Malava, Runda, Zula, Kosa and others); Tunapuko (South American Indian) people in the Andes mountain regions or rural Malawian (small Malawian people, about 19,000 people) communities [20];

– representative group of western (US/European) urban industrialized society[21].

Hunter-gatherer populations mainly prefer starchy foods such as cassava tubers, plants, nuts, game and honey for sustenance. These remote gatherers suffer from multiple gastrointestinal microbial and parasitic infections. They have limited or no access to modern health facilities [22].

In contrast, the diet of traditional agriculturalists is similar to that of Neolithic people, when they moved from a nomadic lifestyle to a sedentary one, followed by the cultivation of food crops, domestication of animals, fishing and trade at a later stage of existence.

The diet of inhabitants of US or European metropolitan areas is high-protein and high-fat. This is accompanied by highly developed diagnostic, therapeutic and preventive medicine.

The results of these studies suggest that the factors of influence in the form of geographical affiliation of individuals can determine the causes of disease of different origins due to the fact that when environmental factors change, the gut microbiota adapts to new conditions by changing its qualitative and quantitative composition almost immediately [23].

The impact of diet on the gut microbiome

Numerous studies suggest that food can rapidly alter the qualitative composition of the gut microbiota. A high-fat diet, or the so-called "Western" diet, is generally considered harmful to the brain [24]. Excessive consumption of high-fat foods is associated with an in the number of microorganisms of the following phyla: *Firmicutes* (mostly Gram-positive bacteria) and *Proteobacteria* (see above) and a decrease in the number of *Bacteroidetes* (consists of three large classes, representatives widely distributed in the environment, including soil, marine sediment, seawater, and animal intestines). Such food also increases plasma and fecal acetate levels, triggers supersynthesis of insulin and ghrelin, and further promotes overeating [25]. The effects of obesity and inflammation caused by fatty foods may be reduced by polyphenols from fruits, accompanied by an increase in the number of *Akkermansia muciniphila* (a type of mucin-degrading human gut bacterium).

Switching to a high-fat or high-sugar diet from a low-fat or high-fiber diet can change the microbiome even in one day. A large numbers of *Bacteroides* are associated with the consumption of animal protein and saturated fats, while an increase in *Prevotella* (*Prevotella* spp. is part of the normal microbiota of the mouth, upper respiratory tract, vagina and other human organs, characteristic of the stomach of healthy people, infected and uninfected *Helicobacter pylori*) is associated with foods rich in carbohydrates and simple sugars. A vegetable-based diet increases the amount of short-chain fatty acids, which is accompanied by increased amounts of *Prevotella* and some *Firmicutes* that degrade fiber [26]. When food with fructose is consumed, the level of *Bacteroidetes* significantly decreases, while the number of *Proteobacteria*, *Firmicutes* and pathogenic *Helicobacteraceae* significantly increases [27].

Food derivatives and low-molecular-weight metabolites fermented by microorganisms are released by the gut microbiota into the bloodstream, which carries them throughout the body and further contributes to various diseases, including brain diseases [28].

The human microbiome and diseases

Determining the etiology of certain diseases associated with general and gut microbiota imbalance is important both in terms of treating these diseases and in terms of identifying the cause and effect. The causes of many diseases according to some researchers are "...physiological interactions between microbial groupings through physiological interactions between individuals..." [29], which is

Table 3: Changes in gut microbiota and pathological status of organs [29]

Organ	Examples of diseases associated with altered microbiota	Microbiota-mediated changes
Brain	Autism spectrum disorders	Increased bacterial toxins,impaired fermentation
Lungs	Asthma, cystic fibrosis	Reduced immunologicaltolerance, altered gene expression
Heart	Cardiovascular diseases	Synthesis of proinflammatory metabolites
Pancreas	Type 1 and 2 diabetes	Reduced insulin sensitivity
Liver	Non-alcoholic fatty liver disease	Altered bile acid metabolism
Fatty tissue	Metabolic syndrome, obesity	Reduced intestinalgluconeogenesis, insulin resistance
Gastrointestinal tract	Inflammatory bowel syndrome, irritable bowel syndrome,intestinal infections	Dysregulated immune response,altered mucociliary barrier
Leather	Acne, eczema, allergic diseases	Increased pathogenic strains, dysregulated immune response

true to some extent. It is further argued that many diseases are associated with a departure from a "healthy" gut microbiome. These include metabolic disorders, inflammatory and autoimmune diseases, neurological conditions and cancer. Certain gut-related conditions (e.g., obesity and inflammatory bowel disease) have been extensively studied in human cohorts and in animal experiments, where significant, and sometimes causal, changes in microbial associations have been found. These studies have stimulated research into a number of complex diseases with unclear etiologies in which the microbiome is suspected to be associated [29]. Table 3 summarizes the data on the effect of altered qualitative composition of the gut microbiota on some diseases.

It is assumed that over the next few years there will be fundamentally new methods of treatment of various diseases caused by an altered gut microbiome. It is likely that the European Medicines Agency and the Food and Drug Administration will require all licensed medicines to be profiled for their effect on the gut microbiota, which is essentially a virtual organ. If a medicine damages this virtual organ, it cannot be licensed [30].

Gut microbiome and malignant tumors

Recently, there have been a growing number of researches on the influence of gut microbial groups on the risk of malignancy. The main purpose of these studies is to answer the question: does a tumor provoke a change in the qualitative composition of the gut microbiota? Numerous studies of this problem have yielded to proposal to divide the relationship between cancer and microbial communities into three categories: primary, secondary and tertiary categories of relationship.

Primary relationships are interactions (or influences of the microbiota) in the proximal (located closer to the center) tumor microenvironment. These interactions are important for understanding the mechanisms of microbiome-tumor relationships. As established for the *H. pylori* model, tumor can arise from microbial infections. In addition, products of the Gram-negative genus *Fusobacterium* (a genus of fibrous, anaerobic bacteria, similar to members of the *Bacteroidetes* fylum; individual species of this genus cause several human diseases) have been shown to be associated with the tumor microenvironment. Oncogenesis caused by *Fusobacterium nucleatum* is thought to result from opportunistic infection followed by chronic inflammation and immunosuppression, making *F. nucleatum* a tumor provoking factor in the primary microbiome-tumor interaction. The microorganism also generates bacterial biofilms that increase adaptation to microbial species; and its products contribute to tumor development and complement the hypoxic tumor microenvironment [31, 32].

Secondary relationships are interactions between tumors and the microbial association of a tissue, organ or organ system. These interactions are most important for identifying potential biomarkers for tissue screening for tumors. The digestive tract can attract some bacteria from the tumor, which can be used as a biomarker in tumor screening.

Tertiary relationships are interactions between the tumor and the distant microbiota. Tertiary interactions include therapeutic (in treatment) modulation by modification by chemotherapeutic drugs and reduction or increase in efficacy or toxicity of these drugs. Despite the physical distance of the individual organ systems that these microbial groupings occupy in relation to the tumor, that groups can have a strong influence on the tumor phenotype, treatment efficacy and outcomes.

Effects of human gut microbiota on drug metabolism

In early 2018, new evidence was demonstrated for a link between the efficacy of anticancer drugs and resident bacteria in the body. Intestinal bacteria contain enzymes that can influence the activation or breakdown of drugs. Gut microorganisms are known to modulate the immune system and this phenomenon may be important in explaining the impact of the gut microbiome on cancer immunotherapy. Researchers have proposed several variations on how this works. For example, gut bacterial antigens may resemble tumor antigens, "training" the immune system to "fight" cancer [33]. It is considered most likely that the commensal microorganisms can activate the immune system.

In addition to linking the microbiome to cancer immunotherapy, scientists have begun to link resident microorganisms to drug efficacy for a wide range of pathological conditions in the body. It is reported that two-thirds of 276 different drugs involved in co-culture with 76 species of human intestinal bacteria were modified by them [34].

Given the wide range of influences of the microbiome, better knowledge of the interactions between resident microorganisms and drugs can change medical practice and will be combined with other types of information, such as genetics, to make treatments more individualized and effective.

The role of human gut microbiota in metabolic diseases

The results of numerous experimental and theoretical studies of the human gut microbiota indicate its significant role in metabolic diseases, including type 2 diabetes (T2DM).

The microbiota modulates inflammation, interacts with food components, influences intestinal permeability, glucose and lipid metabolism, insulin sensitivity, and overall energy homeostasis in the host [35].

Table 4 reflects changes in microbiota composition in type 2 diabetes [36].

However, despite numerous studies supporting the importance of the gut microbiota in the pathophysiology of this disease, this area of knowledge is at an early stage. At present, a point has been reached in the understanding that certain microbial taxa and their associated molecular mechanisms may be involved in the glucose metabolism associated with T2DM.

Table 4: Intestinal dysbiosis in type 2 diabetes

Bacterial type	Changes in species diversity in type 2 diabetes
Bacteroidales	↑*Bacteroides* spp. ↑ *Alistipes* ↑ *Parabacteroides*
Firmicutes	↓↓ *Clostridiales,* ↑*Clostridium* spp. ↓ *Eubacterium rectale* ↓ *Faecalibacterium prausnitzii* ↓ *Roseburia* spp. *Lactobacillus gasseri* ↑ *Streptococcus mutans*
Proteobacteria	*Escherichia coli*
Verrucomicrobia	↓↓ *Akkermansia muciniphila*

Notes. ↑ increasing, ↓ decreasing.

The microbiome–gut–brain axis

There is growing evidence that dynamic changes in the human gut microbiota can alter brain physiology and behavior. Researchers have identified changes in gut microbiota composition associated with several symptoms or diseases, such as pain, cognitive dysfunction, autism, neurodegenerative disorders and cerebral vascular disease [37].

The human microbiota of different localization promotes two-way transmission of brain-intestinal signals through humoral, neural, and immunological pathways. The central nervous system is known to be involved in the regulation of intestinal motility and secretion, as well as in the regulation of mucosal immunity through the neuron-glial-epithelial pathway and visceral nerves, which contributes to changes in the intestinal microenvironment [38]. On the one hand, both external factors such as dietary habits, lifestyle, presence of own specific infections and early influence of environmental microbiota, and internal factors such as genetic determinants, metabolites, immunity and hormones play in the regulation of the qualitative-quantitative composition of gut microbiome. On the other hand, bacteria respond to these changes by producing neurotransmitters and neuromodulators in the gut that affect the host's central nervous system. These chemicals include: bacterial choline, tryptophan, short-chain fatty acids and hormones released from the gut, as well as, ghrelin and leptin. The relationship between brain impairment and corresponding changes in gut microbiota composition, indicating a clear link between gut microbiota and host physiology was summarized in Table 5 [28].

The human gut microbiota and viral infections. Coronavirus and the gut microbiome

The gut microbe regulates to some extent the host's protection against viral infections, including respiratory viruses such as the influenza viruses. This occurs by activating immune antiviral mechanisms and preventing excessive inflammation. Although data on the interaction between normal microbiota and viruses are limited, accumulating evidence with different interventions in the body, such as the effects of antibiotics and microbiota transfer (transplantation), has shown that the microbiota enhances antiviral immunity. The microbiota modulates the immune system by influencing the development of immune cells such as regulatory T-cells and innate lymphoid cells that help to maintain gut and lung homeostasis [39].

The risk of severe COVID-19 infections is most common in people with hypertension, diabetes and obesity, conditions associated with changes in the composition of the gut microbiome. This raises the question of the role that gut microbiome plays in determining COVID-19 severity.

In New York 5,279 patients tested positive for COVID-19 between March 1 and April 8, 2020. Of these, 22.6% were diabetic and 35.3% were obese [40]. SARS-CoV and SARS-CoV-2 have 79.5% nucleotide sequence identity and use angiotensin-converting enzyme 2 (ACE2) receptors to enter host cells. The distribution of ACE2 may determine how SARS-CoV-2 affects the respiratory and digestive tract.

Although coronavirus 2 of severe acute respiratory syndrome (SARS-CoV-2) affects the tissues of the gastrointestinal tract, it is known a little about the role of intestinal commensal microorganisms in the susceptibility and severity of infection.

Patients with COVID-19 have significant changes in fecal microbial groups compared to the control group, that are characterized by an increase in the number of opportunistic microorganisms and depletion of beneficial commensals during hospitalization and at all times after hospitalization. Depleted symbionts and gut dysbiosis persist even after relief from SARS-CoV-2 and respiratory symptoms. The basal abundance in the gut of microorganisms such as *Coprobacillus* (a gram-positive, obligate anaerobic and immobile genus in the family *Erysipelotrichidae*, with one known species), *Clostridium ramnosum* and *Clostridium hathewayi* correlated with COVID-19 severity. Microorganisms such as *Bacteroides dorei*, *Bacteroides thetaiotaomicron*, *Bacteroides massiliensis* and *Bacteroides ovatus* were found in the gut of COVID-19 patients, which inhibit the expression of ACE2 and show an inverse correlation with severity [41].

Gut dysbiosis and epithelial inflammation increase levels of ACE2, a cell surface receptor that plays a key role in dietary amino acid homeostasis,

Table 5: The relationship between brain disorders and changes in gut microbiota [28]

Brain disorders	Dysbacteriosis
Stress and depression	↑*Faecalibacterium, Alistipes, Ruminococcus, Campylobacter jejuni, Firmicutes;* ↓ *Bacteroidetes*
Pain and migraine	↑*H. pylori*; dysbiosis
Autism spectrumdisorders	↓*Faecalibacterium* spp., *Bifidobacteria*, *Akkermansia muciniphila*; ↑*Lactobacillus, Bacteroides, Prevotella, Alistipes*; change in quantity of *Fusobacteria, Verrucomicrobia, Firmicutes/Bacteroides*
Parkinson's disease	↑*H. pylori, E. coli, Ralstonia, Oscillospira, Bacteroides;* ↓*Prevotellaceae, Blautia, Coprococcus, Roseburia*
Alzheimer's disease	Chronic *H. pylori* infection; ↑*E. coli, Salmonella* spp, *Pseudomonas fluorescens, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtills, Streptomyces coelicolor*; *Chlamydia pneumoniae* infection.
Amyotrophic lateral sclerosis	Decreased levels of butyrate-producing bacteria, including *Butyrivibrio fibrisolvens, Escherichia coli, Oscillibacter,Anaerostipes, Lachnospira;*
Multiplesclerosis	↑*Archaea, Psuedomonas, Haemophilus, Blautia, DoreaFusobacteria;* ↓*Bacteroidetes, Firmicutes, Parabacteroides, Adlercreutzia, Prevotella, Bacteroides, Clostridia*
Atherosclerosis	*Lactobacillus rhamnosus, Neisseria polysaccharea,Acidovorax* spp i *H. pylori*; *Collinsella; Roseburia, Eubacterium*
Stroke	*Porphyromonas gingivalis*, Gram-negative bacteria, *Enterobacter, Megasphaera, Oscillibacter;* ↑*Bacteroides,Prevotella, Faecalibacterium*
Arteriovenous malformation	Gram-negative bacteria

Notes. *Unknown genera, ↑ increasing, ↓ decreasing.

innate immunity and gut microbial ecology. ACE2 is a target of SARS-CoV-2 [42]. Elevated levels of ACE2 in patients with an existing pro-inflammatory gut microbiome create conditions favorable for infection by a coronavirus [43], such as SARS-CoV-2, in the gut epithelium, from where it can spread throughout the body [44]. This is consistent with the development of gastrointestinal tract infections and the detection of viral RNA in the feces of many patients with COVID-19 (including persons with the negative PCR test of respiratory secretions) [45].

A recent study in Wuhan, China, confirmed the association between the composition of the gut microbiome and the susceptibility of healthy people to COVID-19 [46]. Elevated levels of microorganisms of *Lactobacillus* species correlate with higher levels of anti-inflammatory IL-10 and improve disease prognosis; elevated levels of pro-inflammatory bacteria including some *Klebsiella* species, *Streptococcus*, and *Ruminococcus gnavus* correlate with increased levels of pro-inflammatory cytokines and disease severity. Kawasaki disease, a condition similar to multisystem inflammatory syndrome in children that is increasingly reported as a complication in young children diagnosed with COVID-19 [47], is characterized by a dysbiotic gut microbiome with increased levels of *Streptococcus* species and decreased levels of *Lactobacillus* species compared to healthy individuals [48]. Consequently, COVID-19 induced changes in the composition of the gut microbiome may contribute to this complication.

Development of new microbiome therapeutic drugs

Most biotechnologies for microbiome recovery (correction) are developed by the US, Canadian and European companies. Recently, however, an increasing number are being located in other regions, such as China, South Korea and Israel. Microbiome-based drug developers are exploring virtually every possible approach to treating and diagnosing disease through the microbiome, using many different technologies.

Consider briefly implemented technologies.

Fecal microbiota transplantation. This approach generates interest in the treatment of disease using the microbiome of a healthy donor and rapidly expanded in the first time of use [49]. However, various regulatory requirements, safety requirements, have made it an afterthought in the industry: 6% of companies use this technology [50].

A specific consortium of microorganisms. This technology is based on treating a patient with a consortium of several bacterial species (usually two or more). While some of these technologies have evolved from further processing and refinement of fecal microbiota transplantation technology, some others have been developed with rational consideration of the ecological properties, metabolic capabilities or other characteristics of the microbial groupings. Given the technical complexity of these constructs, only about 7% of biotechnologies adhere to this strategy for treating disease.

One species (strain). According to this technique, one species of microorganism is entered into the body in order to cause a positive effect. This is a popular approach, followed by about 20% of programs. Most programs use targeted cross-linking between a specific bacterial strain and the immune system to treat inflammatory diseases and cancer (i.e., a personalized approach).

Phages. Using phages for elimination of bacteria and/or changing the composition of the microbiome is a technology used by about 10% of companies. The most obvious application of bacteriophages is the fight against infectious diseases, and this is indeed the area in which most projects are involved.

GMOs. While the metabolic capabilities of microorganisms are almost limitless and not yet fully described, some companies are creating bacteria to turn them into long-term drug delivery systems or expand or enhance their metabolic activity [51]. Due to technical and regulatory complexities of the approach, only 11% of companies use GMOs as therapeutic agents.

Microbiome metabolic products and postbiotics. Bacteria synthesize tens of thousands of different, chemically diverse substances, most of which have not been identified to date. Many of them are thought to have significant physiological effects and, therefore, may have enormous pharmacological potential [52, 53]. Perhaps the most widely described chemicals synthesized by bacteria are antimicrobials and enzymes, and these are the main ones for research [54]. To date, there are several investigational enzymes that aim to break down antibiotics in the gut, which are administered systemically to prevent effects on the gut microbiome (thereby preventing gastrointestinal diseases caused by opportunistic microorganisms), and other enzymes as alternative therapy for certain metabolic diseases.

Chemicals from the gut microbiome. With a greater understanding of the metabolic activity of the microbiome and its relationship to human physiology, there is a growing trend towards the use of external chemicals (mostly small molecules) to alter microbial activity to treat diseases such as immune conditions, irritable bowel syndrome and obesity [55]. This is a growing category (biotechnology) that is currently used by almost 20% of microbiome drug developers, but this proportion is likely to increase in the future.

Conclusions

The human gut microbiome has been attracting increasing attention from physicians and researchers over the past 15 years. The above data indicate that the rapid development of gut microbiome research in recent years is not only due to a better understanding of the function of the microbiome by the scientific community, but is also inseparable from the strategic support of each country. Global investment in research related to the human microbiome has exceeded $1.7 billion over the past decade.

In the human body, the predominant bacterial types, including hundreds of genera and species, are *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Proteobacteria*. The populations of these different bacterial species vary considerably from person to person, and the bacterial composition is perhaps primarily influenced by different environments and diets.

The gut microbiome underlies human health and is associated with many diseases. Current research on the gut microbiome is being transformed from correlation to causation, followed by a study of the mechanisms by which the microbiome affects host health.

The gut microbiome may also influence the action of drugs and their metabolism, affecting the host immune response and disease course.

In addition to discovering pathology and disease mechanisms, the study of the gut microbiome also contributes to the development of new diagnostic methods and therapeutic interventions. 6. When analyzing diseases related to the gut microbiome, such as diarrhea, inflammatory bowel disease, irritable bowel syndrome, cardiovascular disease, autism, Alzheimer's disease, Parkinson's disease, it is important to remember that the gut microbiome is one aspect of disease and therefore its importance should not be overstated.

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МІКРОБІОМ КИШЕЧНИКА ЛЮДИНИ ЯК ІНДИКАТОР ЇЇ ЗДОРОВ'Я

Беззаперечним досягненням у вивченні мікробіому кишечника як об'єднання різних мікроорганізмів, у тому числі вірусів, які населяють різні органи і системи людського організму, є встановлення того факту, що деякі захворювання, які вважалися неінфекційними, можуть бути опосередковані мікроорганізмами. Це привело до того, що мікробіом кишечника назвали "забутим органом", який може слугувати додатковою (і, так би мовити, відсутньою) ланкою для більш об'єктивної та кращої діагностики і лікування багатьох захворювань, які не вважалися інфекційними. Швидкий розвиток досліджень мікробіому кишечника в останні роки не тільки пов'язаний із більш глибоким розумінням науковим співтовариством функції мікробіому, але і невіддільний від стратегічної підтримки кожної країни. Глобальні інвестиції в дослідження мікробіому людини за останнє десятиліття перевищили 1,7 млрд дол. Ці дослідження сприяють розробленню нових методів діагностики і терапевтичних втручань. Наша стаття присвячена аналізу можливостей використання мікробіому кишечника людини для діагностики сучасних захворювань та ролі кишківника у провокації та спричиненні певних захворювань. Показано суттєві відмінності в складі та різноманітності мікробіому людини залежно від географічного розташування і зі зміною суспільно-економічних формацій у бік поступового зменшення різноманітності мікробіому кишечника, що пояснюється трьома стадіями існування людської популяції: видобуток їжі, сільське господарство і промислово розвинене міське життя. Ми аналізуємо вплив на мікробіом кишечника характеру харчування, різноманітних захворювань (у т.ч. і злоякісних новоутворень) та вірусних інфекцій (зокрема, коронавірусу). І навпаки – вплив мікробіому кишечника на дію ліків та їхній метаболізм, що позначається на імунній відповіді господаря і перебігу хвороби.

Ключові слова: кишечник людини; мікробіом кишечника; коронавірус; імунна відповідь; метаболізм ліків.

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МИКРОБИОМ КИШЕЧНИКА ЧЕЛОВЕКА КАК ИНДИКАТОР ЕГО ЗДОРОВЬЯ

Бесспорным достижением в изучении поведения микробиома кишечника как объединения различных микроорганизмов, в том числе вирусов, населяющих различные органы и системы человеческого организма, является установление того факта, что некоторые заболевания, считавшиеся неинфекционными, могут быть опосредованы микроорганизмами. Это привело к тому, что микробиом кишечника назвали “забытым органом”, который может служить дополнительным (и, так сказать, отсутствующим) звеном для более объективной и лучшей диагностики и лечения многих заболеваний, не считавшихся инфекционными. Быстрое развитие исследований микробиома кишечника в последние годы не только связано с более глубоким пониманием научным сообществом функции микробиома, но и неотделимо от стратегической поддержки каждой страны. Глобальные инвестиции в исследования микробиома человека за последнее десятилетие превысили 1,7 млрд долл. Эти исследования способствуют разработке новых методов диагностики и терапевтических вмешательств. Показаны существенные отличия в составе и разнообразии микробиома человека в зависимости от географического расположения и с изменением общественно-экономических формаций в сторону постепенного уменьшения разнообразия микробиома кишечника, что объясняется тремя стадиями существования человеческой популяции: добыча пищи, сельское хозяйство и промышленно развитая городская жизнь. Мы анализируем влияние на микробиом кишечника характера питания, различных заболеваний (в т.ч. и злокачественных новообразований) и вирусных инфекций (в частности, коронавируса). И наоборот – влияние микробиома кишечника на действие лекарств и их метаболизм, что сказывается на иммунном ответе хозяина и течении болезни.

Ключевые слова: кишечник человека; микробиом кишечника; коронавирус; иммунный ответ; метаболизм лекарств.

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Original article

ANTIMICROBIAL ACTIVITY OF *FOMITOPSIS OFFICINALIS* (VILL.) BONDARTSEV & SINGER IN PURE CULTURE

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Background. According to the World Health Organization antibiotic resistance is among the top ten threats to human health, food safety and development. Today antibiotic resistance has reached alarmingly high levels all over the world. Meanwhile, the increase in the synthetic drugs' production has led to the pathogenic mycobiota's rapid adaptation to the created chemicals, which have a narrow focus of application. That is why in modern biotechnology and pharmacology much attention is paid to natural producers of biologically active compounds, in particular – to xylotrophic fungi. It has been experimentally proven that the xylotrophic macromycete *Fomitopsis officinalis* or tinder fungus can be considered to be a promising producer of pharmacological substances with a broad spectrum of action. Studies of active metabolites, contained in the mycelial mass, culture fluid of the medicinal xylotrophic macromycete *F. officinalis,* and determination of their biological action remain relevant.
Objective. The objective was to determine the antimicrobial activity of culture fluid and mycelial mass of *F. officinalis* different strains from the mushrooms collection (*IBK* Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, NAS of Ukraine) against gram-negative and gram-positive bacteria species.
Methods. An *in vitro* study of the antimicrobial activity of ethyl acetate extracts of culture fluid and aqueous-ethyl extracts of mycelial mass for *F. officinalis* strains *IBK*-5004, *IBK*-2497, *IBK*-2498 against gram-positive *Staphylococcus aureus* (B-918), *Bacillus subtilis* (B-901) and gram-negative *Escherichia coli* (B-906), *Bacillus subtilis* (B-900), *Klebsiella pneumoniae* (M-123) bacteria by disc-diffusion method was conducted.
Results. High antimicrobial activity of tinder fungus culture fluid and mycelial mass extracts against *Staphylococcus aureus* was established after the 21st day of cultivation, while on the 28th day the zone of growth retardation was maximal (15–25 mm). The highest indices were recorded in *F. officinalis IBK*-5004 (20–25 mm) and *IBK*-2498 (20–24 mm) strains. Antimicrobial activity against *Klebsiella pneumoniae* in culture fluid extracts was manifested on the 21st and 28th days of cultivation. The highest antimicrobial activity against *Klebsiella pneumoniae* was observed in the culture fluid of the strain *F. officinalis IBK*-5004, the diameter of the growth retardation zone was 18 mm on the 28th day of cultivation. Mycelial mass's extracts showed moderate activity on the 14th day of cultivation (7-8 mm); maximal activity was recorded on the 28th day (12–22 mm). The most active strain was *Fomitopsis officinalis IBK*-2498. No antimicrobial activity against test organisms was detected in the following studied strains: *Escherichia coli, Pseudomonas aeruginosa*, *Bacillus subtilis.*
Conclusions. It has been established that the mycelial mass and culture fluid extracts of *F. officinalis IBK*-5004, *IBK*-2497, *IBK*-2498 strains have high antimicrobial activity against *Staphylococcus aureus* and moderate antimicrobial activity against *Klebsiella pneumoniae* on the 21st and 28th day of cultivation.

Keywords: antibiotic resistance; biologically active substances; mycelium; mycelial mass; culture fluid; gram-negative bacteria; gram-positive bacteria; disk diffusion method; anti-microbial activity.

Introduction

Research and development of new effective biological products for the treatment and prevention of acute and chronic diseases caused by various microorganisms is one of the priorities of modern pharmaceutical mycology. In recent decades, considerable attention has been attracted to antibiotic-producing fungi, which they synthesize in the process of secondary metabolism [1–6]. Synthesis of antibiotics is one of the forms of fungi antagonism against other organisms' species. The antibiotics formation is evolutionary and adaptive in nature and is closely related to the general metabolic processes in fungal cells. In recent years, the increase in the production of synthetic drugs has led to the rapid adaptation of pathogenic mycobiota to the developed chemicals, which have a narrow focus on application, and as a consequence, a significant number of adverse reactions for the human body. Prolonged and not always justified antibiotics application often accelerates the

pathogens evolution in the direction of consolidating their resistance to these drugs. Therefore, it is necessary to replace constantly some types of antibiotics with others. To do this, you need to find the most active organisms – antibiotics producers. That is why in modern medicine and pharmacology much attention is paid to natural producers of biologically active compounds, including xylotrophic macromycetes [1, 3, 7–10]. This is due to the fact that natural compounds from medicinal macromycetes, in contrast to synthetic, have a multifunctional and multifaceted effect on the human body, which significantly reduces the negative effects and addiction. The study of therapeutic activity of medicinal mushrooms different species has shown the feasibility of their application in modern clinical practice [3, 5, 11]. Increased attention to xylotrophic macromycetes is due to the fact that it has been experimentally confirmed that these organisms synthesize secondary metabolites of extremely diverse chemical structure, a significant proportion of which are inhibitors of various cellular processes. Such substances include antibiotics, fungicides, cytostatic compounds, modulators of the immune response, growth regulators. The synthesis of secondary metabolites with antimicrobial activity is more characteristic of wood-destroying basidiomycetes – wood brown rot putrefaction agents. One of such species is the valuable rare macromycete *Fomitopsis officinalis* (Vill.) Bondartsev & Singer, known in medical practice as "tinder fungus" or "larch sponge". The healing properties of larch sponge substances have been known for a long time and are widely used in traditional Chinese and Tibetan medicine [12–15]. Modern research has shown that larch sponge can be considered a promising producer of pharmacological substances with a broad spectrum of action. Unsaturated fatty acids (palmitic, oleic, linoleic, linolenic, arachidonic, etc.), heteropolysaccharides, glucosamines, agaricic acid, phospholipids, carotenoids, sterols, vitamins of B group, vitamins E, A, essential oils, cytokinins, triterpenoids of lanostan type (eburic acid) were isolated from the *F. officinalis* basidiom and mycelium [12, 14, 16–23]. It has been experimentally proven that the antibiotic effect of tinder fungus extracts is not due to individual secondary metabolites, but to the combined action of all biologically active substances [12, 14, 15, 24].

The aim of our work was to study the antimicrobial activity of culture fluid and mycelial mass of *Fomitopsis officinalis* different strains from the mushroom culture collection (*IBK*) against gram-negative and gram-positive bacterial species.

Masterials and methods

The objects of the study was pure cultures of *Fomitopsis officinalis*, which are stored in the *IBK* Mushroom Culture Collection of the M.G. Kholodny Institute of Botany of the National Academy of Science of Ukraine (Table 1).

Table 1: The studied collections of *Fomitopsis officinalis* [25]

IBK number	Origin and date of culture isolation
IBK-2497	Mycoforest Collection of typical cultures (MFTCC), Slovakia, 2016
IBK-2498	Mycoforest Collection of typical cultures (MFTCC), Slovakia, 2016
IBK-5004	V.L. Komarov Botanical Institute, Russian Acadamy of Science (BIN), St.-Petersburg, Russia, 1981

Preparation of mushroom extract. *F. officinalis* cultures were grown superficially on a complex nutrient medium GPE, of the following composition, g/l: glucose – 25.0; peptone – 3.0; yeast extract – 2.0; KH_2PO_4 – 1.0; K_2HPO_4 – 1.0; $MgSO_4 \cdot 7H_2O$ – 0.25. The acidity of the medium was 5.5. Cultivation was performed at a temperature of 26 ± 1 °C for 7, 14, 21, and 28 days. The culture fluid after growing the mycelium was separated from the biomass. Ethyl acetate (2:1 by volume) was added to the culture fluid to concentrate the antimicrobial substances, and the mixture was shaken vigorously for 10 min and left for 20 h at a temperature of 4 °C. Then the ethyl acetate layer was removed, evaporated on a rotary evaporator to dryness. The precipitate was dissolved in 70% ethanol. The solution of the obtained concentrate (10 µl) was applied to standard disks of Bio Merieux firm (6 mm in diameter), dried at 40 °C for 30 min, and placed on the surface of Mueller–Hinton agar seeded with test culture.

Taking into account the amount of concentrate applied to the disk in each sample was 0,10 mg of biologically active substances.

The mycelial mass was dried to constant weight at a temperature of 60 °C. Aqueous-alcoholic 70% extract was prepared at the rate of 20 mg of mycelial mass per 1 ml of solvent. The mycelial mass was crushed, the extraction was performed on an ultrasonic bath at a temperature of 40 °C for 30 min, left for a day in a refrigerator at a temperature of 4 °C, then filtered, centrifuged for 20 min at 13500 g.

Bacterial test organisms. Daily bacterial cultures from the Collection of the Department of Biotechnology and Microbiology of the National University of Food Technologies (Kyiv, Ukraine) were used as test cultures: *Staphylococcus aureus* (B-918), *Pseudomonas aeruginosa* (B-900), *Escherichia coli* (B-906), *Bacillus subtilis* (B-901), *Klebsiella pneumonia* (M-123), which were pre-grown in tubes on Mueller–Hinton slope agar medium (Oxoid). Several same-type clearly isolated colonies of bacteria were selected for inoculum preparation. A small amount of material from the tops of the colonies was transferred by microbiological loop into a test tube with sterile physiological saline, shaken to obtain a homogeneous suspension, bringing the inoculum density to exactly 0.5 according to the McFarland standard ($5 \cdot 10^6$ cells/ml) Mc Farland (No: 0.5) standard. Use the inoculum within 15 min after preparation. A suspension of bacteria in an amount of 0.2 ml was evenly applied to the surface of the Mueller-Hinton agar medium (Oxoid).

Performing of antibacterial screening test. In the study of mycelial mass and culture fluid antibiotic activity, disk diffusion method (DDM) was used [11]. Standard sterile disks were impregnated with extract samples, placed on the surface of Mueller-Hinton agar seeded with test culture. The cultures were incubated at 37 °C for 24 h. Next, the zone of microorganisms' growth inhibition was determined. The results were evaluated by the diameter of the growth retardation zones around the disk: no growth retardation zone – the test culture is not sensitive to this specimen concentration; the diameter of the growth retardation zone is less than 10 mm – moderate sensitivity of culture to the given specimen concentration; the diameter of the growth retardation zone is more than 10 mm – high sensitivity of the test culture to this specimen concentration.

Gentamycin sulphate (40 mg/ml), Ukraine, was used as a positive control. Gentamycin sulphate is a broad-spectrum aminoglycoside antibiotic. It has a bactericidal effect. Actively penetrating the cell membrane of bacteria, it irreversibly binds to the 30S subunit of bacterial ribosomes and, thus, inhibits the synthesis of the pathogen protein. *In vitro* tests confirmed its high activity against aerobic gram-negative bacteria: *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, *Shigella* spp., *Salmonella* spp., *Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Acinetobacter* spp. It is also active against aerobic gram-positive cocci: *Staphylococcus* spp. (including resistant to penicillins and other antibiotics), some strains of *Streptococcus* spp.

Ethyl acetate for culture fluid extracts and ethanol for mycelial mass were used as a negative control.

Statistical processing methods. To obtain reliable results, experimental studies, depending on the conditions of analysis and the requirements of mathematical planning, were performed in 3 replicates. After register the studied indicators, their reliable values were calculated by statistical methods of analysis and found the following indicators: the values of standard deviations, coefficients of variation, confidence intervals. The tables show the average statistically significant data with a 95% probability. Statistical processing of the obtained results was performed using an application program for working with spreadsheets Microsoft Office Excel 2003, 2013 (Microsoft Corporation, USA).

Results

The study of *F. officinalis* strains *IBK*-5004, *IBK*-2497, *IBK*-2498 antimicrobial activity of culture fluid ethyl acetate extracts and mycelial mass aqueous-ethyl extracts against gram-positive *Staphylococcus aureus* (B-918), *Bacillus subtilis* (B-901) and gram-negative *Escherichia coli* (B-906), *Pseudomonas aeruginosa* (B-900), *Klebsiella pneumoniae* (M-123) bacteria by DDM was conducted.

During the experiment it was found that all studied *F. officinalis* strains under these cultivation conditions and in the presence of satisfactory growth did not show antimicrobial activity against test organisms: *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis.*

Antimicrobial activity of mycelial mass and culture fluid extracts was detected against gram-positive bacteria *Staphylococcus aureus* and gram-negative bacteria *Klebsiella pneumoniae* (Table 2, Figs. 1, 2). In relation to *Staphylococcus aureus*, high antimicrobial activity of culture fluid extracts was detected on the 21st day of cultivation, on the 28th day it reached a maximum – the growth retardation zone was 15–24 mm (Table 2). The highest rates were found in *F. officinalis* IBK-5004 (20–24 mm) (Fig. 1b) and IBK-2498 (20–23 mm) strains.

Water-alcohol extracts of mycelial mass showed weak antimicrobial activity after 14 days of cultivation (7-8 mm). However, in strains IBK-5004 and IBK-2498 on the 21st and 28th day of cultivation the growth retardation zone for *Staphylococcus aureus* exceeded the positive control values (Table 2).

Antimicrobial activity against *Klebsiella pneumoniae* in culture fluid extracts of *F. officinalis* *IBK*-5004, *IBK*-2497, *IBK*-2498 strains was mani-

fested on the 21st and 28th day of cultivation. The highest antimicrobial activity against *Klebsiella pneumoniae* was found in the culture fluid of the *F. officinalis* strain IBK-5004, the diameter of the growth retardation zone was 18 mm on the 28th day of cultivation (Table 2, Fig. 1a).

In contrast to the culture fluid, the mycelial mass extracts showed moderate activity on the 14th day of cultivation (7-8 mm), the maximal activity was recorded on the 28th day (12–22 mm). The most active strain was *Fomitopsis officinalis IBK*-2498 (Fig. 2b).

Table 2: Antimicrobial activity of culture fluid ethyl acetate extracts and mycelial mass 70% water-alcohol extracts of strains from mushroom culture collection (*IBK*)

Species, strain	Cultivation day	Microorganisms culture	
		Staphylococcus aureus	*Klebsiella pneumonia*
		Cultural fluid	
		Diameter of microorganisms' growth retardation zone, mm	
		Ethyl acetate extract of the culture fluid	
Fomitopsis officinalis, IBK-5004	7	0 ± 0	0 ± 0
	14	6.1 ± 0.2	0 ± 0
	21	20.3 ± 0.3	8.2 ± 0.1
	28	24.1 ± 0.4	18.1 ± 0.3
	Control+	18 ± 0.1	16 ± 0.1
	Control–	0 ± 0	0 ± 0
Fomitopsis officinalis, IBK-2497	7	0 ± 0	0 ± 0
	14	7.5 ± 0.4	0 ± 0
	21	15.2 ± 0.3	9.4 ± 0.3
	28	19.4 ± 0.5	15.3 ± 0.1
	Control+	18.8 ± 0.1	16.7 ± 0.4
	Control–	0 ± 0	0 ± 0
Fomitopsis officinalis, IBK-2498	7	0 ± 0	0 ± 0
	14	6.1 ± 0.1	0 ± 0
	21	20.3 ± 0.3	8.0 ± 0.1
	28	23.1 ± 0.2	11.5 ± 0.3
	Control+	18.8 ± 0.1	16.7 ± 0.1
	Control–	0 ± 0	0 ± 0
		Water-alcohol extract of mycelial mass	
Fomitopsis officinalis, IBK-5004	7	0 ± 0	0 ± 0
	14	8.1 ± 0	8.2 ± 0.2
	21	20.1 ± 0.3	10.1 ± 0.3
	28	25.3 ± 0.2	12.2 ± 0.3
	Control+	18.8 ± 0.1	16.7 ± 0.1
	Control–	0 ± 0	0 ± 0
Fomitopsis officinalis, IBK-2497	7	0 ± 0	0 ± 0
	14	7.1 ± 0.3	7.1 ± 0
	21	15.4 ± 0.2	10.2 ± 0.2
	28	22.1 ± 0.3	15.2 ± 0.5
	Control+	18.8 ± 0.1	16.7 ± 0.1
	Control–	0 ± 0	0 ± 0
Fomitopsis officinalis, IBK-2498	7	0 ± 0	0 ± 0
	14	7.3 ± 0.2	8.4 ± 0.2
	21	20.4 ± 0.2	14.2 ± 0.2
	28	24.1 ± 0.4	22.3 ± 0.5
	Control+	18.8 ± 0.1	16.7 ± 0.1
	Control–	0 ± 0	0 ± 0

Notes. "Control+" – antifungal-antibiotic solution Gentamycin sulphate. "Control–" – ethyl acetate for experiment with culture fluid, ethanol for experiment with mycelial mass. Growth retardation zone absence – the test culture is not sensitive to a given concentration of the specimen; the diameter of the growth retardation zone is less than 10 mm – the culture is moderately sensitive to a given concentration of the specimen; the diameter of the growth retardation zone is more than 10 mm – high sensitivity of the test culture to a given concentration of the specimen.

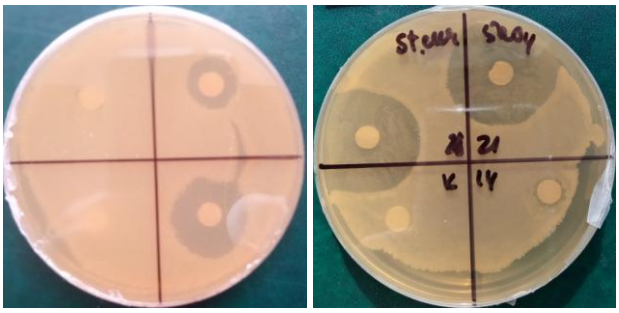

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Figure 1: Antibacterial activity of ethyl acetate extract of *Fomitopsis officinalis* IBK-5004 culture fluid against: (a) *Klebsiella pneumonia* (negative control, the 14th, 21st, 28th days of cultivation); (b) antibacterial activity of ethyl acetate extract of *Fomitopsis officinalis* IBK-5004 culture fluid against *Staphylococcus aureus* (negative control, the 14th, 21st, 28th days of cultivation)

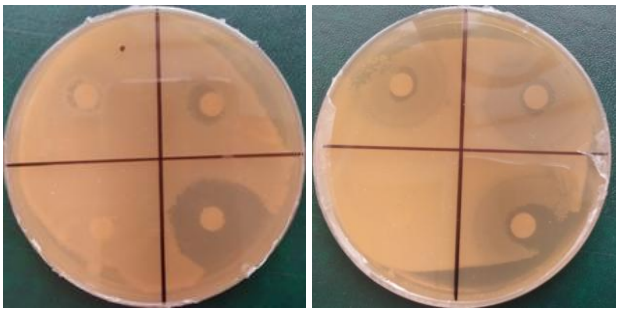

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Figure 2: Antibacterial activity of ethyl acetate extract of *Fomitopsis officinalis* IBK-2498 mycelial mass against: (a) *Staphylococcus aureus* (negative control, the 14th, 21st, 28th days of cultivation); (b) antibacterial activity of ethyl acetate extracts of *Fomitopsis officinalis* IBK-2498 mycelial mass against *Klebsiella pneumonia* (negative control, the 14th, 21st, 28th days of cultivation)

Discussion

The antibiotic formation process is related to the general metabolic processes in fungal cells. Antibiotic biosynthesis occurs in the slow growth phase of the culture (the trophophase end) and reaches a maximum in the stationary growth phase. During this period, the culture fluid is enriched with metabolic products and cell autolysis products, there is an intensive biosynthesis process and maximum antibiotics accumulation. In the process of fungal culture active growth, the cells enzymatic status changes, inducers of secondary metabolism appear and induce mechanisms that inhibit cell proliferation and active growth, stressful situations, activate the antibiotic formation process.

The biological activity of many secondary metabolites which *F. officinalis* is able to synthesize has been experimentally proven [14, 15, 16, 22, 23, 26, 27]. It should be noted that polyresistant mycobacteria, which have been shown to be resistant to two major anti-tuberculosis drugs, isoniazid and rifampicin, are of particular concern. It has been experimentally proven that tinder fungus extracts show high antibacterial activity against the pathogenic bacterium *Mycobacterium tuberculosis* [7, 12, 28, 29], bactericidal activity against *Bacillus anthracis*, *B. subtilis*, bacteriostatic activity against *Micrococcus luteus* and bacteriolytic to *Vibrio* species [12, 14–16]. High antibacterial activity of agaricic acid and lanostane triterpenoids synthesized by *F. officinalis* in the process of metabolism has been established [14, 27, 30]. In the German and Swedish pharmacopoeia, agaricic acid is a part of the drugs used in the treatment of patients with tuberculosis [14]. According to Airapetova *et al.* (2010) lipid fraction from the *F. officinalis* fruiting body has a pronounced antimicrobial effect against gram-positive cocci of the genus *Staphylococcus*: *Staphylococcus aureus* (21–25 mm), *Staphylococcus epidermidis* (22 mm), gram-negative microorganisms *Shigella* and spore-forming microorganisms of the genus *Bacillus*: *Bacillus subtilis* (17 mm), *Bacillus anthracoides* (17 mm). In our experiment, antimicrobial activity against *Bacillus subtilis* was absent. It can be assumed that the studied strains have a low level of antimicrobial substances biosynthesis (below the sensitivity of the applied detection method) and in the future it is necessary to increase the terms and to change conditions of cultivation.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multidrug-resistant *Staphylococcus aureus* that causes nosocomial and community-acquired infections. MRSA infections today pose a serious health care problem [6, 31–34]. It is important to note that the strains studied by us synthesize biologically active substances that are able to overcome resistance to methicillin-resistant *Staphylococcus aureus* and are effective against the test bacterium *Klebsiella pneumoniae*, which is characterized by a significant variety of antibiotic resistance spectra. The variability of *Klebsiella* species resistance to existing antimicrobial drugs and the emergence of strains resistance genes dangerous for the spread confirms the need for continuous monitoring of infectious agents' antibiotic resistance with analysis of their resistance mechanisms, as well as new producers of antimicrobial substances finding.

Conclusions

Thus, it has been found that all studied strains of *F. officinalis* (*IBK*-5004, *IBK*-2497, *IBK*-2498) are able to synthesize substances that inhibit the growth of individual test organisms (*Staphylococcus aureus* and *Klebsiella pneumoniae*). It was found that extracts of both culture fluid and mycelial mass show high antibacterial activity against *Staphylococcus aureus*. The studied cultures of *F. officinalis* have the potential as producers of antimicrobial substances that overcome these forms of bacteria drug resistance, i.e. those forms of resistance, the spread of which is of greatest concern to specialists.

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АНТИМИКРОБНА АКТИВНІСТЬ *FOMITOPSIS OFFICINALIS* (VILL.) BONDARTSEV & SINGER У ЧИСТІЙ КУЛЬТУРІ

Проблематика. За даними Всесвітньої організації охорони здоров'я, стійкість до антибіотиків входить до десятки найбільших загроз для здоров'я людей, розвитку та продовольчої безпеки людства. Сьогодні резистентність до антибіотиків досягла надзвичайно високих рівнів у всьому світі. Своєю чергою збільшення виробництва синтетичних ліків призвело до швидкої адаптації патогенної мікобіоти до створених хімічних препаратів, які мають вузьку спрямованість до застосування. Саме тому в сучасній біотехнології та фармакології значна увага приділяється природним продуцентам біологічно активних сполук, зокрема ксилотрофним грибам. Експериментально доведено, що ксилотрофний макроміцет *Fomitopsis officinalis*, або трутовик лікарський, можна вважати перспективним продуцентом фармакологічних речовин широкого спектра дії. Актуальними залишаються дослідження активних метаболітів, які містяться у міцеліальній масі, культуральній рідині лікарського ксилотрофного макроміцета *Fomitopsis officinalis*, та визначення їх біологічної дії.

Мета. Визначення антимікробної активності екстрактів культуральної рідини та міцеліальної маси різних штамів *Fomitopsis officinalis* із колекції культур шапинкових грибів Інституту ботаніки ім. М.Г. Холодного НАН України (*IBK*) відносно грам-негативних та грам-позитивних видів бактерій.

Методика реалізації. Проведено дослідження *in vitro* антимікробної активності етилацетатних екстрактів культуральної рідини та водно-етилових екстрактів міцеліальної маси для штамів *F. officinalis IBK*-5004, *IBK*-2497, *IBK*-2498 проти грам-позитивних *Staphylococcus aureus* (B-918), *Bacillus subtilis* (B-901) та грам-негативних *Escherichia coli* (B-906), *Pseudomonas aeruginosa* (B-900), *Klebsiella pneumoniae* (M-123) бактерій диско-дифузійним методом.
Результати. Встановлено високу антимікробну активність екстрактів культуральної рідини та міцеліальної маси трутовика лікарського щодо *Staphylococcus aureus* (золотистого стафілококу) після 21-ї доби культивування, на 28-му добу зона затримки росту була максимальною – 15–25 мм. Найвищі показники фіксували у штамів *F. officinalis IBK*-5004 (20–25 мм) та *IBK*-2498 (20–24 мм). Антимікробна активність відносно *Klebsiella pneumoniae* в екстрактів культуральної рідини проявлялась на 21-шу і 28-му добу культивування. Найвищу антимікробну активність щодо *Klebsiella pneumoniae* виявила культуральна рідина штаму *F. officinalis IBK*-5004, діаметр зони затримки росту – 18 мм на 28-му добу культивування. Екстракти міцеліальної маси виявили помірну активність на 14-ту добу культивування (7-8 мм), максимальну активність фіксували на 28-му добу (12–22 мм). Найактивнішим виявився штам *Fomitopsis officinalis IBK*-2498. Не виявлено антимікробної активності в досліджених штамів до тест-організмів: *Escherichia coli*, *Pseudomonas aureginosa*, *Bacillus subtilis.*
Висновки. Встановлено, що екстракти міцеліальної маси та культуральної рідини штамів *F. officinalis IBK*-5004, *IBK*-2497, *IBK*-2498 мають високу антимікробну активність відносно *Staphylococcus aureus*. Помірну антимікробну активність до *Klebsiella pneumoniae* спостерігали на 21-шу та 28-му добу культивування.

Ключові слова: стійкість до антибіотиків; біологічно активні речовини; міцелій; міцеліальна маса; культуральна рідина; грам-негативні бактерії; грам-позитивні бактерії; метод дискової дифузії; антимікробна активність.

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АНТИМИКРОБНАЯ АКТИВНОСТЬ *FOMITOPSIS OFFICINALIS* (VILL.) BONDARTSEV & SINGER В ЧИСТОЙ КУЛЬТУРЕ

Проблематика. По данным Всемирной организации здравоохранения, устойчивость к антибиотикам входит в десятку наибольших угроз для здоровья человека, развития и продовольственной безопасности человечества. Сегодня резистентность к антибиотикам достигла угрожающе высоких уровней во всем мире. В свою очередь увеличение производства синтетических лекарств привело к быстрой адаптации патогенной микобиоты по отношению к синтезированным химическим препаратам, имеющим узкую направленность к применению. Именно поэтому в современной биотехнологии и фармакологии значительное внимание уделяется природным продуцентам биологически активных соединений, в частности ксилотрофным макромицетам. Экспериментально доказано, что ксилотрофный макромицет *Fomitopsis officinalis*, или трутовик лекарственный, можно считать перспективным продуцентом фармакологических веществ широкого спектра действия. Актуальными остаются исследования активных метаболитов, содержащихся в мицелиальной массе, культуральной жидкости лекарственного ксилотрофного гриба *F. officinalis*, и определение их биологического действия.
Цель. Определение антимикробной активности экстрактов культуральной жидкости и мицелиальной массы различных штаммов *F. officinalis* из коллекции культур шляпочных грибов Института ботаники им. Н.Г. Холодного НАН Украины (*IBK*) по отношению к грамотрицательным и грамположительным видам бактерий.
Методика реализации. Проведено исследование *in vitro* антимикробной активности этилацетатных экстрактов культуральной жидкости и водно-этиловых экстрактов мицелиальной массы для штаммов *F. officinalis IBK*-5004, *IBK*-2497, *IBK*-2498 по отношению к грамположительным *Staphylococcus aureus* (B-918), *Bacillus subtilis* (B-901) и грамотрицательным *Escherichia coli* (B-906), *Pseudomonas aeruginosa* (B-900), *Klebsiella pneumoniae* (M-123) бактериям диско-диффузионным методом.
Результаты. Установлена высокая антимикробная активность экстрактов культуральной жидкости и мицелиальной массы трутовика лекарственного относительно *Staphylococcus aureus* (золотистого стафилококка) после 21 суток культивирования, на 28-е сутки зона задержки роста была максимальной – 15–25 мм. Высокие показатели фиксировали у штаммов *F. officinalis* IBK-5004 (20–25 мм) и IBK-2498 (20–24 мм). Антимикробная активность по отношению к *Klebsiella pneumoniae* у экстрактов культуральной жидкости проявлялась на 21 и 28-е сутки культивирования. Наивысшую антимикробную активность в отношении *Klebsiella pneumoniae* проявила культуральная жидкость штамма *F. officinalis* IBK-5004, диаметр зоны задержки роста – 18 мм на 28-е сутки культивирования. Экстракты мицеллиальной массы проявили умеренную активность на 14-е сутки культивирования (7-8 мм), максимальную активность фиксировали на 28-е сутки (12–22 мм). Самым активным оказался штамм *F. officinalis* IBK-2498. Не выявлена антимикробная активность у исследованных штаммов к тест-организмам: *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*.
Выводы. Установлено, что экстракты мицеллиальной массы и культуральной жидкости штаммов *F. officinalis* IBK-5004, IBK-2497, IBK-2498 обладают высокой антимикробной активностью по отношению к *Staphylococcus aureus*. Умеренная антимикробная активность к *Klebsiella pneumoniae* наблюдалась на 21 и 28-е сутки культивирования.

Ключевые слова: стойкость к антибиотикам; биологически активные вещества; мицелий; мицелиальная маса; культуральная жидкость; грамотрицательные бактерии; грамположительные бактерии; метод дисковой диффузии; антимикробная активность.

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Original article

BIOLOGICAL EVALUATION OF MEDICAL DEVICES IN THE FORM OF SUPPOSITORIES FOR RECTAL AND VAGINAL USE

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Background. Programs of preclinical safety studies of the health care products depend on the regulatory status of the investigated products. The classification of such products, in particular suppositories for rectal and vaginal use, is a critical step of developing tactics for their biological evaluation. Adaptation of biological evaluation methods for the medical devices based on the combination of biologically active substances, as well as evaluation of the results of such studies is urgent task of biomedicine.
Objective. To substantiate the regulatory status and to carry out a biological evaluation of medical devices in the form of vaginal suppositories based on octenidine dihydrochloride ("Prodexyn") and in the form of rectal suppositories based on *Saw palmetto*, *Levisticum officinale* and *Calendula officinalis* extracts ("Pravenor").
Methods. Biological evaluation was conducted according to the requirements of ISO 10993 standards using *in vitro* and *in vivo* biological test systems (cytotoxicity in cell culture and the MTT test, sensitizing and irritating effect in guinea pigs).
Results. The cytotoxicity (CC_{50}) of the medical device "Prodexyn" extract in Vero cell culture was 8.35 μg/ml calculated as octenidine dihydrochloride and 416.65 μg/ml calculated as dexpanthenol. "Pravenor" medical device was found to be non-toxic in Vero cell culture. According to the results of MMT assay CC_{50} for octenidine dihydrochloride was 1.67 μg/ml, and 83.33 μg/ml – for dexpanthenol. CC_{50} indicators calculated for the different active ingredients of the medical device "Pravenor" were the following: 50 mg/ml for the dwarf palm berries extract (*Saw palmetto*), 16.67 mg/ml for the lovage roots extract (*Levisticum officinale*), and 16.67 mg/ml for the calendula flowers extract (*Calendula officinalis*). No sensitizing or skin irritating effects were observed in guinea pigs.
Conclusions. Biological evaluation of medical devices in the form of rectal suppositories "Pravenor" and vaginal suppositories "Prodexyn" performed using *in vitro* and *in vivo* biological systems. It was demonstrated an acceptable level of safety of the products. The MTT test was 5 times more sensitive than the Vero cell culture method in determination of cytotoxicity.

Keywords: medical devices; rectal suppositories; vaginal suppositories; antibacterial suppositories; cytotoxicity; sensitizing effects; irritating effects.

Introduction

Today it is impossible to imagine the creation of a modern innovative health care system without the development and implementation of advanced medical technologies in all medical spheres – both preventive and clinical. The wide use of medical devices (MD) allows efficient solving of the issues in medical diagnostics, prevention, and therapy. High-tech and innovative MD became an indispensable mechanism in providing replicability, mass scale and foreseeability of clinical and diagnostic results [1].

Specific features of drugs as a type of products in healthcare system are expressed mostly by their extreme variability of design, origins, methods of manufacture, and use [2, 3]. Such circumstances considerably restrict the development of general (universal) rules for MD standardization, in particular, from the point of view of their quality and safety. Unlike medicinal products with detailed directives on quality and safety (Pharmacopoieias, the Guidelines of the International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, and national regulatory authorities), similar detailed international and national normative documents are absent for medical devices. The mentioned characteristic of MD is also reflected in the systems of MD access for various national markets, which mostly envisage the procedures of compliance assessment, which involve wide range of authorized bodies [4, 5]. The regulatory bodies of developed countries, industry associations, and

international institutions make significant contributions to the international MD standardization system. The Guidelines for Biological Assessment of Medical Devices are developed by International Organization for Standardization, and currently are implemented in the majority of countries, including Ukraine. The series of ISO 10993 standards (Biological evaluation of medical devices) allowed unification of the requirements for various types of MD depending on their route of administration, type of contact with organism, and the contact's duration. The assessment concept, included in standard ISO 10993-1:2018, forms the basis for the development of the medical devices' assessment programs; however, it is not binding, namely due to the extremely wide range of MD peculiarities. MD diversity is supported by the fact that for many MDs the form of manufacture is very similar to medicinal products (for example, eye drops, vaginal and rectal suppositories, skin solution, patches, etc.) In such cases, the primary problem is to meticulously classify the product as a certain medical product's class [6]. The results of class's potential risk determination and MD biological assessment are important incoming information for MD assessment and risk management, which are an integral part of general quality assurance system of any medical devices manufacturer [7–9].

The aim of this article is to carry out a scientific and medical justification of the regulatory status (classification) of the medical devices in the form of vaginal and rectal suppositories, as well as their biological evaluation according to ISO 10993 standards.

Materials and methods

Medical devices. We used the following samples for testing: medical device "Pravenor" (rectal suppositories, further referred to as Pravenor), and medical device "Prodexyn" (vaginal suppositories, further referred to as Prodexyn). Pravenor has the following composition: extract of dwarf palm berries (*Saw palmetto*) – 150 mg, extract of lovage roots (*Levisticum officinale*) – 50 mg, extract of calendula flowers (*Calendula officinalis*) – 50 mg, excipients: hard fat. Prodexyn has the following composition: octenidine dihydrochloride – 2 mg, dexpanthenol – 100 mg, excipients: Macrogol 4000, Macrogol 400.

To dilute the suppositories we used centrifuge tubes and mix of ether (3 ml) and physiological solution (3 ml) in ratio 1:1 (v/v). The suppository was kept in a stoppered tube for 1 h till complete dissolution. At the next stage, the lower phase was collected into another tube, which was left open for 30 min for ether evaporation. The obtained extract (pH 6.8–7.2) was used for the device's biological evaluation. The calculated concentration of active components in the obtained extract was the following: for medical device Prodexyn – 0.67 mg/ml of octenidine dihydrochloride and 33.33 mg/ml of dexpanthenol; for medical device Pravenor – 50 mg/ml of dwarf palm berries extract (*Saw palmentto*), 16.67 mg/ml of lovage root extract (*Levisticum officinale*), and 16.67 mg/ml of calendula flowers extract (*Calendula officinalis*).

Cell culture and its culturing. Vero cell culture (passaged green monkey kidney cell culture), obtained from cell bank of D.I. Ivanovsky Institute of Virology of RAMS (Moscow, Russian Federation) and maintained at the research facility "L.V. Gromashevsky Institute of Epidemiology and Infectious Diseases of the NAMS of Ukraine" (Kyiv, Ukraine) was used.

The cells were maintained in culture via the common method, using complex medium, consisting of 90% RPMI-1640 medium (Sigma, USA) with the addition of 10% inactivated fetal bovine serum (FBS) (Sigma, USA), and antibiotic Kanamycin (50 IU/ml).

The cells were cultured in 50–100 ml glass or plastic vials (Nunc, Denmark) at 37 °C in the 5% CO_2 atmosphere. Every 3-4 days live cells were counted by staining the cells with trypan blue and seeded in the initial cell concentration per 1 ml.

The passaged cells were extracted from vial surface with Gibco® Versene Solution (0.2 g EDTA per litre of phosphate-buffered saline) (Thermo Fisher Scientific, USA), centrifuged and added into 1 ml of medium for precipitation, pipetted and counted in Goryaev's hemocytometer. The cells were seeded into well plates for cell culture (Sigma, США) with estimated number of 200,000 cells in 1 ml of the medium.

Determination of the medical devices' cytotoxic concentration (CC_{50}). To determine CC_{50} of the medical devices, we used at least ten rows of wells in plates with cell culture for each product's dilution. The plates with cell culture were incubated at 37 °C in the 5% CO_2 atmosphere for 5 days. Every day the test and control cultures were monitored for presence or absence of cytopathogenic effect (CPE). CPE degree was determined by changes in cells morphology (rounding, wrinkling of cells, detachment from well surface) and degenerative changes via the following system: "–" – complete absence of cell degeneration; "+" – NMT 25% of cell monolayer is affected; "++" – NMT 50% of

cell monolayer is affected; "+++" – NMT 75% of cell monolayer is affected; "++++" – complete degeneration of cell monolayer. CC_{50} of medical device was defined as the maximal rate, which did not cause degeneration of 50% of cells. Control was defined as a cell monolayer without addition of medical devices' extracts.

MTT assay. The method is based on functioning of the dehydrogenase system in intact cells' mitochondria that convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into formazan. The reaction product may be determined quantitatively by spectrophotometer. The MTT conversion into formazan decreases after death of cells, affected by toxic substances. The cells in concentration 5×10^5 ml^{-1} were cultured in 96-well plates in RPMI-1640 medium with addition of 10% FBS, which contained test substances in various concentrations. Controls were cells that not treated with the studied product. Each concentration was tested in 3 replicates. The plates with cells were incubated at 37 °C in the 5% CO_2 atmosphere for 48 h. MTT substrate (Sigma, USA) was dissolved in sterile phosphate-buffered saline solution (PBS) (0.2 M NaCl, 0.2 M K_2HPO_4, 0.2 M $Na_2H_2PO_4$, 0.2 M KCl), pH 7.2, at room temperature in a concentration of 5 mg/ml. The filtered MTT solution in a volume of 25 μl per 100 μl of cell suspension was put into wells and incubated for 3 h at 37 °C in the 5% CO_2 atmosphere. After incubation the plates with cells were centrifuged for cell precipitation at 1500 rpm for 10 min and rejected the supernatant. 100 μl of 96% ethanol were added to precipitation; it further dissolved crystalline formazin. After 10 min of thorough shaking at 37 °C the optical density of solutions was measured by spectrophotometer at wavelength 540 nm. The percentage of inhibition of cell viability when subjected to the test products was determined by the measuring of optical density of the test samples in comparison with the control cells (CC), which was taken as 100%. For convenience of assessment of the obtained results of cytotoxicity CC_{50} on the basis of MTT assay on the corresponding plot indicated the value corresponding to half of the CC (CC 50%).

Animals. In the study we used random-bred laboratory Guinea pigs, aged 3-4 months, weighing 300–400 g. The animals care was complied with the requirements of international standard ISO 10993-2:2006 "Biological evaluation of medical devices – Part 2: Animal welfare requirements".

Sensitization and skin irritation effects study. The hair was cut from the zones of 2×3 cm on the animals' backs; melted suppositories were applied directly on the skin, fixated with gauze bandage, and left for 4 h. Further monitoring for erythema and oedema was performed 12, 24, 48 and 72 h after the bandage's removal. Photo fixation of results was carried out. Each test group included 6 animals.

Bioethics norms. All work with animals, described in this article, was performed according to the Law of Ukraine "On protection of animals from abuse", European Convention for the Protection of Vertebrate Animals, as well as the Guide for the Care and Use of Laboratory Animals (8^{th} edition). Upon completion of the study the animals were sacrificed via a humane method, aimed at minimal physical and psychological suffering.

Results

Classification of medical devices and justification of biological testing program

Functional characteristics of the medical device Pravenor. To understand the product's functional characteristics, the product's content should be analyzed along with roles of its ingredients in the functioning of male urogenital system should be assessed. Berries of dwarf palm (*Saw palmetto*) contain the following biologically active substances that are necessary for normal functioning of prostate: phytosteroles (hormone precursors, synthesized in a human body), fatty acids (palmitic, linoleic, linolenic acids), lipase (promotes the digestion of fats, fatty acids, fat-soluble vitamins A, D, E) [10]. Lovage roots (*Levisticum officinale*) contain many essential oils (terpineol, cineole, acetic, isovaleric and benzoic acids), which promote urination [11]. Calendula flowers (*Calendula officinalis*) contain essential oils, carotenoids and flavonoids that support regeneration processes of body tissues [12]. Therefore, phytocomponents of this medical device represent a complex of biologically active substances that are necessary for and promote normal functioning of urogenital system in males, mainly – prostate. It is worth mentioning that the above phytocomponents are also included in several medicinal products and dietary supplements (in oral forms). The majority of medicinal products, containing extracts or tinctures of dwarf palm, lovage and calendula, are the so-called traditional medicinal products, which have been developed and marketed in Ukraine for more than 20 years. At present such new oral products are placed on the Ukrainian market as dietary supplements, and this reflects their functional principle of action better, i.e., provision of nutrients for better functioning of

the body's system. The preventive effect of such products is implemented in such a way along with their help in mitigation of respective diseases' course. Therefore, the effect of medical device Pravenor (rectal suppositories) on human body cannot be described as pharmacological or immunological, as well as the one modifying metabolism. Thus, this product may be considered as a medical device in the terms of Technical Regulations for Medical Devices, approved by the Decree No. 753 by the Cabinet of Ministers of Ukraine, dated October, 2, 2013, and Regulation (EU) 2017/745 of the European Parliament and of the Council as of April, 5, 2017 on medical devices.

The medical device Pravenor is classified as a short-term device because its continuous use is envisaged for less than 30 days. Considering rectal route of administration and the device partial absorption, the Rule 21 (Regulation (EU) 2017/745 of the European Parliament and of the Council of April, 5, 2017 on medical devices) may be applied to this medical device, namely: the devices that are composed of substances or substance combinations that are intended to be introduced into in human body via a body orifice or skin application, and that are absorbed or locally dispersed in a human body, and classified as class III, and which achieve their intended purpose in the stomach or lower gastrointestinal tract and which or whose products of metabolism are systemically absorbed by the human body.

Functional characteristics of medical device Prodexyn. To understand the product's functional characteristics. the product's content should be analyzed along with its ingredients's roles in the functioning of female urogenital system. Octenidine dihydrochloride is an antiseptic agent for mucus membranes, skin and wounds. It reacts with microbial cell's wall components and microbial membranes, causing disorders in cell functions. Its antimicrobial mechanism of action also involves the increase of cell membrane's permeability for potassium ions. Octenidine dihydrochloride is not absorbed by the gastrointestinal tract or skin and mucus membranes. Its penetration through placenta may be excluded. Octenidine dihydrochloride is not absorbed by vaginal mucous membrane or by wounds [13, 14]. Dexpanthenol, which is also a component of the product, is converted in tissues into a pantothenic acid, which is an integral part of coenzyme A, and plays important role in epithelium's normal functioning and acceleration of skin and mucous membranes' regeneration. Dexpanthenol is rapidly absorbed by skin and is immediately converted into pantothenic acid, which is widely distributed in organism tissues, mostly in the form of coenzyme A. Pantothenic acid is eliminated predominantly in unchanged form with urine and to a lesser extent – with faeces [15, 16].

Octenidine dihydrochloride in various pharmaceutical forms and methods of use is included also in medicinal and disinfecting products. Dexpanthenol is widely used both by the pharmaceutical and cosmetic industries due to its positive effect on skin and mucous membranes' regeneration. Thus, the medical device Prodexyn contains the antiseptic agent octenidine dihydrochloride, which does not have systemic effects on human organism, and which medical effect lies in inhibition of the development of extraneous microbiota in the vagina and prevention of sexually transmitted diseases. Another device's component, dexpanthenol, plays auxiliary role, aiming at prevention of potential adverse effects of octenidine dihydrochloride, namely – its irritating action. Therefore, the effect of medical device Prodexyn (vaginal suppositories) on human body cannot be described as pharmacological or immunological, as well as the one modifying metabolism. Thus, this product may be considered as medical device in the terms of Technical Regulations for Medical Devices, approved by the Decree No. 753 by the Cabinet of Ministers of Ukraine, dated October, 2, 2013, and Regulation (EU) 2017/745 of the European Parliament, and of the Council as of April, 5, 2017 on medical devices.

The medical device Prodexyn is classified as a short-term device because its continuous use is envisaged for less than 30 days. Considering vaginal route of administration, absence of absorption and systemic effect of octenidine dihydrochloride, the Rule 21 (Regulation (EU) 2017/745 of the European Parliament and of the Council as of April, 5, 2017 on medical devices) may be applied to this medical device, namely: the devices that are composed of substances or of combinations of substances, that are intended to be introduced into the human body via a body orifice or applied to skin, and that are absorbed by or locally dispersed in the human body, and classified as class IIb in all other cases. Taking into account the vaginal route of administration (invasive device) and its intended purpose (prevention of sexually transmitted diseases), the Rule 15 (Regulation (EU) 2017/745 of the European Parliament and of the Council as of April, 5, 2017 on medical devices) may be also applied to this medical device, namely: all devices, used for contraception or prevention of sexually transmitted diseases are classified as class IIb, un-

less they are implantable or long-term invasive devices, and in this case, they are classified as class III.

Program of biological testing. Considering the recommendations of standard ISO 10993-1:2018 "Biological evaluation of medical devices – Part 1: Evaluation and testing within a risk management process" (device group: surface device; type of contact: mucosal membrane; contact duration: 24 h – 30 days), as well as well-established safety profile of the substances in the device content, it was considered sufficient to include three following parameters into the biological testing program: cytotoxicity, sensitizing, and skin-irritating effects.

Biological evaluation of medical devices

Determination of the device cytotoxic concentration (CC_{50}). Vero cells culture (Fig. 1) was used to determine the CC_{50} of the products. At least ten rows of wells in plates with cell culture were used in the experiments for each device dilution in medium. The test and control cultures were monitored every day to determine the presence or absence of CPE. The study results are summarized in the Table and Fig. 1.

The maximum level of dilution of the extract of the product Prodexyn, which led to the CPE of Vero cell culture, was 1:80, which corresponds to the concentration of octenidine dihydrochloride – 8.35 μg/ml, for dexpanthenol – 416.65 μg/ml. At the same time, Pravenor was found to be completely non-toxic when tested in Vero cells culture.

According to MTT assay results (Fig. 2), it was shown that cytotoxic effect CC_{50} was registered for extract dilution 1:400 for medical device Prodexyn (line intersection "CC 50%" and "No. 1 Prodexyn" on Fig. 2). This dilution corresponds to the following values of CC_{50}: for octenidine dihydrochloride – 1.67 μg/ml, for dexpanthenol – 83.33 μg/ml.

According to MTT assay results, medical device Pravenor did not demonstrate a toxic effect, which was confirmed by the obtained ratio of optical density for test product versus optical density for control cell culture (see Fig. 2). Based on the obtained results, it can be concluded that CC_{50} for this product exceeds 50 mg/ml for dwarf palm berried extract (*Saw palmetto*), 16.67 mg/ml for lovage root extract (*Levisticum officinale*), and 16.67 mg/ml for calendula flowers extract (*Calendula officinalis*).

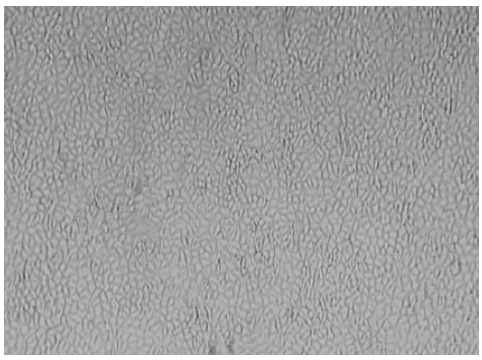


Figure 1: Vero cell culture, used in the experiment (magnification ×10)

Studies of sensitization and skin irritation effects

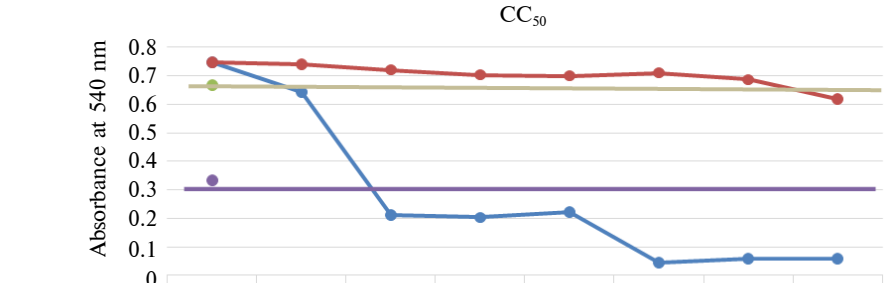
Monitoring of experimental animals have shown that both devices do not cause any skin irritation and are safe to use. The observations were recorded in photographs (Fig. 3).

Discussion

Medical device regulatory status has crucial meaning for the strategy of a scientific research project. There is a considerable difference in studies of the safety and efficiency of medicines and medical devices. Some health care products may have similar or even equivalent forms (solutions for injection, skin solution, nasal sprays, vaginal and rectal suppositories, etc.), however, they may be classified into different regulatory groups. In addition to the aforementioned aspects of preclinical and clinical studies of medical products their regulatory status sometimes influences pharmacoeconomic parameters of the respective projects [17]. In the terms of access of the developed medical products, in particular – medical devices to various segments of global pharmaceutical market the problem of unification of regulatory requirements in various states and supranational structures is very important (for example, EU) for managing quality, safety, and efficiency of such products [18, 19]. Manufacturers of medical devices and other medical products constantly discuss the issues of interconnection between scientific-technical issues in this sphere in terms of regulatory requirements [20–23].

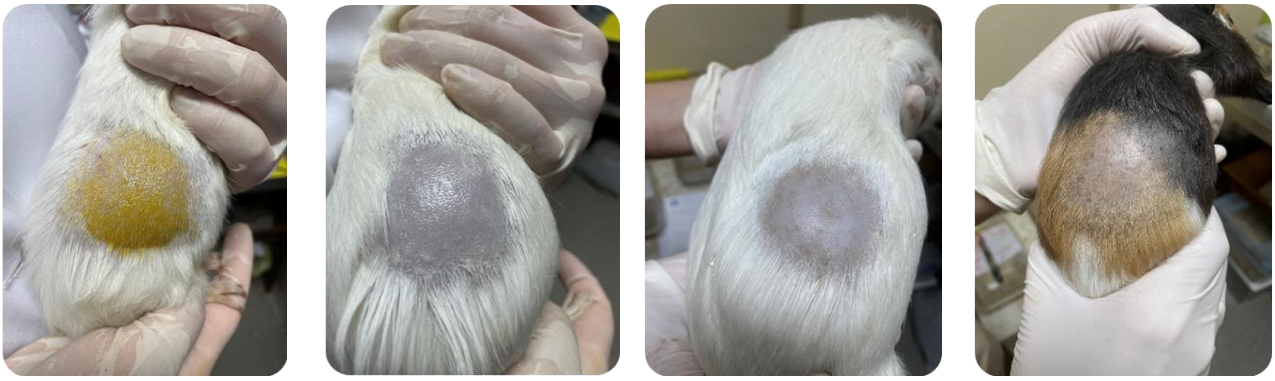
Table: The study results of the device cytotoxic concentration in Vero cell culture

Dilution	Device	
	Prodexyn	Pravenor
1:10	10/10*	0/10
1:20	10/10	0/10
1:40	10/10	0/10
1:80	5/10	0/10
1:160	0/10	0/10
1:320	0/10	0/10
1:640	0/10	0/10
1:1280	0/10	0/10
CC_{50}	1:80	Non-toxic



Dilution	1280	640	320	160	80	40	20	10
№ 1 Prodexyn	0.7475	0.6435	0.2105	0.203	0.2225	0.045	0.0595	0.0595
№ 2 Pravenor	0.7465	0.7405	0.7215	0.704	0.7005	0.711	0.687	0.619
CC	0.6683							
CC 50%	0.334							

Figure 2: Results of cytotoxicity assessment by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay



Guinea pig with the skin zone, on which "Pravenor" was applied

Guinea pig with the skin zone, on which "Prodexyn" was applied

Guinea pig skin after "Pravenor" testing

Guinea pig skin after "Prodexyn" testing

Figure 3: Results of determining of the devices' sensitizing and skin-irritating effects

There are many products for vaginal and rectal use, which are sold on the developed countries markets, and which are classified as medical devices. Usually intra-vaginal medical devices in traditional pharmaceutical forms (suppositories, tablets, capsules, etc.) are developed for correction (restoration) of vaginal microbiota [24–26]. Other indications for such devices include promotion of fertilization [27], delivery of anti-HIV drugs [28], etc. Medical devices in the form of rectal suppositories, creams, and gels are used for treatment and prevention of proctological and urological diseases [29, 30]. In view of this, our medical-scientific justification of classification of the studied products as medical devices is harmonized with regulatory bodies practice (Conformity Assessment Body) of the European Union countries. It is worth noting that at present the current Ukrainian Technical Regulations on Medical Devices and Medical Device Regulations, adopted in the European Union [23, 31], do not match in the part of establishing risk classes for several medical devices, including invasive devices. However, such regulatory disparities between Ukrainian and EU markets do not affect significantly the determination of biological evaluation strategy for such devices.

The obtained data on cytotoxicity of vaginal suppositories (Prodexyn), containing octenidine dihydrochloride and dexpanthenol, correspond to the data of other scientists. Thus, in the study [32] cytotoxic effect of octenidine solution was assessed on fibroblast cell cultures and MCF7 cells (epithelial-like cell line, obtained from invasive adenocarcinoma of human mammary ducts). Octenidine solution in the concentration of 0.5 mg/l significantly inhibited cell growth in 24 h, however, with the concentration of 0.012 mg/l the cytotoxic effect was not observed. Such results allowed authors to recommend octenidine solution for cutaneous use in the treatment of purulent wounds. Similar results were obtained while studying several substances with antiseptic properties as the candidates

for gingivitis treatment: octenidine showed the lowest cytotoxic effect on fibroblasts and epithelial cells [33] among the studied five agents. The modern toxicological studies of dexpanthenol-containing combinations are aimed at determination of the decrease of toxic (in particular, cytotoxic) effects after introduction of dexpanthenol into combination. This approach is justified by dexpanthenol's biological properties, namely – its transformation into pantothenic acid in a body. Pantothenic acid is an integral part of coenzyme A and participates in acetylation, carbohydrate and fat metabolism, synthesis of acetylcholine, corticosteroids, porphyrins; it stimulates regeneration of skin and mucous membranes, normalizes cell metabolism, accelerates mitosis and increases strength of collagen fibres [15, 34]. Most often dexpanthenol is included in drugs for nasal use to improve their safety profile. Thus, the study [35] showed that dexpanthenol significantly decreased the toxic effect of xylometazoline, used on amniotic epithelial cells (human amniotic cell line). Another study [36] proved that dexpanthenol (5%) decreased the toxic effects of xylometazoline and benzalkonium chloride used on amniotic epithelial cell growth. These data suggest the adequacy of the chosen combination in medical device Prodexyn (octenidine + dexpanthenol) both from the point of efficacy and safety profile. The obtained data are absolutely new on the proposed combination and its method of administration.

Phytocombination of medicinal herbal extracts in the medical device Pravenor is original, therefore the study of the cytotoxic effect of this product on cell culture was relevant both from scientific and regulatory point of view. The available literature data [37–42] on cytotoxic effects of extracts from *Saw palmetto*, *Levisticum officinale*, *Calendula officinalis* of biologically active substances, obtained from these medicinal herbs, are, on one hand, addressed by their individual studies (as monopreparations), and, on the other hand, are focused on assessment of anti-cancer activities. Therefore, the obtained data on the absence of cytotoxic effect of the medical device Pravenor are an important prerequisite for safe use of this product as a prostate protector.

Conclusions

Scientific-medical and regulatory justification of classifying Prodexyn and Pravenor as invasive medical devices for prolonged use was performed. The cytotoxic, sensitization and skin irritation studies are sufficient for biological evaluation of such medical devices.

The maximum cytotoxic concentration of the medical device's Prodexyn active ingredients, which led to the CEP of Vero cell culture, was for octenidine dihydrochloride – 8.35 μg/ml and dexpanthenol – 416.65 μg/ml. According to the MTT assay results for the medical device Prodexyn the cytotoxic effect CC_{50} was recorded for dilution that corresponds to the values of CC_{50}: octenidine dihydrochloride – 1.67 μg/ml and dexpanthenol – 83.33 μg/ml.

The medical device Pravenor was completely non-toxic in the study in Vero cell culture. Similar results were obtained in the MTT assay: it was established that the CC_{50} for this product exceeds 50 mg/ml for the extract of dwarf palm berries (*Saw palmetto*), 16.67 mg/ml for lovage root extract (*Levisticum officinale*), and 16.67 mg/ml for calendula flowers extract (Calendula officinalis).

The method of cytotoxicity evaluation, which is based on the assessment of the condition of dehydrogenase system of mitochondrial cells (MTT assay), was 5 times more sensitive if compared to the approach of detecting cytopathic effects on Vero cell culture. Thus, the MTT assay can be considered as a more informative method for the medical devices' cytotoxicity evaluation.

Both medical devices did not demonstrate sensitizing and skin-irritating effects after application on the skin.

The obtained data on safety profile of medical devices Prodexyn and Pravenor allow recommending the products for use in the claimed medical field.

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Disclosure of Interest

Oleksandra Dmytrenko reports being employee of "UA "PRO-PHARMA" LLC; the author declare that she has no competing interests. Alexander Galkin is the Editor-in-Chief of *Innovative Biosystems and Bioengineering* and was not involved in the editorial evaluation or decision to accept this article for publication. The remaining authors have no conflicts of interest to declare.

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БІОЛОГІЧНЕ ОЦІНЮВАННЯ МЕДИЧНИХ ВИРОБІВ У ФОРМІ СУПОЗИТОРІЇВ ДЛЯ РЕКТАЛЬНОГО ТА ВАГІНАЛЬНОГО ЗАСТОСУВАННЯ

Проблематика. Програми доклінічного вивчення безпеки продуктів у системі охорони здоров'я залежать від регуляторного статусу досліджуваних продуктів. Класифікування таких продуктів, зокрема супозиторіїв для ректального та вагінального застосування, є критичним етапом для розробки тактики їх біологічного оцінювання. Актуальними для біомедицини залишаються питання адаптації методів біологічного оцінювання медичних виробів на основі комбінації біологічно активних речовин, а також оцінки результатів такого оцінювання.

Мета. Здійснити обґрунтування регуляторного статусу та біологічне оцінювання медичних виробів у формі вагінальних супозиторіїв на основі октенідину дигідрохлориду ("Продексин") та у формі ректальних супозиторіїв на основі екстрактів рослин *Saw palmetto, Levisticum officinale* і *Calendula officinalis* ("Правенор").

Методика реалізації. Біологічне оцінювання проводили згідно з вимогами стандартів серії ISO 10993 за допомогою біологічних тест-систем *in vitro* та *in vivo* (цитотоксичність у культурі клітин та у МТТ-тесті, сенсибілізуюча та подразнювальна дія на мурчаків).
Результати. Цитотоксичність (CC_{50}) екстракту медичного виробу "Продексин" на культурі клітин Vero становила 8,35 мкг/мл у перерахунку на октенідину дигідрохлорид та 416,65 мкг/мл у перерахунку на декспантенол. Медичній виріб "Правенор" виявився нетоксичним на культурі клітин Vero. За результатами МТТ-тесту CC_{50} для октенідину дигідрохлориду становила 1,67 мкг/мл, декспантенолу – 83,33 мкг/мл. CC_{50} у перерахунку на активні інгредієнти медичного виробу "Правенор" становила 50 мг/мл для екстракту ягід карликової пальми (*Saw palmetto*), 16,67 мг/мл для екстракту коренів любистку лікарського (*Levisticum officinale*), 16,67 мг/мл для екстракту квіток нагідок лікарських (*Calendula officinalis*). Для медичних виробів не було виявлено сенсибілізуючої та шкіроподразнювальної дії на мурчаків.
Висновки. Біологічне оцінювання медичних виробів у формі ректальних супозиторіїв "Правенор" і вагінальних супозиторіїв "Продексин", проведене із використанням біологічних систем *in vitro* та *in vivo*, засвідчило прийнятний рівень безпечності цієї продукції. При визначенні цитотоксичності МТТ-тест виявився у 5 разів чутливішим порівняно з методом на основі культури клітин Vero.

Ключові слова: медичні вироби; ректальні супозиторії; вагінальні супозиторії; антибактеріальні супозиторії; цитотоксичність; сенсибілізуюча дія; шкіроподразнювальна дія.

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БИОЛОГИЧЕСКОЕ ОЦЕНИВАНИЕ МЕДИЦИНСКИХ ИЗДЕЛИЙ В ФОРМЕ СУППОЗИТОРИЕВ ДЛЯ РЕКТАЛЬНОГО И ВАГИНАЛЬНОГО ИСПОЛЬЗОВАНИЯ

Проблематика. Программы доклинического исследования безопасности продуктов в системе здравоохранения зависят от регуляторного статуса исследуемых продуктов. Классифицирование таких продуктов, в частности суппозиториев для ректального и вагинального применения, является критическим этапом разработки тактики их биологического оценивания. Актуальными остаются вопросы адаптации методов биологического оценивания медицинских изделий на основе комбинации биологически активных веществ, а также оценки результатов такого оценивания.
Цель. Произвести обоснование регуляторного статуса и биологическое оценивание медицинских изделий в форме вагинальных суппозиториев на основе октенидина дигидрохлорида ("Продексин"), а также в форме ректальных суппозиториев на основе растительных экстрактов *Saw palmetto*, *Levisticum officinale* и *Calendula officinalis* ("Правенор").
Методика реализации. Биологическое оценивание проводилось согласно требованиям стандартов серии ISO 10993 с помощью биологических тест-систем *in vitro* и *in vivo* (цитотоксичность в культуре клеток и в МТТ-тесте, сенсибилизирующее и раздражающее действие на морских свинок).
Результаты. Цитотоксичность (CC_{50}) экстракта медицинского изделия "Продексин" на культуре клеток Vero составила 8,35 мкг/мл в пересчете на октенидина дигидрохлорид и 416,65 мкг/мл в пересчете на декспантенол. Медицинское изделие "Правенор" оказалось нетоксичным на культуре клеток Vero. По результатам МТТ-теста CC_{50} для октенидина дигидрохлорида составила 1,67 мкг/мл, декспантенола – 83,33 мкг/мл. CC_{50} в пересчете на активные ингредиенты медицинского изделия "Правенор" составила 50 мг/мл для экстракта ягод карликовой пальмы (*Saw palmetto*), 16,67 мг/мл для экстракта корней любистока лекарственного (*Levisticum officinale*), 16,6 мг/мл для экстракта цветков календулы лекарственной (Calendula officinalis). Для медицинских изделий не было обнаружено сенсибилизирующего и кожераздражающего действия на морских свинок.
Выводы. Биологическое оценивание медицинских изделий в форме ректальных суппозиториев "Правенор" и вагинальных суппозиториев "Продексин", проведенное с использованием биологических тест-систем *in vitro* и *in vivo*, удостоверило приемлемый уровень безопасности данной продукции. При определении цитотоксичности МТТ-тест оказался в 5 раз более чувствительным по сравнению с методом на основе культуры клеток Vero.

Ключевые слова: медицинские изделия; ректальные суппозитории; вагинальные суппозитории; антибактериальные суппозитори; цитотоксичность; сенсибилизирующее действие; кожераздражающее действие.

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Original article

QSAR-АНАЛІЗ ВПЛИВУ ІОНІВ МЕТАЛІВ НА АКТИВНІСТЬ ПЕПТИДАЗИ *Bacillus thuringiensis* var. *israelensis* IMV B-7465

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Проблематика. Каталітична активність ензимів, що є їх найважливішою характеристикою, може суттєво змінюватися під впливом ефекторів, наприклад іонів металів, і є предметом спеціальних досліджень, що важливі для біохімії, біотехнології, медицини та інших галузей науки. Зазвичай активність ензимів за наявності металів оцінюють за зміною швидкості ферментативної реакції. Однак проведення подібних експериментальних досліджень, особливо для нових ензимів, як у випадку з пептидазою *Bacillus thuringiensis* var. *israelensis* IMV B-7465, потребує значних ресурсів і розгорнутих кінетичних досліджень. Тому доцільним є застосування методів комп'ютерної хімії, базовою задачею якої є пошук залежності "структура–властивість", для побудови моделі, що матиме змогу з високою вірогідністю оцінити вплив іонів металів на активність пептидази.

Мета. Розробка QSAR-моделей для аналізу і прогнозування впливу іонів металів на активність пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465.

Методика реалізації. Вплив іонів металів вивчали, визначаючи протеолітичну активність пептидази після сумісної інкубації впродовж 30 хв у 0,0167 М буферного розчину трис-HCl (pH 7,5, 37 °C). Кінцева концентрація хлоридів металів Li^+; Na^+; K^+; Cs^+; Cu^{2+}; Be^{2+}; Mg^{2+}; Ca^{2+}; Sr^{2+}; Ba^{2+}; Zn^{2+}; Cd^{2+}; Hg^{2+}; Cr^{3+}; Mn^{2+}; Co^{2+}; Ni^{2+} у буферному розчині становила 4 ммоль/дм^3. Для пошуку кількісного зв'язку "структура–властивість" використовували довідкові дані про властивості іонів металів та методи тренд-вектора і випадкового лісу.

Результати. Дослідження впливу іонів металів на протеолітичну активність пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465 показало, що деякі іони металів (Li^+, Mn^{2+} і Co^{2+}) активували пептидазу, а інші (Cu^{2+}, Be^{2+}, Cd^{2+}, Hg^{2+}, Cr^{3+}) інгібували активність ензиму. Нелінійними методами тренд-вектора та випадкового лісу побудовано адекватні статистичні моделі без помилок класифікації та помилок прогнозу класу активності для тестового набору. Обидві моделі показують, що найважливішими характеристиками іонів металів, які мають вплив на активність ензиму, є електронегативність (EN_{Pol}), перший потенціал іонізації (IP_1), ентропія іонів у водному розчині (S) та енергія спорідненості до електрона (E_{ae}).

Висновки. Методи QSAR-аналізу в сукупності з нелінійними методами тренд-вектора та випадкового лісу дають змогу адекватно описати вплив іонів металів на активність пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465 за рахунок дескрипторів, що відображають певний баланс їхніх електронодонорних і електроноакцепторих властивостей (електронегативність, перший потенціал іонізації, енергія спорідненості до електрона) та ступінь структурованості гідратної оболонки (ентропія сольватації іонів). Обидва статистичних методи дають близькі значення важливості дескрипторів, але тільки метод тренд-вектора дає змогу проаналізувати напрям впливу конкретних характеристик іонів.

Ключові слова: пептидаза *Bacillus thuringiensis* var. *israelensis* IMV B-7465; іони металів; QSAR-аналіз; випадковий ліс; тренд-вектор; активність ензиму.

Вступ

Ензими – унікальні біокаталізатори різноманітних біохімічних процесів – мають високу селективність, каталітичну активність, здатні зберігати свої властивості поза клітинами, що обумовлює їх практичне використання в медицині та різних галузях промисловості: фармацевтичній, харчовій, хімічній. За обсягом виробництва препарати ензимів займають серед біологічно активних речовин ключові позиції після антибіотиків і амінокислот. Найширше використання в промисловості мають гідролітичні, у т.ч. протеолітичні, ензими [1, 2].

Практичне застосування протеїназ (КФ 3.4.21-24) і пептидаз (КФ 3.4.11-15) у медицині різноманітне. Вони чинять тромболітичну та протизапальну дію. Однак найбільш значимим є їх застосування в хірургії для терапії ран різної етіології, що пов'язано з локальністю дії протеаз: швидким відторгненням нежиттєздатної тканини без впливу на здорові ділянки [3].

Особливу увагу дослідників привертають протеолітичні ензими мікробного походження, що мають комплексну протеолітичну активність, здатні до розщеплення нерозчинних білкових субстратів: фібрину, колагену, еластину, завдяки чому є перспективними для лікування ран і опіків. Дослідження цих ензимів мають важливі фундаментальні аспекти в галузі біохімії, мікробіології, медичної біотехнології, біомедицини для визначення їх структури, каталітичної активності, механізму дії, фізико-хімічних та біохімічних властивостей. Як у теоретичному аспекті, так і для створення нових біопродуктів, перспективних для використання у рановій терапії, актуальним є пошук інгібіторів і активаторів нових ензимів, до яких належить пептидаза *Bacillus thuringiensis* var. *israelensis* IMV B-7465, що має комплексну протеолітичну активність.

У роботі [3] розглядається вплив іонів металів на активність протеаз різного походження, який здійснюється за рахунок неспецифічного зв'язування іонів металів молекулами ензиму. Під дією таких ефекторів каталітична активність ензимів може змінюватись і залежати від типу взаємодії іонів металів із біологічною молекулою. Тому нове дослідження або доповнення існуючих знань про вплив подібних ефекторів на активність ензимів має як теоретичне, так і практичне значення. Однак проведення подібних експериментальних досліджень, особливо для нових ензимів, як у випадку з пептидазою *Bacillus thuringiensis* var. *israelensis* IMV B-7465, потребує значних ресурсів, насамперед достатньої кількості ензиму [4]. Тому доцільним є застосування методів комп'ютерної хімії, базовою задачею якої є пошук залежностей "структура–властивість" для побудови адекватних QSAR-моделей, здатних оцінити вплив іонів металів на активність пептидази.

Внесок фізико-хімічних параметрів, що описують властивості іонів металів, у зміну активності пептидази ми дослідили методами QSAR (Quantitative Structure Activity Relationship) моделювання. Раніше подібні дослідження нами були проведені для карбоксилестерази печінки свині, тирозинази та лізоциму, що дало змогу з високим ступенем вірогідності прогнозувати активність ензимів за наявності іонів металів [5, 6].

У цьому дослідженні ми ставимо за мету розробку QSAR-моделей для аналізу та прогнозування впливу іонів металів на активність пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465.

Матеріали і методи

У роботі використовували пептидазу *Bacillus thuringiensis* var. i*sraelensis* IMV B-7465, люб'язно надану проф. Л.Д. Варбанець, Інститут мікробіології і вірусології ім. Д.К. Заболотного НАН України, казеїн за Гаммерстеном, бичачий сироватковий альбумін (Sigma-Aldrich), хлориди металів (МЧП "ТОР").

Визначення вмісту білка проводили методом Лоурі в модифікації Хартрі [7]. Калібрувальний графік будували, використовуючи бичачий сироватковий альбумін, стандартизований за 280 нм, відповідно до $E^{1\%}_{1\text{см}} = 6{,}6$.

Загальну протеолітичну активність визначали за методом Ансона в модифікації Петрової та Вінцюнайте [8], який базується на кількісному визначенні тирозину, що вивільняється при гідролізі казеїну під дією досліджуваного ензиму. За одиницю активності (ПО – протеолітична одиниця) покладали здатність пептидази за 1 хв при 37 °C переводити казеїн у неосаджуваний трихлороцтовою кислотою стан у кількості, що відповідає 1 мкмоль тирозину.

Вплив іонів металів вивчали, визначаючи протеолітичну активність пептидази після сумісної інкубації впродовж 30 хв у 0,0167 М буферного розчину трис-HCl (pH 7,5, 37 °C). Кінцева концентрація хлоридів металів Li^+; Na^+; K^+; Cs^+; Cu^{2+}; Be^{2+}; Mg^{2+}; Ca^{2+}; Sr^{2+}; Ba^{2+}; Zn^{2+}; Cd^{2+}; Hg^{2+}; Cr^{3+}; Mn^{2+}; Co^{2+}; Ni^{2+} у буферному розчині становила 4 ммоль/дм^3.

Для пошуку зв'язку "структура–властивість" використовували довідкові дані про властивості іонів металів [9] та статистичні методи тренд-вектора (Т-вектора) [10, 11] і випадкового лісу [12, 13].

Метод Т-вектора ґрунтується на принциповій ідеї теорії розпізнавання образів – розбиття n об'єктів на два класи відносно середнього значення досліджуваної властивості ($\overline{A}$). Величини ($A_i - \overline{A}$) набувають для одного класу позитивних значень, а для іншого – негативних і тому можуть асоціюватися із зарядами. Звідси, аналогічно вектору дипольного моменту, Т-вектор характеризує в багатовимірному просторі структурних параметрів X_{ij} (i – номер молекули, j – номер структурного параметра) поділ зарядів, що відповідають активному і неактивному класам молекул. Кожна компонента Т-вектора визначається як

$$T_j = \frac{1}{n} \cdot \sum_{i=1}^{n} (A_i - \overline{A}) \cdot X_{ij}$$

і відображає ступінь і напрям впливу *j*-го структурного параметра на величину активності *A*. Для розв'язання оберненої задачі (прогноз активності) використовується таке співвідношення:

$$rank(\hat{A}_i) = rank\left(\sum_{j=1}^{m} T_j X_{ij}\right). \qquad (1)$$

Зазначимо, що кожна компонента Т-вектора розраховується незалежно від інших, і її внесок у модель не налаштовується, тому вплив кількості використовуваних структурних параметрів на надійність моделі не такий критичний, як для регресійних методів. У запропонованому підході якість зв'язку структура–властивість оцінювалась за величиною коефіцієнта рангової кореляції (за Спірменом) між рангом заданої активності та рангом обчисленої активності. Стабільність моделі визначалась в умовах ковзного контролю, коли кожна молекула видалялась із навчальної вибірки і для неї здійснювався прогноз активності на основі отриманої моделі. Отриманий у таких умовах коефіцієнт детермінації Q^2 використовувався як характеристика стійкості моделі.

Також у роботі ми використовували метод випадкового лісу (RF – Random Forest) [10]. Він будується на основі методу класифікаційних дерев і фактично являє собою ансамбль дерев рішень [14]. Статистичний метод RF досить широко використовується для побудови QSAR-моделей завдяки таким своїм перевагам: відсутність проблеми перенавчання моделей і необхідності попереднього відбору змінних; адекватність внутрішньої процедури оцінки якості та прогнозної здатності моделей; стійкість моделей до наявності "шуму" у вихідній вибірці; ефективність роботи з великими базами даних; можливість інтерпретувати одержані моделі; можливість аналізувати вибірки, що включають сполуки з різним механізмом дії. Дані експериментів щодо впливу іонів металів на протеолітичну активність пептидази піддавали статистичній обробці відповідно до [15]. Оцінювали ступінь вірогідності результатів досліджень за кількості повторів $n = 5$ відносно вихідної активності пептидази (8,25 ПО/мг білка за хвилину). Ймовірність відмінностей між середніми значеннями визначали за критерієм Стьюдента на рівні значущості не менше 95 % ($M \pm m$ при $p \leq 0{,}05$).

Результати

На першому етапі досліджень проводили експериментальне визначення протеолітичної активності ензиму (вміст білка 2 мг/см^3) після його інкубації з хлоридами металів у буферному розчині, який не містив у своєму складі додаткових катіонів металів. Інкубація була необхідна для процесу взаємодії ензиму з іонами металів. Концентрація солей металів 4 ммоль/дм^3 була вибрана за результатами проведених досліджень.

Дослідження впливу 18 хлоридів металів на протеолітичну активність пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465 показало, що їх додавання до розчину ензиму та інкубація протягом 30 хв у більшості випадків приводили до зміни ферментативної активності. Так, деякі іони металів (Li^+, Mn^{2+} і Co^{2+}) активували пептидазу, а деякі (Cu^{2+}, Be^{2+}, Cd^{2+}, Hg^{2+}, Cr^{3+}) – інгібували активність ензиму.

З рис. 1, на якому значення активності ензиму (%) під впливом іонів металів впорядковані за зростанням, видно, що вони добре діляться на два класи: неактивні ($A < A_{ср}$) й активні ($A > A_{ср}$), де $A_{ср} = 74{,}9$ – середнє значення активності, причому найбільше підвищення активності (+33,8 %) спостерігається саме в області $A_{ср}$ між іонами Ni^{2+} та La^{3+}. Тому для побудови адекватних QSAR-моделей краще використовувати не величину активності, а її клас, для чого добре підходять класифікаційні статистичні методи. Саме тому далі в роботі для вирішення цього завдання використовували нелінійні методи тренд-вектора [10, 11] і випадкового лісу [12, 16], що добре зарекомендували себе раніше для побудови статистичних моделей на основі класифікаційних оцінок активності.

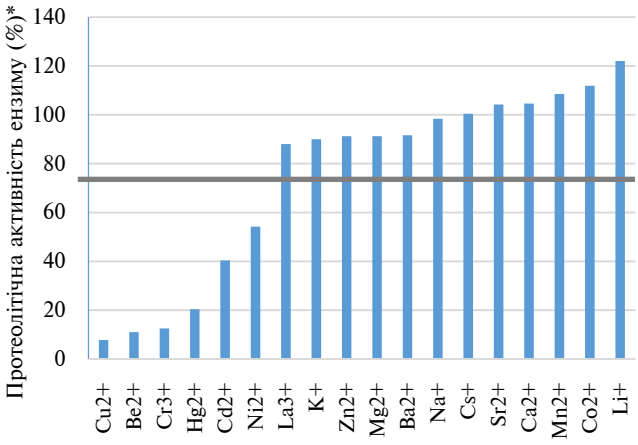


Рисунок 1: Протеолітична активність ензиму (% від вихідної) під впливом іонів металів та їх розподіл відносно середнього значення активності (*$p \leq 0{,}05$ порівняно з контролем, $n = 5$)

Значення протеолітичної активності пептидази (A_{obs}) та класу активності ($A_{obs.\ class}$) наведені в табл. 1.

Таблиця 1: Протеолітична активність пептидази після інкубації з хлоридами металів

Сполука	Іон металу	A_{obs}* ПО/мг білка за хв, $M \pm m$	A_{obs}* %	$A_{obs.\ class}$
1	Li^+	10,06 ± 0,05	122,0	1
2	Na^+	8,1 ± 0,25	98,4	1
3	K^+	7,41 ± 0,29	90,0	1
4	Cs^+	8,27 ± 0,29	100,4	1
5	Be^{2+}	0,91 ± 0,05	11,0	0
6	Ca^{2+}	8,61 ± 0,43	104,6	1
7	Sr^{2+}	8,57 ± 0,43	104,2	1
8	Ba^{2+}	7,54 ± 0,38	91,6	1
9	Zn^{2+}	7,51 ± 0,38	91,2	1
10	Cd^{2+}	3,32 ± 0,17	40,4	0
11	Hg^{2+}	1,68 ± 0,08	20,4	0
12	Cr^{3+}	1,03 ± 0,05	12,5	0
13	Mn^{2+}	8,93 ± 0,44	108,5	1
14	Co^{2+}	9,21 ± 0,46	111,9	1
15	Ni^{2+}	4,46 ± 0,22	54,2	0
16	Cu^{2+}	0,64 ± 0,03	7,8	0
17	Mg^{2+}	7,51 ± 0,38	91,2	1
18	La^{3+}	7,22 ± 0,36	88,0	1

Примітки. Вихідна активність ензиму 8,25 ПО/мг білка за хв, *$p \leq 0,05$ порівняно з контролем; $n = 5$; A_{obs} – значення спостережуваної протеолітичної активності; $A_{obs.\ class}$ – значення спостережуваного класу протеолітичної активності.

Для можливості перевірки здатності моделей до прогнозу вибірку сполук було розділено на навчальну (сполуки 1–15) і тестову (сполуки 16–18). До тестової вибірки увійшли сполуки різних класів активності. Зробити тестову вибірку більш розширеною не було можливості через невелику кількість сполук, що досліджувались.

Для побудови QSAR-моделей аналізували близько 70-ти дескрипторів, що описують властивості іонів металів [9], в тому числі:

- ефективні заряди атомів у основному стані;
- потенціали іонізації елементів;
- енергії дисоціації для солей хлоридів металів;
- середні енергії зв'язків у молекулах типу MCl_x;
- електронегативність елементів за шкалою Полінга;
- термохімічні електронегативності;
- іонізаційні електронегативності елементів;
- довжини зв'язків у галогенідах типу $MeCl_x$;
- атомні радіуси;
- іонні радіуси;
- рекомендовані електронегативності елементів;
- спорідненість до електрона;
- основні термодинамічні характеристики;
- термодинамічні характеристики іонів і нейтральних молекул у водному розчині;
- енергія кристалічної решітки іонних сполук;
- стандартні електронні потенціали у водних розчинах та ін.

У результаті статистичного аналізу кожним зі статистичних методів (тренд-вектора та випадкового лісу) було отримано адекватні QSAR-моделі (М1 та М2 відповідно) **без помилок класифікації та помилок прогнозу** класу активності для тестового набору. В обох випадках тільки чотири дескриптори увійшли до моделей: перший потенціал іонізації (IP_1); ентропія іонів у водному розчині (S, Дж/моль·К); електронегативність елементів за шкалою Полінга (EN_{Pol}); енергія спорідненості до електрона (E_{ae}, еВ). Значення цих дескрипторів для різних іонів металів наведені в табл. 2.

Таблиця 2: Значення дескрипторів, що були відбрані для побудови статистичних моделей [11]

Сполука	Іон металу	IP_1, еВ	S, Дж/моль·К	EN_{Pol}	E_{ae}, еВ
1	Li^+	5,39	13,4	0,98	0,618
2	Na^+	5,14	59	0,93	0,5479
3	K^+	4,34	102,5	0,82	0,5015
4	Cs^+	3,89	52,63	0,7	–0,39
5	Be^{2+}	9,32	75,8	1,57	–0,19
6	Ca^{2+}	6,11	–53,1	1,00	0,0246
7	Sr^{2+}	5,69	–26,4	0,95	1,51
8	Ba^{2+}	5,21	9,6	0,89	0,1446
9	Zn^{2+}	9,39	–112,1	1,65	–0,09
10	Cd^{2+}	8,99	–73,2	1,69	–0,27
11	Hg^{2+}	10,44	146	1,90	–0,19
12	Cr^{3+}	6,77	123	1,66	0,666
13	Mn^{2+}	7,43	–73,6	1,55	–0,97
14	Co^{2+}	7,88	–113	1,88	0,662
15	Ni^{2+}	7,64	97,7	1,91	1,156
16	Cu^{2+}	7,73	40,6	1,9	1,235
17	Mg^{2+}	7,65	–138,1	1,31	–0,22
18	La^{3+}	5,58	–217,6	1,1	0,47

Модель М1, побудована методом тренд-вектора (без помилок класифікації та помилок прогнозу). Для побудованої моделі коефіцієнт детермінації в умовах ковзного контролю становить $Q^2 = 0,70$, що вище допустимого значення 0,5. Внески параметрів (значення див. у табл. 2) у модель, що оцінювали за допомогою компонентів тренд-вектора, мають такі значення:

T (EN_{Pol}) = –0,53 (35 %);

T (IP_1) = –0,50 (33 %);

T (S) = –0,39 (26 %);

T (E_{ae}) = 0,09 (6 %).

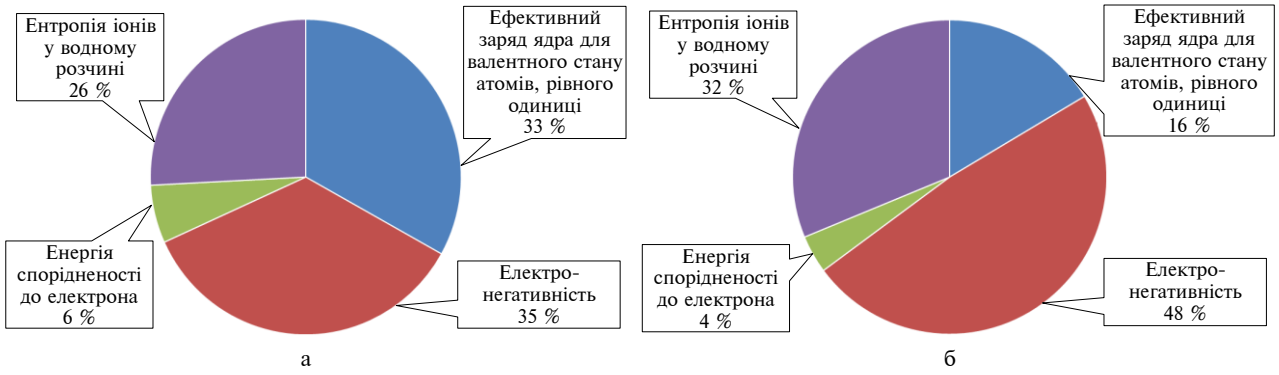
Негативні внески параметрів означають їх обернено пропорційний внесок, тобто збільшення значень цих параметрів призводить до зниження активності.

Аналіз модулів внесків структурних параметрів на досліджувану активність пептидази показав, що характеристики іонів, які визначають їх електронодонорні та електроноакцепторні властивості (EN_{Pol}, IP_1, E_{ae}) більш суттєво впливають на активність ензиму, ніж сольватаційні (S) (рис. 2а). Незважаючи на найменший внесок енергії спорідненості до електрона E_{ae}, виключення дескриптора з моделі призводить до зменшення стійкості моделі, а саме до збільшення помилки прогнозу в умовах ковзного контролю.

Як було зазначено раніше, на основі компонент Т-вектора можливо провести прогноз рангу активності за формулою (1). Якщо провести процедуру багатовимірного шкалювання [17] для відібраних чотирьох структурних параметрів з урахуванням їх внеску (компоненти Т-вектора), то можна отримати візуальну картину, що показує диференціацію іонів металів у двох вимірах (рис. 3а).

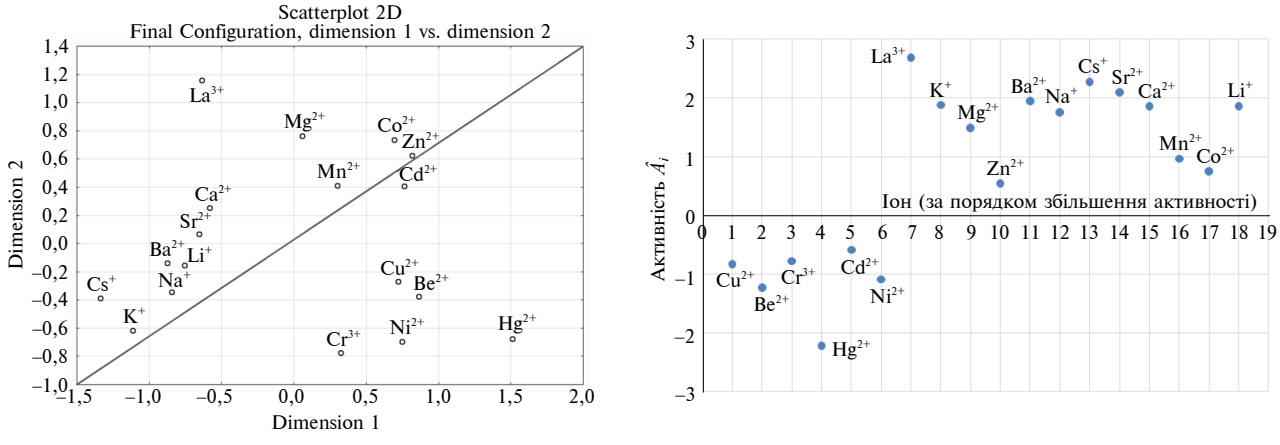
Як видно з рис. 3а, всі менш активні іони металу відносно дії на пептидазу розміщені знизу від діагоналі, а всі більш активні іони – відповідно зверху. Розрахунок прогнозованих значень активності за формулою (1) (рис. 3б) дає аналогічну картину: всі менш активні іони металу мають негативні значення, а всі більш активні іони – позитивні.

Модель M2, побудована методом випадкового лісу (без помилок класифікації та помилок прогнозу). Стійкість моделі: в умовах *out of bag* вибірки [14] помилка класифікації становить S (oob) = 0,33. Інші статистичні характеристики в умовах



а б

Рисунок 2: Оцінка впливу структурних параметрів іонів металів на активність пептидази, отримана на основі інтерпретації моделей M1 (а) та M2 (б)



а б

Рисунок 3: Розподіл іонів металів: (а) при багатовимірному шкалюванні [15] відібраних методом Т-вектора структурних дескрипторів, (б) при розрахунку активності пептидази для різних іонів металів за формулою (1)

out of bag вибірки для цієї класифікаційної моделі є досить задовільними:

$$AC = 0{,}73;\ SP = 0{,}40;\ SE = 0{,}90,$$

де *AC* (точність, accuracy) = $(TP + TN)/(TP + TN + FP + FN)$; *SP* (специфічність, specificity) = $TN/(TN + FP)$; *SE* (чутливість, sensitivity) = $TP/(TP + FN)$; *TP* – кількість збігів для активних, *TN* – кількість збігів для неактивних; *FP* – кількість незбігів класів для активних; *FN* – *FP* – кількість незбігів класів для неактивних.

Важливість змінних, оцінена за алгоритмом, описаним у публікації [14], дає такі результати:

$$\text{T}\ (EN_{\text{Pol}}) = 0{,}062\ (48\ \%);$$

$$\text{T}\ (S) = 0{,}040\ (32\ \%);$$

$$\text{T}\ (IP_1) = 0{,}021\ (16\ \%);$$

$$\text{T}\ (E_{\text{ae}}) = 0{,}005\ (4\ \%).$$

Для моделі M2 (як і для M1) характеристики іонів, що визначають їх електронодонорні та електроноакцепторні властивості (EN_{Pol}, IP_1, E_{ae}), більш суттєво впливають на активність ензиму, ніж термодинамічні (*S*) (див. рис. 2б). Як і у випадку з M1, незважаючи на найменший внесок енергії спорідненості до електрона E_{ae}, виключення дескриптора з моделі призводить до зменшення стійкості моделі, а саме до збільшення помилки передбачення в умовах *out of bag*. Напрям впливу змінних у межах методу лісу дерев оцінити неможливо в силу нелінійності моделі, але, як було показано вище, в межах методу тренд-вектора це можливо.

Обговорення

Найважливіша характеристика ензимів – каталітична активність – може суттєво змінюватися під впливом іонів металів і є предметом численних експериментів для можливості підвищення виходу протеїназ у різних фазах росту бактерій [1], вивчення механізмів дії [2, 3], структури ензимів тощо. Для вирішення цих завдань використовуються фізико-хімічні та біохімічні методи: УФ- й ІЧ-спектроскопія, динамічне світлорозсіювання, флуоресценція, ферментативна кінетика, які потребують значних ресурсів, масштабних експериментів. Одержані нами на першому етапі роботи результати з використанням методів біохімії та спектрофотометрії показали активуючий вплив деяких іонів металів (Li^+, Mn^{2+}, Co^{2+}, Ca^{2+}) відносно протеолітичної активності пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465, а також інгібуючий вплив (Cu^{2+}, Be^{2+}, Cd^{2+}, Hg^{2+}, Cr^{3+}) на активність ензиму. Дані [18] також свідчать про підвищення (або зниження) активності протеолітичних ензимів під впливом іонів двовалентних металів. Так, для глутамілендопептидази 2 *B. intermedius* встановлено, що за наявності 5 мМ іонів Mg^{2+} та Ca^{2+} її питома активність збільшується на 13 і 20 % відповідно; для субтилізиноподібної протеїнази 2 *B. intermedius* Mg^{2+} та Ca^{2+} у такій концентрації збільшують питому активність на 30 та 10 % відповідно. Автори [18] дійшли висновку, що для максимального накопичення протеїназ необхідно вносити у живильне середовище в пізню стаціонарну фазу росту *B. intermedius* 5 мМ Mg^{2+} та Ca^{2+} у концентрації 5 мМ. Подібні результати були отримані відносно біосинтезу глутамілендопептидази 1 штамом *B. intermedius* та рекомбінантним штамом *B. subtilis*.

У роботі [19] встановлено позитивний вплив іонів двовалентних металів відносно активності металопротеїнази MprBi *B. intermedius* 3-19 і негативний – іонів Co^{2+} та Ni^{2+} у концентраціях від 1 до 20 мМ, причому зі збільшенням концентрації це зниження більш значне. Авторами досліджень [20] показано двократне збільшення активності кератинази *Thermoanaerobacter sp.* під впливом 5 мМ Mg^{2+} та її повне пригнічення 5 мМ Cu^{2+}.

Встановлено стабілізуючий вплив катіонів цинку (1 мМ) на активність пептидази *Bacillus thuringiensis* IMV B-7324), що автори [21] пояснюють виникненням оптимальних умов для утворення потрійного комплексу: фермент–ефектор–субстрат і формування каталітично активної конформації ензиму.

Пригнічення активності протеази *Aspergillus niger* KIBGE-IB36 іонами Cs^+, Mn^{2+}, Cu^{2+}, Ni^{2+}, V^{2+}, Co^{2+}, Hg^{2+} і Al^{3+} автори також пояснюють конформаційними змінами білкової молекули [22].

Встановлено вплив іонів металів і механізм їх дії на ферментативний гідроліз лігноцелюлози з використанням комплексу фізико-хімічних методів і ферментативної кінетики [23]. Показано, що ступінь ферментативного гідролізу лігноцелюлози залежить від заряду іона метала та його концентрації. Виявлено, що при додаванні Fe^{3+} максимальний ступінь гідролізу становив 44,5 % за масової концентрації Fe^{3+} 0,4 г/л. Результати використання динамічного світлорозсіювання та флуоресценції показали, що елект-

ростатична взаємодія між Fe^{3+} і карбоксильною групою ферменту сприяла стабілізації його конформації.

Таким чином, іони металів по-різному впливають на активність протеолітичних ензимів, на ступінь ферментативного гідролізу, і для оцінювання їх впливу потребується використання складних методів і ресурсів. Тому перспективним є застосування методів комп'ютерної хімії, основним завданням якої є пошук залежностей "структура–властивість" для побудови адекватних QSAR-моделей та оцінки і прогнозування впливу іонів металів на активність пептидази. В науковій літературі аналогічні відомості практично відсутні, подібні дослідження нами здійснено для карбоксилестерази печінки свині, тирозинази та лізоциму, що дало змогу з високим ступенем вірогідності прогнозувати активність ензимів за наявності іонів металів [24–26], ґрунтуючись на властивостях металів. Саме тому дослідження впливу іонів металів на протеолітичну активність пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465 були проведені в аналогічних умовах (концентрації іонів металів, температура, час інкубації).

У нашій роботі вперше застосовані методи комп'ютерної хімії, що з високою долею вірогідності дають змогу оцінити вплив іонів металів на протеолітичну активність нового ензиму пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465 та спрогнозувати їх дію.

Використання методів комп'ютерної хімії виправдало наші сподівання для пептидази, і в результаті проведених досліджень ми отримали адекватні QSAR-моделі (M1 та M2 відповідно), що дають змогу **без помилок класифікації та помилок прогнозу** відносити іони металів до певного класу активності для тестового набору. Використовуючи обидві моделі та маючи чотири найважливіших дескриптори, а саме: перший потенціал іонізації (IP_1); ентропію іонів у водному розчині (S, Дж/моль·К); електронегативність елементів за шкалою Полінга (EN_{Pol}); енергію спорідненості до електрона (E_{ae}, еВ), можливо прогнозувати вплив металу на каталітичну активність нового ензиму, що може бути корисним для подальших біохімічних і біотехнологічних досліджень. Отримані результати є оригінальними, раніше не були описані в літературі та свідчать про перспективи використання методів комп'ютерної хімії для потреб ензимології.

Висновки

Методи QSAR-аналізу в сукупності з нелінійними методами тренд-вектора та випадкового лісу дають змогу адекватно описати вплив іонів металів на активність пептидази *Bacillus thuringiensis* var. *israelensis* IMV B-7465 за рахунок дескрипторів, що відображають певний баланс їх електронодонорних та електроноакцепторих властивостей (електронегативність, перший потенціал іонізації, енергія спорідненості до електрона) та ступінь структурованості гідратної оболонки (ентропія сольватації іонів). Обидва статистичних методи дають близькі значення важливості дескрипторів, але тільки метод тренд-вектора дає можливість оцінити напрям впливу конкретних характеристик іонів.

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QSAR-АНАЛИЗ ВЛИЯНИЯ ИОНОВ МЕТАЛЛОВ НА АКТИВНОСТЬ ПЕПТИДАЗЫ *Bacillus thuringiensis* var. *israelensis* IMV B-7465

Проблематика. Каталитическая активность энзимов является их важнейшей характеристикой, может существенно изменяться под влиянием эффекторов, например ионов металлов, и является предметом специальных исследований, которые важны для биохимии, биотехнологии, медицины и других отраслей науки. Обычно активность энзимов в присутствии металлов оценивают по изменению скорости ферментативной реакции. Однако проведение подобных экспериментальных исследований, особенно для новых энзимов, как в случае пептидазы *Bacillus thuringiensis* var. *israelensis* IMV B-7465, требует значительных ресурсов и развернутых кинетических исследований. Поэтому целесообразно применение методов компьютерной химии, базовой задачей которой является поиск зависимости “структура–свойство”, для построения модели, которая сможет с высокой долей вероятности оценить влияние ионов металлов на активность пептидазы.
Цель. Разработка QSAR-моделей для анализа и прогноза влияния ионов металлов на активность пептидазы *Bacillus thuringiensis* var. *israelensis* IMV B-7465.

Методика реализации. Влияние ионов металлов изучали, определяя протеолитическую активность пептидазы после совместной инкубации в течение 30 мин в 0,0167 М буферного раствора трис-HCl (pH 7,5, 37 °C). Конечная концентрация хлоридов металлов Li^+; Na^+; K^+; Cs^+; Cu^{2+}; Be^{2+}; Mg^{2+}; Ca^{2+}; Sr^{2+}; Ba^{2+}; Zn^{2+}; Cd^{2+}; Hg^{2+}; Cr^{3+}; Mn^{2+}; Co^{2+}; Ni^{2+} в буферном растворе составляла 4 ммоль/дм^3. Для поиска количественной связи “структура–свойство” использовали справочные данные о свойствах ионов металлов и методы тренд-вектора и случайного леса.
Результаты. Исследование влияния ионов металлов на протеолитическую активность пептидазы *Bacillus thuringiensis* var. *israelensis* IMV B-7465 показало, что некоторые ионы металлов (Li^+, Mn^{2+} и Co^{2+}) активировали пептидазу, а другие (Cu^{2+}, Be^{2+}, Cd^{2+}, Hg^{2+}, Cr^{3+}) ингибировали активность энзима. Нелинейными методами тренд-вектора и случайного леса построены адекватные статистические модели без ошибок классификации и ошибок прогноза класса активности для тестового набора. Обе модели показывают, что важнейшими характеристиками ионов металлов, влияющими на активность энзима, являются электроотрицательность (EN_{Pol}), первый потенциал ионизации (IP_1), энтропия ионов в водном растворе (S) и энергия сродства к электрону (E_{ae}).
Выводы. Методы QSAR-анализа в совокупности с нелинейными методами тренд-вектора и случайного леса позволяют адекватно описать влияние ионов металлов на активность пептидазы *Bacillus thuringiensis* var. *israelensis* IMV B-7465 за счет дескрипторов, отражающих определенный баланс их электронодонорных и электроноакцепторных свойств (электроотрицательность, первый потенциал ионизации, энергия сродства к электрону) и термодинамических свойств в водном растворе (энтропия сольватации ионов). Оба статистических метода дают близкие значения важности дескрипторов, но только метод тренд-вектора позволяет проанализировать направление влияния конкретных характеристик ионов.

Ключевые слова: пептидаза *Bacillus thuringiensis* var. *israelensis* IMV B-7465; ионы металлов; QSAR-анализ; случайный лес; тренд-вектор; активность энзима.

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QSAR ANALYSIS OF THE EFFECT OF METAL IONS ON THE PEPTIDASE *Bacillus thuringiensis* var. *israelensis* IMV B-7465 ACTIVITY

Background. The catalytic activity of enzymes, which is their most important characteristic, can change significantly under the influence of effectors, for example, metal ions, and is the subject of special studies that are important for biochemistry, biotechnology, medicine, and other branches of science. Usually, the activity of enzymes in the presence of metals is assessed by the change in the rate of the enzymatic reaction. However, conducting such experimental studies, especially for new enzymes, as in the case of peptidase *Bacillus thuringiensis* var. *israelensis* IMV B-7465, requires significant resources and extensive kinetic studies. Therefore, it is advisable to use the methods of computational chemistry, the basic task of which is to search for the structure–property relationship, to build a model that can assess the effect of metal ions on peptidase activity with a high degree of probability.
Objective. We are aimed to develop QSAR models for analysis and prediction of the effect of metal ions on the activity of peptidase *Bacillus thuringiensis* var. *israelensis* IMV B-7465.
Methods. The effect of metal ions was studied by determining the proteolytic activity of peptidase after co-incubation for 30 min in 0.0167 M Tris-HCl buffer solution (pH 7.5, 37 °C). The final concentration of metal chlorides Li^+; Na^+; K^+; Cs^+; Cu^{2+}; Be^{2+}; Mg^{2+}; Ca^{2+}; Sr^{2+}; Ba^{2+}; Zn^{2+}; Cd^{2+}; Hg^{2+}; Cr^{3+}; Mn^{2+}; Co^{2+}; Ni^{2+} in the buffer solution was 4 mmol/dm^3. To search for the quantitative structure–property relationship, we used the reference data on the properties of metal ions, as well as trend vector and random forest methods.
Results. A study of the effect of metal ions on the proteolytic activity of peptidase *Bacillus thuringiensis* var. *israelensis* IMV B-7465 showed that some metal ions (Li^+, Mn^{2+} и Co^{2+}) activated peptidase, while others (Cu^{2+}, Be^{2+}, Cd^{2+}, Hg^{2+}, Cr^3) inhibited the enzyme activity. Adequate statistical models without classification errors and activity class prediction errors for the test set were constructed by nonlinear trend vector and random forest methods. Both models show that the most important characteristics of metal ions affecting enzyme activity are electronegativity (EN_{Pol}), the first ionization potential (IP_1), the entropy of ions in aqueous solution (S), and the electron affinity energy (E_{ae}).
Conclusions. QSAR analysis methods in combination with nonlinear trend vector and random forest methods allow adequately describing the effect of metal ions on the peptidase *Bacillus thuringiensis* var. *israelensis* IMV B-7465 activity due to descriptors reflecting a certain balance of their electron-donating and electron-accepting properties (electronegativity, the first ionization potential, the electron affinity energy) and thermodynamic properties in aqueous solution (entropy of solvation). Both statistical methods give similar values of the importance of descriptors, but only the trend vector method allows us to analyze the direction of influence of specific characteristics of ions.

Keywords: peptidase *Bacillus thuringiensis* var. *israelensis* IMV B-7465; metal ions; QSAR analysis; random forest; trend vector; enzyme activity.