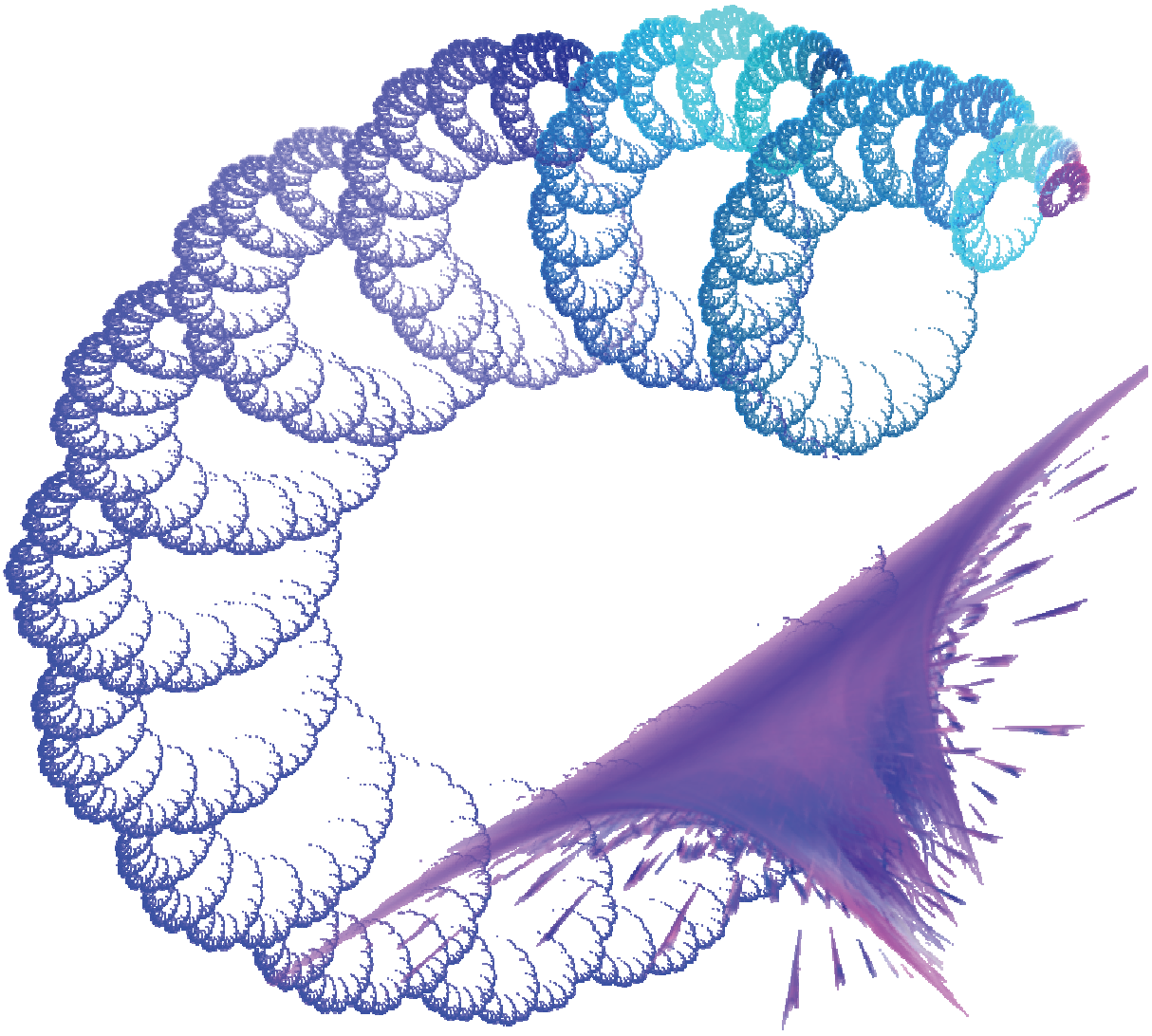
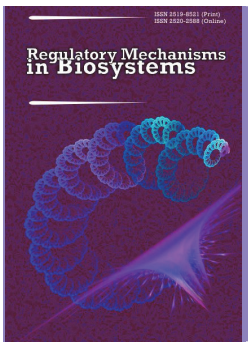
ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)

Regulatory Mechanisms in Biosystems



Volume 9(1)



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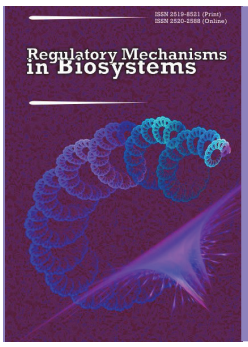
Aims and scope. *Regulatory Mechanisms in Biosystems* publishes peer-reviewed original research and review articles across all aspects of regulatory mechanisms in biological systems from the molecular level of organisation to the level of the organism. This journal mostly focuses on physiological mechanisms of regulation of metabolic processes, biochemical and physiological features of any species including human beings. This journal covers a wide range of regulatory mechanisms in biological systems that are associated both with natural processes and those transformed under the influence of chemicals and drugs, and any other man-made factors. We will publish papers concerned solely with clinical case studies and clinical trials if such articles address important questions in regulatory mechanisms in biosystems. *Regulatory Mechanisms in Biosystem*s focuses on good-quality research, reporting scientifically sound observations and valid conclusions, which bring new and important information to the attention of the wider international scientific community. The journal publishes contributions in the following basic areas: biochemistry, bioinformatics, biophysics, cell biology, endocrinology, genetics, immunology, microbiology, molecular biology, physiology, neuroscience, pharmacology, toxicology.

Publication information. *Regulatory Mechanisms in Biosystems*, ISSN 2519-8521 (Print), ISSN 2520-2588 (Online). Subscription prices are available upon request from the Publisher or from the journal's website (www.medicine.dp.ua). Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by standard mail (surface within Europe, air delivery outside Europe). Priority rates are available upon request. Claims for missing issues should be made within six months of the date of dispatch.

Approved by the Scientific Council of Oles Honchar Dnipro National University, Gagarin Ave., 72, Dnipro, 49010, Ukraine



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ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 3–10
doi: 10.15421/021801

Theoretical and experimental substantiation of a fungal therapeutic drug with immunomodulating and antitumor action according to the authors' prescription

V. A. Kuchmistov*, N. O. Koziko**, O. F. Kuchmistova*,
O. P. Shmatenko*, V. O. Tarasenko*, A. O. Drozdova***

**Ukrainian Military Medical Academy, Kyiv, Ukraine*
***National Medical University named after A. A. Bogomoletz, Kyiv, Ukraine*
****National Medical Academy of Postgraduate Education named after P. L. Shupyk, Kyiv, Ukraine*

Article info

Received 11.01.2018
Received in revised form 29.01.2018
Accepted 02.02.2018

Ukrainian Military Medical Academy, Moskovs'ka st., 45/1, Kyiv, 01015, Ukraine. Tel.: +38-050-334-86-47. E-mail: victor.kuchmistov@gmail.com

Bogomolets National Medical University, Pushkinska st., 22, Kyiv, 01601, Ukraine. Tel.: +38-068-594-67-95. E-mail: nata.koziko@gmail.com

National Medical Academy of Postgraduate Education named after P. L. Shupyk, Dorogozhitska st., 9, Kyiv, 04112, Ukraine. Tel.: +38-066-364-84-14. E-mail: drozdova82@ukr.net

Kuchmistov, V. A., Koziko, N. O., Kuchmistova, O. F., Shmatenko, O. P., Tarasenko, V. O., & Drozdova, A. O. (2018). Theoretical and experimental substantiation of a fungal therapeutic drug with immunomodulating and antitumor action according to the authors' prescription. Regulatory Mechanisms in Biosystems, 9(1), 3–10. doi: 10.15421/021801

The article is devoted to a promising direction in pharmacological mycology and biotechnology, namely the use of Basidiomycetes as a biologically active substance for preparation of pharmaceuticals. The anticancer and immunostimulating properties of Basidiomycetes result from the structural characteristics of the polysaccharides: the presence of β-(1 → 3) connections in the main chain and an additional β-(1 → 6) branched part of glucans. Attention is focused on Shiitake mushrooms (*Lentinula edodes*) as a unique biosystem with a strong ability to be involved in the regulatory mechanism of human immunomodulation. The purpose of the theoretical and experimental study is development of a pharmaceutical preparation from Shiitake biomass (including its immune cells), the effectiveness of which is considered from the standpoint of the chemistry of natural compounds. Having a 3000-year history of cultivation and application, this mushroom is again of interest to scientists around the world because of investigation into its therapeutic and prophylactic potentials. For the first time, the biotechnological method of cultivating *L. edodes* biomass was developed with the use of deep cultivation, adapted to the plant raw material base of Ukraine. Malt wort was used as a nutrient medium without processing *Humulus lupulus*. The analysis of research and preclinical studies of specific activity of the designated biosystem showed a steady increase in human immune status, particularly the antitumor status. This gives special relevance to the evaluation of the properties of the designated biosystem in order to further develop the corresponding fungo-therapeutic immunomodulatory and antitumor drug. According to the results of technological research: physico-chemical characteristics of Shiitake biomass dry powder (including crystallographic) were studied; the possibility of its tableting using only antifriction auxiliaries was substantiated; the optimum technology of tablet manufacturing with an operating name "Shitavit" was presented, an integrated approach to its creation was presented, the qualitative and quantitative composition of the drug was experimentally developed. The direct compression method was rejected due to unsatisfactory performance of all samples of tablets. The chosen technology of using preliminary granulation and the possibility of short-term contact of the *L. edodes* dry powder with a granulation liquid and an adjustable temperature regime allows all the properties of the biologically active substances of the designated raw material to be preserved. The research results for all series of the designated object showed relative stability of parameters for all indicators in laboratory samples. The information presented in this article is a guide to further research, necessary for a better understanding of the healing properties of fungal polysaccharides, increasing the use of broad-spectrum fungi-based preparations,leading to improvement of the quality of human life.

Keywords: Shiitake fungus; *Lentinula edodes*; tablets; immune resistance; antitumor effect

Introduction

Enhancement of immunity to certain diseases remains one of the major problems of modern medicine. Analysing various literature references regarding therapy of such diseases, it is worth mentioning that typically heath-related conditions are not the only factor, but also a complex of simultaneous processes and general overall vulnerability. Clinical settings require combined therapy, thus the purpose of the pharmacotherapy of immunological diseases is to effect different components of the immune system (Shah et al., 2011; Xu et al., 2014). Attention is being increasingly dedicated to the examination of the biological effect of fungi-based preparations and Basidiomycetes in particular. In general, more than 2,000 species of edible and/or medicinal fungi have been identified , many of which are widely consumed, stimulating much research on their health-promoting properties (Friedman, 2016). In the last decades the therapeutic properties of fungus in naturally produced pharmaceuticals have been the center of intensive research (Puchkova & Shcherba, 2006; Rao et al., 2009; Ganeshpurkar et al., 2010; Ren et al., 2012; Mizuno & Nishitani, 2013; Giavasis, 2014; Meng et al., 2016; Zhang et al., 2016).

Accelerated progress in mushroom growing in many countries (Japan, China, South Korea, USA, Canada, and France) within the last

30–40 years is based on modern biotechnologies (Meng et al., 2016). This equates to control of the most important natural functions of fungus and obtaining of large crops of fungi kames as a valuable material to obtain broad-spectrum pharmaceutical substances. Global manufacturing of cultivated fungi-based pharmaceuticals brings in a revenue of $1.2 billion annually (Welbaum, 2015; Krasnopol'skaya et al., 2016). The need to develop similar pharmaceuticals in Ukraine is unquestioned not only from the perspective of clinical implementation but also in terms of the possibility of mainstream use with the purpose of immunodeficiency treatment.

One of five most common edible fungi in the world is Shiitake (*Lentinula edodes*, Pleurotaceae), the natural distribution of which includes the warm and humid climate zones of Southeast Asia. Shiitake fungus leads by volume in fungus cultivation worldwide, and the global production of Shiitake fungal mycelium since the beginning of the 21st century has increased to approximately 800 thousand tonnes annually (Babickaya et al., 2009; Meng, 2016). Only champignon fungus (*Agaricus bisporus*) production volumes can be compared with production of Shiitake (Taufiqur, 2012).

Currently, more than ⅔ of the global Shiitake production is based on extensive rearing, where the fungus is bedded on the freshly-cut deciduous trees stumps (e.g. Chinkapin (Castanopsis), oak, maple tree, hornbeam, poplar, alder, mulberry, etc.) The experiential interest consists in the submerged cultivation methods of Shiitake fungus (Puchkova & Shcherba, 2006; Hearst et al., 2009; Pashev et al., 2009; Zhang et al., 2016). Its advantages include the possibility of growing fungus during the whole year; stable high yield by virtue of creating optimal conditions for obtaining the biomass; usage of a wide range of cellulose herbal substrate and lignin-containing agricultural and industrial wastes; short production cycle (8–10 weeks); automation and mechanisation of the technological process. The submerged cultivation is aimed at obtaining a considerable amount of the good quality seed grain at the transfer from industrial production of fungus kames to the production of the biomass with its further processing into biologically active substances (BAS), pharmaceuticals and separate chemicals desorbing.

Shiitake is considered a medicinal fungus in some forms of conventional medicine. Over the past decade its mineral vitaminous and amino acid profile has been discovered, which defines its high biological value; enhancement of energy metabolism of the human body, emotional lability, hypermnesia, a decrease of chronic fatigue syndrome, improvement of CNS activity and the cardiovascular system, improvement of immune status, etc. (Malitikov, 2013; Friedman, 2016) (Table 1).

Table 1
Complete mineral-vitamin formula of *L. edodes*

In calculation to 100.0 g	54.3 g Carbohydrate; 22.25 g Protein; 19.0 g Water; 2.5g Fiber; 0.75 g Leach; 0.5 g Lipid; 0.217 g Vitamin B_2 (Riboflavin); 304.0 mg Potassium; 230.0 mg Mangan; 142.0 mg Cuprum; 112.0 mg Phosphorus; 20.0 mg Magnesium; 9.0 mg Sodium; 4.0 mg Vitamin B_3 (Niacin, Nicotinic acid or Vitamin PP); 2.0 mg Calcium; 1.5 mg Vitamin B_5 (Pantothenic acid); 1.03 mg Zinc; 0.41 mg Ferrum; 0.293 mg Vitamin B_6 (Pyridoxine); 0.10 mg Copper; 0.015 mg Vitamin B_1 (Thiamine); 325.0 mcg Vitamin A (Acerophotol); 230.0 mcg Manganese; 13.0 mcg Vitamin B_9; (Folic acid); 5.7 mcg Selenium; 4.15 mcg Vitamin B_{12} (Cyanocobalamin); 0.4 mcg Vitamins of group D (Cholecalciferol, Ergocalciferol)

A proteinous strand of this fungus includes, inter alia, a complete set of all 22 essential amino acids, which is an exceptional property in any product (Sreenivasan et al., 2010). According to other sources, using the method of ion-column chromatography, 19 amino acids were found in dry powder of Shiitake fungus biomass (including 7 indispensable amino acids: Lysine, Threonine, Valine, Methionine, Leucine, Phenylalanyl), using the method of atomic emission and atomic adsorption analysis with flame atomisation – 8 micro- and macro elements (Sodium, Potassium, Calcium, Magnesium, Zinc, Ferrum, Mangan, Cuprum) were found (Pashev et al., 2010; Drori et al., 2016; Friedman, 2016). The examined object is richer in amino acid content than soy, beans, and corn. In particular, the immunomodulatory, antitumoral and antiviral activity of Shiitake fungus is of major interest in the XXIst century, taking into account that humanity still has no effective medicine to control certain widespread diseases. Among medicinal fungi with antineoplastic activity, *L. edodes* is the leader according to its immunomodulatory properties and surpasses Reishi fungus (*Ganoderma lucidum*) (Mizuno, 1997; Puchkova & Shcherba, 2006; Hearst et al., 2009; Taufigur, 2012).

Tetsuro Ikekawa (Japan) defined the chemical structure of immunomodulatory 1,6-b-complex of Shiitake kames that are extracted with hot water back in 1969 (Mizuno & Nishitani, 2013). The next decade was dedicated to intensive research on the immunomodulatory activity of touchwood constituents and pure grown fungus of different systemic groups. Research findings on such immuno-amplifiers extracted from Basidiomycetes as lentinan, schizophyllan, krestin, also glucans and mannans of certain types of ascomycetes have been regularly presented, starting from the 1st International Conference on Immunopharmacology (1980, Brighton, UK), International Symposium on Immunomodulators of Microbial Origin (1981, Osaka, Japan), Microbiologists' Association Congress (1982, Budapest, Hungary), and at the 2nd International Conference on Immunopharmacology (1982, Washington, USA) there were numerous submissions on research into some immunostimulants of Basidiomycetes (lentinan, schizophylilan, Krestyn), glucans and mannans, from some types of fungi from the phylum Ascomycota. Scientists arrived at the conclusion that all immuno-amplifiers are polysaccharides, regardless of the primary structure and impact particularities (Minato & Mizuno, 2001; Bisen et al., 2010; Ren et al., 2012). Research in this direction continues to be intensively conducted (Jones, 1995; Feofilova, 2004; Rao et al., 2009; Pashev et al., 2010; Shen et al., 2011; Drori et al., 2016; Zhang et al., 2016). Currently, Shiitake is considered to the most extensively studied medicinal fungus (Welbaum, 2015).

The widespread use of polysaccharides is based on the flexibility of its effect on the immune system as an immunomodulation factor. The general epiphylaxis derives from the direct effect of immunomodulators on maturation, proliferation, and differentiation of immunocytes responsible for humoral and cellular self-protection factors. This permits the use of pharma drugs made of higher basidiomycetes in immuno-correcting therapy (Jones, 1995; Feofilova, 2004; Minato & Mizuno, 2001; Il'inskikh, 2012; Meng et al., 2016).

L. edodes contains a lentinan polysaccharide complex (Lentinan) unique, in its nature, which has no equal in the plant kingdom (Friedman, 2016) (Fig. 1).

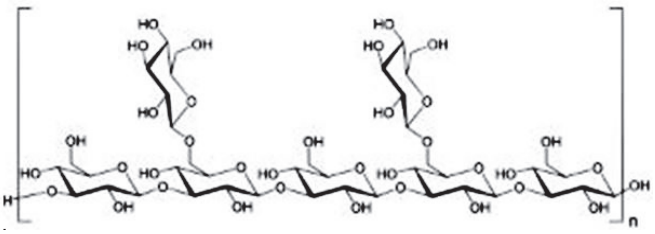

Fig. 1. Basic structure of Lentinan, isolated from the Shiitake fungus

The specified substance stimulates the antineoplastic activity of the immune system, which relies on the ability to increase the generation of perforin polymer protein, attack atypical cells and destroy them, at the same time potentiate the increase of the quantity of natural killer cells (NK-cells), T-lymphocytes fractions (cytotoxic T-cell and T-helpers), as well as cachectin of benign (myoma, fibroma, mastopathy, prostatic hyperplasia) and malignant neoplasms (cancer, sarcoma, lymphoma, melanoma) (Shah et al., 2011). *In vitro* test results show that depending on the concentration of lentinan, the double increase of γ-interferon level in leukocytes occurs, and interferon increases double to fourfold in relation to control (Lindequist et al., 2005; Puchkova & Shcherba, 2006).

High concentration of natural antioxidants sustains radio protective properties, which help to offset the impact of free radicals, which are able to turn normal cells into nosogenic. First, it is L-ergothioneine (a powerful antioxidant) – in its quantitative content, Shiitake fungus is unrivalled (Jones, 1995). Later, scientists were able to isolate an extract from the spawn of this fungus (*L. edodes* mycelium extract, LEM), which also showed outstanding results in therapy of oncological

diseases (Sreenivasan et al., 2010; Xu et al., 2014). The foregoing demonstrates the uniqueness of the selected research target, the effectiveness of which is considered from the standpoint of the chemistry of natural products.

Consequently, taking to account the importance of preventive health care of the population in Ukraine, the matter of obtaining valuable bioproducts and adaptogens is a priority and requires a practical prompt solution. Therefore, the analysis and consolidation of literature data with regard to the modern aspects of the use of the medicinal fungus, *L. edodes*, and, in particular, the technology and formulation development of medication products based on it is a challenging issue.

Materials and methods

Research target – Shiitake fungi biomass dry powder.

Topics examined:

– physiochemical characteristic of the biologically active excipient – *L. edodes* dry powder (crystallographic indicants), the practicability of the possible tabletting of the target;

– evaluation of technological properties of tableting blend on the mentioned vehicle (flowability, compression, tapped density, moisture content);

– in-depth study of the possibility of granulation and compression (main phases of tableting process), selected tableting process validation under codename "Shytavit";

– examination of certain of the tablets' quality attributes (disintegration and solubility, hardness and friability tests) according to modern requirements.

For the purpose of finding solutions to the abovementioned tasks, standard research methods, stipulated in test assessment reference: State Pharmacopoeia of Ukraine (SFU) which is brought up with European Pharmacopoeia (Ph. Eur.) in terms of pharmaceuticals production, were used (Ph. Eur., 2014; Hryzodub et al., 2015).

Crystallographic core data of the research target's parts was evaluated by way of light-optical microscopy method with the use of target's visual analysis.

Crystallinity was investigated with an electron microscope MBI-15 (200x magnification), which gave an opportunity to distinguish the shape and the surface of crystals, as well as particles, average linear dimensions according to procedures developed by the State Scientific Pharmaceuticals Centre (Kyiv, Ukraine). The image was displayed on computer monitor from the microscope via Sony CCD-IRIS camera (Pashev et al., 2009).

For specific particles on photographs, a corresponding regular geometric shape was adjusted and dimensions (length and width) were measured considering the zoom. Form factor indicant, which provides a possibility to outline the isometric level of the powder, it was defined as a ratio of particles' average width (W, mum) to the particles' average length (L, mum), using formula (1):

$$K = \frac{W}{L} \quad (1)$$

It has been established that $K > 0.5$ (up to 1) for isometric particles and $K \leq 0.5$ for anisometric particles.

Flowability is one of the most important parameters which impacts technological processes of processing of loose materials, starting from the transporting process to the production of the end formulation (according to divisions 2.9.16. 2.9.36 of SFU, Hryzodub et al., 2015). The definition was conducted using a standard vibratory device, model VP-12-A manufactured at Plant of Production Manufacturing Machinery (Mariupol, Ukraine), after bumping down for 20 s. Flowability measure (V_c, kg/s) was defined as a ratio of sample weight (m, kg) to the full duration of test run (t, s), using formula (2):

$$V_c = \frac{m}{t \times 20} \quad (2)$$

Direct compression lines the aptitude of the powder to create stable structural systems as a result of particles' attraction under pressure. For determination of dispensing 0.3 g was pressed in a pill 9 mm in diameter using a hydraulic press with 120 MPa compacting pressure. After pressing out, the pill was pushed out from matrix with lower punch and the hardness index was determined (n, N) by the ratio of crushing load (P, Pa) to the basic tablets' parameters: its height (h, mm) and diameter (d, mm), using formula (3):

$$n = \frac{P}{d \times h} \quad (3)$$

Bulk density (mass) is the characteristics of the powder which reflects heaping of its particles thus outlining the powder's aptness to fill the unit of volume, which depends on unit weight and dispersity, form and powder particles' surface texture (according to divisions 2.9.15, 2.9.34 of SFU, Hryzodub et al., 2015).

For determination of exact dispensing of the powder it (approx. 10 g) was placed in a graduated glass cylinder, fixed on 545-AK-3 apparatus manufactured by Plant of Production Manufacturing Machinery (Mariupol, Ukraine). Incremental mechanism lifted the cylinder with powder up to a certain height, from which the cylinder swooped down, hitting into a special device, afterwards was lifted up again to the upper level. By virtue of numerous impulses over the board, powder compaction took place. The vibration amplitude – 35–40 mm, impact frequency – 150 imp./min. After settling the constant powder level in, the tapped density (P, kg/m^3) was calculated by ratio of cylinder mass, filled with powder (P_n + n, kg) and empty cylinder (n, kg) to the cylinder volume after succession (V, m^3), using formula (4):

$$P = \frac{(P_n + n) - n}{V} \quad (4)$$

Testing allowed us to determine using specified criteria, the bulk density and tapped density of the material, which consists of solid particles (powders) to compaction, aptness of material to compaction, as well as its volume and density after compaction (Hryzodub et al., 2015).

Tablets' test was conducted according to modern requirements (according to division 2.9.3 of SFU, Hryzodub et al., 2015).

Tablets' disintegration was conducted from the weighted amount 0.5 g according to SFU using the sieve with 2.0 and 0.5 mm meshes. However , when performing "Dissolve" test Pharmacopeia does not require the obligatory "Disintegration" test (Hryzodub et al., 2015).

Determination of hardness was conducted using TVT of "Erweka" apparatus (Germany) according to modern requirements (according to division 2.9.8 of SFU, Hryzodub et al., 2015).

Determination of friability was conducted using TAP "Erweka" tester on 25 rmp for 5 min (2.9.7, Hryzodub et al., 2015). 20 preweighted tablets were loaded into the drum. Friability index (C, %) was determined by ratio of difference between tablets' mass before abrasion (P, g) and after abrasion (Pk, g) to tablets' weight value before abrasion (P, g), using formula (5):

$$C = \frac{P - P_k}{P} \times 100\% \quad (5)$$

The granules' moisture content was determined as loss on drying. The determination was conducted using type UV-1 express-moisture meter. Sample moisture content (X, %) was calculated as the ratio of the difference between the weight of the sample for testing (P_o, mg) and the sample's constant weight after complete desiccation (P, mg) to the index of the sample for testing (P_o, mg), using formula (6):

$$X = \frac{P_o - P}{P_o} \times 100\% \quad (6)$$

The research was conducted in 5 laboratory samples by the methods described above. The data in the tables is given in the form $x \pm m$, where x is the average value of the indicator, m is the reliable interval at the level $P < 0{,}05$; the results obtained were considered statistically significant (Gubler & Genkin, 1973; Mincer et al., 1991; Pashev et al., 2009). We used an ANOVA, the basic principles of which are developed in the scientific papers Lapach (2002), Kobzar' (2006). Authors of this article analysed selected monographs and research methods on powdered and tableted substances, which are presented in Ph. Eur. 8.8 (2014).

Results

A biotechnological method for obtaining biomass of basidial fungi (Basidiomycetes) used both as a biologically active substance and as

further development of broad-spectrum pharmaceuticals was developed for the first time. A deep cultivation method, adapted to the raw plant material base of Ukraine, was used. Malt wort without hops (*Humulus lupulus*, Cannabaceae) was used as a nutrient medium – a product of soaked and germinated seeds of cereal cultures preheated at a certain temperature, which contributed to the enzymatic destruction of starch in the malt seed and the formation of sugar, mainly maltose. Preclinical studies of specific activity showed a steady increase in human immune status. This confirmed the feasibility of evaluating the properties of the specified biosystem and possible further development of an appropriate fungal therapeutic drug with immunomodulatory and antitumor action.

For the purpose of balanced formula development and industrial technology of a new local tabloid form medicated product codenamed "Shytavit", it was necessary to define and substantiate the sequence of in-process production stages and its pivotal points. This task was completed based on experimental research, conducted using the above mentioned methods. It is commonly known that the production of pills starts with research on the parent's drug properties, which considerably preordain tableting short-cut, selection of available auxiliary substances, which induces the necessary quality of the mentioned formulated product (Dmitrievskij, 2008). Each of factors mentioned above has its certain effect on the pharmacologically active substance. Bulk material in the form of powder-like forms (particle size up to 0.2 mm) or granular (correspondingly 0.1–3.0 mm) is used as the output raw material. They have certain physical, chemical, structural-mechanical and technological properties. In particular, technological properties include flowability, ability to compress, tapped density (mass), fractional (granulometric) composition, moisture content, dispersion, etc. (Gladuh et al., 2016).

At the initial research phase, physicochemical properties of the primary biologically active substance were explored: Shiitake fungi dry powder, its dispersity degree was determined using the sieve method. It is known that the abovementioned properties of the pharmaceutical powder are induced by its crystallographic structure, that is why the shape of *L. edodes* particles was determined first (Fig. 2).

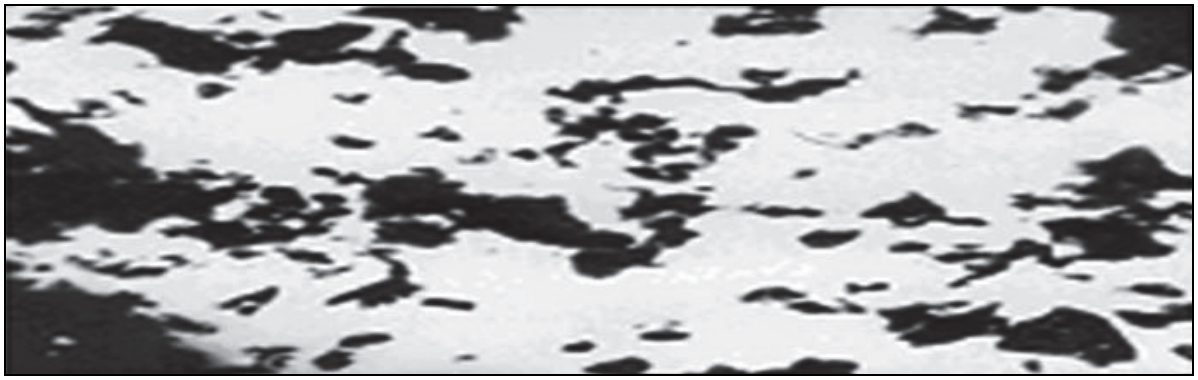

Fig. 2. Microcrystalline structure of *L. edodes*: the image of the object was obtained using MBI-15 electronic microscope (with 200^x magnification) and captured by the camera Sony CCD-IRIS; staining of the fungus biomass samples (in particular, using Lugol' solution) was not performed; in this case, the polysaccharides will react with the micropowder, leading to the change of colour, which is undesirable

Crystallographic analysis of the microcrystalline structure of the powdered target under 200^x magnification showed that it is a medium-dispersed powder with a cuboidal crystalline form with rough and coarse particles surface.

Analysis of the fraction composition of various substances series shows the powder's cryptocrystalline structure with the isodiametric shape of grains (in the form of shapeless lumps), the major portion of which (>90%) had less than 10 microns (Table 2). Based on the obtained data, we can assume that due to the fact that Shiitake fungi biomass dry powder has an isodiametric particles structure, it may have a good flowability. However, to confirm this assumption it was necessary to research its technological characteristics (Pashev et al., 2010). The results of technological characteristics research on all 5 samples of the presented target showed relative stability of all test parameters in laboratory samples.

Table 2
Fractional composition study results of dry powder biomass batches of Shiitake fungus taken as main biologically active substance

Series number	Particles mass percentage, %				
	<40 μm	<20 μm	<10 μm	<5 μm	<1 μm
Series 1	1	8	17	58	16
Series 2	1	9	18	55	17
Series 3	4	9	21	51	15
Series 4	4	12	18	52	14
Series 5	5	5	24	48	18
Average value	2.5	8.6	19.6	52.8	16.0

In the second stage of the research, we tried certain excipients and assessed technological characteristics of Shiitake fungus biomass for the purpose of its possible tableting (Gladuh et al., 2016; Rybachuk et al., 2016). Scientifically proven selection of excipients in each particular case is one of the key factors in obtaining tabloids with maximum therapeutic activity and minimal adverse drug reaction. With the purpose of improvement of the technological parameters of Shiitake fungi biomass dry powder, the following excipients were used: Potato Starch (Solani Amylum), Aerosil (Silica) and Magnesium stearate (Magnesii stearas). At the mixing stage for tableting, the following technological characteristics were evaluated: flowability, compression capability, and tapped density (Table 3).

Table 3
Results of technological characteristics of the Shiitake fungus biomass samples for tableting

No	Mixture composition	Proportion of ingredient, %	Flowability, kg/s	Direct compression N, H	Tapped density, kg/m^3
1	DPSF*	80	2.81 ± 0.14	62.0 ± 3.2	0.48 ± 0.04
	Starch	20			
2	DPSF	80	3.15 ± 0.10	59.0 ± 2.5	0.44 ± 0.02
	Starch	19			
	Aerosil	1			
3	DPSF	80	3.61 ± 0.20	54.1 ± 2.5	0.46 ± 0.02
	Starch	16			
	Aerosil	1			
	Magnesium Stearate	3			
4	DPSF	85	3.51 ± 0.17	51.0 ± 2.6	0.52 ± 0.02
	Starch	11			
	Aerosil	1			
	Magnesium Stearate	3			
5	DPSF	85	3.40 ± 0.16	49.1 ± 2.3	0.57 ± 0.04
	Starch	10			
	Aerosil	2			
	Magnesium Stearate	3			

Notes: * – DPSF – Shiitake fungi biomass dry powder, ** – the number of measurements n = 5, P = 95%, *** – system international units of measurement were used.

During the third stage we conducted a selection of a possible method of Shiitake fungi biomass dry powder tableting, in particular, we analysed the practicability of conducting a direct compression or the necessity of applying pre-granulation. The results of tablets' direct compression impact and their quality are shown in Table 4. During the fourth stage, a tailored composition of tablets was defined under the codename "Shytavit", the external characteristics of which would ultimately meet the compendial requirements (Table 5).

Table 4
Results of qualitative indicators of tablets obtained by direct compression

Index	Mixture 1	Mixture 2	Mixture 3	Mixture 4	Mixture 5
Weight variation, %	8.0 ± 0.40	8.4 ± 0.40	8.3 ± 0.50	7.9 ± 0.40	8.2 ± 0.50
Disintegration time, min	4.9 ± 0.30	5.1 ± 0.30	5.0 ± 0.20	5.2 ± 0.30	5.3 ± 0.40
Abrasion, %	16.0 ± 0.30	15.5 ± 0.07	15.0 ± 0.40	14.0 ± 0.30	15.0 ± 0.40

Notes: the number of measurements n = 5.

Table 5
Results of qualitative and quantitative composition of raw materials for further tableting

Ingredients' Weight	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
DPSF, g	0.5000	0.5000	0.5000	0.5000	0.5000
Aerosil, g	0.0150	0.0150	0.0150	0.0150	0.0150
Starch, g	0.0800	0.1690	0.0567	0.0275	0.0716
Purified water, g	–	0.2300	–	–	–
Starch solution, 5%, ml	–	0.2510	–	–	0.1880
Starch solution, 10%, ml	–	–	0.2630	–	–
Starch solution, 20%, ml	–	–	–	0.2830	–
Magnesium Stearate, g	0.0050	0.0050	0.0050	0.0050	0.0050

Notes: the number of measurements n = 5; in some cases, not all ingredients were used for mixtures (marked with a dash in the table).

At the final stage of the work, the impact of tableting mass moisture effect on the compressing process and the tablets' quality was investigated (Tables 6, 7). The drying of the tableting mass of Sample 2 was performed at a constant temperature.

Table 6
Impact of tableting mass moisture effect on compressing process and tablets' quality

Granules moisture content, %	Granules flowability, kg/s	Tablets hardness, H	Tablets disintegration, min	Characteristics of obtained samples
40.0	2.7	35	1.0	Agglutination of tablets
25.0	2.9	42	1.5	Agglutination of tablets
8.0	3.2	48	2.0	Slight agglutination of tablets
3.5	3.4	60	2.0	Satisfactory appearance of tablets appearance
3.1	4.0	59	2.1	Slight agglutination of tablets

Table 7
Variation of the tablets' samples main quality indicators (n = 5)

Attributes	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Flowability, kg/s	3.9	3.4	2.3	2.0	3.2
Disintegration, min	1.3	2.0	3.5	5.6	1.7
Hardness, H	45.0	62.0	88.0	110.0	51.0
Friability, %	5.0	0.2	0.3	0.3	4.0

Considering test results, it was established that the quality of tablets of the provided formulation and the stipulated process technology under the codename "Shytavit" best meets the compendial requirement .

Discussion

Expanding the range of highly effective medicines for the treatment of a number of diseases is of paramount importance in the medical care of the population. The solution to this problem is facilitated by the search and introduction of new original medicines, the development of scientifically sound technology for their manufacture, based on the latest advances in science and technology. Despite a sufficient number of highly effective modern drugs of synthetic origin, the interest in natural biosystems is not diminished. The problem of the creation of domestically produced medicines based on fungus, with a pronounced pharmacotherapeutic activity, simple manufacturing technology, which are non-toxic in comparison with chemical synthesis products used for immunocorrection therapy, is very relevant for Ukraine. Immune dysfunctions, autoimmune conditions, cancer diseases are clearly the leaders among the causes of death both among the Ukrainian population and on a planetary scale. The Basidiomycetes Shiitake fungus (*L. edodes*) is considered as a unique biological system with a clearly expressed ability to be included in the regulatory mechanism of human immunomodulation. This is confirmed both by literary studies and by our own research results (Pashnev et al., 2010; Shah et al., 2011; Ren et al., 2012; Welbaum, 2015; Drori et al., 2016; Krasnopol'skaya et al., 2016).

Today, the pharmaceutical market is gradually beginning to offer a variety of dosage forms based on biologically active substances, isolated from this fungus. However, most of them, till now have been the product of a simple processing of touchwood and spawn (sometimes together with substrate): extracts, tinctures (water or oil), infusions, ointments, balms, cognac tincture, vodka tincture or flaxseed oil infusion of *Linum usitatissimum*), solutions (1 mg/bottle), drops, capsules, suppositories, powder, syrup, saps. Furthermore, it is a plant product for many biologically active dietary supplements (Sotnikova, 2001; Pashnev et al., 2010; Bisen et al., 2010; Taufiqur, 2012; Giavasis, 2014; Drori et al., 2016; Krasnopol'skaya et al., 2016). For example: "Shiitake mushroom extract No. 40" in the form of capsules and tablets 0.25 g, No. 40 (production of Ukraine); "Shiitake mushroom extract No. 80" in the form of capsules and tablets 0.25 g, No. 80 (Ukraine); "Immunomax" represents freeze-dried powder in ampoules and vials of 100 or 200 IU (Finland); "Fungimax" tablets of 0,4 g No. 30 in the form of capsules, 0.2 g No. 60 or in powder form in bags of 1.0 g (Russia); "Shiitake" represents tablets with a skin of 0.56 g No 20 and No. 60 (Russia); "Mushroom triple" (drug fungus Shiitake, Reishi and Maitake) represents tincture at 250,0 g (Russia); "Shiitake Fung Chi" in the form of capsules, 75 mg No. 60, cream in tubes of 50 mg of powder in bags No. 30 (Russia), etc. Clear recommendations regarding the use of these drugs were suggested. For example, the cream "Shiitake Fung-Chi" is designed for external use in diseases of the joints, oncology (in cases of metastasis to bone tissue), ulcers and wounds that are difficult to heal, benign formations on the skin (papillomas, lipomas, warts).

To achieve the goal of this research in the course of theoretical and experimental study of the mechanism of operation of the polysaccharide Shiitake fungi complex on the immune system of tissue of the human large intestine , we reached the conclusion that the most effective is the usage of a micro powder obtained from the touchwood of Shiitake fungi, rather than, for example, its extract or an extract from its kames. According to data from the literature, there is a direct proportion between the length of glycan in medication and intensity of its effect on immune cells. On the other hand, molecules in extracts are short. In those cases when it is necessary to use 1–2 g in conversion to Shiitake extract to reach the desired effect, only 200 mg of the micro pow-

der is enough (Malitikov, 2013). Consequently, a medicinal product based on the said powdered biologically active substance, shows more apparent therapeutic benefit and is economically more affordable.

In our view, the optimal tableting technologies of Shiitake micro powder have still not been fully examined. A detailed survey in this field may contribute to the emergence of highly effective tableted immunocorrecting drugs for prevention and complex therapy of immune diseases.

The choice of the dosage form (Tabulettae) relates to a number of its known advantages (medical pharmaceutical, manufacturing, operational) and high bioavailability when used orally (*per os*). This leads to the increase of worldwide production of tabloids by 10–15% annually (Gladuh et al., 2016). Occasional studies have been dedicated to the topic of development of *L. edodes* based tableted forms. In particular, according to Feofilova (2004), tablets were produced from the dry extract of these fungi. Dry concentrate contained sufficiently high percentage of BAS, including lentinan, the anti-tumour effect of which depended heavily on the dose. Based on the results of the preclinical studies, the recommended therapeutic dose of the dry Shiitake extract was 2.0 in one pill, and 2–4 pills were to be taken daily. The pills provided were covered with the special soluble (sugar) coating (Feofilova, 2004). Some manufacturers added vitamins, for example, D_3 (Cholecalciferol) and C (Ascorbic acid), medicinal herbs like redberry (*Panax schinseng*, Araliaceae). The abovementioned studies intended the development of biologically active substances only, as noted above. We did not find any publications devoted to the development of tablets on the basis of Shiitake fungus powder.

Our studies were aimed to the development of a pharmaceutical drug of pluripotential treatment-and-prophylactic action. Theoretical and experimental confirmation of the tailored composition and technology of the tablets codenamed "Shytavit" included the analysis of physicochemical and technological properties of the ingredients. The choice of excipients was determined primarily based on physicochemical and technological properties of BAS and environmental stability factors. Biopharmaceutical studies prove that excipients are not form-building vehicles, but have multiple impacts on the pharmaceutical drug and its therapeutic effect (Dmitrievskij et al., 2010). In particular, the conditions for a direct compression of tablet formulation have been investigated, and the influence of the type and concentration of binders and lubricants on compressibility and the quality of the tablets obtained have been previously studied. The optimal concentrations of binders and the force of tablet compression have been determined (Rybachuk & Rybachuk, 2016). One of the problems of drug manufacturing is how to obtain satisfactory powder flowability in tableting machine feeders (hoppers, batchers). Obtained granules or powder have an irregular surface, which hinders its absorption from the hopper to the matrixing cavity. Moreover, granules can stick to the walls of the matrix and punches as a result of adhesion in contact areas of the particles in tablet tooling. To minimise such adverse effects, friction-proof substances, presented by the glidant and lubricants, are used (Gladuh et al., 2016).

In view of the above, the following excipients (in this case friction proof substances, lubricants) of the pharmaceutical composition (tabletting mass) were chosen:

– Aerosil (Silicii dioxydum, Aeroperl) as glidant substance, the quantity of which cannot exceed 10% of the whole mass;

– Amylum Solani (Potato starch) as glidant and anti-adherent substance the quantity of which is not regulated by the State Pharmacopoeia of Ukraine;

– Magnesii stearas as a glidant, lubricant, and anti-adherent substance, the quantity of which cannot exceed 1% of the whole mass (Hryzodub et al., 2015).

The quality of the above mentioned is subject to special conditions stipulated in European Pharmacopoeia, in particular, Aerosil Ph. Eur. Monograph 0434 ЕФ 6 2.9.2, Magnesii stearas Ph. Eur./E572 Monograph ЕФ 7 2.6.8, 2.6.14 (Ph. Eur., 2014).

Excipients must provide their necessary functional properties under minimum content with minimal content in the product. In this regard, empirically, a certain content of each mentioned ingredient was matched to achieve the optimal technological characteristics of tableting mass with Shiitake fungi biomass dry power. In our view, Series No.4 has the best ratio of ingredients in tableting mass (Table 2), namely: the amount of dried and ground biologically-active substance of *L. edodes* is 85%, Solani amylum (Potato starch) is 11%, Magnesii stearas (Magnesium stearate) is 3% and Silicii dioxydum (Aerosil, Aeroperl) is 1%. Such amounts are consistent with the requirements of Pharmacopeia.

Technological development always requires appropriate scientifically based technological schemes of production. With the development of biopharmaceutics, more attention is being paid to the technological aspects of the drug formulation. Choice of tableting method is the critically important task for *L. edodes* dry powder based drug technology development in the form of pills. As the analysis of the literature showed, research in this direction continues. For example, in the article by Rybachuk (2016) the technology of manufacturing tablets by direct compression of natural zeolite is described (Rybachuk & Rybachuk, 2016). Without a doubt, a major pragmatic interest is the production of directly compressed tablets, as it allows high production efficiency to be achieved and shortens the time of technological processes through excluding some technological operations. It is known that the capacity for direct compression (without granulation) is typical, first of all of the substances which belong to the cubic system (Dmitrievskij et al., 2010). The object of our study possesses precisely such characteristics (medium dispersed powder of cubic crystalline form), as described in the Results section. Furthermore, powdery substances with the abovementioned physicochemical properties are able to be tableted directly without such auxiliary excipients as Sodium chloride and Potassium bromide (Gladuh et al., 2016).

However, the quality of all tablet samples, obtained using the abovementioned method, appeared unsatisfactory (Table 2), namely: (a) on simple examination we noticed contaminations and the surface of all tablet samples was not unscored; (b) during the tableting process the tableting mass of sampled No. 2 and No. 4 stuck to the tablet tooling and the obtained tablets had irregular edges; (c) obtained tablet samples did not pass the friability test and mass deviation indicator according to the requirements of SPU. All of the above-mentioned factors impelled us to reject the direct extrusion method.

For further study, we proposed a pre-granulation method for powder compaction and obtaining uniform crystal granules which have to improve the flowability and the appearance of tablets. Since we are using BAS as an active substance, we chose the method of wet granulating, which implies obtainment of granules with the possibility of short-term contact of *L. edodes* dry powder with granulating liquid and adjustable temperature scenario. It is worth mentioning that in the process of "Shiitake Mushroom Powder" drug production in tablets of 0.4 g, developed by State Chemical-Pharmaceutical Academy (St. Petersburg, Russia), these medicinal fungi were not cooked/heated at all. This ensured the preservation of polysaccharides in its native state (Malitikov, 2013).

We propose a production technology of the medicinal product, which also allows preservation of all BAS of the provided materials. More specifically, the granulate was prepared with the pre-sifted powders of Shiitake, starch, aerosol and magnesium stearate. Purified water, as well as 5%, 10% and 20% of starch paste, were used as humectants. The humectant was calculated on the basis that the water is 44 ± 1%. This refers to the fact that if the concentration of the moisturiser is less than 38%, granulated structure is not observed and at the concentration of 46% and more, adhesion of the granulate takes place.

Based on the obtained measured data (Tables 2, 4) the optimal formulation of the product in tablet form under the codename "Shytavit" was determined: Shiitake fungi biomass dry powder – 0.4 g, Aerosil – 0.015 g, Starch – 0.08 g, Magnesium stearate – 0.0005 g per tablet. The resulting tablets with mentioned qualitative-quantitative formula complied with pharmacopeia requirements for its terminal characteristics.

Consequently, with the flowability of 3.4–3.5 kg/s, compression capacity 51.0 H and tapped density 0,52 kg/m^3, the highest accuracy of the dose delivery was achieved for tablets for feeder units of the tableting machine (under observance of the Shiitake fungus biomass storage regulations).

Obviously, the quality of tablets is significantly affected also by the granules moisture index, which ensures the necessary flowability of the powder or tableting mass. Excessive moisture content of the powder decreases its flowability through forming the adsorbed layer on the particles. Therefore, the tableting material has to have optimal moisture. There is a positive correlation between the given moisture of the tableting mass and its compression capacity. The better the compressibility, the higher the hardness of the tablet. If the compressibility is low, the tablet will tend to partial or event full collapsibility when stripping off the tableting machine matrix (Gladuh et al., 2016).

The moisture content of the powders used in tablet production must be between 3–5% (Dmitrievskij et al., 2008). The tableting mass (3.5%) moisture content determined by us, also proves that the selected experimental formulation agents the high quality of tablets (Tables 5, 6). Analysis of *L. edodes* processability discovered marginal moisture-sorptive properties and medium flowability.

The set of studies conducted by us showed that physicochemical and technological properties of Shiitake fungi biomass dry powder comply with the requirements for powders which can be tableted using the pre-granulation method (with the possibility of the short-term contact of *L. edodes* dry powder with a liquid for granulation along with adjustable temperature) without adding auxiliary excipients (Sodium chloride and Potassium bromide), which shortens the time of the technological process and lowers the product cost.

With this in mind, Shiitake fungus according to analysis of the literature is currently the most superpotent innovative plant-based medication with immunomodulatory effect. Todays scientists are using the protective properties of *L. edodes* more often ,combining it with chemo- and radiation therapy. By dint of such fungi based medicines, patients even with stage II–III cancer can reach remission from three months to 10 years (when combined with the traditional cancer treatment) (Shen et al., 2011; Giavasis, 2014; Meng et al., 2016; Zhang et al., 2016). In addition, Shiitake remains one of the most biologically active non-hazardous fungi.

The tabletted Shiitake fungi biomass dry powder based therapeutic developed by the authors of this article is a highly effective drug, which, with no doubt will enrich the domestic pharmaceutical market. Even in the absence of treatment indication (immune dysfunction, including AIDS, oncology diseases, autoimmune and neurological diseases, ecological allergies, mycotic and virus infections, multiocular sclerosis, post-infarct and post-apoplectic conditions, vascular diseases, viral upper respiratory tract infection), it is recommended to take "Shytavit" tablets twice per year as a preventive measure, in spring and in autumn, when the human immune system is most vulnerable and requires help from the outside. It is worth mentioning that "Shytavit" tablets work well with most nutraceuticals and potentiate the effect of medicinal herbs. Exceptions are Echinaceae (Asteraceae) and *Aconitum napellus* (Ranunculaceae). It is not recommended to combine fungi therapy with ASA (Aspirin).

The foregoing shows that the dried biomass of *L. edodes* is a prospective product for developing pharmaceutical drugs and is capable of significantly strengthening human immune defences.

Conclusion

Based on examination of the literature , we explored the main tendencies of pharmacological mycology development, in particular with regard to Shiitake (*L. edodes*). We conducted a comparative assessment of other mushrooms, champignons (*Agaricus bisporus*) and Lingzhi fungus (*Ganoderma lucidum*). We consider the evaluation of technological properties of Shiitake fungi with the purpose of the further potential development of a convenient immunomodulation medicine drug to be of current interest.

We researched and presented the relationship between the strong correlation between the chemical makeup of *L. edodes* powdered biomass, as research target, and its pronounced immunomodulation and antitumor effect with application of modern methods of scientific analysis. A biotechnological method of Basidionycetes biomass production was developed for the first time. The deep cultivation method, adapted by the authors to Ukraine's plant resource base, was used. Preclinical studies confirmed an increase inf patients' immune status.

The literature review did not found publications on the development of tableted drugs using Shiitake fungi powder basis. Previous researchers focused only on development of nutritional supplements. One may assume that the authors are the initiators of research in the abovementioned direction, as the search for the optimal technology for micropowder tableting of this biosystem was conducted for the first time.

Based on the research undertaken , we proposed a technology of tablets under the codename "Shytavit", as well as a theoretical and experimental proven integrated approach to its formulation:

– we provided the reasons for preference for the tableted form as the most optimal in the given case, as well as rationale for tableting;

– we outlined physical and chemical parameters of Shiitake fungi biomass dry powder as working quality indexes (crystallographic property and fractional makeup), which induce the ability for compression;

– based on the technological research findings (flowability – 3.40–3.51 kg/s, compressibility – 51 H, tapped density – 0,52 kg/m^3, moisture content – 3.5%) we substantiated the possibility of tableting of dried powder of Shiitake fungus biomass with the use of antifriction excipient only;

– the direct compression method was rejected as a result of the unsatisfactory quality of all tables samples;

– we selected tableting technology with the use of pre-granulation, with the possibility of short-term contact of the *L. edodes* dry powder with granulating liquid and adjustable temperature scenario;

– we determined the optimal qualitative-quantitative formula of the medicine per tablet (Shiitake fungi biomass dry powder – 0.4 g, Aerosil – 0.015 g, potato starch – 0.08 g, and magnesium stearate – 0.005 g), which is in full compliance with compendial requirements.

The authors developed a new tableted drug codenamed "Shytavit" with tablet strength 0.5 g for fungi therapeutic correction of immune dysfunctions, the production technology of which ensures the presservation of all biologically active properties of the raw material in its original condition.

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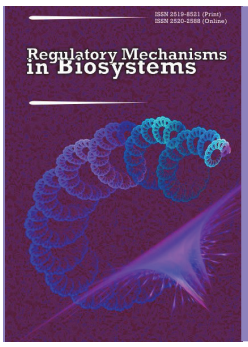

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 11–14
doi: 10.15421/021802

The role of neuro-specific dihydropyrimidinase-related protein 2 (dpyl2) in spatial memory formation in teleosts

D. V. Garina*, V. V. Bol'shakov*, I. Y. Toropygin**, A. A. Mekhtiev***, A. M. Andreeva*

**I. D. Papanin Institute for Biology of Inland Waters, Russian Academy of Sciences, Borok, Russia*
***V. N. Orekhovich Research Institute of Biomedical Chemistry, Russian Academy of Sciences, Moscow, Russia*
****A. I. Karayev Institute of Physiology, National Academy of Sciences of Azerbaijan, Baku, Azerbaijan*

Article info

Received 02.01.2018
Received in revised form 28.01.2018
Accepted 30.01.2018

I. D. Papanin Institute for Biology of Inland Waters, Russian Academy of Sciences, 109, Borok, 152742, Russia. Tel.: +7910-824-24-76. E-mail: dvgarina1976@yandex.ru

V. N. Orekhovich Research Institute of Biomedical Chemistry, Russian Academy of Sciences, Pogodinskaya st., 10/8, Moscow, 119121, Russia. Tel.: +7499-246-69-80.

A. I. Karayev Institute of Physiology, National Academy of Sciences of Azerbaijan, Sharif-Zade st., 2, Baku, AZ1100, Azerbaijan. Tel.: +99-450-336-06-84. E-mail: arifmekht@yahoo.com

Garina, D. V., Bol'shakov, V. V., Toropygin, I. Y., Mekhtiev, A. A., & Andreeva, A. M. (2018). The role of neuro-specific dihydropyrimidinase-related protein 2 (dpyl2) in spatial memory formation in teleosts. Regulatory Mechanisms in Biosystems, 9(1), 11–14. doi: 10.15421/021802

This article presents the results of an experiment on the influence of serotonin-modulating anticonsolidation protein (SMAP) on the spatial memory formation of juvenile goldfish *Carassius auratus* (L.) in a maze with food reinforcement. Three experimental fish groups were formed: (1) intact animals, (2) experimental group (fish injected ICV with SMAP in 24 h before the beginning of training; 2 µl, 1.5 mg·ml^{-1}), (3) active control group (fish injected ICV with inactivated SMAP). Goldfishes of the experimental group demonstrated the lowest capability for spatial recognition: the maximum level of performance of the task was on 4th day of the training – 38%, while the values of this index in fishes of the control and intact groups were 70% and 63% respectively. In general, throughout the period of the training the average value of task performance was 16% in the SMAP-injected fish (in the control and intact groups – 42% and 53%, respectively). By using Ds-Na-polyacrylamide gel electrophoresis SMAP composite-on has been revealed. It was found that it consists of 10–12 protein components, among which four proteins dominated. They were identified by mass spectrometry MALDI-TOF: spectrin, dihydropyrimidinase-related protein 2 (DPYL2), tubulin and actin. It has been suggested that the most likely candidate responsible for the negative effects of SMAP on fish memory formation is DPYL2. It was hypothesized that anticonsolidation effect of SMAP is caused by the effect of DPYL2 which blocks the growth of axons or its cytostatic activity which leads to disorders in formation of new neurons in the brain as a result of learning.

Keywords: teleosts; serotonin-modulating anticonsolidation protein; SMAP; learning; spatial memory

Роль нейроспецифичного дигидропиримидиназа подобного белка-2 (dpyl2) в формировании пространственной памяти у костистых рыб

Д. В. Гарина*, В. В. Большаков*, И. Ю. Торопыгин**, А. А. Мехтиев***, А. М. Андреева*

**Институт биологии внутренних вод имени И. Д. Папанина РАН, Борок, Россия*
***Научно-исследовательский институт биомедицинской химии имени В. Н. Ореховича РАН, Москва, Россия*
****Институт физиологии имени А. И. Караева НАН Азербайджана, Баку, Азербайджан*

Исследовали влияние серотонин-модулируемого антиконсолидационного белка (СМАБ) на формирование пространственной памяти у молоди карася *Carassius auratus* (L.) в лабиринте с пищевым подкреплением. Опытной группе рыб за 24 ч до начала обучения вводили в область четвертого желудочка мозга 2 мкл СМАБ (1,5 мг/мл), контрольной группе – СМАБ, инактивированный нагреванием, интактная группа не подвергалась инъекции. Караси опытной группы продемонстрировали наихудшую способность к пространственному распознаванию: максимальный уровень обученности отмечался на четвертый день и составил 38% (в контрольной и интактной группах – 70% и 63%, соответственно), в целом же за все время обучения средний показатель в опытной группе рыб составил 16% (в контрольной и интактной группах – 42% и 53%, соответственно). С помощью SDS-электрофореза в полиакриламидном геле установили состав СМАБ: он представлен девятью белковыми компонентами, среди которых по относительному содержанию доминируют четыре. С помощью масс-спектрометрии MALDI проведена их идентификация: спектрин, дигидропиримидиназа-подобный белок-2 (DPYL2), тубулин и актин. Поскольку три из четырех указанных белков – структурные, мы предположили, что наиболее вероятный кандидат, ответственный за негативный эффект СМАБ на формирование долговременной памяти у рыб, – это дигидропиримидиназа-подобный белок-2 (DPYL2). Выдвинута гипотеза, что антиконсолидационный эффект СМАБ обусловлен эффектом DPYL2, блокирующим прорастание аксонов или его цитостатической активностью, нарушающей образование в результате обучения новых нейронов в головном мозге.

Ключевые слова: рыбы; серотонин-модулируемый антиконсолидационный белок; СМАБ; пространственная память

Введение

Память и обучение рыб в раннем онтогенезе, а также приобретенные взрослыми особями навыки лежат в основе успешного существования вида, размножения и освоения им экологической ниши. В настоящее время установлено, что у рыб существует как кратковременная, так и долговременная память (Csányi et al., 1989; Zion et al., 2011), способность к простым (классический условный рефлекс) и довольно сложным формам научения (пространственное распознавание) (Rodriguez et al., 2005). Молекулярные механизмы долговременной памяти рыб в последние десятилетия привлекают все больше внимания исследователей. Получены доказательства участия холинергической и глутаматергической нейротрансмиттерных систем в процессах обучения и памяти у *Danio rerio* (Cognato et al., 2012). Показана важная роль теленцефалона и его отделов (латерального и медиального паллиума) в формировании эмоциональной, временно́й и пространственной памяти у лучеперых рыб (Broglio et al., 2005, 2010). Дорзолатеральное удаление теленцефалона золотой рыбки приводит к ухудшению пространственного распознавания, сходному с таковым при повреждении гиппокампа млекопитающих (Portavella et al., 2002). Нейрофизиологические исследования свидетельствуют также о вовлечении мозжечка рыб в процессы памяти и обучения, как это наблюдается у высших позвоночных (Rodriguez et al., 2005). Обнаружено, что острый стресс, вызванный феромоном тревоги или кайромоном хищника и примененный непосредственно перед тестированием рыб, значительно ухудшает пространственную и сигнальную память у *Danio rerio* (Gaikwad et al., 2011). Вместе с тем, в этой области физиологии низших позвоночных существует немало белых пятен.

Серотонин-модулируемый антиконсолидационный белок, или СМАБ, первоначально выявлен в затылочной области коры головного мозга крысы после аппликации серотонина (Mekhtiev, 2000). Название белка обусловлено, с одной стороны, установленной зависимостью его содержания в нервных клетках головного мозга млекопитающих от уровня серотонина (Gasanov & Mekhtiev, 1991; Mekhtiev et al., 2003), а с другой – негативным влиянием СМАБ на формирование долговременной памяти у животных (Mekhtiev, 2000). Нарушение консолидации следов памяти под действием СМАБ наблюдалось как у высших позвоночных (млекопитающих) в различных условно-рефлекторных моделях обучения (Guseinov & Mekhtiev, 2012; Mekhtiev et al., 2015), так и у низших (костистых рыб) при введении им СМАБ в желудочек мозга перед обучением (Garina & Mekhtiev, 2014). Кроме того, описан ряд других эффектов СМАБ, важных для жизнедеятельности организма: участие в обезвреживании токсинов (Movsum-Zadeh et al., 2013), регуляции процессов эмбриогенеза и метаморфоза (Mekhtiev et al., 2016), антиоксидантный (Bakhshalieva et al., 2010), антимутагенный (Mekhtiev et al., 2006) эффекты и некоторые другие.

Участие СМАБ в регуляции столь значимых для нормального функционирования организма процессах привело к мысли о том, что он задействован в одном из ключевых сигнальных каскадов клетки. Целью данной работы являлось 1) исследовать влияние СМАБ на формирование пространственной памяти у рыб; 2) выяснить состав СМАБ с помощью электрофореза в полиакриламидном геле и затем идентифицировать выявленные белковые фракции с помощью масс-спектрометрии MALDI.

Материал и методы исследований

Схема поведенческого эксперимента. Эксперимент по исследованию влияния серотонин-модулируемого антиконсолидационного белка (СМАБ) на формирование пространственной памяти у рыб проводили на молоди серебряного карася *Carassius auratus* (L.), выращенной на экспериментальной прудовой базе ИБВВ РАН (п. Борок) и затем содержавшейся в аквариальных условиях в течение 4 месяцев. Возраст молоди на момент начала опыта составил 6–7 месяцев, средняя масса – 7,8–9,7 г, средняя длина 8,5–9,0 см. За 7 суток до начала эксперимента рыб взвешивали, измеряли и помещали в индивидуальные контейнеры объемом 4 л с принудительной аэрацией. Процедуру внутричерепной инъекции препаратов проводили под наркозом по ранее апробированной методике (Garina & Mekhtiev, 2014). Опытной группе рыб (n = 8) вводили в область четвертого желудочка мозга 2 мкл нативного СМАБ (1.5 мг/мл), контрольной группе (n = 10) – СМАБ в той же дозе, инактивированный нагреванием на водяной бане в течение 40 мин при температуре 55 ºС. Интактную группу карасей (n = 8) не подвергали каким-либо инъекциям. Через 24 ч после инъекции регистрировали динамику формирования навыка – нахождение корма, находящегося в определенном месте лабиринта (рис. 1), в течение 10 сеансов обучения.

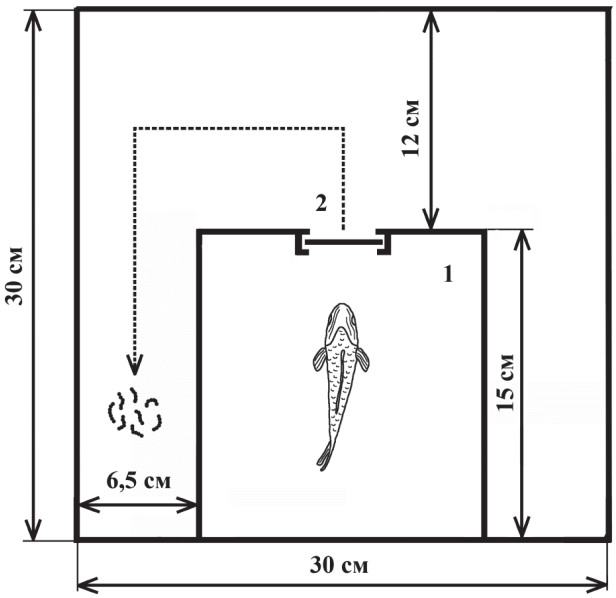

Рис. 1. Схема экспериментальной установки для исследования пространственной памяти у рыб: *1* – стартовая камера; *2* – заслонка, закрывающая выход из стартовой камеры

В течение суток обучение проводили однократно (один сеанс). Регистрировали факт выполнения или невыполнения задания (нахождение и поедание корма) особью в течение 10 мин после открытия заслонки в стартовой камере.

Статистическую обработку полученных результатов проводили с помощью однофакторного дисперсионного анализа ANOVA (Statistica 7.0, StatSoft Inc., USA) при уровне значимости $P < 0,05$.

Препарат. Серотонин-модулируемый антиконсолидационный белок (СМАБ) получен в Институте физиологии имени А. И. Караева НАН Азербайджана; выделен из мозга быка с использованием методов высаливания сульфатом аммония и колоночной гель-хроматографии на сефадексе G-150, под контролем твердофазного иммуноферментного анализа. Препарат доводили физиологическим раствором для холоднокровных животных (pH 7,3) до концентрации 1,5 мг/мл и хранили до начала работы при температуре –80 ºС.

Исследование состава СМАБ и идентификация белков. Исследование состава СМАБ проводили в 12,5% Ds-Na-ПААГ в восстанавливающих условиях (Laemmli, 1970). В качестве маркеров молекулярной массы использовали набор белков PageRulerTM Prestained Protein Ladder Plus (10, 15, 27, 35, 55, 70, 100, 130, 250 кДа) (Fermentas). Расчет величин молекулярных масс белков (MW) осуществляли с помощью программы ONE-Dscan, Ver 1.31 (Scananalytic Inc.). После электрофореза из геля вырезали участки, содержавшие мажорные фракции белков, подвергали их трипсинолизу и затем идентифицировали белки с помощью масс-спектрометрии MALDI. Масс-спектры регистрировали на времяпролетном масс-спектрометре BRUKER Ultraflex II (“Bruker Daltonics”, Германия). Идентификацию белков проводили в системе Mascot (опция «пептидный фингерпринт», www.matrixscience.com). Поиск

проводили в базе данных NCBI.nr среди белков всех организмов. Кандидатов, имеющих параметр достоверности score > 92 ($P < 0,05$), считали достоверно определенными.

Результаты

Влияние ICV-инъекции СМАБ на пространственное распознавание в лабиринте у карасей. Караси интактной группы обучались достаточно быстро: уже в первом сеансе обучения корм находили 3 особи из 8 (38%) (рис. 2).

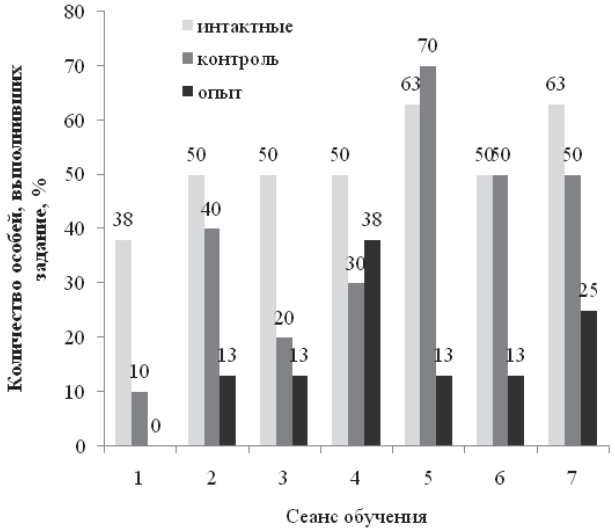

Рис. 2. Динамика формирования навыка у карасей находить корм в лабиринте

Уровень обученности рыб постепенно увеличивался и достигал максимума на пятые сутки (63%). Рыбы из группы активного контроля обучались в целом хуже, однако на пятые сутки у них выполняли задание 70% особей. У карасей опытной группы максимальный уровень обученности наблюдался на четвертый день и составил 38%, в целом же за все время обучения средний показатель составил 16% (в контрольной и интактной группах – 42 и 53% соответственно) ($P < 0,05$).

Электрофоретический анализ состава СМАБ. Исследование состава СМАБ с помощью электрофореза в денатурирующих условиях показало, что он представлен девятью компонентами, среди которых по относительному содержанию доминировали четыре (рис. 3, фракции 1–4). Все четыре фракции отобраны для масс-спектрометрического анализа.

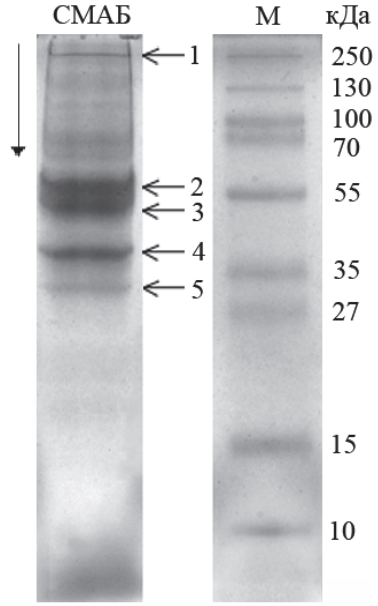

Рис. 3. SDS-электрофорез СМАБ: слева (столбик «СМАБ»): электрофореграмма СМАБ; стрелки справа указывают на локализацию фракций СМАБ (1–5), взятых на последующий анализ MALDI-TOF; стрелка слева – направление электрофореза; справа (столбик «М»): локализация маркеров молекулярной массы PageRulerTM Prestained Protein Ladder Plus (Fermentas) на электрофореграмме; цифрами обозначены молекулярные массы стандартных белков-маркеров (кДа)

Масс-спектрометрический анализ белков в составе СМАБ. Среди идентифицированных в составе СМАБ четырех белков оказались спектрин (1), дигидропиримидиназа подобный белок-2 (2), тубулин (3) и актин (4) (табл. 1).

Таблица 1
Идентификация белков в составе СМАБ

Номер фракции белка на электрофореграмме	Кандидатный белок (NCBI)	Accession number (NCBI)	MW, Da calc[1]/obs[2]	Score[3]	Coverage,[4] %
1	Spectrin beta chain, non-erythrocytic 1 isoform X2 [*Bos taurus*]	gi|528968379	272512/241840	126	14
2	Dihydropyrimidinase-related protein 2 [*Bos taurus*]	gi|115496400	62239/56540	188	45
3	Tubulin beta-4A chain isoform X10 [*Orcinus orca*]	gi|821404056	40540/52110	158	53
4	Actin, cytoplasmic 2 [*Canis lupus familiaris*]	gi|924442847	41711/39570	109	40
5	Actin, cytoplasmic 2 [*Macaca fascicularis*]	gi|54896078	41722/32260	119	37

Примечание: [1] – расчетная величина молекулярной массы; [2] – экспериментальная величина молекулярной массы; [3] – величина достоверности; [4] – перекрывание аминокислотных последовательностей.

Обсуждение

Таким образом, нами впервые получены свидетельства негативного влияния серотонин-модулируемого антиконсолидационного белка на формирование пространственной памяти у костистых рыб. Анализ состава СМАБ показал, что он состоит из нескольких белковых компонентов, при этом по относительному содержанию преобладают четыре белка. Параметр достоверности (score) для всех белков выше 92 (109–188); величины перекрывания аминокислотных последовательностей (coverage) четырех белков и их гомологов удовлетворительны (14–53); величины расчетных и экспериментальных молекулярных масс белков (MW) близки. Кроме того, экспериментальный и кандидатные виды животных, у которых обнаружены белки со сходной структурой, принадлежат одному классу Mammalia. В целом указанные факты позволяют утверждать надежность проведенной идентификации белков.

Поскольку спектрин, тубулин и актин являются структурными белками, а все остальные фракции в составе СМАБ представлены в слишком низкой концентрации, можно предположить, что наиболее вероятный кандидат, ответственный за эффекты СМАБ – это дигидропиримидиназа-подобный белок (dihydropyrimidinase-related protein 2, DPYL2, или CRMP2) (www.uniprot.org/uniprot/O02675). Это внутриклеточный нейроспецифичный 62 кДа белок, относится к небольшому семейству цитозольных фосфопротеинов, известных как медиаторы Sema3A сигналинга и нейрональной дифференциации. После активации Sema3A плексин А связывается с белками CRMP, вследствие чего они подвергаются фосфорилированию протеинкиназами Cdk5, GSK3beta и Fes. У CRMP блокируется способность связываться с димерами тубулина, что вызывает деполимеризацию F-актина и в конечном счете – прекращение роста аксона. Биологическая функция белков CRMP – участие в развитии и поляризации нейронов, регуляции роста ак-

сонов и клеточной миграции (Schmidt & Strittmatter, 2007; Nakamura et al., 2000). Присутствие в составе СМАБ тубулина и актина, повидимому, обусловлено их взаимодействием с CRMP2 в клетке. Поскольку фракционирование СМАБ происходит в неденатурирующих (в отсутствии детергентов) условиях, дигидропиримидиназа подобный белок-2 элюируется с гель-хроматографической колонки G-150 в комплексе с тубулином и актином, одним пиком.

Поскольку в основе обучения животных лежит образование новых связей между нейронами, а внутримозговое введение СМАБ млекопитающим и рыбам перед выработкой у них условных рефлексов приводило к нарушению формирования у них памяти (Guseinov & Mekhtiev, 2012; Garina & Mekhtiev, 2014; Mekhtiev et al., 2015), можно предположить, что негативный эффект СМАБ на формирование пространственной памяти у рыб в настоящей работе обусловлен эффектом DPYL2, блокирующим прорастание аксонов, вовлеченных в формирование памяти нейронов. Кроме того, поскольку показано, что в результате обучения происходит образование новых нейронов в ряде структур головного мозга животных (Sherstnev et al., 2010, 2016; Deng et al., 2010; Yau et al., 2015), то антиконсолидационные эффекты СМАБ также могут быть связаны с цитостатической активностью DPYL2.

Заключение

В настоящем исследовании впервые установлено, что серотонин-модулируемый антиконсолидационный белок (СМАБ) оказывает выраженное негативное влияние на формирование пространственной памяти у костистых рыб. В состав препарата СМАБ входят девять белковых компонентов, среди которых доминируют четыре: спектрин, дигидропиримидиназа подобный белок-2, тубулин и актин. Полученные результаты позволяют предположить, что антиконсолидационный эффект СМАБ обусловлен эффектом DPYL2, блокирующим прорастание аксонов, или его цитостатической активностью, нарушающей образование в результате обучения новых нейронов в головном мозге рыб. Для подтверждения этой гипотезы необходимы дополнительные исследования с очищенным препаратом DPYL2.

Благодарности

Авторы выражают благодарность И. П. Рябцевой за техническую помощь при проведении работы.

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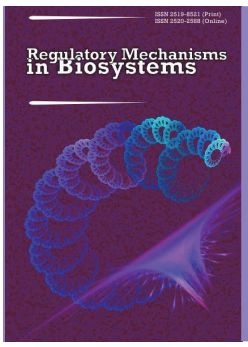

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 15–22
doi: 10.15421/021803

Content of chemical elements in the liver of cattle with fasciolosis and dicrocoeliosis

O. V. Kruchynenko*, M. P. Prus**, M. V. Galat**, S. M. Mykhailiutenko*, O. S. Klymenko*, L. M. Kuzmenko*

**Poltava State Agrarian Academy, Poltava, Ukraine*
***National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine*

Article info

Received 14.01.2018
Received in revised form 22.02.2018
Accepted 27.02.2018

Poltava State Agrarian Academy, Skovorody st., 1/3, Poltava, 36003, Ukraine.

National University of Life and Environmental Sciences of Ukraine, Heroiv Oborony st., 15/3, Kyiv, 03041, Ukraine. Tel.: +38-099-062-64-96. E-mail: oleg.kruchynenko@pdaa.edu.ua

Kruchynenko, O. V., Prus, M. P., Galat, M. V., Mykhailiutenko, S. M., Klymenko, O. S., & Kuzmenko, L. M. (2018). Content of chemical elements in the liver of cattle with fasciolosis and dicrocoeliosis. Regulatory Mechanisms in Biosystems, 9(1), 15–22. doi: 10.15421/021803

The concentration of chemical ements (Pb, Cd, Cu, As, Zn, Hg, Fe, Co, Mn) in the liver of healthy cattle and those affected by *Fasciola hepatica* and *Dicrocoelium lanceatum* in Poltava region (central part of Ukraine) was determined. The research was carried out by the method of atomic and absorption spectrometry carried out at the Regional State Laboratory of Veterinary Medicine in Poltava region. The liver samples (n = 30) from healthy cattle black-and-white breed and those affected by *F. hepatica* and *D. lanceatum* were taken at the meat processing plant. The ages of the cattle ranged from 6 to 8 years. The samples were immediately cooled, transported to the laboratory and stored at –20 °C for further analysis. The results of the research determined the average indicators of concentration of some toxic elements in the livers of healthy cattle and those infected by the trematodes. The content of chemical elements in the liver of healthy animals and those affected by fasciola can be represented in the form of a decreasing rank number: Zn > Fe > Cu, and for dicrocoeliosis, respectively, Fe > Zn > Cu. It has been established that Cu and Zn are involved in the metabolic processes of the body of trematodes, which is confirmed by our research. The presence of *F. hepatica* and *D. lanceatum* in the body of cattle significantly reduces the level of copper and zinc, with a high inverse correlation dependence on the intensity of infection, thus indicating the possibility of their accumulation by helminths. Concentration of Cu and Zn in the liver of cattle with fasciolosis was 6.82 ± 0.29 and 35.77 ± 1.93 mg/kg, while for animals with dicrocoeliosis it was 3.90 ± 0.25 and 41.91 ± 2.22 mg/kg. The content of cobalt and manganese in the liver of healthy animals was, respectively, 0.05 ± 0.01 and 1.95 ± 0.06 mg/kg. In the case of *Fasciola* parasitising in the liver tissue, the level of cobalt (0.10 ± 0.02) and manganese (2.55 ± 0.16) significantly increased, positively correlating with the intensity of the infection, indicating no effect on the exchange and bioaccumulation of these elements by helminths.

Keywords: microelements; heavy metals; correlation; *Fasciola hepatica*; *Dicrocoelium lanceatum*

Вміст хімічних елементів у печінці великої рогатої худоби за фасціольозу та дикроцеліозу

О. В. Кручиненко*, М. П. Прус**, М. В. Галат**,
С. М. Михайлютенко*, О. С. Клименко*, Л. М. Кузьменко*

**Полтавська державна аграрна академія, Полтава, Україна*
***Національний університет біоресурсів та природокористування України, Київ, Україна*

На території Полтавської області (центральна частина України) визначено концентрацію хімічних елементів (Pb, Cd, Cu, As, Zn, Hg, Fe, Co, Mn) у печінці великої рогатої худоби від здорових тварин і уражених *Fasciola hepatica* та *Dicrocoelium lanceatum*. Дослідження проведено методом атомно-абсорбційної спектрометрії на базі Регіональної державної лабораторії ветеринарної медицини в Полтавській області. На м'ясокомбінаті відбирали зразки печінки (n = 30) від здорових корів чорно-рябої породи та уражених *F. hepatica* та *D. lanceatum*. Вік великої рогатої худоби – 6–8 років. Зразки негайно охолоджували, транспортували в лабораторію та до подальшого аналізу зберігали за –20 °С. Встановлено середні показники концентрації окремих хімічних елементів у печінці здорових та інвазованих трематодами тварин. Уміст хімічних елементів у печінці здорових тварин і уражених фасціолами можна навести у вигляді спадного ранжувального ряду: Zn > Fe> Cu, а за дикроцеліозу – Fe > Zn > Cu. Мідь та цинк беруть участь в обмінних процесах організму трематод, що підтверджується нашими дослідженнями. Присутність *F. hepatica* та *D. lanceatum* в організмі великої рогатої худоби достовірно знижує рівень міді та цинку, маючи високу зворотну кореляційну залежність від інтенсивності інвазії. Концентрація Cu та Zn у печінці за фасціольозу складає 6,82 ± 0,29 і 35,77 ± 1,93 мг/кг, а за дикроцеліозу – 3,90 ± 0,25 та 41,91 ± 2,22 мг/кг. Вміст кобальту та марганцю у печінці здорових тварин становить, відповідно, 0,05 ± 0,01 і 1,95 ± 0,06 мг/кг. За паразитування фасціол у тканині печінки достовірно підвищується рівень кобальту (0,10 ± 0,02) та марганцю (2,55 ± 0,16), позитивно корелюючи з інтенсивністю інвазії, що свідчить про відсутність впливу на обмін та біоакумуляцію гельмінтами цих елементів.

Ключові слова: мікроелементи; важкі метали; кореляція; *Fasciola hepatica*; *Dicrocoelium lanceatum*

Вступ

До незамінної групи речовин, що виконують важливі біологічні функції, відносять мікро- та макроелементи. Ці мінеральні речовини мають високу біологічну активність, містяться у продуктах харчування, питній воді, тканинах людини та тварини. Мідь, кобальт, цинк, марганець, залізо беруть участь майже в усіх біологічних процесах, що відбуваються у тканинах організму, та мають досить специфічну дію (Ekici et al., 2004; Mohammadifard et al., 2017). Мідь виступає необхідним компонентом дихального ферменту цитохромоксидази. За відсутності хімічних елементів неможливі: дихання, утворення крові, білковий, вуглеводний та жировий обміни (Minatel & Carfagnini, 2002; Stern, 2010).

Кобальт бере активну участь у ферментативних процесах організму (Orjales et al., 2017). Відмічено його гальмівний вплив на окисно-відновні процеси, він знижує активність цитохром- і холіноксидази, каталази крові, активує дипептидазу, аргіназу, кісткову та кишкову фосфатази (Leskinen et al., 2016).

Біологічна роль цинку пов'язана з вуглеводним обміном. Цей мікроелемент разом із ферментами, гормонами та вітамінами впливає на фундаментальні життєві процеси: розмноження, ріст і розвиток організму, обмін білків, жирів, окисно-відновні процеси та енергетичний обмін (Grosskopf et al., 2017).

Марганець – незамінний елемент для життя рослин і тварин. Він стимулює синтез холестерину та жирних кислот, виявляючи тим самим гіпотронні дії. Крім впливу на процеси кровотворення, марганець впливає на антитілогенез, прискорюючи утворення антитіл. Більшість елементів, у тому числі Mn, має характерний для нього діапазон безпечної експозиції (Botle et al., 2004; Griffiths et al., 2007; Bowman et al., 2011).

В організмі тварин ферум присутній в усіх тканинах, проте найбільша його частина зосереджена в кров'яних тілах. Атоми заліза займають центральне положення в молекулах гемоглобіну, завдяки чому останні можуть приєднувати та відщеплювати кисень (Hansch & Mendel, 2009). Недостатнє надходженння перерахованих елементів до організму з кормами чи неповне засвоєння у разі захворювань супроводжуються патологічними змінами, викликає дисбаланс мінеральних речовин, порушення обмінних процесів, зниження якості продукції (Berger, 2005; Dermauw et al., 2013; Alzaheb & Al-Amer, 2017).

До мікроелементів належать також важкі метали, які акумулюються в різних наземних і водних екосистемах. Вони – невід'ємна складова рослин, організму тварин і людей, оскільки багато сполук цих елементів входять до складу вітамінів, гормонів та різних тканин (Kabata-Pendias, 2004; Tchounwou et al., 2012; Ramos et al., 2014). Однак надмірне накопичення цих елементів викликає тяжкі наслідки, навіть захворювання (Zeng et al., 2016).

Відомо, що важкі метали стають високотоксичними у разі підвищення їх порогових концентрацій. Індивідуальна потреба живих організмів у цих елементах мізерна, а надходження із зовнішнього середовища їх надлишкових кількостей спричиняє порушення функцій різних органів та їх систем. Кадмій в організмі активує цинкзалежні ферменти, входячи до складу деяких білків, бере участь у метаболізмі заліза, міді та кальцію, впливає на вуглеводний обмін. Разом із цим особливої необхідності в його надходженні немає, що підтверджено дослідженнями: згодовування бугайцям хлориду кадмію у дозах 0,03 і 0,05 мг/кг маси тіла протягом 30 діб спричинило розвиток хронічного кадмієвого токсикозу. Задавання з кормом хлориду кадмію у дозі 0,05 мг/кг зумовило вірогідне зниження рівня неензимної та ензимної системи антиоксидантного захисту організму тварин, на що вказує зниження активності супероксиддисмутази, каталази, глутатіонпероксидази, вмісту відновленого глутатіону, селену, вітамінів А та Е в їх крові (Gutyj et al., 2016). За даними Lazarus et al. (2008), кадмій належить до найтоксичніших речовин, що мають властивість накопичуватися. Період його напіввиведення коливається в межах 10–35 років. Цей елемент накопичується в нирках, печінці, трубчастих кістках, підшлунковій залозі, селезінці та інших тканинах і органах, спричиняє підвищення тиску, порушення роботи нервової системи, легень, нирок та появу злоякісних новоутворень (Skalny, 2004). Свинець досить повільно виводиться з організму, переважно з калом і сечею. Він стимулює процеси росту та відновлення тканин; бере участь у процесах обміну кальцію та заліза; регулює вміст гемоглобіну у крові; активує або пригнічує активність деяких ферментів. Однак вже у разі потрапляння 1 мг свинцю в організм реєструється його передозування, що спричиняє захворювання кісток, гіпертонію, анемію, атеросклероз, виснаження, ниркову недостатність, послаблення імунітету, зниження рівня мікро- та мікроелементів та інші патології. Основні органи накопичення катіонів свинцю – нирки та мозок (Shefa & Héroux, 2017).

Арсен поліпшує кровотворення, регулює засвоєння азоту та фосфору, чинить послаблювальну дію на окисні процеси, взаємодіє з деякими групами білків, а також із цистеїном, глутатіоном, ліпоєвою кислотою; бере участь у ферментативних реакціях, діє як замінник фосфату (Datta et al., 2010). Перевищення порогової концентрації елемента спричиняє дратівливість, алергію, екзему, дерматити, виразки, кон'юктивіти. Також уражається дихальна система, відмічається порушення функції печінки. Реєструють підвищення ризику виникнення онкологічних захворювань, пригнічення функцій нервової системи. Токсична доза в діапазоні 5–50 мг/доба, а збільшення до 50 мг і вище може призвести до летальних наслідків (Skalny, 2004; Datta et al., 2012).

За останні роки зросли викиди токсичних елементів у довкілля різних країнах світу. Забруднення навколишнього природного середовища важкими металами стало глобальною проблемою. До головних факторів, що спричинили загрозливий стан довкілля, відносять застарілу технологію виробництва у промисловості, несучасне обладнання, низький рівень упровадження ресурсо- та енергозаощаджувальних, екологічно чистих технологій; високий рівень концентрації промислових об'єктів; відсутність належних природоохоронних систем; низький рівень експлуатації існуючих природоохоронних об'єктів; відсутність належного правового та економічного механізмів регулювання; прогресуючу урбанізацію населення, розростання гігантських мегаполісів, відсутність належного контролю за охороною довкілля, виверження вулканів (Davydova, 2005; Shakir et al., 2017).

Екологічні наслідки таких геохімічних змін привертають увагу, оскільки, на відміну від інших речовин, що забруднюють середовище, метали у природних умовах не руйнуються, а лише змінюють форму знаходження залежно від низки факторів. Крім того порушується природний кругообіг хімічних елементів (Sokolenko & Sokolenko, 2015).

Тому, інтерес до концентрації токсичних елементів у живих організмах зріс (Li et al., 2015). Відомості про вміст важких металів і особливості їх локалізації у тканинах тварин, птиці, безхребетних мають велике практичне значення (Gašparík et al., 2010; Ihedioha & Okoye, 2013; Brygadyrenko & Ivanyshyn, 2015). Актуальність моніторингу полягає в тому, що надходження значної частини токсичних елементів в організм людини в основному відбувається за ланцюгом «ґрунт – рослина (корм, раціон) – тварина – продукт тваринництва – людина» (Roggeman et al., 2014; Sachko et al., 2016).

Для визначення особливостей розташування вмісту важких металів у живих організмах, знаючи їх властивість до акумуляції, науковці дослідили тварин-біоіндикаторів. Вони дозволяють визначити ступінь небезпеки тих або інших речовин для живої природи та людини; дають можливість контролювати дію будь-яких металів, сполук тощо. До переваг «живих індикаторів» відносять можливість оцінити стан популяції в цілому, включаючи токсичні елементи та антропогенні фактори. Вони показують тенденції, швидкість змін, співвідношення, що відбуваються в навколишньому середовищі, вказують на шляхи та місця накопичення в екосистемах важких металів (López Alonso et al., 2002; Yarsan et al., 2014).

Аналіз показників накопичення токсичних елементів у тваринах-індикаторах дозволяє дати санітарну характеристику стану середовища відносно факторів промислового забруднення, оцінити ступінь ризику від дії нових антропогенних чинників у біосфері та, відповідно, скласти коротко- та довгострокові прогнози зміни екології (Saltyikova, 2011; Peterson & Schulte, 2016). Біомоніторин-

гу важких металів в органах і тканинах риб присвячено праці Le et al. (2016) та Javed & Usmani (2016). Доступні літературні дані свідчать також і про акумуляцію важких металів паразитами риб, що локалізуються в її різних органах (Nachev et al., 2010; Bayoumy et al., 2015; Hassan et al., 2016). Автори доводять, що паразити здатні накопичувати важкі метали в концентраціях, які у багато разів вищі, ніж у тканинах господаря (Sures, 2004; Leite et al., 2017; Sures et al., 2017). Через здатність паразита до біоакумуляції важких металів у своїй системі вони можуть служити потенційними показниками якості навколишнього середовища (Nachev & Sures, 2016; Vidal-Martínez & Wunderlich, 2017). Акантоцефали – найбільш вивчена група паразитів у водних екотоксикологічних дослідженнях (Sures et al., 1997; Podolska et al., 2016). Представники інших видів гельмінтів вивчені меншою мірою.

Тому мета цієї статті – визначити вміст токсичних елементів у печінці великої рогатої худоби за фасціольозу та дикроцеліозу.

Матеріал і методи досліджень

Дослідження проводили на базі Регіональної державної лабораторії ветеринарної медицини в Полтавській області, яка акредитована Національним агентством з акредитації України (НААУ). На м'ясокомбінаті відібрано зразки печінки (n = 30) від здорових корів чорно-рябої породи та уражених *F. hepatica* та *D. lanceatum*. Вік великої рогатої худоби становив 6–8 років. Зразки негайно охолоджували, транспортували у лабораторію та до подальшого аналізу зберігали за температури –20 °C.

Вміст міді, цинку, кадмію, свинцю, заліза, кобальту та марганцю визначали методом атомно-абсорбційної спектрометрії з атомізацією у полум'ї атомно-абсорбційного спектрофотометра Varian AA 240-FS (ГОСТ 30178-96). Рівень миш'яку визначали за допомогою спектрофотометра Cary 50 та фотоелектроколориметра КФК-2 (ГОСТ 26930-86). Концентрацію ртуті визначали за допомогою аналізатора ртуті DMA-80 (EPA Method 7473 "Mercury in solids and solutions by thermal decomposition amalgamation, and atomic absorbtion spectrophotometry" & ISO 11212-2:1997(E) Part 2 "Determination of mercury content by atomic absorbtion spectrometry"). Гранично допустимий вміст токсичних елементів узято згідно з наказом Державного департаменту ветеринарної медицини № 107 від 27.09.2004 року.

Статистичне опрацювання отриманих результатів проводили з використанням програми Statistica 10 (StatSoft Inc., USA, 2011). Усі параметри розглядали як непараметричні дані, виражали як середнє значення ± SE (стандартна помилка). Для попарного порівняння результатів використовували критерій Манна – Уїтні. Значущими вважали відмінності між показниками у групах за $P < 0,05$. Коефіцієнт кореляції Спірмена (r_s) застосовували для визначення залежності між змінними: інтенсивністю інвазії та концентраціями мікроелементів у печінці.

Результати

Установлено концентрації хімічних елементів (Pb, Cd, Cu, As, Zn, Hg, Fe, Co, Mn) у досліджених зразках печінки великої рогатої худоби. Проаналізовано вміст цих металів у печінці агельмінтних тварин і уражених фасціолами та дикроцеліями. Концентрація миш'яку та ртуті в усіх дослідних зразках не перевищувала гранично допустимі норми та не мала достовірної різниці. Встановлено перевищення вмісту міді у здорових тварин (27,3 ± 5,0 мг/кг) порівняно з гранично допустимою концентрацією, що свідчило про надмірне його надходження з кормом. За паразитування трематод концентрація міді у печінці достовірно знижувалася. Її рівень у досліджених зразках за наявності фасціол становив 6,82 ± 0,29 мг/кг, а за дикроцеліозу – 3,90 ± 0,25 мг/кг ($P < 0,0001$).

Одержані результати свідчать, що вміст свинцю не перевищував гранично допустимі концентрації в усіх пробах. У печінці за відсутності збудників паразитарних хвороб його концентрація становила 0,19 ± 0,01, тоді як в органі за ураження фасціолами сягала 0,23 ± 0,01 мг/кг. Слід зазначити, що за ураження печінки дикроцеліями, навпаки, реєстрували зниження вмісту Pb до 0,14 ± 0,01 ($P < 0,004$) порівняно з контролем. Концентрація кадмію у печінці здорових тварин (група H) склала 0,122 ± 0,006 мг/кг, у хворих на фасціольоз (група F) – 0,057 ± 0,007 мг/кг ($P < 0,0002$), на дикроцеліоз (група D) – 0,037 ± 0,003 мг/кг ($P < 0,0001$). У зразках органа агельмінтної великої рогатої худоби вміст цинку становив 94,3 ± 2,5 мг/кг. У хворих на фасціольоз і дикроцеліоз жуйних цей показник достовірно ($P < 0,0001$) знижувався (35,8 ± 1,9 та 41,9 ± 2,2 мг/кг, відповідно). Вміст заліза також зменшувався у другій та третій групі: концентрація цього елемента у групі F склала 34,2 ± 2,1 мг/кг ($P < 0,0001$), а у групі D – 52,6 ± 0,6 мг/г, порівняно з показником контрольної групи – 55,9 ± 1,4 мг/кг (рис. 1).

Таким чином, концентрація у печінці кадмію, міді, цинку та заліза за ураження трематодами нижча, ніж у контрольній групі. Крім перерахованих елементів, достовірно знижується у групі D також вміст свинцю. За результатами досліджень (табл. 1), у печінці великої рогатої худоби, ураженої фасціолами та дикроцеліями, відбулося достовірне відносно контрольної групи підвищення концентрації кобальту до 0,103 ± 0,015 ($P < 0,1$) та 0,143 ± 0,009 мг/кг ($P < 0,0001$) та марганцю до 2,547 ± 0,160 ($P < 0,004$) та 2,210 ± 0,078 мг/кг ($P < 0,100$). Вміст кобальту та марганцю у печінці здорових тварин становив, відповідно, 0,049 ± 0,009 та 1,951 ± 0,060 мг/кг.

Таблиця 1

Оцінка різниці показників між двома вибірками за порівняння груп H та F і H та D(n = 10, U-тест Манна – Уїтні)

Досліджувані елементи	z-значення за порівняння груп H та F	P value за порівняння груп H та F	z-значення за порівняння груп H та D	P value за порівняння груп H та D
Pb	–1,58745	0,1124	2,8725	0,0040
Cd	3,6662	0,0002	3,7418	0,0001
Cu	3,7418	0,0001	3,7418	0,0001
Zn	3,7418	0,0001	3,7418	0,0001
Hg	3,7418	0,0001	–1,8142	0,0696
Fe	3,7418	0,0001	1,8142	0,0696
Co	–2,5323	0,0113	–3,7418	0,0001
Mn	–2,8725	0,0040	–2,4945	0,0126

У печінці здорових тварин ранжувальний ряд за рівнем хімічних елементів представлений таким чином: Zn > Fe > Cu та Mn > Pb > Cd > Co у співвідношенні 3,45 : 2,05 : 1,00 й 39,82 : 3,96 : 2,49 : 1,00, відповідно. У досліджуваному органі спостерігали високий вміст цинку та низький – кобальту. Концентрацію арсену та ртуті не брали до уваги, оскільки ці показники в усіх дослідних зразках достовірно не корелювали. Разом із цим, вміст токсичних елементів у печінці великої рогатої худоби, ураженій фасціолами, можна навести у вигляді спадних ранжувальних рядів: Zn > Fe > Cu у співвідношенні 5,23 : 5,02 : 1,00 та Mn > Pb > Co > Cd – 44,68 : 3,96 : 1,81 : 1,00. У печінці корів, уражених збудником дикроцеліозу, ранжувальний ряд мав іншу послідовність: Fe > Zn > Cu та Mn > Co > Pb > Cd, з таким співвідношенням: 13,49 : 10,75 : 1,00 та 59,73 : 3,86 : 3,73 : 1,00.

Інтенсивність фасціольозної інвазії (II) в середньому становила 43,4 ± 6,41 екз./гол. (min–max: 19–74). У групі D, відповідно, II = 42,6 ± 6,5 екз./гол. (min–max: 16–87).

Підрахунок коефіцієнта кореляції хімічних елементів підтвердив високу залежність вмісту міді, цинку, кобальту та марганцю від інтенсивності фасціольозної інвазії та кількості міді та цинку – від дикроцеліозної (табл. 2). Установлено також, що концентрація свинцю мала високу пряму кореляційну залежність від інтенсивності фасціольозної ($P < 0,05$) та високу обернену – від інтенсивності дикроцеліозної інвазії ($P < 0,05$). Рівень кадмію позитивно корелював із присутністю трематод роду *Fasciola*, негативно – за дикроцелій, але не мав достовірної залежності. Вміст кобальту та марганцю залежав лише від інтенсивності фасціольозної інвазії з найвищими коефіцієнтами кореляції ($P < 0,05$).

Як видно з таблиці 2, коефіцієнти кореляції концентрації ртуті у печінці тварин груп F і D не мали достовірної різниці. Показники вмісту цинку мали високу обернену кореляційну залежність від інтенсивності фасціольозної ($P < 0,05$) та дикроцеліозної ($P <$

0,05) інвазій. Концентрація міді обернено залежала від присутності фасціол ($P < 0,05$) та дикроцелій ($P < 0,05$). Вміст заліза достовірно не корелював з показниками ураження трематодами.

Проведені дослідження свідчать, що паразитування фасціол достовірно підвищує у печінці тварин рівень свинцю, кобальту та марганцю, позитивно корелюючи з інтенсивністю інвазії; знижує вірогідно концентрацію міді та цинку з високою оберненою кореляційною залежністю від інтенсивності інвазії. Встановлено обернено достовірний кореляційний зв'язок між умістом свинцю, міді, цинку та показником кількості дикроцелій у печінці хворих тварин.

Таблиця 2

Кореляція між інтенсивністю трематодозних інвазій та вмістом хімічних елементів

Групи, n = 10	Pb	Cd	Cu	Zn	Hg	Fe	Co	Mn
Коефіцієнт кореляції (r_s) групи F	0,76*	0,60	–0,85*	–0,70*	0,53	–0,54	0,91*	0,79*
Коефіцієнт кореляції (r_s) групи D	–0,72*	–0,47	–0,81*	–0,84*	0,42	–0,24	0,59	0,25

Примітка: * – $P < 0,05$.

Обговорення

Ми дослідили вміст хімічних елементів у печінці великої рогатої худоби, ураженої збудниками фасціольозу та дикроцеліозу, на території Полтавської області (центральна частина України). Виявлено різні концентрації мікроелементів, важких металів і токсичних елементів у дослідних зразках. Порівнюючи їх вміст у печінці здорових та інвазованих трематодами тварин, установили, що рівень мінеральних елементів у досліджуваному органі змінювався за присутності паразитів. Водночас концентрація арсену та ртуті в усіх дослідних зразках не перевищувала гранично допустимі норми.

Серед металів, що відносно рівномірно розподіляються в організмі, зазвичай реєструється найвищий їх вміст в органах, де зосереджені інтенсивні біохімічні процеси: у печінці, залозах внутрішньої секреції, нирках (López-Alonso et al., 2000; Suttle, 2010; Nwude et al., 2011). Дослідники повідомляють (Jarzyńska & Falandysz, 2011), що печінку слід розглядати як потужне джерело таких мікроелементів, як Co, Cr, Cu, Mo, Mn, Se і Zn, які накопичуються в організмі жуйних тварин у межах трофічного ланцюга.

Автори наголошують, що печінка великої рогатої худоби стає потенційно небезпечним органом для здоров'я людей у разі акумуляції в ньому важких металів. Крім того, науковці зазначають, що виявлення концентрації одного металу може свідчити про рівень накопичення іншого (Nwude et al., 2011). На думку науковців, печінка акумулює максимальну кількість таких мікроелементів як Cu, Mn і Md, тому її використовують як орган-мішень для виявлення цих металів (Oymak et al., 2017). Про важливість дослідження печінки та нирок у тварин на вміст елементів задля оцінювання забруднення навколишнього середовища повідомляє низка дослідників (Paßlack et al., 2014; López-Alonso et al., 2017). Автори рекомендують визначати в цих органах Sr, Ba, Cd, Cu, Zn, Mn, Cr, Sb, Se та Pb.

Результати наших досліджень свідчать, що присутність фасціол достовірно підвищує вміст кобальту у печінці тварин на 52,4%. Це положення підтверджують інші автори. Зокрема, російські дослідники у хворих на опісторхоз людей виявляли підвищене накопичення у тканинах печінки хрому, ртуті, цезію, лантану та кобальту (Il'inskikh et al., 2006). Під час дослідження кормових добавок у щурів, уражених фасціолами, дослідники встановили, що рівні марганцю та кобальту у печінці суттєво не змінюються і залежать від форми захворювання: за гострої стадії кількість марганцю підвищувалась, а за хронічної – незначно знижувалась (Tsocheva-Gaitandjieva et al., 2002). Це збігається з результатами наших досліджень, якими встановлено, що рівень марганцю, маючи позитивну кореляцію з інтенсивністю інвазії, підвищувався у хворих на фасціольоз на 23,4%. Водночас у тварин, уражених дикроцеліями, вміст марганцю та кобальту не мав кореляційної залежності. Зміни вмісту кобальту, міді, цинку та магнію відмічали турецькі вчені у дітей, хворих на лямбліоз і ентеробіоз (Culha & Sangün, 2007).

Проведені нами дослідження також встановили, що паразитування фасціол сприяло достовірному зниженню концентрації заліза у печінці тварин до 34,2 ± 2,1 мг/кг, маючи високу обернену кореляційну залежність від інтенсивності інвазії. Інші дослідники також повідомляють про зменшення рівня заліза у сироватці крові хворих на фасціольоз тварин (Lotfollahzadeh et al., 2008). Про кореляційну залежність вмісту мікроелементів у тканинах організму та сироватки крові повідомляє Petukhova (2013), що свідчить про високу інформативність рівня мікроелементів у печінці тварин.

Minguez et al. (2011) зазначають, що аналіз концентрації мікроелементів у безхребетних в окремих екосистемах може слугувати індикатором забруднення навколишнього середовища. Інші науковці доводять, що паразити хребетних організмів накопичують мікроелементи з навколишнього середовища. Отже, дослідження вмісту мікроелементів у їх тканинах може свідчити про рівень забруднення того регіону, де перебувала тварина (Nachev & Sures, 2016). За даними Suleyman et al. (2006), елементи, що беруть участь в обмінних процесах організму – магній (Mg), цинк (Zn), мідь (Cu) – здатні накопичуватися у *F. hepatica* та *D. dendriticum*.

Згідно з нашими дослідженнями, присутність фасціол достовірно знижує рівень купруму у печінці хворих тварин учетверо, за дикроцеліозу – усемеро порівняно з агельмінтними тваринами ($P < 0,0001$). Інші автори подібну тенденцію пояснюють накопиченням міді, цинку та заліза самими гельмінтами. Іранські дослідники наводять дані щодо акумуляції міді фасціолами, тому це дає можливість використання трематоди роду *Fasciola* як маркерів забруднення навколишнього середовища важкими металами (Lotfy et al., 2013). У той же час автори зазначають, що коефіцієнт біоконцентрації міді у *D. lanceatum* найвищий серед порівнюваних видів трематод, що узгоджується нашими даними, оскільки вміст купруму у печінці хворих на дикроцеліоз був мінімальним. Про те, що *F. gigantica* – хороший біоакумулятор міді та цинку, повідомляють Acosta et al. (2017).

Нашими дослідженнями встановлено, що рівень цинку, маючи високу обернену кореляційну залежність (r_s – –0,70 та –0,84, $P < 0,05$) від інтенсивності фасціольозної та дикроцеліозної інвазії, становив 35,77 ± 1,93 та 41,91 ± 2,22 мг/кг, що значно нижче, ніж у здорових тварин (94,28 ± 2,49 мг/кг). Враховуючи думку наведених авторів, можна стверджувати, що трематоди добре накопичують і цинк (Lotfy et al., 2013; Acosta et al., 2017).

Проведено низку досліджень (Nachev et al., 2013), в яких вивчено, що кишкові паразити накопичують в основному токсичні елементи (кадмій, арсен, свинець), а тканинні – основні біологічно важливі для організму тварин (мідь, залізо, селен та цинк). Паразити шлунково-кишкового каналу конкурують за поживні речовини та метали з навколишніми тканинами, можливо в результаті переривання ентерогепатичного циклу. Метали, пов'язані з жовчю, поглинаються в кишечнику гельмінтами і не доступні для реабсорбції кишечником (Thielen et al., 2004). Зокрема, турецькі вчені встановили, що за паразитування кишкових нематод у дрібних жуйних у сироватці крові концентрація марганцю та заліза нижча за норму, а вміст цинку та кальцію перебував на нижній фізіологічній межі. У той же час кількість кадмію підвищувалася та мала позитивну кореляцію з інтенсивністю інвазії (Unubol Aypak et al., 2016).

За даними Brázová et al. (2015), вміст мікроелементів у тканинах паразита та господаря залежить від характеру (моно- чи полі-) та інтенсивності інвазії. Інші дослідники зазначають, що біоакумуляція паразитами мікроелементів залежить від виду металу та гельмінта. Зокрема, Lotfy et al. (2013) зазначають, що коефіцієнт біотрансформації хрому перебував на вищому рівні у *F. hepatica*, а цинку – у *F. gigantica*. Під час досліджень головня європейського концентрації Cu, Mn, Ag, Cd, Pb мали вищі значення у кишкових паразитів, порівняно із тканинами шлунково-кишкового каналу, а рівні металів Fe та Zn були нижчими у паразитів відповідно (Marijić et al., 2013). Різні профілі накопичення важких металів гельмін-

тами вчені пов'язують зі специфічністю мікробіотів, морфологією кутикули та міжвидовою конкуренцією. Накопичення важких металів залежить також від пори року та погодних умов (Mazhar et al., 2014; Nachev & Sures, 2016).

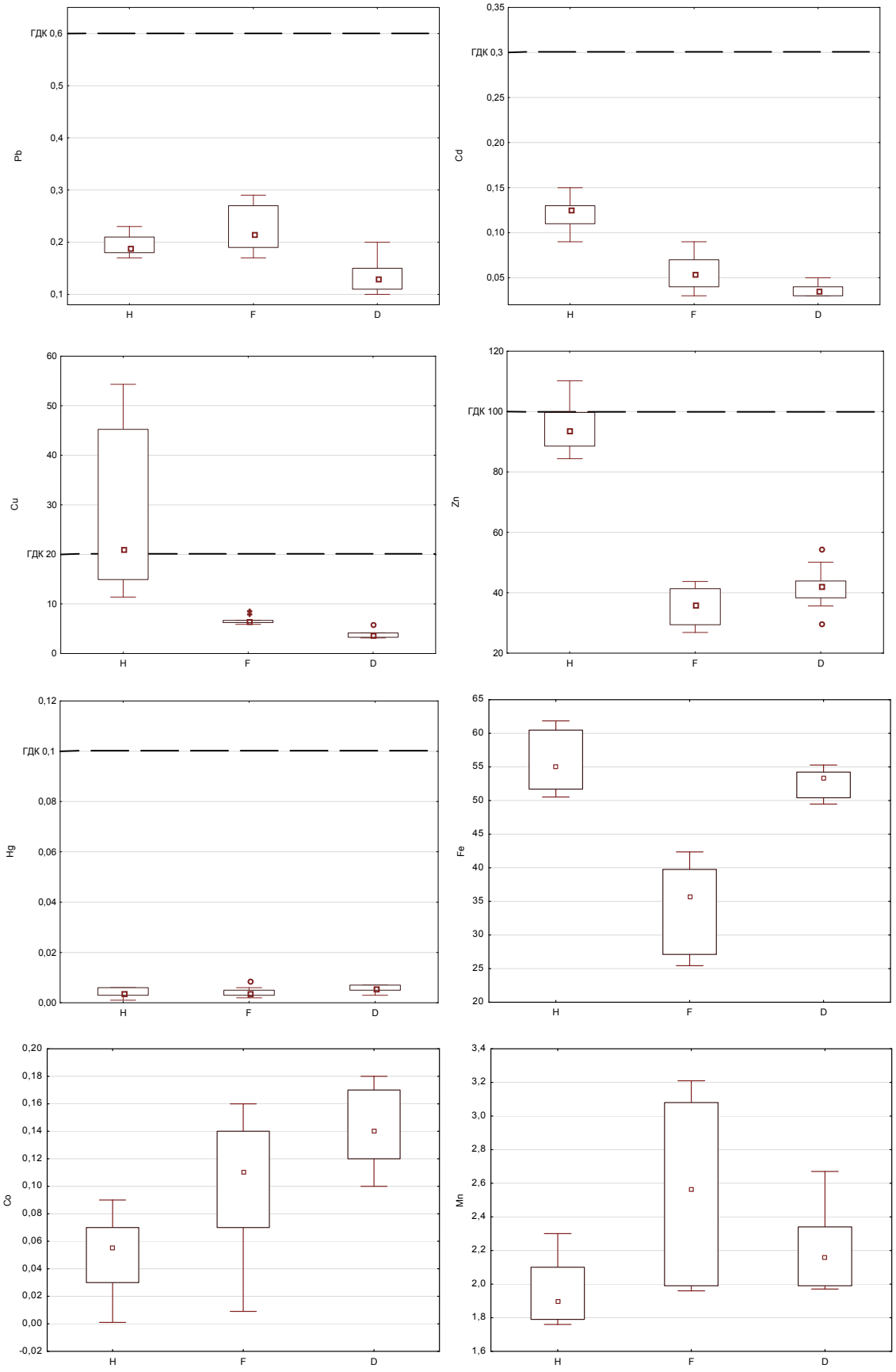

Рис. 1. Вміст (мг/кг) Pb, Cd, Cu, Zn, Hg, Co, Mn і Fe у печінці агельмінтних (H), уражених фасціолами (F) та дикроцеліями тварин (D): ГДК – гранично допустима концентрація

Про біоакумуляцію металів у тканинах гельмінтів повідомляють також Sures et al. (1998), які вивчали накопичення Pb й Cd у *F. hepatica*. За даними Sures et al. (1998), які вивчали коефіцієнт біоакумуляції свинцю та кадмію, встановлено більшу здатність до накопичення саме *F. hepatica*. Вони вказують, що марити трематод накопичували свинець до концентрації, що значно перевищувала таку у м'язах, нирках і печінці спонтанно заражених тварин. Розрахований нами коефіцієнт кореляції між інтенсивністю фасціольозної інвазії та рівнем свинцю становив (r_s – 0,76, P < 0,05), що свідчило про високу позитивну залежність. У той же час, за паразитування дикроцелій, зареєстровано високу обернену кореляційну залежність між II та вмістом Pb (r_s – –0,72, P < 0,05). Різницю між нашими результатами та результатами авторів можна пояснити відносно невеликим розміром вибірки (непараметричні дані).

Разом із тим, Sures et al. (1998) доводять, що серед важких металів, саме вміст кадмію вищий у тканинах великої рогатої худоби, але нижчий у паразита. Аналогічні результати отримано під час наших досліджень. Установлено низьку концентрацію кадмію у печінці хворих на фасціольоз і дикроцеліоз тварин порівняно з контрольною групою (P < 0,0002; P < 0,0001). Інші важкі метали не виявили кореляційної залежності від інтенсивності ураження трематодами.

Висновки

З'ясовано середні показники концентрації деяких хімічних елементів у печінці здорової великої рогатої худоби. Розподіл хімічних елементів за вмістом в агельмінтних тварин можна навести у вигляді спадного ранжувального ряду Zn > Fe > Cu > Mn > Pb > Cd > Co. Доведено, що наявність трематод викликає перерозподіл хімічних елементів у тканинах печінки. За паразитування фасціол у тканині печінки достовірно підвищується рівень кобальту та марганцю, позитивно корелюючи з інтенсивністю інвазії. Це свідчить про відсутність впливу на обмін та біоакумуляцію гельмінтами цих елементів. Присутність фасціол і дикроцелій в організмі великої рогатої худоби достовірно знижує рівень міді та цинку, маючи високу обернену кореляційну залежність від інтенсивності інвазії, вказуючи тим самим на можливість накопичення їх трематодами.

Ми висловлюємо слова вдячності офіційному лікарю ветеринарної медицини у Полтавській області С. М. Канівцю за допомогу у відбиранні зразків для досліджень і завідувачу хіміко-токсикологічного відділу, заступнику директора Регіональної державної лабораторії ветеринарної медицини у Полтавській області, лікарю ветеринарної медицини О. О. Ісаєвій за допомогу у виконанні досліджень.

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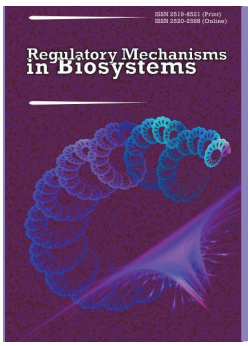

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 23–40
doi: 10.15421/021804

Promising new fixed combination for the treatment of diseases of the hepatobiliar system: Substantiation of pharmacotherapeutic properties and pharmaceutical quality profile

L. B. Bondarenko*, N. O. Gorchakova**, O. I. Golembiovska*, O. Y. Galkin***, ****

**Institute of Pharmacology and Toxicology, National Academy of Medical Sciences of Ukraine, Kyiv, Ukraine*
***O.O. Bogomolets National Medical University, Kyiv, Ukraine*
****UA PRO-PHARMA LLC, Kyiv, Ukraine*
*****National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute", Kyiv, Ukraine*

Article info

Received 15.01.2018
Received in revised form 24.02.2018
Accepted 27.02.2018

Institute of Pharmacology and Toxicology, National Academy of Medical Sciences of Ukraine, Anton Tsedik st., 14, Kyiv, 03057, Ukraine. Tel. +38-044-456-42-56.

O.O. Bogomolets National Medical University, Shevchenko blvd., 13, Kyiv, 01601, Ukraine. Tel. +38-044-234-40-62.

UA PRO-PHARMA LLC, Kozatska st., 120, build. E, Kyiv, 03680, Ukraine. Tel.: +38-044-422-50-70. E-mail: info@pro-pharma.com.ua

National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute", Peremogy av., 37, Kyiv, 03056, Ukraine. Tel.: +38-044-240-90-36. E-mail: a.galkin@kpi.ua

Bondarenko, L. B., Gorchakova, N. O., Golembiovska, O. I., & Galkin, O. Y. (2018). Promising new fixed combination for the treatment of diseases of the hepatobiliar system: Substantiation of pharmacotherapeutic properties and pharmaceutical quality profile. Regulatory Mechanisms in Biosystems, 9(1), 23–40. doi: 10.15421/021804

In this review article an analysis of literature data on the pharmacological and clinical study of a fixed combination of medicinal substances (artichoke leaf extract, ursodeoxycholic acid, taurine, and *Angelica sinensis* roots extract), as well a scientific substantiation of the pharmaceutical quality profile of the corresponding finished solid dosage form has been conducted. Chronic diseases of the hepatobiliary system are some of the most common human diseases and inferior only to atherosclerosis . The fact that cholecystectomy is the most common surgical operation in the abdominal organs is evidenced by the widespread distribution of the pathology of the biliary system. The fact that there is an increasing number of diagnoses of cholelithiasis in children and infants is a cause for concern. Diseases of the biliary system are closely related to violations of the functional state of the liver. Synthesis of cholesterol supplemented bile with reduced bile acid content significantly increases the risk of gallstones, as well as gallbladder cholesterol. We have substantiated that the developed preparation is a fixed combination of medicinal substances with well-researched medical applications in the treatment of dyspeptic disorders with functional disorders of the biliary system, biliary dyskinesia of the hypokinetic type, and gastritis with reflux of bile. Each of the components of the fixed combination has an important influence on the human hepatobiliary system. The effect of ursodeoxycholic acid is due to the relative replacement of lipophilic toxic bile acids, improving the secretory capacity of hepatocytes and immunoregulatory processes, which is especially important in liver and cholestatic diseases. Taurine is a synergist of ursodeoxycholic acid, since it forms biliary conjugates in the liver. The artichoke extract has choleretic, hepatoprotective and diuretic effects, while the *A. sinensis* roots extract demonstrates moderate spasmolytic and anti-inflammatory properties. We conducted a general description of active pharmaceutical ingredients and a review of biopharmaceutical tests, analyzed relevant pharmacokinetic and pharmacodynamic studies, and summarized the results of clinical efficacy and safety trials. Particular attention is paid to the results of clinical studies of the developed fixed combination. It should be noted that artichoke leaf extract, ursodeoxycholic acid, and taurine are widely used throughout the world in official medicine, at the same time, *A. sinensis* roots extract is more widely used in traditional Chinese medicine. The fixed combination has a favorable safety profile, is investigated in clinical conditions *in vivo* both in the form of individual components and in the form of a single drug. A fixed combination pharmaceutical profile is based on the general requirements for solid dosage forms, as well as experimentally substantiated specific indicators and research methods.

Keywords: ursodeoxycholic acid; artichoke; taurine; *Angelica sinensis*; clinical trials; pharmaceutical quality

Introduction

Chronic diseases of the hepatobiliary system are some of the most common human diseases and inferior only to atherosclerosis only. According to the WHO, there are more than 2 billion people in the world who suffer from liver disease, which is 100 times the prevalence of HIV infection. Over the past 20 years, there has been a clear tendency towards global increase in the number of patients with hepatobiliary diseases. There is an increase in the frequency of pathology of the hepatobiliary system in young patients, in women 4–7 times more often than in men. According to WHO experts, every 5th woman and every 10th person in Europe suffers from liver and biliary tract pathologies (Cong et al, 2011; Beuers et al., 2015).

The fact that cholecystectomy is the most common surgical operation in the abdominal organs is evidenced by the widespread distribution of the pathology of the biliary system. The increasing number of diagnoses of cholelithiasis in children and infants is a matter for concern. Diseases of the biliary system are closely related to violations of the functional state of the liver. Synthesis of cholesterol supplemented bile with reduced bile acid content significantly increases the risk of gallstones, as well as gallbladder cholesterol (Özkardeş et al., 2014; Coats et al., 2015; Tsai et al., 2015; Chhabra et al., 2016).

In recent years, there has been a search for the creation of polyfunctional action drugs. This is due to an increase in the number of patients with polymorphic pathology. At present, doctors are approached by patients who have not only one pathology, but several. According to

modern data, the number of patients who present to their doctor with polymorbid pathology is about 80%. The causes of polymorbidity are often the anatomical proximity of the affected organs, the common pathogenesis, and causal relationship. This is especially true of the hepatobiliary system, as well as diseases caused by metabolic disorders such as metabolic syndrome, diabetes mellitus, and atherosclerosis. The central place in the development of these diseases is the liver – the main organ of metabolism. Polymorbidity always involves the use of several media, which leads to polypharmacy, therefore the use of natural safe agents that have a multifunctional normalizing effect on the pathogenesis of the disease and allow reducing the use of drugs and avoiding undesirable effects of polyproagram is advisable (Kim et al., 2014; Chan et al., 2014; Uvarova el al., 2014; Pallayova et al., 2015; Tomeno el al., 2015).

As part of previous work the scientific substantiation of the safety of the pharmaceutical combination of artichoke leaf extract (200 mg), ursodeoxycholic acid (100 mg), taurine (100 mg), and Angelica sinensis roots extract (50 mg) was conducted. The following preparation is designed for the treatment of dyspeptic disorders with functional disorders of the biliary system, biliary dyskinesia of the hypokinetic type, and gastritis with reflux of bile (Anokhina et al., 2014; Zvyagintseva et al., 2014; Gorchakova et al., 2017).

The aim of this work was a scientific substantiation of the pharmacodynamic and pharmacokinetic properties of the fixed combination, as well as the pharmaceutical profile of the quality of the appropriate dosage form.

General characteristics of active pharmaceutical ingredients

The proposed pharmaceutical composition is a fixed combination of drugs with well-studied medical applications.

Ursodeoxycholic acid (UDCA) is one of the native bile acids synthesized during the normal exchange of bile acids in the human body. It is an epimer of chenodesoxycholic acid and is a hydrophilic, non-cytotoxic bile acid. Ursodeoxycholic acid is the least aggressive bile acid – a natural component of human bile, its content is 1–5% of the total amount of bile acids in the human body. Applied as a medicinal product in world medicine for more than 30 years, including in Ukraine – more than 20 years (Blikhar et al., 2012; Wisher, 2012).

From the beginning, UDCA has been recommended for the dissolution of gallstones and for the treatment of reflux gastritis, and is now considered a standard for the treatment of cholestatic liver disease with an autoimmune component, such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis cholangitis (PSC), etc. Currently, in the State Form of Medicines of Ukraine, it refers to the pharmacotherapeutic group of drugs used in diseases of the liver and bile ducts; the ATC code: A05A A02 (Blikhar et al., 2012; Wisher, 2012).

A small amount of UDCA is constantly contained in human bile. After oral administration, it reduces bile cholesterol saturation, slowing down the absorption of cholesterol in the small intestine and reducing cholesterol secretion into bile. Obviously, as a result of the dissipation of cholesterol and the formation of liquid crystals, partial dissolution of cholelithiasis occurs. According to modern concepts, UDCA is believed to be effective in hepatitis and cholestatic diseases due to the relative replacement of lipophilic, toxic detoxification toxic bile acids with hydrophilic cytoprotective non-toxic ursodeoxycholic acid, as well as the improvement of the secretory capacity of hepatocytes and immunoregulation processes (Blikhar et al., 2012; Wisher, 2012). It stabilizes membranes of hepatocytes and cholangiocytes, has a direct cytoprotective effect. By reducing the intake of cholesterol in the intestine and other biochemical effects, it causes hypocholesterolemic effects. It suppresses the process of cell death, caused by the action of toxic bile acids. Due to the high polarity of its molecule, UDCA is capable of producing non-toxic mixed micelles with apolar (toxic) bile acids, which reduces the ability of gastric refluxes to damage cell membranes in biliary reflux gastritis and reflux esophagitis. In addition, UDCA forms dual molecules that can be incorporated into cell membranes, stabilize them and make them insensitive to the action of cytotoxic micelles. It reduces the saturation of bile with cholesterol by inhibiting its absorption in the intestine, inhibiting the synthesis of the liver and reducing the secretion of bile; increases the solubility of cholesterol in bile, forming liquid crystals with it; reduces the lithogenic index of bile. The result is the dissolution of cholesterol gallstones (as a result of changes in the ratio of cholesterol/bile acids in bile) and preventing the formation of new concretions (as a result of a decrease in the content of bile cholesterol). In addition, ursodeoxycholic acid induces cholera, enriched with bicarbonates, which leads to an increase in the bile passages and stimulates the excretion of toxic bile acids through the intestine (Blikhar et al., 2012; Wisher, 2012).

The immune modulating effect of UDCA is due to inhibition of expression of HLA-antigens on hepatocyte and cholangiocyte membranes, normalization of the natural killer activity of lymphocytes, etc. UDCA is capable of delaying the progression of fibrosis in patients with primary biliary cirrhosis, cystic fibrosis and alcoholic steatohepatitis, reducing the risk of developing varicose veins in the esophagus (Borum et al., 1990; Cheng et al., 2012; Kotb, 2012; Buko et al., 2014).

The second component of the drug is taurine – sulfonic acid, which is formed in the body from cysteine amino acid (Blikhar et al., 2012; Wisher, 2012). Taurine is normally present in small amounts in the tissues and bile of humans and animals (Tiedemann et al., 1827; van Stijn et al., 2012).

It is synthesized in the body by enzymatic oxidation of the sulfhydryl group of cysteine with the participation of cysteine deoxygenase to cysteine sulfinic acid:

$$HSCH_2CH(NH_2)COOH \rightarrow HO_2SCH_2CH(NH_2)COOH,$$

followed by decarboxylation of cysteine sulfic acid in hypotaurine:

$$HO_2SCH_2CH(NH_2)COOH \rightarrow HO_2SCH_2CH_2NH_2,$$

and oxidation of hypotaurine in taurine (Sumizu, 1962):

$$HO_2SCH_2CH_2NH_2 \rightarrow HO_3SCH_2CH_2NH_2.$$

Taurine forms conjugates with bile acids (acylating by bile acids on the amino group), and the formed conjugates (for example, taurochole and taurodeoxycholic acids) are part of bile, and, as surfactants, contribute to the emulsion of fats in the intestine. It participates in lipid metabolism, improves energy and metabolism, stimulates healing with dystrophic diseases and processes accompanied by significant metabolic disorders of tissues (Blikhar et al., 2012; Wisher, 2012; van Stijn et al., 2012). Under conditions of systemic exposure, taurine also has hepatoprotective, cardiotonic and antihypertensive properties. It is used in cardiovascular failure, cardiac glycoside poisoning and in type 1 and type 2 diabetes mellitus (Loseva et al., 2010; Gebara et al., 2015; Gu et al., 2015; Sagara et al., 2015; Toyoda et al., 2015). Taurine is often introduced into complex medicinal products. Pharmacotherapeutic group: amino acids; the ATC code: S01X A 21. Like ursodeoxycholic acid, it has been used as a medicinal product in world medicine for more than 30 years (Blikhar et al., 2012; Wisher, 2012).

In the composition of the drug as the third pharmacologically active substance is artichoke leaves extract (*Cynara cardunculus* var. *scolymus*). A herbal remedy, a pharmacotherapeutic group: agents used in diseases of the liver and bile ducts; the ATC code: A 05A X 10. Detects choleretic, diuretic, hepatoprotective and hypolipidemic effects. Increases bile excretion, promotes excretion of nitrogen-containing substances (urea, creatinine) and toxins from the body, reduces lipid and total cholesterol in the blood, and also reduces the feeling of overflow of the stomach, relieves spasm. Pharmacological effect is caused by a complex of biologically active substances of the preparation: cynarine, chlorogenic acid, ascorbic acid, carotene, B-group vitamins, and inulin. The main active ingredient – cynarine – has a choleretic effect. The hole-kinetic effect is less. Available in artichokes ascorbic acid, carotene, vitamins B_1 and B_2, and inulin contribute to the normalization of metabolic processes. Indications for the use of an atrix extract are dyspeptic phenomena; biliary outflow, gallbladder hypokinesia, chronic hepatitis, chronic intoxication, chronic renal insufficiency, urolithiasis, urate, atherosclerosis, obesity (as part of complex therapy) (Kraft, 1997; Guarrera et al., 2005; Mootoosamy et al., 2014; Giacosa et al., 2015). Like UDCA and taurine, artichoke extract has been used as a medicinal product in world medicine for more than 30 years (Blikhar et al., 2012; Wisher, 2012). In the composition of the drug as the fourth pharmacologically active substance is *Angelica sinensis* extract (commonly

known as Danggui). A herbal remedy, a pharmacotherapeutic group: other agents applied with functional disorders from the side of the gastrointestinal tract; the ATC code: A 03 A X 20.

Angelica sinensis (Oliv.) Diels is a traditional medicinal and edible plant that has long been used for tonifying, replenishing, and invigorating blood as well as relieving pain, lubricating the intestines, and treating female irregular menstruation and amenorrhea. *A. sinensis* has also been used as a health product and become increasingly popular in China, Japan, and Korea (Wei et al., 2016). The yellowish brown root of the plant is harvested in fall and is a well-known Chinese medicine used over thousands of years.

Review of biopharmaceutical trials

UDCA is a native component of the normal metabolism of bile acids in the body, whose physico-chemical properties and localization of the implementation of the physiological action predetermine the oral administration of this compound and the optimality of the capsules or tablets dosage form. Studies have shown that, in general, the degree of biliary enrichment of UDCA does not depend on the dosage form or the number of doses administered per day, but rather on the total daily dose (Blikhar et al., 2012; Wisher, 2012).

However, research on new UDCA dosage forms is ongoing. A preparation based on UDCA was developed, which is coated with a newly developed capsule film that dissolves in the stomach and releases the active substance only at pH ≥ 6.5 (Simoni et al., 1995). In 12 healthy volunteers, UDCA serum levels were measured after a single oral dose of 450 mg of UDCA in three different dosage forms: enteric-clearing capsules, solid, non-drip gelatin capsules and conventional gelatin capsules. The drug was administered after eating. The area under the curve [AUC, mmol/l (8 hours)] after oral administration of the enteric-derived UDCA capsule (39.0 ± 8.5) was significantly higher than that for conventional capsules (30.5 ± 4.9), both solid and non-drowning capsules (29.3 ± 3.4). In addition, the maximum serum UDCA concentration was significantly higher in the case of enteric coating capsules compared to the other two formulations, while achieving maximum UDCA concentrations in serum was observed later. These results may be explained by the assumption that the new capsule releases its contents into the intestine at the stage of the maximum alkaline pH in it, caused by the secretion of bile and pancreatic secretions. This improves the solubility of UDCA at alkaline pH, thus giving it a higher concentration gradient that facilitates passive absorption.

Physico-chemical properties of UDCA have determined the specificity of biopharmaceutical studies of its dosage forms for the needs of pediatrics (Santoveña et al., 2014). Due to the very low solubility and low dose in the formula (1.5%) UDCA for the administration of appropriate doses for infants and children was converted into a suspension with minimal use of excipients, avoiding the use of both complex supplements and those not recommended by the European Agency for the Medicines (EMA). It was obtained 2 stable (within 30–60 days) of a composition with a certain particle size.

Regarding the quantitative content of ursodeoxycholic acid in capsules, a number of clinical studies were conducted on dosage regimens. Clinical studies have shown that the administration of UDCA in the range of 10 to 15 mg/kg/day, improves liver function in chronic liver disease. With PBC, PSC, and chronic hepatitis (CH), ursodeoxycholic acid was used at doses of 250, 500 and 750 mg/day for two months, with the treatment regimen assigned to each patient. Significant decrease in serum levels of marker enzymes was observed in all groups at doses of 250 mg/day, which corresponds to approximately 4–5 mg/kg/day. Two higher doses induced further improvement in the studied parameters, especially in patients with PBC, but no significant differences were found between dose levels of 500 and 750 mg/day (Podda et al., 1989).

In a short-term, randomized, double-blind, controlled, cross-study, different UDCA doses regimens (for total dose 600 mg/day) were studied: a course of 3 months, followed by placebo for 3 months or placebo for 3 months followed by UDCA course for 3 months and long-term UDCA courses up to 20 months (Hwang et al., 1993). Dosage regimens of ursodeoxycholic acid up to 8.7 mg/kg/day at PBC have been studied in double-blind, multicenter trials (Battezzati et al., 1993). A comparison of three doses of ursodeoxycholic acid in the treatment of PBC was performed in another randomized study (Angulo et al., 1999). A total of 155 patients were randomized to receive low (5–7 mg/kg/day), normal (13–15 mg/kg/day) and high (23–25 mg/kg/day) UDCA doses. In this case, there were no statistically significant differences between the effects of different doses. Higher doses of UDCA (25–30 mg/kg/day) have been studied in clinical trials for patients with PSC (Harnois et al., 2001). It has been shown that the use of UDCA at a dose of 25–30 mg/kg/day may be beneficial for patients with PSC and this mode deserves further evaluation in long-term, randomized, placebo-controlled studies. A randomized, double-blind, multi-dose (300, 600, 900 mg/day) UDCA efficacy study for bile acid metabolism regimen over a period of 21 days showed a higher average UDCA dose efficiency (Hess et al., 2004). A multicentre double-blind, randomized controlled trial of UDCA prophylactic use (15 mg/kg/day) in patients after liver transplantation did not confirm the optimism of the initial report of the beneficial effect of UDCA prophylactic treatment in acute rejection after liver transplantation (Keiding et al., 1997).

Taurine is also a native component of the human body, readily soluble in water and permeable through bivarials. Since taurine implements its hepatoprotective effect together with UDCA, the choice of dosage forms in this case is limited by its physical and chemical properties. Regarding the quantitative content of taurine in capsules, a number of clinical studies were conducted on dosing regimens (Colombo et al., 1996; Heubi et al., 2002; Vettorazzi et al., 2017). In particular, taurine at a dose (30 mg/kg/day) was administered together with UDCA (15 mg/kg/day) during the year, resulting in an increase in its pharmacological effect in the treatment of liver disease in patients with severe pancreatic dysfunction and in poor condition nutrition (Colombo et al., 1996). Tauroursodeoxycholic acid (TUDCA) at doses of 10–13 mg/kg/day administered for 3 months induced hepatocyte proliferation in humans (Vettorazzi et al., 2017). The high dose regimens of TUDCA (30 mg/kg/day) during enteral administration were also studied for the pediatric population (Heubi et al., 2002). However, the complex of taurine from UDCA was ineffective in preventing the development or treatment of cholestasis in newborns.

The third component of the drug is an artichoke extract. Given the herbal origin of this component and its complex chemical content, it is clear that the methods of obtaining dosage forms can play a fundamental role in the realization of its pharmacological action (Betancor-Fernández et al., 2003; Lupattelli et al., 2004; Ferracane et al., 2008). In particular, the study of the effect of different methods of processing artichoke herbal material significantly affects its antioxidant profile, antioxidant activity and physical characteristics (Lupattelli et al., 2004; Ferracane et al., 2008). Screening of artichoke leafy pharmaceutical preparations showed that antioxidant activity was higher in aqueous fractions than lipophilic fractions, and correlated with the total content of phenols in extracts (Betancor-Fernández et al., 2003). Regarding the quantitative content of artichoke extract in capsules, a number of clinical studies were conducted on dosage regimens (Sannia, 2010; Barrat et al., 2013). Thus, in the treatment of functional dyspeptic symptoms with oral administration of artichoke extract (15% for chlorogenic acid, 150 mg extract per capsule) for 60 days, the positive clinical effect, which was defined as reducing the total score of all symptoms by 50%, was recorded in 38% of patients during 30 days, and 79% – within 60 days. After 60 days, in patients, the level of total cholesterol, low density lipoprotein and serum triglyceride decreased by 6–8% compared with baseline values ($P < 0.001$), and the activity of transaminases and gamma-glutamyltransferase was 13–20 units per liter ($P < 0.01$) in comparison with higher initial values (Sannia, 2010). A randomized, double-blind, placebo-controlled study of low-density lipoprotein cholesterol effects in large doses of artichoke extract in patients with primary moderate hypercholesterolemia showed that doubling of the daily dose from 3 to 6 tablets for 4 weeks did not lead to additional statistically significant increase in the pharmacological effect (Barrat et al., 2013).

Radix *Angelica sinensis*, the dried root of *A. sinensis* (Danggui), is a herb used in Chinese medicine to enrich blood, promote blood circula-

tion and modulate the immune system. It is also used to treat chronic constipation of the elderly and debilitated as well as menstrual disorders. Research has demonstrated that Danggui and its active ingredients, as anti-arthrosclerotic, anti-hypertensive, antioxidant anti-inflammatory agents which would limit platelet aggregation, are effective in reducing the size of cerebral infarction and improving neurological deficit scores (Hirata et al., 1997; Wu et al., 2011). Dong quai contains a chemical compound called butylidenephthalide which has antispasmodic activity in vitro and might relieve muscle cramps (Ko, 1980). Since 1970s, 165 chemical constituents, including phthalides, phenylpropanoids, terpenoids and essential oils, aromatic compounds, alkaloids, alkynes, sterols, fatty acids, and polysaccharides have been isolated or detected from the various parts of the title plant (Ma et al., 2015).

Pharmacokinetic studies

The literature does not provide data on the effects of UDCA, taurine and artichoke extract on enzymes 1A2, 2A6, 2C9, 2C19, 2D6, 2E1 of cytochrome P450 systems in human biomaterials. It has been shown that UDCA is an inducer of CYP3A, but the clinical significance of this effect is still unknown (Blikhar et al., 2012; Wisher, 2012). Investigations using quantitative polymerase chain reaction and Western blot methods of reversing the effects of ursodeoxycholic acid on proteins included in the exchange of bile acids and detoxification processes of xenobiotics in the intestine of healthy people and patients with PBC showed that UDCA induced in the gut the expression of genes of the key protein intestine pumps, as well as breast cancer resistance proteins (BCRP) and P-glycoprotein (multidrug resistance protein 1) (Dilger et al., 2012). There was no direct reverse effect on the enzymes involved in the exchange of bile acids (Wang et al., 2009). There is also data on the influence of taurine on the biosynthesis of a number of transport proteins, which are included in the cytochrome P450 2E1-dependent catabolism of xenobiotics (Miyazaki et al., 2014).

Absorption. UDCA, as a rule, is present in the form of a small fraction of the total amount of bile acids in the human body (about 5%). After oral administration, most part of UDCA is absorbed in the small intestine and to a small extent in the colon by passive, non-ionogenic diffusion and absorption is incomplete. Subsequently, 50% of UDCA from the portal blood when it is first passed through the liver is absorbed to conjugate with amino acids. Following a single oral administration of UDCA in a dose of 500 mg to healthy volunteers, peak plasma concentrations were from 2.7 to 6.3 μg/ml. The maximum concentration was reached after 60 min, and the second peak concentration in plasma – after 180 min. Following a single oral administration of UDCA at doses of 250, 500, 1000, and 2000 mg, the corresponding absorption rates were 60.3%, 47.7%, 30.7%, and 20.7%. Binding to proteins in plasma is 96–98% (Blikhar et al., 2012; Wisher, 2012).

Taurine, a sulfur-free amino acid, is a normal component of the human diet. Until recently, the pharmacokinetics of taurine in humans after oral administration had not been sufficiently studied. The study of pharmacokinetic parameters of taurine (given oral administration in the morning at 4 g) was performed on 8 healthy male volunteers (mean age 27.5, range 22–45 years). Blood samples were taken at certain intervals and the concentration of taurine in plasma was measured using a modified high-performance liquid chromatography method. Oral taurine was absorbed from the gastrointestinal tract for 1.0–2.5 hours after administration. The maximum concentration of taurine in plasma (C_{max}) was 0.69 ± 0.15 mmol in 1.5 ± 0.6 hours after administration (T_{max}).

The plasma half-life varied from 0.7 to 1.4 h (an average of 1.0 ± 0.3), the volume of distribution – from 19.8 to 40.7 liters (an average of 30.0 ± 7.6), the ratio of clearance/bioavailability – from 14.0 to 34.4 l/h (an average of 21.1 ± 7.8), and the area under the curve for the interval 0–8 hours (AUC) is 116.0 to 284.5 mg/l (an average of 206.3 ± 63.9) (Ghandforoush-Sattari et al., 2010). In another report, pharmacokinetic parameters were investigated in the presence of intravenous injection of 200 mg of taurine in 6 hypertensive patients and six healthy volunteers. In this study, the half-life and the distribution of taurine were 3.85 ± 0.05 min and 9.6 ± 3.2 l, respectively. However, they monitored the concentration of taurine in blood plasma for only 20 min, and therefore probably considered the alpha phase, which is masked by the absorption phase of taurine in the oral route of administration (De Luca et al., 2015).

Artichoke leaf extracts are traditionally used in the treatment of dyspeptic disorders and liver diseases. In order to obtain more detailed information on the absorption, metabolism and utilization of artichoke leaf extracts, two different extracts were administered to 14 healthy volunteers (Wittemer et al., 2005). Each participant in the study received doses of both extracts. The first extract – the content of caffeoylquinic acids is equivalent to 107.0 mg of carbohydrate and the content of luteolin glycosides is equivalent to 14.4 mg of luteolin. The second extract – the content of caffeoylquinic acids is equivalent to 153.8 mg of coffee acid and the content of luteolin glycosides is equivalent to 35.2 mg of luteolin. Urine and plasma tests were performed using a validated method of high-performance liquid chromatography. Peak concentrations in the blood plasma of carbohydrate, ferulic acid and isofuric acid were achieved within 1 hour. In contrast, the maximum concentrations of dihydrofuranic acid and dihydroisopropylacetate were observed only after 6–7 hours, indicating two different metabolic pathways for different caffeoylquinic acids. The peak concentration of luteolin glycosides in plasma was rapidly reached within 0.5 hours.

Regarding *A. sinensis* extract, there are some works dedicated directly to its pharmacokinetics in normal and pathological animals (Li et al., 2012; Jin et al., 2017). Several studies also has been conducted to observe both drug-drug interactions and pharmacokinetics (Lo et al., 1995; Abebe et al., 2002). The main pharmacokinetic parameters of *A. sinensis* extract were studied and summarized by Chinese authors (Table 1) (Jin et al., 2017). A validated ultra-performance liquid chromatography-triple quadruple mass spectrometry (UPLC-TQ/MS) method was employed to quantify the a content of the main constitutes in Gui-Hong extracts (ancient and classic formula comprised of *A. sinensis* and *Carthamus tinctorius* L.). The results showed that Gui-Hong extracts (0.405 g/ml) contained 144.075 μg/ml of hydroxysafflor yellow A (HSYA), 3.758 μg/ml of caffeic acid, 4.350 μg/ml of p-coumaric acid, 33.844 μg/ml of kaempferol-3-*O*-rutinoside, 31.647 μg/ml of ferulic acid, 1.583 μg/ml of 3-n-butylphthalide, and 3.175 μg/ml of ligustilide.

Table 1
Pharmacokinetic parameters of seven components of Gui-Hong extracts (n = 6) (by Jin et al., 2017).

Components	C_{max} (ng/mL)	T_{max} (h)	$T_{1/2z}$ (h)	MRT_{0-t} (h)	AUC_{0-t} (ng×mL^{-1}×h)	$AUC_{0-\infty}$ (ng×mL^{-1}×h)
Hydroxysafflor yellow A	25 ± 9	1.80 ± 0.40	2.60 ± 0.30	3.65 ± 0.27	116 ± 31	127 ± 32
Caffeic acid	20 ± 5	0.75 ± 0.00	2.70 ± 1.20	2.21 ± 0.21	41 ± 3	53 ± 11
p-Coumaric acid	750 ± 29	0.17 ± 0.00	1.04 ± 0.37	0.76 ± 0.11	430 ± 34	434 ± 35
Kaempferol-3-*O*-rutinoside	17 ± 3	0.75 ± 0.00	1.00 ± 0.53	1.73 ± 0.21	31 ± 8	32 ± 8
Ferulic acid	341 ± 34	0.17 ± 0.00	2.38 ± 1.10	1.43 ± 0.19	273 ± 65	312 ± 77
3-*n*-Butylphthalide	21 ± 11	0.17 ± 0.00	7.60 ± 1.73	2.55 ± 0.08	48 ± 9	109 ± 25
Ligustilide	136 ± 25	0.08 ± 0.00	6.33 ± 1.45	5.69 ± 0.76	432 ± 71	463 ± 79

Pharmacokinetic parameters of HSYA of blood stasis rats exhibited higher C_{max}, $T_{1/2z}$, AUC_{0-t}, MRT_{0-t}, and $AUC_{0-\infty}$, and lower T_{max}. Among these data, T_{max}, AUC_{0-t}, $AUC_{0-\infty}$, and $T_{1/2z}$ had a significant difference. As previously work indicated that HSYA was mainly absorbed through the small intestine, while poor blood circulation might prolong the retention time of HSYA in the small intestine, thus eventually led to an increased bioavailability of HSYA. Additionally, slowed blood circulation may decrease the liver perfusion, which may lead to decreased hydroxylation, methylation, acetylation, and glucuronidation of the HSYA in the liver. This decreased drug metabolism may significantly increase the bioavailability of HSYA in blood stasis rats (Tian et al., 2010; Jin et al., 2016; Jin et al., 2017).

For caffeic acid, the plasma samples from the blood stasis rats showed higher C_{max}, $T_{1/2z}$, AUC_{0-t}, MRT_{0-t}, and $AUC_{0-\infty}$. T_{max} of caffeic acid in normal and blood stasis rats was same. Meanwhile, the C_{max}, AUC_{0-t}, MRT_{0-t}, and $AUC_{0-\infty}$ of caffeic acid had significant difference. Catechol-*O*-methyltransferase (COMT) mediated *O*-methylation plays an important role in the metabolism of caffeic acid in rat hepatocytes. Blood stasis syndromes induced by adrenaline hydrochloride or occlusion of the left anterior descending coronary artery have shown decreased COMT activity in liver plasma and the heart. The increased bioavailability of caffeic acid may partially contribute to the decreased liver and blood metabolism (Grohmann, 1987; Azuma et al., 2000; Lafay et al., 2006; Jin et al., 2017).

For *p*-coumaric acid, the model samples revealed higher C_{max}, $T_{1/2z}$, AUC_{0-t}, MRT_{0-t}, and $AUC_{0-\infty}$. And the C_{max}, AUC_{0-t}, MRT_{0-t}, and $AUC_{0-\infty}$ of *p*-coumaric acid had a significant difference (Jin et al., 2017). Another work has demonstrated that the pharmacokinetic behavior of *p*-coumaric acid may be altered after compatibility in rats (Zeng et al., 2016). While in this study, the result indicated that the absorption of p-coumaric acid could be increased and the process of elimination was slowed in the pathological state.

As for kaempferol-3-*O*-rutinoside, the model samples showed higher C_{max}, $T_{1/2z}$, AUC_{0-t}, MRT_{0-t}, and $AUC_{0-\infty}$ compared with normal rats, and the $T_{1/2z}$, AUC_{0-t}, MRT_{0-t}, and $AUC_{0-\infty}$ of kaempferol-3-*O*-rutinoside had significant difference. Kaempferol-3-*O*-rutinoside is a kind of flavonoid glycoside. A series of reasons such as high polarity, large molecules, etc., will result in the low bioavailability of kaempferol-3-*O*-rutinosideafter oral administration (Jin et al., 2017).

For ferulic acid with similar chemical structure of p-coumaric acid, the model rats implied higher C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$, and lower $T_{1/2z}$ and MRT_{0-t}. The C_{max} of ferulic acid had a significant difference. Additionally, T_{max} of ferulic acid in these two groups was the same, which implied that ferulic acid absorbed more rapidly in blood stasis rats (Jin et al., 2017).

When looking at 3-*n*-butylphthalide, the model rats showed lower $T_{1/2z}$ and MRT_{0-t}, and higher C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$. Moreover, the $T_{1/2z}$ and AUC_{0-t} of 3-*n*-butylphthalide had a significant difference. Since 3-*n*-butylphthalide was mainly metabolized by CYP2E1, 2C11 and 3A1/2 in rats, which referred that model rats could change the eliminated time of 3-*n*-butylphthalide may be due to the changes in the enzymes (Jin et al., 2017).

Finally, for ligustilide, the model rats exhibited higher C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$, and lower MRT_{0-t} and $T_{1/2z}$. The result implied that blood stasis model rats could speed up the time and extent of absorption of ligustilide; however, there was no significant difference between normal and model rats (Jin et al., 2017).

There are also data on pharmacokinetics in animal models, for substances of known therapeutic activities (Dymowski, 2013). In a pharmacokinetic study (Luo et al., 2003) ferulic acid and paeoniflorin were detected in the serum of mice after intra-gastric administration of a combination Angelic-Paeonia root powder. The concentrations of both substances at different times were determined in serum, using HPLC. The pharmacokinetic parameters of ferulic acid in the experiment were: T_{peak} = 2.606 ± 0.586 h, C_{max} = 6.372 ±1.510 mg/l, $t_{1/2(ka)}$ = 1.249 ± 0.365 h, $t_{1/2(ke)}$ = 2.101 ± 0.665 h, AUC = 41.399 ± 11.763 mg × h/l, K_e = 0.330 ± 0.085 h^{-1}, K_a = 0.555 ± 0.133 h^{-1}.

Other authors (Ru et al., 2007) observed low oral bioavailability of senkyunolide A in rats. The pharmacokinetics of senkyunolide A, of the essential oil of Rhizoma Chuanxiong (*Ligusticum chuanxiong*), which is commonly used for the treatment of cardiovascular diseases, was studied in rats. After intravenous administration, senkyunolide A was extensively distributed (V_d/F: 6.74 ± 0.73 l/kg) and rapidly eliminated from the plasma (CL/F: 7.20 ± 0.48 l/h/kg and $t_{1/2}$: 0.65 ± 0.06 h). Hepatic metabolism was suggested as the major route of senkyunolide A elimination as indicated by the results of an in vitro S9 fraction study. After intraperitoneal administration, senkyunolide A exhibited dose independent pharmacokinetics. The absorption after IP administration was rapid (T_{max}: 0.04 ± 0.01 h), and the bioavailability was 75%. After oral administration, senkyunolide was also absorbed rapidly (T_{max}: 0.21 ± 0.08 h) however its oral bioavailability was low, about 8%. Moreover as contributing factors were determined the instability in the gastrointestinal tract (accounting for 67% of the loss) and a hepatic first-pass metabolism (accounting for another 25%). Pharmacokinetics of senkyunolide A were unaltered when the extract was administered, which suggests that components in the extract have insignificant effects on senkyunolide A pharmacokinetics.

Distribution. In healthy people, at least unconjugated, 70% UDCA binds to plasma proteins (Rost et al., 2004). UDCA binds to one site with a protein molecule responsible for biliary bile acid binding in the ileum (the ileal bile acid-binding protein, IBABP) and increases the affinity of binding other human bile acids on the second site of IBABP. Since UDCA is one of the binding sites on IBABP, this reduces the the effect of co-operative binding, which is often observed for the major bile acids in humans. In addition, IBABP is essential for the full activation of farnesoid-X alpha receptor (FXRα) bile acids, including ursodeoxycholic acid. There is no information on binding of conjugated UDCAs to plasma proteins in healthy subjects or patients with primary biliary cirrhosis. However, since the effectiveness of UDCA is due to its concentration in bile, but not in plasma, its serum levels are not an indicator of bioavailability in clinical conditions. The volume of distribution of UDCA has not been determined, however, it is believed to be small, since the compound is mainly distributed in the bile and small intestine. In bile, the concentration of UDCA reaches a peak in 1–3 hours (Fang et al., 2012).

Taurine can both come from outside to the body, and synthesize in the human body itself. The administered dose of taurine is rapidly absorbed and distributed over the tissues of the body. Most taurine is noted in the brain, the retina, the heart muscle, the liver and kidneys. Taurine passes through the hemathenecalfal and placental barrier (van Stijn et al., 2012).

The pharmacological activity of the artichoke extractas well as *Angelica sinensis* extract is due to the total effect of its components, therefore detailed pharmacokinetic studies of its distribution in human tissues have not been conducted (Blikhar et al., 2012; Wisher, 2012).

Metabolism. Approximately 50–70% of UDCA from portal blood when it is first passed through the liver is absorbed to conjugate with amino acids (Blikhar et al., 2012; Wisher, 2012). UDCA is conjugated to glycine and taurine, and then excreted to bile and into the small intestine. In the intestine, some UDCA conjugates may be subjected to reverse processes of deconjugation and reabsorption of the ileum. UDCA conjugates may also be dehydroxyl to lithocholic acid, part of which is absorbed, sulfated in the liver and excreted from the body by the bile ducts.

In an adult, about one-quarter of bile acids are conjugated to taurine and a small portion of taurine is also converted to isotonate with the participation of either bacterial or tissue enzymes. Subsequent metabolism proceeds to the formation of sulfate, CO_2, water and ammonia, the latter being converted into urea (Sturman et al., 1975).

The artichoke extract components are completely metabolized in the human body to methylated derivatives of carbohydrate (ferulous and iso-fructose acids) and to products of their hydration (dihydric and dihydrofuranic acids). With the exception of dihydrofurylic acid, all these compounds are present in the human body in the form of sulfates or glucuronides. Luteolin is completely metabolized in the human body to sulfate or glucuronide (Wittemer et al., 2005).

There are several reports on metabolism of the main bioactive ingredient of *A. sinensis* extract. Ligustilide is one of the most abundant bioactive ingredients in this plant. The study (Yan et al., 2007) reported, for the first time, the pharmacokinetics of ligustilide, administered in its pure form and in an herbal extract, in rats. After i.v. administration of pure ligustilide, it was distributed extensively (V_d, 3.76 ± 1.23 l/kg) and eliminated rapidly ($t_{1/2}$, 0.31 ± 0.12 h). The i.v. clearance (CL) of ligustilide after extract administration was significantly higher than that dosed in its pure form [CL, 20.35 ± 3.05 versus 9.14 ± 1.27 l/h/kg, $P < 0.01$; area under the curve (AUC), 0.79 ± 0.10 versus 1.81 ± 0.24 mg × h/l, $P < 0.01$], suggesting significant interaction between ligustilide and components present in the extract. Dose-dependent pharmacokinetics was observed after i.p. administration, and a significantly higher dose-normalized AUC (1.77 ± 0.23 mg × h/l) at 52 mg/kg was obtained than

that at 26 mg/kg (0.93 ± 0.07 mg × h/l, $P < 0.05$). Oral bioavailability of ligustilide was low (2.6%), which was partly because of extensive first-pass metabolism in the liver. Seven metabolites of ligustilide were identified, and three of them were unequivocally characterized as butylidenephthalide, senkyunolide I, and senkyunolide H. These three compounds also occurred naturally in the herb and were reported to be bioactive.

In the article (Moridani et al., 2001), evidence is presented showing that caffeic acid (CA) form plant extract when oxidized by peroxidase/H_2O_2 or tyrosinase/O_2. Mass spectrometry analyses of the metabolites formed with peroxidase/H_2O_2/glutathione (GSH) revealed that mono- and bi-glutathione conjugates were formed, which formed a bi-glutathione conjugate only when GSH was present. In the absence of GSH, hydroxylated products and *p*-quinones of CA were formed by peroxidase/H_2O_2. NADPH also supported rat liver microsomal-catalyzed CA-glutathione conjugate formation, which was prevented by benzylimidazole, a cytochrome P_{450} inhibitor. Furthermore, the cytotoxicity of CA toward isolated rat hepatocytes was markedly enhanced by hydrogen peroxide or cumene hydroperoxide-supported cytochrome P_{450} and was prevented by benzylimidazole. Cytotoxicity was also markedly enhanced by dicumarol, an NADPH/oxidoreductase inhibitor. These results suggest that dihydroxycinnamic acids were metabolically activated by P_{450} peroxidase activity to form cytotoxic quinoid metabolites. Metabolism of phenolic compounds has been described in detail in several articles (Lafay et al., 2006; Zhao et al., 2010; Pei et al., 2016).

Elimination. UDCA is eliminated primarily via the faeces. In the treatment of urinary excretion, UDCA increases but remains below 1%, except for severe cholestatic liver disease. In healthy volunteers receiving once orally 500 mg of C^{14}-spotted UDCA, 30% to 44% of the dose is excreted in the faeces during the first three days, in the form of unchanged UDCA (2–4%), lithocholic acid (37%), and 7-keto-lithocholic acid (5%). The half-life, determined by the use of the radioactive label, is approximately 3.5 to 5.8 days in the case of oral administration of UDCA due to the effective enterohepatic circulation of UDCA in the body. In patients with severe liver disease, renal excretion becomes one of the main ways of eliminating bile acids (Blikhar et al., 2012; Wisher, 2012).

In the case of one-time oral administration of taurine at a dose of 4 g, its plasma concentration returns to the normal range after 8 h (elimination phase). The process of taurine elimination from plasma is realized in a mechanism with first-order kinetics (Ghandforoush-Sattari et al., 2010). The total content of taurine in the body is regulated by the kidneys. Taurine is one of the major urinary tract amino acids in the human body, since the ability of the kidneys to its re-absorption is low (Jacobsen et al., 1968; Chesney et al., 1985). The daily amount of taurine that is excreted in urine, although it depends on the diet, is usually in the range of 65 to 250 mg (0.5–2.0 mmol).

Elimination of the metabolic products of the artichoke extract occurs mainly through the kidneys, with urine. The elimination profile of most products is bi-phase (Wittemer et al., 2005). Elimination of the metabolic products of the *A. sinensis* extract was reported in works we have already described (Moridani et al., 2001; Yan et al., 2007; Pei et al., 2016; Jin et al., 2017).

Pharmacokinetics in special patient groups. Pharmacokinetics and bioavailability of UDCA are independent of sex. Also, there were no clinically significant inter-racial differences in its pharmacokinetic parameters among individuals of the European and Mongolian races. Relative to the Negroid race, data is insufficient (Blikhar et al., 2012; Wisher, 2012).

Pregnant women. In the case of UDCA, taurine and artichoke extract, there are no valid and well-controlled studies in pregnant women. Since studies of the effect on animal reproductive function are not always predictive of the response in the human body, these compounds should not be used in women who are or may become pregnant. If such preparations are used during pregnancy or if the patient becomes pregnant while taking this drug, she should be aware of the potential risk to the fetus (Blikhar et al., 2012; Wisher, 2012). In the case of taurine, there are separate indications that during pregnancy it is accumulated in the tissues of the mother, and then in the perinatal period, it goes to the fetus through the placenta and the newborn through the mother's milk. It accumulates in the brain of newborns. Low levels of taurine in the maternal body lead to low levels of taurine in the fetus and to slow the growth of offspring (Naismith et al., 1987).

Breastfeeding women. It was not investigated whether orally administered UDCA, taurine and artichoke extract in maternal milk were excreted (Blikhar et al., 2012; Wisher, 2012).

Older patients (geriatrics). In the case of UDCA, taurine and artichoke extract, there are no adequate or well-controlled studies in the geriatric population (Blikhar et al., 2012; Wisher, 2012).

Children (pediatrics). In the case of UDCA, taurine and artichoke extract, there are no adequate and well-controlled studies in the pediatric population. There are only individual studies available on various aspects of the pharmacological action of these compounds in children of all ages and newborns. (Santoveña et al., 2014).

The pharmacokinetic parameters of UDCA in newborns were studied using an alpha magnetic spectrometer to quantify the concentration of sub-therapeutic doses of ^{14}C-labeled UDCA (37, 120 and 370 Bq) in minimal fractions (5–25 ml) of biological liquids. However, the limited statistical sample (data from 3 newborns) and the high variability of data do not allow any definite conclusions (Santoveña et al., 2014). Since absorption in the intestine and the transition of bile acids through the portal vein of the liver are immature in young children, the pharmacokinetic parameters of UDCA established in adults may vary significantly in the pediatric population.

In the case of taurine, this amino acid is conventionally irreplaceable in humans and is now added to many baby nutrition mixtures as a precautionary measure to provide improved nutrition in premature infants and children with cystic fibrosis, as well as a positive effect on the auditory trunk of the brain caused by reactions in premature newborns (Aerts et al., 2002; Raphael et al., 2012).

Pharmacodynamic studies

When taken inside, UDCA is absorbed in the intestine due to passive diffusion, and in the ileum by means of active transport. In the liver, UDCA binds to glycine and taurine. The resulting conjugates are secreted into the bile, the acid is included in the system of hepatic-intestinal circulation. With systematic administration, UDCA becomes the main bile acid in the blood serum – it accounts for about 50% of the total amount of bile acids. There is a dose-dependent increase in its share in the bile acid pool (Chernobrovy, 2013).

The enrichment of bile by UDCA makes bile more hydrophilic and less cytotoxic. UDCA modulates apical secretion in hepatocytes – phosphorylation/dephosphorylation of transport proteins (activation / inactivation), which improves the excretory function of the liver. Increasing the content of UDCA in bile reduces the degree of cholangiocellular damage, portal inflammation and proliferation of the ducts. UDCA stimulates the secretion of bile acids and other organic anions (glucuronides of bilirubin, glutathione conjugates) and prevents cholestasis caused by hydrophobic bile acids. Patients with PBC and PSC treated with UDCA had less inflammatory response to the bile ducts. At the moment, the existence of three main mechanisms of action of UDCA is assumed: protection of cholangiocytes from cytotoxicity of hydrophobic bile acids; stimulation of hepatobiliary secretion; protection of hepatocytes from apoptosis caused by bile acids.

The mechanisms of UDCA cytoprotection are not fully understood. However, it is known that excessive accumulation of toxic bile acids causes apoptosis of hepatocytes. UDCA suppresses apoptosis by blocking mitochondrial dysfunction. Laboratory studies have shown that UDCA prevents apoptosis caused not only by deoxycholic acid, but also by other damaging factors (ethanol, transforming growth factor, Fas-ligand and okadaic acid). The mechanism of action of UDCA is associated with a decrease in mitochondrial depolarization with subsequent inhibition of the release of cytochrome C and activation of caspases (Lazaridis et al., 2001; Pelletier et al., 2003; Venneman et al., 2006; Guarino et al., 2007; Roma et al., 2011; Portincasa et al., 2012; Guarino et al., 2013; Chernobrovy, 2013). The mechanism of inhibition of apoptosis by UDCA and tauroursodexycholic acid (TUDCA) is

shown in Fig. 1. UDCA suppresses mitochondrial processes, namely: inhibits translocation of Bax, ROS synthesis, cytochrome C secretion, and caspase-3 activation. According to available data, UDCA may interfere with pathway of the death receptor by receptors by inhibiting caspase-3 activation. In addition, TUDCA inhibits apoptosis processes associated with EP stress by regulating intracellular levels of calcium and inhibiting calpain and activating caspase-12. In this case, it is important to note that UDCA interacts with NSAs, which leads to the dissociation of NSR/hsp90 and nuclear translocation of the complex of UDCA/NDS. Once in the nucleus, UDCA modulates the pathway of E2F-1/p53/Bax, thus preventing apoptosis. In addition, UDCA reduces cyclone D1 and Apaf-1 levels, further inhibiting the mitochondrial apoptotic cascade (Amaral et al., 2009).

In pharmacological doses UDCA significantly reduces (by 40–60%) the saturation of bile with cholesterol by inhibiting the absorption of cholesterol in the intestine and suppressing its secretion in bile (Roma et al., 2011). UDCA reduces the toxicity of bile acids, which can damage cell membranes and cause cholestasis. This involves various mechanisms: inhibition of absorption of endogenous hydrophobic bile acids from the small intestine, choleretic effect, which causes dilution of endogenous bile acid salts in the bile ducts and thus protects hepatocytes. The recommended dose of UDCA for the treatment of cholelithiasis is 8–10 mg/kg/day, with larger doses there are no additional benefits. The dissolution rate is about 1 mm reduction in the diameter of a gallstone per month. The diameter of gallstone more than 20 mm negatively affects the speed of its dissolution and the overall result of litholithic therapy. Lack of dynamics or minimal reduction of concrement after 6–12 months of UDCA treatment indicate a poor dissolution prediction. The chance to reduce the diameter of large (more than 20 mm) or multiple gallstones by means of litholithic therapy does not exceed 40–50% after the first year of treatment (Portincasa et al., 2012).

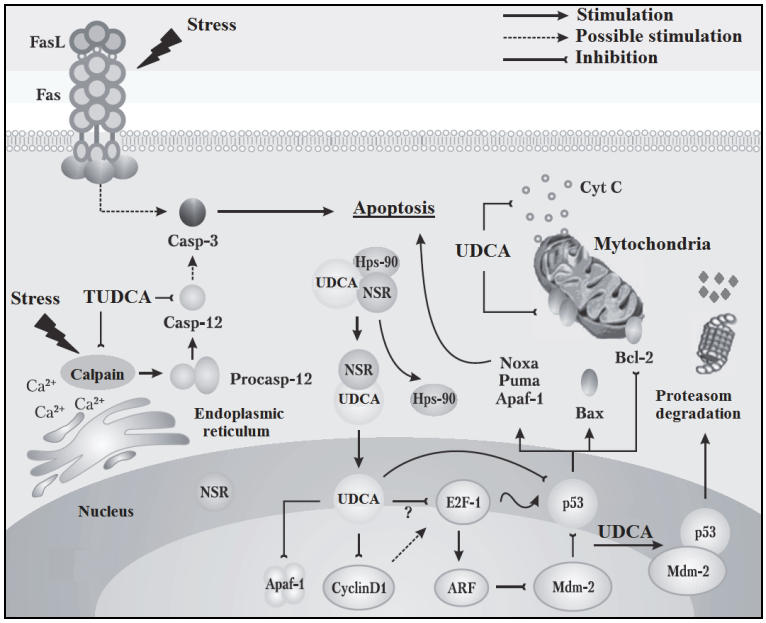

Fig. 1. Mechanisms of UDCA and TUDCA inhibition of apoptosis (by Amaral et al., 2009):
Apaf-1 – apoptotic protease activating factor 1; ARF – ARF (alternative reading frame) cell suppressor; Bax, Bcl-2 – negative (Bcl-2) and positive (Bax) regulators of apoptosis; Casp-12, Casp-3 – caspases; Cyt C – cytochrome C; Fas – Fas cell surface death receptor; Fas L – ligand of Fas-receptor; Hsp-90 – heat shock protein; MDM-2 – mouse double minute 2 homolog; NSR – nuclear steroid receptors; Procasp-12 – procaspase

Gallbladder sludge is considered as another therapeutic target for UDCA. Its appearance can be provoked, for example, by rapid weight loss, pregnancy, parenteral nutrition, organ transplantation. A positive UDCA effect has been demonstrated in a clinical study in which patients with idiopathic acute pancreatitis and concomitant microcholletises or gallbladder sludge were taking UDCA preparations for 3–6 months. This helped to prevent relapses of cholelithiasis and pancreatitis during 44 months of observation in most patients (Lazaridis et al., 2001). UDCA also has anti-inflammatory effects. Prolonged administration of UDCA preparations significantly reduces the incidence of complications of cholelithiasis. It was found that treatment with UDCA in patients with cholelithiasis reduced the incidence of biliary pain and exacerbations of cholecystitis during a 18-year period (Tomida et al., 1999). In the first four years of receiving UDCA, relapse was less than 10%, compared with 40% in the placebo group. Interestingly, this therapeutic effect did not depend on dissolution of gallstones, but was associated with a decrease in inflammatory processes in the body. Other studies (Guarino et al., 2007) indicate that UDCA treatment recovers the contractile function of the gallbladder, improves oxidative-reduction processes, eliminates oxidative stress and inflammation, and thus has a positive effect on biliary symptoms irrespective of lysis of gallstones.

The possible mechanism of reduction of inflammation is considered to be related to the antioxidant properties of UDCA, a decrease in the concentration of prostaglandin E_2 and the activity of catalase. In clinical trials involving patients with severe cholecystitis and frequent biliary colic, there was no significant positive effect on the clinical course of UDCA for 100 days (Venneman et al., 2006).

The above properties of UDCA are also used in the treatment of liver parenchymal lesions. Recent experimental data indicate the expressed hepatoprotective properties of the drug in the case of the most common toxic agent – alcohol. UDCA improves functionally-morphological state of the liver. However, clinical trials of UDCA intake of 13–15 mg/kg/day for 6 months in persons with progressive alcoholic cirrhosis of the liver (Classes B and C for the Child-Pugh classification) did not show improvement in the survival of these patients (Pelletier et al., 2003). Using the modern method of diagnosis (ultrasound elastography of the liver), it was shown that patients who were taking UDCA additionally in the abstinence state had a more pronounced decrease in organ density (Mayev et al., 2010). Clinical trials of UDCA efficacy in ethanolled liver damage have not been conducted sufficiently to determine the site of this drug in the treatment of various forms of alcoholic liver disease. Perhaps this is due to the considerable heterogeneity of the clinical variants of alcoholic liver disease and the lack of generally accepted recommendations for dosage and duration of treatment. The diversity of biochemical and immunological effects of UDCA suggests that its use is possible in any clinical form of alcoholic liver disease.

An important aspect of using UDCA is the treatment of biliary reflux. The damaging effect of bile acids depends on their concentration, conjugation and pH of the environment. Taurine conjugates of bile acids dissolve even at pH 2. Thus, at low absolute values of the pH of

the mucous membrane they only damage the taurine conjugates. On the contrary, at high pH values (for example, in the stomach coke after surgery), unconjugated bile acids have a damaging effect. The bile acids, which have detergent properties, promote the solubilization of lipids in the membranes of the surface epithelium cells. Soluble conjugated bile acids (pH 2–4) penetrate into epithelial cells. Intracellular concentrations of bile acids can be 8 times higher than extracellular. Such excessive accumulation leads to increased permeability of cell membranes, their destruction, damage to intercellular contacts and, ultimately, loss of cells. This detrimental effect depends not only on the concentration of bile acids in reflux, but also on the duration of the period during which the mucous membrane is exposed to bile. Under the influence of pancreatic phospholipase A from biliary lecithin, lysolecithin is formed (catalyzing this reaction bile acids and trypsin). The presence of bile acids and lysolecithin in the mucous membrane of the stomach is accompanied by an increase in the inverse diffusion of hydrogen ions, as well as an increase in the release of histamine and gastrin. The negative effect of duodenogastroesophageal reflux on the mucous membrane of the esophagus has been proved. In this case, the esophagus is damaged by hydrochloric acid and pepsin, and damage to bile acids is pH-dependent. Conjugated bile acids have a negative effect on acidic pH values (2–4), and unconjugated – at pH 5–8. At alkaline pH values, negative effects were inherent not only on unconjugated bile acids, but also on trypsin (Sital et al., 2006).

The hydrophilicity of UDCA and the choleretic effect are important for esophagogastric protection. UDCA does not have a negative effect on the cell membrane – UDCA micelles are practically insoluble in them due to hydrophilicity. UDCA displaces toxic hydrophobic bile acids due to competitive capture of receptors. Important is the induction of cholerexis, when bile-rich bicarbonate contributes to the elimination of toxic bile acids through the intestine. The share of UDCA in gastric contents is increased to 50% with decreasing of the content of gastric and deoxycholic acids, the concentration of chenodeoxycholic acid (CDCA) does not change. In experiments and clinical studies, the cytoprotective properties of UDCA for the protection of the mucous membrane of the stomach and esophagus have been proved. This is due to UDCA in the phospholipid layer of the cell membrane, which contributes to its stabilization and increased resistance to damaging factors. In addition, the prescription of UDCA drugs reduces the subjective findings of gastric dyspepsia (Chernobrovy, 2013).

UDCA prevents the development of tumors by counteracting the stimulatory effect of other bile acids, such as deoxycholic acid (DCA). UDCA and DCA have a multidirectional effect on the epidermal growth factor (EGFR) and the expression of cyclooxygenase-2, which can play a key role in tumorigenesis in the colon. Based on convincing theoretical foundations, some clinical studies have shown that UDCA can reduce the risk of colorectal cancer, but today there are few trials with high-quality design, they are predominantly retrospective. UDCA negatively modulates the mitochondrial activation pathway by inhibiting the translocation of Bax, the formation of active forms of oxygen, the release of cytochrome C and caspase-3 (Huo et al., 2011).

Taurine is a part of the main components of bile. Conjugation is necessary to maintain the solubility of bile acids in the water environment of the intestinal contents. The presence of sulfur in tauroconjugates facilitates their ionization, increasing the detergent action and solubility, as well as reabsorption. The bile acids play an important role in preserving the function of the intestinal barrier and preventing enterobacterial invasion into tissues. In addition, bile acid tauroconjugates have choleretic effects and prevent cholestasis, unlike glycine-conjugated bile acids (Gérard, 2014). *In vitro*, at physiological concentrations, glycolithoholic acid is easily precipitated with calcium, unlike taurolitoholic acid. Thus, taurine is essential for increasing bile turnover, increasing bile acid production and preventing cholestasis. The introduction of taurine is likely to lower cholesterol, triglycerides, low density lipoprotein, and body weight. It reduces the amount of cholesterol in the aorta wall, the number of lipid peroxidation products, while increasing glutathione levels. It has been shown that administration of taurine inhibits cell proliferation by inhibiting the expression of mitogen-activated protein kinase (Sheybak et al., 2005). In addition, taurine manages cellular membranes. It has membrane protection and osmoregulating effects, positively affects the phosphorlipid composition of membranes, and normalizes the electrolyte balance, holding potassium and magnesium inside the cells, and sodium – from the outside. Taurine plays a very important role in the movement of calcium ions through membranes (Sheybak et al., 2005).

In particular, taurine can increase or reduce the level of calcium in the heart. It is precisely with the stabilizing effect of taurine on the membrane that its regulatory effect on the normalization of protein, carbohydrate, electrolyte metabolism, activity of a number of enzymes and hormones, energy and regenerative processes in the body, and the strengthening of the immune system are associated with its regulatory effect. Additional administration of taurine has a beneficial effect on the parameters of antioxidant defense and reduces in the experiment manifestations of diabetic neuropathy, nephropathy and retinopathy.

Taurine gives warning of a decrease in the activity of membrane-bound Na^+/K^+-ATPase and an excessive Ca^{2+} outcome. At the same time, the level of glycosylated hemoglobin and the intensity of lipid peroxidation processes in erythrocytes, which improved glucose utilization, emphasizing the potential therapeutic value of taurine in diabetes (Sheybak et al., 2005).

Another function of taurine is the preservation of euglycemia by increasing the effectiveness of binding of insulin to receptors. Use of taurine in diabetes can normalize the function of platelets and raise the level of amino acids in the blood plasma. The experiment showed that taurine improves the metabolism of glucose and lipids, reducing insulin resistance and hypercholesterolemia. Taurine warns of developing microangiopathy as a result of lowering the degree of apoptosis in endothelial cells (Sheybak et al., 2005).

It has been established that taurine can act as an antioxidant, binding active forms of oxygen. The metabolic precursor of taurine – hypotaurine also has antioxidant properties. Preventive administration of taurine was reported by acute bronchiolitis induced by inhalation of NO_2. Probably, in this case, taurine acts as a stabilizer for cell membranes, regulating the flow of potassium, sodium, calcium and magnesium ions (Marcinkiewicz et al., 2014). In vitro it has been shown that taurine, forming a taurochloramine, binds hypochloric acid, a strong oxidant that causes DNA damage. Taurochloramines may also play a regulatory role in inflammatory processes, inhibiting the production of interleukins 6 and 8, possibly due to a decrease in the activity of gene transcription of cytokines (Marcinkiewicz et al., 2014). The functional activity of macrophages is largely associated with the transport of taurine through a cell membrane. Treatment of macrophages with lipopolysaccharide (0.1 and 10 μg/ml) leads to a 60% decrease in the transport of taurine ($P < 0.01$). Transport of taurine after 24 hours did not differ from the control values in case of simultaneous treatment with lipopolysaccharide and g-interferon (150 units/ml). It was shown that inositol restores the processes of taurine transport in macrophages in conditions of its suppression (Sheybak et al., 2005). Moreover, conjugates of taurine, secondary bile acids, retinoids and some xenobiotics, due to their increased polarity after binding to taurine, become more water-soluble, which increases their clearance. This indicates the potential role of taurine in detoxification processes. The effectiveness of this amino acid in liver cirrhosis, depression and infertility in men is shown (Sheybak et al., 2005). The beneficial effects of taurine on gastric and intestinal mucosa have been described. When cystic fibrosis supplements taurine reduce the severity of steatorrhea. In Alzheimer's disease, memory decline is accompanied by a decrease in the concentration of acetylcholine.

Thus, performing numerous physiological functions in the tissues, taurine successfully modulates them in a variety of pathophysiological conditions, confirming the need for it to be found at high concentrations in the most energetically dependent cells.

The pharmacological properties of the artichoke extract are due to the effect on the patient's body of the entire complex of biologically active substances that are part of the artichoke leaf (*Cynara scolymus* L.). Thus, the phenolic compound of cynarine in combination with phenol acids and bioflavonoids contained in the artichoke provides choleretic, diuretic and hepatoprotective effects of the preparation. The expressive

diuretic effect of artichoke preparations helps to increase the amount of urea that is excreted from the body and eliminate toxic substances, including so-called "middle molecules" with urine. Applying an artichoke extract provides a clear detoxifying effect, in particular, reduces the intensity of the endogenous that is, the so-called metabolic intoxication. Phenolic acids that are part of the artichoke leaf extract (coffee, chlorogenic, neochlorogenic, and caffeine) have high biological activity and cause a pronounced immunoactive action of artichoke preparations (Ben Salem et al., 2017).

Thus, the positive effect of artichoke extract and its combination with vitamin E (tocopherol acetate) on the lipid peroxidation indicators (LIPs) in patients with non-alcoholic steatohepatitis (NASH) is associated with chronic non-calculous cholecystitis (CNCC) against a background of secondary immunodeficiency states (SIDSs). Along with the normalization of the LIPs, namely the decrease in lipoproxidation products in the blood serum, intermediate, ie, diene conjugates (DC) and finely malone dialdehyde (MDA), there was also an improvement in the clinical and biochemical parameters that characterize the functional state of the liver and the decrease in the manifestations of SIDSs, that is, the degree of T-lymphopenia and the imbalance in the subpopulation composition of T-lymphocytes (Babak et al., 2006). In this study, it was found that when administering an artichoke extract together with vitamin E, anti-oxidant and immunomodulatory properties of this combination of drugs are implemented.

Data on the antioxidant activity of the artichoke extract, also obtained in the treatment of patients with NASH, combined with the CNCC against the background of abdominal obesity. This study demonstrated a decrease in the effect of an artichoke extract in the blood serum of products of lipid peroxidation products – MDA and DC in patients with NASH combined with CNCC in the phase of unstable remission or moderate exacerbation of the chronic inflammatory process in the gall bladder against obesity (Frolov et al., 2009). The positive effect of artichoke extract and its combination with the immune active preparation (galavit) on LIPs indices in patients with NASH combined with osteoporosis was also established.

It is known that the activity of the lipid peroxidation processes against the suppression of the activity of antioxidant defense is one of the most universal pathological mechanisms in the formation of chronic pathology of the liver and gall bladder. At the same time, phyto substances, in particular artichokes, are considered as natural antioxidant agents, established in the experiment and confirmed in clinical conditions (Ben Salem et al., 2017). Proceeding from this, it can be assumed that the use of artichoke preparations may be useful in a variety of chronic pathologies of the liver and gall bladder.

The pharmacological properties of the *Angelica sinensis* extract are due to the effect on the patient's body of the entire complex of biologically active substances. At the moment, the following pharmacological properties of this plant are known: effects on cardio- and cerebro-vascular systems, anti-inflammatory effect, antifibrotic action, antispasmodic activity, anti-oxidant activities, neuroprotective action, immune support and hematopoiesis (Fang et al., 2012). Proceeding from the pharmacotherapeutic profile of the complex preparation being developed, let us look in detail on the anti-inflammatory effect and antispasmodic activity.

In 1986, L. Li (Fang et al., 2012) found that sodium ferulate could regulate prostacyclin (PGI2)/thromboxane A2 (TXA2) ratio by inhibitting TXA2 activity without affecting PGI2. Ligustilide (LIG) showed a concentration-dependent anti-inflammatory effect in lipopolysaccharides (LPS) activated microglia, without causing cytotoxicity. Pretreatment with LIG at 2.5, 5, 10, and 20 μmol/l decreased LPS-induced NO production to 75.9%, 54.4%, 43.1%, and 47.6%; TNF-α content to 86.2%, 68.3%, 40.1%, and 39.9%; Interleukin-1β (IL-1β) content to 31.5%, 27.7%, 0.6%, and 0 ($P < 0.01$); and MCP-1 content to 84.4%, 50.3%, 45.1%, and 42.2%, respectively, compared with LPS treatment alone. LIG (10 μmol/l) significantly inhibited LPS stimulated immunoreactivity of activated nuclear factor κB (NF-κB), cyclooxygenase-2 (Prostaglandin-endoperoxide synthase 2), and inducible nitric oxide synthase (iNOS). LIG exerted a potent anti-inflammatory effect on microglia through inhibition of NF-κB pathway. The data provide direct evidence of the neuroprotective effects of LIG and the potential application of LIG for the treatment of the neuroinflammatory diseases characterized by excessive microglial activation (Wang et al, 2010; Fang et al., 2012).

Su et al. (2011), sought to determine the effects of LIG on LPS-induced inflammation in RAW 264.7 macrophages. LIG significantly suppressed the production of nitric oxide, prostaglandin E2 (PGE2), and TNF-α. The inhibition of NO was concomitant with a decrease in the protein and mRNA levels of LPS-induced iNOS. Furthermore, activation of activator protein-1 (AP-1) and NF-κB in the nucleus and the cytosolic degradation of IκBα were abrogated by LIG. LIG also inhibited the phosphorylation of IκB kinase (IKK) and mitogenactivated protein kinases (MAPKs), including p38 MAPK, extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK). The intracellular reactive oxygen species (iROS) level was also significantly decreased. These results suggest that LIG exhibits anti-inflammatory activities by blocking the activation of MAPKs/IKK and the downstream transcription factors AP-1 and NF-κB, which may result from LIG's down-regulation of iROS production (Su et al, 2011; Fang et al., 2012). LIG significantly decreased neurological deficit score, infarct volume, and RTP801 expression, increased EPO transcription in I/R rats, and induced a significant increase in cell viability and EPO and a decrease in LDH and RTP801 in I/R neurons. Also, LIG increased ERK phosphorylation (p-ERK) and the positive effects of LIG on p-ERK as well as cell viability and EPO could significantly be blocked by PD98059, but not LY294002 and SB203580. In addition, transfection of SH-SY5Y cells with RTP801 plasmid DNA induced a significant increase in RTP801 as well as LDH release, while LIG significantly inhibited the effects of transfection on RTP801 expression and also increased cell viability. Therefore, it suggests that LIG has a significant neuroprotecting role against I/R injury by promoting EPO transcription via a ERK signaling pathway and inhibiting RTP801 expression and has the potential to be developed into a therapeutic agent in preventing and treating ischemic disorders (Wu et al, 2011; Fang et al., 2012).

Antispasmodic activity of the *A. sinensis* extract has been studied for several decades. Methods or techniques of cell culture were used to explore the mechanism of *Angelica* polysaccharide (APS) inhibiting proliferation of HaCaT cells (a spontaneously transformed aneuploid immortal keratinocyte cell line from adult human skin) from the angulation of apoptosis. The effect of APS on proliferation of HaCaT cells was examined by trypan blue staining and flow cytometry. The cell growth curve showed that a dose range of 25–2500 mg/l APS had significant inhibition action on HaCaT cells in a dose dependent manner. The flow cytometry result showed a decrease in the S phase and G2/M phase HaCaT cells, while a phemomenal increase in the G0/G1 phase HaCaT cells was observed at 250 mg/l APS. This study suggested that APS could significantly inhibit proliferation of HaCaT cells; by impairing the mechanism of DNA synthesis in preventing HaCaT cells from entering the S phase (Fang et al., 2012).

Other studies had found that intrauterin hypoxia could stimulate proliferation of neural stem cells (NSCs) of neonatal rats. The proliferation reached a peak during 6 h hypoxia; Proliferation also expressed in 9 h, but the ability began to decline. However, the NSCs showed necrosis or apoptosis in a 12 h hypoxia (Chen et al, 2010). To study the effect of intrauterin hypoxia on the proliferation and differentiation of NSCs from neonatal rats and the protective role of angelica injection on NSCs under hypoxia, the authors used immunohistochemistry and an image processing system to analyze the expression of glial fibrillary acidic protein (GFAP) and neuron specific enolase (NSE). The following results were obtained from the study: (1) Expression of GFAP-positive cells in the hippocampus of neonatal rats in the hypoxia group was higher than control group; (2) Expression of NSE-positive cells was less in the hypoxia group than in the control group; (3) Expression of GFAP-positive cells in the hippocampus of neonatal rats was less in the angelica group than in the hypoxia group, whereas expression of NSE-positive cells was higher in the angelica group than in the control group. These results indicated that hypoxia could stimulate the proliferation of NSCs of neonatal rats and differen-

tiation of NSCs into glial cells. Meanwhile, the number of neurons in hippocampus CA3 area was decreased. The ability of proliferation and differentiation of NSCs into glial cells after hypoxia was attenuated by angelica injection, which was also effective in relieving neuron decrement. Therefore, it was suggested that angelica injection has a certain protective effect on the nervous system of neonatal rats with intrauterine hypoxia (Fang et al., 2012). LIG, butylidenephthalide, and butylphthalide were found to have antispasmodic activity against rat uterine contractions and in other smooth muscle systems. The components were characterized as non-specific antispasmodics with a mechanism different from papaverine (Fang et al., 2012).

A recent study was dedicated to the hepatoprotective effect of polysaccharides from different preparations of *A. sinensis* (Hua et al., 2014). The authors report that polysaccharides are important chemical substances of *A. sinensis*. These compounds effectively treat liver diseases, show hepatoprotectivity, and contribute directly to the therapeutic effect of *A. sinensis*. However, the precise molecular mechanism of the effects of the different *A. sinensis* products polysaccharide has not been comprehensively explored. The following investigation was designed to assess the effects and possible mechanisms of polysaccharides in the different *A. sinensis* products against carbon tetrachloride-induced liver injury. Liver injury was induced by intraperitoneal injection with Carbon tetrachloride (CCl_4) in the mice. Gas chromatography-mass spectrometry (GC–MS) combined with pattern recognition approaches, namely, principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), were used to determine differentiating metabolites in plasma and liver tissue. PCA and PLS-DA score plots of the liver injury group clustered separately from that of the control, while groups treated with polysaccharides from charred *A. sinensis* (ASTP), parching *A. sinensis* with soil (ASTUP), parching *A. sinensis* with wine (ASJP), parching *A. sinensis* with Sesame Oil (ASYP) clustered closely with the control. This result indicates that the metabolic profiles of the ASTP, ASTUP, ASJP, and ASYP groups are almost similar to those of the control. Potential metabolite biomarkers (six in the liver homogenates and seven in the plasma) were identified. These biomarkers include citric acid, succinic acid,glycine, palmitelaidic acid, arachidonic acid, fumaric acid, malic acid, valine, ananine, and hexadecanoic acid. Functional pathway analysis revealed that alterations in these metabolites are associated with lipid, amino acid, and energy metabolism. Notably, ASTP exhibited a potential pharmacological effect by regulating multiple perturbed pathways to the normal state. It is likely that ASTP, ASTUP, ASJP, ASYP intervene in the metabolic process of liver injury mice by affecting the lipid and amino acid metabolism. Metabonomics is robust and promising for the identification of biomarkers and elucidation of the mechanisms of a disease, thereby highlighting its importance in drug discovery (Hua et al., 2014).

Efficiency and safety studies

Ursodeoxycholic acid has assumed a leading position in the treatment of liver and bile duct diseases during the last 30 years. The scope of UDCA use (including PBC, PSC, chronic hepatitis with cholestatic component, especially alcoholic and medicinal, cystic fibrosis, biliary tract atresia, posttransplant cholestasis, cholestasis in parenteral nutrition, intrahepatic cholestasis in pregnant women, chronic viral hepatitis – in the absence of antiviral therapy or in combination with it, NASH), has expanded in recent years and now includes the treatment of biliary reflux gastritis and reflux esophagitis, postcholecystectomy syndrome, pores antroduodenal motility ants in various diseases of the upper gastrointestinal tract, atherogenic dyslipidemia. Formal indications are lysis of cholesterol gallstones in the gall bladder and gastritis caused by bile reflux, as well as symptomatic treatment of PBC in the compensation phase. UDCA is often used in pharmacotherapy for other clinical conditions (acute and chronic hepatitis of different etiology, cholestasis) (Blikhar et al., 2012; Wisher, 2012). After the report on the dissolution of gallstones with UDCA, it was used as an alternative to surgical methods for the treatment of cholelithiasis (Roma et al., 2011; Zaretskiy et al., 2011).

The effectiveness and safety of UDCA drugs were studied in particular by Ukrainian clinicians (Zaretskiy et al., 2011). In one study, it was shown that after UDCA prescription at a dose of 15 mg/kg/day in 2–3 doses during the month, complete dissolution of concrements in patients was observed. In all observations, concrements were small – from a fine dispersed suspension to 7–8 mm in diameter and in plural – from 2 to 5.

In another case, a clinical study was conducted on the efficacy and safety of UDCA drugs in terms of their oral administration to 70 patients. Oral litholithic therapy is by far the only truly non-invasive method for treatment of patients with gallstone disease (GSD). The benefits of such therapy include: the absence of pronounced side effects, the lack of lethality, the possibility of outpatient treatment. However, as shown by clinical studies (Zaretskiy et al., 2011), it is possible to count on the successful dissolution of gallstones only with strict selection of patients: the size of concrements should not exceed 15 mm; it should be pure cholesterol, that is, do not give shadows on the X-ray, and should not give an "acoustic path" to the ultrasound; the gall bladder must fully maintain its function, and the bubble ducts must be passable; the gall bladder should be less than half filled with stones; the bile duct should be free from gallstones. Patients were warned that during the period of litholithic therapy they had no right to take clofibrate, estrogens, cholestiramine, antacids, because they contain compounds that bind acid bile. The most important condition for the successful dissolution of gallstones was the regularity of UDCA taking. The selection of patients also proceeded from the fact that the most favorable conditions for oral lithotripsy are in the early stages of the disease, with an uncomplicated course of GSD, rare episodes of bile duct, moderate pain syndrome.

Under supervision there were 70 patients with GSD, including 18 men and 52 women. Ultrasonography of the abdominal cavity was performed using Toshiba-33, Aloka-630 (Japan). Cholelithiasis in the examined patients was an accidental finding, the patients did not know about its existence. Their complaint was gravity in the right hypochondrium. The examination and treatment of patients was conducted from 2002 to 2007. The average age of the men was 56.4 ± 2.9 years, and the women – 48.7 ± 3.4 years. In 20 people the body weight was 60–70 kg, in another 30 patients – 71–80 kg, and in the rest 20 patients – 81–90 kg. Excessive body mass was registered in half of the patients (35 people), among them women predominated. Most of the patients (65 people) used refined food. For all patients the functional state of the liver was evaluated on the basis of the following biochemical parameters: level of bilirubin, total protein and protein fractions, activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), and alkaline phosphatase (ALP). Biochemical studies of blood and ultrasound were performed after 1, 3, 6, 9, and 12 months from the beginning of treatment.

In the biochemical study, some increase in the content of total bilirubin (in 8 patients) was noted, and other indicators were within the normal range. All patients had cholesterol gallstones that were usually round or oval, and were identified on the basis of appropriate criteria (Zaretskiy et al., 2011). The number of concrements in the gall bladder was from 2 to 5. In 30 patients, the gallstones were up to 5 mm ib size, in another 30 – to 6–8 mm, and in the rest 10 – to 10 mm.

UDCA was administered in dosage of 10 mg/kg/day. The course of treatment ranged from 1 month to 1 year. UDCA was taken in capsules without chewing, before bedtime. This form of drug administration ensured its uniform release during the day. Patients were informed of the pharmacological properties of UDCA, the duration of treatment and the expected results. The diet No. 5 (Pevzner's diet) was prescribed for all patients.

One month after the beginning of the treatment, complete dissolution of gallstones was noted in 10 patients with small and single concrements, after 3 months – in 26 patients, after 6 months – in 21 patients, after 9 months – in 4 patients, and after 12 months – in 3 patients. And in 6 patients, who had gallstones of up to 10 mm, their reduction to 2–5 mm was identified. In one patient developed calcinosis of gallstones was stopped with an acoustic path according to the ultrasound, and UDCA treatment. Persons with insoluble stones had an additional 2-month treatment course, after which the concrements completely dissolved.

In general, in the world to date, only one meta-analysis of 23 randomized controlled trials (RCTs) has been conducted in which the efficacy of cholelithiasis treatment with UDCA and CDCA was studied (May et al., 1993). It covered 1949 patients who took medications at recommended doses for more than 6 months. The best result was achieved when UDCA was administered at a dose greater than 7 mg/kg/day. In 37.3% of patients, lysis of concretions was observed.

Although gallstones consist mainly of cholesterol and should in theory dissolve, a large proportion of gastroenterologists still genuinely perceive the results of this meta-analysis and consider the actual level of gallstone dissolution to be 10%. Applicants for UDCA treatment should have cholesterol (non-bilirubin) concrements without signs of measuring less than 20 mm in diameter and free-swallowing choledoch. The majority of clinicians consider UDCA to be the optimal dose for the treatment of cholelithiasis at rates not exceeding 10 mg/kg/day, since further dose increases do not significantly increase the effect. The dissolution rate is about 1 mm reduction in the diameter of a gallstone per month. The diameter of the concrement more than 20 mm negatively affects the speed of its dissolution and the overall result of litholithic therapy. Lack of dynamics or minimal reduction of concrement after 6–12 months of treatment with UDCA suggests a poor dissolution prognosis (Zaretskiy et al., 2011). The chance of reducing the diameter of large (more than 20 mm) or multiple stones with the help of litholithic therapy does not exceed 40–50% after the first year of treatment (Portincasa et al., 2012).

In 2012, a meta-analysis of the hepatoprotective properties of UDCA was performed on the results of 3 RCTs with microscopic evaluation of biopsy specimens. In particular, a reduction in globular inflammation and a level of GGT in serum was observed (Wu et al., 2012).

The most pronounced therapeutic effect of UDCA is observed in patients with PBC and PSC. The performed meta-analyzes of the RCTs showed that the drug administration not only improved the patients' well-being and biochemical parameters, reduced their itching, but also influenced survival. However, a new systematic review did not confirm this (Rudic et al., 2012). The review covered 16 RCTs (1,447 patients) and did not demonstrate significant benefits of UDCA adding to standard circuits at endpoints such as death from the underlying disease, death from all causes, and the need for liver transplantation. The intensity of skin itching and fatigue were also not significantly improved. The possible cause of inconsistency with previous outcomes could be the predominance of patients with severe and terminal stages of the disease when UDCA treatment could not be effective.

A similar situation was observed with the PSC patients. A group of Greek researchers led by S. Triantos in 2011 conducted a meta-analysis of 8 placebo-controlled studies that covered the treatment outcome of 567 patients (Triantos et al., 2011). The study was completed in the last decade. The effect on the course of the disease of receiving high (>15 mg/kg) doses of UDCA was studied. The drug did not contribute to reducing mortality, intensity of itching, general weakness, the risk of cholangiocarcinoma. The histological picture also did not change significantly. These data were confirmed in the following year by the meta-analysis of G. Poropat and co-authors which, despite the probable improvement in biochemical parameters, did not show a reduction in the risk of death and improvement of the histological picture (Chen et al., 2003). UDCA did not reduce the need for transplantation, did not prevent portal hypertension and encephalopathy.

A double-blind, double-simulated, randomized (1 : 1), cross-linked, multi-center clinical trial was conducted in accordance with a two-stage, group-level, adaptive design in eight centers in Germany and one in the Netherlands. Two different UDCA drugs (pills and capsules) were studied in this clinical trial (Hopf et al., 2013). A total of 65 patients were recruited and randomized in the trial. The therapeutic equivalence of UDCA tablets and UDCA capsules with the effect on serum biochemical parameters was equal. Regarding safety, the total number of registered adverse events (AE) was 98. AEs were reported in 43/64 (67.2%) patients. The overall incidence of AEs occurring during treatment with UDCA tablets was very similar to their frequency when treated with UDCA capsules: 28/62 (45.2%) patients and 29/61 (47.5%) patients respectively. Most AEs were digestive disorders (upper abdominal pain, bloating, abdominal distension, nausea, vomiting). No deaths were recorded during the study. However, two serious side effects were reported due to hospitalization, which were assessed as not related to UDCA therapy. One patient had a nasal septum operation and one patient had the aortic valve replaced as a result of stenosis. In general, both drugs were well tolerated, and the safety analysis showed the same UDCA safety profile for UDCA tablets and capsules. Three clinically relevant parameters were chosen in this study to assess therapeutic efficacy (ALT, AST, and GGT). In general, the maximum UDCA effect on liver function parameters was observed after 8 weeks. Clinical efficacy of UDCA capsules and tablets in patients with PBC was demonstrated, and confirmed by the results of a pharmacokinetics study at a daily dose of 15 mg/kg. Both UDCA dosage forms were well tolerated and had similar security profiles. However, almost three times as many patients preferred to take pills (45.3%), compared with only 15.6% of patients who preferred capsules.

According to evidence-based medicine, the hepatoprotective effect of UDCA is currently considered ambiguous, but the use of hard endpoints (death, organ transplant, etc.) may not always be able to demonstrate the potency of hepatotoxic agents. Systematic reviews of almost all authors dedicated to UDCA pharmacotherapy emphasize the lack of high-quality randomized, high-quality randomized trials without risk of systematic error, random errors, and shifting results. Consequently, it will be possible to prove or disprove the hepatoprotective properties of UDCA only when conducting such studies in the future.

Recently, a relatively new and detailed study for UDCA – biliary sludge (initial manifestation of GD) – has being conducted (Gubska, 2013). It is the possibility of prescribing UDCA for this category of patients which makes UDCA preparations particularly interesting not only for gastroenterologists, but also for doctors-physicians, family physicians, etc. Sludge is defined as a viscous suspension in the cystic bile, which includes dense structures (0.05–1.2 mm crystals) and small concrements – up to 2–3 mm in diameter. Therefore, the synonym of biliary glucose is biliary microlithiasis (presence in the cavity of the gallbladder of thickened bile, which creates a clearly defined echostructure by ultrasound, slowly moves when the patient's position changes). The clinical significance of biliary sludge has been debated, as there are numerous observations of its involuntary disappearance of the latter in a certain (18–70%) category of patients. However, biliary sludge is rarely asymptomatic. It has been shown that in 25–70% of patients, the presence of the biliary sludge, one way or another, is accompanied by symptoms of biliary dyspepsia, pain syndrome (colic in 9–15% of patients) and dysfunction of the sphincter of Oddi. Perhaps one of the most unfavorable and terrible complications of biliary sludge, in addition to the abovementioned attacks of biliary colic, episodes of cholecystitis, cholangitis (including purulent), the formation of gallstone disease and stenotic papillitis, is acute pancreatitis. According to various authors, it is biliary sludge that causes the formation of acute idiopathic pancreatitis in 33–90% of patients (Gubska, 2013).

In accordance with the classification of GSD by the Russian Gastroenterologists Association (2003), biliary sludge is an early manifestation of the disease, which is noted in its first (initial) stage (Hohlacheva et al., 2016). Therefore, in most cases, in the presence of biliary sludge, a drug correction is required, for example with UDCA preparations. It is not only possible but also necessary to carry out a successful medical prophylaxis of gallstone disease. First of all, it is actual for overweight people who are eager to lose weight.

In 2007, recommendations were adopted in Germany for the diagnosis and treatment of GSD, according to which the administration of UDCA in a daily dose of not less than 500 mg for 3–6 months significantly reduces the risk of developing gallstone disease in the face of rapid decline body weight (over 1.5 kg per week) (Lammert et al., 2007).

UDCA preparations are interesting not only for their hepatotropic properties, but also for a large number of extrahepatic clinical effects. Relatively new indications for the use of UDCA are reflux gastritis and esophagitis due to alkaline biliary reflux. The hydrophilicity of UDCA and the choleretic effect are important for esophagogastric protection. UDCA displaces toxic hydrophobic bile acids due to competitive capture of receptors. Important is the induction of cholerexis, when bile-

rich bicarbonate contributes to the elimination of toxic bile acids through the intestine.

It has been established that alkaline gastroesophageal reflux occurs in 5–20% of patients with gastroesophageal reflux disease (GERD). During the pathological rejection of duodenal reflux, the destruction of the mucin barrier of the esophagus mucosa is accompanied by aggressive pancreatic enzymes (especially trypsin) and bile acids, which complicates the course of the GERD and ultimately – may be complicated by the development of the Barrett esophagus and the adenocarcinoma of the esophagus (Gubska, 2013).

Recent studies have shown that with bile reflux, the optimal dose of UDCA is 500 mg per day (250 mg in 2 doses) due to the ability of the bile acids contained in the refluxate to pass through the UDCA in a water-soluble form that to a lesser extent irritates the mucous membrane of the stomach and esophagus. The course of treatment for biliary reflux is at least two months. The share of UDCA in gastric contents is increased to 50% with decreasing of the content of gastric and deoxycholic acids, the concentration of CDCA does not change. In experiments and clinical studies, the cytoprotective properties of UDCA for the protection of the mucous membrane of the stomach and esophagus have been demonstrated (Thao et al., 2008; Chernobrovy et al., 2013). This is due to UDCA in the phospholipid layer of the cell membrane, which contributes to its stabilization and increased resistance to damaging factors. In addition, the prescription of UDCA drugs reduces the subjective findings of gastric dyspepsia.

It should be noted that extrahepatic UDCA effects are related to inflammatory bowel disease, as well as primary and secondary prevention of colorectal cancer as a result of inflammatory bowel disease. Thus, the UDCA prophylactic effect on colorectal cancer is that patients with poorly differentiated dysplasia who received UDCA for 2 years did not show deterioration, while 22.2% of patients who did not take UDCA had progression to dysplasia up to the need for colectomy. In the study of Alberts et al. (2005) the effectiveness of secondary prevention of oncogenesis in 661 patients who received UDCA for 6 months has been proved.

Clinical trials in patients with primary biliary cirrhosis indicate that the conjugate of ursodeoxycholic acid with taurine (TUDCA) has metabolic properties that can promote its long-term use as an alternative to ursodeoxycholic acid for patients with chronic cholestatic liver disease. However, direct comparison of TUDCA and UDCA in primary biliary cirrhosis has not been carried out for a long time (Larghi et al., 1997).

Finally, the effects of ursodeoxycholic and tauroursodeoxycholic acids were compared in 23 patients with PBC in a cross-linked clinical trial (Larghi et al., 1997). Both drugs were administered randomly, at a daily dose of 500 mg for two 6-month periods separated by a 3-month washout period. The biochemical parameters of blood serum (levels of activity of enzymes reflecting the functional state of the liver and associated with cholestasis and cytolysis) consistently improved compared to baseline values, both when administered with ursodeoxycholic and with the administration of taurouxedoxycholic acid, but no significant differences were found between the pharmacological effects of these two bile acids. Both preparations were well tolerated and none of the patients complained of side effects. In the short term, tauroursodeoxycholic acid appears safe and, at least as effective as ursodeoxycholic acid, as a means of PBC treatment (Larghi et al., 1997).

Another group of researchers also showed interest in tauroursodeoxycholic acid, which, due to its high hydrophilicity, may have a significant therapeutic value in the treatment of chronic cholestatic liver disease. They conducted a study of the dependence of the pharmacological effect of TUDCA on its dose in 24 patients with PBC that were randomly assigned to receive 500, 1000, and 1500 mg of TUDCA per day for six months. It was shown that the level of UDCA bile saturation was in the range from 15% to 48% and was not related to the dose of the drug. The biochemical parameters of blood serum (levels of activity of enzymes reflecting the functional state of the liver and related to cholestasis and cytolysis) decreased significantly after the first month of treatment with all three doses of TUDCA. There were no significant differences between the three doses at the time of interim control, but further further reduction of biochemical parameters occurred in patients with the administration of 1000 and 1500 mg of TUDCA per day. The level of total cholesterol and cholesterol in high density lipoprotein decreased significantly in patients who received two higher doses. Diarrhea was the only side effect. The final analysis of all data obtained from a clinical study indicated that the TUDCA dose of 10 mg/kg/day is optimal for use in long-term studies in patients with primary biliary cirrhosis (Crosignani et al., 1996).

As shown by these studies, the conjugation of taurine with bile acids not only increases their hydrophilicity and solubility, but also significantly affects the solubility of cholesterol, increasing its excretion. The introduction of not only TUDCA, but also itself, of taurine, has led to reduction of serum cholesterol levels in sick people. In a clinical blind placebo-controlled study, 22 healthy male volunteers aged 18–29 were randomly assigned to two groups that received a high-fat/high cholesterol diet for three weeks to raise serum cholesterol levels. The experimental group received 6 grams of taurine every day. At the end of the trial period, the control group had significantly higher levels of total cholesterol and cholesterol in the low density lipoprotein group than in the taurine group (Mizushima et al., 1996). The ability of taurine to improve the lipid profile was also studied by other scientists (Zhang et al., 2004; de la Puerta et al., 2010).

A number of clinical trials investigated the choleretic and hypolipidemic properties of artichoke extract and its effects in patients with symptoms of dyspepsia. A randomized, double-blind, placebo-controlled, cross-sectional study involving 20 volunteers was conducted to evaluate the choleretic effect of a single administration of an artichoke extract at a dose of 1.92 g. Monitoring of intra-ocular secretion of bile was carried out using multichannel probes starting from 30 minutes after the administration of the drug and within 4 hours after it. Increases in bile secretion were observed in both groups: with the introduction of artichoke extract and placebo. The maximum increase in bile secretion in the group with the administration of the artichoke extract was 152% and was reached 60 minutes after taking the drug, and in the placebo group – 40% and reached in 30 minutes. Differences between the groups with artichoke extract and placebo were statistically significant 30, 60, and 90 minutes after the administration of the drug ($P < 0.01$), and 120 and 150 minutes after taking the drug ($P < 0.05$) (Kirchhoff et al., 1994). The results of other clinical studies (both placebo-controlled and uncontrolled) regarding the choleretic effects of artichoke extract were summarized in the review (Kraft, 1997).

The effect of artichoke extract was also investigated in several studies in patients with non-specific complaints about the gastrointestinal tract, including dyspepsia, functional disorders of the bile ducts, constipation and stomach irritation. Patients were given up to six capsules of artichoke extract daily for six weeks (during the first study) or six months (during the second study). One capsule contains 320 mg of standardized artichoke water extract. Both studies noted improvements in clinical symptoms and decreased total cholesterol and triglycerides levels (compared with baseline values) in patients' blood serum. An analysis of the subgroup of 279 patients with at least three out of five symptoms of irritable bowel syndrome indicated a significant reduction in the severity of symptoms after taking artichoke extract (Bundy et al., 2004). The effectiveness of artichoke extract in patients with hyperlipoproteinemia was evaluated in a randomized, double-blind, placebo-controlled, multicenter study involving 143 patients with initial concentrations of total cholesterol > 7.3 mmol/l. Participants received artichoke extract at a dose of 1800 mg per day in two divided doses, or placebo for six weeks. At the end of the study, the average concentration of total cholesterol decreased in the group with the introduction of artichoke extract 18.5% and in the placebo group at 8.0% respectively ($P < 0.0001$) (Englisch et al., 2000).

Treatment with artichoke extract also led to a significant reduction in low density lipoprotein cholesterol compared to placebo ($P = 0.0001$) following a randomized, double-blind, placebo-controlled study of artichoke extract (Sahebkar et al., 2017). The average baseline total cholesterol for participants in this study was low. Analysis of results in subgroups revealed hypolipidemic effects of artichoke extract. However, the number of participants included in this trial was small and insufficient. A series of three open, uncontrolled studies on the administration

of concentrated artichoke juice (obtained from fresh leaves and flower buds) at doses of 10 ml three times a day for 12 weeks to 84 patients with secondary hyperlipidemia (Wider et al., 2009) was also conducted. Six weeks after treatment, the concentration of total cholesterol, low density lipoprotein cholesterol and triglycerides decreased, while the high-density lipoprotein cholesterol tended to increase.

A number of clinical trials investigated treatment of gynecological and even oncological diseases (concomitant therapy) by preparations based on *A. sinensis* extract. But we focus on those studies in which the anti-spasmolytic effect was important as well as its application in gastroenterology. One study described the retrospective observation of the treatment of a group of 200 gynaecological outpatients with dysmenorrhea, irregular menstruations, aged 16–46 and treated with the product 'Concentrated Danggui Wan' and another combination of *Angelica* and *Astragalus*. One hundred and forty-eight patients were in the treatment group, 52 in the comparator group. Diseases persisted from 6 months to 12 years, average 5 years. Inclusion criteria covered three groups of symptoms, defined as following. Dysmenorrhea: premenstrual and menstrual abdominal pain affecting work and daily activities, unstable effects of antispasmodic treatment. Irregular menstrual cycle: menstrual cycle shorter than 20 days or longer than 35 days, or in 2 consecutive months the menstruation lasted for more than 7 days. Reduced menstrual flow: menstrual period less than 2 days or progressive decline of menstrual flow. The treatment group (148 cases) was treated daily with product Concentrated Danggui Wan. Each dose unit "pill" contained 0.25 g of the drug. Twice a day 10–20 pills were taken each time with lukewarm water. Each treatment lasted for 4 weeks, and each patient received 2–3 treatments. The control group (52 cases) took large honey-based *Angelica* pills daily (ingredients of the pills were *Angelica* and *Astragalus*), twice a day, 9 g each time. Each treatment lasted for 4 weeks, and each patient normally received 2–3 treatments. During the treatment, all other medications for dysmenorrhea and irregular menstruation were prohibited. Effects of the treatment and the side-effects were measured. Results of treatment were assessed in a 3-step scale. Significantly effective: abdominal pain is reduced after the treatment and it no longer affects daily activities and work; menstrual cycle becomes largely normal, i.e., less than 5 days early or late; the flow increases at least one third as compared to before the treatment; menstruation lasts for 5–7 days, and other symptoms have disappeared or been alleviated. Effective: abdominal pain is reduced. With the help of painkillers, a patient can remain at his/her job. Symptoms of menstrual problems have become less severe but improvements are limited as compared to the "very effective" result. Ineffective: no improvements with abdominal pain and other problems. Menstrual cycle and flow have shown no changes. Therapy in the treatment group was assessed as significantly effective in 59/148 patients (39%), effective in 81/148 (54%) ineffective 7/148 (4%). Therapy in the control group was assessed as significantly effective in 27/52 patients (52%), effective in 18/52 (34%) ineffective in 7/52 (13%) patients. The author states that "there have been no clear side-effects in using Concentrated Danggui Wan for treating dysmenorrhea and irregular menstruation". In a few cases the patients developed mild nausea but the symptom quickly disappeared when the treatment was halted; there is no more detailed data (Dymowski, 2013).

The aim of another study (Dong et al., 2004) was to explore the abnormal function of platelets and the role of *Angelica sinensis* injection (ASI) in patients with ulcerative colitis (UC). In 39 patients with active UC, 25 patients with remissive UC and 30 healthy people, α-granule membrane protein (GMP-140) and thromboxane B2 (TXB2) were detected by means of enzyme-linked immunosorbent assay (ELISA), 6-keto-PGF1a was detected by radioimmunoassay, platelet count (PC) and 1 min platelet aggregation rate (1 min PAR) were detected by blood automatic tester and platelet aggregation tester respectively, and von Willebrand factor related antigen (vWF:Ag) was detected by the means of monoclonal-ELISA. The 64 patients with UC were divided into two therapy groups. After routine treatment and *Angelica sinensis* injection (ASI) + routine treatment respectively for 3 weeks, all these parameters were also detected. The PC, 1 min PAR and levels of GMP-140, TXB2, and vWF:Ag in active UC were significanrly higher than those in remissive UC and normal controls ($P < 0.05$–0.01). Meanwhile, 1 min PAR and levels of GMP-140, TXB2, and vWF:Ag in remissive UC were still significantly higher than those in normal controls ($P < 0.05$). Furthermore, 6-keto-PGF1a level in active and remissive UC was remarkably lower than that in normal control ($P < 0.05$–0.01). These parameters except 6-keto-PGF1a were significantly improved after the treatment in the ASI therapy group ($P < 0.05$–0.01), whereas they all were little changed in the routine therapy group ($P > 0.05$). The authors conclude that platelets can be significantly activated in UC, which might be related to vascular endothelium injury and imbalance between TXB2 and 6-keto-PGF1a in the blood. ASI can significantly inhibit platelet activation, relieve vascular endothelial cell injury, and improve microcirculation in UC.

The use of *Angelica sinensis* preparations for stopping bleeding from haemorrhoids has been reported (Gan et al., 2010). Limited, weak evidence showed that some herbal formulae, when including *Radix Sanguisorbae*, *Radix Rehmanniae, Fructus Sophorae, Radix Angelicae Sinensis, Radix Scutellariae*, etc., may alleviate some symptoms caused by haemorrhoids. These include hematochezia, congestive haemorrhoidal cushions and inflammation of perianal mucosa in the short term.

Own clinical studies of the preparation

Over the past few years, a certain experience of use of Choloplant-Tau capsules preparation (artichoke leaf extract 200 mg, ursodeoxycholic acid 100 mg, taurine 100 mg, and *Angelica sinensis* extract 50 mg) (UA Pro-Pharma LLC, Ukraine) in the treatment of biliary pathology has been accumulated (Anokhina et al., 2014; Zvyagintseva et al., 2014). In particular, the use of the preparation in combination therapy in patients with dysfunction of the sphincter of Oddi was investigated (Zvyagintseva et al., 2014).

Usage of this drug substances complex allows effectively restoration of the drainage function of the biliary tract, improvement of the bile drainage, reduction of the lithogenic properties of bile, which helps to prevent the formation of concrements, normalization of the motor function, biliary tract, restoration of the tone of the sphincter of Oddi, and increases the antioxidant protection the body. The clinicians examined 23 patients with Oddi's sphincter dysfunction after cholecystectomy with a disease duration of up to 2 years, aged 19–74 (mean age 54.05 ± 3.22 years), and evaluated the clinical efficacy of the preparation. The diagnosis was verified using clinical, laboratory and instrumental research methods. Before treatment, moderate pain and feeling of heaviness in the right hypochondrium was observed in 21 patients (91.3%), bitterness in the mouth – in 14 (60.9%), nausea – in 18 (78.2%), flatulence – in 16 (69.5%), a stroke violation – in 12 (52.1%). The biochemical parameters of cholestasis before treatment were bilirubin – 34.6 ± 2.7 μmol/l, cholesterol – 8.3 ± 0,6 mmol/l. With ultrasound, the enlargement of the choledochus was detected in 22 (95.6%) patients. Patients took the drug in 2 capsules 3 times a day. The efficacy of the drug was evaluated on the 21st day of treatment. After treatment, pain and severity in the right hypochondrium disappeared in all patients who had complaints (100%); bitterness in the mouth and nausea – in 16 (69.5%) and 20 (86.9%), flatulence – in 14 (60.8%) patients, normalization of the stool occurred in 11 (47.8%) patients. In assessing the biochemical parameters in all patients, the normalization of the indicators was observed. The level of total bilirubin in the blood serum decreased 2-fold and amounted to 17.3 ± 2.7 μmol/l on average, and cholesterol level was 5.8 ± 0.3 mmol/l ($P < 0.05$ compared with the corresponding indicator before treatment). According to the ultrasound, the choledoch dimensions reached norm in 19 (82.6%) patients. The obtained results testify to the expediency of the use of the drug in the treatment of Oddi's sphincter dysfunction after cholecystectomy, since it has a pronounced therapeutic effect in this pathology, contributes to the reduction of lithogenic properties of bile, prevents the development of gallstone formation.

Pharmaceutical quality profile for new preparation

When developing the quality specification for a new drug, the results of our previous studies and the experience of other authors regar-

ding the analytical standardization of UDCA based drugs and phytopharmaceutical products (Dhami et al., 2015; Galkin et al., 2011; Galkin et al., 2013; Gontova et al. 2016; Lutsenko et al., 2017; Ramaekers et al., 2017), as well as regulatory requirements (European Pharmacopoeia, Ph. Eur. 9th Edition) were used.

The specification for the preparation has been developed in accordance with the requirements of the "Note for Guidance Specifications: Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products: Chemical Substances" (CPRM/ICH/367/96) and the manufacturer's specifications for the UDCA, taurine and artichoke extract.

Identification test. This specification test is proposed as additional to the determination of the quantitative content of the main active substances. Identification is carried out in accordance with the following methods.

The identification of the artichoke extract is carried out by the determination of hydroxycholic acids, which are the main active ingredients of dry extract of artichoke leaves (*Cynara scolymus* L.). This is done by adsorption spectrophotometry in the ultraviolet region, according to the requirements of Ph. Eur., 2.2.25, in assay test. The ultraviolet absorption spectrum of the test solution in the region from 250 nm to 400 nm should have a maximum absorption at the wavelength 327 ± 2 nm and a shoulder in the region 300 ± 2 nm, since this spectrum is characteristic of the chlorogenic acid, which is the dominant hydroxychric acid in dry extract of artichoke leaves.

The identification of the ursodeoxycholic acid is carried out by liquid chromatography, according to the requirements of Ph. Eur., 2.2.29, at the same time as assay test. The retention time of the main peak of the ursodeoxycholic acid obtained on the chromatogram of the test solution was compared. It should correspond to the time of holding the peak of the ursodeoxycholic acid on the comparison solution chromatogram.

The identification of taurine is carried out by thin-layer chromatography according to the requirements of Ph. Eur., 2.2.27, simultaneously with the test "Impurities of taurine". On the chromatogram of the solution tested, the taurine spot by location, form and color must correspond to the spot of the taurine of the comparison solution.

The identification of polyphenolic compounds to the *A. sinensis* extract is carried out by qualitative reaction with phosphoric-molybdenum-tungsten reagent P (the appearance of a blue color).

Test "Uniformity of mass". No more than 2 individual masses deviate from the average mass by more than 7.5%, and none of them will deviate by more than 15.0%. This regulation is proposed on the basis of experimental data.

Test "Related impurities". Requirements for impurities are set according to "Note for Guidance on Impurities in New Drug Products" (CPMP/ICH/2738/99).

Related impurities of ursodeoxycholic acid. Impurity A (xenodeoxycholic acid) and impurity C (lithocholic acid) are specific impurities of the ursodeoxycholic acid. The tests are carried out by the HPLC method (impurity A, unidentified impurities, amount of impurities) in accordance with the requirements of Ph. Eur., 2.2.29, and by TLC (impurity C) according to the requirements of Ph. Eur., 2.2.27. On the chromatogram of the solution to be tested, the peak area of the impurity A (xenodeoxycholic acid) should not exceed 10 times the area of the main peak on the chromatogram of the comparison solution (1.0%); the area of any other impurity should not exceed the area of the main peak on the chromatogram of the comparison solution (0.1%). The sum of the areas of the peaks of all impurities should not exceed 15 areas of the main peak on the chromatogram of the comparison solution (1.5%). An impurity C (lithoacid acid) can be detected by the HPLC method, but in the determination of other contaminant impurity of ursodeoxycholic acid, the lithoholic acid has a retention time of more than 90 minutes on the chromatogram.

Thus, the determination of the impurity is proposed to be carried out by the TLC to the requirements of Ph. Eur., 2.2.27, according to the method of the corresponding monograph on the ursodeoxycholic acid on a silica gel plate in a mobile phase ice acetic acid : acetone : methylene chloride (1 : 30 : 60) after treatment with a solution of phosphoric molybdic acid in a mixture of sulfuric acid and acetic acetic acid and heating. Stains of vaginal acids acquire a blue color. In this case, the stain of the impurity C with the intensity and size is not more intense than the acid stain of the ursodeoxycholic acid on the comparison solution (less than 0.1%).

Related impurities of taurine. The determination is carried out by the TLC according to the requirements of Ph. Eur., 2.2.27, on Silicagel 60 F 254 plate in the mobile phase butanol : ice acetic acid : water (60 : 20 : 20) after treatment with ninhydrin solution and heating. Solutions of amino acids, polypeptides, peptones and primary amines when heated with ninhydrin (1,2,3-indanthion) acquire a blue or violet color. In this case, no stain of the impurity should exceed the size and intensity of the staining of the taurine spot on the comparison solution (less than 0.5%).

Dissolution test. Tests are conducted in six units in accordance with the requirements of the Ph. Eur., 2.9.3, with the use of a blade device. The amount of ursodeoxycholic acid, which has been transferred to the solution, is determined by HPLC according to the requirements of Ph. Eur., 2.2.29. For the preparation, it is proposed to use one study point for the release of active substances, since it relates to a normal release dosage forms with normal release containing rapidly soluble active substances. The pH of the dissolution medium (synthetic gastric juice, pH 8.0, without pancreatin) given in the United States Pharmacopeia monograph for tablets of ursodeoxycholic acid is not suitable for its determination in the presence of other active substances and concomitant substances (the effect of the physicochemical properties of the substance and the auxiliary substances) – there is no peak of the ursodeoxycholic acid on chromatogram of the test solution. The same is observed for the dissolution medium – the synthetic intestinal juice, pH 6.8, without pancreatin. However, taurine is reliably determined in these conditions.

In order to optimize the technique of dissolution test and simultaneously determine the ursodeoxycholic acid and taurine in the preparation as a dissolving medium, it is proposed to use water P. In this case, the ursodeoxycholic acid and taurine that have been transferred to the solution are reliably determined, which is confirmed by the validation data and experimental data obtained in the step pharmaceutical development. In this way, the tests are carried out in water for 45 minutes. The drug can withstand the test if the amount of ursodeoxycholic acid and taurine that has passed into the solution in 45 minutes is at least 85% (Q+5%) of the nominal content. This regulation is proposed on the basis of experimental data and Ph. Eur., 2.9.3, for solid dosage forms with traditional release.

Disintegration test. The time of full disintegration in water (disc method) should be no more than 30 minutes. This regulation is proposed on the basis of experimental data (Ph. Eur., 2.9.1, test A).

Test "Uniformity of dose units". The determination is made by the calculation and weighting method for taurine, since the content of this component is greater than 25 mg and is more than 25% of the weight of the dosage form. The determination is carried out by a direct method for the artichoke extract and the ursodeoxycholic acid, since the drug substances content is less than 25% of the total weight of the medicinal product. Calculate the acceptance value AV using the reference number M for case 1. The AV for the first 10 units is less than or equal to L1 = 15, or the final AV calculated from 30 units is less than or equal to L1 = 15 and no individual contents in the dosage unit is not less than 0.75 M and not more than 1.25 M. The result must meet the requirements of the Ph. Eur., 2.9.40. This parameter is controlled during the release of the series and is not critical for the stability studies.

Microbiological purity test. In accordance with the requirements of the general article Ph. Eur., 5.1.4, the eligibility criteria for the microbiological purity of the finished non-sterile administered medicinal products for oral use are included in the specification. Tests are conducted in accordance with the requirements of Ph. Eur., 2.6.12, 2.6.13. The following conditions were established: the total number of aerobic microorganisms (TAMS) $\leq 10^3$ CFU/g, the total number of yeast and mold (TYMS) $\leq 10^2$ CFU/g, the absence of *Escherichia coli* in 1 g.

Assay test. It is proposed to set the limit of quantitative content ± 5% to the declared quantity of active substances during release and ± 10% during the storage period. The quantitative content of the ursodeoxycholic acid is determined by HPLC according to the requirements of

Ph. Eur., 2.2.29, based on the average weight of the dosage form with refractometric detection on a column filled with 5 μm octadecyl silicagel, 4.6 × 150 mm. The content of the ursodeoxycholic acid should be 95–105% of the normal amount at release and from 90% to 110% up to the shelf life. This regulation is proposed on the basis of experimental data and requirements of Ph. Eur.

The quantity of artichoke extract is determined by the content of the hydroxycholic acid, based on the average weight of the dosage form, by the absorption spectrophotometry method in the UV region at 327 ± 2 nm, using the optical absorption index of chlorogenic acid. The content of hydroxycinnamic acids should be not less than 5.0 mg, in terms of chlorogenic acid on average weight. This regulation is proposed on the basis of experimental data (Ph. Eur., 2.2.25).

The quantitative content of taurine is determined by the acid-base titration method. Taurin, due to the amphoteric nature, can not directly titrate with alkaline solution. Titration is possible if the amino group is blocked by the action of formaldehyde. The formed compound can be titrated alkalimetrically with a phenolphthalein indicator. The content of taurine should be 95–105% of the normal amount at release and from 90% to 110% up to the shelf life.

Conclusions

An overview of our own data and data from the literature on the pharmacological and clinical study of a fixed combination of medicinal substances (artichoke leaf extract 200 mg, ursodeoxycholic acid 100 mg, taurine 100 mg, and *Angelica sinensis* roots extract 50 mg), as well a scientific substantiation of the pharmaceutical quality profile of the corresponding finished solid dosage form has been conducted. It is substantiated that this drug is a fixed combination of medicinal substances with well-researched medical applications in the treatment of dyspeptic disorders with functional disorders of the biliary system, biliary dyskinesia of the hypokinetic type, and gastritis with reflux of bile. Each of the components of the fixed combination has an important influence on the human hepatobiliary system. The effect of ursodeoxycholic acid is due to the relative replacement of lipophilic toxic bile acids, improving the secretory capacity of hepatocytes and immunoregulatory processes, which is especially important in liver and cholestatic diseases. Taurine is a synergist of ursodeoxycholic acid, since it forms biliary conjugates in the liver. The artichoke extract has choleretic, hepatoprotective and diuretic effects, while the *A. sinensis* roots extract demonstrates moderate spasmolytic and anti-inflammatory properties. The fixed combination has a favorable safety profile, has been investigated in clinical conditions in vivo both in the form of individual components and in the form of a single drug. A fixed combination pharmaceutical profile is based on the general requirements for solid dosage forms, as well as experimentally substantiated specific indicators and research methods.

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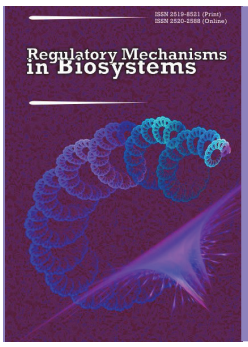

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 41–46
doi: 10.15421/021805

Hereditary tubulopathies including the associated bone disease

M. O. Ryznychuk, T. V. Khmara, M. I. Kryvchanska, I. I. Zamorskii

Higher State Educational Institution of Ukraine "Bukovinian State Medical University", Chernivtsi, Ukraine

Article info

Received 28.12.2017
Received in revised form 26.01.2018
Accepted 29.01.2018

Higher State Educational Institution of Ukraine "Bukovinian State Medical University", Teatralna Sq., 2, Chernivtsi, 58001, Ukraine.
Tel.: +38-050-192-09-53.
E-mail: rysnichuk.mariana@gmail.com

Ryznychuk, M. O., Khmara, T. V., Kryvchanska, M. I., & Zamorskii, I. I. (2018). Hereditary tubulopathies including the associated bone disease. Regulatory Mechanisms in Biosystems, 9(1), 41–46. doi: 10.15421/021805

Tubulopathy is a heterogeneous group of diseases combined by the nephron functions disorders of one or more enzyme proteins in the tubular epithelium that cease to function as a reabsorption of one or several substances filtered from the blood through the glomeruli into tubules, which determines the development of the disease. This review addresses the tubulopathies accompanying bone disease, namely: de Tony-Debre-Fanconi syndrome (autosomal dominant, autosomal recessive, X-linked), renal distal metabolic acidosis type I (classic, autosomal dominant, autosomal recessive inheritance), renal distal tubular metabolic acidosis I (autosomal dominant, autosomal recessive inheritance) and type II (autosomal recessive inheritance accompanying delayed mental development and eye disorders), combined distal and proximal renal tubular metabolic acidosis type III (autosomal recessive inheritance characterized by osteoporosis), hypophosphatemia rickets (X-linked dominant, autosomal dominant, primary hypercalciuria, autosomal recessive inheritance). However, the diagnosis of tubulopathy remains complex and requires expensive laboratory equipment and specialist expertise; it can be diagnosed in children showing the following symptoms: impaired growth, vitamin D resistant rickets (lower limb deformities between 2 and 3 years of age). In the evaluation of such patients urine analysis is commonly used (levels of calcium, phosphorus, pH, bicarbonate, sodium, potassium, glucose, creatinine, protein, amino acids), blood count (levels of creatinine, uric acid, alkaline phosphatase, glucose, pH and sodium, bicarbonate, potassium, chloride, calcium, phosphorus ions), ultrasound of the kidneys to detect nephrocalcinosis. Determination of serum parathyroid hormone concentration, vitamin D metabolites, aldosterone and plasma renin activity, cysteine lymphocyte concentration (suspicion to diagnose cystinosis) and ophthalmologist examination may also be used as additional diagnostic methods. Despite the fact that most tubulopathies can be diagnosed clinically, molecular genetic studies are needed to clarify the type of inheritance and prognosis. The use of calcitriol will help in the management of phosphorous levels in the blood. Correction of vitamin D deficiency state is not required. Calcitriol supplementation may prevent secondary hyperparathyroidism resulting from increased phosphate intake.

Keywords: vitamin D resistant rickets; children; hypophosphatemia; genes

Introduction

Tubulopathies form a heterogeneous group of diseases combined by the presence of disorders in the tubular epithelium of the nephron functions of one or more enzyme proteins that cease to function as reabsorption of one or several substances filtered from the blood through the glomeruli into tubules, which determines the development of the disease. By origin they are classified into primary and secondary tubulopathies. The primary ones involve a hereditary defect of the genes that regulate the function of a particular tubular enzyme, resulting in the development of pathology usually from the first months or years of life of the child. Currently, not all genes are known, the mutation of which leads to the development of hereditary tubulopathies.

Based on the etiology, primary tubulopathy is divided into proximal (different forms of Fanconi syndrome, glycineuria, cystonuria, phosphate diabetes, renal tubular acidosis type II (in children), renal glucoseuria, etc.), distal (renal tubular acidosis type I, nephrogenic diabetes insipidus, pseudogiopoaldosteronism) and mixed, and by the number of disturbed functions, such as isolated (monosymptomatic) and combined (polysymptomatic).

For a practical physician a classification based on the isolation of the leading clinical symptom complex is considered optimal. At present, more than 30 different primary tubulopathies are known, their number increases with further study of the pathophysiology of the kidneys. According to some authors, it is advisable to classify tubulopathies according to the leading clinical manifestation. The classification below does not purport to represent all existing hereditary tubulopathies and is limited to the most common diseases.

According to the main syndromes, tubulopathies can be divided into three groups:

1. Tubulopathies accompanying bone diseases (primary de Toni-Debre-Fanconi syndrome (autosomal dominant, autosomal recessive, X-linked), renal distal metabolic acidosis type I (classic, autosomal dominant, autosomal recessive inheritance), renal distal tubular metabolic acidosis I (autosomal dominant, autosomal recessive inheritance) and type II (autosomal recessive inheritance accompanied by delayed mental development and eye disorders), combined distal and proximal renal tubular metabolic acidosis type III (autosomal recessive inheritance characterized by osteoporosis), hypophosphatemia rickets (X-linked dominant, autosomal dominant, primary hypercalciuria, autosomal recessive inheritance).

2. Hereditary tubulopathies accompanying polyuria (renal glycosuria, nephrogenic diabetes insipidus, pseudo hyperaldosteronism).

3. Hereditary tubulopathies characterized by nephrolithiasis (cystinuria, glycosuria, primary hyperoxaluria, xanithinuria, alkapthonuria, Dent disease).

We provide a brief overview of the tubulopathies associated with bone disease.

Primary de Toni-Debre-Fanconi syndrome (OMIM 134600, 613388, 615605, 616026)

Syn.: Fanconi syndrome, glucoaminophosphate diabetes, glucosephamide diabetes, rickets hereditary vitamin D-resistant, idiopathic Fanconi syndrome, Fanconi hereditary syndrome, Renal Fanconi syndrome, Fanconi syndrome, Primary de Toni-Debre-Fanconi syndrome, Inherited Fanconi syndrome.

Fanconi syndrome is divided into four types: type I (OMIM 134600), type II (OMIM 613388), type III (OMIM 615605), type IV diabetes with MODY (OMIM 616026).

In its complete form, Fanconi syndrome (disease) is characterized by a triad of symptoms: hypophosphatemia associated with bone disease, excessive renal wasting of glucose and amino acids, arising as a result of violations in the proximal segment of the nephron (Bacconi et al., 2005).

Is associated with the most severe types of prolapsed tubulopathies. The disease is genetically predisposed (Bai et al., 2004; White et al., 2005).

Among other researchers, a Swiss pediatrician Fanconi was the first to describe the particular signs of the disease. In 1931, he described a child with dwarfism, rickets, glycosuria and albuminuria. Two years later, de Toni found hypophosphatemia to be further clinical evidence and, later on, Debré determined elevated levels of organic acids in urine, called aminoaciduria (Younes et al., 2003; Zivičnjak et al., 2011).

Aetiopathogenesis

It is believed that the genetically determined defects of enzymatic phosphorylation in the renal tubules (combined tubulopathy); deficiency of enzymes from the complexes II and III (succinate dehydrogenase and cytochrome oxidase) of the respiratory chain are the basis of the disease. Some authors believe that the basis of the disease is mitochondrial genesis (Lichter-Konecki et al., 2001; Watanabe, 2017).

These mutations lead to various defects in the renal proximal tubules leading to excessive urinary waste of phosphates, glucose and amino acids, as well as to the acid-base imbalance. Metabolic acidosis and insufficiency of phosphorus compounds can also contribute to bone deformities (osteomalacia (adults) and rickets (children)) (Pishak et al., 2015).

Based on its etiology, the syndrome can be divided into two main categories: primary (hereditary) and secondary (acquired). The secondary one is the most common.

The primary (hereditary) syndrome resulting from a genetic mutation occurs in approximately 1 in 20.000 births, de Tony-Debre-Fanconi disease occurs in approximately 1 in 40.000 births. This disease is believed to be caused by the damage to the transport systems in the proximal tubules resulting in disruption of phosphate, glucose and amino acids transportation. Typical episodic features include dehydration, symptoms of rickets and delayed growth. Sometimes the disorder manifests itself at an older age as a renal failure (Tasic, 2008; Besouw et al., 2017). Damage to the sodium transport systems in the proximal tubules (for example, in acute renal failure) leads to a pronounced sodium reabsorption and tubular acidosis disorder, hydrogen ion transport and proximal tubules reabsorbed substances can be disrupted: glucose, phosphate, uric acid, amino acids.

An acquired Fanconi syndrome develops associated with other hereditary disorders or kidney diseases, namely: congenital metabolism or transport disorders (cystitis, tyrosineemia type I, glycogenosis type XI, galactosemia, congenital intolerance to fructose, Wilson disease, oculocerebrorenal syndrome (Lowe syndrome), vitamin D-resistant rickets, impaired energy metabolism, McArdle-Schmid-Pearson disease, cytochrome C oxidase deficiency (COX deficiency), pyruvate carboxylase (PC) deficiency, carnitine palmitoyltransferase I (CPT I) deficiency); chronic diseases (paraproteinemia (multiple myeloma), tubulointestitial nephropathies, nephrotic syndrome, nephropathy in renal transplant allografts, malignant tumors (paraneoplastic disease)); heavy metal salts intoxication (mercury, lead, cadmium, uranium); organophosphate poisoning (toluene, maleic acid, lysol); drug-induced toxicity (platinum-based agents, expired tetracycline and gentamicin), severe burns. The syndrome may be complete if these three symptoms (glycosuria, phosphaturia and aminoaciduria) are observed and incomplete if there are only two of them:

– glycosophosphamide diabetes without acidosis (described by Dent and Kyle);

– phosphoglucide diabetes (described by Mac Cune);

– aminophosphoric diabetes (described by Jonxin, Wallgren and Nicola);

– glucosamine diabetes (described by Juillard and Fischer).

Clinical evidence

The severity of clinical manifestations and metabolic disorders may differ depending on two clinical and biochemical variants of the disease: in children (early) and adults (late). The pediatric form arises during the 1st year of life and manifestations can include frequent vomiting, loss of appetite, mental and physical developmental delay, a tendency to severe infectious diseases. Gradually there is a proportional dwarfism, rickets and renal insufficiency.

Early dwarfism: intense increase in the rate of growth in height and weight (up to 30%) that occurs during the 5–6 of normal growth and weight gain.

Median age at diagnosis of rickets is 10–12 months; it is characterized by a topographical specificity: the skull is deformed by localized impact; in contrast to fractures in the thoracic (mid back) spine and limbs. Bone pain of moderate intensity primarily tends to be localized in the limbs and spine. It is associated with severe hypocalcaemia (1.6–1.8 mmol/L) and reduced intestinal calcium absorption.

Polydipsia and polyuria are common for the beginning of the disease, progressively intensifying and systematically regressing at different age periods, but never go away completely.

General symptoms of chronic inflammatory myopathy include slow but progressive muscle weakness and transverse abdominal disruption. It is marked by frequent constipation.

Eye disorders: pigment retinitis, congenital cataracts. Renal failure progresses into a chronic kidney disease between 8–14 years.

The late syndrome is generally noticed between 3 and 6 years of age; it is accompanied by the delayed changes in general medical condition, osteomalacia augmentation and hypokalemic paralysis. Characterized by polyuria and polydipsia, moderate developmental delay, severe genu valgum deformities; low level of phosphate, potassium and calcium in the blood, normal amino acids and glucose concentration. Low plasma bicarbonate concentration is common in the early stages of the disease, later on hyperchloremic acidosis develops. It is characterized by disturbances in the concentration of renal function (hipostenuria, polyuria), sometimes moderate proteinuria, generalized hyperaminociduria, elevated excretion of phosphates, calcium, glucose, citrates in patients. Urine reaction is neutral or alkaline (Lichter-Konecki et al., 2001; Tasic et al., 2008; Watanabe, 2017).

In patients with de Toni-Debre-Fanconi syndrome the following laboratory abnormalities:

– hypophosphatemia;

– hypocalcemia;

– hypokalemia;

– hyponatremia;

– elevated alkaline serum phosphatase;

– metabolic acidosis (pH: 7.25–7.35; base excess BE (elevated level of alkalinity): –12– –10 mmol/L) secondary to reduced proximal tubular reabsorption of bicarbonate:

– increased Pyruvic and Lactic Acid Content of Blood;

– hypophosphaturia;

– calciuria;

– polyuria;

– decreased serum uric acid with an increased uric acid clearance;

– glycosuria (above 20–30 g/L);

– development of generalized hyperaminociduria (less than 2.0–2.5 g/24 h) in all the amino acid types;

– failure of amino acid genesis – reduce titratable acidity;

– increased urine pH (higher than 6.0);

– tubulin-like proteinuria - the presence of immunoglobulins in the urine of the light chains, lysozyme, β_2-microglobulin.

Radiological method features has proven to be useful in detecting pronounced osteoporosis with severe disorders in metaphyseal areas

with characteristic bowing of the bones, accompanied by a delayed bone age relative to chronological age of a child.

Additionally, type II is characterized by increased serum 25-hydroxyvitamin D levels in children and decreased – in adults (Levtchenko et al., 2006; Magen et al., 2010). In type III, varus angulation of the lower extremities, while renal failure does not occur (Klootwijk et al., 2014). Type IV could be suspected in infants who are large for their gestational age (more than 4 kg), subject to neonatal hypoglycemia, hyperinsulinism and hepatomegaly. There is a risk of development of insulin dependent diabetes mellitus (maturity-onset diabetes of the young (MODY)) accompanied by nephrocalcinosis and renal failure (Hamilton et al., 2014).

Treatment

Dietary restrictions:

– in galactosemia: milk;

– in fructose intolerance: sugar, honey, apples, pears, watermelons, carrots;

– in cystonesis: protein foods, high-methionine foods, kitchen salt;

– in tyrosinemia: high-tyrosine and methionine foods;

Recommended foods (Novikov et al., 2004; Savenkova and Leviashvili, 2004):

– in pyruvate carboxylase deficiency: low carb high fat diet (LCHF diet);

– potassium-, calcium-, phosphorus-rich foods;

– liquid intake is typically not restricted.

Correction for renal tubular metabolic acidosis:

– 2% or 4% sodium bicarbonate solution (5 ml/kg/day) in 4 divided doses (intravenous, oral, rectal administration) and calcium supplements;

– citrate mixture to reduce the dose of sodium bicarbonate;

– treatment of hypokalaemia (potassium supplements);

Correction for hypophosphatemic rickets with normal calcium level and/or hypocalcemia, osteoporosis:

– calcium supplements (calcium carbonate, calcium phosphate, calcium citrate, calcium glycerophosphate). Phosphate buffer (continuously);

Active metabolites of vitamin D: oxide; calcidiol; calcitriol; or calcium-, phosphorus- and calcitriol- containing binding agents.

Recombinant human growth hormone treated with 0.6–0.7 IU/kg/week of rhGH administered daily for 3 months.

Renal tubular acidosis (RTA)

Several bone deformities in children with tubulopathies are associated with a number of factors; metabolic acidosis should be considered a sign of an underlying disease process. The most vital parameter affecting protein binding of calcium is the pH. Since bone responds to overacidity, chronic metabolic acidosis of any origin can cause growth retardation. In addition, metabolic acidosis causes alterations in the bone reabsorptive capacity for calcium and therefore increases urinary calcium excretion. The development of metabolic acidosis is caused by a violation of reabsorption of bicarbonates and secretion of hydrogen ions, as well as a violation of the activity of carbonic anhydrase with respect to hydration of CO_2 (this enzyme also stimulates proton secretion not only in renal proximal tubules and collecting ducts, but also in osteoclasts) (Kartamyisheva et al., 2011).

The disease is inherited by both auto dominant and auto recurrent types; and is clinically characterized by hyperchloremic acidosis and baseline deficiency in the serum.

There are two types of disease: distal renal tubular acidosis (dRTA) type I is characterized by an impairment of the normal urinary acidification process in the distal part of the nephron; Proximal renal tubular acidosis (pRTA) type II is characterized by a defect in the ability to reabsorb bicarbonates in the proximal tubule. Type III is a combination of isolated proximal (type 2) or distal (type 1) tubular pathologies.

Type I dRTA

There are distinguished two types of the disease by the pattern of inheritance: autosomal dominant or autosomal recessive.

Classical type I dRTA autosomal dominant (OMIM 179800)

The syndrome is caused by mutations in the SLC4A1 (MIM 109270) gene, found in a place on the long arm of chromosome 17 called 17q21.31 (Bergwitz et al., 2006). Clinically it is characterized by osteomalacia, plastic deformity of the long tubular bones and growth retardation. It is caused by the disorder of the tubular acidogenesis, when the kidneys fail to reduce the urine pH associated with the increase in hydrogen ion concentration as a result of the increased reverse diffusion of hydrogen ions through the tight junctions that hold the tubular epithelial cells. The distal canal is unable to create a concentration gradient between the tubular fluid and the blood. This finding suggests that bicarbonate ions have been effectively replaced by chloride ions and the hyperchloremic metabolic acidosis arises (Fry & Karet, 2007; Kraut et al., 2010).

First, in people with this syndrome in their teens or adulthood the following signs and symptoms are observed : poor appetite, polyuria, polydipsia, rapid fatigability and delayed physical development. Next bone deformities commonly associated with rickets (lower-limb valgus deformity, "rachitic rosary", widening of wrist, frontal and parietal lobe), as well as with the pronounced muscular hypotonia. The first manifestations of the renal tubular acidosis usually appear in children two years of age. People with more severe and prolonged rickets may experience permanent bone deformities (Rodriguez, 2002; Karet, 2002; Civitelli & Ziambaras, 2011).

Laboratory studies have revealed metabolic acidosis, low plasma bicarbonate- and increased plasma chloride concentration, hypocalcemia, hypokalemia, hypophosphatemia, increased alkaline phosphatase activity, secondary hyperparathyroidism and decreased intestinal calcium absorption. High-resolution ultrasound has been found to be a sensitive and reliable method for the detection of nephrocalcinosis. A significantly decreased renal function (urine specific gravity from 1001 to1008) is observed, a persistently low urine pH (<5.5), as well as normal bicarbonate levels. Hypercalciuria is associated with excessive urinary calcium excretion (as a compensation for a metabolic acidosis) (Bergwitz & Jüppner, 2010; Escobar et al., 2013) mediated by the renal Ca^{2+} transport proteins (Laing & Unwin, 2006; Nijenhuis et al., 2006) and increased renal sodium reabsorption. This association may implicate increased renal blood flow as a contributory cause of urinary hyperexcretion of insoluble mineral salts, which can lead to recurrent kidney stones or nephrocalcinosis. These factors, together with high urine pH, contribute to abnormal accumulation of calcium and the development of nephrocalcinosis and / or renal stones, which may lead to further deterioration of renal function (Karet, 2002; Loymana et al., 2010).

Type I autosomal recessive dRTA with deafness or with preserved hearing (OMIM 602722) Syn.: RTADR.

Defects in the ATP6V0A4 (7q34) or ATP6V1B1 (2p13.3) genes cause autosomal recessive dRTA with deafness and with preserved hearing, respectively. However, several patients with ATP6V0A4 mutations have developed hearing loss, usually in young adulthood.

Clinical features. This syndrome occurs in early childhood associated with frequent vomiting and development of dehydration followed by growth retardation and nephrocalcinosis, preceded by chronic renal insufficiency. Often the syndrome is associated with the development of neurosensory deafness. Laboratory findings are the same as in the autosomal dominant form. Parents are usually married (Leung, 2014).

Type II proximal renal tubular acidosis with ocular abnormalities and mental retardation (OMIM 604278)

The syndrome is caused by the function mutations in the SLC4A4 gene (MIM 603345) located on chromosome 4 (4q13.3) (Igarashi et al., 2001).

It is associated with the immature nephrons, low carbonic anhydrase II(c) and I(b) activity; as well as low HCO3- ATPase activity in mitochondria of renal tubular cells.

Clinical features. Reduced proximal tubular reabsorption of bicarbonate, resulting in impaired capacity for net acid excretion and persistent hyperchloremic metabolic acidosis. In the first few months of life a history of vomiting, thirst, subfebrile temperature, marked delay in physical growth, rickets-like changes in the skeleton may be present. Developmental delay, nystagmus, congenital cataracts, corneal stromal opacities, glaucoma and permanent enamel hypoplasia (Pettifor, 2008).

Laboratory diagnosis: increased osmotic fragility of erythrocytes with slightly acid urine (pH less than 6). Hydrogen ions (H^+) excretion remains within normal limits and corresponds to nutrition. The bicarbonate threshold for bicarbonate reabsorption is decreased, while its excretion is sharply increased.

Treatment: Dietary restriction of oxalate intake (sorrel, spinach, tomato juice, chocolate, etc.), alkaline mineral water, administration of sodium bicarbonate to restore normal acid base status; or diluted citrate solution (Shohla solution) at a dose 5–30 mmol HCO_3^-/kg/day. Adding more potassium is typically needed. Shohla solution (pharmacy – prepared) containing in 1000 mL not less than 140 g of citric acid and 90 g of sodium citrate (1 g of $NaHCO_3$ = 12 mg of alkalosis, 10 ml of Shohla solution = 10 mg of alkalosis). Vitamin D treatment in patients with osteoporosis and osteomalacia.

Combined proximal and distal renal tubular acidosis (Type III RTA) (Autosomal recessive inheritance associated with osteoporosis) (OMIM 267200)

The syndrome is almost invariably associated with increased bicarbonate excretion.

Clinical features. Metabolic acidosis in early infancy associated with hypokalemic paralysis, osteomalacia with subsequent skeletal deformities and growth retardation and early osteoporosis.

Radiographic evaluation of nephrocalcinosis, abdominal calcifications, osteoporosis and bone deformation. Autosomal recessive inheritance, much more prevalent in males.

Hypophosphatemic rickets (hypophosphatemia)

The maintenance of normal phosphate homeostasis constitutes the basic physiologic function of the kidneys (Bastepe & Jüpper, 2008; Natochin, 2008). Serum phosphate concentration exists in three major forms: free ionized (84–85%), protein-bound (10%), and calcium-, magnesium- and sodium compounds (1%) (Escobar et al., 2013). If urine pH is >7.4, approximately 80% of total phosphate concentration is in the divalent form (HPO_4^{2-}), while 20% will be in the monovalent form (H_2PO^{2-}) (Kartamyisheva et al., 2011). Usually, about 90% of phosphate in the glomerular filtrate is reabsorbed in the proximal tubule, and 80% reabsorbed proximally (Bastepe & Jüpper 2008; Natochin, 2008; Escobar et al., 2013). The currently known main regulators of phosphate homeostasis include parathyroid hormone (PTH) and vitamin D_3 (calcitriol) (Escobar et al., 2013) and leads to a higher plasma phosphorus concentration (Baroncelli et al., 2012).

Calcitriol or biologically active form of vitamin D_3 stimulates phosphate reabsorption. Phosphatonins include fibroblast growth factor 23, frizzled-related protein-4 and phosphoglycoprotein extracellular matrix. Fibroblast growth factor-23 (FGF-23) is a 26-kDa protein activating the specific cell surface receptors(FGFRs) (Perwad & Portale, 2011).

The ENPP1 (173335), PHEX (300550), DMP1 (600980) and FGF23 genes stimulate the elevation of fibroblast growth factor 23 (FGF-23). The FGF-23 gene is located on chromosome 12p13.3. Fibroblast growth factor 23 (FGF-23) is the gene identified as causative for autosomal dominant hypophosphatemia rickets (Bai et al., 2004; Ben-Dov et al., 2007).

The biological activity and physiological role of FGF-23 have recently been clarified. Several animal models (mice with excess FGF-23 activity as a result of *in vivo* forced overexpression) exhibit hypophosphatemia and increased P excretion of 1.25-dihydroxyvitamin D (Sitara et al., 2004, Shimada et al., 2004a; Shimada et al., 2004b). FGF-23 deficient mice are characterized by a severe aging-like phenotype associated with ectopic calcifications organ atrophy, and osteomalacia. Mice lacking FGF-23 were characterized by severe vascular- and soft tissue calcification (Kuro-o et al., 1997). Needless to mention that extensive vascular and soft tissue calcification in both FGF-23 and klotho ablated mice are associated with severe hyperphosphatemia, and increased serum level of hydroxyvitamin D. The FGF-23 biology was studied on mouse models treated with recombinant FGF-23 or overexpression of FGF-23. FGF-23 suppresses the expression of the types IIa and IIc sodium-phosphate cotransporters on the apical membrane of renal proximal tubular cells, thus inducing phosphaturia (Shimada et al., 2005). The phosphatidic action of FGF-23 is not expressed in the absence of sodium-hydrogen exchanger regulatory factor-1 (NHERF-1) and increases in the presence of parathyroid hormone (PTH). In addition, FGF-23 suppresses the formation of $1,25(OH)_2D$, suppressing 1-alpha-hydroxylase (CYP27B1), which converts 25-hydroxyvitamin D [25(OH)D] to 1,25(OH)2D and stimulates the formation 24-hydroxylase (CYP24), which converts $1,25(OH)_2D$ into inactive metabolites in the proximal tubule of the kidneys. In addition, FGF-23 impairs the production of renal 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] by inhibiting the expression of CYP27B1, the enzyme that converts 25-(OH)D to inactive metabolites in the proximal tubule. FGF23 also reduces the expression of interstitial sodium-phosphate conveyor NPT2b (Saito et al., 2003), reducing the intestinal phosphate absorption.

FGF-23 acts directly on the parathyroid gland to inhibit PTH synthesis and secretion. It has been shown that FGF-23 activates the mitogen-activated protein kinase pathway leading to a decrease in parathyroid hormone (PTH) secretion both *in vivo* rats and *in vitro* shingles (Ben-Dov et al., 2007). FGF-23 has also been shown to increase expression of parathyroid 1-alpha-hydroxylase (Krajisnik et al., 2007), which converts 25-hydroxyvitamin D [25(OH)D] to $1,25(OH)_2D$.

FGF-23 secretion is regulated by local bone-derived factors, such as phosphate-regulating gene with homologies to endopeptidases and dentin matrix protein-1 (Lorenz-Depiereux et al., 2006a; Lorenz-Depiereux et al., 2006b). $1,25(OH)_2D$ affects FGF-23 secretion both *in vivo* and *in vitro* through the activation of FGF-23 mediated vitamin D (Liu et al., 2006).

1. Factors decreasing phosphate reabsorption:
– parathyroid hormone;
– atrial natriuretic peptide;
– glucocorticoids;
– dopamine.

Phosphauric factors:
– fibroblasts 23 growth factor;
– fibroblasts 7 growth factor;
– matrix extracellular phosphoglycoprotein (MEPE);
– other.

2. Factors increasing phosphate reabsorption:
– parathyroidectomy;
– $11\alpha25(OH)_2D_3$;
– growth hormone;
– insulin-like growth factor;
– food regulation;
– acute (minutes, hours);
– chronic (hours, days);
– several system factors.

X-linked recessive hypophosphatemia rickets (Dent's disease) (OMIM 300554)

The disease is caused mainly by mutations in the CLCN5 gene located on chromosome Xp11.22.

X-linked recessive hypophosphatemia rickets is a form of X-linked hypercalciuric nephrolithiasis, which comprises a group of disorders characterized by proximal renal tubular reabsorptive failure, hypercalciuria, nephrocalcinosis, and renal insufficiency.

Clinical features: rickets or osteomalacia, hypercalciuria, hypophosphatemia and proteinuria in children. Progressive calcification and renal failure in adult patients (Gambaro et al., 2004). Clinically, it may show bone pain, fatigue, muscle weakness, and repeated bone fractures. Symptoms are related to bone pain, fatigue, muscle weakness and recurrent bone fractures.

Autosomal dominant hypophosphatemia rickets (OMIM 193100)

Autosomal dominant hypophosphatemia rickets (ADHR) results from activating mutations in a fibroblast growth factor 23 (FGF-23) gene in chromosome 12p13 encoding a phosphate-regulating hormone (Sun et al., 2012; Wöhrle et al., 2013).

Small amounts of the gene originate in the brain, thymus, small intestine, heart, liver, lymph nodes, thyroid-shaped and pterygoid glands, bone marrow and in large quantities in tumors with oncogenic osteomalacia. No expression in the bones. Elevated levels of FGF-23 are associated with inhibition of reabsorption of phosphates in the renal tubule and hypophosphatemia. FGF-23 can physiologically function as a locally active factor secreted in excessive amounts in conditions of pathology, and may cause renal phosphate loss.

Less than 100 cases have been described.

Clinical manifestations depend on the age of onset and on the severity of hypophosphatemia.

Clinical features: ADHR shows incomplete penetrance and variable age at onset (childhood to adult). Phosphate excretion can be evaluated by measuring the maximum tubular reabsorption per glomerular filtration rate.

Laboratory diagnosis: It is characterized by severe hypophosphatemia arising from a defect in the renal reabsorption of filtered inorganic phosphorus (P_i), elevated serum alkaline phosphatase activity and fibroblast growth factor 23 (FGF-23), inappropriately low-normal serum concentration of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] levels for the degree of prevailing hypophosphatemia (Econs et al., 1997).

Treatment: aimed at improving growth, enhancing mineralization of bones, and preventing skeletal deformities caused by rickets. It consists of daily oral administration of phosphate and calcitriol and is associated with frequent monitoring of calcium, alkaline phosphatase and parathyroid hormone, and phosphate serum concentrations, as well as urinary calcium and creatinine.

Autosomal recessive hypophosphatemia rickets type 1 (ARHR1) (OMIM 241520)

ARHR1 is caused by homozygous loss-of-function mutations in the DMP1 (Dentin matrix protein 1) gene in chromosome 4q22.

Clinical features: Lower-extremity deformities. No response to vitamin D therapy (vitamin D resistant rickets), high bone density. Back pain, restricted joint motion. Premature fusion of the skull bones. Deafness (aplasia of the vestibulocochlear nerve that results in ipsilateral congenital sensorineural hearing loss). Dental defects and early caries. It is accompanied by muscle weakness and pathologic fractures.

Radiographic evaluation: early osteosclerosis and skull thickening, trabecular bone density in ribs (Feng et al., 2006; Lorenz-Depiereux et al., 2006a).

Laboratory diagnosis: there are no symptoms of hypophosphatemia.

Autosomal recessive hypophosphatemia rickets type 2 (ARHR2) (OMIM 613312)

ARHR2 is caused by homozygous loss-of-function mutation in the ENPP1 gene in chromosome 6 (6q). Mutations in ENPP1 gene are also responsible for generalized arterial calcification of infancy.

Clinical features: hypophosphatemia rickets, sometimes generalized arterial calcification of infancy (Lorenz-Depiereux et al., 2010).

Laboratory diagnosis: hypophosphatemia.

X-linked, dominant, hypophosphatemia rickets (XLHR) (OMIM 307800)

Inactivating mutations in PHEX gene with homologies to endopeptidase on the X chromosome (Xp22) have been identified as a cause of XLHR. This endopeptidase is mainly expressed in bones and teeth, regulating FGF-23 synthesis. The disease occurs as an X-linked dominant disorder with complete penetrance often complicated by variable expressivity.

PHEX revealed possible alternative regulatory mechanisms for phosphate homeostasis, bone mineralization, and vitamin D metabolism. It controls sodium-dependent phosphate transport proteins in intestinal and renal proximal tubular epithelial cells. The genetic disorder is associated with inability of the renal proximal tubule to reabsorb phosphate, which affects intestinal phosphate absorption. PHEX is primarily expressed in osteoblasts, odontoblasts, lung, ovary, parathyroid gland, brain and muscle. We found no correlation between the location or type of mutation and the disease severity.

In XLHR osteoblast is likely to produce some inhibitor. Moreover, it was reported that cross-transplantation of kidneys in hyp-children results in transfer of the mutant phenotype. It can be associated with the primary defect in osteoblasts, as the correction of hyperphosphatemia and calcitriol in patients were observed low mineralization zones around osteolytic lacunae. XLH is the most frequent form of hypophosphatemia rickets, with a prevalence of 1/20.000. The disease affects both sexes. Patients with early onset disease have phosphate wasting, rickets, and lower extremity deformities in childhood.

Characteristics heritable dental developmental anomalies: enamel hypoplasia, dentinogenesis imperfecta, enlarged dentinal tubules, leading to tooth abscess. Characteristic cranial base abnormalities: thickening of outer cortical table of frontal bone and slightly sunken median line between the eyes at the forehead. Osteoarthritis of the lower extremities is developed in adults, osteophytes are formed and in some cases hearing loss may occur. Muscular weakness and hypotension are not observed. Other clinical manifestations, such as enthesopathy (calcification of ligaments and their attachment to bone), which is accompanied by joint pain and joint mobility disorders (Baroncelli et al., 2012).

Laboratory diagnosis: hypophosphatemia with low renal phosphate reabsorption, normal serum calcium values, normal or low vitamin D serum level ($1,25(OH)_2D_3$ or calcitriol), normal serum parathyroid hormone levels and increased serum alkaline phosphatase activity.

Therapy aimed at normalization of PHT levels with calcitrol supplementation and calcitriol. Correction of vitamin D deficiency state is not required. Calcitriol supplementation may prevent secondary hyperparathyroidism resulting from increased phosphate intake (Gaucher et al., 2009).

Conclusions

Although the diagnosis of tubulopathy remains complex and requires expensive laboratory equipment and specialist expertise; it can be diagnosed in children showing the following symptoms: impaired growth, vitamin D resistant rickets (lower limb deformities between 2 and 3 years of age). In the evaluation of such patients urine analysis is commonly used (levels of calcium, phosphorus, pH, bicarbonate, sodium, potassium, glucose, creatinine, protein, amino acids), blood count (levels of creatinine, uric acid, alkaline phosphatase, glucose, pH and sodium, bicarbonate, potassium, chloride, calcium, phosphorus ions) and ultrasound of the kidneys to detect nephrocalcinosis. Determination of serum parathyroid hormone concentration, vitamin D metabolites, aldosterone and plasma renin activity, cysteine lymphocyte concentration (suspicion to diagnose cystinosis) and ophthalmologist examination may also be used as additional diagnostic methods. Despite the fact that most tubulopathies can be diagnosed clinically, molecular genetic studies are needed to clarify the type of inheritance and prognosis.

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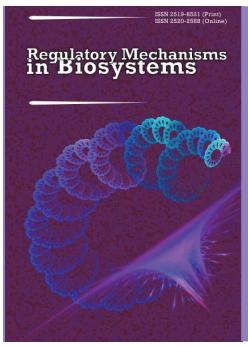

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 47–55
doi: 10.15421/021806

Modern magnetic immunoassay: Biophysical and biochemical aspects

O. Y. Galkin, O. B. Besarab, M. O. Pysmenna, Y. V. Gorshunov, O. M. Dugan

National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute", Kyiv, Ukraine

Article info

Received 10.10.2017
Received in revised form 15.11.2017
Accepted 19.11.2017

National Technical University of Ukraine "Igor Sikorsky Kyiv Polytechnic Institute", Peremogy av., 37, Kyiv, 03056, Ukraine.
Tel.: +38-044-240-90-36.
E-mail: a.galkin@kpi.ua

Galkin, O. Y., Besarab, O. B., Pysmenna, M. O., Gorshunov, Y. V., & Dugan, O. M. (2018). Modern magnetic immunoassay: Biophysical and biochemical aspects. Regulatory Mechanisms in Biosystems, 9(1), 47–55. doi: 10.15421/021806

In this review article an analysis of the biochemical and biophysical aspects of modern magnetic immunoassay (MIA) is conducted and additionally the problems and perspectives of its application in biology, biotechnology and medicine are defined. Magnetic immunoassay should be considered as an evolutionary extension of the classical immunoassay. MIA can have many variants of modifications, similar to the classic immunoenzymatic assay. The key distinctive element of the MIA is the use of magnetic particles (MPs), which are usually nanoparticles. MPs in the MIA can act as a marker for detection, or the solid phase at which the immunochemical reaction takes place. MIA possesses basic advantages over classical immunoassay methods: thanks to the unique magnetic properties of the MPs and the ability to manipulate it in the external magnetic field, it is possible to increase the informative value of the analysis (first of all, sensitivity and specificity), as well as the rigid requirements for "purity" of tested samples. For the purposes of immunoassay, magnetic particles of size from 10 to 200 nm are important, since such particles are in a superparamagnetic state, in the absence of strong magnetic fields; they are not agglomerated in a liquid medium. The size of the spherical particle determines the rate of sedimentation and mobility in the solution. The outer polymeric membrane serves as a matrix in which the surface functional groups are added, and also protects the core of the metal from the external environment. The outer shell may also consist of agarose, cellulose, porous glass, silicon dioxide etc. There are several strategies for the synthesis of nanoparticles: mechanical (dispersion), physical (gas phase deposition), wet chemical methods (chemical comprecipitation, thermal decomposition, methods of micro emulsion, hydrothermal reactions) and physico-chemical methods. Also used are magnesite nanoparticles of biogenic origin. Magnetic particles may function, and this is important for immunoassay. Surface functional groups include carboxylic, amino, epoxy, hydroxyl, tosyl, and N-hydroxysuccinate-activated groups. Magnetic spherical particles usually interact with surface molecules such as streptovidine, biotin, protein A, protein G, and immunoglobulin etc. Directions and prospects of the development of methods of magnetic immunoassay are determined, mainly, by the development of methods for detecting or influencing magnetic particles. In this case, the classical methods of detection are electrochemical methods, electrochemiluminescence, fluorescence. More modern ones include giant magnetoresistance, superconducting quantum interference devices, surface-enhanced Raman spectroscopy, biosensors based on nonlinear magnetization, magneto-PCR immunoassay. The current trend is to combine or integrate the application of various biochemical, physical, molecular and genetic, physico-chemical detection methods. In fact, all of these benefits undoubtedly open up broad prospects for the practical application of MIA in biology, biotechnology and medicine.

Keywords: serologic diagnostics; magnetic particles; biochemical and physical methods

Introduction

Among the whole complex of methods of clinical laboratory diagnostics, methods of serologic diagnostics were among the first to be proposed and implemented in practical medicine. Serologic diagnostics remains extremely relevant to the present day. Serologic methods are used for diagnostics of infectious (bacterial, viral, fungal, parasitic), and non-infectious (oncological, endocrine, allergic) diseases. A significant proportion of diagnostic examinations carried out by the laboratory service relates precisely to serological tests. Serologic methods remain an indispensable part of the provision of sanitary and epidemiological well-being of the population (Galkin, 2014a).

One of the modern trends in laboratory medicine is the intensive use of various nanotechnologies, which should include methods using magnetic nanoparticles (MNPs). MNPs have already become an important tool in clinical laboratory diagnosis and medical imaging in vivo. Important prerequisites for the successful use of MNPs for medical purposes are their high "bioavailability", which is achieved both by the size of the particles, and the possibility of their functionalization (using covalent and non-covalent methods) and purposeful targeting under the influence of external magnetic fields, as well as the stability of physical characteristics (magnetization, size) and the possibility of their modeling depending on specific medical and biological tasks. The size of the magnetic particles (MPs) and the intensity of the external magnetic fields can be selected so that the effect of such MPs in living objects will be "physiological" (force can vary from 10^{-12} to 10^{-9} N) (Aseri et al., 2015). Magnetic materials used in this case (compounds of iron, cobalt, nickel, etc.) are "technological", yet not all of them are characterized by an acceptable level of biocompatibility (when it comes to their in vivo use) (Wu et al., 2015; Foglia et al., 2017). All of the above, combined with the internal permeability of magnetic fields in human and animal tissues, offers an extremely wide range of possibilities for using MPs in biomedicine (Ghodbane et al., 2013; Issa et al., 2013). The use of magnetic nanotechnologies in laboratory diagnostics allows the elimination of the imperfections and limitations that are typical of traditional immuno-assays (enzyme-linked immunosorbent assay, immunofluorescence, etc.) (Mani et al., 2011; Day et al., 2015; Wang et al., 2017a; Liao et al., 2017). In particular, it becomes possible to disclaim strict requirements to the "purity" of the tested material, but also expand the range of

materials that can be tested; it is possible to increase the analytical sensitivity of the analysis, to improve other bioanalytical characteristics, etc. (Tsai et al., 2007; Lin et al., 2013; Li et al., 2014; Manera et al., 2017; Nie et al., 2017).

The purpose of our work is to analyze the current state of the use of magnetic immunoassay (MIA) in fundamental and applied research in biology, biotechnology and medicine.

Problems and benefits of using MPs in immunoassay

Widespread use of magnetic particles in immunoassay is due to the following circumstances. With the help of MPs, it is possible to increase the sensitivity and reduce the time of analysis by manipulating the particles with an external magnetic field, magnetic laundering and magnetic separation. In addition, as detected labels, MPs have advantages over traditional fluorescent and enzyme markers, whose application in opaque or highly dispersed biological media has a number of severe limitations. There are two main directions of use of MPs in immunoassay: firstly, MPs can act as a solid phase for immune complex formation, and, secondly, MPs can act as labels, for providing detection in the analysis (Fig. 1).

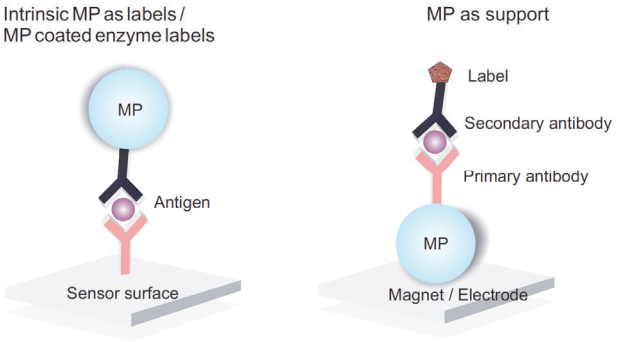

Fig. 1. Principal approaches to the use of MP in immunoassay (by Mani et al., 2011)

It should be noted that the studied bioanalysts (for example, biological fluids or food samples) are often complex colloidal systems, which, in turn, often leads to false-positive or false-negative results of immunoassay due to nonspecific reactivity of antibodies. Also it should be noted that in optical detection the color or autofluorescence of the sample can contribute to the recorded signal, increasing the noise and thus reducing the signal to noise ratio.

The above circumstances determine the widespread use of MPs for preliminary purification of the analyte (magnetic separation) (Choi et al., 2001; Nagasaki et al., 2007). With this approach, at the first stage, MPs with immobilized antibodies are added to the samples, and the antibodies recognize the wanted antigen. An external magnetic field is then applied and the antigen-enriched MPs can be used as a purified solid phase for further analysis (Fig. 1). Such a technique reduces the probability of non-specific binding and reduces the time of analysis (Gehring et al., 2004; Orlov, 2014). The use of magnetic particles as a solid phase increases the antigen's stability during immunoassay because, firstly, the number of washing steps is reduced and, secondly, the laundering process itself becomes more efficient due to the possibility of using an external magnetic field for solid phase manipulation (Choi et al., 2001; Orlov, 2014).

Fluorescent and enzyme labels used in immunoassay have a number of methodological limitations, which sometimes require laborious detection methods, which do not always have high reliability and reproducibility; therefore, it is very promising to use MPs in immunoassay as labels for detection (Orlov, 2014). Magnetic labels, as a rule, consist of nanoparticles of iron oxide in the size of 5–50 nm, which are enclosed in a polymeric membrane and form a particle in the size from 20 nm to 5 μm. Magnetic nanoparticles of such sizes have a unique magnetic property – lack of residual magnetization (so-called superparamagnetic nanoparticles). The phenomenon of superparamagnity is widely used for biomedical purposes, e.g., in magnetic resonance tomography (Smith-Bindman et al., 2012), but the idea of using it for the detection of magnetic nanoparticles in immunoassay is relatively new (Luo et al., 2017; Xue et al., 2017; Sood et al., 2017). Magnetic particles have a number of advantages over standard optical labels. Firstly, since the level of the magnetic background in biological samples is usually negligible, using MPs can produce a very high signal to noise ratio. Secondly, the opacity or color of the samples does not affect the magnetic properties of MPs. Thirdly, the magnetic tags are stable, and their magnetic properties do not change. Fourth, with the help of an external magnetic field, it is possible to manipulate of MPs, to increase efficiency and reduce analysis time by magnetic stirring, washing and separating (Morozov et al., 2007; Nikitin et al., 2008a; Dittmer et al., 2010; Orlov, 2014). Currently, the use of magnetic nanoparticles as labels for immunoassay in combination with the active influence on it is popular.

Note that using MPs as a label has its own peculiarities and disadvantages. The most significant of them is the difficulty in achieving satisfactory analytical (validation) characteristics of the method, which primarily concerns linearity. Linearity is known to represent the ability of the technique (within the range of application) to give a value directly proportional to the concentration (amount) of the analyte in the sample (Galkin et al., 2015; Lutsenko et al., 2017). Such a situation, for example, occurs when determining staphylococcal enterotoxins in complex biological fluids. The use of MPs in combination with the fluidic force discrimination method for detecting enterotoxins using a flat chip and optical reading allowed high sensitivity to be achieved: the detection limit was 1 pg/ml and 1 fg/ml for multistage and semi-homogeneous analysis formats, respectively. However, with an increase in the concentration of antigen by 10 orders of magnitude, there was only a fourfold increase in the recorded signal (the signal increased by only 15% with a tenfold increase in the concentration of enterotoxins). The authors note that it is difficult to distinguish concentrations that differ by less than 10 times (Mulvaney et al., 2007; Mulvaney et al., 2009). Magnetic particle manipulation in combination with electrophoretic concentration of a toxin on the surface during active analysis leads to an increase in the optical signal by 1.7 times with a tenfold increase in the antigen concentration. Taking into account the distribution of observed signals, the authors conclude that the analysis can only provide a quailtative result, determining the presence or absence of a toxin. In addition, active analysis requires pre-centrifugation and desalting complex mediums (Shlyapnikov et al., 2012).

In this case it should be noted that all immunochemical methods are not always characterized by a satisfactory linearity, even providing mathematical transformation of the results of the study. Under these circumstances it is possible to move from the linearity as such to finding the proper concentration of analysis function in the samples under study (Chen et al., 2016; Giannetto, et al., 2017). One of the ways to overcome such a disadvantage is the following. When detecting enterotoxins, the number of MPs-labels can be determined not only optically, but also using biosensors based on the giant magnetic resistance (GMR). The successes in the development of GMR-biochips made them three orders of magnitude more sensitive than the enzyme-linked immunosorbent assay (ELISA). However, in order to double the signal, it is usually necessary to increase the concentration of antigen in order, and the full range of signal changes is one and a half order. Some approaches, such as enhancement of the GMR-signal by moving the magnetic particles into a zone with the highest sensitivity or optical reading of the magnetically activated particles, can increase the range of detected signals to two orders of magnitude (Kurlyandskaya et al., 2017; Rizzi et al., 2017; Wang et al., 2017a; Salek-Maghsoudi et al., 2018).

Characteristics of magnetic particles and methods for obtaining them

For the purposes of immunoassay, magnetic particles of size from 10 to 200 nm are important, since such particles are in a superparamagnetic state, in the absence of strong magnetic fields; they are not agglomerated in a liquid medium. Properties of magnetic nanoparticles and polymer clusters containing nanoparticles largely depend on the pro-

perties of the magnetic material. One of the main properties is magnetic susceptibility χ_m, which characterizes the dependence of the magnetization of a substance on the intensity of the external magnetic field H (Tygai et al., 2014; Wu et al., 2016; Tang et al., 2017):

$$M = \chi_m \times H.$$

The characteristic magnetization curve for MPs is shown in Fig. 2. At the same time, it is important that such value of magnetic field intensity HS corresponds to magnetic saturation MS. For immunoassay purposes, it is advisable to use MPs markers with greater magnetic susceptibility and higher magnetization of saturation with the same initial magnetic susceptibility of the particle (Orlov, 2014). This kind of characterization of the particles of iron oxides, and in particular of magnetite, is determined mainly by their crystalline structures and essentially depends on the conditions for the reaction to produce them (Rajput et al., 2016; Vidojkovic et al., 2017). The best magnetic properties of magnetite are achieved with an equal molar ratio of iron oxides (II) and (III).

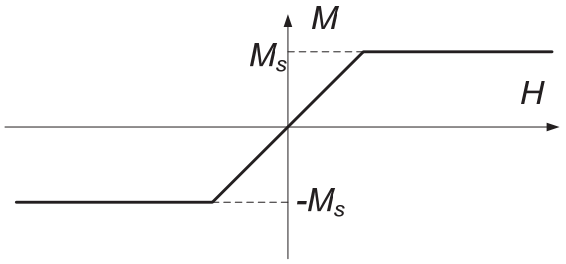

Fig. 2. Simplified MPs magnetization characteristic

It should be noted that at present, magnetic spherical particles of various types are available as commercial products for use in scientific and applied applications. Paramagnetic particles are most applicable to systems whose purpose is magnetic separation and transportation, since they acquire magnetism in the presence of a magnetic field but have zero magnetization in the absence of a magnetic field.

The most common examples of paramagnetic particles are magnesium oxide nuclei and non-magnetic polymer shells. It is the polymeric surfaces of such particles that provide the functional ability to chemically attach biomolecules. Metal oxides are used to create magnetic core more often than pure metals (Fe, Co, Ni), since they have higher oxidation resistance (Table 1). Polymer shells also stabilize magnetic particles, giving them elasticity and ability to swell.

The magnetic core can also consist of a set of paramagnetic nanoparticles located in the core of the polymer. Typically, nanoparticles of a size range from 100 nm to 50 μm in diameter are commercially available. The size of the spherical particle determines the rate of sedimentation and mobility in the solution. The outer polymeric membrane serves as a matrix in which the surface functional groups are added, and also protects the core of the metal from the external environment. The outer shell may also consist of agarose, cellulose, porous glass or silicon dioxide (Day et al., 2015).

There are several strategies for the synthesis of nanoparticles: mechanical (dispersion), physical (gas phase deposition), wet chemical methods (chemical comprecipitation, thermal decomposition, methods of micro emulsion, hydrothermal reactions) and physico-chemical methods. Also used are magnesite nanoparticles of biogenic origin, forming certain types of bacteria.

Table 1
Saturation magnetization and magnetization susceptibility of some magnetic metal oxides (Philippova et al., 2011)

Oxides	Magnetization saturation, emu/g	Magnetization susceptibility
γ-Fe_2O_3	74	-5×10^{-6}
Fe_3O_4	84	$+18 \times 10^{-6}$
Fe_2O_3–Fe_3O_4	~80	$+7 \times 10^{-6}$
$CoO \cdot Fe_2O_3$	65	-110×10^{-6}

Wet chemical methods have been studied more widely than physical ones, since they can provide a higher level of control over the size, composition, magnetic properties, and the form of magnetic nanoparticles. This is especially important for screening cells in a liquid medium (Zhu et al., 2016).

The water-in-oil microemulsion (or reverse micelle) method provides the following: nanoparticles are formed by an isotropic dispersion or by mixing two liquids that form microdermas. Such micro domains are stabilized by the interphase film of the surface-active substance. This method can be considered as a derivative of deposition or a method of recovery with the difference that the reaction occurs in small droplets of water stabilized in an organic solvent. When mixing two microemulsions containing the necessary reagents, the micro domains come in contact and collapse again when stirred, resulting in the formation of a precipitate containing nanoparticles (Zhang et al., 2017; Beshkar et al., 2017). The low yield of nanoparticles, compared with other methods, as well as the need for a large amount of solvent, jeopardizes the efficiency and production scale.

Hydrothermal synthesis allows a wide range of nanostructured forms and compositions to be obtained. This synthesis method is based on phase change and separation, which occurs at the interface between liquid, solid and soluble phases (Wu et al., 2016). An example of the use of hydrothermal synthesis for the manufacture of monodispersed magnetic nanoparticles in the range from 200–800 nm is described. A mixture of iron salts (e.g., $FeCl_3$), a high boiling point boiling substance (e.g. ethylene glycol), an electrostatic stabilizer (for example, sodium acetate) and a surfactant (for example, polyethylene glycol) are heated to 200 °C, and maintained at this temperature for 8–72 hours in a sealed autoclave made of stainless steel (Han et al., 2012).

Nanoparticles of metal oxides, in particular, magnetite Fe_3O_4 and magnetite γ-Fe_2O_3, are often synthesized with the use of alkaline co-precipitation of iron and iron salts (Mohapatra et al., 2010).

Synthesis of iron oxide nanoparticles with carboxymethyldextran and polyethylenimine polymeric membranes. Nanoparticles of iron oxide are synthesized by the method of co-precipitation (co-precipitation) of iron salts $FeCl_3$ and $FeCl_2$. The most common protocol for such synthesis involves the following. Use 5.9 g $FeCl_3 \times 6H_2O$ and 2.15 g $FeCl_2 \times 4H_2O$, mixed in 100 ml of degassed water, followed by addition of 12.5 ml of 30% NH_4OH. The solution is heated to 85 °C, and incubated for 2 hours. The formed suspension of particles is washed with 2M HNO_3 for peptizing the particles and also three times by degassed water. Then the aggregates are removed using a magnet, and the nanoparticles in the supernatant are covered with polymers. A solution of carboxymethyldextran (CMD) or polyethylenimine 25 kDa at a concentration of 300 g/l is added to nanoparticles to a final concentration of 50 g/l and incubated for 4 hours at 80 °C. The resulting particles are washed off the free polymer by centrifugation at 16,800 g for 1–3 hours (Mohapatra et al., 2010).

Synthesis of ferritic nitride nanoparticles coated with a carboxymethyldextran polymer shell. Nanoparticles of ferritic acid are most often synthesized according to the method (Shevchenko et al., 2017), which provides the following. 8.85 g $FeCl_3 \times 6H_2O$ is used in 100 ml of degassed water; the salt is precipitated with addition of 12.5 ml of 30% NH_4OH and incubated for 2 h at 90 °C. Subsequently peptizing the particles using 0.6 M HNO_3 for 10 minutes is performed and washed with HNO_3 by three-times centrifugation, after which the particles are coated by CMD at 80 °C for 4 hours, followed by three-times centrifugation to wash off the unbound polymer.

Synthesis of golden nanoparticles. Golden nanoparticles are synthesized by the reduction of the hydrochloric acid of $HAuCl_4$ with sodium citrate (Santhoshkumar et al., 2017).

Biosynthesis of magnetic particles. Intracellular biogenic magnetic nanoparticles (BMNPs) in the form of crystals of magnetite, magma and grazhite are found in many organisms, including bacteria, insects, mushrooms, fish, birds, animals and others (Gorobets et al., 2017). BMNPs are also found in normal tissues of the brain, liver, heart, spleen, and also in human tumor tissues (Gorobets et al., 2014a). The genetic regulation of the synthesis of these nanoparticles is thoroughly studied solely for magnetotaxis bacteria. The so-called genes of the magnetosomal islet of such bacteria responsible for the synthesis of magnetos are revealed. The use of natural magnetic properties of microorganisms containing BMNPs, as well as the development of new

technologies for the creation of synthetic analogues of BMNPs *in vitro*, using biomineralization proteins, will allow the acquisition of magnetic nanoparticles with controlled parameters, which is an extremely important task for many technologies: for immunoassay, purposeful delivery of medicinal preparations, magnetic separation of biological media (Gorobets et al., 2013a, 2013b, 2014b).

Enzymatic synthesis of magnetic nanoparticles. The first in vitro enzymatic synthesis of paramagnetic and antiferromagnetic nanoparticles toward magnetic ELISA reporting has been reported (Kolhatkar et al., 2015). With our procedure, alkaline phosphatase catalyzes the dephosphorylation of l-ascorbic-2-phosphate, which then serves as a reducing agent for salts of iron, gadolinium, and holmium, forming magnetic precipitates. The nanoparticles were found to be paramagnetic at 300 K and antiferromagnetic under 25 K. Although weakly magnetic at 300 K, the room-temperature magnetization of the nanoparticles found here is considerably greater than that of analogous chemically-synthesized samples. This approach of enzymatically synthesizing magnetic labels reduces the cost and avoids diffusional mass-transfer limitations associated with pre-synthesized magnetic reporter particles, while retaining the advantages of magnetic sensing.

Methods of magnetic particles functionalization

Surface functional groups include carboxylic, amino, epoxy, hydroxyl, tosyl, and N-hydroxy succinate-activated groups (Fig. 3). Magnetic spherical particles usually interact with surface molecules such as streptovidine, biotin, protein A, protein G, IgG, IgE, and IgM.

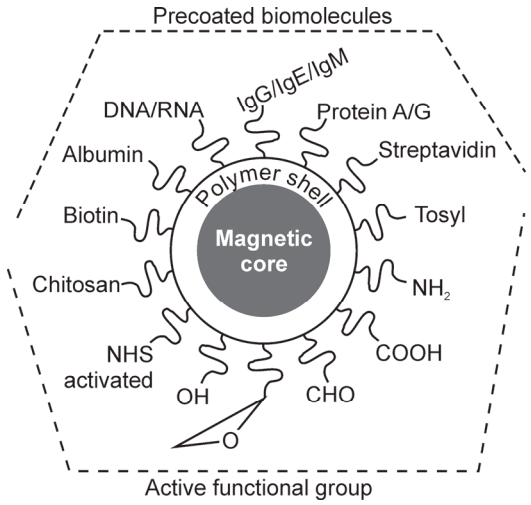

Fig. 3. Structure of MPs and surface functional groups (by Mani et al., 2011)

Surface functional groups can be activated by EDC-coupling chemistry for carboxylates and glutaraldehyde for amines in order to further interact with functional groups of biomolecules (EDC = l-ethyl-3-(3-dimethylaminopropyl) carbodiimide). Surface tosyl, N-hydroxy succinate-activated and epoxy groups can be used to attach biomolecules without the use of cross-linking agents. Particles pre-coated with streptavidin can interact with biotinyled biomolecules. Magnetic particles coated with protein A can selectively bind to Fc-regions of antibodies for the purpose of targeted immobilization (Mani et al., 2011).

Magnetic particles consisting of paramagnetic nanoparticles that are in the polymer matrix of the nucleus can have a multi-domain magnetic structure with residual magnetic moments. Such a structure of the nucleus of a magnetic nanoparticle can lead to magnetic clusterization due to the induced magnetism of neighboring particles. At room temperature, real paramagnetic particles, whose nuclei are constructed of iron oxide, should have radii in the low range (~ 10^{-9} m). In this way, usually, particles of 0.1–3.0 μm in diameter, whose nuclei are constructed of nanoparticles of iron oxide, are coated with a polymer. Such particles are characterized by clusterization in the dispersion due to magnetic interactions between particles (Hermanson, 2008; Mani et al., 2011).

Covalent conjugation of nanoparticles with proteins. The nanoparticles coated with the polymers with HOOC-groups covalently bind to protein molecules using EDC as a crosslinking agent (crosslinker). During this reaction, an intermediate product, the derivative of O-acylizoic acid is formed, and it undergoes a nucleophilic attack on the amino group of the protein, resulting in the formation of a stable amide bond between the amino group and the carboxyl group on the surface of the particle. To increase the stability of the active intermediate and reduce the probability of hydrolysis EDC should be used together with N-hydroxysulfosuccinimide (Nhydroxysulfosuccinimide, sulfo-NHS). This reaction is carried out in two steps: first, the nanoparticles are activated with EDC/sulfo-NHS in the MES buffer (based on 2-(N-morpholino)ethanesulfonic acid), then, after removing excess crosslinkkers centrifuged or using a magnetic tripod, the protein is added in the appropriate buffer. Optimal protein ratio: nanoparticles: EDC/sulfo-NHS should be selected experimentally for each type of conjugate. In order to prevent the aggregation of the nanoparticles during the reaction, it is advisable to periodically process the ultrasound bath. The reaction is usually carried out for at least two hours, after which the excess of the unreacted protein is removed by centrifugation (for particles < 150 nm) or by magnetic separation (for particles > 150 nm) (Rusling et al., 2010; Shipunova et al., 2013).

Methods for detecting of systems based on MPs

Requirements for magnetic particles and their synthesis depend on the methods by which they are planned to be used in bioanalysis. Moreover, the control of the synthesis of magnetic particles and the selection of conditions of immunoassay should be carried out using the registration device. Below we provide characteristics of the main approaches for detecting magnetic particles in immunoassay – when MPs are a label (giant magnetoresistance, superconducting quantum interference devices, electrochemical methods et al.), and when MPs are a basis for the immune complex formation (electrochemical methods, electrochemiluminescence, fluorescence, magneto-PCR immunoassay et al.).

Giant magnetoresistance (GMR) is the quantum and mechanical effect observed in thin metal films, consisting of ferromagnetic and conductive non-magnetic layers alternating between them. The effect is a significant change in the electrical resistance of such a structure when the mutual direction of magnetization of the neighboring magnetic layers is changed. The direction of magnetization can be controlled, for example, by the influence of the external magnetic field (Fert, 2008). In the absence of an external field, the magnetic moments of the neighborring ferromagnetic layers are oriented antiparallelly, which ensures high electrical resistance of the structure. If the GMR sample is placed in an external magnetic field, the magnetic moments of all ferromagnetic layers are aligned along the directions of this field, which causes a decrease in the structure's resistance. At zero value of the external field the resistance is maximal. With increasing field, the resistance initially falls linearly, and then goes to saturation. Depending on the conditions and material, the maximum resistance of the sensor may be more than minimum value by 1.2–5.0 times. Thus, by changing the resistance of the GMR-sensor one can judge the presence of an external magnetic field on it.

For the detection of magnetic particles, the GMR-structure is placed in an external magnetic field perpendicular to the surface of the sensor. Such an external field does not affect the structure's resistance, since the resistance change can only be caused by the field that lies in the sensor plane. The magnetic particle on the surface of the GMS structure causes a change in the distribution of the magnetic field so that the component of the external field appears along the sensor plane. Consequently, the resistance of the GMR-structure decreases when MPs are on its surface. Thus, using the GMR-structure, the presence of magnetic particles on the surface of the sensor can be detected. For immunoassay, the surface of the GMR sensor is modified to immobilize antibodies that will specifically bind to the antigen tested when the test samples are passed (Fig. 4). After that, a solution of MP, coated with antibodies to another antigen epitope, is applied to the sensor surface. By changing the resistance of the GMR structure, the amount of MPs that are connected

to the sensor surface (Kim et al., 2013) is calculated. It should be noted that the bioanalytical application of GMR sensors has a number of limitations. A magnetic sensor detects distortion of the external magnetic field by a particle. However, the spatial scale of such distortion can be compared with the size of the particle. Therefore, with the standard technologies of GMR-sensors creating, only magnetic clusters of micron-size, comparable to the dimensions of the sensitive sensor element, are used. Such clusters are much larger compared to molecules that are recognized or revealed during the analysis, which imposes a number of restrictions on the use of such technology. In addition, the magnetization of small magnetic particles is small enough, so the signal is recorded with a low signal to noise ratio (Orlov, 2014; Rizzi et al., 2017; Crespo et al., 2018).

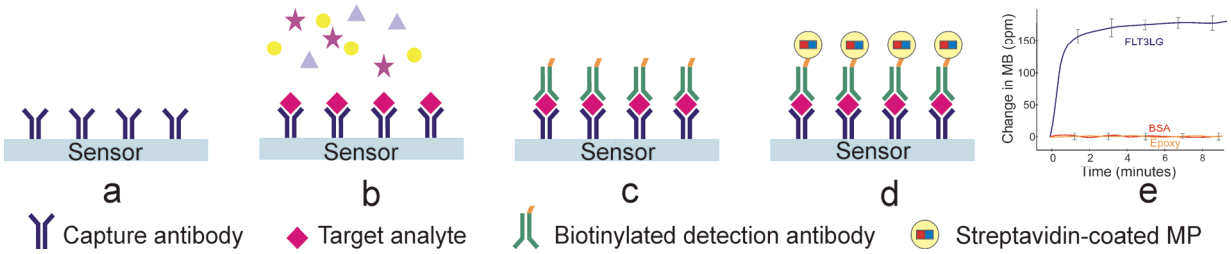

Fig. 4. A schematic of magneto-nanosensor biochip immunoassay: (*a*) Capture antibodies are immobilized covalently on the sensor surface. (*b*) Target antigens are captured and noncomplementary antigens are subsequently washed away. (*c*) Addition of biotinylated detection antibody forms a sandwich structure. (*d*) Streptavidin-coated magnetic nanoparticles bound to the biotinylated detection antibody produce stray magnetic field. (*e*) An example of real-time binding curve showing the change in magnetoresistance (MR) in parts per million (ppm) over time for 500 pg/ml Flt3lg (blue) compared with BSA negative control (orange) and epoxy reference (red) (by Kim et al., 2013)

Superconducting quantum interference devices (SQUIDs). SQUIDs are ultrasensitive magnetometers used to measure very weak magnetic fields. SQUIDs have a record high sensitivity that reaches $5 \cdot 10^{-33}$ J/Hz (sensitivity to a magnetic field of about 10^{-13} T). For long-term measurements of averaged values within a few days, sensitivity values of $5 \cdot 10^{-18}$ T can be achieved (Drung et al., 2007). There are two types of SQUIDs: based on direct current and high-frequency devices. The work of SQUIDs on alternating current is based on the non-stationary Josephson effect and uses only one Josephson contact. Many of the experiments in fundamental physics and measurements in biomagnetism, including the measurement of excessive signals, are performed using alternating current SQUIDs. The exceptionally high sensitivity to the magnetic flux relies on the entire spectrum of medical applications of SQUIDs (magnetoencephalography, magnetogastrography, magnetic marker monitoring, and heart research). There are also considerations regarding the application of SQUIDs in a quantum computer as qubits (Vesanen et al., 2013).

Unlike traditional magnetometers in which SQUIDs are used as passive low-frequency or permanent magnetic field sensors, an alternating current of the microwave frequency circulating around the SQUID ring is used in a scanning SQUID microscope when a constant voltage occurs on its Josephson junctions (a non-stationary Josephson effect). The basic principle of the fact that the microwave current flows in the SQUIDs ring is easier when a corresponding sample is next to it (Vesanen et al., 2013).

Recently SQUIDs-magnetometers have been used for immunoassay (Nakatani et al., 2012; Saari et al., 2015; Park, 2016; Rong et al., 2016; Liao et al., 2017). Thus, the use of magnetic markers in combination with the detection of SQUIDs allowed reducing detection in a standard ELISA. Another example of the application of SQUID technology is the detection of bacteria by the time of magnetic relaxation. In this approach, the magnetic particles are added to the model under study, immobilized antibodies on the surface, bacteria specifically recognized for the recognition. Further, this template is placed in a homogeneous external magnetic field. The magnetic moments of the parts while guided parallel to the field. If you remove the source of the external magnetic field, the particles will return to the state of minimum energy, when the total magnetic moment of the system will be zero. In this case, two mechanisms of relaxation of the magnetic moment will compete: Brownian and Néel. Brunov's mechanism is associated with the physical turn of himself, and Néel's – with the turning of the magnetic moment inside the stationary particle (Wang et al., 2015, 2016).

For free particles, a fast Brownian relaxation mechanism will dominate, but for particles that bind to bacteria, the slower Néel mechanism becomes predominant, since the rotation of such particles is complicated. Thus, the total time of magnetic relaxation will depend on the number of parts that are associated with the bacteria, and hence the concentration of the bacteria themselves in the sample. With SQUIDs, the dependence of the magnetic signal on time is measured and the relaxation time is calculated. At the time of magnetic relaxation, the presence and concentration of bacteria in the sample are judged. An essential advantage of using the method of magnetic relaxation is that the analysis is carried out in one step, without any blurring steps. Nevertheless, there are significant difficulties: the need for cryogenic cooling, labor-intensive calibration of the method, etc., which does not allow wide use of this approach in biochemical diagnostics (Eberbeck et al., 2008; Orlov, 2014).

Biosensors based on nonlinear magnetization. The method of detecting magnetic nanoparticles by their nonlinear magnetization is based on the effect on the particles of the external alternating magnetic field at two frequencies. For the construction of such biosensors, the main consideration is the choice of frequency and amplitude of the corresponding components of the external field. The amplitude of field intensity H1 for the fields with a lower frequency should be greater than the magnitude of field intensity of saturation HS of MPs core. In this way, the low-frequency component of the magnetic field will periodically block the ability of the magnetic particles to further magnetization. The equivalent field intensity H (t) (Fig. 5c) consists of both the sum of the low-frequency components H1 (t) (Fig. 5a) and the high-frequency component H_2 (t) (Fig. 5b). Under conditions of the MPs magnetization for the simplified characteristic (Fig. 5), and no consider the magnetic hysteresis phenomenon, we obtain a graph of MPs magnetization (Fig. 5d), which is vertically limited by the values of saturation magnetization ± MS. The resultant induction signal associated with the presence of MPs will be nonlinearly modulated by both frequencies (Manera et al., 2017; Nikitin et al., 2017). Subsequently, the mathematical analysis of the results recorded by the sensor, the decomposition of the signal in the Fourier series, the selection of the component of high-frequency oscillations, and the corresponding idealized (no-noise) $m < 2 > (t)$ curve are shown in Fig. 5e.

In real conditions, the high-frequency component of the signal will have significant noise, caused, in particular, by the presence of constructive elements of the sensor, having magnetic properties. One of the approaches to noise isolation is the use of MPs with standardized magnetization characteristics, which will have an average value of the existence of a high frequency component of the detected signal for half the period of the low frequency signal. What makes it possible to isolate from the signal only the high-frequency component, which has the required duration (caused by MPs itself). This approach is a rather reliable method for registering minor changes, even in the presence of significant background noise – in fact, there is a possibility to significantly increase the sensitivity of the analysis. The promise of such a method is due to the fact that it can be used *in vivo* (Nikitin et al., 2008b).

Surface-enhanced Raman spectroscopy (SERS) is a surface-sensitive technique that enhances Raman scattering by molecules adsorbed on rough metal surfaces or by nanostructures such as plasmonic-magnetic silica nanotubes. The enhancement factor can be as much as 10^{10} to 10^{11} which mean the technique may detect single molecules (Xu et al., 2013).

Surfaces with nanoparticles, prepared for the detection of Raman scattering, are used to detect biomolecules, and therefore can determine the presence of proteins and biological fluids. This technique was used to detect urea in plasma, and can be considered as a candidate for a new generation of cancer diagnostic methods. The ability to analyze the composition of the mixture at the nanoscale makes the surfaces prepared for SERS promising in environmental studies, pharmaceutics, in the analysis of real experiments, for the detection of narcotic substances and explosives, the analysis of food quality, the detection of individual algal cells, etc. SERS in combination with plasmonic elements can be used for highly sensitive methods for detecting the interaction between biomolecules (Yang et al., 2013; Han et al. 2014; Li et al., 2014; Pallaoro et al., 2015; Lin et al., 2016; Xu et al., 2016).

Electrochemical biosensors containing capture antibodies and secondary antibodies with a label (Fig. 1) are characterized by high indicators of informative analysis (Mani et al., 2011). Electrochemical sensors based on MPs are widely used in medical devices for *in vitro* diagnosis, and most of them are based on capture ELISA (Tang et al., 2007; Tsai et al., 2007).

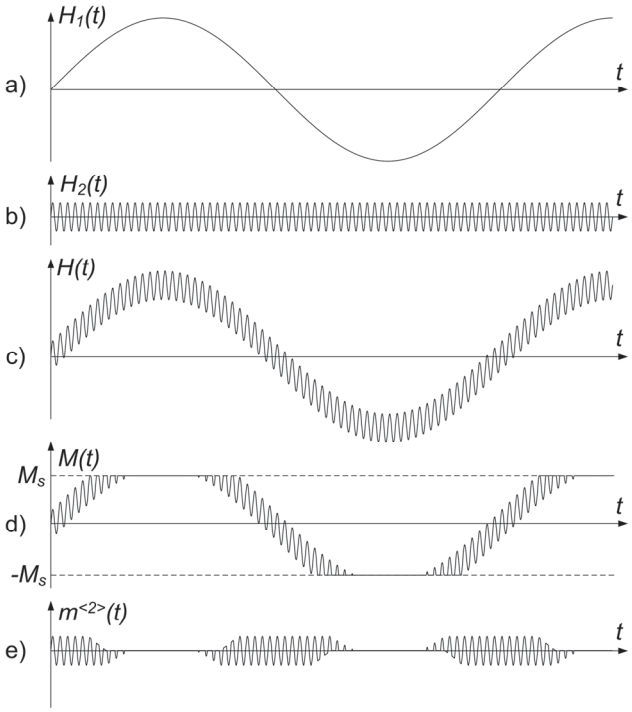

Fig. 5. The principle of detecting MPs on the basis of non-linear remagnetization

Electrochemiluminescence (ECL) detection envisages electrochemically generated light emission. As luminescent label most often are used tris (2,2′-bipyridyl) ruthenium (II). Often, such analytical systems contain avidin-biotin reagents based on the capture ELISA principle and have a quite satisfactory bioanalytical characteristic, which brings opportunities for their wide practical use (De Roeck et al., 2017; Nie et al., 2017; Wang et al., 2017b).

Magneto-PCR based immunoassay. This method can be considered as an analogue of ELISA in various modifications (including using the avidin-biotin signal amplification system) in which the label is not an enzyme but a DNA sequence. MPs function as a solid phase. Obviously, such an analysis is more cumbersome, compared with immuno-PCR, but it can provide even more impressive indicators of informativity, in particular, analytical sensitivity (Malou et al., 2011). For example, the authors managed to reach the limits of detecting the surface antigen of hepatitis B virus at 320 pg/ml (traditional ELISA test-kits have a sensitivity of about 10 ng/ml) (Wacker et al., 2007). Consequently, such analytical systems are designed to determine the small concentrations of bioanalys, including in complex materials.

Magnetic immunoassay automation. An important precondition for the widespread use of MIA in clinical laboratory diagnostics is possibility to automate and standardize of the analysis. The latter is related with the standardization of hardware implementation of the method. The most original automated application of MIA addresses the technology of epitopon mapping. Epitope mapping enables one tos obtain genetic engineering peptides (proteins) and their subsequent study as potential antigenic determinants. At present, many variants of this methodological approach have been developed, in particular: phage and bacterial display, site-directed mutagenesis, mapping of recombinant proteins with tag (Galkin, 2014b). The methodological principles of use of MPs in epitope mapping technologies were formed at the end of the 20th century (Kala et al., 1997; McConnell et al., 1999). Library-based display technologies have been staggeringly optimized since their appearance in order to mimic the process of natural molecular evolution. Display technologies are essential for the isolation of specific high-affinity binding molecules (proteins, polypeptides, nucleic acids and others) for diagnostic and therapeutic applications in cancer, infectious diseases, autoimmune, neurodegenerative, inflammatory pathologies etc. Applications extend to other fields such as antibody and enzyme engineering, cell-free protein synthesis and the discovery of protein–protein interactions. Phage display technology is the most established of these methods but more recent fully in vitro alternatives, such as ribosome display, mRNA display, cis-activity based display and covalent antibody display, as well as aptamer display and in vitro compartmentalization, offer advantages over phage in library size, speed and the display of unnatural amino acids and nucleotides. Altogether, they have produced several molecules currently approved or in diverse stages of clinical or preclinical testing and have provided researchers with tools to address some of the disadvantages of peptides and nucleotides such as their low affinity, low stability, high immunogenicity and difficulty to cross membranes (Galán et al., 2016).

When setting MIA usually requires the presence of a microtiter plate, a reader, a magnetic separator (used for washing the wells of the tablet), a thermostat, a multichannel pipette. An original example is the use of a pin-based magnetic particle processor to automate the method. The processor can accommodate several microtiter plates filled with different washing buffers and with different incubation periods. Consequently, the processor application ensures the standardization of such parameters as washing conditions, incubation time, and parallel tests in different buffers. Separate stages of magnetic separation occur when magnetic particles are transferred between the wells and rod-shaped magnets, coated with a plastic coating through successive seizure and release movements. Operating mode of magnetic particle processors: the rod-shaped magnet is covered by a plastic cap and moves into a solution containing suspended magnetic beads; moving slowly up and down, the beads are attracted to the cover, and by moving the covered magnet to the next position, the beads are transferred to a new solution; once the magnet is removed from the cap, the beads are slowly suspended again; the magnet head and plastic covers are raised to the starting position to proceed to the next stage of the process (Konthur et al., 2010).

Comparative characteristics of MIA and its prospects

The use of magnetic particles provides a number of advantages, including the ease of separation and the suitability for automation. After coating the magnetic particles with the ligand, they become suitable for selective capture and distribution of various molecular particles. In this case, undesired components, large or fibrous particles, and a viscous matrix of the sample can be washed after a simple stage of magnetic particle distribution. Thus, the high efficiency of magnetic separation prevents the effect of non-target molecules that create the background and provides the most sensitive detection of target molecules.

Magnetic particles are often used to detect antibodies/antigens for several reasons. The use of MPs makes it possible to increase the

surface area relative to the stable fluid volume in the well of a 96-well plate, thereby facilitating the interaction between antigen and antibody in a small volume (Lin et al., 2013). Increasing the area of the surface and uniform distribution of the particles throughout the sample provides the speed and sensitivity of determining the content of the molecules in question at low concentrations. In addition, magnetic spherical particles can be easily and quickly absorbed at the bottom of the well plate, and can be separated from the medium using a magnetic field (Lin et al., 2013). The method of magnetic ELISA is simple and fast, and, due to the very low non-specific binding background, it requires very small amounts of magnetic particles and a ligand. Preparation of magnetic parts is carried out easily and quickly. Magnetic ELISA also provides the availability of certain epitopes on the surface of the granules, that is, the availability of primary antibodies, in the case of setting sandwich variants of the method (Kourilov et al., 2002). The limitation in the formulation of a magnetic ELISA still is the interpretation of the results obtained at very low concentrations of target substances in the samples (Burgos-Ramos et al., 2012).

The main advantages of MIA compared with the traditional ELISA are as follows. First, the kinetics of the reaction of homogeneous small particles of magnesium added to the solution of the sample occurs quickly and effectively. The kinetics of the reaction when formulated with the usual ELISA is much slower, since only the bottom layer of the solution, captured on the surface of the polystyrene plate, directly contacts the test substance in the sample. Secondly, removing unbound reagents is more thorough when using homogeneous MPs, since the whole surface of the magnetic particles in the suspension is washed out. This approach provides the ability to remove most unbound reagents, which helps to achieve a lower nonspecific background, which in turn, improves the sensitivity of the analysis. Thirdly, even coverage ensures the delivery of particles with similar properties to all wells. During adsorption of molecules on the surface of the plate the phenomenon of sticking to the walls of the hole tablet may occur, which will create a background. Fourthly, the surface properties of the magnetic particles can be modified to maximize and/or orientate the molecules attached to their surface. Different molecules can be attached to the surface of the particles by passive adsorption or by covalent binding as needed. Fifthly, only magnetic particles can capture certain of the molecules analyzed in the suspension, ignoring other components in the solution. As a result, the target molecules can be concentrated in the precipitate for a few minutes without centrifugation. The sediment of magnetic particles with entrapped molecules analyzed can be transferred to the well of the plate for analysis.

Magnetic particles are characterized by numerous properties that make them suitable for widespread use in various fields – from visualization to drug delivery: easy to fabricate, manipulate in fluid, as well as a wide range of commercially available diameters of magnetic particles, ranging from nanoparticles (50 nm) to microparticles (up to ten microns) (Svobodova et al., 2015).

Magnetic particles have a wide range of applications: positive and negative selection of cells, allocation of molecular complexes. Due to the speed and ease of staging magnetic ELISA, this type of ELISA has found its application for clinical purposes (Hoyoung et al., 2013). Magnetic ELISA has been used for quantitative evaluation of immunoglobulins, rapid detection of circulating antigens, determination of cyclosporin A, and for other purposes. Selection of cells by means of magnetic particles has a certain advantage. The method is fast and easy to execute and does not require complicated hardware design. In addition, the method practically does not affect the viability of cells, it requires only very small amounts of the ligand, and can be performed in sterile conditions. Activated balls adsorb ligands, are economically affordable and can bind a large variety of molecules: antibodies, antigens, hormones, DNA and RNA. Thus, the MIA absorbed on the surface of the magnetic particle by a specific antibody is an excellent way to test the adsorption of the appropriate ligand and verify the availability of the appropriate epitopes. Another application of MIA is the detection of mutations in medical genetics. This method has been used for genotyping in samples from patients with predisposition to thrombophilia and detecting fusion transcripts of chromosomal translocations in children with acute lymphoblastic leukemia (Burgos-Ramos et al., 2012). MIA can also be used to detect pathogens and toxins present in food, water analysis (Orlov et al., 2013).

Conclusions

Magnetic immunoassay should be considered as an evolutionary extension of the classical immunoassay. MIA can have many variants of modifications, similar to the classic immunoenzymatic assay. The key distinctive element of the MIA is the use of magnetic particles, which are usually nanoparticles. MPs in the MIA can act as a marker for detection, or the solid phase at which the immunochemical reaction takes place.

MIA possesses two basic advantages over classical immunoassay methods: thanks to the unique magnetic properties of the MPs and the ability to manipulate it in the external magnetic field, it is possible to increase the informative value of the analysis (first of all, sensitivity and specificity), as well as the rigid requirements for "purity" of tested samples.

Directions and prospects of the development of methods of magnetic immunoassay are determined, mainly, by the development of methods for detecting or influencing magnetic particles. In this case, the classical methods of detection are electrochemical methods, electrochemiluminescence, fluorescence. More modern ones include giant magnetoresistance, superconducting quantum interference devices, magneto-PCR immunoassay. The current trend is to combine or integrate the application of various biochemical, physical, molecular and genetic, physico-chemical detection methods. Such complex approaches, on the one hand, allow one to achieve the best bioanalytical characteristics of the analysis, and, on the other hand, complicate the hardware design of the methods, which is not always favorable for practical use. In fact, all of these benefits undoubtedly open up broad prospects for the practical application of MIA in biology, biotechnology and medicine.

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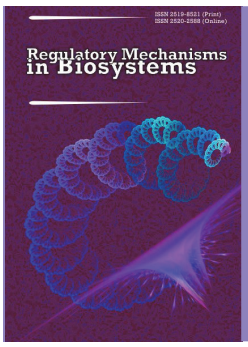

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 56–61
doi: 10.15421/021807

Variability of the antioxidant properties of *Berberis* fruits depending on the plant species and conditions of habitat

N. O. Khromykh*, Y. V. Lykholat*, I. M. Kovalenko**, A. M. Kabar*, O. O. Didur*, M. I. Nedzvetska*

**Oles Honchar Dnipro National University, Dnipro, Ukraine*
***Sumy' National Agricultural University, Sumy, Ukraine*

Article info

Received 14.01.2018
Received in revised form 07.02.2018
Accepted 09.02.2018

Oles Honchar Dnipro National University, Gagaryn Ave., 72, Dnipro, 49010, Ukraine. E-mail: khromykh58@rambler.ru

Sumy' National Agricultural University, Kondratyeva st., 160/5, Sumy, 40021, Ukraine.

Khromykh, N. O., Lykholat, Y. V., Kovalenko, I. M., Kabar, A. M., Didur, O. O., & Nedzvetska, M. I. (2018). Variability of the antioxidant properties of Berberis fruits depending on the plant species and conditions of habitat. Regulatory Mechanisms in Biosystems, 9(1), 56–61. doi: 10.15421/021807

Plant fruits, leaves, stems, and other parts are of high nutritional value, and are the source of physiologically active compounds, which can contribute to the treatment of many diseases caused by oxidative stress. Enrichment of the species spectrum of the fruit plants expands the possibilities of their use in dietary nutrition and human treatment. Unfortunately, the introduction of the new fruit plant species in industrial gardens in the Steppe Dnieper is limited to a large extent by the unfavorable climate. In this regard, the assessment of the possibility of realizing the genetic potential of fruit plants from different geographical areas in the steppe climate acquires both scientific and practical significance. The study was conducted on the basis of the fruit plants collection collection of the Botanical Garden of the DNU including four introduced species and one natural species from the genus *Berberis*. The anomalous weather conditions during the growing season of 2017 (snowfall in April followed by a drought in June) were accompanied by an earlier stage appearance of leaves, flowering and fruit ripening of all introduced plants, especially the Asian species *B. amurensis* and *B. koreana*, compared to the native species *B. vulgaris*. In accordance with the results obtained, fresh weight of the ripe fruits of *Berberis* species decreased in the order of *B. amurensis* > *B. vulgaris* > *B. canadensis* > *B. koreana* > *B.* x *declinata*. The highest total phenolics content, determined in the isopropanolic fruit extracts by Folin – Ciocalteau assay, was found in the fresh ripe fruits of *B. koreana* (1362 ± 66 mg GAE/100 g WW), followed by *B.* x *declinata* and *B. vulgaris* fruits (91% and 77% of the *B. koreana* phenolics content respectively). The highest total flavonoids content determined using the aluminum chloride method was revealed in the fruits of *B. koreana* (210 ± 6 mg RE/100 g FW) exceeding the content in fruits of other *Berberis* species by 1.1–2.1 times, while the lowest value (103 ± 4 mg RE/100 g FW) was found in the fruits of *B. amurensis*. The total reducing power, determined by RP assay, varied in the range from 5.0 to 9.6 mg AAE/100 g DW, and the highest levels were found in the fruits of *B. koreana* and *B.* x *declinata* (respectively, 9.6 ± 0.6 and 8.6 ± 0.5 mg AE/100 g DW) exceeding the reducing capacity of other *Berberis* species by 1.7–1.9 times. In the fruits of genus *Berberis* species strong positive correlation was found between the total reducing power and the total content of phenols (r = 0.87), as well as between the reducing power and the total content of flavonoids (r = 0.84). High correlation coefficients confirm the significant contribution of the *Berberis* fruit phenolic compounds, including the flavonoids, to the antioxidant capacity. So, the study results showed that fruits of all examined Berberis species can be an easily accessible source of antioxidants, however, the antioxidant capacity of fruits decreased in order of *B. koreana* > *B.* x *declinata* > *B. vulgaris* > *B. amurensis* > *B. canadensis*.

Keywords: fruit plants; *Berberis*; antioxidants; phenols; flavonoids; total reducing power

Introduction

The introduction of fruit plants enriches the variety of the species composition of the regional flora and at the same time creates an opportunity to expand the vegetable raw material base to meet the nutritional needs of human health. To date, more than 400 species of fruit and berry plants have been introduced in different regions of Ukraine, but they have been introduced insufficiently in industrial gardens in the Steppe Dnieper due to the unfavorable climate. Efficiency of the adaptation of useful fruit species to the adverse regional climatic conditions can be increased, in particular, due to the use of synthetic plant growth regulators (Shcherbina et al., 2017). Recently the technogenic impact and changes of climate have affected the state of living organisms: plants (Lykholat et al., 2017), animals (Brygadyrenko, 2015) and people (Lykholat et al., 2016). Plant fruits, leaves, stems and other parts are of high nutritional value and are the source of physiologically active compounds. This predetermines their long-term use in traditional medicine and today presents a number of important objects of numerical research to obtain new substances for the creation of therapeutic agents (Bak et al., 2010; Orhan, 2012; Imenshahidi & Hosseinzadeh, 2016; Rahimi-Madiseh et al., 2017). Thanks to the ability to biosynthesis and the accumulation of the components with antioxidant properties, fruit plants can contribute to the treatment of many diseases caused by oxidative stress (Sahan et al., 2012). For instance, the antioxidant ability of the phenolic compounds contained in plants is associated with anti-carcinogenic, anti-mutagenic and anti-inflammatory effects, as well as the effect on signaling pathways of carcinogen metabolism (Huang et al., 2010). Extracts from fruits, leaves, roots and other plant organs are now widely used in medical research. In particular, the high potential of the extracts from *Berberis vulgaris* for inhibiting lipid peroxidation has been demonstrated, indicating promising use in the treatment of hepatic oxidative stress, idiopathic male factor infertility and Alzheimer's disease (Abd El-Wahab et al., 2013).

In the recent decades, Alzheimer's disease has become one of the most threatening diseases in the elderly, especially because of the lack of an effective therapeutic agent against this disease. According to the generally accepted cholinergic hypothesis of the origin of the disease, its appearance is associated with a deficiency of the neurotransmitters acetylcholine and butyrylcholine; consequently, the inhibition of the acetylcholinesterase and butyrylcholinesterase enzymes, that cleave acetylcholine and butyrylcholine, has become the common approach to the treatment of Alzheimer's disease (Orhan, 2012). Since the necessary properties possess metabolites of plant origin, in this regard, the establishment of a wide range of pharmacological effects of the natural alkaloids has become of great importance. In the methanol extracts from roots and stems of different plants of the *Berberis* genus, four alkaloids were identified by HPLC, including berbamine, jatroizine, berberine and palametin (Di et al., 2003). The most well-defined are the properties of isoquinoline alkaloid berberine, which is contained in plants of different species of the genus *Berberis*. The berberine effects include an inhibitory effect on acetylcholinesterase and butyrylcholinesterase, inhibition of monoamine oxidase, reduction of amidoid-peptide levels and lower cholesterol levels, making berberine a promising agent against Alzheimer's disease (Ji & Shen, 2011).

Among all the species of *Berberis* genus, the phytochemical composition of *B. vulgaris* is the most widely studied at present, including a large number of components, such as ascorbic acid, vitamin K, several triterpenoids, more than 10 phenolic compounds and more than 30 alkaloids. Thus, three phenolic compounds were identified in the extracts from root cortex of *B. vulgaris*, including N-(p-trans-Cumaroyl) tyramine, cannabisine G and (+/-)-lyonirisinol; of these, cannabisin exhibits high antioxidant activity (Tomosaka et al., 2008). In addition to alkaloid berberine, the most important components of the roots, bark, leaves and fruits of *B. vulgaris* are isoquinoline alkaloids, beramines and palmatin. As a result, extracts from almost all parts of *B. vulgaris* can have anti-inflammatory, antioxidant, antidiabetic, antibacterial, analgesic and hepatotoxic effects (Imanshahidi and Hosseinzadeh, 2008).

Recent studies have confirmed the known effects of *B. vulgaris* in traditional medicine and justified the use of fruits and other organs of *B. vulgaris* for the development of new drugs (Rahimi-Madiseh et al., 2017). In particular, it has been shown that *B. vulgaris* extracts are safe and non-toxic and can induce the death of cancer cells due to their potent antioxidant activity (Abd El-Wahab et al., 2013; Hoshyar et al., 2016). In addition, based on clinical trials, the suitability of *B. vulgaris* extracts and berberine has been demonstrated as well for the treatment of tumors, diabetes, cardiovascular diseases, hyperlipidemia, inflammation, bacterial and viral infections, mental illnesses, Alzheimer's disease, osteoporosis (Imenshahidi & Hosseinzadeh, 2016).

Other species of the *Berberis* genus require detailed phytochemical studies; however, data on the chemical composition of extracts from the roots of *B. aetnensis* and *B. libanotica* indicate the possibility of using these plants as promising species for the treatment of Alzheimer's disease (Bonesi et al., 2013). The analysis of methanolic extracts from the bark of *B. darwinii* for inhibition of acetylcholine esterase *in vitro* has confirmed the therapeutic potential of the plants for the treatment of Alzheimer's disease (Habtemariam, 2011).

The numerous studies of the plant's physiologically active compounds have shown the dependence of their accumulation both on the plant properties and on the environment (Vagiri et al., 2013). For instance, in the *B. asiatica* plants in the western Himalayas, the berberine content was significantly higher in the populations growing at low altitudes and in all populations was higher in the roots than in the stems. In addition, moisture and potassium content of the soil signifycantly affected the berberine content (Andola et al., 2010). It was shown that the total content of alkaloids in the stems and roots of different plants of the *Berberis* genus depends on the origin of plants, their species and various organs (Di et al., 2003). Consequently, the content of physiologically active metabolites in fruits and other parts of the plant is determined genetically and simultaneously has a high dependence on the microclimatic and edaphic conditions in which the ontogenetic development of the fruit plants took place. In this paper, we aimed to study the implementation of the genetically determined antioxidant potential of fruits of the *Berberis* species during vegetation in the climatic conditions of the Steppe Dnieper.

Materials and methods

Study area. The research was conducted in 2017 in Dnipro city (steppe zone of Ukraine) in the Botanical Garden of Oles Honchar Dnipro National University (48°26'14'' N, 35°02'35'' E). The climate of the region has distinct continental features, including seasonal droughts with high temperatures and dry hot winds. The low average amount of precipitation (472 mm) decreases in arid years to 250 mm, and the total evaporation for a year exceeds the amount of precipitation by 2–3 times. The weather conditions during the period of research were characterized by abnormal features, in particular, the precipitation of 4 cm of snow with the simultaneous decrease in temperature to 3–6 °C from April 19 to 22, and thereafter the heat and droughts observed during June (Table 1).

Table 1
Local weather conditions in June 2017 (city of Dnipro)

Period	Air temperature, °C		Temperature on the surface of the soil, °C		Relative humidity, %		Rainfall, mm	
	average	norm	min	max	min	average	actual	norm
I decade	20.1	19.1	6.3	57.0	23	52	0.3	14.0
II decade	19.0	19.1	8.8	58.2	28	64	17.1	27.0
III decade	23.2	20.6	8.8	60.3	27	60	19.7	18.0

Data collection. The study objects were the fruits of the *Berberis* plants from the collection of the Botanical Garden of DNU, among which there was a natural species *B. vulgaris*, as well as four introduced species from different geographic regions. The *Berberis* genus has up to 500 species of plants and belongs to the Barberry family (Berberidaceae). All plants of this genus are ornamental shrubs that are spectacular during flowering and fruiting, have smart leaves and spiny shoots. Their areas of natural growth are Transcaucasia, Southern and Eastern Europe, and Asia, where the plants prefer to settle in dry and light areas near forests, on mountain slopes, infertile soils.

B. vulgaris (European barberry) has a natural distribution within the Near East, Transcaucasia, Central, Eastern and Southern Europe, where it grows on the forest edges, slopes, lawns; in the mountains reaches up to 2000 m. Barberry prefers light and dry areas. It is also found on chalk outcrops and river gravels. This species has shoots of up to 2–3 m in height, blooms in the period from April to May; the fruits ripen during September – October. The main advantage over other fruit barberries is its high winter hardiness; it is able to survive frosts down to –35 °C.

B. amurensis is common in the Far East, Korea, and China, where it grows on the fringes of forests and the banks of mountain streams, on stony ground. This is a thorny shrub with a sprawling crown up to 3.5 m in height. The shoots have a yellowish tint; they turn gray-yellow by the autumn. The color of the leaves also varies depending on the season: in summer they are bright green, and in autumn red or golden-red. The fruits of this species are red, shiny, and edible, ripen in October.

B. canadensis (American barberry) grows in the valleys and on the banks of the rivers of North America, where it also grows on mountain slopes. Brown and purple shoots of the bush reach a height of 2.0–2.5 m. The species blooms in June, the fruits have an elliptical shape, up to 1 cm long. This species bloomsis abundantly from mid-May to June, unpretentious, easily tolerates drought and winter frosts. This species has sufficient winter hardiness and heat resistance, but suffers from dryness.

B. koreana grows on the Korean peninsula. This species has became known as a cultivar only relatively recently, at the beginning of the 20th century, and has not yet widely spread. The height of the shrub does not exceed 2 m, the leaves are larger, stiffer, almost leathery. This plant blooms from late May to the second decade of June, approximately in the course of two weeks. Fruits ripen in September and have an almost spherical shape. This species is characterized by high winter hardiness; it prefers light, but it tolerates a partial shade, it is not exacting to the fertility of soils, it is drought-resistant. This species prefers alkaline soils, although it also grows on slightly acidic soils, but it does not survive the stagnant moistening and compaction of soils.

B. x *declinata* is a hybridogenic species, which is a spontaneous hybrid of *B. canadensis* and *B. vulgaris*. This is a shrub of up to 2 m in height with a densely curved crown, which has spines up to 1.5 cm in length. Plant leaves appear in April, flowering begins at the end of May and continues in June. Fruits up to 1 cm in length ripen during August-September. This species is one of the most winter-hardy species and can withstand frost up to –34 °C. The plants of this species do not require watering, however they need intensive lighting. The observations conducted during 2017 characterized the phenological peculiarities of the studied species of the genus *Berberis*, so the selection of fruits is carried out in accordance with the terms of their maturation for the different plant species (Table 2).

Table 2
Characteristics of the phenological differences of genus *Berberis* species in 2017

Species	Introduction time	Source of the introduction	Phenological phases of plants		
			leaves appearance	flowering	ripe fruit appearance
B. vulgaris L.	1954	M.M. Gryshko NBG, Ukraine, Kyiv	10.04	27.04 – 07.05.	28.09.
B. amurensis Rupr.	1956	Finland, Helsinki	03.04.	20.04 – 02.05.	16.09.
B. canadensis Mill.	1952	Canada, Ottawa	06.04.	25.04. – 05.05.	25.09.
B. koreana Palib.	1950	Denmark, Copenhagen	05.04.	21.04. – 02.05.	19.09.
B. x declinata Schrad.	1950	State Nykytskyj Botanical Garden, Crimea, Yalta	08.04.	25.04. – 04.05.	26.09.

It should be noted that in April 2017 anomalous snowfall together with a sharp drop in temperature coincided with the flowering phase in the two studied species *B. amurensis* and *B. koreana*, and these species were the first to form ripe fruit. All other species started the flowering phase later, after the temperature had risen. The strong drought, which was observed in the first two decades of June, created unfavorable conditions for plants of all the species in the initial period of fruit formation.

Data analysis. The antioxidant compounds of fruits of all *Berberis* species were extracted using 80% isopropanol. The extracts intended to determine the total content of phenolic compounds and flavonoids were obtained by boiling 1 g of pulp of fresh fruits (without peel and seeds) in 10 ml of isopropanol for 1 hour with a reflux condenser. After this, the crude extracts were cooled, filtered, and then the volume of isopropanol was adjusted to 10 ml. To determine the total antioxidant capacity (by FRAP assay) of the fruits, extracts obtained by holding the air-dried powdered vegetable material (200 mg) in 5 ml of isopropanol for 24 hours at room temperature were used.

The total phenolic content of the fruit extracts was measured by the Folin – Ciocalteau method described by Singleton et al (1999) in modification (Nwanna et al., 2013). Briefly, 0.2 ml of the plant extract diluted with 0.2 ml of distilled water was oxidized with 1 ml of 10% Folin – Ciocalteau reagent, and neutralized by 0.8 ml of 7.5% sodium carbonate solution in three minutes. Next, the reaction mixture was incubated for 40 minutes at 45 °C, and cooled, after which the optical density of the samples was measured at a wavelength of 726 nm. The total content of the phenolic compounds was calculated using a calibration graph prepared on the solutions of Gallic acid (GA) in the range 2.5–20.0 μg/ml. The study results were expressed as mg Gallic acid equivalents per 100 g of fruit wet weight (mg GAE/100 g WW).

Aluminium chloride spectrophetometric method in the modification of Pękal and Pyrzynska (2014) was used for the measurement of the total flavonoids content in the *Berberis* fruits. In brief, to 2 ml of isopropanol extract from the fresh fruits, 1 ml of a 2% solution of aluminum chloride ($AlCl_3$), and 1 ml of 1 M sodium acetate solution were added. The reaction mixture was maintained for 10 minutes at room temperature, filtered and the optical density measured at 425 nm. The quantitative content of flavonoids in the samples was calculated using a calibration graph prepared on the routine solutions with different concentrations (7.5, 15, 30, 50, 75, and 90 μg/ml), and the result was expressed as mg routine equivalents per 100 g of fruit wet weight (RE/100 g WW).

The reducing power (RP) of the *Berberis* fruits was determined according to the procedure described by Pulido et al. (2000). Briefly, 1 ml of extract was mixed with 1 ml of a 0.2 M sodium phosphate buffer (pH = 6.6) and 1 ml of 1% potassium ferricyanide ($K_3Fe(CN)_6$), and the reactive mixture was incubated at 50 °C for 20 min. After that, 1 ml of 10% trichloroacetic acid was added to the mixture which was centrifuged at 3000 rpm for 10 min. Then, to 1 ml of the supernatant 1 ml distilled water and 0.2 ml of 0.1% ferric chloride ($FeCl_3$) solution were added, and the absorbance was measured at 700 nm. Ascorbic acid was used as reference standard, and the calibration graph was constructed by solutions of ascorbic acid (AA) in the range of concentrations 10–100 μg/ml. The total reduction power of the crude fruit extracts was expressed as ascorbic acid equivalents per g of dry fruits weight (mg AAE/g DW) in accordance with the method of Augustus et al (2015).

Data were expressed as mean ± standard deviation (SD) of three measurements. The differences were considered to be statistically significant at $P < 0.05$. Statistical analyses were performed with used T-test and F-test for independent samples. Interspecific differences in the antioxidant properties of the *Berberis* fruits were evaluated in comparison with the indices of the natural species *B. vulgaris*. Pearson correlation was used to analyze the relation between environmental variables of *Berberis* pooled for all sampling dates.

Results

The average mass of raw ripe fruits of the different species of *Berberis* measured at the time of ripe fruit selection varied considerably in the different species of the genus (Table 3).

Table 3
The weight of the fresh *Berberis* fruits (average of 100 fruits) collected in 2017

Species	Fruit weight, g, x ± SD	P
B. vulgaris	24.99 ± 8.374	–
B. amurensis	39.45 ± 6.050	0.0001
B. canadensis	19.09 ± 3.356	0.0171
B. koreana	16.58 ± 1.394	0.0007
B. x declinata	11.66 ± 1.643	<0.0001

A comparative analysis of fruit fresh weight of the introduced species with the weight of *B. vulgaris* fruits showed a significant ($P < 0.05$) difference in the characteristics. The greatest average weight was found for the fruits of *B. amurensis* (39.45 ± 2.211 g per 100 fruits), and the smallest weight was found for the fruits of the hybrid species *B.* x *declinata* (11.22 ± 1.076 g per 100 fruits).

The total phenolic compounds in the ripe fruits of the different *Berberis* species as determined by Folin – Ciocalteau assay, was expressed as Gallic acid equivalents (GAE) by reference to a standard curve ($y = 304.15x$, and $R^2 = 0.99$). The total flavonoids content of various species of *Berberis* was determined using aluminum chloride method, and is expressed as routine equivalent (equation of regression $y = 0.0139x$, $R^2 = 0.999$). The total reducing power (RP) of the fruits of *Berberis* different species was expressed as the equivalent of ascorbic acid (equation of linear regression $y = 252.05x - 32.316$, $R^2 = 0.993$). Then, the study results (mean ± standard deviation) were statistically processed using the *B. vulgaris* indexes as a control (Table 4).

Discussion

In accordance with the results obtained, average fruit weight of the different *Berberis* species decreased in the order of *B. amurensis* > *B. vulgaris* > *B. canadensis* > *B. koreana* > *B.* x *declinata*. The size and weight of the fruits is a specific feature of a certain genotype, as well as important attributes of fruit quality, which, along with other characteristics, help to correctly evaluate new or introduced species (McGhie et al., 2005; Kaldmae et al., 2013). The weight of the *B. amurensis* fruits exceeded the weight of the fruits of all other *Berberis* species by 1.6–3.4

times, which can be advantage of this species at the estimation of fruit harvest. In addition, *B. amurensis* was the first of all the species to undergo the flowering and ripening of the fruits in less favorable conditions in 2017 (Table 2).

Table 4
Statistical estimate of total phenolic, total flavonoids content, and the reducing power of the *Berberis* fruits

Species	Index, x ± SD	t-value	df	P	F-ratio	P
	Total phenolic content (mg Gallic Acid Equivalents/100 g WW)					
B. vulgaris	1052.3 ± 54.34	–	–	–	–	–
B. amurensis	923.2 ± 45.89	3.15	4	0.0347	1.40	0.833
B. canadensis	899.2 ± 33.96	4.14	4	0.0144	2.56	0.562
B. koreana	1362.8 ± 66.11	–6.28	4	0.0033	1.48	0.806
B. x *declinata*	1243.2 ± 27.43	–5.43	4	0.0056	3.92	0.406
	Total flavonoids content (mg Routine Equivalents/100 g WW)					
B. vulgaris	142.5 ± 6.38	–	–	–	–	–
B. amurensis	102.8 ± 4.13	9.04	4	0.0008	2.39	0.590
B. canadensis	109.8 ± 4.86	7.06	4	0.0021	1.73	0.734
B. koreana	210.4 ± 6.10	–13.31	4	0.0002	1.09	0.955
B. x *declinata*	188.2 ± 7.83	–7.84	4	0.0014	1.50	0.799
	Total reducing power (mg Ascorbic Acid Equivalents/g DW)					
B. vulgaris	7.6 ± 0.39	–	–	–	–	–
B. amurensis	7.1 ± 0.54	–1.38	4	0.2395	1.93	0.683
B. canadensis	5.0 ± 0.41	–8.03	4	0.0013	1.45	0.815
B. koreana	9.6 ± 0.56	5.08	4	0.0071	2.10	0.646
B. x *declinata*	8.6 ± 0.50	2.59	4	0.0607	1.69	0.744

In our study, the total phenolic content of the crude extracts of the *Berberis* fruits varied in a relatively wide range, and the content of the phenols in the *B. vulgaris* was significantly ($P < 0.05$) different from those of all other species (Table 4). The study results showed that the highest phenolics concentration was revealed in the fresh ripe fruits of *B. koreana* (1362.8 ± 66.1 mg GAE/100 g WW), followed by *B.* x *declinata* fruits (91% of the *B. koreana* phenolics content). The phenolics concentration in the fruits of the native species *B. vulgaris* (1052.3 ± 54.3 mg GAE/100 g WW) reached 77% of the highest content in *B. koreana* fruits. Similar content of the polyphenols was found by Pyrkosz-Biardzka et al. (2014) in the methanolic crude extracts of *B. vulgaris* fruits, where it reached 1024.3 ± 15.2 mg GAE/100 g FM. The aqueous and alcoholic extracts of *B. vulgaris* fruits contained the total phenols at the levels 184.1 ± 5.3 and 291.2 ± 2.5 mg GAE/g of dried extract respectively (Hoshyar et al., 2016). In total, the results obtained showed the fairly high total phenolic content in the fruits of *Berberis* species compared to other fruit plants. Thus, Wolfe et al. (2003) evaluated the highest total phenolic content in different varieties of apples as 589 ± 83.2 and 500 ± 13.7 mg GAE/100 g of crude mass.

The smallest value of the phenolic compounds was revealed in the fresh fruits of the northern species *B. canadensis* (899.2 ± 34.0 mg GAE/100 g WW), and it was one and a half times lower than that of the southern species *B. koreana*. Significant differences in the content of phenolic compounds in the investigated fruits of *Berberis* species are consistent with other data on the variation of total phenolic content, depending on the genotype and plant tissues as well. Thus, a study of small fruits from 107 genotypes of three genera (*Vaccinium* L., *Rubus* L., and *Ribes* L.) demonstrates the wide diversity of phytochemical levels (total phenols and anthocyanins), and antioxidant capacities within and across genera (Moyer et al., 2002). The variation in the content of polyphenols in the range from 523 to 2724 mg GAE/100 g dry mass was found in a group of 67 different apple varieties (Wojdyło et al., 2008). In the leaves and flowers of *Crataegus azarolus* the total content of the phenols varied from 2.83 mg to 111.96 GAE/g of dried extract (Lakache et al., 2016). The HPLC analysis showed that among four cultivars of saskatoon berry (*Amelanchier alnifolia* Nutt.) grown in Finland, the total phenolic content varied 410.6–822.1 mg/g FW (Lavola et al., 2016). The total level of the polyphenols of four different honeysuckle (*Lonicera caerulea* L.) genotypes identified by LC/MS method was 775–2005 mg/100 g dry matter (Wojdyło et al., 2013).

The natural phenolic compounds are the secondary plant metabolites, which form a large and diverse group of the phytochemicals including simple phenols, lignans, phenylpropanoids, flavonoids, coumarins and other compounds (Jafari et al., 2014). Phenols carry out important physiological functions in the plant organism, in particular, increased accumulation of phenolic compounds in the leaves positively correlated with the improvement of the stability of the clover plants to drought (Nichols et al., 2015). Flavonoids are polyphenolic compounds, whose potential is not fully revealed, although they have been studied for a long time (Eghdami & Sadeghi, 2010). In our study, the highest total content of the flavonoid compounds revealed in fruits of *B. koreana* (210.4 ± 6.1 mg RE/100 g WW) exceeded the content in fruits of other *Berberis* species by 1.1–2.1 times, and the lowest value (102.8 ± 4.1 mg RE/100 g WW) was found in the fruits of *B. amurensis* (Table 4). The content of flavonoids determined by us in the isopropanolic extracts of *B. vulgaris* fruits (142.9 ± 6.4 mg RE/100 g WW) was higher than the content in methanol extract (86.0 ± 1.8 mg RE/100 g FM), reported by Pyrkosz-Biardzka et al. (2014). The highest flavonoids share in total phenolics content was found in the fruits of *B. koreana* and *B.* x *declinata* (15.5% and 15.1% respectively), while it decreased to 13.6%, 12.5%, and 11.1%, respectively, in the fruits of species *B. vulgaris*, *B. canadensis* and *B. amurensis*. Much higher percentages of flavonoids were revealed in the fruits of different apple genotypes, where the flavonoids accounted for 52–60% (Wolfe et al., 2003), or even 80% of the total content of phenols (Wojdyło et al., 2008). The highest amount of flavonoids in the leaves and flowers of *Crataegus azarolus* reached 5.9 mg QE/g of dried extract (Lakache et al., 2016).

The reducing power reflects the antioxidant capacity of plant extracts, and it can be characterized by different methods and expressed in different units, which complicates comparison of results. The antioxidant properties of fruits are due to both the ability to trap free radicals and to form metal chelates (Brewer, 2011), which suggests differences in the estimation of the reduction potential. In our study, the highest levels of the total reducing power (determined by RP assay) was found in the fruits of *B. koreana* and *B.* x *declinata* (respectively, 9.6 ± 0.6 and 8.6 ± 0.5 mg AE/100 g DW) exceeding the indices of other species by 1.7–1.9 times (Table 4). Deepa et al. (2013) reported the total reducing power of the methanolic extracts of different Indian spice herbs variation in range 0.57–6.0 mg AAE/g DW. In the fruits of two different genotypes of eggplant (Solanaceae) the reducing power was determined at the levels 48.8 ± 0.6 and 56.7 ± 1.4 mg AE/g of dried extract (Nwanna et al., 2013). The reducing power of fresh *Thymus vulgaris* leave extracts varied between 0.39–0.94 mg AAE/g WW (Eghdami et al., 2013). The reducing capacity of the extracts from the plants of *Cyperus erectus* in the range 5.6–20.0 mg AAE/g DW was estimated as high (Augustus et al., 2015). Therefore, the reducing power of the *Berberis* fruits defined in the range 5.0–9.6 mg AAE/100 g DW may be deemed sufficiently high. Since the flavonoids are the major contributors to the total reducing power in different fruit species (Borges et al., 2010), it is possible that antioxidant capacity of fruits of some *Berberis* species could be reduced due to the adverse effects of abnormal weather conditions during the growing season in 2017 (Table 2). This assumption is consistent, in particular, with the data of Bettaieb et al. (2011) on the dependence of the antioxidant level of cumin (*Cuminum cyminum* L.) on the power of stress in experimental drought conditions.

In our study,a strong positive correlation was found between the total reducing power and the total content of phenols in the fruit extracts of *Berberis* species ($r = 0.87$, $P < 0.001$), and the total content of flavonoids and the total reducing power as well ($r = 0.84$, $P < 0.001$). High correlation coefficients confirm the significant contribution of phenolic compounds, including flavonoids, to the antioxidant capacity of the fruits of all examined *Berberis* species, which can be an easily accessible source of antioxidants. The obtained results comport with data (Konćić et al., 2010) on the correlation between the antioxidant capacity and phenolic compounds content in fruits of *B. vulgaris* and *B. croatica*.

Conclusion

The study results confirmed the remarkable effect of genotype on the nature of phenology and accumulation of the phenolic compounds in the fruits of different *Berberis* species. Regional unfavorable weather

conditions during the growing season stimulated the earlier stage appearance of leaves, flowering and fruit ripening of all introduced plants compared to the native species *B. vulgaris*. However, the most accelerated were the phenological rhythms of the Asian species *B. amurensis* and *B. koreana*, while the phenological phases of the northern species *B. canadensis* and the hybrid species *B.* x *declinata* were closer to *B. vulgaris*. The fruit weight of the different *Berberis* species decreased in the order of *B. amurensis* > *B. vulgaris* > *B. canadensis* > *B. koreana* > *B.* x *declinata*. Antioxidant capacity was determined as relatively high in the fruits of all *Berberis* species, with a significant predominance of *B. koreana* and *B.* x *declinata*. The total phenolic content of the fruits and also the total reducing power decreased in the order of *B. koreana* > *B.* x *declinata* > *B. vulgaris* > *B. amurensis* > *B. canadensis*, while the total flavonoids content – in the order of *B. koreana* > *B.* x *declinata* > *B. vulgaris* > *B. canadensis* > *B. amurensis*. The relatively low concentration of the antioxidants in the fruits of *B. amurensis* can be compensated for by the largest fruit weight of this species. Results showed that all the studied species of the genus *Berberis* are sufficiently rich sources of natural phenolic antioxidants. Species *B. koreana* and *B.* x *declinata* could potentially be the most promising in the unstable climatic conditions of the Steppe Dnieper.

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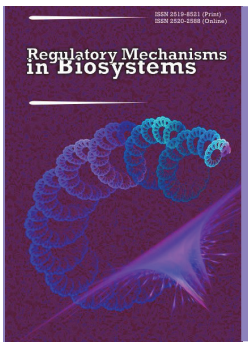

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 62–68
doi: 10.15421/021808

Strains of lactic acid bacteria isolated from traditional Carpathian cheeses

I. M. Slyvka*, O. Y. Tsisaryk*, G. V. Dronyk**, L. Y. Musiy*

**Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies, Lviv, Ukraine*
***Chernivtsi National University named after Yuriy Fedkovich, Chernivtsi, Ukraine*

Article info

Received 17.01.2018
Received in revised form 19.02.2018
Accepted 21.02.2018

Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies, Pekarska st., 50, Lviv, 79010, Ukraine. Tel.: +38-097-986-15-44. E-mail: slyvka.88@ukr.net

Chernivtsi National University named after Yuriy Fedkovich, Kotsyubinsky st., 2, Chernivtsi, 58012, Ukraine.

Slyvka, I. M., Tsisaryk, O. Y., Dronyk, G. V., & Musiy, L. Y. (2018). Strains of lactic acid bacteria isolated from traditional Carpathian cheeses. Regulatory Mechanisms in Biosystems, 9(1), 62–68. doi: 10.15421/021808

Traditional national fermented products and cheeses are a source for the search for species and strains of lactic acid bacteria (LAB) which are not within the range of bacterial agents used in the dairy industry. Classical and modern genetic-molecular methods are used to identify LAB isolated from such products. The purpose of our work was isolation and identification of LAB from traditional Carpathian cheeses made from ewe's milk and the study of their technological properties. Three samples of cheese were selected for our research – one sample of brine cheese bryndza and one sample of budz (bryndza before salting), produced in the highlands of the Carpathians and one sample of buts, produced in the foothills zone. 106 cultures were isolated from the samples of cheese. Genus and species identification was completed using classical microbiological and molecular genetics methods. Based on the complex of tinctorial, cultural, physiological and biochemical indices, the LAB isolated were assigned to the following genera and species: *Lactococcus* spp. (26 cultures), including *L. lactis* (13 cultures) and *L. garvieae* (13 cultures); *Lactobacillus* spp. – *L. plantarum* (31 cultures); *Enterococcus* spp. – *E. faecium* (25 cultures); *Leuconostoc* spp. – *L. mesenteroides* (24 cultures). These results were confirmed by molecular genetics methods. The largest range of species was found in a sample of bryndza from the Carpathian highlands. The isolated cultures were studied according to technological properties – milk-coagulation activity, acid-forming ability and resistance to different concentrations of kitchen salt. Most strains of *L. lactis* ssp. *lactis*, *L. plantarum* and *L. mesenteroides* were active acid-forming agents and coagulated milk in 3–9 hours, while *L. garvieae* and *E. faecium* coagulated milk after more than 24 hours. More than 80% of cultures showed resistance to 4% of kitchen salt solution, *E. faecium* was observed to have the highest salt tolerance. The results of RAPD typing showed significant intra-species heterogeneity, which indicates the need for further research on identification of individual strains. In all samples of cheese, *L. lactis*, *L. garvieae*, *E. faecium* were detected, which shows that they are typical representatives within the traditional Carpathian bryndza. Particular attention was paid to *E. faecium*, as many researchers have indicated probiotic properties of individual strains, as well as the ability to synthesize volatile substances that enrich the flavor bouquet of cheeses. Today strains of *E. faecium* are involved in the bacterial composition of starter cultures for cheeses.

Keywords: cheese bryndza; cheese buts; tinctorial properties; polymerase chain reaction; RAPD-typing

Introduction

Traditional dairy products are made from raw milk using non-industrial methods, and contain specioes and strains of lactic acid bacteria (LAB), which are currently not included in fermentation starter preparations used in the dairy industry. These bacteria may be capable of valuable technological (Zhong et al., 2016) and probiotic properties (Natarajan and Parani, 2015; Zhang et al., 2016, 2017). Therefore, in recent years, researchers have focussed on these bacteria, first of all, as strains which are promising for industrial use (Ortakci et al., 2015; Oberg et al., 2016; Culumber et al., 2017). Strains with probiotic properties are included in bacterial preparations for producing dairy products with functional properties, and also for producing medical preparations (Chaharovskyy & Zholkevskaya, 2003; Kigel', 2003; Diduh et al., 2008). Microbiota of traditional dairy products are a subject of interest for research from the perspective of natural populations, which were formed over a prolonged period in certain geographical regions (Bao et al., 2012; Liu et al., 2012). Between the cultures in such populations, symbiotic interrelations occur (Kimoto-Nira et al., 2012); this can be a model for constructing new microbial compositions. It is important not to lose such natural populations. Unfortunately, the composition and properties of the bacteria of domestic traditional fermented milk products and cheese, made in non-industrial conditions in Ukraine remain unresearched, and the number of such products continues to decrease.

One of the most popular national dairy products is bryndza, brined cheese made in the Carpathians from raw sheep milk directly in the pastures where the sheep are grazed from May to October. Creating bacterial preparations for industrial production of bryndza out of pasteurized milk would allow inoculation of bryndza using pure cultures, which are typical for raw milk. This, in turn, would allow one to obtain a safe product while maintaining the taste and the aroma of the traditional cheese (Neviani et al., 2009; Zhong et al., 2016), which is an daily product of consumption for the inhabitants of the Carpathians and a favorite product for many consumers. We should emphasize the fact that using an industrial bacterial preparation is a precondition for controlling the technological processes and receiving the product with the desired properties.

Until now, the selection of strains for bacterial preparations have used mostly classical microbiological methods. The identification and assessment of the natural ecological niches isolated from the microbiological cultures were made studying their morphological, cultural, physical-chemical and technological properties (Ao et al., 2012). The

achievements of the past decades in microbiology, genetics and molecular biology allow the genetic diversity of certain species of microorganisms to be characterised. The development of the systematics of microorganisms on the basis of molecular-genetic methods oriented towards studying the bacterial genome, and accumulation of experimental data enable a number of controversial problems to be solved regarding the taxonomical position of particular groups of microorganisms (Kovalenko & Lashhevskyj, 2003; Giraffa et al., 2010; Vasyljuk et al., 2014). Modern biotechnology is inextricably linked to using new approaches to selecting the natural strains of microorganisms for the composition of bacterial preparations (Podgorskyj & Kovalenko, 2002; Naumenko, 2005; Mayo et al., 2014). The new approaches include the selection of strains, for the strains possess features which are essential for industrial use in food products. These features, first of all, include safety, i.e. no pathogenic factors and no signs of resistance to antibiotics (Amaral et al., 2016; Zhang et al., 2016). Some features of the strains are related to the probiotic properties, i.e. ability to synthesize the bacteriocins (Ao et al., 2012; Goh & Philip, 2015), survival in the conditions of the gastrointestinal tract and manifestation of adhesive properties (Amaral et al., 2016) and decrease in the level of cholesterol in the blood (Zhang et al., 2017). The objective of our research was the isolation and identification of LAB in traditional Carpathian cheese, made of sheep milk, and study of their properties.

Materials and methods

The article analyzes LAB, isolated from three samples of traditional sheep cheese, bryndza and budz, selected in different geographical regions of the Carpathians and made from milk of different sheep breeds (Table 1).

Table 1
Origin of cheese samples

Kind of cheese	Designations of samples hereinafter	Location of cheese selection	Sheep breed
Bryndza	A	Putyla, Chernivtsi Oblast (highland)	Ukrainian Carpathian
Budz*	B	Putyla, Chernivtsi Oblast (highland)	Ukrainian Carpathian
Budz*	C	Dana farm, Koteleve village, Chernivtsi Oblast (pre-mountain area)	Bucovina type of Karakul breed

Note: * – Budz sheep cheese according to the technology is Bryndza before salting.

For isolating the bacteria, we selected one gram of cheese from each sample, and homogenized it in 9 ml of sterile saline solution. The bacteria were inoculated onto solid growth mediums M_{17} and MRS (Himedia, India) using the method of ten-fold serial dilutions (10^{-1} to 10^{-6}) of the analyzed material. The inoculations were carried out in three parallels. They were cultivated in a thermostat at the temperatures of 30, 42 ºC over 48 hours. All studied cultures were inoculated at least twice into MRS and M_{17} agar. The strains of lactate bacteria were maintained in 0.5 ml of MRS or M_{17} nutrient broth, which included LAB cultures, which were mixed with 50% sterile glycerol and frozen to –80 °C. The numberof lactate microorganisms were calculated using the standard method according to GOST 104444.11-89. Food products. Methods for identification of the lactic acid bacteria.

The study of morphological characteristics of the LAB cultures used preparations stained using Gram's method. Microscopy was made using immersion oil and ×1350 zoom. We determined the size, pattern of staining, cell position, absence of unfavourable (external) microflora and changed forms in the smear.

We chose only gram-positive and catalase-negative isolates, which were maintained at the temperature –80 °C in sterile MRS broth with addition of 15% glycerine. Frozen bacterial cultures were used for further identification. The working cultures were revived from frozen into working cultures by making two inoculations in MRS broth at the temperature of 45 °C.

The differential characteristics for determination of genus were conducted using the complex of tinctorial, cultural and physical-biochemical features of the studied LAB strains. The main criteria for the differentiation were: the ability to release CO_2 when the media is cultivated with glucose, fermentation of a particular range of carbohydrates, hydrolysis of arginine (Harrigan & McCance, 1976), growth at different temperature regimes 10, 15 and 45 °C, and also capability of growth in the presence of 2.0, 4.0, 6.5% NaCl (Kvasnikov & Nesterenko, 1975).

Biochemical properties of the LAB were studied in accordance with a range of fermentation of carbohydrates using Hiss culture media (Himedia, India). The cultures were inoculated to the half-liquid media using the method of inoculation loop. For the determination of biochemical properties, an amount of 2% of the following carbohydrates was added to the Hiss media: arabinose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, raffinose, sucrose, sorbitol, trehalose and xylose.

For accurate identification of LAB of "wild" microbiota of the traditional Carpathian cheese, which was studied for the first time, we used polymerase chain reaction (PCR). The isolation of genome DNA was made using a Genomic Mini (A&A Biotechnology) set in accordance with the manual. The DNA samples were analyzed qualitatively and quantitatively, using a NanoDrop 2000 (Thermo Scientific, USA) spectrophotometer. The DNA samples were maintained at the temperature of 20 °C for further use.

The DNA fragments, which included the 16S pPHK gene, were amplified using the following universal primers:

EGE1 (5-AGAGTTTTGATCCTGGCTCAG-3),
1492R: 5'-TACGGYTACCTTGTTACGACTT-3'.

Every reaction mixture for PCR contained 2 µl (50 ng) of DNA, 1 µl 10 µm of dNTP mixture, 1 µl 10 µm of every primer, 5 µl of 10xPCR-buffer (Fermentas, Lithuania) and 1.25 U Taq DNA-polymerase (Fermentas) with addition of up to 50 µl of sterile Milli-Q water.

The DNA amplification programme consisted of primary denaturalization at the temperature of +96 °C for 5 min, 35 cycles (96 °C for 30 s, 52 °C for 45 s, 72 °C over 1.5 min). The final stage of polymerization was carried out at the temperature of 72 °C during 10 min.

Disintegration into fragments was carried out using restrictive endonucleases, according to the manual. For the disintegration into restrictive fragments, we used the following ferments: HinfI, RsaI (Roche, Switzerland), Sau3A (Fermentas) i HhaI (Takara); they were used in separate reactions. All disintegrations were carried over a period of 2 hours at 37 °C. The fragments were divided in 2 agarose gel.

RAPD-PCR was carried out in a volume of 25 µl, which included 1 µl (~ 25 ng) of each DNA, 1 µl 10 µm of dNTP, 1 µl 10 µm of primer 1254 (5'-CCGCAGCCAA-3'), 2.5 µl of 10xPCR (Fermentas), 1.5 µl 50мM of $MgCl_2$ and 0.75 U of Taq DNA polymerase (Fermentas). The PCR programme consisted of 4 cycles (5 min at the temperature of 96 °C, 5 min at 36 °C and 5 min at 72 °C), then 30 cycles (1 min at 96 °C, 1 min at 36 °C, and 2 min at 72 °C). The final 10-minute extension was conducted at 72 °C. After the amplification 20 µl of PCR product was divided in 2 agarose gel.

The 16S r RNA area was amplified using polymerase chain reaction (PCR) as described previously. The amplified fragments were cleaned using Gel Purification GPB Mini Kit (GenoPlast, Poland) according to the manufacturer's instruction and sequenced in a Genomed (Poland) commercial service. The determination of sequencing was made in both directions with the same primers, which were used for amplifying 16S r DNA. The sequencing of 16S rRNA was compared with the sequencing of the fragments from the NCBI base (www.ncbi.nlm.nih.gov). The search for similarities between the sequenced fragments was conducted using the BLAST algorithms, available at the www.ncbi.nlm.nih.gov/blast.

The statistical analysis was made using the Statistica 6 (StatSoft Inc., USA) software. The difference between the data was considered significant at $P < 0.05$ (ANOVA).

Results

We established that the total number of LAB (Table 2), isolated from the sample of bryndza (A), was 1.5–1.9 times less than for the samples of budz (B and C). Such difference is explained by the effect on the

survivability of the LAB caused by the table salt, which composed 5.5% of the bryndza. The samples B and C were unsalted budz cheese.

Table 2
The number of lactate bacteria which
were isolated from samples (CFU/g, x ± SE, n = 3)

Bryndza (A)	Budz (B)	Budz (C)
$4.4 \pm 0.12 \cdot 10^5$	$6.7 \pm 0.13 \cdot 10^5$	$8.5 \pm 0.12 \cdot 10^5$

Lactate bacilli were grown on a MRS dense growth media, forming white or grey colonies of 1 to 5 mm diameter, sometimes of lenticular or starlike shape. The surface of the colonies was mostly smooth and shiny (S-shape), though in some cases there were rugged colonies (R-shape). The incubation was made at the temperature of 37 °C. The lactic acid cocci were observed to have a distinctive growth on a dense media M_{17} in the form of rounded and cymbiform colonies. The rounded colonies with distinct edges were formed on the surface of the growth media, whereas the cymbiform colonies grew slightly into the agar. The bacteria were incubated at 25 and 42 °C.

For studying the cheese samples, we selected 106 isolates of lactic acid bacteria. All isolated cultures were gram-positive and catalase-negative. The bacterial cultures were identified at the level of genus on the basis of their cell morphology, fermentation of carbohydrates, the ability to release CO_2 when cultivated in the media with glucose, hydrolysis of arginine, growth at the temperature of 10, 15 and 45 °C, and also capability of growth in the presence of 2.0, 4.0, 6,5% NaCl according to Wood & Holzapfel (1995). We found that the isolated cultures belong to four genera (Table 3).

Table 3
Tinctorial, cultural and physical-biochemical features
of lactate cultures isolated from traditional sheep cheese

Indicators	Genus			
	Lactobacillus	*Lactococcus*	*Leuconostoc*	*Enterococcus*
The number of isolated cultures	31	26	24	25
Morphology	bacilli	cocci	cocci	cocci
Staining	gram-positive	gram-positive	gram-positive	gram-positive
Catalase activity	absent	absent	absent	absent
Release of CO_2 while growing in a media with glucose, number of cultures / %	0	0	24 / 100	25 / 100
Arginine hydrolysis, number of cultures / %	0	26 / 100	0	25 / 100
Fermentation of carbohydrates, number of cultures / %				
arabinose	24 / 77	0 / 0	16 / 67	12 / 48
fructose	31 / 100	26 / 100	24 / 100	25 / 100
galactose	31 / 100	26 / 100	24 / 100	25 / 100
glucose	31 / 100	26 / 100	24 / 100	25 / 100
lactose	31 / 100	26 / 100	24 / 100	25 / 100
maltose	31 / 100	17 / 65	24 / 100	25 / 100
mannitol	31 / 100	0 / 0	0 / 0	23 / 92
mannose	31 / 100	26 / 100	24 / 100	25 / 100
raffinose	31 / 100	0 / 0	24 / 100	6 / 24
sucrose	31 / 100	4 / 15	24 / 100	25 / 100
sorbitol	26 / 84	0 / 0	0 / 0	0 / 0
trehalose	31 / 100	26 / 100	24 / 100	25 / 100
xylose	8 / 26	0 / 0	12 / 50	0 / 0

According to a complex of tinctorial, cultural and physical-biochemical features, we found that the cultures which grew at the temperature of 10 and 15 °C in the presence of 6.5% NaCl, but not at 45 °C, with no gas release when grown in the media with glucose and capability of arginine hydrolysis were lactate acid bacilli. Cocci-like bacteria, but with prolonged body shape, which often occur in pairs or as short chains, grew at the temperature of 10 °C, but not at 45 °C and had no capability of arginine hydrolysis; they released CO_2 while grown in the media with glucose, which indicates heterofermentative fermentation. These bacterial cultures were identified as *Leuconostoc*. All other cocci hydrolyzed agrinine, not releasing CO_2 when grown in a medium with glucose and grew at the temperature of 10 °C and in the presence of 4% NaCl. Some of them were able to grow at the temperature of 45 °C, and in the presence of 6.5% NaCl, therefore were classified as *Enterococcus*, whereas the isolates, which did not grow at the temperature of 45 °C, and in the presence of 6.5% NaCl were classified as lactate cocci.

From the bryndza sample, we isolated 68 cultures, 31 of them belong to the following genera: *Lactobacillus* (46%), 5 – *Lactococcus* (7%), 24 – *Leuconostoc* (35%), 8 – *Enterococcus* (12%). From the sample of budz prepared in the highlands, we isolated 10 cultures of the *Lactococcus* genus, which were 56% of the total and 8 cultures of *Enterococcus* genus (44%). The LAB of the budz sample prepared in pre-mountain area also belong to two genera: *Lactococcus* (11 cultures, 55%) and *Enterococcus* (9 cultures, 45%). In the sample of bryndza, we found representatives of four genera, whereas in two other samples, we found only two. It should be noted that all samples of cheese had a high content of *Enterococcus* bacteria.

According to the results of set of bacteriological methods, the studied LAB cultures were classified as follows: *L. lactis* ssp. *lactis* (13 cultures) and *L. garvieae* (13 cultures); *L. plantarum* (31 cultures); *E. faecium* (25 cultures); *L. mesenteroides* ssp. *mesenteroides* (24 cultures).

It was found that about 90% and 93% strains of *L. plantarum* grew at the temperature of 10 °C and 15 °C, whereas no growth was observed at 45 °C. Similar growth was observed in *L. lactis* ssp. *lactis* − 83% and 87% at the temperature of 10 and 15 °C respectively; in *L. garvieae* − 81% and 89% and in *L. mesenteroides* – 85% and 92%. It should be noted that the highest activity of growth was observed among *E. faecium* strains (87% and 90% at the temperature of 10 and 15 °C, and also 90% at 45 °C). The results of the tests are given in Table 4.

Most strains of *L. lactis* ssp. *lactis* (over 90%) were active acidifiers and coagulated milk in 3–9 hours, whereas *L. garvieae* and *E. faecium* were less active, most of them coagulated milk in more than 24 hours (Table 5).

Table 4
Growth of lactate acid bacteria in relation
to the temperature and concentration of NaCl (number of strains in %)

Species of lactate bacteria	Growth at the temperature			Growth at the concentration of NaCl, %		
	10 °C	15 °C	45 °C	2.0	4.0	6.5
L. plantarum (n = 31)	90.3	93.4	0.0	97.6	86.6	58.8
L. lactis ssp. *lactis* (n = 13)	82.8	87.5	0.0	95.9	83.7	0.0
L. garvieae (n = 13)	80.6	89.4	0.0	96.4	84.6	0.0
E. faecium (n = 25)	87.5	89.7	98.6	100.0	98.5	93.3
L. mesenteroides (n = 24)	85.1	92.3	0.0	97.3	89.2	43.5

Table 5
Milk-coagulating and acidifying activity of strains of lactate bacteria

Species of lactate bacteria	Milk-coagulating activity		Acidifying activity		
	the speed of bunch formation, hours	number of strains, %	titrated acidity, °T	active acidity, IU pH	number of strains, %
L. plantarum (n = 31)	3	5.5	100–120	4.8	19.4
	6	30.0	90–100	5.0	41.9
	9	42.9	80–90	5.3	35.5
	>24	22.6	60–80	5.5	3.2
L. lactis ssp. *lactis* (n = 13)	3	15.2	100–120	4.8	15.3
	6	25.5	90–100	5.0	46.2
	9	51.8	80–90	5.3	30.8
	>24	7.5	60–80	5.5	7.7
L. garvieae (n = 13)	3	0.0	100–120	4.8	7.6
	6	7.7	90–100	5.0	23.1
	9	23.1	80–90	5.3	38.5
	>24	69.2	60–80	5.5	30.8
E. faecium (n = 25)	3	0.0	100–120	4.8	8.0
	6	20.4	90–100	5.0	19.8
	9	26.5	80–90	5.3	35.2
	>24	53.1	60–80	5.5	37.0
L. mesenteroides (n = 24)	3	0.0	100–120	4.8	8.3
	6	12.5	90–100	5.0	12.5
	9	41.7	80–90	5.3	25.0
	>24	45.8	60–80	5.5	54.2

However, it is important that all isolated cultures manifested a milk-coagulating property. The bordering acidity of *L. lactis* ssp. *lactis* and

L. plantarum was 120 °T, over 61% of strains of these species caused the acidity of milk up to 90–120 °T. Only 30%, 28% and 21% of *L. garvieae*, *E. faecium* and *L. mesenteroides* strains relatively brought the acidity of milk to the mentioned values. However, the composition of these cultures is capable of providing milk acidity sufficient for formation of a bunch during the cheese making.

Figure 1 provides an electrophoretogram of disintegration of amplified DNA fragments by restrictive enzymes. The analysis of profiles of fingerprints of the studied cultures' DNA fragments amplified by the HinfI, RsaI, Sau3A and HhaI enzymes showed the presence of five species: *L. plantarum*, *L. lactis*, *L. garvieae*, *L. mesenteroides*, *E. faecium*. Every enzyme proved the presence of these species. The results of RAPD typing coincide with the results received using bacteriological methods.

Conducting the polymerase chain reaction using the 1254 primer allowed isolation of five primary clusters (I–V) with the level of similarity of 51–85%. The results of RAPD typing found significant intraspecific heterogeneity of the studied LAB, which indicates the necessity of further studies in identification of LAB at the level of strains. The RAPD-PCR results are provided in Figure 2.

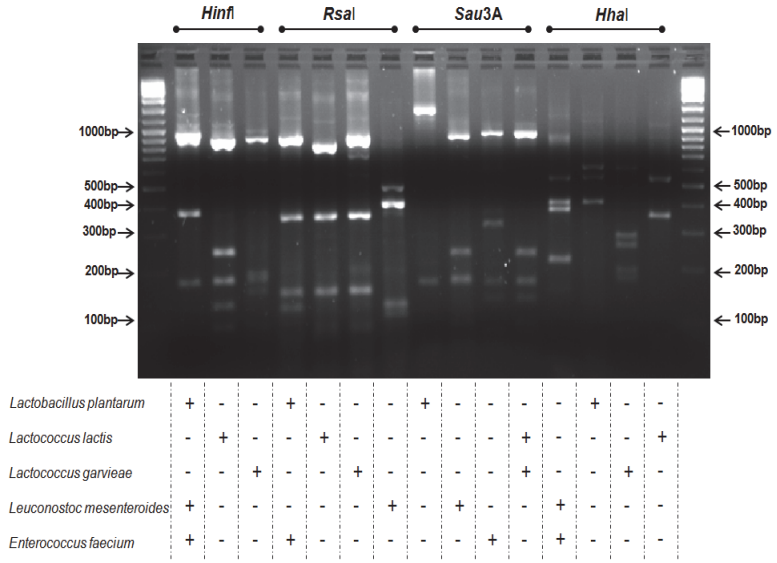

Fig. 1. Electrophoretogram of disintegration of amplified DNA fragments by Hinf1, Rsa1, Sau3A, Hhal restrictive enzymes: the first and the last tracks are markers of molecular mass of the DNA fragments, every track divides the mixture of DNA; "+" indicates the presence of the species

Discussion

Bryndza and budz cheese, which have been made from sheep milk in the Carpathians for centuries, have a specific taste and aroma, which are formed due to fermentative activity of the bacteria involved in the cheese making. Milk with natural content of LAB is highly rated in the production of different kinds of cheese made out of either raw or pasteurized milk. Raw sheep milk is highly polluted bacterially, which is related to the conditions in which it was obtained. Apart from the LAB content, it usually contains undesirable bacteria. For safety of sheep cheese, the milk should be pasteurized; however this causes death also to the LAB bacteria, which could have valuable technological and probiotic properties. Rendering the LAB composition which is typical for original Carpathian cheese in bacterial preparations for industrial production, is a subject of interest and an important issue.

Out of three samples of Carpathian cheese made in the highlands and in pre-mountain zone, 106 LAB cultures were isolated. The total number of LAB was the lowest in bryndza, which included 5.5% of salt, whereas the two other samples did not contain salt. However, the sample of bryndza is distinctive for the highest bacterial diversity, including four genera (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*), which were represented by five species (*L. lactis* ssp. *lactis*, *L. garvieae*, *L. plantarum*, *E. faecium*, *L. mesenteroides* ssp. *mesenteroides*). LAB isolated from the samples of non-salted budz cheese were represented by only three species (*L. lactis* ssp. *lactis*, *L. garvieae*, *E. faecium*) and two genera respectively (*Lactococcus* and *Enterococcus*). We did not isolate cultures of other two species (*L. plantarum* and *L. mesenteroides* ssp. *mesenteroides*). This, in our opinion, is related to the high salt-resistance. Low salt content and insignificant representation in fresh cheese allowed the bacteria to survive and successfully compete with less salt-resistant species during salting, ripening and maintaining of bryndza in 18% brine, which is part of the production technology. The impact of salt was also proven by the lower overall of LAB in bryndza. It should be noted that the two samples of budz have similar representation: in two samples, we isolated 55–56% cultures of *Lactococcus* genus and 44–45% of *Enterococcus*. This leads us to think that there are no significant differences in the LAB content of raw sheep milk between the highland and pre-mountain areas of the Carpathians. However, the study in this direction should be continued due to reports on the differences in the content of "wild" microbiota in relation to the geography of the making of traditional fermented products, though on a significantly larger scale regarding both the products and location – China, Mongolia, and Russia (Zhong et al., 2016).

LAB isolated from all samples of cheese belong to mezophilic and thermophilic cultures, which coincides with the reports by Poznanski et al. (2004), who studied the bacterial composition of the traditional national cheese made in the conditions of alpine tundra.

Representatives of the *Enterococcus* genus made up a significant part of the total LAB content of all samples of cheese. Currently, the representatives of *Enterococcus* genus are used in the content of fermentation starter preparations for many fermented products (Goh & Philip, 2015), mostly cheese (Neviani et al., 2009; Natarajan et al., 2015), made in South and North Europe out of either raw or pasteurized milk. The presence of *Enterococcus* during the ripening of cheese has a positive effect on their sensory properties (Gatti, 2014), they improve the taste and aroma and prolong the time of maintenance (Franz et al., 2003). The most common species of *Enterococcus* isolated from the cheese were the following species: *E. faecium*, *E. faecalis* and *E. durans* (Belicov et al., 2007; Serio et al., 2007; Veljovic et al., 2007). Some *E. faecium* strains manifest probiotic properties (Saarela et al., 2000; Ammor et al., 2007; Franz et al., 2011; Amaral et al., 2016).

It is worth mentioning that Ukrainian scientists were among the first to determine that *E. faecium* strains manifest probiotic properties. This led to the creation with their participation of Gerolakt, a lactate gerontological nutrition product (Kovalenko et al., 1994). However, the *E. faecium* strains include pathogenic and antibiotic-resistant strains; therefore the study of priobiotic properties should be preceded by study of the safety of separate strains for humans. It should be noted, that there are clear differences in the safety of certain *Enterococcus* strains in relation to the source of isolation, the strains with virulent genes are isolated from ill people, and not from food products (Moreno et al., 2006). Currently, the domestic industry does not use bacterial pre-

parations with representatives of the *Enterococcus* genus, unlike West-European countries, where, as mentioned before, they are included in microbial compositions for cheese production.

According to the results of biochemical fermentation of carbohydrates, all strains of *Enterococcus* fermented galactose. Decreasing the level of galactose is one of modern trends of the dairy industry, for galactose is undesirable in food products for various reasons. Galactose is badly metabolized in the human organism, which could have a negative effect on the well-being of people who suffer lactase deficiency (Novelli & Reichardt, 2000). Accumulation of galactose in dairy products can cause negative effects: darkening of cheese, release of CO_2 by bacteria which are not included in a fermentation starter preparation, development of purulent or pathogenic microflora (Vaillancourt et al., 2004). Therefore, selection of LAB for producing dairy products prove significance of their both technologic and probiotic properties (Goh & Philip, 2015; Natarajan & Parani, 2015; Zhang et al., 2017).

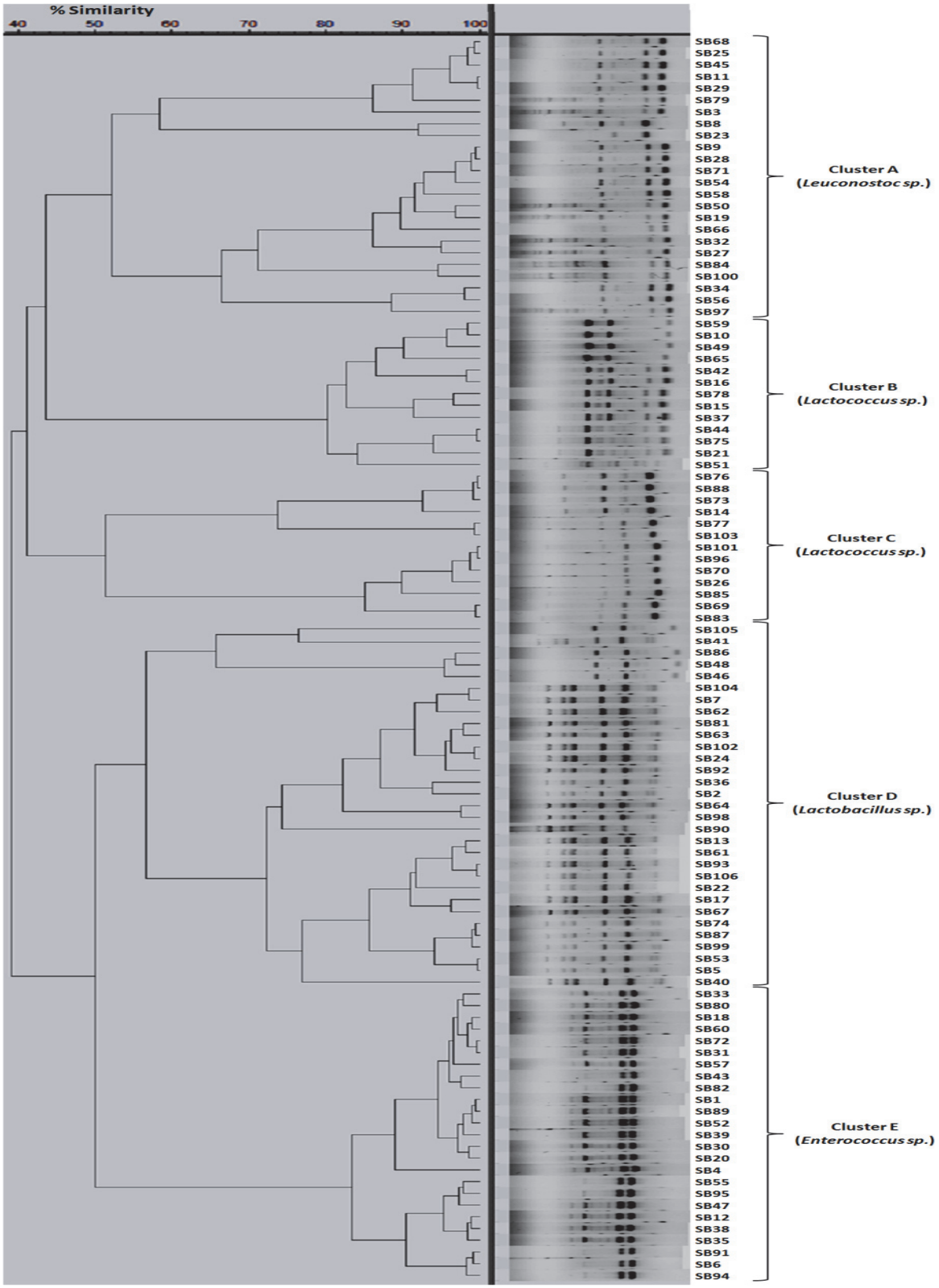

Fig. 2. Electrophoretogram of the products of DNA amplification using 1254 primer

Bacteria of heterofermentative *L. mesenteroides* is typical for home-made cheese of different countries. Their presence is explained by their role in the process of cheese ripening due to proteolytic and lipolytic activity and synthesis of aromatic compounds (Cocolin et al., 2004; Giraffa et al., 2010). The presence of *L. garvieae* can be considered an important part of microbial compound of bryndza, which is related to natural fermentation. The presence of *L. garvieae* is typical for traditional dairy products in different countries, especially Italy (Fortina et al., 2007). *L. garvieae* is a typical culture for production of home-made cheese in this country. Differential characteristic, based only on bacteriological methods, does not always provide the possibility of accurate identification of strains, and sometimes of microorganism species. The ability of some LAB strains to manifest fermentative properties which differ from the properties described for a given species, is a common phenomenon, which complicates the definition of their taxonomic position (Kovalenko & Lashhevskij, 2003; Mayo et al., 2014). The objective of the study was the traditional Carpathian cheeses, previously unstudied , therefore we used molecular-genetic methods, which a number of researchers consider necessary (Yu et al., 2011, 2012), to prove our results of the LAB identification.

To summarize, the microflora of cheese A was determined to be the most diverse in representatives of the LAB species and genera, involved in its making. It should be noted that out of three samples of cheese made from milk of different sheep breeds in different regions with different climatic conditions, we isolated three species of lactate acid bacteria: *L. lactis* ssp. *lactis*, *L. garvieae* and *E. faecium*. Therefore, we consider this microflora compound distinctive for traditional Carpathian bryndza.

Conclusions

We conducted genus and species level identification of LAB isolated from traditional Carpathian cheeses, bryndza and budz, using classic microbiological and molecular-genetic methods. Using a set of tinctorial, cultural and physical-biochemical features, we identified the following LAB bacteria: *L. lactis*, *L. garvieae*, *L. plantarum*, *E. faecium*, *L. mesenteroides*. This was proven by the results of molecular-genetic studies. Using polymerase chain reaction with 1254 primer, we determined significant intraspecific diversity of LAB strains isolated from the samples of cheese. This indicates the necessity of identification of strains and studying their properties.

The sample of bryndza from the Carpathian highlands had the largest species representation. These species can be considered as promising for developing a bacterial preparation for making brined cheese in industrial conditions, for they manifest high resistance to 4% concentration of NaCl and are quite active acidifiers – titrated acidity of milk after their inoculation is 60 to 120ºT.

The study was funded by a grant of the President of Ukraine (“Molecular-genetic identification of lactate acid microflora of national dairy products and biotechnology of developing bacterial preparations”) according to the project F 70/122-2017 State Fund of Fundamental Research of Ukraine.

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Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 69–74
doi: 10.15421/021809

Strains of soil microorganisms promising for the creation of a complex plant protection product against mycoses and harmful insects

O. A. Drehval, A. A. Dreus, N. V. Cherevach, T. V. Sklyar

Oles Honchar Dnipro National University, Dnipro, Ukraine

Article info

Received 17.01.2018
Received in revised form 12.02.2018
Accepted 26.02.2018

Oles Honchar Dnipro National University, Gagarin ave., 72, Dnipro, 49010, Ukraine.
Tel.: +38-056-760-85-14.
E-mail: microviro@ukr.net

Drehval, O. A., Dreus, A. A., Cherevach, N. V., & Sklyar, T. V. (2018). Strains of soil microorganisms promising for the creation of a complex plant protection product against mycoses and harmful insects. Regulatory Mechanisms in Biosystems, 9(1), 69–74. doi: 10.15421/021809

We evaluated the antagonistic activity of 23 strains of *Bacillus* spp. against phytopathogenic fungi *Fusarium oxysporum*, *F. culmorum*, *F. moniliforme*, *Cladosporium herbarum*, *Alternaria alternata* and *Aspergillus niger*. The antagonistic activity was tested by agar diffusion (the method of blocks). For determining the influence of bacteria on barley plants , ardent seeds were treated by cultural liquid (dilution 1 : 10) for 2 hours and germinated in Petri dishes on moist filter paper. The fungistatic effect of *Bacillus* spp. separately and in combination with entomopathogens (in equal ratio) was determined by the level of inhibition of the fungi *Fusarium* spp. on a solid nutrient medium with 5% of the culture liquid. Insecticidal activity of microorganisms was determined in the model experiments by the percentage of death of the caterpillar *Archips podana* Scop. Strains of *Bacillus* sp. KMB-3 and *Bacillus* sp. KMB-6 inhibited the growth of all test cultures (zones of growth inhibition 11.4–30.6 and 11.5–29.4 mm, respectively). We established the absence of antagonism between selected strains and entomopathogenic bacteria *Bacillus thuringiensis* IMB-7186, fungi *Beauveria bassiana* IMB-F-100043. We found that treatment of barley seeds with culture liquids of *Bacillus* sp. KMB-3 and *Bacillus* sp. KMB-6 didn't have a negative effect on the morphometric indices and dry weight of seedlings. We established that the highest percentage of growth inhibition of *F. culmorum* IMB-F-50716 was provided by a complex of *Bacillus* sp. KMB-3, *B. bassiana* IMB-F-100043 and *B. thuringiensis* IMB-7186, whose action was at the same level as the action of monoculture *Bacillus* sp. KMB-3 (85.4% and 84.7%, respectively). The highest percentage inhibition of growth of *F. oxysporum* IMB-F-54201 was provided by a complex of strains of *Bacillus* sp. KMB-3 and *B. bassiana* IMB-F-100043, whose effect was slightly inferior to that of the monoculture *Bacillus* sp. KMB-3 (68.4% and 75.1%, respectively). The insecticidal activity of complexes *Bacillus* sp. KMB-3, *B. bassiana* IMB-F-100043, *B. thuringiensis* IMB-7186 or *Bacillus* sp. KMB-6, *B. bassiana* IMB-F-100043, *B. thuringiensis* IMB-7186 insignificantly differed from that of the complex entomopathogens *B. bassiana* IMB-F-100043 and *B. thuringiensis* IMB-7186 (71.1%, 73.3% death versus 80.0%). The selected microbial complexes can be considered as promising for the development of a preparation for the protection of plants against fungal diseases and harmful insects.

Keywords: antifungal action; *Bacillus* sp.; biocontrol; plant diseases; phytotoxicity; insecticidal activity

Штами ґрунтових мікроорганізмів, перспективні для створення комплексного препарату захисту рослин від мікозів та шкідливих комах

О. А. Дрегваль, А. А. Дреус, Н. В. Черевач, Т. В. Скляр

Дніпровський національний університет імені Олеся Гончара, Дніпро, Україна

Оцінено антагоністичну активність 23 штамів *Bacillus* spp. відносно фітопатогенних грибів *Fusarium oxysporum*, *F. culmorum*, *F. moniliforme*, *Cladosporium herbarum*, *Alternaria alternata* та *Aspergillus niger*. Антагоністичну активність перевіряли методом дифузії в агар за діаметром зон затримання росту навколо блоків. Для визначення впливу бактерій на рослини ярого ячменю насіння обробляли культуральною рідиною (розведення 1 : 10) упродовж двох годин і пророщували в чашках Петрі на зволоженому фільтрувальному папері. Фунгістатичну дію *Bacillus* spp. окремо та в комплексі з ентомопатогенами (в однаковому співвідношенні) визначали за рівнем пригнічення грибів *Fusarium* spp. на щільному живильному середовищі, до складу якого входило 5% культуральної рідини. Інсектицидну активність мікроорганізмів визначали в модельних експериментах за відсотком загибелі гусениць *Archips podana* Scop. Відібрано штами *Bacillus* sp. KMB-3 та *Bacillus* sp. KMB-6, що пригнічували ріст усіх досліджуваних тест-культур. Установлено відсутність антагонізму між відібраними штамами та ентомопатогенними бактеріями *Bacillus thuringiensis* IMB-7186 і грибами *Beauveria bassiana* IMB-F-100043. Показано, що обробка насіння ярого ячменю культуральними рідинами *Bacillus* sp. KMB-3 та *Bacillus* sp. KMB-6 негативно не впливала на морфометричні показники та суху вагу проростків. Найбільший відсоток інгібування росту *F. culmorum* IMB-F-50716 забезпечив комплекс *Bacillus* sp. KMB-3, *B. bassiana* IMB-F-100043. і *B. thuringiensis* IMB-7186, дія якого була на рівні дії монокультури *Bacillus* sp. KMB-3 (85,4% та 84,7% відповідно). Найбільше пригнічення росту *F. oxysporum* 54201 забезпечив комплекс *Bacillus* sp. KMB-3 і *B. bassiana* IMB-F-100043, дія якого незначно поступалася дії

монокультур (68,4% та 75,1% відповідно). У модельних дослідах інсектицидна активність комплексів *Bacillus* sp. KMB-3, *B. bassiana* IMB-F-100043, *B. thuringiensis* IMB-7186 або *Bacillus* sp. KMB-6, *B. bassiana* IMB-F-100043, *B. thuringiensis* IMB-7186 відносно гусениць *Archips podana* Scop. незначно відрізнялася від дії ентомопатогенів *B. bassiana* IMB-F-100043 і *B. thuringiensis* IMB-7186 (71,1% та 73,3% загибелі проти 80,0%). Підібрані мікробні комплекси можуть бути використані для розроблення біопрепарату захисту рослин від грибкових хвороб і шкідників.

Ключові слова: антифунгальна дія; *Bacillus* spp.; біоконтроль; хвороби рослин; фітотоксичність; інсектицидна активність

Вступ

Хімічні пестициди широко застосовують для захисту сільськогосподарських рослин від збудників захворювань і комах-шкідників. Це один із найважливіших елементів інтенсивних сільськогосподарських технологій, спрямованих на отримання високих і стабільних врожаїв рослин (Iutynska & Ponomarenko, 2010). Проте хімічні препарати екологічно небезпечні, оскільки повільно розкладаються, продукти їх розпаду потрапляють у ґрунт і негативно впливають на біоту (Meena & Kanwar, 2015). У результаті інтенсивного застосування пестицидів знижується чисельність і життєздатність ґрунтових сапрофітних мікроорганізмів, і навпаки, підвищується вміст шкідливих організмів, що викликає поступове зниження родючості ґрунтів та зменшення виробництва сільськогосподарської продукції. Необхідність отримання екологічно чистої продукції вимагає розширення досліджень, пов'язаних із розробленням систем біологічного захисту, що не порушують екологічної рівноваги ґрунту, сприяють поліпшенню його фітосанітарного стану (Iutynska & Ponomarenko, 2010; Hollensteiner et al., 2017).

Серед антагоністів фітопатогенних мікроорганізмів слід відмітити представників роду *Bacillus*, які продукують різні антимікробні речовини, такі як циклічні ліпопептиди, а також літичні ферменти та хітинази (Alvarez et. al., 2012; Ji et al., 2013; Meena & Kanwar, 2015; Yamamoto et al., 2015; Bodhankar et al., 2017; Dimkić et al., 2017; Molinatto et al., 2017; Rishad et al., 2017). Ліпопептиди, які продукуються *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus* (міксубтилін, фенгіцини A і B, ітурин), проявляють антифунгальну дію, тоді як сурфактин має широкий спектр антибактеріальної дії (Ji et al., 2013; Khong et al., 2013; Meena & Kanwar, 2015). Крім того, широко розповсюджені у ґрунті бактерії *B. amyloliquefaciens* синтезують амілолізин, який не належить до ліпопептидів і пригнічує ріст переважно грампозитивних бактерій (Chen et al., 2016).

Переваги циклічних ліпопептидів порівняно з хімічними засобами захисту рослин – низька токсичність, високий ступінь біодеградації та безпечність для навколишнього середовища (Meena & Kanwar, 2015; Chen et al., 2016). Серед інших представників роду *Bacillus* слід відмітити широко відомий ентомопатогенний мікроорганізм *B. thuringiensis*, деякі штами якого здійснювали антагоністичний вплив на фітопатогенні гриби роду *Verticillum*. Автори припускають, що значний внесок в антифунгальну дію, крім вторинних метаболітів, вносять міколітичні хітинази, які продукуються цими штамами (Hollensteiner et al., 2017). Із ризосфери кукурудзи нещодавно виділено штам *Lysinibacillus sphaericus*, який крім ларвіцидних метаболітів продукував 2-пентил-4-хінолінкарбонову кислоту, що проявляє антифунгальну дію різного ступеня проти фітопатогенних грибів *Alternaria alternata*, *Curvularia lunata*, *Aspergillus* sp., *Sclerotinia* sp., *Bipolaris spicifera*, *Trichophyton* sp. (Naureen et al., 2017). Ґрунтові штами бацил входять до складу ризосфери та не тільки захищають кореневу систему від збудників хвороб, а і продукують біологічно-активні речовини, що стимулюють ріст і розвиток рослин, такі як індолілоцтова кислота, сприяють солюбілізації фосфатів і силікатів, а також індукують підвищення резистентності рослин до збудників мікозів (Khong et al., 2013; Yamamoto et al., 2015; Hollensteiner et al., 2017; Naureen et al., 2017). На їх основі розробляються біологічні препарати для захисту та поліпшення живлення сільськогосподарських рослин. Більшість розроблених мікробних препаратів для захисту рослин від хвороб і шкідників створено на основі монокультур мікроорганізмів. Останнім часом зусилля науковців спрямовані на створення мікробних препаратів комплексної дії на основі асоціацій мікроорганізмів (Srivastava et al., 2010; Egamberdieva et al., 2016, 2017).

Автори статті розробили комплексний інсекто-акарицидний мікробний препарат Бактофунгін-LS на основі ентомопатогенних мікроорганізмів *B. thuringiensis* IMB-7186 та *Beauveria bassiana* IMB-F-100043, який показав високу активність проти широкого спектра комах-шкідників (Drehval et al., 2015). Враховуючи потреби сільськогосподарського виробництва щодо збереження врожаю, доцільно розширити сфери застосування цього препарату не тільки проти шкідників, а і проти збудників грибних хвороб.

Мета цієї статті – виділення бактерій роду *Bacillus* – антагоністів фітопатогенних грибів, перевірка відсутності фітотоксичності виділених культур, визначення взаємовідносин бактерій-антагоністів з ентомопатогенними мікроорганізмами, що входять до складу бактофунгіну, дослідження фунгістатичної дії комплексів антагоністів фітопатогенів та ентомопатогенів відносно грибів роду *Fusarium*, а також інсектицидної активності відносно гусениць *Archips podana* Scop.

Матеріал і методи досліджень

Антагоністичну активність штамів ґрунтових бактерій відносно фітопатогенних грибів перевіряли методом дифузії в агар за діаметром зон затримання росту навколо блоків. Як тест-культури використано штами фітопатогенних грибів із колекції відділу фізіології та систематики мікроміцетів ІМВ НАН України *Fusarium oxysporum* IMB-F-54201, *F. culmorum* IMB-F-50716, *Cladosporium herbarum* IMB-F-16878, а також штами з колекції культур мікроорганізмів кафедри мікробіології, вірусології та біотехнології ДНУ імені Олеся Гончара, виділені зі зразків ґрунту, ураженого насіння та плодів: *F. oxysporum* KMB-F-12, *F. moniliforme* KMB-F-23, *Alternaria alternata* KMB-F-16, *Aspergillus niger* KMB-F-25. Фітопатогенні гриби вирощували на картопляному агарі з 1% глюкози. Взаємовідносини бактерій-антагоністів з ентомопатогенними мікроорганізмами визначали вищезгаданим методом дифузії в агар.

Для перевірки відсутності фітотоксичної дії штамів на рослини ярого ячменю сорту Кристалія бактерії вирощували у м'ясопептонному бульйоні (МПБ) на мікробіологічній качалці (200 об./хв) за 29 ºС три доби. Насіння ячменю (100 насінин) обробляли культуральною рідиною у розведенні 1 : 10 упродовж двох годин і пророщували в чашках Петрі на зволоженому фільтрувальному папері. На четверту добу досліду визначали відсоток пророслого насіння, довжину та суху масу коренів і надземної частини рослин. Як контроль використовували насіння, оброблене стерильною водопровідною водою.

Для визначення фунгістатичної дії антагоністів та ентомопатогенів окремо кожного та в комплексі (у рівних співвідношеннях) культуральні рідини мікроорганізмів вносили у розплавлене живильне середовище Чапека (5% від об'єму) в чашки Петрі, на поверхню якого після застигання розміщували блок 10-добової культури грибів *F. oxysporum* IMB-F-54201 або *F. culmorum* IMB-F-50716. Визначали відсоток інгібування росту грибів на шосту добу порівняно з контролем (середовищем без культуральної рідини).

Інсектицидну активність культуральних рідин мікроорганізмів (розведення 1 : 10) визначали у модельних дослідах за відсотком загибелі гусениць *Archips podana* Scop. Контролем слугували комахи, корм яких обробляли водопровідною водою. Статистичну обробку даних здійснювали за допомогою комп'ютерної програми Statistica 6 (StatSoft Inc., USA). Достовірність відмінностей результатів визначали із застосуванням ANOVA.

Результати

Зі зразків ґрунту чорнозему звичайного виділено 23 ізоляти грампозитивних аеробних і факультативно-анаербних споротвірних бактерій, які попередньо віднесено до роду *Bacillus*, та досліджено на антагоністичну активність відносно штамів фітопатоген-

них грибів – збудників фузаріозу (*F. oxysporum* КМВ-F-12, *F. oxysporum* ІМВ-F-54201, *F. culmorum* ІМВ-F-50716, *F. moniliforme* КМВ-F-23), альтернаріозу (*A. alternata* КМВ-F-16), чорної цвілі (*A. niger* КМВ-F-25), кладоспоріозу (*C. herbarum* ІМВ-F-16878). Із 23 ізолятів 14 проявили фунгістатичну дію (табл. 1).

Найперспективнішими за спектром дії виявилися штами КМВ-3 та КМВ-6, які пригнічували ріст усіх перевірених тест-культур, також із дещо меншим спектром високу антагоністичну дію проявили штами КМВ-5 та КМВ-8. Серед перевірених тест-культур найчутливішими до дії виділених штамів бактерій виявилися *A. alternata* КМВ-F-16, *A. niger* КМВ-F-25 та *C. herbarum* ІМВ-F-16878, найстійкішими – *F. oxysporum* КМВ-F-12 і *F. oxysporum* ІМВ-F-54201 (рис. 1).

Оскільки основна мета дослідження – пошук мікробів-антагоністів для створення комплексного біопрепарату для захисту рослин від шкідників і хвороб, здійснено перевірку взаємовідношення відібраних вищезгаданих штамів антагоністів та ентомопатогенних бактерій *B. thuringiensis* ІМВ-7186 та грибів *B. bassiana* ІМВ-F-100043 – компонентів біопрепарату Бактофунгін-LS (рис. 2).

Усі досліджені штами не пригнічували ріст *B. thuringiensis* ІМВ-7186, три штами (КМВ-3, КМВ-5, КМВ-6) також не пригнічували ріст *B. bassiana* ІМВ-F-100043, штам КМВ-8 показав незначне інгібування росту *B. bassiana*. Враховуючи активність і спектр дії виділених антагоністів, для подальшої роботи обрано два штами бацил (КМВ-3 та КМВ-6).

Таблиця 1

Антагоністична активність штамів бактерій роду *Bacillus* до фітопатогенних грибів (n = 8)

Штами бактерій	Діаметр зони пригнічення росту тест-культур, мм						
	Fusarium xysporum КМВ-F-12	*Fusarium oxysporum* ІМВ-F-54201	*Fusarium culmorum* ІМВ-F-50716	*Fusarium moniliforme* КМВ-F-23	*Alternaria alternata* КМВ-F-16	*Aspergillus niger* КМВ-F-25	*Cladosporium herbarum* ІМВ-F-16878
КМВ-1	0	13,2 ± 0,3	0	11,2 ± 0,3	0	0	0
КМВ-2	0	0	20,6 ± 1,0	20,4 ± 0,8	24,0 ± 0,7	20,2 ± 0,5	26,1 ± 1,1
КМВ-3	13,3 ± 0,3	11,4 ± 0,3	18,5 ± 0,7	21,5 ± 0,6	30,6 ± 1,2	24,2 ± 0,7	28,3 ± 2,0
КМВ-4	12,4 ± 0,4	0	13,3 ± 0,5	0	0	0	15,2 ± 0,8
КМВ-5	0	17,5 ± 0,7	21,4 ± 1,2	19,6 ± 0,5	28,5 ± 0,9	25,1 ± 0,9	27,4 ± 1,0
КМВ-6	11,5 ± 0,2	15,2 ± 0,9	23,2 ± 1,5	20,5 ± 0,7	27,2 ± 0,6	29,4 ± 0,6	23,5 ± 0,9
КМВ-7	15,3 ± 0,8	15,1 ± 0,6	0	19,3 ± 0,2	27,3 ± 0,8	19,5 ± 0,3	26,5 ± 0,5
КМВ-8	0	18,5 ± 0,8	20,0 ± 0,9	20,7 ± 0,3	25,5 ± 0,4	25,5 ± 0,8	28,1 ± 0,6
КМВ-9	16,2 ± 0,9	0	10,5 ± 0,1	0	0	0	0,
КМВ-10	12,0 ± 0,3	0	0	0	0	0	0
КМВ-11	0	0	0	0	12,5 ± 0,2	9,5 ± 0,2	13,2 ± 0,3
КМВ-12	10,5 ± 0,2	10,3 ± 0,2	14,5 ± 0,6	0	12,0 ± 0,5	0	0
КМВ-13	0	11,2 ± 0,3	0	12,0 ± 0,4	0	10,4 ± 0,1	0
КМВ-14	12,2 ± 0,4	12,4 ± 0,5	13,3 ± 0,5	10,0 ± 0,6	0	0	13,3 ± 0,4

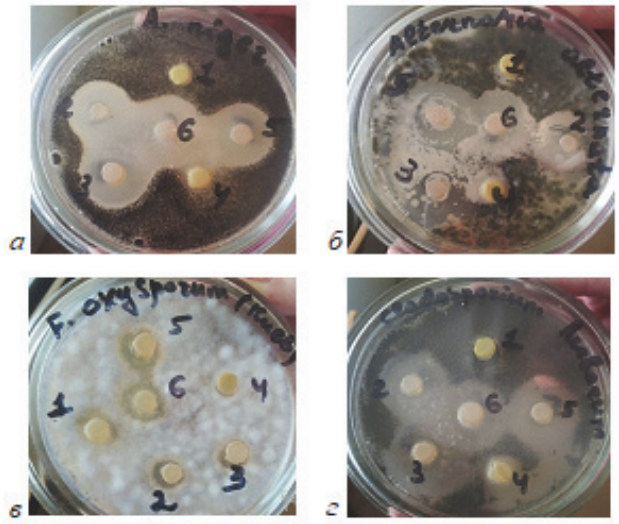

Рис. 1. Вплив штамів бактерій роду *Bacillus* на ріст фітопатогенних грибів: *а – Aspergillus niger* КМВ-F-25; *б – Alternaria alternata* КМВ-F-16; *в – Fusarium oxysporum* ІМВ-F-54201; *г – Cladosporium herbarum* ІМВ-F-16878; *1–6* – номери штамів-антагоністів

Таблиця 2

Вплив штамів бактерій-антагоністів на формування проростків ярого ячменю сорту Кристалія (n = 100)

Варіант обробки	Частка пророслого насіння, %	Середня довжина пагонів, мм	Середня довжина коренів, мм
Bacillus sp. КМВ-3	91,0 ± 1,9	38,8 ± 2,0	261,0 ± 6,3
Bacillus sp. КМВ-6	84,0 ± 6,6	38,3 ± 2,8	246,7 ± 18,4
Контроль	95,0 ± 2,2	43,1 ± 2,9	264,7 ± 26,4

Розробляючи біопрепарати для захисту рослин, необхідно здійснювати випробування на фітотоксичність мікробних культур. Наші дослідження на рослинах ячменю ярого сорту Кристалія показали відсутність фітотоксичної дії *Bacillus* sp. КМВ-3 та *Bacillus* sp. КМВ-6 за морфометричними показниками та сухою вагою проростків. Незначні різниці відсотка пророслого насіння, довжини пагонів і коріння проростків та їх сухої ваги статистично не достовірні (табл. 2, рис. 3).

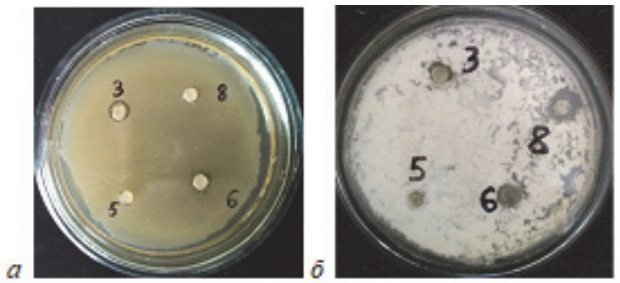

Рис. 2. Вплив найактивніших штамів-антагоністів фітопатогенних грибів на ріст ентомопатогенних мікроорганізмів: *а – Bacillus thuringiensis* ІМВ-7186; *б – Beauveria bassiana* ІМВ-F-100043; *3*, *5*, *6*, *8* – номери штамів-антагоністів

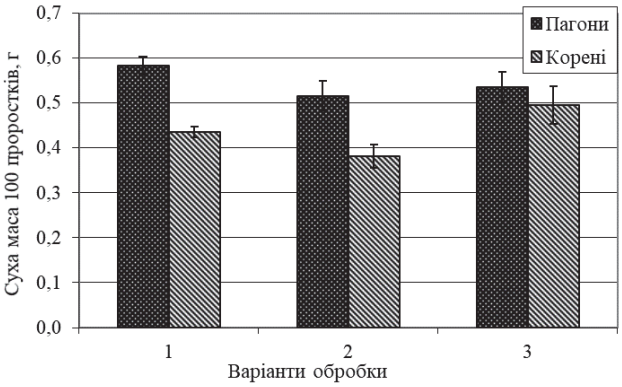

Рис. 3. Суха маса коренів та пагонів ярого ячменю сорту Кристалія за обробки культуральною рідиною бактерій: *1 – Bacillus* sp. КМВ-3; *2 – Bacillus* sp. КМВ-6; *3* – контроль (n = 100)

Для створення комплексного препарату важливо дослідити спільну дію всіх компонентів мікробного комплексу на збудників грибних хвороб рослин. Випробувано дію комплексів на гриби роду *Fusarium*, оскільки, з одного боку, вони завдають найбільших збитків сільськогосподарському виробництву, з іншого – ці гриби виявилися менш чутливими до дії виділених антагоністів.

Попередня перевірка антагоністичних властивостей ентомопатогенних мікроорганізмів показала відсутність фунгістатичної дії *B. thuringiensis* IMB-7186 і, навпаки, суттєве інгібування росту фузаріїв культурою *B. bassiana* IMB-F-100043. Тому дію мікробних комплексів, що складалися зі штаму-антагоніста та ентомопатогена *B. bassiana* IMB-F-100043 і штаму-антагоніста та штамів *B. bassiana* IMB-F-100043 і *B. thuringiensis* IMB-7186 порівнювали з дією монокультур штамів-антагоністів (табл. 3, 4).

Найбільший відсоток інгібування росту *F. culmorum* IMB-F-50716 забезпечив комплекс *Bacillus* sp. KMB-3, *B. bassiana* IMB-F-100043 і *B. thuringiensis* IMB-7186, дія якого була на рівні дії монокультури *Bacillus* sp. KMB-3. Найбільше пригнічення росту *F. oxysporum* IMB-F-54201 забезпечив комплекс *Bacillus* sp. KMB-3 і *B. bassiana* IMB-F-100043, дія якого дещо поступалася дії монокультур.

Таблиця 3

Комплексна дія штамів-антагоністів та *B. thuringiensis* IMB-7186 і *B. bassiana* IMB-F-100043 на лінійний ріст *F. culmorum* 50716 (n = 8)

Варіант досліду	Діаметр колонії, мм третя доба	Інгібування росту,% третя доба	Діаметр колонії, мм шоста доба	Інгібування росту, % шоста доба
Контроль	29,7 ± 1,4	–	78,5 ± 1,9	–
Bacillus sp. KMB-3	12,0 ± 0,3**	59,6	12,0 ± 0,3**	84,7
Bacillus sp. KMB-6	11,4 ± 0,3**	61,6	11,1 ± 0,2***	85,9
B. thuringiensis IMB-7186	30,1 ± 1,5	0,0	83,2 ± 1,1	0,0
B. bassiana IMB-F-100043	10,7 ± 0,3***	64,0	15,5 ± 1,3*	80,3
Bacillus sp. KMB-3 + *B. bassiana* IMB-F-100043	11,3 ± 0,3**	62,0	11,5 ± 0,3***	85,4
Bacillus sp. KMB-6 + *B. bassiana* IMB-F-100043	14,2 ± 0,9*	52,2	13,7 ± 0,3*	82,5
Bacillus sp. KMB-3 + *B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186	11,3 ± 0,7**	62,0	11,3 ± 0,2***	85,6
Bacillus sp. KMB-6 + *B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186	15,2 ± 0,4*	48,8	14,8 ± 1,2*	81,1

Примітка: * – P < 0,05, ** – P < 0,01, *** – P < 0,001 відносно контролю – ростом гриба за відсутності культуральних рідин мікроорганізмів у середовищі.

Таблиця 4

Комплексна дія штамів-антагоністів та *B. thuringiensis* IMB-7186 і *B. bassiana* IMB-F-100043 на лінійний ріст *F. oxysporum* IMB-F-54201 (n = 8)

Варіант досліду	Діаметр колонії, мм третя доба	Інгібування росту, % третя доба	Діаметр колонії, мм шоста доба	Інгібування росту, % шоста доба
Контроль	44, 3 ± 0,6	–	85,4 ± 0,4	–
Bacillus sp. KMB-3	23,4 ± 2,6*	47,2	21,3 ± 1,3***	75,1
Bacillus sp. KMB-6	23,9 ± 1,7*	46,1	25,6 ± 1,6**	70,0
B. thuringiensis IMB-7186	45,3 ± 2,3	0,0	87,5 ± 1,2	0,0
B. bassiana IMB-F-100043	18,9 ± 0,7**	57,3	21,2 ± 3,3**	75,2
Bacillus sp. KMB-3 + *B. bassiana* IMB-F-100043	25,5 ± 2,9*	42,4	27,0 ± 2,7*	68,4
Bacillus sp. KMB-6 + *B. bassiana* IMB-F-100043	29,8 ±1,4*	22,7	29,4 ± 2,0*	65,6
Bacillus sp. KMB-3 + *B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186	33,2 ± 0,9*	25,1	31,4 ± 1,3*	63,2
Bacillus sp. KMB-6 + *B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186	35,2 ± 1,1*	20,6	35,4 ± 1,5*	58,5

Примітка: див. табл. 3.

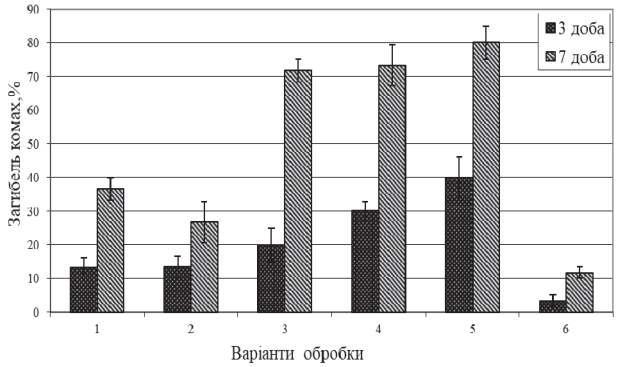

Рис. 4. Інсектицидна активність мікробних культур та їх комплексів відносно гусениць *Archips podana* Scop.: *1 – Bacillus* sp. KMB-3; *2 – Bacillus* sp. KMB-6; *3 – Bacillus* sp. KMB-3 + *B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186; *4 – Bacillus* sp. KMB-6 + *B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186; *5 – B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186; *6* – контрольні (незаражені) комахи (n = 8)

Розробляючи склад поліфункціонального мікробного препарату, доцільно перевірити інсектицидну активність комплексів ентомопатогенів із *Bacillus* sp. KMB-3 або *Bacillus* sp. KMB-6. Дію комплексів порівнювали з інсектицидною активністю *B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186 (Бактофунгін-LS) та дією штамів бацил відносно гусениць листовійки всеїдної (рис. 4).

Інсектицидна активність комплексів *Bacillus* sp. KMB-3, *B. bassiana* IMB-F-100043, *B. thuringiensis* IMB-7186 та *Bacillus* sp. KMB-6, *B. bassiana* IMB-F-100043, *B. thuringiensis* IMB-7186 незначно відрізнялася від дії ентомопатогенів *B. bassiana* IMB-F-100043 + *B. thuringiensis* IMB-7186. Невисоку інсектицидну дію встановлено також для штаму *Bacillus* sp. KMB-3 (36,7% загибелі личинок, P < 0,01 порівняно з контролем).

Обговорення

Ґрунтові штами *Bacillus* spp. характеризувалися різним ступенем і спектром антифунгальної дії. Із даних літератури відомо, що бактерії роду *Bacillus* здатні продукувати різні антимікробні речовини, наприклад, циклічні ліпопептиди, які пригнічують ріст міцелію грибів і проростання конідій мікроміцетів. Зокрема, *B. amyloliquefaciens* CNU114001 продукував ліпопептид ітурин, який характеризувався активністю проти широкого спектра фітопатогенних грибів *Alternaria panax*, *Botrytis cinerea*, *Colletotrichum orbiculare*, *Penicillium digitatum*, *Pyricularia grisea* та *Sclerotinia sclerotiorum* (Ji et al., 2013). В іншому дослідженні показано, що бактерії того самого виду бацил продукують циклічні ліпопептиди сурфактин С, фенгіцини А і В, які пригнічували ріст *S. sclerotiorum*. Штами *B. amyloliquefaciens* пропонується використовувати для біоконтролю склеротиніального захворювання стовбуровою гнил-

лю (Alvarez et al., 2012). Бактерії, які продукують сурфактин, зазвичай характеризуються антибактеріальною дією. Так, *B. subtilis* 6051 пригнічував ріст *Pseudomonas syringae*, а *B. amyloliquefaciens* KPS46 – *Xanthomonas axonopodis* pv. *glycines* (Meena & Kanwar, 2015). У цьому дослідженні широкий спектр антифунгальної дії показали штами *Bacillus* sp. KMB-3 і *Bacillus* sp. KMB-6, які пригнічували ріст усіх протестованих культур фітопатогенів та не проявляли фітотоксичності.

Останнім часом у рослинництві замість препаратів на основі монокультур дослідники пропонують використовувати комплекс різних мікроорганізмів із додатковими або синергічними властивостями. Передпосівна інокуляція комплексом, що складався із симбіотичного азотфіксатора *Mesorhizobium ciceri* IC53 та ендофітного штаму *B. subtilis* NUU4, ефективніше стимулювала ріст бобової рослини *Cicer arietinum* L., а також утворення бульбочок, формування стручків та урожаю порівняно з інокуляцією одним азотфіксатором. Позитивний вплив мікробного комплексу автори пояснюють такими додатковими властивостями *B. subtilis* NUU4 як здатність до утворення індолілоцтової кислоти, солюбілізація фосфатів і зменшення прояву інфекції, спричиненої фітопатогенним грибом *Fusarium solani* (Egamberdieva et al., 2017). Egamberdieva et al. (2016) повідомили про синергетичний вплив комбінованої інокуляції *Mesorhizobium* sp. та *Pseudomonas extremorientalis* TSAU20 на ростові показники лікарської бобової рослини *Glycyrrhiza uralensis* Fish. за сольового стресу. В іншому дослідженні сумісне застосування *Pseudomonas fluorescens*, *Trichoderma harzianum* і ендомікоризних грибів краще знижувало інфікування томатів *F. oxysporum* f. *lycopersici*, ніж інокуляція одним із цих мікроорганізмів. Комбінована інокуляція знизила важкість захворювання на 74% у польових умовах, урожайність культури при цьому підвищилась на 20% порівняно з контролем (Srivastava et al., 2010). У нашому дослідженні підібрані комплекси мікроорганізмів проявили фунгістатичну та інсектицидну дію та можуть застосовуватись для захисту рослин від грибкових хвороб і комах-шкідників.

Перевірка антагоністичних властивостей ентомопатогенних мікроорганізмів показала відсутність фунгістатичної дії досліджуваного штаму *B. thuringiensis* IMB-7186 і, навпаки, суттєве інгібування росту фузаріїв культурою *B. bassiana* IMB-F-100043. З даних літератури відомо, що деякі штами *B. thuringiensis*, крім інсектицидної дії, характеризуються високою антагоністичною активністю щодо фітопатогенних мікроміцетів родів *Venturia*, *Verticillium* тощо. Це зумовлено синтезом протигрибкових речовин (бацилібактину, цвітерміцину А), а також міколітичних хітиназ, що спричиняють лізис, зміни щільності, товщини та напрямку росту міцелію (Hollensteiner et al., 2017; Patyka et al., 2017). Внесений у щільне живильне середовище очищений препарат ендохітинази *B. thuringiensis* subsp. *tenebrionis* DSM-2803 пригнічував радіальний ріст *Colletotrichium gloeosporioides*, збудника антракнозу рослин. Автори спостерігали пряму кореляцію між концентрацією ендохітинази та рівнем інгібування росту фітопатогену (De la Fuente-Salcido et al., 2016). Крім хітинази штам *B. thuringiensis* HD1 утворював хітинзв'язувальний білок, локалізований в оболонках спор. Цей білок посилював інсектицидну дію кристалічного білка Cry 1Ac та інгібував ріст грибів *Culvularia oryzae*, *Aspergillus oryzae*, *Aspergillus parasiticus*, *Verticillium dahliae*. Хітинзв'язувальний білок діє як синергіст хітинази, внаслідок чого відбувається інтенсивніше пригнічення росту грибів (Arora et al., 2013). Також відомо, що *B. bassiana*, крім активності проти комах, може виступати антагоністом відносно збудників хвороб рослин. Механізм антагоністичної дії цього гриба дослідники пов'язують зі здатністю продукувати антибіотичні речовини, а також конкуренцією за субстрат та індукцією системної резистентності рослин проти збудників захворювань рослин (Shahid et al., 2012). Отримані нами дані підтверджують здатність штаму *B. bassiana* IMB-F-100043 пригнічувати ріст фітопатогенних грибів, проте використаний нами штам *B. thuringiensis* IMB-7186 не пригнічував досліджувані гриби роду *Fusarium*.

Дані наших досліджень свідчать про невисоку інсектицидну активність культуральних рідин *Bacillus* sp. KMB-3. Із літературних джерел відомо, що деякі антагоністи фітопатогенних грибів, що продукують хітинолітичні ферменти, характеризуються ентомоцидною дією. Rishad et al. (2017) повідомили про виділення та очищення хітинази із *B. pumilus* MCB-7, що проявляла міколітичну активність відносно *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Ceratorhiza hydrophila* та *Fusarium oxysporum*. Цей фермент показав також ентомоцидну активність відносно личинок *Scirpophaga incertulas* Walker (Lepidoptera: Pyralidae), шкідника рису. Meena & Kanwar (2015) також встановили, що супернатанти культуральних рідин штамів *B. subtilis*, що продукують ліпопептид сурфактин, показали високу смертність личинок і пупаріїв комарів видів *Culex quinquefasciatus*, *Anopheles stephensi*, *Aedes aegypti*.

Висновки

Зі зразків ґрунту чорнозему звичайного виділено 23 ізоляти бактерій, віднесених до роду *Bacillus*. Найвищу антагоністичну активність відносно фітопатогенних грибів *Fusarium culmorum*, *F. moniliforme*, *F. oxysporum*, *Cladosporium herbarum*, *Aspergillus niger*, *Alternaria alternate* показали штами *Bacillus* sp. KMB-3 та *Bacillus* sp. KMB-6. Установлено відсутність антагонізму між відібраними штамами та ентомопатогенними бактеріями *Bacillus thuringiensis* IMB-7186 та грибами *Beauveria bassiana* IMB-F-100043. Обробка насіння ярого ячменю культуральними рідинами *Bacillus* sp. KMB-3 та *Bacillus* sp. KMB-6 негативно не впливала на морфометричні показники та суху вагу проростків. Підібрано мікробні комплекси, до складу яких входять один із відібраних штамів-антагоністів та ентомопатогени *B. thuringiensis* IMB-7186 і *B. bassiana* IMB-F-100043, що проявляють фунгістатичну дію до фітопатогенних грибів роду *Fusarium* та інсектицидну активність відносно гусениць *Archips podana* Scop.

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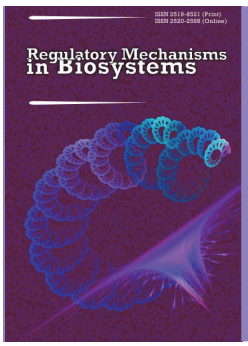

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 75–79
doi: 10.15421/021810

Morphological features of development of *Strongyloides westeri* (Nematoda, Rhabditida) *in vitro*

Y. A. Gugosyan*, V. A. Yevstafyeva**, O. A. Gorb**, V. V. Melnychuk**, I. O. Yasnolob**, C. M. Shendryk*, M. A. Pishchalenko**

**Dnipro State Agrarian and Economic University, Dnipro, Ukraine*
***Poltava State Agrarian Academy, Poltava, Ukraine*

Article info

Received 21.01.2018
Received in revised form 17.02.2018
Accepted 18.02.2018

Dnipro State Agrarian and Economic University, Serhii Efremov st., 25, Dnipro, 49600, Ukraine. Tel.:+38-066-112-9-505. E-mail: y.gugosyan@gmail.com

Poltava State Agrarian Academy, Skovorody st., 1/3, Poltava, 36003, Ukraine. E-mail: evstva@ukr.net

Gugosyan, Y. A., Yevstafyeva, V. A., Gorb, O. A., Melnychuk, V. V., Yasnolob, I. O., Shendryk, C. M., & Pishchalenko, M. A. (2018). Morphological features of development of Strongyloides westeri (Nematoda, Rhabditida) in vitro. Regulatory Mechanisms in Biosystems, 9(1), 75–79. doi: 10.15421/021810

Strongyloides westeri (Ihle, 1917), a parasitic horse nematode, has an unusual lifecycle, which allows it to exist for a long time in the environment. Morphometric features of eggs, larvae and free-living *S. westeri* were studied *in vitro* under different temperature regimes. The optimal temperature for their embryonic development is 25 °C, under which 90% of the first stage rhabditiform larvae are formed and released within 7 hours of cultivation. The temperatures of 20 and 30 °C are less favorable for their development. Embryonic development of *Strongyloides* has four stages that differ in morphology and size. The eggs of a parthenogenetic female are 3.7% longer and 19.6% wider than eggs isolated from free-living females of *S. westeri*. In embryogenesis, the eggs shorten by 4.4 μm (6.5%) and widen by 5.35 μm (8.3%). New data were obtained on postembryonic development of *S. westeri*. The differential morphometric features of stage 1 and 2 rhabditiform larvae which grow both in length and width (33.7% and 30.4% respectively) are established. The development of filariform larvae is associated with loss of bulbous thickening and formation of cylindrical oesophagus. Simultaneously, the body elongates, and the gut becomes shorter. Differential morphometric features of free-living males and females of *S. westeri* are the length and width of body, length of oesophagus, gut, tail end, and size of spicules. Postembryonic development of the free-living and parasitic generations from rhabditiform larvae is temperature-dependent. Most of the free-living generations of *Strongyloides* (54.0%) are formed at 20 °C, and filariform larvae mostly (70.0%) develop at 30 °C. The obtained results of morphological studies improve differential diagnostics of the nematode at various stages of development and further advance the study of its intraspecific variability.

Keywords: *Strongyloides*; horses; nematode eggs; larvae; biological properties; morphometry

Introduction

Among the world parasitic fauna, parasitic worms are a most impressive group (Levine, 1980; Anderson, 2000; Kennedy & Harnett, 2013). Wild and domestic animals are well-known reservoirs of helminths, and prevalence of infection depends on a number of factors, such as the species composition and population sizes of the hosts, environmental conditions, anthropogenic impact, and biological properties of the helminth (Lee et al., 2002; John et al., 2011; Goater et al., 2014; Boyko & Brygadyrenko, 2016, 2017; Carlson et al., 2017).

Nematodes *Strongyloides westeri* Ihle, 1917 are widely distributed equine helminths. According to the literature, levels of equine infection depend on the animals' age, living conditions, prophylactics, climatic conditions. Prevalence can reach 90% (Lyons et al., 2007; Araujo et al., 2012; Ricardo et al., 2012; Lyons and Tolliver, 2014, 2015; Miller et al., 2017).

Nematodes of the genus *Strongyloides* (Grassi, 1879) are of specific interest because of their development cycle, which has alternative parasitic and free-living generations. The parasitic stage is represented only by parthenogenetic females living in the upper sections of the equine small intestine. Free-living nematodes are not parasitic and represented by both males and females living outside the animal host. There is evidence that depending on environmental factors, in particular the air temperature and humidity, eggs in faeces of sick animals or laid by a free-living female can develop differently. In case of direct development, the egg releases a rhabditiform larva that further transforms into a filariform one, which upon maturing can infect the host. Under indirect development, the rhabditiform larvae develop into either males or females. Postembryonic development of *Strongiloides* has distinct morphological traits by which its stages are identified (Lyons et al., 1973; Dewes and Townsend, 1990; Grant et al., 2006; Viney, 2006; Santos et al., 2010; Thamsborg et al., 2016).

Such specific biological properties of *Strongyloides* nematodes indicate the appearance of parasitism in non-parasitic species, followed by the evolution of relevant adaptations. The regressive morphological and biological changes lead to parthenogeny in the parasitic female. Meanwhile, the free-living larvae have the possibility of variable biological adaptations (Blaxter et al., 1998; Dorris et al., 2002; Thompson et al., 2006; Eberhardt et al., 2007).

The establishment of a helminth faunistic complex in certain environmental conditions is also heavily influenced by a number of factors. The most important are the biological properties of parasites that are so far not sufficiently studied in *Strongyloides* species of equines. Hence, investigating the morphological properties of embryonic and postembryonic stages of *S. westeri* outside its host will allow us to complement the already known facts of its biology and to better understand its parasitic adaptations. The aim of present work is to investigate the specifics of morphometric structure and biological properties of the embryonic and postembryonic stages of *S. westeri* nematode *in vitro*.

Materials and methods

Research was carried out in 2016–2017 in the laboratories of Parasitology and Veterinary-Sanitary Expertise of the Department of Veterinary Medicine of Poltava State Agrarian Academy and Dnipro State Agrarian and Economic University. Morphological and size parameters of the eggs of *S. westeri* were obtained from different substrates: gonads of free-living females and faeces of infected horses. The shape and shell features, including thickness, length and width of eggs were studied.

The development of *S. westeri* was investigated by culturing eggs, isolated from the faeces of infected horses and from free-living females, in a thermostat at 20, 25 and 30 °C for 10 hours. The culture was examined hourly under a microscope to count the percentage of released larvae and study the egg morphometry.

Postembryonic stages of *S. westeri* were measured in experimental culture *in vitro* at 25 °C for 10 days. The parameters of rhabditiform larvae L_1 and L_2, filariform larvae, and free-living adult males and females were investigated.

The percentage of rhabditiform larvae developing into filariform larvae (directly) and into free-living males and females (indirectly) was established at different temperatures (20, 25 and 30 °C).

Morphometric parameters of embryonic, postembryonic and adult stages of *S. westeri* were measured using ImageJ for Windows® (version 2.00) in interactive mode using ×10 and ×40 objective, and ×10 photo eyepiece. To calibrate the image analyzer, ruled scale of ocular micrometer was coincided with the scale of stage micrometer included in MikroMed microscope kit. Microphotographs were taken using a 5 Mpix digital camera of MikroMed microscope. The material and significance levels were analyzed using standard methods of statistical processing. All the data are reported as the sample mean ± the standard deviation (SD).

Results

Differences in the formation of rhabditiform larvae (L_1) were found at different temperatures. Most of the larvae (more than 56%) are released within 3–6 hours. Meanwhile, embryonic development can be divided into four stages: blastomere cleavage, larval formation, formation of mobile larvae, and release. The optimal temperature for development of rhabditiform larvae and their release from eggs was 25 °C (Table 1).

Table 1
Embryonic development of *S. westeri* eggs at different temperatures (%, n = 100)

Developmental stage	T °C	Culture time, hours												
		before culture	1	2	3	4	5	6	7	8	9	10	11	12
Blastomere cleavage	20	100	23	15	9	8	8	8	8	8	8	8	8	8
	25	100	20	11	8	6	6	6	6	6	6	6	6	6
	30	100	19	10	9	8	8	8	8	8	8	8	8	8
Larva formation	20	–	77	8	6	1	–	–	–	–	–	–	–	–
	25	–	80	9	3	2	–	–	–	–	–	–	–	–
	30	–	81	9	1	1	–	–	–	–	–	–	–	–
Mobile larva formation	20	–	–	57	28	6	1	–	–	–	–	–	–	–
	25	–	–	63	22	6	2	1	–	–	–	–	–	–
	30	–	–	66	13	5	3	2	2	2	2	2	2	2
Release of larvae from eggs	20	–	–	–	56	19	8	6	3	3	3	3	3	3
	25	–	–	–	62	20	6	6	4	4	4	4	4	4
	30	–	–	–	59	18	8	5	4	4	4	4	4	2
End of development	20	–	–	–	–	8	8	8	13	13	13	13	13	13
	25	–	–	–	–	6	6	6	10	10	10	10	10	10
	30	–	–	–	–	8	8	10	14	14	14	14	14	14

At the start of the experiment, 100% of eggs were at the blastomere cleavage stage (Fig. 1a). Later, the percentage of eggs at this stage decreased and after a 4-hour-long exposure more than 90% of them started the next stage of development (Fig. 1b). The percentage of eggs that stopped developing at this stage was 8% at 20 and 30 °C, and 6% at 25 °C. Larval formation begins quite early, within the first hour of the experiment more than 77% of *Strongyloides* eggs contained an immobile larva. The next stage of embryogenesis was characterized by the formation of a mobile rhabditiform larva (Fig. 1c). It peaked at the sixth hour of culturing. The percentage of eggs that stopped developing was 11 at 20 °C, 8 at 25 °C, and 10 at 30 °C.

Larval release from eggs was first registered at the third hour of culturing (Fig. 1d), and at the sixth hour it peaked (to 90%). Meanwhile the percentage of arrested larvae and undeveloped eggs was 13 at 20 °C, 10 at 25 °C and 14 at 30 °C. Thus the lowest mortality of *S. westeri* eggs was at 25 °C.

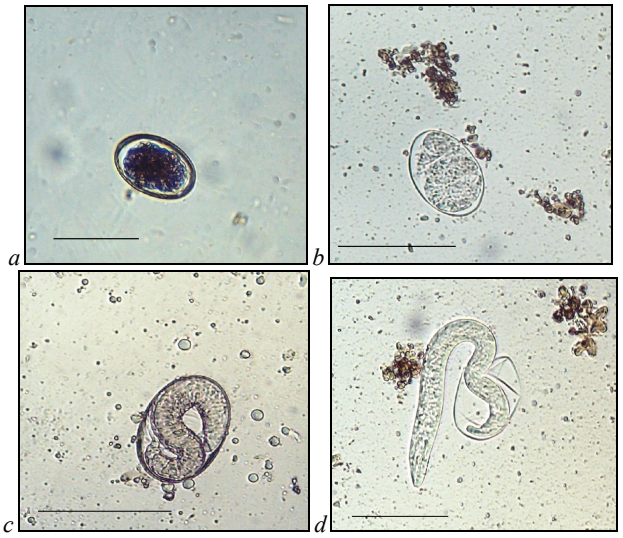

Fig. 1. Embryonic development of *S. westeri in vitro*: *a* – blastomere cleavage; *b* – larval formation; *c* – mobile larva formation; *d* – release of rhabditiform larva (L_1) from egg; bar – 50 µm

The eggs were oval with wide flat poles and thin shells, grey and half-transparent (Fig. 1). The size parameters of the eggs isolated from the gonads of free-living females and from the faeces of infected horses were significantly different (Table 2).

Table 2
Size parameters of *S. westeri* eggs, isolated out of various substrates, n = 10

Parameters, µm		Min	Max	x ± SD
Length	from free-living female gonads	39.76	52.71	47.71 ± 4.61
	from faeces of infected horses	41.88	52.33	49.21 ± 2.90
Width	from free-living female gonads	23.64	31.65	27.50 ± 2.44
	from faeces of infected horses	29.95	39.13	34.24 ± 3.77***
Shell thickness	from free-living female gonads	0.87	1.30	1.07 ± 0.13
	from faeces of infected horses	0.98	1.32	1.16 ± 0.09

Note: *** – P < 0.001 compared to values of eggs isolated from free-living female gonads.

The eggs isolated from the faeces of infected horses, were slightly longer – by 3.1% than the ones isolated from the gonads of the females (47.7 ± 4.6 and 49.2 ± 2.9 µm). The most pronounced differences were in the width dimension. The eggs isolated from faeces were wider by 19.6% (P < 0.001), and their shells thicker by 7.7% compared to the same parameters in eggs isolated from gonads (27.5 ± 2.4 and 1.1 ± 0.1 µm, respectively).

During *in vitro* embryogenesis of *S. westeri* larva, there were changes not only in their internal structure, but in the parameters of the egg length, width and shell thickness (Table 3).

In embryogenesis the egg length and shell thickness significantly decreased, while width increased. Thus, during blastomere cleavage the egg length decreased significantly by 4.4%, during larva formation by 6.1% (P < 0.05), during mobile larva formation by 6.5% (P < 0.01). Egg width increased by 5.3% during blastomere cleavage, by 7.6% during larva formation, by 8.3% during mobile larva formation (P < 0.05). Eggshell thickness also changed and at the mobile larva formation stage was the least (thinner by 19.4% compared to before culture, P < 0.001).

Thus, eggs of *S. westeri* differ by morphology and size parameters depending on the embryogenesis stage and the substrate they were isolated from. Such features should be taken into consideration in species identification.

In the first day of observations, stage 1 rhabditiform larvae (L_1) of *S. westeri* developed, with subsequent transition into stage 2 on the second to third day of development (L_2). Rhabditiform larvae had their own specific features: bulbous thickening of oesophagus, gut filled by pigmented grainy mass in two rows (Fig. 2a, b). On the fourth day of culture, we found filariform larvae with long cylindrical oesophagus and thinner tail end. Morphometrically, the rhabditiform and filariform larvae of *S. westeri* are distinctly different (Table 4).

Table 3
Size parameters of embryonic development of *S. westeri in vitro* (n = 10)

Parameters, μm	Before culture		Developmental stage					
			blastomere cleavage		larval formation		formation of mobile larva	
	x ± SD	Min – Max	x ± SD	Min – Max	x ± SD	Min – Max	x ± SD	Min – Max
Length	48.25 ± 1.93	45.12 – 51.23	46.12 ± 2.52	41.35 – 50.15	45.29 ± 3.12*	41.13 – 49.36	45.11 ± 2.02**	41.35 – 47.21
Width	34.33 ± 3.75	29.05 – 40.19	36.27 ± 2.60	32.14 – 40.42	37.17 ± 2.33	32.02 – 39.85	37.46 ± 1.59*	35.02 – 40.12
Shell thickness	1.13 ± 0.11	0.94 – 1.32	1.06 ± 0.11	0.83 – 1.21	0.99 ± 0.08**	0.85 – 1.14	0.91 ± 0.09***	0.74 – 1.03

Note: * – P < 0.05, ** – P < 0.01, *** – P < 0.001 compared to pre-cultivation values.

Fig. 2. Larvae of *S. westeri*: *a* – rhabditiform (L_1), *b* – rhabditiform (L_2), *c* – filariform; bar – 100 μm

Table 4
Size parameters of rhabditiform and filariform larvae of *S. westeri in vitro* (n = 10)

Parameters, μm	x ± SD	Min	Max
First stage rhabditiform larva (L_1)			
Length	313.48 ± 28.54	264.19	347.25
Width	15.52 ± 2.21	12.03	18.75
Oesophagus length	115.71 ± 8.32	104.01	130.12
Gut length	167.63 ± 26.09	134.10	211.15
Tail end length	30.95 ± 2.05	26.95	34.57
Second stage rhabditiform larva (L_2)			
Length	473.23 ± 28.37	421.21	521.10
Width	22.3 ± 5.46	16.40	31.35
Oesophagus length	119.22 ± 9.91	102.16	134.75
Gut length	310.21 ± 30.64	257.02	354.16
Tail end length	44.52 ± 6.85	35.14	58.13
Filariform larva			
Length	516.42 ± 19.38	484.26	541.43
Width	15.08 ± 1.38	12.46	17.01
Oesophagus length	261.80 ± 11.59	241.26	284.25
Gut length	160.11 ± 8.97	144.35	173.22
Tail end length	95.24 ± 6.53	81.03	102.41

The average body length of L_2 was 473.23 ± 28.37 μm, which is 33.7% more than length of L_1 (313.48 ± 28.54 μm). Body width of L_2 was also 30.4% greater than in L_1. Comparing L_2 and filariform larvae we found the latter to be slightly longer (by 8.3%) and thinner (by 32.3%). The most typical trait of developing filariform larvae was oesophagus formation and loss of the bulbous tip. The process was accompanied by oesophagus growth by 54.4% and gut shortening by 48.3%, which is evidently linked to larvae becoming parasitic.

Postembryonic development of L_2 was followed by their transformation either into filariform larvae or into free-living males and females. In culture, free-living generations appeared from Day 4. They have distinct morphological features; male *S. westeri* has weakly delineated buccal capsule, and on the tail end two spicules of the same size, gubernaculum and pre- and postnatal papillae (Fig. 3a, b, c). The female has a thinner anterior end, straight tail end, vulva in the middle of the body, eggs in the uterus (usually 2–4, sometimes 5) (Fig. 3). The oesophagus had two thickenings, the frontal one elongated and the tail-end one a bulb with a valve apparatus (Fig. 4a, b, c).

Morphometric studies found sex dimorphism in free-living generations of *Strongyloides* (Table 5). Average female length was 934.84 ± 59.37 μm, which is 18.9% longer than average male (757.72 ± 60.04 μm). Females were also 18.5% wider.

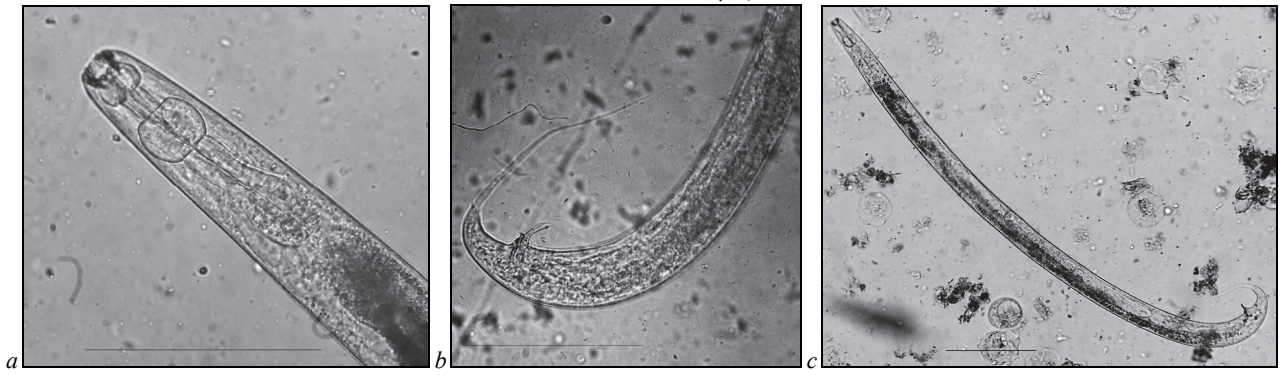

Fig. 3. *S. westeri* (♂): *a* – anterior end, *b* – tail end, *c* – whole specimen; bar – 100 μm

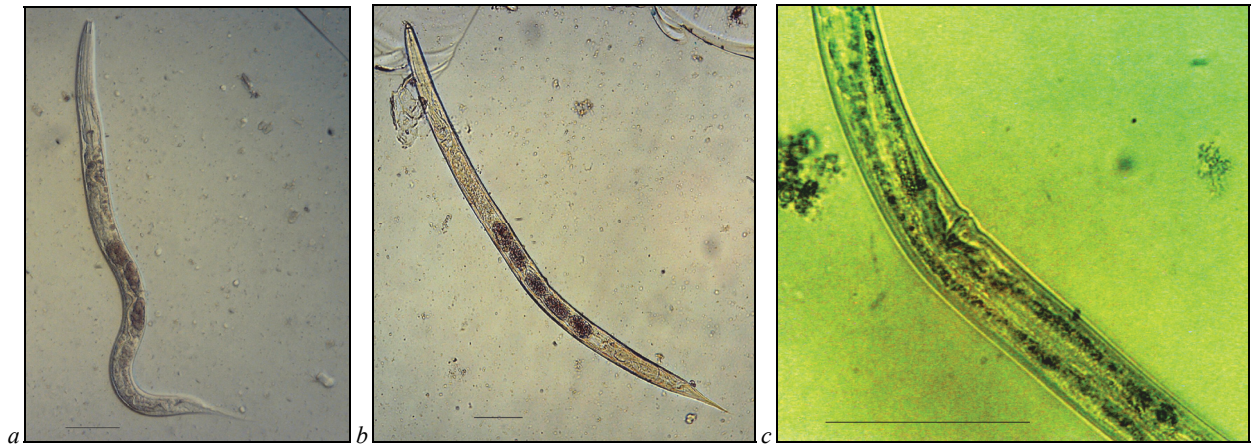

Fig. 4. *S. westeri* (♀): *a*, *b* – general appearance of females with different numbers of eggs; *c* – vulval region; bar – 100 μm

Table 5
Size parameters of *S. westeri* free-living generations *in vitro* (n = 10)

Parameters, μm	♂			♀		
	x ± SD	Min	Max	x ± SD	Min	Max
Body length	757.72 ± 60.04	645.9	834.5	934.84 ± 59.37	846.0	1007.3
Body width	28.31 ± 3.75	22.7	34.7	37.9 ± 5.23	28.3	45.8
Oesophagus length	126.34 ± 10.81	104.5	137.5	148.43 ± 8.71	138.4	165.5
Gut length	577.69 ± 63.68	472.8	654.4	689.36 ± 49.28	611.3	751.0
Tail end length	54.67 ± 11.62	34.7	72.5	98.19 ± 11.32	80.8	122.9
Spicule length	3.61 ± 0.65	2.89	4.73	–	–	–
Eggs in uterus length	–	–	–	39.86 ± 2.09	36.45	42.54
Eggs in uterus width	–	–	–	22.83 ± 1.93	20.25	26.75
Number of eggs	–	–	–	2.80 ± 1.08	1	5

In free-living males, the tail end length was less than 44.3% shorter than in females (98.19 ± 11.94 μm). The ratio of oesophagus to gut in males and females were almost the same (1 : 4.5 and 1 : 4.6, respectively). In culture and postembryonic development of *S. westeri* rhabditiform larvae, the majority of filariform larvae were formed at 30 °C (70 %), fewer larvae were observed at 25 °C (63%) (Table 6).

Table 6
Postembryonic development of rhabditiform larvae of *S. westeri in vitro* (n = 100)

Developmental stage	T, °C	Day of culture										Total
		0	1	2	3	4	5	6	7	8	9	10	
Rhabditiform larvae	20	100	100	100	95	72	41	12	3	3	3	3	3
	25	100	100	100	89	63	29	9	3	2	2	2	2
	30	100	100	98	81	52	20	6	3	3	3	3	3
Filariform larvae	20	–	–	–	5	10	11	16	1	–	–	–	43
	25	–	–	–	8	16	22	12	4	1	–	–	63
	30	–	–	2	15	20	21	10	2	–	–	–	70
Free-living males	20	–	–	–	–	8	12	7	4	–	–	–	31
	25	–	–	–	1	4	6	3	2	–	–	–	16
	30	–	–	–	1	5	6	2	–	–	–	–	10
Free-living females	20	–	–	–	–	5	8	6	4	–	–	–	23
	25	–	–	–	2	6	6	5	–	–	–	–	19
	30	–	–	–	1	4	5	2	1	–	–	–	17

The filariform larvae developed faster in cultures at 30 °C and were found from Day 2, while the percentage of free-living generations was the least observed (27%). Cultures at 25 °C had 63% of filariform larvae and 35% free-living males and females. The highest percentage of free-living generations (54%) was found at 20 °C. Thus, our research supports the dependence of the alternation of *Strongyloides* generations on temperature regimes.

Discussion

Analyzing the obtained data, we should note that abiotic factors greatly affect the development and morphometric parameters of embryonic and postembryonic stages of *S. westeri*. We established that the optimal temperature for culturing eggs of equine *Strongyloides* is 25 °C. It was found that the embryogenesis of *S. westeri* takes 4 to 6 hours at 20 to 30 °C. We also obtained novel data on the morphometric structure of eggs isolated from different substrates during their embryonic development. Our morphometric results are insignificantly different from those previously published (Ivashkin & Dvojnos, 1984), according to which the egg length of equine *Strongyloides* is 39 to 60 μm, and width 39 to 42 μm (compared to the 41.9–52.3 μm and 29.9–39.1 μm, respectively in the present study). Such data are in agreement with the findings of Ihle (1918) and others. Also, morphometric changes during embryogenesis were found, in particular the decrease in length (by 4.4 μm or 6.5%, $P < 0.01$) and thickening (by 5.3 μm or 8.3%, $P < 0.05$), and the thinning of eggshells (by 19.4%, $P < 0.001$).

Rhabditiform and filariform larvae and free-living generations of *S. westeri* were described quite a while ago (Ihle, 1918; Blieck & Baudet, 1920; Schuurmans-Stekhoven, 1930), yet there are no detailed descriptions of these helminths and their variability in Ukraine. We found morphometric parameters of rhabditiform larvae of the first and second stages. In the available literature we found general descriptions of *S. westeri* rhabditiform larvae regardless of developmental stages. Our research allows one to identify separate morphometric parameters of L_1 and L_2: mean length of L_1 was 313.5 ± 28.5 μm, width 15.5 ± 2.2 μm, and those of L_2 473.2 ± 28.4 and 22.3 ± 5.5 μm, respectively. We also measured larval oesophagus, gut and tail end. During the development of filariform larvae they grow slightly in length, with the most typical changes occurring in the structure of the oesophagus and its ratio to gut length. In L_2 the ratio was 1 : 2.60, and in filariform larvae it was 1.63 : 1. Thus, the larval ontogenesis is characterized by important morphometric changes that should be taken into account when identifying *Strongyloides* species.

We obtained new differential data on the morphometry of the free-living *S. westeri* generations. The free-living female mean length was 934.8 ± 59.4 μm, width 37.9 ± 5.2 μm. Males were smaller by 18.9–18.5% (length – 757.7 ± 60.0 μm, width – 28.3 ± 3.8 μm). The parameters are in accord with most of the previous findings, which in its turn indicates adaptability of the helminths. However, one should note that most authors report seeing 5 to 7 eggs in the gonads of free-living females, which is more than what we observed in most cases (2.8 ± 1.1 eggs).

The developmental biology of the helminths is characterized by their high adaptability and survival rates in unfavorable conditions. Tsuji & Fujisaki (1993) in their studies on culturing *S. venezuelens in vitro* prove that changing temperature from 25 to 37 °C is the main factor influencing the development of invasive larvae. Also, filariform larvae were found in extreme temperatures, as high as as 30 °C in the cultures of *S. stercoralis* (Shiwaku et al., 1988). The same was found for *Strongyloides* species in culture (Minato et al., 2008). In a study of the effect of temperature on L_1 stage of *S. ratti*, the larvae kept at 4 or 10 °C for 120 hours could not develop due to the arrested or delayed growth. However, L_1 could develop after transfer to the culture at 25 °C during

48 hours. The larvae stimulated by cold (4 or 10 °C) developed directly into invasive L_3 stages and it took as little as one minute of exposure to the low temperatures to induce direct development. Correspondingly, *Strongyloides* sp. can survive growth arrest or delay (Sakamoto & Uga, 2013).

Our studies showed that culturing rhabditiform larvae at 20 °C favored the formation of a greater number of free-living generations, and at 25–30 °C that of filariform larvae. It is in accordance with the findings of field biology of *S. westeri* (Malygin, 1957; Vislobokov, 2008). Our research proves that males and females develop in different quantities at different temperatures, yet the overall numbers are practically the same – 57 males and 59 females. Thus, our data, as well as the literature, show the significant effect of the environment on the development of different generations of *Strongyloides* sp.

Conclusions

Size parameters of the embryonic development stages of *S. westeri* have significant differences and depend on the substrate and the developmental stage. The process of embryogenesis of *S. westeri* in vitro has four stages: blastomere cleavage, larval formation, mobile larva formation, and release from egg; the stages have morphometric and significant size changes. Embryonic development of *S. westeri* occurs at 20 to 30 °C in 4–6 hours, and average survival rates is 87.7%.

Postembryonic development of *Strongyloides* is characterized by the formation of rhabditiform larvae (L_1 and L_2), filariform larvae, free-living generations of males and females, whose development is accompanied by morphometric changes. The main differential features of *S. westeri* at the discussed developmental stages are body length and width, structure and size of oesophagus and gut and their ratio, length of the tail end. It is possible to regulate the formation of filariform larvae and free-living generations of males and females by adjusting the temperature regime of the culture.

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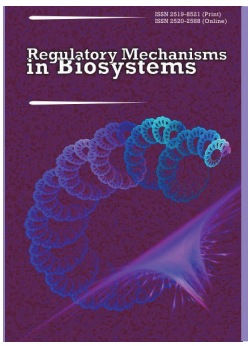

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 80–84
doi: 10.15421/021811

The kinetic properties of arginase in sperm cells of inferile men

R. V. Fafula, U. P. Iefremova, O. K. Onufrovych, H. V. Maksymyuk, D. Z. Vorobets, Z. D. Vorobets

Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

Article info

Received 27.01.2018
Received in revised form 16.02.2018
Accepted 18.02.2018

Danylo Halytsky Lviv National Medical University, Pekarska st., 69, Lviv, 79010, Ukraine.
Tel.: +38-032-275-58-76.
E-mail: roman_fafula@ukr.net

Fafula, R. V., Iefremova, U. P., Onufrovych, O. K., Maksymyuk, H. V., Vorobets, D. Z., & Vorobets, Z. D. (2018). The kinetic properties of arginase in sperm cells of inferile men. Regulatory Mechanisms in Biosystems, 9(1), 80–84. doi: 10.15421/021811

Nowadays the role of NO in the development of male infertility is actively studied. Arginase (EC 3.5.3.1) is a manganese metalloenzyme which converts L-arginine to L-ornithine and urea and reciprocally regulates NO production. Although arginase activity has usually been detected in the reproductive tract, including spermatozoa, no data relating to the kinetic properties of the enzyme in ejaculated spermatozoa has been reported. This study was designed to study the kinetic parameters of arginase of spermatozoa of infertile men. Spermatozoa arginase activity was measured by determining levels of urea production. Kinetic analysis of the enzyme reaction was performed in a standard incubation system with modified physical and chemical characteristics or the respective components (the substrate concentration, Mn^{2+} concentration, incubation time and protein content). Pathobiochemical and kinetic properties of sperm arginase obtained from human normozoo- and pathospermic samples were compared. The maximum rate of L-arginine hydrolysis (detrermined by L-arginine) for arginase of spermatozoa obtained from men with preserved fertility was 2.0, 1.8 and 1.9 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. However, affinity constants for L-arginine was not significantly different between fertile and infertile men. The maximum rate of L-arginine hydrolysis (deretmined by Mn^{2+}) for arginase of spermatozoa obtained from men with preserved fertility was 1.6, 1.7 and 1.7 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. However, affinity constants for Mn^{2+} were not significantly different between fertile and infertile men. In the whole range of time, the urea production by arginase in sperm cells obtained from oligozoospermic samples is much lower compared to value in healthy donors. The results of kinetic analysis indicate that urea production by arginase is much more intense in the control group than in patients with various forms of pathospermia. The initial (instantaneous) reaction rate of arginase reaction was lower for oligozoospermic samples compared to normozoospermic samples. It has been found that inhibition of arginase activity in sperm cells of infertile men occurs by non-competitive type and was related to marked decrease in maximum reaction rate while affinity of arginase to L-arginine and Mn^{2+} was unaffected.

Keywords: arginase activity; L-arginine; enzyme inhibition; spermatozoa; pathospermia

Introduction

Infertility is an important worldwide socio-demographic and medical-biological problem for most developed countries. It affects 10–15% of couples globally and approximately up to 40–50% of infertility is caused by the male factor (Agarwal et al., 2015; Luc et al., 2015). According to the trends observed, the problem of male infertility is predicted to increase (Winters et al., 2014). Nowadays the role of NO in the development of male infertility is actively studied. It is a crucial biological messenger involved in a variety of physiological and pathophysiological processes in different cells, including spermatozoa. Disturbances in the NO-signaling system are considered as key pathogenetic links in the development of male infertility (Kullisaar et al., 2013).

Arginase (EC 3.5.3.1) is a manganese metalloenzyme which converts L-arginine to L-ornithine and urea and reciprocally regulates NO production by competing with NO-synthase for common substarte (L-arginine) (Vanhoutte et al., 2008; Venkatakrishnan et al., 2010). Two isoforms of arginase exist in mammals. Arginase I (cytosolic form) is expressed in hepatocytes and is thought to be primarily involved in ureagenesis, whereas arginase II (mitochondrial form) is expressed extrahepatical tissues. Type II arginase plays an important role in regulating the nitric oxide production and is involved in the biosynthesis of polyamines and aminoacids. Also, arginase plays an important role in regulating the cellular synthesis of NO and modifies its biological effects (Mori et al., 2007).

Data regarding arginase activity (expression) in the sperm cells of infertile men are limited (Hadwan et al., 2014). Arginase II-deficient mice show a reduction in fertility (Stephen et al., 2004). We have demonstrated previously inhibition of the arginase pathway of the L-arginine metabolism, which was not significantly dependent on the type of disruption of spermatogenesis (Fafula et al., 2016). Arginase activity was detected not only in spermatozoa, bu also in seminal plasma. A positive correlation has been shown between arginase activity and semen volume, semen mass activity, sperm motility and sperm concentration (Gür et al., 2012). It was found that plasma arginase activity was significantly lower than in the non-stress situation and during stress there was a negative correlation between the percentage of rapid progressive motility and arginase activity (Eskiocak et al., 2006). Similar relationships between the plasma arginase activity and the sperm concentration and sperm motility were detected in sheep and bulls (Gür et al., 2012; Türk et al., 2011). Although arginase activity has usually been detected in the reproductive tract, including spermatozoa, no data relating to the kinetic properties of enzyme in ejaculated spermatozoa has been reported. Therefore, this study was designed to study the kinetic parameters of arginase of spermatozoa of infertile men.

Materials and methods

Subjects. This study involved 16 infertile men with different forms of pathospermia. A detailed medical history was compiled for all studied cases. Exclusion criteria: subjects currently on any medication or antioxidant supplementation were not included. In addition, subjects with infertility lasting over 10 years, azoospermia, testicular varicocele, genital infection, chronic illness and serious systemic diseases, smokers and alcoholic men were excluded from the study because of their well-known high seminal reactive oxygen species levels and decreased antioxidant activity (Atig et al., 2012).

Ejaculates from a 16 infertile and 10 fertile healthy individuals were obtained. Infertile men were age-matched to fertile control cases. Subjects were classified into three groups as having different forms of pathospermia (oligozoospermia, asthenozoospermia, oligoasthenozoospermia). Semen samples of fertile men represent the control group, which consisted of 10 healthy men with somatic fertility, normozoospermia and confirmed parenthood (married for 3–10 years and have healthy 1–3 children). Semen samples were obtained by masturbation and collected into sterile containers, following 3–5 days' abstinence from sexual activity. After liquefaction at 37 ºC with 5% CO_2 in air, semen samples were examined for volume, sperm concentration, pH, morphology and motility according to the World Health Organization guidelines (WHO Laboratory Manual for the Examination and processIng of Human Semen, 2010).

Ethical approval. Before becoming involved in the study, all the men were made aware of patient information leaflets and gave informed consent to participate in research. Terms of sample selection meet the requirements of the principles of Helsinki Declaration on Pprotection of Human Rights, Convention of Europe Council on Human Rights and Biomedicine and the provisions of laws of Ukraine. Approval for the study was taken from the Ethics Committee of Danylo Halytsky Lviv National Medical University. All patients and healthy donors gave written informed consent to participate in the research (Ethical Committee Approval, protocol No 6 from March 29, 2017).

Cell preparation. Sperm cells were washed from semen plasma by 3 times centrifugation at 3000 g for 10 min in media which contained (mM): 120 NaCl, 30 KCl, 30 Hepes (pH 7.4). The content of total protein in the samples was determined by Lowry method (Lowry et al., 1951) using a kit to determine its concentration ("Simko Ltd"). Determination of arginase activities was carried out in permeabilized spermatozoa. The detergent saponin in a final concentration of 0.5% was added to sperm suspension for permeabilization of sperm membranes.

Arginase activity assay. Spermatozoa arginase activity was measured by determining levels of urea production. Briefly, incubation media of the following composition (mmol/ml): L-arginine – 100, $MnCl_2$ – 2, Tris-HCl – 20 (pH 9.5) was used. The protein concentration usually did not exceed 50–100 mg. The mixture was incubated at 37 °C for 90 min, and the reaction was stopped by adding 1 ml 50% trichloroacetic acid. After centrifugation, the urea was determined in the supernatant spectrophotometrically by measuring absorbance at 520 nm according to the assay kit "Simko Ltd". Arginase activity was expressed as nmol urea per min per mg protein.

Kinetic analysis. Kinetic analysis of the enzyme reaction was performed in a standard incubation system (as described above) with modified physical and chemical characteristics or the respective components (the substrate concentration, Mn^{2+} concentration, incubation time and protein content). The apparent affinity constant for L-arginine (KL-Arg) and maximum reaction rate (V_{max}) were determined by Lineweaver-Burk plot {1/V; 1/[S]}. The dynamics of urea production in arginase reaction in sperm cells of fertile and infertile men were determined according to the paper (Kosterin et al., 1987). The kinetic parameters characterizing arginase reaction – the initial (instantaneous) reaction rate (V_0), maximum amount of the reaction product (P_{max}) and characteristic reaction time (time half saturation) τ were determined in coordinates {P/t; P}.

Statistical analyses. The data are expressed as means ± standard error (M±SE). One-way ANOVA was performed to detect statistical significance. Differences with $P < 0.05$ were considered as significant. Kinetic and statistical calculations were carried out using the software MS Office computer programs. The equation of the straight line that approximates the experimental data the best was calculated by method of least squares. The absolute value of the correlation coefficient r was from 0.80 to 0.95.

Results

Pathobiochemical and kinetic properties of sperm arginase obtained from human normozoo- and pathospermic samples were compared. Different methodological approaches (studies on purified enzymes, isolated subcellular structures, on whole cells or on homogenates) are used for studying arginase activity. Enzymes might be in latent state and inaccessible to substrates in whole cells. Therefore, testing their activities is possible after prior disturbance of integrality of spermal membranes. This can be achieved by introducing a substance leading to perforation of plasma membranes (detergent) in the incubation medium. Using a suspension cells pretreated with detergent (saponin) is an adequate model for correct testing of arginase activity. Under these conditions the natural interrelation of intracellular structures is obeyed.

Kinetic analysis of arginase activity on L-arginine concentration. Since arginase is an enzyme that hydrolyzes L-arginine, changes in its concentration in the incubation medium affect the rate of arginase reaction. The dependence of the arginase activity on the substrate concentration in the incubation medium was determined by the apparent affinity constant to the substrate KL-Arg. For its determination L-arginine was added to the incubation medium in concentrations ranging from 10 to 150 mM (at constant concentration of $MnCl_2$ – 2 mM). We observed a monotonic increase in the enzyme activity of sperm cells obtained from both normo- and pathozoospermic samples reaching a plateau at 100 mM (Fig. 1). As can be seen from Fig. 1 the arginase activity in pathozoospermic samples was reduced in comparison with normozoospermic samples in the whole range of L-arginine concentrations. However, the maximal arginase activity was observed in presence of 100 mM L-arginine in incubation medium for both normo- and pathozoospermic samples.

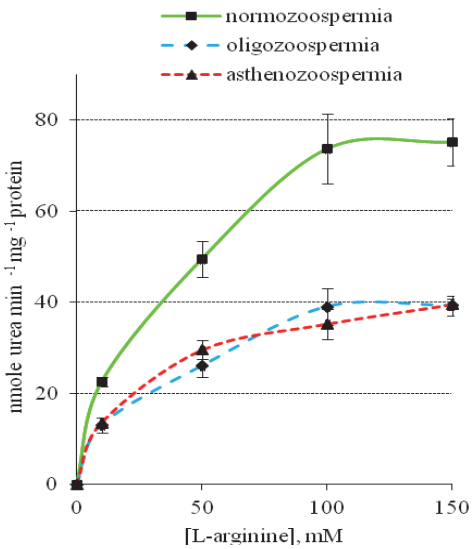

Fig. 1. Dose-dependent effect of extracellular L-arginine concentration on arginase activity in sperm cells of fertile and infertile men (x ± SE, n = 6–8)

In order to elucidate the possible mechanism of change in arginase activity in patients with oligo- and asthenozoospermia, the main kinetic parameters of L-arginine hydrolysis were determined in Lineweaver-Burk plot (Fig. 2).

As can be seen, the concentration curves {1/[S]; 1/[V]} differ by angle of inclination for normo- and pathozoospermic patients. The main kinetic parameters of arginase of sperm cells of fertile and infertile men are presented in Table 1.

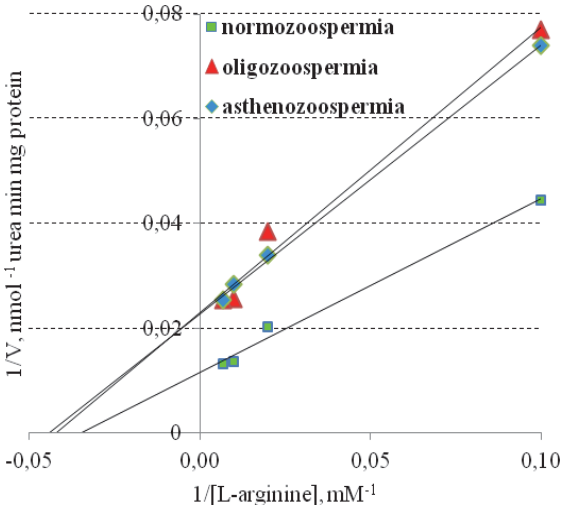

Fig. 2. Linearization of concentration curves represented on Fig. 1 in Lineweaver-Burk plot (n = 6–8; r > 0.80)

Table 1
Kinetic parameters of arginase in sperm cells of fertile and infertile men determined by L-arginine (x ± SE, n = 6–8)

Kinetic parameters	Normozoospermic men	Pathozoospermic men		
		oligozoospermia	asthenozoospermia	oligoasthenozoospermia
$K_{L\text{-}Arg}$, mM	21.0 ± 1.8	20.8 ± 2.6	23.8 ± 1.5	24.2 ± 2.0
V_{max}, nmol urea per min per mg protein	74.6 ± 5.2	37.7 ± 3.6*	41.6 ± 3.9*	40.2 ± 4.2*

Note: * – P < 0.001 compared to normozoospermic men (with preserved fertility).

The data in Table 1 show that the maximum rate of L-arginine hydrolysis for arginase of spermatozoa obtained from men with preserved fertility was 2.0, 1.8 and 1.9 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. However, affinity constants for L-arginine were not significantly different between fertile and infertile men. Thus, in patients with oligo-, astheno- and oligoasthenozoospermiza, the inhibition of arginase activety in sperm cells occurs by noncompetitive type, by reducing the reaction rate (value of Vmax decreases).

Kinetic analysis of arginase activity on Mn^{2+} concentration. Since arginase activity is Mn^{2+}-dependent enzyme, the changes in the Mn^{2+} concentration in the incubation medium affect the rate of arginase reaction. To determine the influence of Mn^{2+} ions on arginase activity, Mn^{2+} ions at varying concentrations (at constant concentration of L-arginine – 100 mM) were added during preincubation (Fig. 3). While preincubation with a Mn^{2+} concentration of 3–4 mM fully activated sperm arginase of human pathospermic samples, a Mn^{2+} concentration of 2 mM fully activated that of normozoospermic samples.

In order to elucidate the possible mechanism of change in arginase activity in patients with oligo- and asthenozoospermia the main kinetic parameters of L-arginine hydrolysis were determined in Lineweaver-Burk plot (Fig. 4).

The main kinetic parameters arginase of sperm cells of fertile and infertile men are presented in Table 2.

Table 2
Kinetic parameters of arginase in sperm cells of fertile and infertile men determined by Mn^{2+} (x ± SE, n = 6–8)

Kinetic parameters	Normozoospermic men	Pathozoospermic men		
		oligozoospermia	asthenozoospermia	oligoasthenozoospermia
K_{Mn2+}, mM	1.3 ± 0.4	2.5 ± 0.8	1.5 ± 0.5	2.1 ± 0.7
V_{max}, nmol urea per min per mg protein	88.2 ± 10.5	55.0 ± 5.8*	52.3 ± 9.2*	53.5 ± 6.2*

Note: see Table 1.

Data in Table 2 show that the maximum rate of L-arginine hydrolysis for arginase of spermatozoa obtained from men with preserved fertility was 1.6, 1.7 and 1.7 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. However, affinity constants for Mn^{2+} were not significantly different between fertile and infertile men. Thus, in patients with oligo- and asthenozoospermiza, the inhibition of arginase activity in sperm cells occurs by noncompetitive type, by reducing the reaction rate (value of V_{max} decreases significantly).

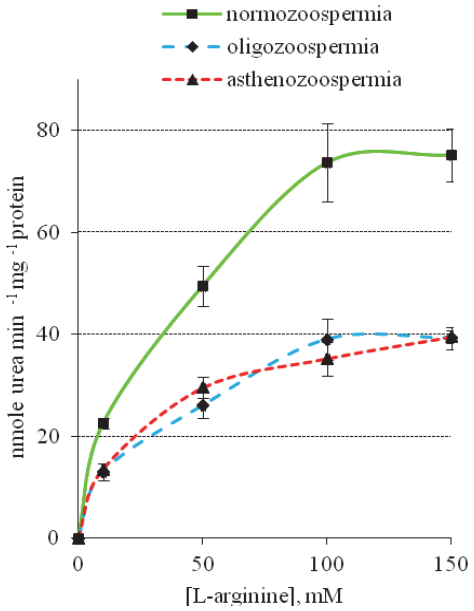

Fig. 3. Dose-dependent effect of extracellular Mn^{2+} concentration on arginase activity in sperm cells of fertile and infertile men (x ± SE, n = 6–8)

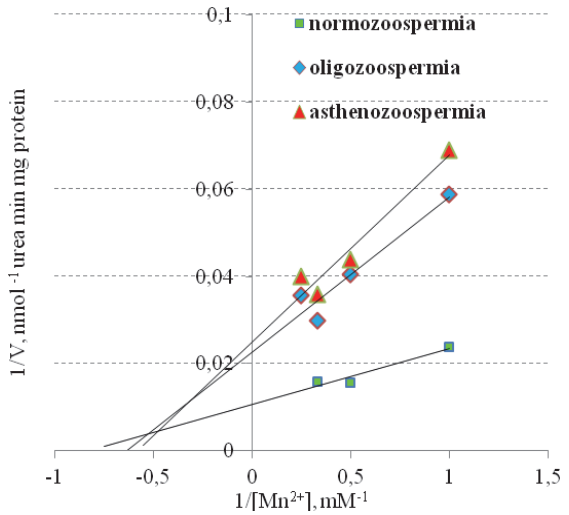

Fig. 4. Linearization of concentration curves represented on Fig. 3 in Lineweaver-Burk plot (n = 6–8; r > 0.85)

Kinetic analysis of arginase reaction on time. The kinetics of urea production in arginase reaction was examined. Suspension of spermatozoa was incubated in the standard incubation medium (concentrations (at constant concentration of L-arginine – 100 mM and $MnCl_2$ – 2 mM) for different periods of time (0–150 min). These experiments show that kinetics of arginase reaction by saponin-permeabilized spermatozoa is reflected by curves which tend to saturation (Fig. 5). Analysis of the results shows that kinetics of urea production by arginase is consistent with the first-order reaction in the range 0–60 min. In this time interval the dependence of urea production on the incubation period is almost linear.

As can be seen from Fig. 5 in the whole range of time, the urea production by arginase in sperm cells obtained from oligozoospermic samples is much lower compared to the value in healthy donors. From linearization of the curves in the coordinates {P/t; P} it can be seen that maximum amount of urea production by arginase in normozoospermic samples exceeds this value in infertile men (Fig. 6). The dynamics of urea production in arginase reaction and its linearization in the coordinates {P/t; P} for astheno- and oligoasthenozoospermic patients had an identical character (not represented in this article).

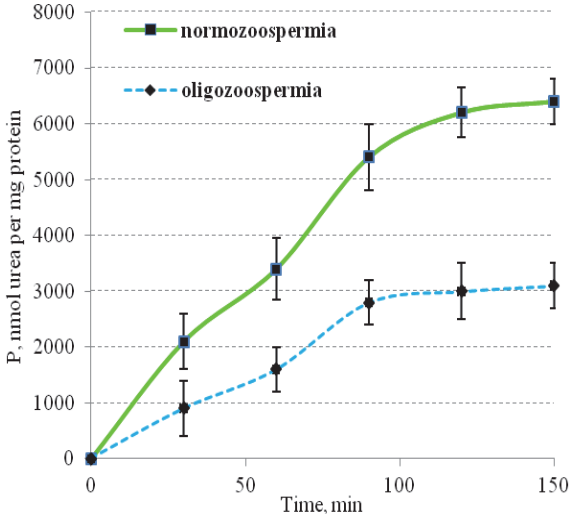

Fig. 5. Dynamics of urea production in arginase reaction in sperm cells of fertile and infertile men (x ± SE, n = 6–8)

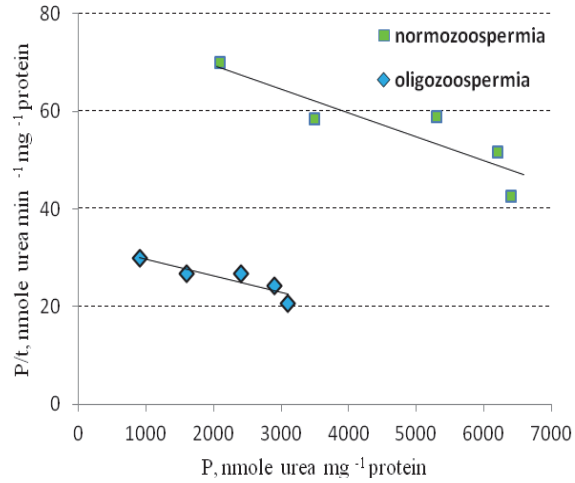

Fig. 6. Linearization of concentration curves represented on Fig. 5 in coordinates [P/t; P] (n = 6–8; r > 0.80)

By linearization of the data in the coordinates P/t on P the main kinetic characteristics of arginase reaction in sperm cells of fertile and infertile men were calculated (Table 3).

Table 3

Kinetic parameters of arginase in sperm cells of fertile and infertile men determined by time of incubation (x ± SE, n = 6–8)

Kinetic parameters	Normozoospermic men	Pathozoospermic men		
		oligozoospermia	asthenozoospermia	oligoasthenozoospermia
V_0, nmol urea per min per mg protein	77.4 ± 1.9	35.5 ± 1.9 ***	37.5 ± 4.9 **	33.8 ± 6.0 **
P_{max}, nmol urea per mg protein	15995 ± 928	10408 ± 1047*	11544 ± 1156*	11250 ± 960*
τ, min	207.4 ± 16.8	291.7 ± 15.8*	322.6 ± 61.8	298.5 ± 16.0 *

Note: * – P < 0.05, ** – P < 0.01, *** – P < 0.001 compared to normozoospermic men (with preserved fertility).

The maximum instantaneous rate of arginase reaction for spermatozoa obtained from men with preserved fertility was 2.2, 2.0 and 2.3 times greater than this value for oligo-, astheno- and oligoasthenozoospermic samples respectively. Maximum amount of reaction product (urea) in the control group exceeds this value in patients with all forms of pathospermia by 1.4–1.5 times. The results of kinetic analysis indicate that urea production by arginase is much more intense in the control group than in patients with various forms of pathospermia. The characteristic reaction time (time half saturation) of arginase reaction for spermatozoa obtained from men with preserved fertility was 1.4–1.5 times lower than this value for pathospermic samples.

Kinetic analysis of arginase activity on protein concentration. Taking into account that enzyme activity depends on the protein content in incubation medium, the arginase reaction was initiated by adding protein with concentrations ranging from 25 to 150 μg/ml in suspension of sperm cells (Fig. 7). It was found that a gradual increase in sperm protein concentration in the incubation medium led to an increase in V_0 of arginase reaction. The V_0 of arginase reaction was lower for oligozoospermic samples compared to normozoospermic samples. The dependence of the urea production on the protein content in incubation medium has the same character for astheno- and oligoasthenozoospermic samples (not represented in this article).

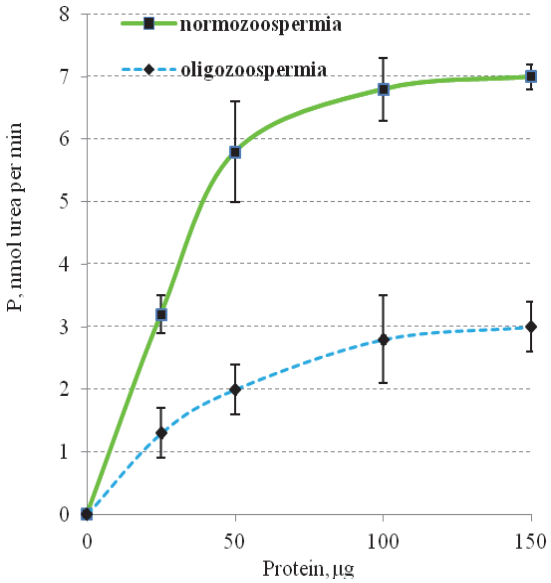

Fig. 7. Dependence of the initial rate of arginase reaction in sperm cells of fertile and infertile men on protein content (x ± SE, n = 6–8)

Discussion

Nitric oxide is a biological molecule which is involved in many physiological functions and pathophysiological outcomes (Bonavida et al., 2006). Several data suggest a crucial role of NO in sperm cell physiology. Altered NO production has been implicated in the pathogenesis of the male infertility (Kullisaar et al., 2013). Overproduction of NO can lead to oxidative and nitrosative stress and may have a potential pathogenetic implication in the reduction of sperm motility (Balercia et al., 2004).

Since arginase is an arginine-depleting enzyme, it affects NOS activity and regulates NO production. This regulatory mechanism is realized by reducing L-arginine availability for NOS to produce NO (Racke et al., 2010). The proper balance between NOS and arginase activity (expression) is essential for maintenance of NO homeostasis (Porro et al., 2014). Therefore, study of arginase activity and its kineric properties may have important clinical significance.

Previously, the arginase activity in sperm cells obtained from fertile and infertile men has been reported by our laboratory. We have found an inhibition of arginase pathway of L-arginine metabolism, which was not significantly dependent on the type of disruption of spermatogenesis

(Fafula et al., 2016). We found an inhibition of arginase pathway of L-arginine metabolism, which is not significantly dependent on the type of disruption of spermatogenesis (Kullisaar et al., 2013). We also have shown that in patients with decreased fertility potential the arginase / NOS ratio was shifted towards predominance of iNOS-derived NO production (Fafula et al., 2018). In this study, we investigated the kinetic parameters of arginase of spermatozoa of infertile men. Obtained values of affinity contant were in millimolar range which in agreement with other studies (Dillon et al., 2002). However, kinetic parameters of enzyme were determined in a closed system with isolated enzymes and do not take into account enzyme coupling, non-freely diffusible substrate pools, intracellular localization of the enzymes and substrate transporter expression and activity, diffusion gradients, and potential sequestration (Shen et al., 2005; Topal et al., 2006; Jiang et al., 2011). In the present study we used permeablized sperm cells in which functioning of enzyme correspond to intact cells.

There are some limitations in the present study. Firstly, our control group (normozoospermic men with proven fertility) and pathospermic patients contained a highly heterogeneous population, with large variations in spermogram parameters and infertility histories. Secondly, it is therefore essential to validate our findings with greater sample sizes and to determine the disease specificity (secretory or excretory infertility, varicocele or others) by comparing spermogram parameters. Nevertheless, the present study extends previous work and provides further evidence of altered L-arginine metabolism in sperm cells in pathospermia.

Conclusions

It has been found that inhibition of arginase activity in sperm cells of infertile men occurred by non-competitive type and was related to marked decrease in maximum reaction rate while affinity of arginase to L-arginine and Mn^{2+} was unaffected.

The publication contains the results of studies conducted under the President of Ukraine grant for competitive projects (project No Ф63/97-2016 from 10.08.2016 "Molecular biological regulatory mechanisms of disturbance of fertilizing ability spermatozoa and the development of new immuno-biochemical diagnostic methods of fertility in men" of the State Fund for Fundamental Research (The President's Order No 97/2016-рп dated: April 13, 2016).

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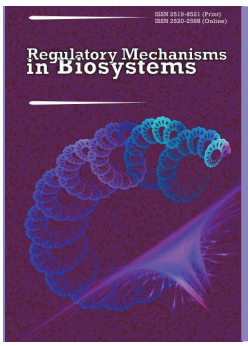

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 85–89
doi: 10.15421/021812

Characterization of Ca^{2+},Mg^{2+}-ATPase of blood lymphocytes in women with ovarian cancer

R. V. Barylyak, U. P. Iefremova, O. K. Onufrovych, O. V. Melnyk, D. Z. Vorobets, Z. D. Vorobets

Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

Article info

Received 14.01.2018
Received in revised form 12.02.2018
Accepted 16.02.2018

Danylo Halytsky Lviv National Medical University, Pekarska st., 69, Lviv, 79010, Ukraine. Tel.: +38-032-275-76-32. E-mail: kaf_medicalbiology@meduniv.lviv.ua

Barylyak, R. V., Iefremova, U. P., Onufrovych, O. K., Melnyk, O. V., Vorobets, D. Z., & Vorobets, Z. D. (2018). Characterization of Ca^{2+},Mg^{2+}-ATPase of blood lymphocytes in women with ovarian cancer. Regulatory Mechanisms in Biosystems, 9(1), 85–89. doi: 10.15421/021812

Ionized Ca^{2+} is crucial for regulation of practically all intracellular processes, including tumor growth, cell proliferation, apoptosis, etc. The plasma membrane Ca^{2+},Mg^{2+}-ATPase plays an important role in maintaining intracellular Ca^{2+} homeostasis. The function of this enzyme is to reduce the Ca^{2+} concentration in the cytosol, namely its transport against a concentration gradient in the extracellular medium. We have investigated the activity of plasma membrane Ca^{2+},Mg^{2+}-ATPase of lymphocytes of practically (clinically) healthy women of different age groups and also patients with ovarian cancer stage III and IV. It was found that the enzyme activity in women of the older age group was not significantly reduced in relation to the activity of the younger age group. Thus, the value of the maximum rate of ATP hydrolysis by plasma membrane Ca^{2+},Mg^{2+}-ATPase of blood lymphocytes in practically healthy women under the conditions of physiological norm was 1.1 times higher than under of pre-nosological state. In patients with ovarian cancer (stages III and IV), plasma membrane Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes significantly differed from the physiological norm and decreased by 1.6 and 1.8 times, compared with the physiological norm. The decrease of the plasma membrane Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes in patients with ovarian cancer indicates an increase of Ca^{2+} in the cytosol of lymphocytes. Determination of affinity constants showed that these values were in the submillimolar range of concentration, corresponding to the physiological concentration in the cell cytoplasm (0.5–5.0 mM). In healthy persons, under the condition of physiological norm, the affinity constant of plasma membrane Ca^{2+},Mg^{2+}-ATPase to the ATP was 0.16 ± 0.02 mM and at pre-nosological state – 0.19 ± 0.02 mM. The affinity constant of plasma membrane Ca^{2+},Mg^{2+}-ATPase of lymphocytes to ATP in patients with ovarian cancer (stage III) was 0.32 ± 0.03 mM and with ovarian cancer (stage IV) 0.35 ± 0.03 mM. That is, the affinity constant of plasma membrane Ca^{2+},Mg^{2+}-ATPase of lymphocytes to ATP in patients with ovarian cancer was 2.0–2.1 times higher than this value for the blood lymphocytes in the control group (physiological norm). The kinetic analysis of Ca^{2+}-activated, Mg^{2+}-dependent hydrolysis of ATP in blood lymphocytes in women showed that the decrease in the activity of Ca^{2+},Mg^{2+}-ATPase was due to a decrease in the affinity of the enzyme to the substrate (KATP increases 2-fold).

Keywords: plasma membrane; Ca^{2+}-ions; ATPase activity; ATP hydrolysis rate; affinity constant; Ca^{2+}-pump; lymphocytes

Introduction

It is known that ovarian cancer (OC) occupies a leading place among the causes of mortality from malignant formations (Howlader et al., 2013). Specifically, according to the International Agency for Research of Cancer, more than 165,000 newly diagnosed cases of ovarian cancer are reported annually in the world. It is the cause of death of more than 100,000 women (Howlader et al., 2013).

Ovarian cancer refers to severe pathology of the female reproductive system (Buys et al., 2011; Paryzhak et al., 2014). This pathology manifests itself especially through its high ability to proliferate and metastasize, which determines the clinical course of the disease (Paryzhak et al., 2014; Vovchuk, 2014). It is widely studied with the aim of both improving the methods of diagnosis and detection of the tumor process in the early stages, as well as optimizing the treatment based on modern ideas about its pathogenesis. Probably, the violation of proliferative processes and the development of OC is preceded by a pre-nosological state that is clinically asymptomatic. Data from the literature show that the greater the age of a woman, the greater the probability of developing ovarian cancer (Markman et al., 2004; Lukianova et al., 2006; Vovchuk, 2014; Yakubets et al., 2016).

Therefore, an important direction in physiological, biochemical and other biomedical investigations is the elucidation of mechanisms regulating the functioning of the cell both in practically healthy individuals and those with pathological conditions (Radchenko, 2004; Gzhegotsky et al., 2008). However, the limits of the majority of physiological processes in practically healthy individuals are quite broad and can conditionally correspond to both the physiological norm and the pre-nosological state (Radchenko, 2004; Gzhegotsky et al., 2008; Yakubets et al., 2016). It is believed that the state of the physiological norm is characterized by the balance of the work of many regulatory and functional systems of the body, while in the pre-nosological state, the mobilization of functional resources and the tension of regulatory systems is necessary (Gzhegotsky et al., 2008). In this regard, the recognition of intermediate, that is, prenosological states preceding nosological-definite forms of diseases is a highly topical issue. Often the term "practically (clinically) healthy" corresponds to clinically asymptomatic conditions at the border of norm and pathology, which may require preventive correction (Radchenko, 2004; Gzhegotsky et al., 2008).

Currently, there are no clear criteria to differentiate between the state of the physiological norm and the pre-nosological state, which makes it difficult to use them in medical and biological research and in

clinics. To identify the earliest preclinical stages of pathological processes, study of cellular regulatory systems and the search for new biochemical and other markers is carried out. In this aspect, the role of Ca^{2+} ions as a universal intracellular messenger in the regulation of cellular functions is indisputable (Feske, 2007; Monteith et al., 2007; Bergner et al., 2008; Monteith et al., 2012; Pinto et al., 2015; Dang et al., 2016; Padanyi et al., 2016; Peters et al., 2016; Monteith et al., 2017).

Specifically, Ca^{2+} is one of the major determinants of invasiveness and metastatic potential of transformed cells (Feske, 2007; Monteith et al., 2007; Paryzhak et al., 2014). It regulates the transcription of genes, metabolism, proliferation, apoptosis, etc. Malignant growth is accompanied by increased proliferation and decreased apoptosis. Therefore, in the study of tumor growth, it is particularly important to study the Ca^{2+} homeostasis. The increase in Ca^{2+} concentration in the cytoplasm is the result of its transport from the extracellular medium and release from the intracellular stores. Two main structures involved in maintaining and controlling intracellular Ca^{2+} homeostasis are plasma membrane Ca^{2+},Mg^{2+}-ATPase and Ca^{2+},Mg^{2+}-ATPase of the endoplasmic reticulum (Monteith et al., 2007; Bergner et al., 2008; Monteith et al., 2012; Padanyi et al., 2016).

On the other hand, it is known that T-lymphocytes play a central role in the antitumor protection of the body. They carry out antitumor protection by destroying cancer cells, as well as synthesizing substances that activate other cells in the immune system. Some T-lymphocytes kill cancer cells (T-killers). Others cells help the latter kill cancer cells (T-helpers). It is believed that the presence or absence of certain groups of T-lymphocytes, is associated with important differences in the prediction of the development of ovarian cancer in patients (Gavalas et al., 2010; Knutson et al., 2015; Krishnan et al., 2017).

Also, peripheral blood lymphocytes can serve as an adequate model for studying pre-nosological conditions and the development of ovarian cancer and objectively reflect changes of the genetic and metabolic homeostasis of an organism (Davtian et al., 2001; Krishnan et al., 2017). That is, they can be test systems for study of regulatory mechanisms of the cell, in particular Ca^{2+}-transporting systems for ovarian cancer. Thus, in spite of existing research, which is devoted to pre-nosological states, different age aspects, the functioning of blood lymphocytes of different age groups and so on, the role of a number of regulatory systems, in particular, ATP-hydrolase in both practically healthy individuals and in neoplastic transformations of organs and tissues, still remains unclear.

The purpose of present work was to determine the activity and characterize the kinetic properties of plasma membrane Ca^{2+},Mg^{2+}-ATPases of blood lymphocytes in practically healthy women of different age groups and in women with ovarian cancer.

Materials and methods

Patients. The research was ccarried out on blood lymphocytes isolated from practically healthy women and patients with neoplastic changes in the ovary. The total number of practically (clinically) healthy women, representative by age (mean age 53.8 ± 5.4 years) was 44 persons. This group was formed from volunteers from among the employees of Danylo Halytsky Lviv National Medical University and also of the employees of the Lviv State Regional Oncology Treatment and Diagnostic Center. In turn, a group of practically healthy women was conditionally divided into two subgroups: FN (26 people, 20–40 years old, physiological norm) and PS (18 people, 41–60 years old, pre-nozological state). Such a conditional division was based on the fact that with increasing age of women, the probability of ovarian cancer increases and the concentration of the tumor marker CA-125 increases in the blood (Yakubets et al., 2016).

The group of women with neoplastic ovarian changes was 32 women aged 24–75 years (average age 55.4 ± 5.3 years) who were receiving inpatient treatment at the Lviv Regional State Oncology Treatment and Diagnostic Center in the period 2013–2017 and passed the complete clinical and laboratory diagnostic. The study included patients with an established diagnosis of ovarian cancer without the presence of concomitant diseases at the start of the study.

The research group was divided into two subgroups, depending on the stage of development of ovarian cancer: OC 1 – the patients with III stage of ovarian cancer, a tumor is distributed on one or two ovaries and gives metastases on the peritoneum beyond the pelvis (or metastases in retroperitoneal lymph nodes) (n = 22); OC 2 – the patients with IV stage of ovarian cancer, a tumor is extended on one or two ovaries with distant metastases (n = 10).

Appropriate diagnoses were established on the basis of a wide range of general-clinical, laboratory, special oncology, instrumental research methods. In addition, for the differentiation of practically healthy women and diagnosis of ovarian cancer, the level of the tumor marker of glycoprotein CA-125 in blood serum was determined (Paryzhak et al., 2014; Yakubets et al., 2016). All patients with ovarian cancer and practically healthy persons were well informed about the purpose, tasks and term of the study and provided written informed consent to participate in conducting research on blood samples. All patients and healthy donors gave written informed consent to participate in research (Ethical Committee Approval, protocol No 4, April 18, 2016).

Cell preparation. Blood sampling by means of venipuncture was carried out from the elbow vein in the morning hours under conditions of physiological rest, on an empty stomach, in a quantity of 20 ml in test tubes, and stabilized with heparin (final dilution 1 : 100). Whole blood diluted in the ratio 1 : 1 by physiological solution was layered in a density gradient of the ficol triambraust (ρ = 1.08 g/cm^3) and centrifuged for 20 min at 500 g. The removed interphase rings of mononuclear cells were washed twice within 10 min with a physiological solution (Boyum, 1968; Pidkovka et al., 2002). After the last centrifugation, a small amount of saline solution was added to the precipitate, resuspended and using a trypan blue, the count of the number of live and dead cells in the Goryaev cell (Mishell, 1980) was measured. The integrity and viability of blood lymphocytes in all researches was not less than 95%.

For permeabilization of blood lymphocyte membranes and disclosure of enzyme latent activity, saponin was added to the suspension. This technique is based on work previously performed on lymphocytes. Blood lymphocytes were incubated for 10 min at moderate shaking in a solution containing saponin at a concentration of 0.2% (optimal concentration) (Pidkovka et al., 2002; Vorobets et al., 2006; Fafula et al., 2011).

Assay of Ca^{2+},Mg^{2+}-ATPase activity. Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes was determined by registering the process of ATP hydrolysis by accumulation of inorganic phospahate (P_i). The determination of the total Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes was carried out at 37 °C in an incubation medium (volume – 1 ml) of the following composition (mM): 150 KCl, 0.05 $CaCl_2$, 5 $MgC1_2$, 5 ATP, 1 $NaNO_3$ (mitochondrial ATPase inhibitor); 1 ouabain (inhibitor Na^+,K^+-ATPase) (Pidkovka et al., 2002; Fafula et al., 2011), 20 Hepes-Tris buffer (pH = 7.4). For division the total Ca^{2+},Mg^{2+}-ATPase activity into components: thapsigargin-insensitive plasma membrane Ca^{2+},Mg^{2+}-ATPase and thapsigargin-sensitive Ca^{2+},Mg^{2+}-ATPase membranes of the endoplasmmic reticulum (EPR) the inhibitor an Ca^{2+},Mg^{2+}-ATPase EPR-thapsigargin (0.1 μM) was added to the standard Ca^{2+} and Mg^{2+}-containing incubation medium.

Plasma membrane Ca^{2+},Mg^{2+}-ATPase was calculated as the difference between total Ca^{2+},Mg^{2+}-ATPase activity and Ca^{2+},Mg^{2+}-ATPase activity in the presence of thapsigargin. Ca^{2+},Mg^{2+}-ATPase activity was expressed in μmoles of P for 1 min per 1 mg of protein.

The kinetic parameters characterizing the Ca^{2+}-activated Mg^{2+}-dependent ATP-hydrolysis – the affinity constant (KATP) and the maximum rate of ATP hydrolysis determined by ATP (VATP) were calculated by the Lineweaver-Burk plot. The resulting concentration dependences of the rate of ATP-hydrolysis reaction on the substrates of reaction were plotted in the coordinates: {1/V; 1/[S]}, where S is the substrate concentration and V is the rate of enzymatic hydrolysis of ATP at a given concentration of the substrate.

Results

It is known that Ca^{2+},Mg^{2+}-ATPase transport Ca^{2+} ions across the membrane against their electrochemical gradient. This process is conju-

gated with ATP hydrolysis. Ca^{2+},Mg^{2+}-ATPase of blood lymphocytes has been demonstrated by researchers earlier (Pidkovka et al., 2002; Vorobets et al., 2006; Fafula et al., 2011). The physiological role of this enzymatic system in the regulation of Ca^{2+}-homeostasis of the cell is determined by its high affinity to the substrate of transporting, which is Ca^{2+} (Fafula et al., 2011; Padanyi et al., 2016).

Disturbances of the activity of Ca^{2+}-dependent ATP-hydrolysis systems indicate structural and functional changes in biological membranes in the development of pathological processes. Changes in the activity of these systems of the cell lead to the redistribution of ions between the cytoplasm and the extra-cellular medium, changes in cell membrane potential. With growth of tumor, disturbances of the functional activity of the membrane-bound enzymatic systems acquires a general (systemic) character (Monteith et al., 2012; Pinto et al., 2015; Padanyi et al., 2016; Peters et al., 2016).

As a result of the performed studies, it was found that plasma membrane Ca^{2+},Mg^{2+}-ATPase activity of lymphocytes in practically healthy women aged 20–40 years (FN) was 2.97 ± 0.26 μmol P_i/min·mg of protein (Fig. 1).

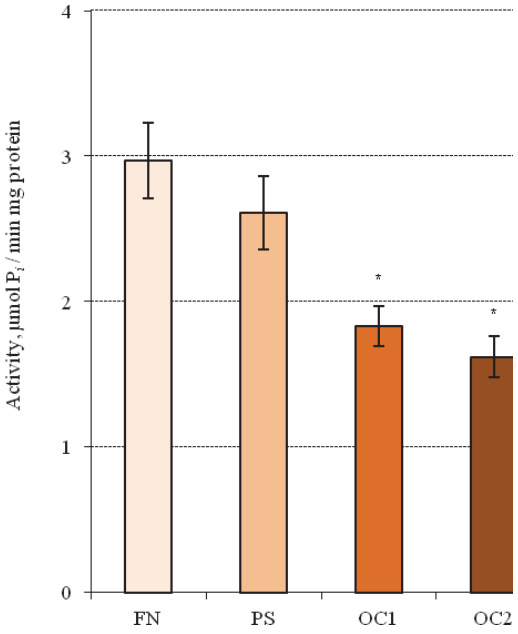

Fig. 1. Plasma membrane Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes of women at the physiological norm (FN), pre-nosological state (PS), in patients with ovarian cancer III (OC1) and IV (OC2) stages: X ± m, n = 8–12; * – P < 0.05 compared to physiological norm

In practically healthy women 40–60 years old (PS) this value was 2.61 ± 0.25 μmol P_i/min•mg of protein. In patients with OC (Stages III and IV), plasma membrane Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes significantly differed from the physiological norm and was 1.83 ± 0.14 and 1.62 ± 0.14 μmol P_i/min•mg of protein. Enzyme activity decreased by 1.6 and 1.8 times, respectively (P < 0.05), compared with the physiological norm.

The decrease of the plasma membrane Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes in patients with OC indicates the increase in $[Ca^{2+}]_i$ in the cytosol of lymphocytes. Ca^{2+},Mg^{2+}-ATPase and Na^+,K^+-ATPase use the energy of ATP hydrolysis to transport ions against their electrochemical gradient. Therefore, changes in the ATP concentration in the incubation medium will affect the rate of ATP hydrolysis.

The dependence of Ca^{2+},Mg^{2+}-ATPase activity on the substrate concentration (ATP) in the incubation medium was determined by the affinity constant to the substrate (KATP). It was calculated by determining the plasma membrane Ca^{2+},Mg^{2+}-ATPase activity in the incubation medium which contained the substrate in the concentration range from 1 to 5 mM (with a constant concentration of Ca^{2+} ions – 0.05 mM and Mg^{2+} ions – 5 mM).

It was shown that an increase in the ATP concentration in an incubation medium in the range from 1.0 to 4.0 mM leads to a gradual increase in plasma membrane Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes of practically healthy persons reaching a plateau (Fig. 2).

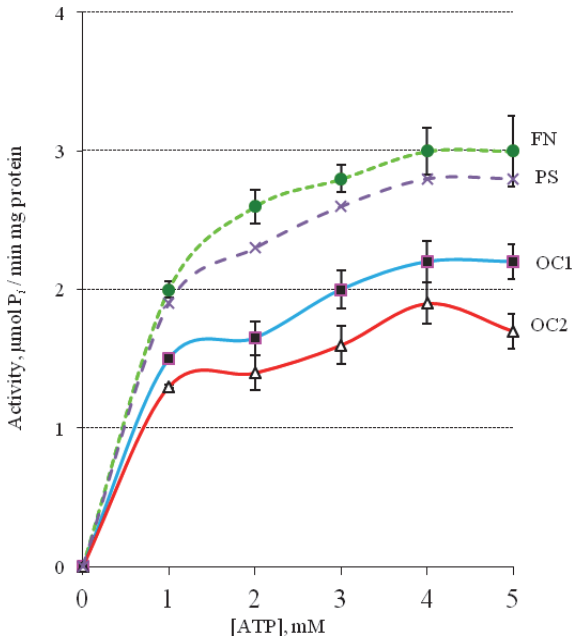

Fig. 2. Dependence of plasma membrane Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes of women at the physiological norm (FN), pre-nosological state (PS), patients with ovarian cancer III (OC1) and IV (OC2) stages on the ATP concentration in the incubation medium (x ± m, n = 8–12)

The maximum values of the hydrolase activity of plasma membrane Ca^{2+},Mg^{2+}-ATPase of the blood lymphocytes in healthy subjects and in patients with OC were noted at 4 mM ATP in the incubation medium. The study of the concentration dependence of Ca^{2+},Mg^{2+}-ATPase activity on ATP shows that the activity of plasma membrane Ca^{2+},Mg^{2+}-ATPases in patients with OC was decreased in comparison with control groups throughout the range of studied concentrations of the substrate.

For clarification of possible mechanisms of changes in plasma membrane Ca^{2+},Mg^{2+}-ATPase activity, the main kinetic parameters of ATP hydrolysis in immunocompetent cells in healthy persons and patients with OC were determined (Fig. 3). The dependence curves (1/V; 1/[ATP]) differ by angle of inclination for physiological norm and patients with ovarian cancer. Curves (1/V; 1/[S]) at the normal physiological state and at pathology cross the X and Y axes at different points. This dependence corresponds to a mixed type of inhibition of the enzyme.

To determine the main kinetic parameters of ATP hydrolysis with the participation of plasma membrane Ca^{2+},Mg^{2+}-ATPase of blood lymphocytes in patients with OC and the elucidation of the possible mechanism of enzymatic activity change, the curves of concentration dependences were linearized in the Lineweaver-Burk plot.

It was established that the values of the maximum rate of ATP hydrolysis by plasma membrane Ca^{2+},Mg^{2+}-ATPase of blood lymphocytes of practically healthy women in the conditions of FN was 3.02 ± 0.26 and in the conditions of PS 2.76 ± 0.22 μmol P_i/min•mg of protein (Table 1). The maximum rate of ATP hydrolysis by plasma membrane Ca^{2+},Mg^{2+}-ATPase of lymphocytes in patients with OC of III stage was 1.95 ± 0.20 and IV stage 1.77 ± 0.15 μmol P_i/min•mg of protein. It can be seen that the maximum rate of ATP hydrolysis by Ca^{2+},Mg^{2+}-ATPase of blood lymphocytes in patients with OC of both stages and controls group was different and this difference was statistically significant (P < 0.05).

Determination of affinity constants showed that these values were in the submillimolar range of concentration, corresponding to the physiological concentration in the cytoplasm of cells (0.5–5.0 mM). In healthy persons, under the condition of FN, the affinity constant of plasma membrane Ca^{2+},Mg^{2+}-ATPase to the ATP was 0.16 ± 0.02

mM, and at PS – 0.19 ± 0.02 mM. The affinity constant of plasma membrane Ca^{2+},Mg^{2+}-ATPase of lymphocytes to ATP in patients with OC (stage III) was 0.32 ± 0.03 mM and at OC (stage IV) 0.35 ± 0.03 mM.

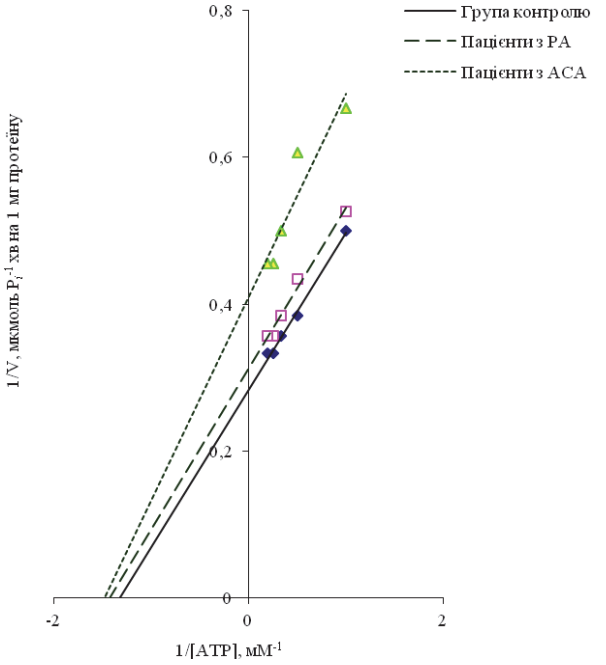

Fig. 3. Linearization of concentration curves in Lineweaver-Burk plot, here V is ATP-hydrolase activity of plasma membrane Ca^{2+},Mg^{2+}-ATPase of women at the physiological norm (FN), patients with ovarian cancer III (OC1) and IV (OC2) stages (n = 8–12; r > 0,85)

Table 1

Kinetic parameters of Ca^{2+}-activated, Mg^{2+}-dependent ATP hydrolysis in blood lymphocytes of women at physiological norm (FN), pre-nosological state (PS), patients with ovarian cancer III (OC1) and IV (OC2) stage defined by ATP (x ± m, n = 8–12)

Kinetic parameters	Control group		Patients with OC	
	FN	PS	III stage (OC1)	IV stage (OC2)
V_{ATP}, μmole P_i/ min 1 mg protein	3.02 ± 0.26	2.76 ± 0.22	1.95 ± 0.20*	1.77 ± 0.15*
K_{ATP}, мМ	0.16 ± 0.02	0.19 ± 0.02	0.32 ± 0.03*	0.35 ± 0.03*

That is, the value of the affinity constant to ATP for plasma membrane Ca^{2+},Mg^{2+}-ATPase of lymphocytes in patients with OC was 2.0–2.1 times higher than this value for the blood lymphocytes in the group of physiological norm. It can be concluded that the inhibition of enzyme activity occurs both by reducing the maximum rate of ATP hydrolysis (V_{max} was decreased) and by reducing the affinity of plasma membrane Ca^{2+},Mg^{2+}-ATPase to the substrate (the affinity constant to ATP was increased).

It is known that the extrusion of Ca^{2+} from the cell through the plasma membrane is carried out by two main mechanisms: Ca^{2+},Mg^{2+}-ATPase and Na^+/Ca^{2+}-exchanger. However, most researchers demonstrate that under carcinogenesis, the main mechanism is the Ca^{2+},Mg^{2+}-ATPase, which supports the concentration of cytosolic Ca^{2+} at a level of ~ 100 nM (Monteith et al., 2012; Monteith et al., 2017). In order to achieve precise Ca^{2+} control over several processes in the same cell, it is paramount that the Ca^{2+}-homeostasis is strictly controlled in time and space. The amplitude-temporal and spatial aspects of the Ca^{2+}-signal should be precisely regulated to achieve specific results, such as, for example, the cell cycle regulation, apoptosis or the cell proliferation (Monteith, 2007). Plasma membrane Ca^{2+},Mg^{2+}-ATPase is not only itself involved in controlling the intracellular Ca^{2+}-concentration, but also controls the formation of inositol-1,4,5-triphosphate and, accordingly, a decrease of Ca^{2+}-efflux from the endoplasmic reticulum (Padanyi et al., 2016). Changes of the expression of plasma membrane Ca^{2+},Mg^{2+}-ATPase in the process of tumor growth, resulting in unbalanced homeostasis in tumor cells were shown (Padanyi et al., 2016). Our data concerning the decrease of Ca^{2+},Mg^{2+}-ATPase activity agree that in cases of ovarian cancer there is an increase in the Ca^{2+} concentration in cytosol and even hypercalcemia (Pinto et al., 2015; Padanyi et al., 2016; Peters et al., 2016). The increase of the Ca^{2+}-concentration is due primarily to the fact that extrusion of Ca^{2+} from the cell decreases due to a decreased Ca^{2+},Mg^{2+}-ATPase activity. The increase in Ca^{2+} concentration by inhibiting the ATPases activity in ovarian cancer also induces apoptosis (Monteith et al., 2007; Monteith at al., 2017).

The data on the expression of endoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase in cells of patients with colon carcinoma and breast cancer (Papp et al., 2012) also indicate about a decrease in the Ca^{2+} efflux from cytosol in tumor growth. It was shown that the expression of this enzyme was reduced and the search for therapeutic drugs was directed at their effect by reducing the Ca^{2+} concentration in cytosol.

By transferring Ca ions to the extracellular medium across the plasma membrane, the Ca^{2+}-pump reduces the cytosolic Ca^{2+} level and thus helps control over cells activity.

Ca^{2+}-pumps can be not only the biomarkers, they can also serve as anticancer therapeutic targets (Monteith et al., 2007). One of the important features of the Ca^{2+}-pump in comparison with other existing or potential therapeutic targets in cases of cancer is the presence of pharmacological activators and inhibitors of Ca^{2+}-pumps. From the chemogenic point of view, many Ca^{2+}-pumps are potential pharmacological targets, and from the biological point of view, they are modulators of Ca^{2+}-signals that can affect to tumorigenic regulatory pathways. By modulating the activity of Ca^{2+}-channels and pumps that are expressed in cancer cells, their activators or inhibitors can purposefully change Ca^{2+} homeostasis in cancer cells (Monteith et al., 2012; Monteith et al., 2017).

Conclusion

It was found that Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes in women of different age groups was not significantly different. However, in women of the older age group, this activity had a tendency to decrease. With ovarian cancer, the Ca^{2+},Mg^{2+}-ATPase activity of blood lymphocytes was significantly decreased in relation to the control group, but there was no significant difference in the aenzyme ctivity between the 3rd and 4th stages of ovarian cancer. The kinetic analysis of Ca^{2+}-activated, Mg^{2+}-dependent ATP hydrolysis in plasma membrane of blood lymphocytes of women showed that decrease in Ca^{2+},Mg^{2+}-ATPase activity was due to a decrease in affinity to the substrate (KATP increases 2-fold).

The work is a part of the research work "Investigation of functional and metabolic reserves of stress-limiting systems of organism by extreme conditions in order to identify effective ways of their correction" (state registration number 0116U004510) and "Development and introduction of immuno-biochemical methods of early diagnostics of the development of pathological processes in the body" (Grant of the President of Ukraine No. 1039/2014-рп, September 24, 2014).

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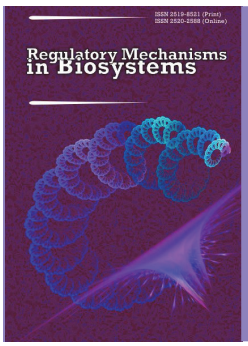

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 90–94
doi: 10.15421/021813

Serum paraoxonase activity in patients with rheumatoid arthritis, its relationship with the clinical course and cardiovascular compications

S. V. Shevchuk, Y. S. Seheda, O. Y. Galyutina, N. P. Masik, O. V. Shevchuk

National Pirogov Memorial Medical University, Vinnytsia, Ukraine

Article info

Received 24.01.2018
Received in revised form 14.02.2018
Accepted 17.02.2018

National Pirogov Memorial Medical University, Khmelnitske shosse,104, Vinnytsia, 21100, Ukraine. Tel. +38-068-952-52-07. E-mail: masikoi@i.ua, iuliia.segeda@gmail.com

Shevchuk, S. V., Seheda, Y. S., Galyutina, O. Y., Masik, N. P., & Shevchuk, O. V. (2018). Serum paraoxonase activity in patients with rheumatoid arthritis, its relationship with the clinical course and cardiovascular compications. Regulatory Mechanisms in Biosystems, 9(1), 90–94. doi: 10.15421/021813

Nowadays low paraoxonase activity is generally recognized as an independent risk factor of cardiovascular diseases involved in pathologic remodeling of the heart and vessels as well as thrombosis in the general population. But the role of paraoxonase activity in RA patients is unknown. Based on the above, the aim of the work was to study serum paraoxonase activity in patients with rheumatoid arthritis, to evaluate its association with clinical course and structural and functional status of the cardiovascular system. 67 patients with RA, 18 males and 49 females were studied. The control group consisted of 25 apparently healthy individuals. Rheumatoid arthritis was diagnosed according to international classification criteria ACR 2012. The indices of total cholesterol (TC), high density lipoprotein cholesterol (HDLC) and triglycerides (TG) in blood serum were determined by standard conventional methods. Low density lipoprotein cholesterol (LDLC) values were calculated by Friedwald formula. Serum paraoxonase activity was measured by spectrophotometric method. High resolution ultrasound and Doppler ultrasonography of the brachial artery were performed to study endothelium function. Sonographic B-mode scanning and pulsed Doppler ultrasound of heart and blood flow spectra were done on ultrasound scanner. Serum paraoxonase activity was found to be about 18.8% lower in the patients with RA than in the control group. Serum paraoxonase activity was shown to decrease proportionally to the increase of the age in RA patients. In the group of patients over 45, the level of the enzyme was 13.0% lower than in the patients over 30. The study established that the increase of systolic and diastolic arterial pressure is associated with decrease of serum paraoxonase activity in RA patients. The patients with RA combined with arterial hypertension had significantly (by 10.9%) lower activity of the studied enzyme than those with no arterial hypertension. However, no significant relationship between paraoxonase activity and duration of the disease, obesity and smoking was revealed. Paraoxonase activity in RA patients was demonstrated to be dependent on lipid levels. The lowest paraoxonase activity was recorded in individuals with the highest levels of TC, LDLC and the lowest HDLC indices. Paraoxonase activity in RA patients is associated not only with atherosclerotic vascular damage (IMT, decreased FMDBA) but also with structural and functional heart status (systolic and diastolic functions, left ventricular myocardial hypertrophy). Decreased serum paraoxonase level is suggested to be the predictor of early development of cardiovascular complications in RA patients.

Keywords: enzyme activity; autoimmune disease; metabolic risk factors; changes of the cardiovascular system

Introduction

Cardiovascular diseases are acknowledged as the leading cause of mortality among patients with rheumatoid arthritis (RA) (Ladak et al., 2017; Meissner et al., 2017; Myasoedova et al., 2017). Framingham risk factors (age, sex, atherogenic lipid levels, history of arterial hypertension, smoking) are considered to have a decisive role in early development of cardiovascular complications. But accelerated atherogenesis cannot be explained exclusively by the action of traditional risk factors in RA patients. Currently much attention is focused on metabolic and immunologic markers which are likely to play one of the key roles in atherogenesis of autoimmune diseases. Recent studies provide convincing evidence that anti-cyclic citrullinated peptide antibodies (anti-CCP), rheumatoid factor IgM, circulating immune complexes, anti-inflammatory cytokines (TNF-alfa, IL-6), Th0/Th1 of T-cells, homocysteine, dyslipidemia, decreased folic acid level, impaired vitamin metabolism as well as disturbances in paraoxonase activity can be involved in the development of cardiovascular diseases in RA (Yang et al., 2015; Batún Garrido et al., 2016; Rodríguez-Carrio et al., 2016; Tocci et al., 2016; Bernardes et al., 2017; Herly et al., 2017). Nowadays low paraoxonase activity is generally recognized as an independent risk factor of cardiovascular diseases involved in pathologic remodeling of the heart and vessels as well as thrombosis in the general population (Kerekes et al., 2008; Tang et al., 2012; Patra et al., 2013; Kovalenko et al., 2014; Wang et al., 2015; Kunutsor et al., 2016). Changes in paraoxonase activity inevitably lead to decreased defense antioxidant function of HDL and increased oxidant stress (Kim et al., 2016; Kulka, 2016). In RA patients the following factors can decrease serum paraoxonase activity: excessive rheumatoid factor, anti-CCP, systemic inflammatory process (El-Banna & Jiman-Fatani, 2014; Shahmohamadnejad et al., 2015). Ethnic and race differences in serum paraoxonase activity have been detected as well (Bounafa et al., 2015; Sayın Kocakap et al., 2015). Despite a large number of studies addressed to cardio-vascular complications in RA, the role of low paraoxonase activity has not been established yet. It should be noted that in the Ukrainian patient population with RA this enzyme activity also has not been studied. The relationship of paraoxonase activity with other metabolic cardiovascular risk factors as well as structural and functional status of the heart has not

been established either. Based on the above, the aim of the work was to study serum paraoxonase activity in patients with rheumatoid arthritis, to evaluate its association with the clinical course and structural and functional status of the cardiovascular system.

Materials and methods

67 patients with RA, 18 males and 49 females were studied. The control group consisted of 25 apparently healthy individuals. Rheumatoid arthritis was diagnosed according to international classification criteria ACR 2012 (Kay & Upchurch, 2012). The analysis of traditional risk factors of atherosclerotic vascular damage was done in RA patients. Body mass index (BMI, kg/m^2) was calculated in all patients with RA. Obesity was estimated in case of BMI > 30. The indices of total cholesterol (TC), high density lipoprotein cholesterol (HDLC) and triglycerides (TG) in blood serum were determined by standard conventional methods. Low density lipoprotein cholesterol (LDLC) values were calculated by Friedwald formula: LDLC = TC – HDLC – 0.45·TG. All detected values were divided into normal, marginally increased and high values of lipid profile according to the Third Report of the National Cholesterol Education Program (2002). High activity of inflammatory process in RA patients was estimated according to increase of ESR, CRP, TNF-α levels and calculated DAS 28 score, Pain index score, Articular index and Edema index score. Serum paraoxonase and arilesterase activity was measured by spectrophotometric method (Connelly et al., 2004). A persistent analytic system was used for the evaluation of serum paraoxonase activity in RA patients and control group. High resolution ultrasound and Doppler ultrasonography of the brachial artery by Celermajer et al. (1992) were performed to study endothelium function. Flow-mediated vasodilation of the brachial artery (FMDBA) was assessed according to changes in its diameter and measured before and after temporary occlusion of the vessel with blood preasure cuff (reactive hyperemia). Location of the brachial artery was associated with visualization of its internal diameter and was measured in the middle third of the shoulder. Sonographic B-mode scanning and pulsed Doppler ultrasound of blood flow spectra were done on ultrasound scanner Sonoline 6000 C (Medisson, Southern Korea) at the 30, 60 and 90th s after cuff decompression. Brachial artery dilation by more than 8% from baseline diameter within 30 s after decompression was considered to be the criteria of adequate endothelial response to ischemia. All measurements of endothelial relaxation were done from 8 to 10 AM.

The thickness of the intima-media complex (IMT) of the common carotid artery (CCA) was determined at the time of B-mode ultrasonography of the carotid artery in diastole 2 cm from bifurcation at maximum magnification. The area of atherosclerotic plaques of the carotid artery (cAP) was measured in all the patients, and the extent of vascular atherosclerotic damage was evaluated (Wendelhag et al., 1993). Echocardiography (EchoCG) was done for 63 patients with RA on ultrasound scanner Sonoline 6000 C (Medisson, Southern Korea) Statistical processing of the obtained results was carried out on a personal computer using the standard statistical programs. The results are presented as the mean ± standard error (x ± SE). All values follow a normal distribution. The average value, standard errors, reliability of the differences were evaluated according to Student's t-criterion. Pearson's correlation coefficient test was used to measure the strength of a linear association between two variables. The statistical significance was determined if P < 0,05.

Results

Serum paraoxonase activity was found to be about 18.8% lower in the patients with RA than in the control group (107.8 mmol/l·h and 132.9 mmol/l·h, respectively) (Table 1). Moreover, according to percentile distribution, serum paraoxonase activity level ranged from 94.5 to 172.6 mmol/l·h in apparently healthy persons, while in the patients with RA it was 80.9–129.4 mmol/l·h. The analysis of traditional risk factors of atherosclerotic vascular damage on the basis of paraoxonase activity in the patients showed no significant differences in the indices of serum paraoxonase activity between males and females, but there was significant decrease of that index with the increase of age (Table 2).

Table 1
Serum paraoxonase activity in RA patients and in the control group

Group	Median	Paraoxonase, mmol/l·h					
		P_5	P_{10}	P_{25}	P_{75}	P_{90}	P_{95}
Control, n = 25	132.9	94.5	118.0	127.9	154.2	161.8	172.6
RA patients, n = 67	107.8	80.9	85.8	89.6	123.3	126.5	129.4

Note: $P_{5,\ 10,\ 25,\ 75,\ 90,\ 95}$ – frequency of serum paraoxonase activity occurence (5%, 10%, 25%, 75%, 90%, 95% respectively) below the specified value.

Table 2
Analysis of serum paraoxonase activity depending on traditional risk factors in RA patients (x ± SE)

Index		Paraoxonase, mmol/l·h	
		control group	RA patients
Females, n = 49		108.2 ± 2.51	109.6 ± 2.26
Males, n = 18		105.9 ± 5.38	101.1 ± 4.44
Age	below 30 years, n = 11	113.4 ± 2.71	113.6 ± 2.82
	30–45 years, n = 27	115.7 ± 2.86	113.7 ± 3.54
	>45 years, n = 29	109.2 ± 5.89	99.1 ± 2.68***
Disease duration	<5 years, n = 26	–	109.5 ± 3.65
	5–10 years, n = 18	–	105.8 ± 4.34
	>10 years, n = 23	–	106.0 ± 2.95
With no AH, n = 50		112.6 ± 4.11	110.3 ± 2.34
With AH, n = 17		108.8 ± 3.69	98.2 ± 3.90**
Non-smokers, n = 55		107.0 ± 2.89	107.8 ± 2.37
Smokers, n = 12		102.9 ± 6.31	105.3 ± 5.27
BMI > 30 kg/m^2, n = 57		112.9 ± 2.71	107.4 ± 2.26
BMI < 30 kg/m^2, n = 10		109.2 ± 7.12	106.9 ± 5.44

Note: * – statistical significance in comparison of examined parameters P < 0.05, ** – P < 0.01, *** – P < 0.001.

In the patients over 45, paraoxonase level was 13% lower than in the patients before 30. A similar tendency was observed in RA combined with arterial hypertension (AH). In such patients paraoxonase activity was decreased nearly 1.12 times. But no significant relationship between paraoxonase activity and duration of the disease, obesity and smoking was revealed. The relationship between paraoxonase activity and lipid metabolism indices (Table 3) was evaluated. For this purpose, all the patients were divided into three groups: those with normal indices, marginally increased and high indices of lipid profile according to the Third Report of the National Cholesterol Education Program (2002). RA patients with high levels of TC, LDLC and low levels of HDLC were found to have significantly lower average paraoxonase level than the individuals with optimal lipid levels. In the patients with high levels of TC and LDLC paraoxonase activity was significantly lower – by 24.0% and 14.5%, respectively, and in those with low levels of HDLC – by 9.4% less than in the patients with optimal levels of those indices. No significant decrease of paraoxonase activity in the patients with hypertriglyceridemia was revealed.

Table 3
Analysis of serum paraoxonase activity depending on lipid level in RA patients (x ± SE)

Indices	Lipid level	Paraoxonase activity, mmol/l·h	
		absolute value	r
TC, mmol/l	optimal, n = 35	115.8 ± 2.47	
	marginally elevated, n = 23	101.4 ± 3.20***	–0.62#
	high, n = 7	88.0 ± 2.91***	
HDLC, mmol/l	normal, n = 24	110.2 ± 3.35	
	subnormal, n = 17	114.0 ± 3.62	0.24
	low, n = 24	99.8 ± 3.38*	
LDLC, mmol/l	normal, n = 39	113.3 ± 2.45	
	marginally elevated, n = 10	101.2 ± 6.58	–0.48#
	high, n = 16	96.8 ± 4.03***	
TG, mmol/l	normal, n = 20	113.2 ± 3.48	
	marginally elevated, n = 20	106.3 ± 4.42	–0.21
	high, n = 25	105.1 ± 2.95	

Note: * – statistical significance in comparison of examined parameters P < 0.05, ** – P < 0.01, *** – P < 0.001, # – strong correlation between the variables.

The relationship between paraoxonase activity and lipid metabolism was further verified by correlation analysis. In RA patients there

was close inverse association between paraoxonase activity and HDLC, and close direct association between paraoxonase activity and LDLC.

The next task was to evaluate the relationship between inflammatory process and enzyme system activity in RA patients (Table 4). The patients with low enzyme activity (≤89.7 mmol/l·h) were found to have higher levels of ESR, CRP and TNF-α by 14–15% than those with relatively normal paraoxonase activity (>89.7 mmol/l·h). A similar tendency was observed in total index of DAS 28 activity, pain index, articular index and edema index (r = 0.30–0.36).

Table 4
The relationship between paraoxonase activity and inflammatory process activity in RA patients (x ± SE)

Indices	Paraoxonase activity		r
	> 89.7 mmol/l·h, n = 48	≤ 89.7 mmol/l·h, n = 19	
ESR, mm/h	34.7 ± 0.30	40.4 ± 0.42***	–0.35#
CRP, mg/l	13.7 ± 0.16	16.4 ± 0.18***	–0.31#
TNF-α, ng/ml	164.7 ± 1.59	195.5 ± 1.78***	–0.33#
DAS_{28}, scores	5.3 ± 0.02	5.9 ± 0.03***	–0.36#
Pain index, scores	29.0 ± 0.41	37.5 ± 0.46***	–0.32#
Articular index, scores	24.9 ± 0.45	32.6 ± 0.48***	–0.38#
Edema index, scores	7.1 ± 0.16	10.1 ± 0.15***	–0.30#

Note: see Table 3.

The thickening of common carotid artery (IMT) walls, decreased FMDBA and severity of atherosclerotic damage in RA patients were revealed to be associated with decreased paraoxonase activity (Table 5). The patients with low paraoxonase level (≤ 89.7 mmol/l·h) had considerably lower FMDBA value (by 16.1%), and considerably larger cIMT (by 23.9%) than those with relatively normal paraoxonase level. Besides, the proportion of patients with decreased FMDBA and increase of IMT was 29.7–32.0% higher in the patients with low paraoxonase level than in those with a relatively normal level. The AP size and the extent of atherosclerotic damage of carotid arteries increased proportionally to paraoxonase activity decrease. Atherosclerotic damage of carotid arteries was revealed in 68.4% of patients with low paraoxonase activity as compared to 4.2% of patients with relatively normal indices of the studied enzyme.

Table 5
The relationship between paraoxonase activity and subclinical manifestations of atherosclerotic vascular damage (FMDBA, IMT, cAP) in RA patients (x ± SE)

Indices	Paraoxonase activity		r
	> 89.7 mmol/l·h, n = 48	≤ 89.7 mmol/l·h, n = 19	
IMT, mm	0.73 ± 0.01	0.96 ± 0.01***	–0.35#
Individuals with cIMT >0.90 mm, n (%)	11 (22.9%)	10 (52.6%)§	–0.31#
FMDBA, %	5.27 ± 0.10	4.42 ± 0.10***	–0.36#
Individuals with FMDBA ≤8.0%, n (%)	20 (41.7%)	14 (73.7%)§	–0.32#
Presence of cAP	2 (4.2%)	13 (68.4%)§	–0.38#
Extent of atherosclerotic CCA damage	0.15 ± 0.02	1.47 ± 0.04***	–

Note: * – statistical significance in comparison of examined parameters P < 0.05, ** – P < 0.01, *** – P <0.001, # – strong correlation between the variables, § – statistical significance in comparison of examined parameters.

The study of structural and functional heart status in RA patients showed its definite relationship with serum paraoxonase activity (Table 6). While the diameter of the aorta and the left atrium was 31.6 ± 0.09 and 36.3 ± 0.33 mm, respectively, in the patients with normal paraoxonase activity, it was 34.9 ± 0.14 and 38.9 ± 0.22 mm, respectively, in the patients with its decreased activity, i.e. it was larger by 9.4% and 6.6%, respectively. In the group of patients with impaired function of the enzyme system, left ventricular posterior wall thickness (LV posterior wall thickness) and intraventricular septum thickness (Septal thickness) appeared to be significantly higher as well. The most evident differences were found in left ventricular mass index: 173.4 ± 2.77 g/m in the patients with normal paraoxonase level, and 259.9 ± 2.57 g/m in those with decreased paraoxonase level (≤ 89.7 mmol/l·h), i.e. higher by 33.3% . In the latter group significant increase in sizes and volumes of the left ventricle were observed: LV diastolic diameter, LV systolic diameter, LV diastolic volume, LV systolic volume, LV mass/height.

Impaired systolic and diastolic functions of the left ventricle were associated with reduced paraoxonase activity in the blood as well. In the patients with low paraoxonase activity the relationship between E/A ratio was by 27% less and ejection fraction – by 16% less than in the patients with normal enzyme activity.

Table 6
Analysis of serum paraoxonase activity depending on structural and functional myocardial changes according to EchoCG findings and in RA patients (x ± SE)

Index	Paraoxonase activity	
	> 89.7 mmol/l·h, n = 43	≤ 89.7 mmol/l·h, n = 18
Aorta, mm	31.6 ± 0.09	34.9 ± 0.14***
LA diameter, mm	36.3 ± 0.33	38.9 ± 0.22***
LV diastolic diameter, mm	47.4 ± 0.27	51.5 ± 0.28**
LV systolic diameter, mm	31.0 ± 0.18	35.9 ± 0.28***
LV posterior wall thickness, mm	9.6 ± 0.09	12.2 ± 0.10***
Septal thickness, mm	10.3 ± 0.08	12.7 ± 0.09***
LV diastolic volume, ml	126.8 ± 1.07	132.6 ± 0.80***
LV systolic volume, ml	46.5 ± 0.77	55.6 ± 1.03***
Ejection fraction, %	62.9 ± 0.35	54.1 ± 0.47***
LV mass/height, g/m	173.4 ± 2.77	259.9 ± 2.57***
E, m/c	64.8 ± 0.82	50.6 ± 0.53***
A, m/c	60.8 ± 0.71	61.9 ± 0.42
E/A ratio	1.14 ± 0.01	0.84 ± 0.01***

Note: see Table 2.

Discussion

The study revealed the Ukrainian patient population with RA to have considerable reduction (by 18.8%) of serum paraoxonase activity as compared to apparently healthy individuals. The data obtained in the study confirmed those presented by other authors (Tanimoto et al., 2003; Isik et al., 2007). Nevertheless, some reports in the modern literature deny the increase of paraoxonase level in RA patients (Hashemi et al., 2010). Serum paraoxonase activity was shown to decrease proportionally to the increase of the age in RA patients. In the group of patients over 45 the level of the enzyme was 13% lower than in the patients over 30. According to reports from the literature, paraoxonase activity in the patients with autoimmune pathology decreases in the process of reproductive aging (Kiss et al., 2007). At the same time, no sex differences in serum paraoxonase levels in RA patients were found.

The study established that the increase of systolic and diastolic arterial pressure is associated with decrease of serum paraoxonase activity in RA patients. The patients with RA combined with AH had significantly (by 10.9%) lower activity of the studied enzyme than those with no AH. However, no significant relationship between paraoxonase activity and duration of the disease, obesity and smoking was revealed. There was only a tendency to decrease of the studied enzyme in the presence of the abovementioned risk factors.

Paraoxonase activity in RA patients was demonstrated to be dependent on lipid levels. The lowest paraoxonase activity was recorded in individuals with the highest levels of TC, LDLC and the lowest HDLC indices. In RA patients with optimal TC level, paraoxonase activity was 24% lower than in those with hypercholesterolemia . Correlation analysis provided additional evidence of the association of enzyme activity with impaired lipid metabolism. Such relationship between paraoxonase activity and lipid metabolism is not surprising as paraoxonase-1 is known to be a part of high density lipoproteins (HDL), it has potent antioxidant properties and protects HDL and LDL against overoxidation under the influence of active oxygen forms (Mkhitarian et al., 2016).

Serum paraoxonase level was found to be associated with increased levels of proinflammatory mediators ESR, CRP and TNF-α, as well as

articular syndrome indices. For instance, in the patients with low paraoxonase activity (≤ 89.7 mmol/l·h) serum levels of proinflammatory mediators were on average 15% higher than in the patients with relatively normal paraoxonase activity (> 89.7 mmol/l·h). The pathogenic role of systemic inflammatory process in impaired function of enzyme systems in RA patients has been previously reported in other studies (Kerekes et al., 2008; Shahmohamadnejad et al., 2015).

The data received in the study suggested low paraoxonase activity to be an adverse factor in progression of structural and functional alterations of the heart and vessels in RA patients. Significant increase of LV mass/height, the diameter of aorta and the left atrium, sizes and volumes of the left ventricle, IMT was recorded in the patients with low paraoxonase level as compared to patients with optimal paraoxonase level. Decrease in systolic and diastolic functions of the cardiac muscle was proportional to the decrease of serum paraoxonase level in RA patients. The area and severity of atherosclerotic damage of CCA had the tendency to increase with the decrease of serum paraoxonase activity. The adverse effect of that risk factor on the clinical course of cardiovascular pathology in RA patients has been previously confirmed in other studies. A low paraoxonase level in RA patients was shown to cause early formation of atherosclerotic vascular damage, to determine the level of FMDBA and IMT (Kerekes et al., 2008; Charles-Schoeman et al., 2013; El-Banna et al., 2014).

Thus, significant decrease of serum paraoxonase level is a common occurrence in RA patients. The concentration of this enzyme decreases proportionally to age, it is associated with arterial hypertension, dyslipidemia and disease activity but is not significantly influenced by the disease duration, obesity or smoking. A close relationship between paraoxonase activity and structural and functional heart status is indicative of potential involvement of this enzyme in the development of atherosclerotic alterations in the vessels.

Conclusions

RA patients have decreased serum paraoxonase activity (by 18%) as compared to apparently healthy individuals. Low paraoxonase activity is associated with age, arterial hypertension, dyslipidemia and disease activity and is not dependent on disease duration, obesity or smoking.

Paraoxonase activity in RA patients is associated not only with atherosclerotic vascular damage (cIMT, decreased FMDBA) but also with structural and functional heart status (systolic and diastolic functions, left ventricular myocardial hypertrophy).

Directions for future research. Further studies can determine the place of paraoxonase among other metabolic and traditional risk factors of cardiovascular complications in RA patients.

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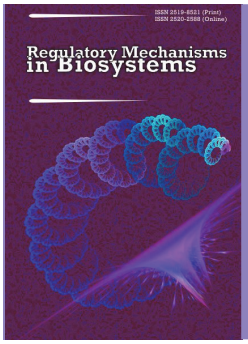


Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 95–104
doi: 10.15421/021814

Prenatal morphogenesis of compartments of the parenchyma of the lymph nodes of domestic cattle (*Bos taurus*)

P. N. Gavrilin, M. A. Lieshchova, O. G. Gavrilina, T. F. Boldyreva

Dnipro State Agrarian and Economic University, Dnipro, Ukraine

Article info

Received 12.01.2018
Received in revised form 14.02.2018
Accepted 15.02.2018

Dnipro State Agrarian and Economic University, Serhii Efremov st., 25, Dnipro, 49600, Ukraine. Tel. +38-056-268-54-17. E-mail: morphologagro@gmail.com, lieshchova.m.o@dsau.dp.ua

Gavrilin, P. N., Lieshchova, M. A., Gavrilina, O. G., & Boldyreva, T. F. (2018). Prenatal morphogenesis of compartments of the parenchyma of the lymph nodes of domestic cattle (Bos taurus). Regulatory Mechanisms in Biosystems, 9(1), 95–104. doi: 10.15421/021814

The article analyzes the regularities of the formation and development of the lymphoid lobules of the parenchyma of the somatic (*Limphonodi cervicales superficiales*) and visceral (*L. jejunales*) lymph nodes in domestic cattle in the fetal period of ontogenesis. We used routine histological techniques and author's modification of the impregnation of total median sections with silver nitrate. Visualization of various zones of lymphoid lobules was carried out taking into account the specific for different cell zones architectonics of reticular fibers. It has been established that signs of morphological heterogeneity of parenchyma of lymph nodes are first found in three month-old fetuses, which is associated with the concentration of lymphoid tissue along the marginal sinus. Separation of lymphoid lobules and their structural and functional differentiation are first detected in five month fetuses. In the lymphoid lobules of five month-old fetuses all structural and functional cell zones are observable, among which the regions of clonal proliferation of T and B lymphocytes are the least developed, and regions of the transit corridors for lymphocytes migrating medullary and the cords (zone of potential accumulation of plasmocytes and antibody formation) are the most developed. Structural and functional transformations of compartments in the prenatal period of ontogenesis are accompanied by a predominantly moderate increase of the relative volume of specialized T- and B-dependent zones of lobules, against a background of a gradual decrease of the volume of transit corridors for lymphocytes migrating and zone of potential accumulation of plasmocytes and antibody formation. Due to the small volume and relatively low rates of development of the lymphocytes clonal proliferation zones, the quantitative ratios of the cellular zones in lymphoid lobules of the lymph nodes of domestic cattle in prenatal ontogenesis remain relatively stable, while maintaining the maximum indices of the development of transit corridors for lymphocytes migration and medullary cords. Among the zones of lymphocytes clonal proliferation throughout the fetal period, T-dependent zones predominate, the relative volume of which is 5.0–7.5 times greater than the volume of B-dependent zones. Lymphoid lobules in the lymph nodes of the domestic cattle fetuses of all age groups are arranged along the marginal sinus in one row and have a polar structure due to the formation of lymph nodes at one pole of the lobules in the interfollicular zone. In the visceral lymph nodes (*L. jejunum*) of 8–9 month-old fetuses, individual lymph nodes can form in paracortical strands, on the border with the interfollicular zone.

Keywords: lymphoid tissue; deep cortex units; interfollicular cortex; lymphatic nodules; lymphatic sinuses; paracortical and medullaru cords

Пренатальный морфогенез компартментов паренхимы лимфатических узлов быка домашнего (*Bos taurus*)

П. Н. Гаврилин, М. А. Лещева, Е. Г. Гаврилина, Т. Ф. Болдырева

Днипровский государственный аграрно-экономический университет, Днипро, Украина

Проанализированы закономерности формирования и развития субъединиц (лимфоидных долек) паренхимы соматических (*Limphonodi cervicales superficiales*) и висцеральных (*L. jejunales*) лимфатических узлов у быка домашнего в плодном периоде онтогенеза. Использовали рутинные гистологические методики и авторскую модификацию импрегнации тотальных срединных срезов азотнокислым серебром. Визуализацию различных зон лимфоидных долек осуществляли с учетом специфической для той или иной зоны архитектоники ретикулярных волокон. Признаки морфологической гетерогенности паренхимы лимфатических узлов впервые обнаружены у трехмесячных плодов, что связано с концентрацией лимфоидной ткани вдоль краевого синуса. Обособление лимфоидных долек и их структурно-функциональная дифференциация впервые выявляются у пятимесячных плодов. В лимфоидных дольках пятимесячных плодов различаются все структурно-функциональные зоны, среди которых наименее развитыми являются участки клональной пролиферации Т- и В-лимфоцитов, а наиболее – зона транзита лимфоцитов и

мозговые тяжи. Структурно-функциональные преобразования компартментов в пренатальном периоде онтогенеза сопровождаются преимущественным умеренным увеличением объема специализированных Т- и В-зависимых зон долек, на фоне постепенного уменьшения объема зон транзита лимфоцитов и потенциальной аккумуляции плазмоцитов и антителообразования. Из-за незначительного объема и относительно низких темпов развития зон клональной пролиферации лимфоцитов, количественные соотношения клеточных зон в дольках лимфатических узлов быка домашнего в пренатальном онтогенезе остаются относительно стабильными, с сохранением максимальных показателей степени развития зон транзита и мозговых тяжей. Среди зон клональной пролиферации лимфоцитов на протяжении всего плодного периода преобладают Т-зависимые зоны, относительный объем которых в 5,0–7,5 раза превышает объем В-зависимых зон. Лимфоидные дольки в лимфатических узлах плодов быка домашнего всех возрастных групп располагаются в один ряд вдоль краевого синуса и имеют полярную структуру из-за формирования лимфатических узелков на одном полюсе долек в интерфолликулярной зоне. В висцеральных лимфатических узлах (тощей кишки) 8–9-месячных плодов отдельные лимфатические узелки могут формироваться в паракортикальных тяжах, на границе с интерфолликулярной зоной.

Ключевые слова: лимфоидная ткань; единицы глубокой коры; интерфолликулярная зона; лимфатические узелки; лимфатические синусы; паракортикальные и мозговые тяжи

Введение

На сегодня очевидным является тот факт, что паренхима лимфатических узлов млекопитающих имеет не слоистую, а дольчатую (компартментную) структуру (Kelly et al., 1975; Konenkov et al., 2008; Butler et al., 2016; Gavtilin et al., 2017a). Лимфоидные дольки в лимфатических узлах разных видов животных устроены по единому принципу и представлены четырьмя основными составляющими: зоны клональной пролиферации Т- и, соответственно, В-лимфоцитов; зона транзита лимфоцитов и межклеточного взаимодействия; зона аккумуляции плазмоцитов и синтеза антител (Kowala & Schoefi, 1986; Margaris & Black, 2012; Ikomi et al., 2012).

Зона транзита представляет собой биологический канал (транспортный коридор) для лимфоцитов, которые мигрируют через стенку вен с высоким эндотелием в паренхиму узлов (Ikomi et al., 2012; Ager, 2017). Данные структуры формируются вокруг соответствующих венозных сосудов (вен с высоким эндотелием), большая часть которых локализируется в интерфолликулярной зоне и паракортикальных тяжах (De Bruyn & Cho, 1990; Platt & Randolph, 2013; Ruddle, 2016). Интерфолликулярные зоны располагаются в непосредственной близости от главных коллекторов лимфы, которые в лимфатических узлах млекопитающих представлены двумя основными типами: лимфатическими пространствами между капсулой и паренхимой узлов (коллекторами I типа) и внутритрабекулярными лимфатическими цистернами (коллекторами II типа) (Gavrilin et al., 2017c). Зона акуммуляции плазмоцитов и синтеза антител в виде мозговых тяжей находится в лимфоидной дольке на противоположном полюсе от главных лимфатических коллекторов и граничит с терминальным участком внутриузлового лимфатического русла – воротным синусом (Von Andrian & Mempel, 2003; Sixt et al., 2005; Konenkov et al., 2008; Olson et al., 2012; Houston et al., 2016).

Зона клональной пролиферации Т-лимфоцитов (центральные зоны единиц глубокой коры) находится между интерфолликулярной зоной и мозговыми тяжами. Т-зоны в лимфатических дольках являются единичными образованиями, формирующими основу каждого компартмента. Главной их структурно-функциональной особенностью является то, что они никогда непосредственно не контактируют с внутриузловыми лимфатическими пространствами, а миграция лимфоцитов из синусов в соответствующую зону происходит через каналы ретикулярного остова транзитных зон (Kaldjian et al., 2001; Katakai et al., 2004; Palm et al., 2016). Зоны клональной пролиферации В-лимфоцитов (лмфатические узелки), в отличие от соответствующих Т-зон, являются достаточно многочисленными, рассеянными диффузно (мозаично) в пределах каждой дольки и формируются исключительно вдоль внутриузловых лимфатических синусов (краевого, паракортикальных, иногда мозговых) (Katakai, 2004; Capece & Kim, 2016; Nurken & Marzhan, 2016; Gavrilin et al., 2017c).

В лимфатических узлах с коллекторами I типа, к которым также относятся соответствующие органы человека и лабораторных животных, большая часть лимфатических узелков формируется в интерфолликулярной зоне, в самом поверхностном слое паренхимы, вдоль краевого синуса (Rouse et al., 1984; Katakai, 2004; Ohtani & Ohtani, 2008; Capece & Kim, 2016; Iwasaki et al., 2016). В лимфатических узлах с коллекторами II типа узелки концентрируются в виде гнезд в глубоких участках паренхимы ближе к вершинам трабекул, где внутритрабекулярные синусы формируют расширения (Hoshi et al., 1986; Gavrilin et al., 2014; Gavrilin et al., 2017c). Тот или инной тип расположения лимфатических узелков в паренхиме узлов возможно связан с неравномерной концентрацией антигенпрезентирующих клеток во внутриузловых лимфатических пространствах, максимальное количество которых выявляется в лимфе внутриузловых лимфатических коллекторов, а минимальное – во второстепенных, более мелких, особенно мозговых и воротных синусов (Jia et al., 2012; Houston et al., 2016; Iwasaki et al., 2016).

Абсолютно доказанным, на сегодняшний день, является факт реактивного характера развития всех без исключения структурно-функциональных зон компартментов паренхимы лимфатических узлов. Указывается, что интенсивная антигенная стимуляция приводит к 3–5-кратному увеличению объема лимфатических долек и, соответственно, ее составляющих компонентов (Gretz et al., 1997; Sapin, 2006; Sainte-Marie, 2010). Установлено также, что в условиях естественной умеренной антигенной стимуляции объемные соотношения компонентов лимфатических долек в лимфатических узлах различных видов млекопитающих являются относительно стабильными. В соответствующих органах половозрелых животных максимальный объем характерен для зон клональной пролиферации Т-лимфоцитов (25–40% совокупного объема паренхимы) и, несколько меньше, зон аккумуляции плазмоцитов и синтеза антител (20–30%). При этом относительный объем зон транзита лимфоцитов в 2–3 раза (10–15%), а зон клональной пролиферации В-лимфоцитов в 3–5 раз (5–15%) меньше вышеуказанных зон компартментов с максимальной степенью развития (Nicander et al., 1991; Gavrilin et al., 2017c). Отмечается также, что в лимфатических узлах мозоленогих млекопитающих (верблюда одногорбого), с характерным многоуровневым типом расположения компартментов, зоны пролиферации обоих основных клонов лимфоцитов развиты в одинаковой степени (Gavrilin et al., 2017b).

Имеющиеся на сегодня сведения о закономерностях структуры долек в паренхиме лимфатических узлов млекопитающих в основном получены при исследовании данных органов в постнатальном периоде онтогенеза у половозрелых и физиологически зрелых особей в условиях «естественного» комплекса воздействия на органы иммунной системы антигенов как экзо-, так и эндогенного характера (Bélisle & Sainte-Marie, 1981; Vyrenkov et al., 1995; Willard-Mack, 2006; Ikomi et al., 2012; Butler et al., 2016).

Формирование целостного представления о дискретной структуре паренхимы лимфатических узлов у млекопитающих невозможно без данных о развитии лимфатической дольки как основной структурно-функциональной единицы паренхимы узлов на всех этапах онтогенеза.

Антигенный гомеостаз как одно из ключевых условий сохранения биологической индивидуальности организма обеспечивается у млекопитающих комплексом различных механизмов резистентности и реактивности на всех этапах индивидуального развития, в том числе и в пренатальном онтогенезе (Hlystova, 1987; Sinkora et al., 2002; Jeklova et al., 2007; Ekman & Iivanainen, 2009; Benezech et al., 2010). При этом структурно-функциональная состоятельность периферических лимфоидных органов у плодов млекопитающих формируется по данным ряда исследователей уже в плодном периоде онтогенеза, что особенно выражено у плодов

зрелорождающих видов копытных животных, и характеризуется адекватной реакцией на антигены с формированием полноценного иммунного ответа (Nishikawa et al., 2003; Randall et al., 2008; Grigor'ev & Moljanova, 2009).

Учитывая особый эпителио- и десмохориальный тип строения плаценты у большинства видов копытных, которая в норме является абсолютно непроницаемой для макромолекул с потенциальными антигенными свойствами, основным фактором антигенной стимуляции паренхимы периферических лимфоидных органов являются антигены эндогенного происхождения, концентрация которых в организме плодов возрастает пропорционально степени развития соматических систем (Sapin et al., 1978; Aagaard et al., 2014; Parker & Makori, 2017). Время появления признаков дефинитивного строения паренхимы лимфатических узлов у млекопитающих является дискуссионным. В работах разных авторов оно варьирует от средины плодного периода до начальных этапов постнатального онтогенеза (Sminia et al., 1986; Emelyanenko, 1987; Šinkora & Butler, 2009; Grigor'ev & Moljanova, 2009). При этом спектр исследуемых в лимфатических узлах плодов морфологических маркеров иммунокомпетентности в основном ограничивается такими показателями как общее количество лимфоидной паренхимы, признаки ее дифференцировки на корковое и мозговое вещество, наличие в паренхиме узлов лимфатических узелков (Grigor'ev & Moljanova, 2009; Chuchkova et al., 2016).

Что касается аспектов формирования и морфогенеза лимфоидных долек в лимфатических узлах плодов, они на сегодняшний день исследованы недостаточно. Дольчатая структура паренхимы узлов обусловлена особенностями морфогенеза данных органов вследствие внедрения сразу нескольких мезенхимальных скоплений почек в просвете лимфатических синусов (Mebius, 2003). Однако дальнейшие механизмы морфогенеза и формирования зональной дольчатой структуры лимфоидной паренхимы в лимфатических узлах млекопитающих в условиях крайне ограниченного антигенного воздействия при автономном внутриутробном развитии в литературе практически не описаны. Прежде всего это относится к зрелорождающим видам копытных животных, у которых в силу высокой степени зрелости аппарата движения при рождении взаимодействие реактивных структур организма с факторами внешней среды нарастает лавинообразно с первых часов постнатального онтогенеза, что требует соответствующего уровня развития и компетентности антигенреактивных структур.

Цель данной статьи – определить особенности морфогенеза лимфоидных долек (компартментов) паренхимы в лимфатических узлах зрелорождающих видов млекопитающих с лимфатическими коллекторами простого типа на примере плодов быка домашнего на макро-микроскопическом и тканевом уровнях организации.

Материал и методы исследований

Материал для исследований отбирали от плодов быка домашнего (*Bos taurus* Linnaeus, 1758), полученных от беременных особей после убоя (по причинам, не связанным с инфекционными и инвазионными заболеваниями) в условиях мясоперерабатывающего предприятия «Юбилейный» города Днипро. Для исследований путем анатомического препарирования отбирали соматические – поверхностный шейный (*Limphonodi cervicales superficiales*) и висцеральные – тощей кишки (*L. jejunales*) лимфатические узлы от плодов разного возраста: 2-месячных (n = 4), 3- (n = 5), 4- (n = 10), 5- (n = 12), 6- (n = 5), 7- (n = 7), 8- (n = 6) и 9-месячных (n = 6) (Zelenevsky, 2013). Всего исследовано 110 органов. Возраст плодов определяли по массе, длине тела и степени развития производных кожи (Studencov, 2000).

Исследования проведены в Днипровском государственном аграрно-экономическом университете. Отобранные органы фиксировали в 10% растворе нейтрального формалина 5–10 суток. После фиксации целые органы или их серединные сегменты, полученные в плоскости, перпендикулярной их воротам, промывали под проточной водой в течение 6–12 часов для удаления формалина. Для получения обзорных гистологических препаратов часть органов и сегментов заливали в парафин, согласно общепринятым методикам, а часть использовали для получения тотальных срединных замороженных срезов с использованием микротома-криостата. Из парафиновых блоков на санном микротоме изготовляли серийные гистосрезы толщиной 5–8 мкм с дальнейшей окраской гематоксилином (Эрлиха) – эозином, азур II – эозином и пикрофуксином по Ван-Гизон. На микротоме-криостате МК-25М готовили тотальные срединные замороженные срезы лимфатических узлов с последующей импрегнацией их нитратом серебра по методике Фута в модификации Gavrilin (1999). Этот метод окраски использовали для определения особенностей структурных преобразований ретикулярной стромы органов и одновременной визуализации как лимфоидных долек, так и их отдельных зон.

Гистологические препараты исследовали с использованием световых микроскопов Olimpus CX-41 и Leica DM 1000 (окуляр ×10, объективы ×4, ×10, ×40). В гистологических препаратах, окрашенных гематоксилином и эозином, по Ван-Гизон, импрегнированных нитратом серебра, определяли качественные и количественные показатели степени развития и дифференцировки тканевых компонентов (стромы, паренхимы) и внутриорганных лимфатических синусов, а также особенности формирования лимфоидных долек и их отдельных функциональных зон: межклеточного взаимодействия и транзита – периферия единиц глубокой коры (паракортекса); клональной пролиферации Т-лимфоцитов – центр единиц глубокой коры (паракортекса); клональной пролиферации В-лимфоцитов – лимфатические узелки; накопления плазматических клеток и синтеза антител – мозговые тяжи). Количественный анализ тканевых компонентов стромы, лимфоидной паренхимы с ее структурными зонами и лимфатических синусов проводили методом «точечного счета» с использованием окулярных тестовых систем и нанесенными равноудаленными точками (100 точек) на всей площади гистопрепарата (Avtandilov, 1990). Относительный объем вышеуказанных компонентов и зон определяли по формуле:

$$V_i = P_i/P_t \cdot 100\%,$$

где V_i – относительный объем исследуемого компонента, P_i – число точек, попавших на структурный компонент, P_t – общее число точек тестовой системы, попавших на гистопрепарат.

Необходимое число точек для получения достоверных данных ñ (минимальный размер выборки, при котором выборочные наблюдения отклоняются от значений для генеральной совокупности не больше чем на 5%), определяли по формуле:

$$ñ = 400(100 – n) / n,$$

где n – число точек, приходящихся на анализируемый компонент при предварительном подсчете 100 точек (Avtandilov, 1990).

Микрофотографии получали с помощью микроскопа Leica DM1000 (окуляр ×4, объективы ×10/0,25, ×40/0,65), интегрированного с персональным компьютером.

Статистический анализ результатов проведен в Statistica 10.0 (StatSoft Inc., USA). Различия между выборками определены с использованием ANOVA и считались значимыми при $P < 0,05$.

Результаты

Лимфатические узлы у быка домашнего до начала плодного периода представлены мезенхимальными тканевыми зачатками и отдельными группами клеток гематогенного происхождения (рис. 1).

Выраженная дифференцировка лимфатических узлов на строму и паренхиму определяется начиная только с 3-месячного возраста. С данного возраста и до конца пренатального периода онтогенеза, паренхима лимфатических узлов является наиболее развитым тканевым компонентом (табл. 1). У 3-месячных плодов паренхима лимфатических узлов представлена лимфоидной тканью, а соединительнотканная строма – плотной волокнистой неоформленной соединительной тканью с соответствующим клеточным составом и структурой межклеточного вещества. В данный период развития строма четко подразделяется на капсулу и воротное утолщение с многочисленными кровеносными и лимфатическими сосудами, но капсулярные трабекулы выражены незначительно (рис. 2).

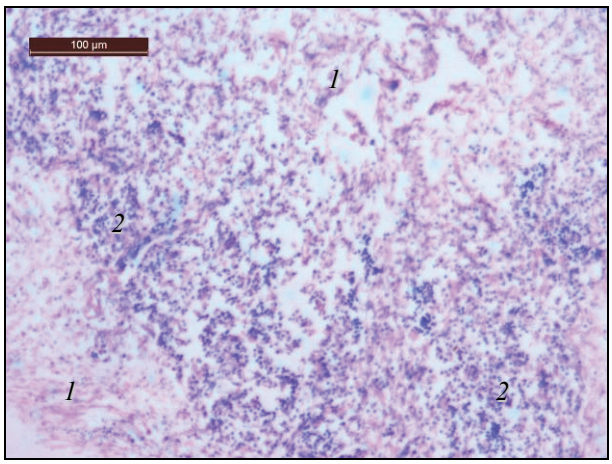


Рис. 1. Скопление мезенхимальных (*1*) и гематогенных (*2*) клеток в зачатке поверхностного шейного лимфатического узла: азур II и эозин

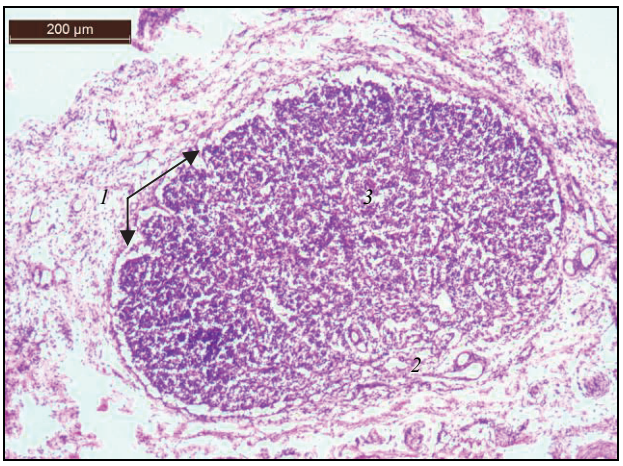


Рис. 2. Капсула (*1*), воротное утолщение (*2*), лимфоидная паренхима (*3*) лимфатического узла тощей кишки 3-месячного плода: гематоксилин и эозин

Таблица 1

Динамика относительного объема (%) лимфоидной паренхимы и соединительнотканной стромы лимфатических узлов быка домашнего в пренатальном периоде онтогенеза (x ± SD)

Узел	Тканевый компонент (%)	Возраст, мес.						
		3, n = 5	4, n = 10	5, n = 12	6, n = 10	7, n = 7	8, n = 6	9, n = 6
Поверхностный шейный	соединительнотканная строма	9,90 ± 1,52[a]	13,3 ± 2,92[b]	14,56 ± 2,48[b]	14,92 ± 2,45[b]	16,24 ± 1,49[b]	17,18 ± 0,97[b]	17,28 ± 1,06[b]
	лимфоидная паренхима	90,10 ± 1,52[c]	86,67 ± 2,92[d]	76,79 ± 4,39[e]	76,04 ± 2,75[e]	73,67 ± 2,38[e]	73,85 ± 0,75[e]	68,38 ± 1,45[f]
Тощей кишки	соединительнотканная строма	19,16 ± 2,31[g]	18,31 ± 1,71[g]	14,23 ± 2,03[h]	13,73 ± 2,11[h]	13,40 ± 2,23[h]	10,92 ± 2,05[h]	15,08 ± 1,93[i]
	лимфоидная паренхима	80,84 ± 2,31[j]	81,69 ± 1,71[j]	78,65 ± 1,68[k]	78,10 ± 2,81[k]	75,26 ± 6,43[k]	74,47 ± 4,97[k]	72,43 ± 5,82[k]

Примечание: разными латинскими буквами обозначены выборки достоверно отличающиеся одна от другой (P < 0,05) по результатам теста Тьюки.

Для лимфатических узлов плодов данного возраста характерно значительное развитие лимфоидной ткани, относительный объем которой, на фоне отсутствия четко выраженных синусов узлов, в 4–9 раз больше объема соединительнотканной стромы (табл. 1).

Начиная с третьего месяца пренатального развития плодов в лимфатических узлах наблюдается разделение лимфоидной паренхимы на две зоны: большую центральную (мозговое вещество) и меньшую периферическую (корковое вещество) (рис. 3а). Центральная зона имеет однородную структуру, менее плотную, ее ретикулярная основа представлена густой сетью извитых волокон, которые, объединяясь между собой, образуют равномерные ячейки средних размеров (рис. 3б). Периферическая зона густо заполнена малыми лимфоцитами, имеет вид тонкой полоски, размещенной вдоль капсулы. Сеть ретикулярных волокон в этой зоне более разрежена, а большинство волокон преимущественно ориентировано перпендикулярно капсуле.

Соединительнотканная строма в разных лимфатических узлах развита неравномерно. В поверхностном шейном лимфатическом узле относительный объем соединительнотканной стромы при этом минимальный, до 4-месячного возраста отмечено достоверное его увеличение и стабилизация до конца плодного периода. В лимфатическом узле тощей кишки относительный объем соединительнотканной стромы значительно выше, он максимален, затем наблюдается постепенное его уменьшение (начиная с 5-месячного возраста) и незначительное увеличение (к концу плодного периода). В первой трети плодного периода (2–4 месяца) относительная площадь лимфоидной паренхимы имеет максимальное значение, начиная с 4-месячного возраста в поверхностном шейном и 5-месячного в лимфатических узлах тощей кишки этот показатель постепенно снижается, достигая своего минимума у 9-месячных плодов. Динамика относительного объема лимфоидной паренхимы у плодов быка связана с процессами формирования системы лимфатических синусов, развитие которых сопровождается ее снижением на фоне выраженной морфологической сегрегации паренхимы узлов.

В 4-месячном возрасте наиболее значимо изменяется соединительнотканная строма.

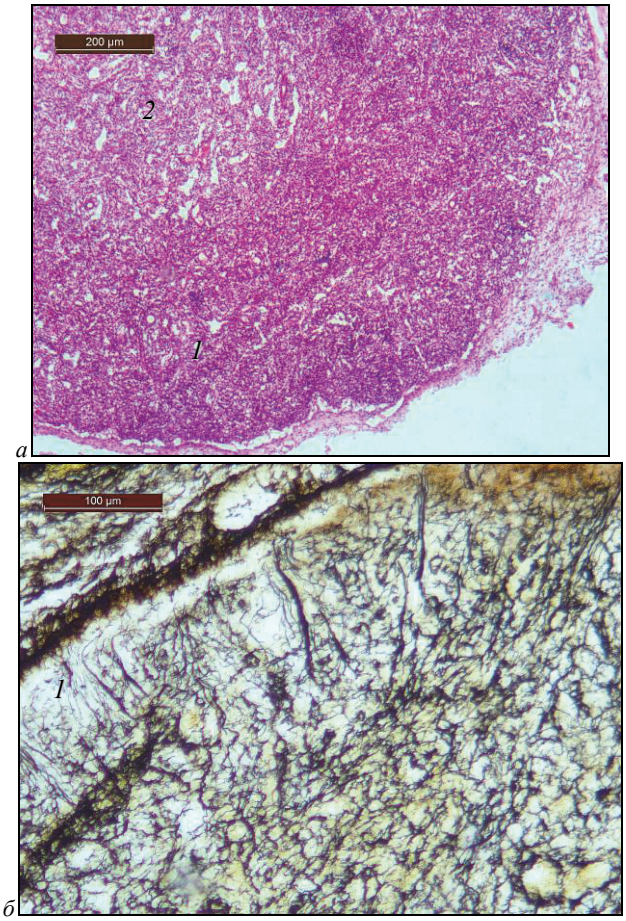


Рис. 3. Корковое (*1*) и мозговое (*2*) вещество паренхимы поверхностного шейного лимфатического узла 3-месячного плода: *а* – гематоксилин и эозин, *б* – импрегнация азотнокислым серебром

В поверхностных шейных узлах выявляется относительно неширокая соединительнотканная капсула с тонкими капсулярными трабекулами. В отличие от соматических, в висцеральных лимфатических узлах соединительнотканная капсула тоньше, капсулярные трабекулы менее выражены, воротное утолщение имеет небольшую площадь и овальную форму.

Как в соматических, так и в висцеральных лимфатических узлах 4-месячных плодов впервые выявляется подкапсулярный (краевой) синус (рис. 4а). Лимфоидная паренхима изменяется в меньшей степени, но именно в это время впервые появляются признаки ее функциональной специализации. Периферическая зона лимфоидной паренхимы расширяется, плотность размещения в ней клеток увеличивается. Капсулярными трабекулами и промежуточными (корковыми) синусами паренхима начинает разделяться на отдельные дольки. Центральная зона лимфатических узлов существенно не изменяется, но отмечается начало формирования системы мозговых синусов и мозговых тяжей, с соответственной архитектоникой ретикулярных сетей (рис. 4б).

В лимфатических узлах 5-месячных плодов наиболее выраженные изменения характерны для лимфоидной паренхимы. Из-за утолщения капсулярных трабекул и развития вокруг них промежуточных корковых синусов дольчатая структура паренхимы становится четко выраженной. В срединных срезах лимфатических узлов 5-месячных плодов лимфоидные дольки расположены в один слой и развиты относительно равномерно.

У плодов быка домашнего, как и у новорожденных особей данного вида и половозрелых лабораторных животных, лимфатические дольки паренхимы лимфатических узлов состоят из комплекса клеточных тяжей: периферические зоны единиц глубокой коры и интерфолликулярной зоны (транзита лимфоцитов и межклеточного взаимодействия), сферических структур – центры единиц глубокой коры и лимфатические узелки (клональной пролиферации лимфоцитов) и мозговых тяжей (зон потенциального накопления плазматических клеток и синтеза антител). В данный период практически в каждой лимфоидной дольке лимфатического узла определяются все функциональные зоны, но степень их развития значительно отличается как в пределах долек одного узла, так и долек соматических и висцеральных лимфатических узлов.

Наиболее развитыми зонами лимфатических долек паренхимы лимфатических узлов 5-месячных плодов являются единицы глубокой коры и мозговые тяжи, относительный объем которых практически одинаков как в соматических, так и в висцеральных лимфатических узлах (табл. 2).

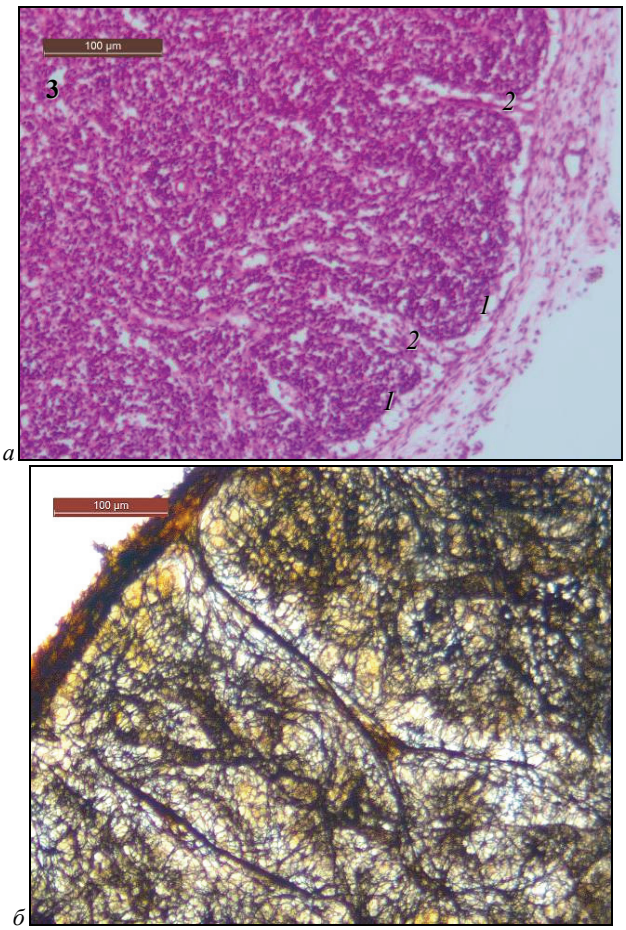


Рис. 4. Подкапсулярный (*1*), промежуточные (*2*), мозговые (*3*) лимфатические синусы в поверхностном шейном лимфатическом узле 4-месячного плода: *а* – гематоксилин и эозин; *б* – импрегнация азотнокислым серебром

Таблица 2

Динамика относительного объема (%) компонентов лимфоидной паренхимы лимфатических узлов быка домашнего в пренатальном периоде онтогенеза (x ± SD)

Узел	Компоненты лимфоидной паренхимы	Возраст, мес				
		5, n = 12	6, n = 10	7, n = 7	8, n = 6	9, n = 6
Поверхностный шейный	интерфолликулярная зона	4,83 ± 2,47[a]	6,10 ± 2,30[a]	6,21 ± 1,99[a]	8,60 ± 2,13[a]	9,48 ± 3,14[a]
	центральные зоны единиц глубокой коры	2,45 ± 0,71[b]	4,63 ± 1,15[c]	5,46 ± 1,21[c]	5,48 ± 0,72[c]	6,43 ± 0,92[c]
	паракортикальные тяжи	34,79 ± 2,47[d]	35,85 ± 2,31[d]	22,43 ± 1,85[e]	21,13 ± 2,15[e]	16,88 ± 3,24[f]
	лимфатические узелки	0,52 ± 0,41[g]	0,70 ± 0,37[g]	0,71 ± 0,27[g]	1,07 ± 0,40[g]	1,27 ± 0,45[g]
	мозговые тяжи	34,21 ± 4,12[h]	29,10 ± 4,37[i]	38,86 ± 2,48[j]	37,58 ± 2,61[j]	34,33 ± 3,82[j]
Тощей кишки	интерфолликулярная зона	6,40 ± 1,28[k]	5,67 ± 1,50[k]	7,33 ± 1,45[l]	7,62 ± 2,28[l]	9,90 ± 2,19[l]
	центральные зоны единиц глубокой коры	2,70 ± 1,13[m]	6,76 ± 1,67[m]	9,07 ± 1,42[n]	10,41 ± 1,51[n]	11,82 ± 1,88[n]
	паракортикальные тяжи	33,00 ± 2,68[o]	23,20 ± 3,97[p]	20,93 ± 2,79[p]	22,53 ± 2,90[p]	25,12 ± 3,32[p]
	лимфатические узелки	1,41 ± 0,60[q]	1,75 ± 0,43[q]	2,43 ± 0,36[r]	2,42 ± 0,39[r]	3,10 ± 0,47[s]
	мозговые тяжи	35,14 ± 2,63[t]	40,73 ± 3,28[u]	35,50 ± 7,29[u]	31,50 ± 5,60[u]	22,48 ± 5,47[v]

Примечание: см. табл. 1.

Единицы глубокой коры являются основой каждой лимфоидной дольки и имеют четкую полярную дифференциацию. Расширенный полюс непосредственно граничит с подкапсулярным синусом, а суженный направлен к воротному утолщению, боковые поверхности ограничены промежуточными корковыми синусами, расположенными преимущественно вдоль капсулярных трабекул. Каждая единица глубокой коры, начиная с 5-месячного возраста плодов, четко разделяется на периферическую и центральную зоны. Периферическая зона представлена паракортикальными тяжами и интерфолликулярной зоной (корковым плато). Максимальный относительный объем имеют паракортикальные тяжи, которые в 5–7 раз превышают объем интерфолликулярной зоны (табл. 2). Наименее развитыми зонами лимфоидных долек являются зоны размножения Т- и В-лимфоцитов, при этом центры единиц глубокой коры, которые являются зонами клональной пролиферации Т-лимфоцитов, более развиты, нежели зоны пролиферации В-лимфоцитов (лимфатические узелки).

Лимфатические узелки в лимфатических узлах 5-месячных плодов выявляются в незначительном количестве и только первичные (без центров размножения), их относительный объем менее 1% в соматических и менее 2% – в висцеральных. Они преимущественно формируются на основе интерфолликулярной зоны

и в этом возрасте локализируются вдоль краевого синуса. Во всех исследуемых лимфатических узлах плодов данного возраста лимфоидные дольки располагаются в один ряд, но развиты неравномерно в соматических и висцеральных лимфоузлах. В поверхностном шейном лимфатическом узле дольки четко разделены между собой хорошо развитыми капсулярными трабекулами, вдоль которых расположены промежуточные синусы (рис. 5а). В лимфоузлах тощей кишки из-за менее развитого трабекулярного аппарата и из-за незначительного просвета промежуточных синусов дольковая структура паренхимы выражена не четко (рис. 5б).

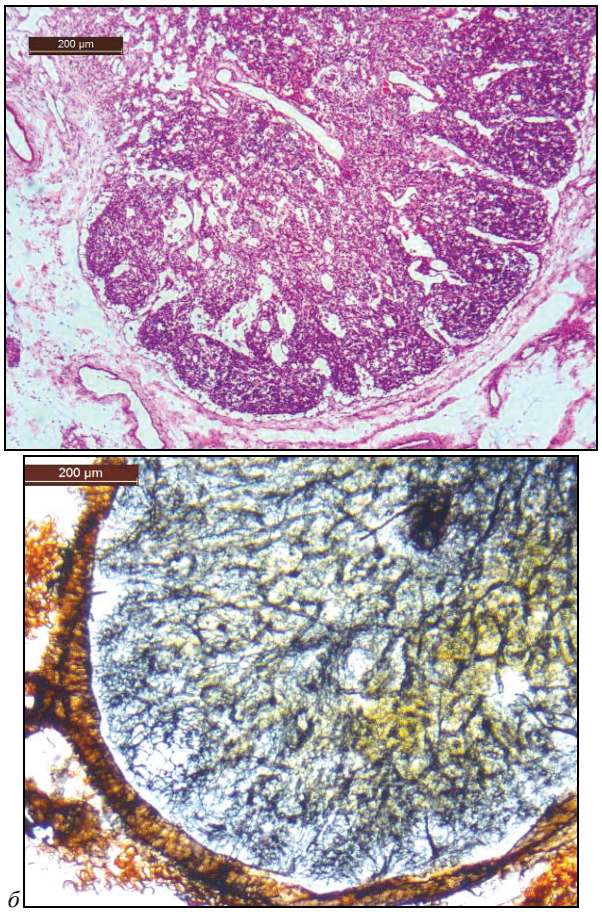


Рис. 5. Лимфоидные дольки лимфатических узлов 5-месячных плодов быка домашнего: *а* – в поверхностном шейном (гематоксилин и эозин), *б* – в лимфатическом узле тощей кишки (импрегнация азотнокислым серебром)

При исследовании гистопрепаратов лимфатических узлов, импрегнированных азотнокислым серебром, определено, что для каждой функциональной зоны характерна специфическая архитектоника ретикулярных волокон. Для центральных зон единиц глубокой коры это крупнопетлистая сотообразная, для паракортикальных тяжей и итерфолликулярной зоны – мелкопетлистая со специфической плетеноподобной структурой (рис. 6а). Для лимфатических узелков характерна равномерная среднепетлистая сеть, а для мозговых тяжей – плотные мелкопетлистые сети, которые окружают кровеносные сосуды (рис. 6б).

Также начиная с 5-месячного возраста плодов значительного развития достигает система лимфатических синусов в лимфатических узлах, которая состоит из подкапсулярного (краевого), воротного, межуточных корковых и хорошо выраженных мозговых синусов (рис. 7).

Наиболее развитыми функциональными зонами лимфоидной паренхимы узлов быка домашнего на протяжении плодного периода остаются единицы глубокой коры и мозговые тяжи. В единицах глубокой коры большую часть объема занимают паракортикальные тяжи. Динамика их относительного объема в паренхиме поверхностного шейного узла характеризуется стабильностью этого показателя на протяжении 5–6-го месяца, достоверным снижением начиная с 7-месячного возраста плодов с достижением минимальных значений к концу плодного периода онтогенеза. В лимфатических узлах тощей кишки данный показатель достоверно снижается уже с 6-месячного возраста плодов, в последующем стабилизируется и до конца плодного периода не превышает 20–25% (табл. 2).

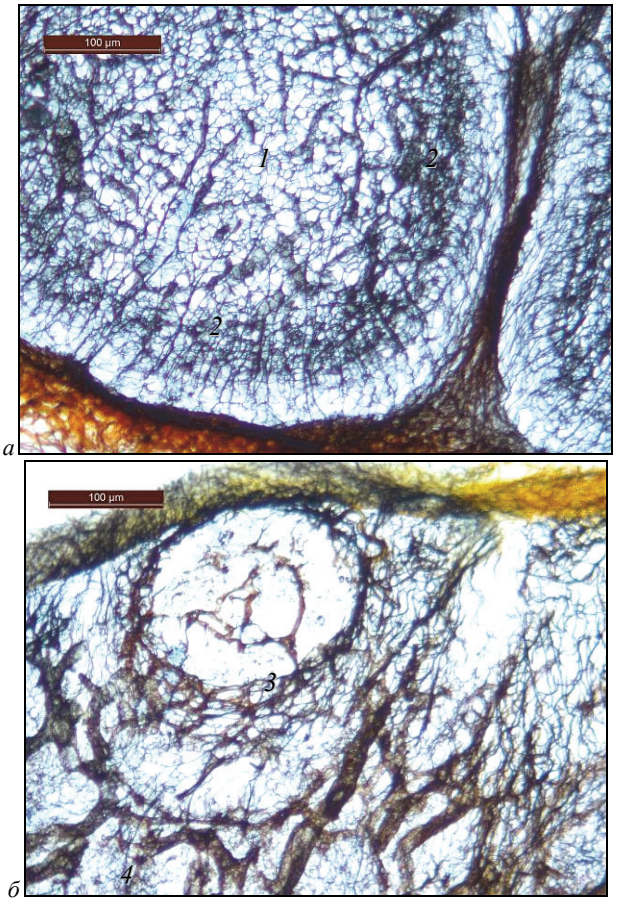


Рис. 6. Архитектоника ретикулярных волокон центра единицы глубокой коры (*1*), паракортикальных тяжей и интерфолликулярной зоны (*2*), лимфатического узелка (*3*), мозговых тяжей (*4*) поверхностного шейного лимфатического узла 8-месячного плода быка домашнего (импрегнация азотнокислым серебром)

В отличие от паракортикальных тяжей, относительный объем центральных участков единиц глубокой коры на протяжении всего плодного периода возрастает. В соматических лимфатических узлах их относительный объем достоверно увеличивается, начиная с 6-месячного, а в висцеральных – с 7-месячного возраста. Соотношение относительного объема паракортикальных тяжей и центров единиц глубокой коры в начале плодного периода составляет в поверхностном шейном узле 1/14, а к концу плодного периода только 1/2,6, а в лимфатических узлах тощей кишки – 1/12,0 и 1/2,3 соответственно.

Интерфолликулярная зона в поверхностном шейном лимфатическом узле в начале плодного периода составляет 1/7, а в лимфатическом узле тощей кишки 1/6 относительного объема единиц глубокой коры. На протяжении плодного периода ее относительный объем практически не изменяется и не превышает 10%. Но в связи с тем, что относительный объем единиц глубокой коры постепенно снижается, соотношение объема интерфолликулярной зоны к объему единиц глубокой коры возрастает до 1/2 в соматических узлах и 1/4 – в висцеральных.

Лимфатические узелки в лимфоидной паренхиме лимфатических узлов, на протяжении пренатального периода онтогенеза, остаются недостаточно развиты. Их совокупный относительный

объем в соматических лимфатических узлах достигает 1% только к 8-месячному возрасту плодов, а к концу пренатального развития не превышает 1,3%. Более развиты лимфатические узелки в висцеральных лимфатических узлах, где до конца 9-месячного возраста их относительный объем достигает более 3% и среди них выявляются узелки с центрами размножения.

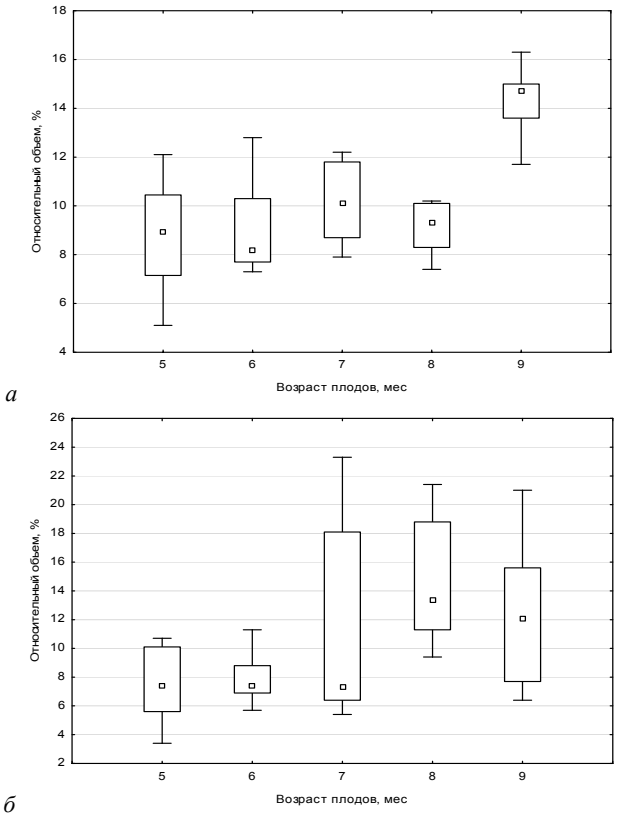


Рис. 7. Динамика относительного объема лимфатических синусов лимфатических узлов быка домашнего на протяжении 5–9 месяцев пренатального периода онтогенеза: *а* – поверхностный шейный, *б* – лимфоузел тощей кишки

Лимфатические узелки в лимфатических узлах плодов преимущественно располагаются вдоль подкапсулярного синуса на основе интерфолликулярной зоны, но начиная с 8-месячного возраста в некоторых лимфатических узлах тощей кишки они были выявлены на боковых поверхностях единиц глубокой коры, что свидетельствует о возможности их формирования на основе перакортикальных тяжей (рис. 8).

Мозговые тяжи разной конфигурации полностью формируются, начиная с 5-месячного возраста, занимая в соматических и висцеральных узлах около 35% относительного объема лимфоидной паренхимы. Динамика развития относительного объема мозговых тяжей в соматических лимфатических узлах характеризуется достоверным его уменьшением до 6-месячного возраста, увеличением и стабилизацией показателя с 7-месячного возраста и до конца пренатального развития. В висцеральных лимфатических узлах до 6-го месяца развития плодов отмечен достоверный рост относительного объема мозговых тяжей и резкое его уменьшение до 9-месячного возраста.

Таким образом, к концу плодного периода лимфатические узлы быка домашнего имеют высокую степень структурной дифференциации и представлены достаточно сформированными лимфатическими дольками (компартментами). На протяжении всего пренатального периода онтогенеза лимфатические дольки лимфатических узлов располагаются однослойно вдоль хорошо выраженного подкапсулярного синуса. Между собой лимфатические дольки разделяются капсулярными трабекулами и промежуточными корковими (паракортикальными) синусами. В связи с тем, что капсулярные трабекулы более развиты в соматических узлах, то и степень разделения долек между собой больше выражена в поверхностном шейном узле (рис. 9).

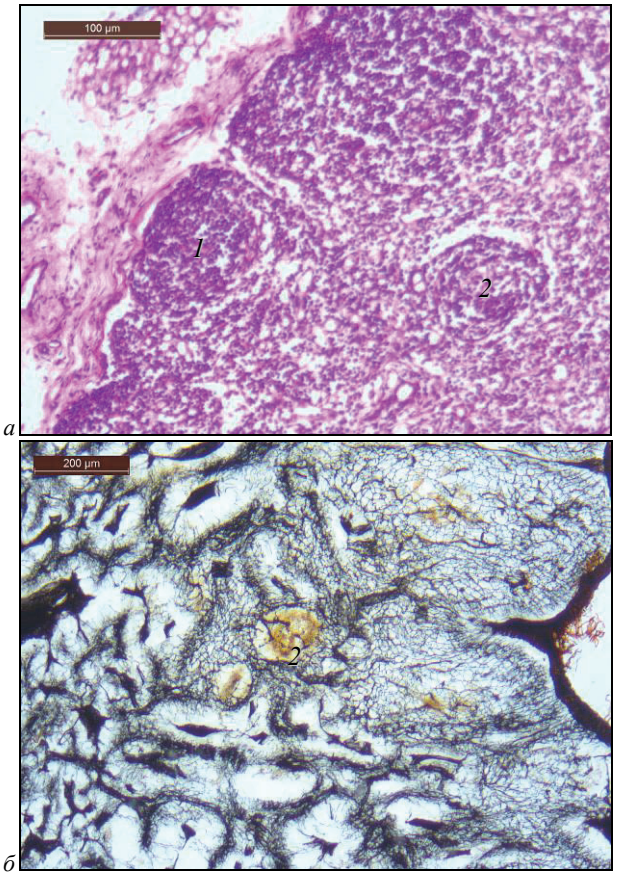


Рис. 8. Лимфатические узелки на основе интерфолликулярной зоны (*1*) и паракортикальных тяжей (*2*) в лимфатическом узле тощей кишки: *а* – гематоксилин и эозин, *б* – импрегнация азотнокислым серебром

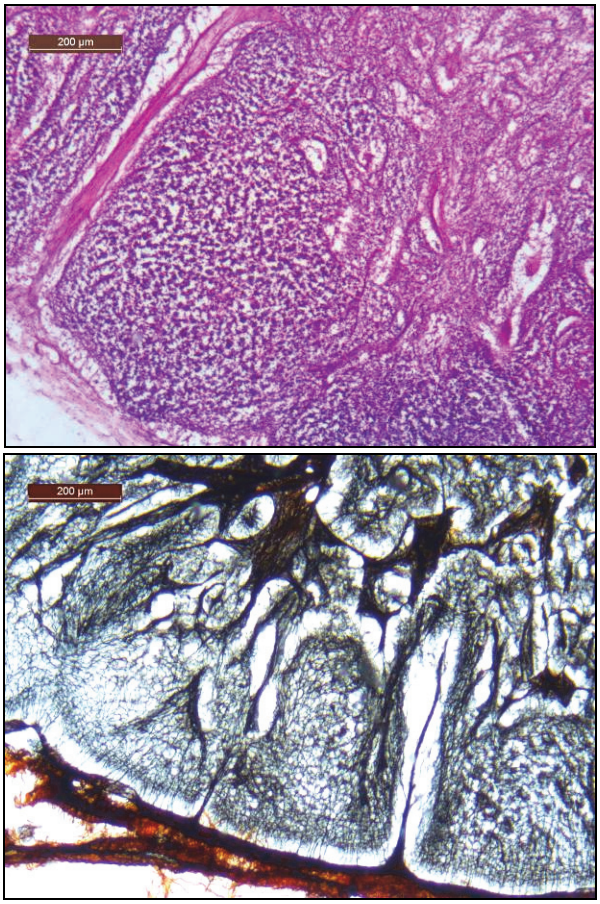
К концу плодного периода онтогенеза практически в каждой лимфатической дольке паренхимы лимфатических узлов плодов выражены все основные структурно-функциональные зоны как транзита лимфоцитов и их клональной пролиферации, так и потенциального накопления плазмоцитов и синтеза антител.

Обсуждение

Результаты наших исследований свидетельствуют, что у зрелорождающих копытных млекопитающих формирование основных дефинитивных черт строения лимфатических узлов как периферических лимфоидных органов происходит в плодном периоде пренатального онтогенеза поэтапно, с постепенным увеличением степени структурно-функциональной дифференциации лимфоидной паренхимы и, как следствие, формирования комплекса морфологических маркеров иммунокомпетенции.

Дольчатая структура в лимфатических узлах млекопитающих закладывается у предплодов, а основой каждой субъединицы данных органов является отдельная мезенхимальная почка, количество которых в тех или иных узлах детерминировано генетически (Mebius, 2003). В то же время окончательное формирование дольчатой структуры паренхимы с четкой визуализацией данных структур связано с такими факторами как развитие в дольке полного комплекса микроциркуляторного русла, в том числе венул с высоким эндотелием, дифференцировка внутриузловых лимфатических пространств и, как следствие, заселение долек иммунокомпетентными пулами Т- и В-лимфоцитов и включение узлов в общую систему рециркуляции лимфоцитов (De Bruyn & Cho, 1990; Rjabchikov et al., 2002; Ekman & Iivanainen, 2009; Benezech et al., 2010; Platt & Randolph, 2013; Ager, 2017).

Значительное увеличение в паренхиме лимфатических узлов 3-месячных плодов быка домашнего количества лимфоцитов с концентрацией их вдоль краевого синуса, вероятно, является свидетельством того, что именно в этот период микроциркуляторное русло данных органов приобретает дефинитивные черты строения, а центральные органы иммунной системы продуцируют достаточное для заселения периферических лимфоидных органов количества иммунокомпетентных лимфоцитов (Rjabchikov et al., 2002).



а

б

Рис. 9. Лимфатические дольки в паренхиме поверхностного шейного лимфатического узла: *а* – гематоксилин и эозин, *б* – импрегнация азотнокислым серебром

Морфологические маркеры наличия в организме плода функции иммунологической реактивности (адаптивного специфического иммунитета) впервые выявляются у 5-месячных плодов. Именно в этот период формируется полнокомпонентное внутриузловое лимфатическое русло, которое включает в себя основной лимфатический коллектор (краевой синус) и ряд их производных (паракортикальные и мозговые синусы).

Зона клональной пролиферации Т- и В-лимфоцитов в лимфатических дольках паренхимы лимфатических узлов 5-месячных плодов развита крайне незначительно на фоне гораздо более существенно развитых зон миграции лимфоцитов (транспортных коридоров), а также мозговых тяжей, которые состоят преимущественно из фибробластических ретикулярных клеток и их волокон (Katakai et al., 2004; Benezech et al., 2010).

Минимальные морфометрические характеристики зон клональной пролиферации лимфоцитов в лимфатических узлах плодов связаны с тем, что у плацентарных млекопитающих антигенный прессинг на органы на ранних этапах онтогенеза является крайне неравномерным, он лавинообразно нарастает непосредственно после рождения, в период постнатальной адаптации и имеет высокие стабильные показатели на дальнейших этапах постнатального развития (Gavrilin et al., 2017a, 2017c).

У плодов млекопитающих, особенно у видов с эпителио- и десмохориальным типом плаценты, в норме абсолютно непроницаемой для каких либо относительно крупных молекул, которые могут потенциально иметь антигенные свойства, антигенная «нагрузка» на периферические лимфоидные органы весьма незначительна (Sapin, 2006).

Единственным стимулом для развития антигенреактивных структур в периферических лимфоидных органах у млекопитающих с наиболее совершенным плацентарным барьером считаются эндогенные макромолекуры (эндогенные антигены), концентрация которых в организме плода возрастает пропорционально масштабам формирования соматических систем организма плода (Emelyanenko, 1987; Sapin et al., 1978).

Нами установлено, что в лимфоидных дольках паренхимы плодов быка домашнего на протяжении всего плодного периода масштабы развития (относительный объем) зон клональной пролиферации Т-лимфоцитов значительно преобладает соответствующий показатель В-зависимых зон (лимфатических узелков). Известно также, что у половозрелых и физиологически зрелых млекопитающих два основных типа специфического реагирования относительно уравновешены, а количественные морфометрические характеристики структур, обеспечивающих соответствующие типы иммунологической реактивности, не имеют существенной разницы (Gavrilin et al., 2017c).

Возможно, именно особенности антигенного спектра и свойства аутоантигенов в организме плодов быка домашнего влияют на поляризацию иммунного ответа по Th-1 типу, а цитотоксический иммунный ответ является основным типом специфического реагирования у зрелорождающих млекопитающих с непроницаемым для макромолекул плацентарным барьером (Emelyanenko, 1987).

Характерно также, что морфологические маркеры Th-2 зависимого иммунного ответа больше выражена в висцеральных лимфатических узлах плодов, потенциальным источником антигенов для которых являются как соматические макромолекулы, так и аутоантигены, образующиеся в полости кишечной трубки.

Таким образом, результаты наших исследований свидетельствуют, что автономное развитие плода у быка домашнего и изоляция его от материнских антигенов и, соответственно, антигенов внешней среды не являются преградой для дифференцировки паренхимы лимфатических узлов на лимфоидные дольки с отдельными структурно-функциональными зонами, которые являются морфологическими маркерами функции адаптивного специфического иммунитета. В то же время лимфоидные дольки лимфатических узлов на момент рождения плодов имеют ряд «эмбриональных» черт (относительная стабильность объема всех функциональных зон, минимальная степень развития зон клональной пролиферации Т- и В-лимфоцитов, что обусловлено условиями их существования.

Эмбриональная лимфоидная долька имеет характерную однополярную гистоархитектонику и специфическую слоисто-дискретную структуру без мозаичности, присущей соответствующим образованиям паренхимы узлов в постнатальном онтогенезе (Gavrilin et al., 2017a). Слоистым компонетом в лимфоидной дольке лимфатических узлов плодов являются интрафолликулярная зона (корковое плато), а также зона мозговых тяжей, а дискретной – единицы глубокой коры с выраженным сферообразным центром (зона клональной пролиферации Т-лимфоцитов). Лимфатические узелки, которые в лимфоидной дольке «постэмбриональных» лимфатических узлов расположены диффузно от интерфолликулярной зоны до зоны мозговых тяжей, у плодов концентрируются преимущественно вдоль краевого синуса в интрафолликулярной зоне и, как исключение, отдельные из них могут развиваться в начальных участках паракортикальных тяжей в лимфатических узлах тощей кишки. Возможно, это является следствием весьма умеренных иммунореактивных свойств внутриутробных аутоантигенов (Sapin, 2006).

Выводы

В лимфатических узлах зрелорождающих копытных млекопитающих (быка домашнего) с лимфатическими коллекторами

простого типа морфологические признаки структурно-функциональной дифференциации и специализации паренхимы впервые выявляются у 3-месячных плодов, а степень выраженности структурной гетерогенности лимфоидной ткани до конца плодного периода постоянно усиливается. В развитии лимфоидной паренхимы лимфатических узлов у плодов выделяется ряд последовательных этапов: формирование лимфоидного матрикса вдоль краевого синуса узлов без выраженной дольчатости (3–4-месячные плоды); обособление лимфоидных долек в целом и формирование их основных функциональных зон (транзита лимфоцитов, клональной пролиферации Т- и В-лимфоцитов, аккумуляции плазматических клеток и антителообразования) (5-месячные плоды); развитие компонентов лимфоидных долек преимущественно за счет увеличения объема зон транзита лимфоцитов и клональной пролиферации Т- и В-лимфоцитов.

В лимфоидных дольках лимфатических узлов быка домашнего с момента формирования данных органов до конца плодного периода наиболее развитыми, с максимальным относительным объемом, являются зоны транзита лимфоцитов (интерфолликулярная зона и паракортикальные тяжи), а также зона потенциальной аккумуляции лимфоцитов и антителообразования (мозговые тяжи). Среди зон клональной пролиферации лимфоцитов на всех этапах развития долек с момента их обособления преобладают Т-зависимые зоны, относительный объем которых в 5,0–7,5 раза превышает соответствующие показатели В-зависимых зон. Пренатальное развитие компартментов у плодов быка домашнего характеризуется постепенным увеличением, от незначительного до умеренного, относительного объема зон клональной пролиферации Т- и В-лимфоцитов. При этом относительный объем зон транзита лимфоцитов, а также аккумуляции плазматических клеток имеет тенденцию к уменьшению.

Гистоархитектоника лимфоидных долек на протяжении всего плодного периода существенно не изменяется и характеризуется однослойностью расположения компартментов, их выраженной полярной структурой, формированием лимфатических узелков преимущественно вдоль основного лимфатического коллектора (краевого синуса), за исключением висцеральных узлов брюшной полости, где лимфатические узлы образуются на основе паракортикальных тяжей.

Дальнейшие исследования особенностей пренатального морфогенеза компартментов лимфатических узлов быка домашнего будут направлены на выяснение закономерностей их цитогенеза с выявлением основных маркеров дифференцировки и клональной пролиферации лимфоцитов.

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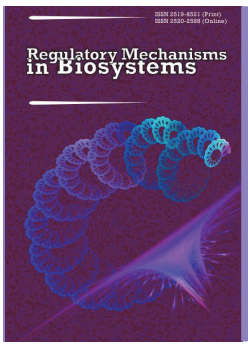

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 105–111
doi: 10.15421/021815

The significance of hepatic transaminases and ultrasound in the diagnosis of non-alcoholic fatty liver disease

Y. M. Stepanov, N. V. Nedzvetskaya, V. B. Yagmur, I. S. Konenko

Institute of Gastroenterology of National Academy of Medical Sciences of Ukraine, Dnipro, Ukraine

Article info

Received 17.02.2017
Received in revised form 14.03.2017
Accepted 16.03.2017

Institute of Gastroenterology of National Academy of Medical Sciences of Ukraine, Slobozhansky ave., 96, Dnipro, 49074, Ukraine.
Tel.: +38-056-760-33-05.
E-mail: gastrodnepr@i.ua

Stepanov, Y. M., Nedzvetskaya, N. V., Yagmur, V. B., & Konenko, I. S. (2018). The significance of hepatic transaminases and ultrasound in the diagnosis of non-alcoholic fatty liver disease. Regulatory Mechanisms in Biosystems, 8(2), 105–111. doi:10.15421/021815

Non-alcoholic fatty liver disease is characterized by fatty liver infiltration without any another common cause of steatosis (severe alcohol, drugs, etc.). Non-alcoholic fatty liver disease is associated with metabolic risk factors, which are diabetes type 2, dyslipidemia, obesity, and in some cases, it has a genetic predisposition as a main cause. The liver biopsy remains the "gold standard" for assessing the degree of steatosis, necrosis and liver fibrosis. However, non-invasive investigations, especially biochemical markers and visualization methods remain the first-line diagnostic analyses, as well as assessment of the response to treatment. In view of this, the aim of our research was to evaluate the validity of biochemical parameters of liver function and ultrasound in the diagnosis of non-alcoholic fatty liver disease. Patients diagnosed with non-alcoholic fatty liver disease were studied in this research Every patient underwent to both an examination and treatment in the Department of Liver and Pancreatic Diseases at the Institute of Gastroenterology, NAMS of Ukraine. All patients were exposed to ultrasound visualization of the abdominal organs, standard biochemical studies (content analyses of alanine aminotransferase, aspartate aminotransferase, total bilirubin and its fractions, activity of alkaline phosphatase, gammaglutamyltranspeptidase, X-lipoproteins, total protein, albumin, fibrinogen, international normalized ratio) were performed in the blood serum . Increased echogenicity of the liver and distal decrement of ultrasound, as the main ultrasonographic symptoms of liver steatosis, were determined with high incidence in all patients with non-alcoholic fatty liver disease. A number of symptoms (heterogeneity of the echo-structure of the liver of medium and coarse-grained nature, roundness of the lower edge of the liver, inequalities in the liver contour), the frequency of which is more closely related to the severity of inflammatory, as well as fibrotic changes, were observed more often in patients with non-alcoholic steatohepatitis and cirrhosis compared with steatosis. The deterioration in the visualization of small branches of the liver veins was determined as a result of the smoothness of the vascular pattern and its depletion. Moreover, the results showed an increment of the spleen volume, along with the enlargement of the splenic vein of patients with cirrhosis. All observed changes were considered as a component of portal hypertension and were induced with fibrotic transformation of the liver. The lack of correlation of the degree of fibrosis with the content of transaminases confirms the low diagnostic significance of these indicators. Nevertheless, the moderate direct correlation of the determined ultrasonographic indexes with degree of the fibrosis in the liver indicates the possibility of using this method for screening non-alcoholic fatty liver disease.

Keywords: liver steatosis; nonalcoholic steatohepatitis; alanine aminotransferase; aspartate aminotransferase; ultrasound examination

Значення печінкових трансаміназ і ультразвукового дослідження в діагностиці неалкогольної жирової хвороби печінки

Ю. М. Степанов, Н. В. Недзвецька, В. Б. Ягмур, І. С. Коненко

Інститут гастроентерології НАМН України, Дніпро, Україна

Неалкогольна жирова хвороба печінки – стан жирової інфільтрації печінки за відсутності іншої загальної причини стеатозу (важкого вживання алкоголю, ліків тощо). Неалкогольна жирова хвороба печінки пов'язана з факторами метаболічного ризику, такими як ожиріння, цукровий діабет другого типу, дисліпідемія та, в деяких випадках, генетична схильність. Біопсія залишається золотим стандартом для оцінювання ступеня стеатозу, некрозапалення та фіброзу печінки. У той же час, використання візуалізаційних і неінвазивних досліджень біохімічних маркерів – методи першої лінії для встановлення діагнозу та оцінювання ефективності лікування. Оцінено значення біохімічних показників функціонального стану печінки та ультразвукового дослідження в діагностиці неалкогольної жирової хвороби печінки. Досліджено пацієнтів із неалкогольною жировою хворобою печінки, які проходили обстеження та лікування у відділенні захворювань печінки та підшлункової залози ДУ «Інститут гастроентерології НАМН України». Всім хворим проводили ультразвукове

дослідження органів черевної порожнини, а також стандартні біохімічні дослідження (визначення в сироватці крові аланінамінотрансферази, аспартатамінотрансферази, загального білірубіну та його фракції, активність лужної фосфатази, гаммаглутамілтранспептидази, Х-ліпопротеїдів, загального білка, альбуміну, фібриногену, міжнародне нормалізоване відношення). Підвищена ехогенність печінки та дистальне згасання ультразвуку, як основні ультрасонографічні симптоми стеатозу печінки, визначені практично в усіх хворих на неалкогольну жирову хворобу печінки. Низка симптомів (неоднорідність ехоструктури печінки середньо- та крупнозернистого характеру, заокругленість нижнього краю печінки, нерівність контуру печінки), частота яких більшою мірою пов'язана з ураженістю запальних, а також фіброзних змін, зареєстрована частіше у пацієнтів зі стеатогепатитом і цирозом печінки, ніж зі стеатозом. Виявлені погіршення візуалізації дрібних гілок печінкових вен унаслідок згладженості судинного малюнка та його збіднення, збільшення селезінки в розмірах, поряд із розширенням селезінкової вени, у хворих на цироз печінки, розглядались як компонент портальної гіпертензії та були наслідком фібротичної трансформації печінки. Відсутність кореляції ступеня фіброзу з рівнем трансаміназ підтверджує низьку діагностичну значимість цих показників. Помірний прямий кореляційний зв'язок окремих ультрасонографічних показників печінки зі ступенем фіброзу вказує на можливість використання цього методу для скринінгу неалкогольної жирової хвороби печінки.

Ключові слова: стеатоз печінки; неалкогольний стеатогепатит; аланінамінотрансфераза; аспартатамінотрансфераза; ультразвукове дослідження

Вступ

Неалкогольна жирова хвороба печінки – загальне захворювання, яке невпинно зростає в усьому світі. Реальна поширеність її невідома, оскільки неалкогольна жирова хвороба печінки часто не діагностується, й у більшості пацієнтів, навіть хворих із діабетом, лікарі не підозрюють патологію печінки через нормальний рівень трансаміназ (Chalasani, 2012; Gaggini, 2013). Дані щодо поширеності неалкогольної жирової хвороби печінки коливаються від 6,3% до 33,0% (Ong & Younossi, 2007; Chalasani, 2012; Loomba & Sanyal, 2013). Цей показник збільшується до 57% у пацієнтів з ожирінням, до 70% – у пацієнтів із діабетом і до 90% – в осіб із важким ожирінням (Gastaldelli, 2007). Неалкогольна жирова хвороба печінки вражає чоловіків більше, ніж жінок. Порівняно з європейцями латиноамериканці мають вищу поширеність, тоді як у чорношкірих вона нижча порівняно з обома. Однак в азіатів неалкогольна жирова хвороба печінки проявляється за нижчих індексів маси тіла, багато з пацієнтів не мають резистентності до інсуліну (Ong & Younossi, 2007; Gastaldelli, 2007; Chalasani, 2012; Loomba & Sanyal, 2013).

Неалкогольна жирова хвороба печінки включає дві патології: стеатоз та неалкогольний стеатогепатит. Стеатоз характеризується як ожиріння печінки без значного запалення та зазвичай вважається доброякісним і оборотним, з мінімальним ризиком прогресування цирозу або печінкової недостатності. На відміну від стеатозу, для неалкогольного стеатогепатиту характерне запалення та пошкодження печінки у вигляді балонування гепатоцитів і клітинного некрозу. Оскільки неалкогольний стеатогепатит – важча стадія неалкогольної жирової хвороби печінки, ризик прогресування цирозу, печінкової недостатності та гепатоцелюлярної карциноми вищий, але незначно (Farrell & Larter, 2006; Bellentani & Marino, 2009; Loomba & Sanyal, 2013).

Патогенез неалкогольної жирової хвороби печінки активно вивчається в усіх розвинутих країнах. Інсулінорезистентність – найпоширеніший ключовий механізм, що веде до стеатозу печінки, а згодом – до стеатогепатиту. Багато дослідників підтримує гіпотезу двох ударів, для прогресування стеатозу до неалкогольного стеатогепатиту, і в кінцевому підсумку до цирозу печінки. Початковий перший удар – накопичення жиру в гепатоцитах, яке зазвичай відбувається за макровезикулярним типом. Більшість авторів вважають, що це пов'язано з метаболічними факторами: резистентністю до інсуліну, абдомінальним ожирінням та дисрегуляцією метаболізму жирних кислот. Другий удар – оксидативний стрес, що вважається причиною запалення та розвиненого фіброзу. Цей процес передбачає активацію ферментів цитохрому P_{450}, які сприяють генерації окисного стресу, перекисному окисненню ліпідів та дисфункції мітохондрій (Paschos & Paletas, 2009; Liu et al., 2015).

Останніми роками запропоновано складніші гіпотези «багаторазових ударів» із метою пояснення впливу метаболізму жирних кислот, через декілька послідовних або паралельних цитотоксичних шляхів, на прогресування неалкогольної жирової хвороби печінки. Окрім ліпотоксичності, опосередкованої жирними кислотами, ці гіпотези також розглядають вплив резистентності до інсуліну, гормонів жирової тканини, харчових факторів, мікробіоти кишечника, генетичних та епігенетичних чинників (Berlanga et al., 2014; Buzzetti et al., 2016; Hansen et al., 2017). Вважається також, що накопичення заліза в гепатоцитах відіграє роль пошкоджувального фактора, спричиненого оксидативним стресом. Це можна вважати можливим поясненням того, чому чоловіки хворіють частіше, ніж жінки, які мають менший загальний запас заліза в організмі через менструації та, як правило, дієту з меншим умістом заліза порівняно з чоловіками (Shim, 2012; Machado & Cortez-Pinto, 2014).

Макровезикулярне відкладення жиру – початкова патологічна ознака за неалкогольної жирової хвороби печінки. Згодом, під час розвитку патології, спостерігається картина гепатоцелюлярного пошкодження, відома як балонна дистрофія. Ця клітинна дегенерація спрямовує запальні клітини (наприклад, нейтрофіли) до паренхіми органа. У свою чергу, запалення запускає механізм репарації, що включає відкладання білків у міжклітинному матриксі. Прогресія фіброзу супроводжується патологічними змінами, які виглядають як перисинусоїдальний / перицелюлярний фіброз. У результаті поширення фіброзу у поєднанні з утратою гепатоцитів розвивається печінково-клітинна недостатність. З прогресом фіброзу та зменшенням кількості гепатоцитів ступінь запалення зрештою знижується та розвивається цироз (Takahashi & Fukusato, 2014; Sharma et al., 2015; Magee et al., 2016).

Для неалкогольної жирової хвороби печінки характерний безсимптомний перебіг. Деякі пацієнти можуть скаржитися на неспецифічні симптоми (нездужання, втома та дискомфорт у правому підребер'ї). Найпоширеніша клінічна ознака – абдомінальне ожиріння та гепатомегалія. У пацієнтів із цирозом можуть виникати асцит або симптоми портальної гіпертензії (Brunt & Tiniakos, 2009; Abd El-Kader & El-Den Ashmawy, 2015). Діагноз, як правило, підозрюється на основі випадкових знахідок підвищених ферментів печінки під час скринінгових лабораторних досліджень, що дає підстави для подальшого обстеження (Bayard et al., 2006).

Діагноз неалкогольної жирової хвороби печінки вимагає підтвердження стеатозу візуалізаційними або гістологічним методами та виключення інших причин стеатозу та хронічного захворювання печінки (відсутність значного вживання алкоголю, вірусні гепатити, хвороба Вільсона, аутоімунні захворювання та ятрогенні пошкодження печінки). Проведення біопсії печінки суперечливе на ранніх стадіях захворювання, однак на пізніх стадіях необхідне для встановлення ступеня тяжкості пошкодження печінки на основі гістологічного дослідження, що визначає тактику лікування (Sharma et al., 2015; Magee et al., 2016).

Аналіз крові у багатьох випадках недостатній для діагностики, тому що рівень печінкових трансаміназ може бути нормальним за наявності неалкогольної жирової хвороби печінки (Shim, 2012). Аланінамінотрансфераза (АЛТ) та аспартатамінотрансфераза (АСТ) присутні у гепатоцитах і виділяються у кровоток у відповідь на пошкодження або смерть гепатоцитів. Обидва ферменти характерні для різних типів тканин, але АЛТ вважається більш специфічною для печінки. АСТ може бути чутливішим показником пошкодження печінки за алкогольного та, у деяких випадках, аутоімунного гепатитів (Newsome et al., 2018). На ранніх стадіях захворювання зміни АСТ і АЛТ – найпоширеніша аномалія, причому обидва ферменти підвищені у співвідношенні АСТ/АЛТ менше ніж 1 (Mofrad et al., 2003). Підвищення гаммаглутамілтранспептидази корисне для підтвердження печінкового походження підвищення АЛТ. Цей показник найчастіше підвищується

внаслідок ожиріння, надмірного споживання алкоголю та наркотиків. Незважаючи на те, що підвищений рівень гаммаглутамілтранспептидази має низьку специфічність, це один із найточніших показників смертності від патології печінки (Donnan et al., 2009). Рівні лужної фосфатази та тригліцериди також можуть бути підвищеними. Такі показники як білірубін, альбумін та міжнародне нормоване відношення, як правило, перебувають у межах норми, якщо хвороба не прогресувала до розвинутого фіброзу чи цирозу (Charatcharoenwitthaya et al., 2012).

Нині тільки біопсія печінки дає можливість точно відрізнити стеатоз, неалкогольний стеатогепатит і фіброз на ранніх стадіях. Роль біопсії полягає, перш за все, у визначенні ступеня захворювання, а саме фіброзу, активності запалення та стеатозу. Вона також необхідна у випадках, коли діагноз піддають сумніву. Це найкорисніше для ідентифікації фіброзу, перш ніж хвороба прогресувала до розвинутої стадії та клінічних проявів, таких як портальна гіпертензія. Біопсія повинна призначатися пацієнтам із більшою ймовірністю прогресування неалкогольної жирової хвороби печінки, що визначається супутніми захворюваннями, такими як діабет, ожиріння, похилим віком, підвищениим сироватковим феритином або тим, що мають постійне підвищення трансаміназ, незважаючи на модифікацію способу життя. У хворих із тяжким перебігом хвороби – як допоміжний засіб для визначення необхідності трансплантації печінки. Незважаючи на те, що біопсія в цілому безпечна, можуть виникати біль та ускладнення (кровотеча), що вимагає чіткого обґрунтування її проведення та згоди пацієнта (Chalasani, 2012; Shim, 2012).

Клінічне значення неалкогольної жирової хвороби печінки та обмеження біопсії збільшили потребу в точних і неінвазивних методах візуалізації для оцінювання структурних змін печінки. Нині вони включають ультрасонографію, комп'ютерну томографію, магнітно-резонансну томографію та магнітно-резонансну спектроскопію (Lee & Park, 2014; Lee et al., 2016; Berzigotti et al., 2018). Стеатоз печінки, виявлений комп'ютерною томографією, зазвичай діагностується випадково (КТ-сканування, призначене з інших причин). Комп'ютерна томографія має підвищену специфічність для виявлення інших причин захворювання печінки, але її застосування обмежене високою вартістю (Bayard et al., 2006). Проте цей метод корисний для оцінювання стеатозу під час обстеження кандидата-донора печінки. Висока просторова роздільна здатність комп'ютерної томографії, напевне, необхідна для оцінювання стану судин донорської печінки, включаючи печінкову артерію, портальну та печінкову вени за умов контрастування (Lee, 2017). Магнітно-резонансна томографія – найточніша для визначення кількісного вмісту жиру у печінці, але вона дорого коштує та доступна тільки у великих медичних установах. Як і інші методи візуалізації, обмежується неможливістю диференціювати стеатоз, стеатогепатит і навіть пізні стадії фіброзу або цирозу (Lee et al., 2016; Berzigotti et al., 2018). Ультразвукове дослідження вважається дослідженням першого ряду в діагностиці неалкогольної жирової хвороби печінки, що пояснюється загальною доступністю, низькою вартістю, вищою чутливістю, ніж комп'ютерна томографія. Ультразвуковим зображенням стеатоз печінки визначається як неоднорідність та гіперехогенність паренхіми печінки внаслідок накопичення ліпідів, стертість судинного малюнка. Ультразвукова діагностика не визначає вміст жиру, але може застосовуватись для розрізнення м'якого та розвиненого ураження печінки (Lee & Park, 2014).

Мета цієї статті – оцінити валідність біохімічних показників функціонального стану печінки та ультразвукового дослідження за допомогою діагностики неалкогольної жирової хвороби печінки.

Матеріал і методи досліджень

Використано результати лабораторних досліджень і клінічні дані 122 хворих на неалкогольну жирову хворобу печінки віком 19–74 років, середній вік – 45,1 ± 11,9 років, які проходили обстеження та лікування у відділенні захворювань печінки та підшлункової залози ДУ «ІГ НАМН України» протягом 2010–2016 років. Серед них 57 жінок (46,7%) середнім віком 47,8 ± 9,6 років та 65 чоловіків (53,3%) середнім віком 42,7 ± 13,3 років.

Діагностику неалкогольної жирової хвороби печінки здійснювали за загальноприйнятими критеріями з обов'язковим виключенням вживання алкоголю. Обстежених розподілили на три групи: I група – 15 хворих на стеатоз печінки, II група – 92 пацієнти зі стеатогепатитом та III група – 15 хворих на цироз печінки неалкогольної етіології. Серед біохімічних показників цитолітичного синдрому вивчали активність аланінамінотрансферази, аспартатамінотрансферази у сироватці крові. Холестатичний синдром характеризували вміст лужної фосфатази, гаммаглутамілтранспептидази, вміст загального білірубіну та співвідношення його фракцій, Х-ліпопротеїдів. Білково-синтетичну функцію печінки оцінювали за вмістом загального білка, альбуміну, фібриногену, міжнародного нормалізованого відношення.

Ультразвукове дослідження структури печінки проводили на апараті SH-2000 Honda Electronics в реальному масштабі часу натще. Аналіз структурних змін містив оцінювання розмірів, контурів, акустичної структури та ехогенності печінки, стан біліарної системи загальноприйнятим методом. Особливу увагу приділяли таким ультразвуковим характеристикам: дистальне затухання ультразвуку, змінення ехогенності, візуалізації судин, заокругленість краю печінки.

Обробку результатів здійснювали у пакеті Statistica 6.0 (StatSoft Inc., USA). Оскільки більшість даних мали нормальний розподіл, використовували показники параметричної статистики. Вірогідність різниці між вибірками оцінювали за t-критерієм Стьюдента, розбіжності вважали вірогідними за $P < 0,05$. Усі кількісні показники наведені у вигляді $x \pm SD$, де x – середнє арифметичне, SD – середньоквадратичне відхилення. Кореляційний аналіз проводили за допомогою коефіцієнта кореляції Пірсона.

Результати

Аналіз клінічної картини показав, що абдомінальний біль превалював у пацієнтів всіх груп і виявлявся в 11 (73,3%) хворих на стеатоз, у 63 (68,5%) пацієнтів зі стеатогепатитом і у 8 (53,3%) хворих із цирозом. Переважала локалізація болю у правому підребер'ї, що може бути наслідком гепатомегалії та розтягування капсули печінки, а також дисфункціональних біліарних порушень. Крім цього, з достовірною перевагою серед осіб зі стеатозом печінки виявляли такі симптоми як біль у лівому підребер'ї, частота яких склала 53,3%. Також майже у третини всіх хворих траплялися біль в епігастральній ділянці (33,3%, 29,3% та 20,0% для I, II та III груп, відповідно) (рис. 1). Наявність такого болю, ймовірно, зумовлена супутніми патологіями (хронічний гастродуоденіт, хронічний холецистит, хронічний панкреатит).

Таким чином, абдомінальний біль однаково часто траплявся в усіх хворих, в основному за рахунок болю у правому підребер'ї. Значимо більша частота болю в лівому підребер'ї у групі зі стеатозом, можливо, зумовлена супутньою гастродуоденальною та панкреатичною патологією.

Аналогічно больовому абдомінальному синдрому, відчуття важкості у правому підребер'ї як прояв гепатомегалії та дисфункціональних біліарних розладів зазначалося рівномірно в усіх групах: за стеатозу в 5 (33,3%) випадках, за стеатогепатиту – у 35 (38%) та у 5 (33,3%) – за цирозу. Досить високу частоту скарг на гіркоту в роті та здуття живота відмічали в усіх хворих, із незначним переважанням у групі зі стеатозом печінки (рис. 2). Цей факт свідчив про вираженість біліарної диспепсії за неалкогольної жирової хвороби як наслідок дисфункціональних розладів жовчовивідних шляхів. У той же час, частота інших проявів диспепсії у спостережуваних хворих значуще не різнилася між групами.

Результати біохімічних досліджень активності АЛТ показали, що в усіх хворих цей показник перевищував дані контрольної групи. При цьому активність АЛТ у хворих I (68,2 ± 6,2 од./л) і III груп (82,8 ± 16,4 од./л) достовірно вища, ніж у здорових людей (25,4 ± 9,8 од./л), що свідчить про підвищення проникності мембран гепатоцитів та їх руйнування. Достовірних відмінностей між

групами хворих не виявлено. Аналізуючи ступінь активності АЛТ, визначили мінімальну активність у 3 (20%) та високу в одному (6,7%) випадку у групі зі стеатозом. У хворих зі стеатогепатитом підвищення АЛТ характеризувала у 49 (53,3 %) – мінімальну, у шести (6,5%) – помірну та у чотирьох (4,3%) – високу активність; у групі з цирозом: у восьми (53,3%) – мінімальну та по одному (6,7%) випадку помірної та високої активності.

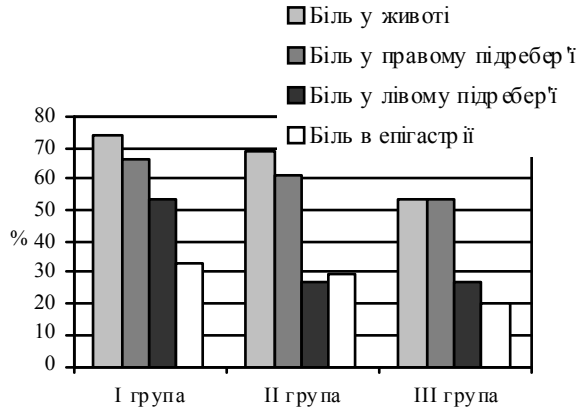

Рис. 1. Особливості больового абдомінального синдрому в обстежених хворих (%)

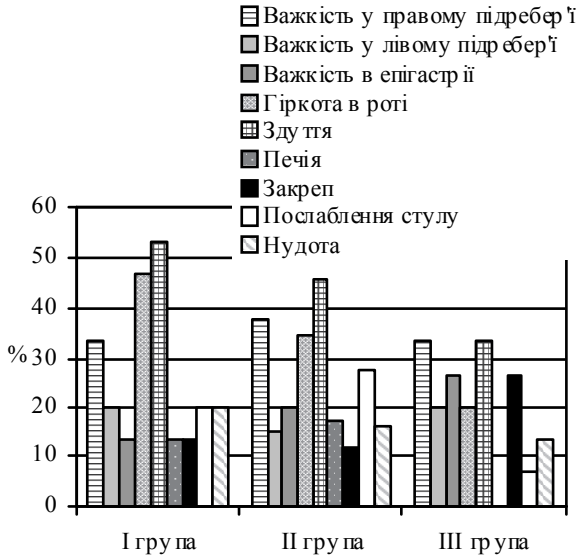

Рис. 2. Особливості диспепсичного синдрому в обстежених хворих (%)

Активність АСТ мала подібні тенденції, проте достовірні відмінності встановлені між пацієнтами з цирозом (64,5 ± 16,4 од./л) і стеатозом (32,4 ± 2,7 од./л), стеатогепатитом (46,6 ± 3,8 од./л) і контрольною групою (19,6 ± 7,4 од./л). Серед особливостей холестатичного синдрому виявлені гіпербілірубінемія (переважно за рахунок збільшення непрямої фракції) у хворих III групи. Достовірність відмінностей виявлена як з контрольною, так і з I та II групами (39,7 ± 8,5 проти 12,2 ± 0,7 та 15,7 ± 1,0 мкмоль/л, відповідно). Цьому факту не суперечить і значимо вища активність гаммаглутамілтранспептидази у пацієнтів із цирозом (228 ± 62,1 од./л), ніж за стеатозу (42,6 ± 5,7 од./л) та стеатогепатиту (85,5 ± 24,9 од./л). Активність лужної фосфатази незначно зростала в групі із цирозом (116,6 ± 17,6 од./л), що підтверджувало наявність холестазу, але без достовірних відмінностей між групами та контролем. Гіпота гіперпротеїнемія як прояви гепатоцелюлярної недостатності та імунозапального синдрому, визначені в поодиноких випадках у хворих II та III груп, не мали достовірних відмінностей від контрольного значення. При цьому визначено достовірно нижчий рівень альбуміну в III групі (табл. 1).

Гепатомегалія виявлена у більшості хворих I (95,7%) та в усіх хворих II та III груп. Переважно збільшення печінки визначене за рахунок правої частки, без достовірних відмінностей між групами. Товщина лівої частки також мала тенденцію до збільшення, з достовірною різницею між групами зі стеатозом і цирозом печінки. Товщина хвостатої частки зберігалась у межах норми у більшості хворих, із достовірністю відмінностей між групами зі стеатогепатитом і цирозом печінки. Такі симптоми як підвищена ехогенність печінки та дистальне згасання ультразвуку показані з високою частотою (60–100%) в усіх хворих на неалкогольну жирову хворобу (табл. 3). Ці ознаки визнають як основні ультрасонографічні симптоми стеатозу печінки. З іншого боку, низка симптомів (неоднорідність ехоструктури печінки середньо- та крупнозернистого характеру, заокругленість нижнього краю печінки, нерівність контуру печінки), частота яких більшою мірою могла бути пов'язана з вираженністю запальних, а також фіброзних змін, навпаки, реєструвалися дещо частіше у пацієнтів зі стеатогепатитом і цирозом, ніж зі стеатозом.

Таблиця 1
Показники функціонального стану печінки обстежених хворих (x ± SD)

Показник	I група n = 15	II група n = 92	III група n = 15	Контроль n = 20
АЛТ, од./л	51,9 ± 14,8	68,2 ± 6,2*	82,8 ± 16,4**	25,4 ± 9,8
АСТ, од./л	32,4 ± 2,7	46,6 ± 3,8	64,5 ± 16,4**,',''	19,6 ± 7,4
Білірубін загальний, мкмоль/л	12,2 ± 0,7	15,7 ± 1,0	39,7 ± 8,5**,',''	13,2 ± 2,2
Білірубін прямий, мкмоль/л	2,9 ± 0,2	3,8 ± 0,3	14,8 ± 5,5**,',''	4,1 ± 0,6
Лужна фосфатаза, од./л	98,6 ± 17,2	125,2 ± 27,8	116,6 ± 17,6	78,6 ± 33,5
Гаммаглутамілтранспептидаза, од./л	42,6 ± 5,7	85,5 ± 24,9	228,0 ± 62,1**,',''	39,7 ± 18,2
Х-ліпопротеїди, ммоль/л	3,15 ± 2,81	3,08 ± 1,43	3,35 ± 3,08	2,13 ± 0,81
Білок загальний, г/л	73,5 ± 6,6	74,9 ± 7,2	72,3 ± 6,2	75,5 ± 8,6
Альбумін, г/л	57,3 ± 1,4#	47,2 ± 0,5	36,6 ± 1,1**,',''	59,1 ± 5,1
МНВ	1,10 ± 0,03	1,15 ± 0,02	1,34 ± 0,08',''	1,10 ± 0,13
Фібриноген, г/л	2,9 ± 0,25	3,16 ± 0,11	4,06 ± 0,51''	2,7 ± 0,8

Примітки: P < 0,05 – достовірність розходжень між показниками II групи хворих і контрольною групою (*), III та контрольною групою (**), II та III груп ('), III та I груп (''), I та II груп (#) за t-критерієм Стьюдента.

Результати комплексного сонографічного дослідження органів черевної порожнини у хворих на неалкогольну жирову хворобу печінки показали характерні відмінності у групі хворих із цирозом (табл. 2).

Таблиця 2
Ультрасонографічні особливості розмірів печінки (x ± SD)

Показник	I група n = 15	II група n = 92	III група n = 15
Товщина правої частки печінки, мм	161,5 ± 9,9	156,7 ± 17,4	160,5 ± 20,2
Товщина лівої частки печінки, мм	76,7 ± 9,9	78,0 ± 15,9	85,9 ± 12,6*
Товщина хвостатої частки, мм	32,5 ± 6,8	32,8 ± 6,4	38,6 ± 9,7**
Гепатомегалія, n (%)	15 (100)	88 (95,7)	15 (100)

Примітки: * – P < 0,05, достовірність розходжень між показниками III та I груп за t-критерієм Стьюдента; ** – P < 0,05, достовірність розходжень між показниками III та II груп за t-критерієм Стьюдента.

Подібним співвідношенням характеризувалися зміни ангіоархітектоніки печінки (діаметр ворітної вени, візуалізація печінкових вен). Погіршення візуалізації дрібних гілок печінкових вен через згладженість (меншу виразність) судинного малюнка та його збіднення виявляли достовірно частіше за цирозу (80,0%), ніж за стеатогепатиту (47,8%). У 60 хворих виконано черезшкірну трепанбіопсію печінки під ультразвуковим контролем. Процедуру про-

водили під місцевою анестезією. Ускладнень, які вимагали оперативної корекції, не спостерігали. У 5 (8,3%) пацієнтів відмічено короткочасний біль в області пункції, слабкість, запаморочення, що не потребували медикаментозної корекції. В одному випадку (1,7%) больовий синдром після біопсії купірували внутрішньом'язовим уведенням анальгетика. Для аналізу отриманого матеріалу використовували систему E. M. Brunt (Brunt & Tiniakos, 2010).

Результати морфологічного дослідження показали відсутність фіброзу у 24 (40,0%), слабкий фіброз (F_1) мав місце у 23 (38,3%), помірний (F_2) – у 5 (8,3%), виражений (F_3) – у 4 (6,7%), цироз (F_4) виявлено у 4 (6,7%) хворих (рис. 3, 4). У більшості хворих визначено мінімальний (A1) та помірний (A2) ступінь запального процесу та помірний ступінь жирової дистрофії (S2) (рис. 5).

За даними морфологічного дослідження, у 8 (13,3%) хворих виявлено простий стеатоз печінки, у 4 (6,7%) цироз і у 48 (80,0%) стеатогепатит, з яких 26 (54,2%) з мінімальною, 19 (39,6%) – з помірною та 3 (6,3%) – з високою активністю.

Таблиця 3

Акустична характеристика печінки в обстежених хворих

Показник	I група n = 15	II група n = 92	III група n = 15
Підвищення ехогенності, n (%)	12 (80)	87 (94,6)	15 (100)
Дистальне згасання ультразвуку, n (%)	15 (100)	84 (91,3)	9 (60)*
Нерівність контуру, n (%)	1 (6,7)	2 (2,2)	7 (46,7)*
Закруглений край, n (%)	7 (46,7)**	79 (85,9)	13 (86,7)
Неоднорідність структури			
Дрібнозерниста, n (%)	8 (53,3)	54 (58,7)	3 (20)*
Середньозерниста, n (%)	2 (13,3)**	33 (35,9)	5 (33,3)
Крупнозерниста, n (%)	1 (6,7)	0	6 (40)*
Зміни ангіоархітектоніки печінки			
Воротна вена, мм (x ± SD)	12,0 ± 1,1	12,3 ± 1,4	14,2 ± 1,7
Згладженість судинного малюнка, n (%)	6 (40)	44 (47,8)	12 (80)*

Примітки: * – P < 0,05, достовірність розходжень між показниками III та I і II групами за t-критерієм Стьюдента; ** – P < 0,05, достовірність розходжень між I та II і III групами за t-критерієм Стьюдента.

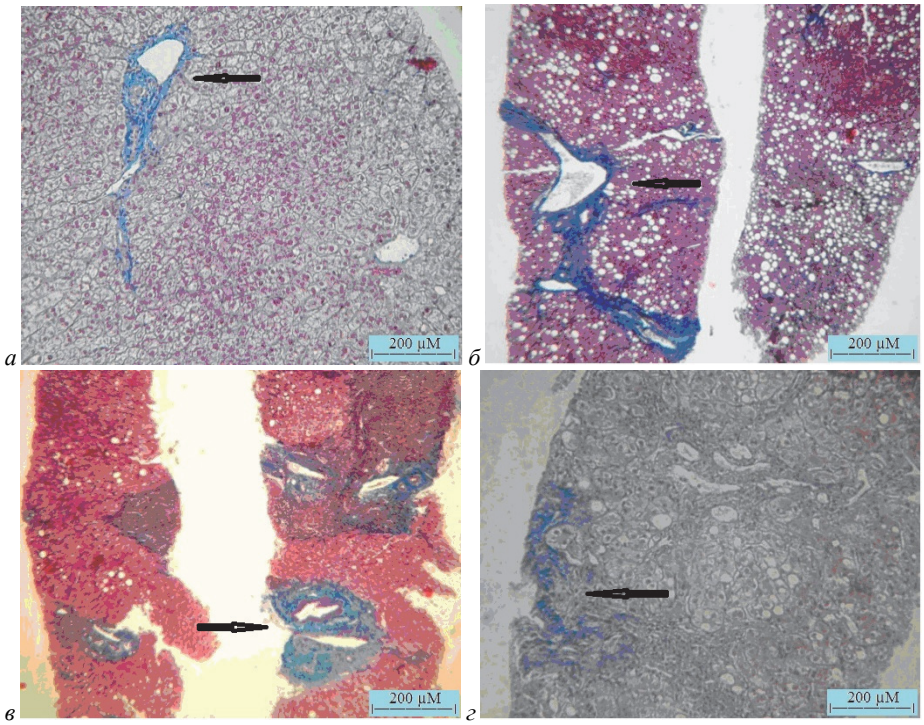

Рис. 3. Стадії фіброзу за E. M. Brunt: *а* – хворий З., ступінь фіброзу F_1; *б* – хвора В., ступінь фіброзу F_2; *в* – хворий В., ступінь фіброзу F_3; *г* – хворий Д., ступінь фіброзу F_4; забарвлення за Маллорі в модифікації Слінченко

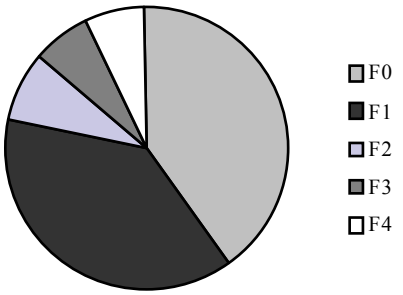

Рис. 4. Результати морфологічного дослідження: ступінь фіброзу F

Таблиця 4

Рівні печінкових трансаміназ залежно від стадії фіброзу

Показник	Ступінь фіброзу				
	F0 n = 24	F1 n = 23	F2 n = 5	F3 n = 4	F4 n = 4
АЛТ, од./л	67,7 ± 15,8	54,8 ± 7,3	109,2 ± 49,7	94,9 ± 32,7	66,8 ± 26,7
АСТ, од./л	42,7 ± 9,7	35,3 ± 3,4	53,0 ± 16,5	56,0 ± 6,5*	90,7 ± 38,1**

Примітки: * – P < 0,05, достовірність розходжень між показниками F_1 та F_3; ** – P < 0,05, достовірність розходжень між показниками F_1 та F_4.

За результатами кореляційного аналізу не виявлено зв'язку між ступенем фіброзу, стеатозу, активністю запального процесу та активністю печінкових трансаміназ, а достовірні відмінності щодо показників АЛТ і АСТ спостерігали між слабким (F_1) і вираженим (F_3) фіброзом і слабким фіброзом (F_1) і цирозом печінки (F_4) (табл. 4).

Стадія фіброзу безпосередньо корелювала з частотою низки показників ультрасонографії печінки: підвищена ехогенність (r = 0,56, P = 0,04), неоднорідність ехоструктури печінки (r = 0,58, P = 0,04), зниження візуалізації печінкових вен (r = 0,64, P = 0,01), які можна розглядати як клінічні еквіваленти фіброзу.

Обговорення

Таким чином, для хворих на неалкогольну жирову хворобу печінки характерна наявність цитолітичного синдрому через підвищення активності трансаміназ, до того ж, відмічали вищу активність АЛТ, ніж АСТ. Подібні тенденції спостерігали у хворих усіх груп, але зміни були значнішими у хворих на цироз печінки. Підвищення концентрації загального білірубіну (переважно за рахунок збільшення непрямої фракції) та активності гаммаглутамілтранспептидази у пацієнтів із цирозом порівняно зі хворими на стеатоз і стеатогепатит указує на розвиток внутрішньо-печін-

кового холестазу, що пояснюється порушенням структури печінки через фіброзну трансформацію. Підвищення фібриногену, визначене у групі з цирозом, показує дисбаланс у системі гемостазу: схильність до гіперкоагуляції та ризик утворення тромбів.

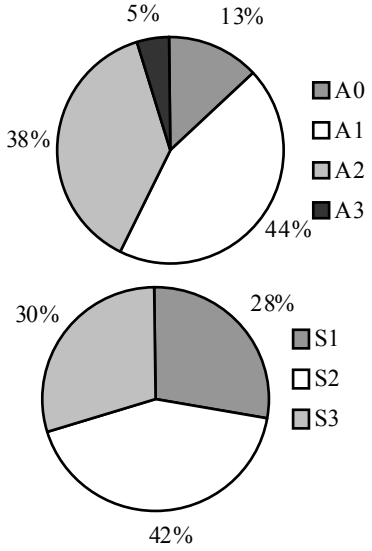

Рис. 5. Результати морфологічного дослідження: ступінь активності запального процесу (A) та стеатозу печінки (S)

У цілому, активність трансаміназ характеризувала цитоліз гепатоцитів, характерний для неалкогольної жирової хвороби печінки. Також мінімальна активність АЛТ та АСТ у групі зі стеатозом, підтверджує дані, що рівень трансаміназ не завжди відображає морфологічну активність запального процесу в печінці (Shim, 2012).

У дослідженні 440 осіб, пацієнти з нормальним та підвищеним показником АЛТ мали подібну тяжкість неалкогольної жирової хвороби. Це свідчить, що рівні амінотрансфераз плазми – недосконалі параметри для її діагностики (Maximos et al., 2015). Навпаки, у статті корейських вчених (Lee et al., 2017) активність АЛТ визначена як незалежний предиктор розвитку тяжкого фіброзу. Також у дослідженні, проведеному в Іспанії, за даними регресійного аналізу, рівень АЛТ у сироватці крові визначений як головний показник неалкогольної жирової хвороби (незалежно від статі, віку, індексу маси тіла та окружності талії). Крім того, крива ROC мала AUC 0,93 і показала, що значення АЛТ у сироватці крові ≥23 од./л передбачало наявність неалкогольної жирової хвороби печінки з чутливістю 0,94 та специфічністю 0,72 (Martin-Rodriguez et al., 2017).

Діагностична значущість ультразвукового дослідження щодо виявлення стеатозу змінюється залежно від наявності супутньої патології печінки. У пацієнтів без супутнього захворювання печінки цей метод пропонує достатньо точну діагностику середньоважкого стеатозу печінки, з показником чутливості 81,8–100,0%, а специфічність 98,0%. На відміну від цього, ультразвукове дослідження менш точне за діагностики стеатозу печінки, коли враховували всі його ступені (тобто ≥ 3% або 5%), із зареєстрованою чутливістю від 53,3% до 66,6% та специфічністю від 77,0% до 93,1% (Lee et al., 2010). Оскільки печінковий фіброз також збільшує ехогенність печінки, наявність хронічного захворювання цього органа знижує точність ультразвукового дослідження за діагностики стеатозу. Наприклад, одне дослідження, яке включало пацієнтів із гепатитом С (Hepburn et al., 2005), виявило, що ультразвукове дослідження мало чутливість 60% та специфічність 73% у процесі виявлення середньоважкого ступеня стеатозу печінки.

Наше дослідження показало, що такі симптоми як підвищена ехогенність печінки та дистальне згасання ультразвуку (основні ультрасонографічні симптоми стеатозу печінки) характеризувалися високою частотою в усіх хворих на неалкогольну жирову хворобу печінки. Низка симптомів (неоднорідність ехоструктури печінки середньо- та крупнозернистого характеру, заокругленість нижнього краю, нерівність контуру), частота яких більшою мірою пов'язана з вираженістю запальних, а також фіброзних змін, виявлені незначно частіше у пацієнтів зі стеатогепатитом і цирозом, ніж зі стеатозом. Погіршення візуалізації дрібних гілок печінкових вен внаслідок згладженості судинного малюнка та його збіднення, збільшення селезінки в розмірах, поряд із розширенням селезінкової вени у хворих на цироз печінки розглядалися як компонент портальної гіпертензії та були наслідком фібротичної трансформації печінки.

Висновки

Відсутність кореляції ступеня фіброзу з рівнем трансаміназ підтверджує низьку діагностичну значимість цих показників. Навпаки, виявлений у наведеному дослідженні помірний прямий кореляційний зв'язок окремих ультрасонографічних показників печінки зі ступенем фіброзу вказує на можливість використання цього методу для скринінгу неалкогольної жирової хвороби печінки.

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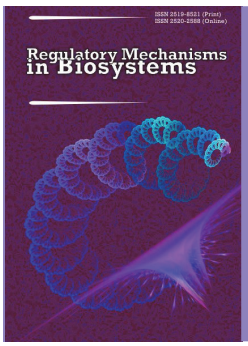

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 112–117
doi: 10.15421/021816

Clostridium perfringens in foods and fish

A. D. J. Cortés-Sánchez

Consejo Nacional de Ciencia y Tecnología (CONACYT), Centro de Investigaciones Biológicas del Noroeste, Unidad Nayarit (UNCIBNOR+).Tepic, Nayarit, México

Article info

Received 14.02.2018
Received in revised form 27.02.2018
Accepted 28.02.2018

Consejo Nacional de Ciencia y Tecnología (CONACYT), Centro de Investigaciones Biológicas del Noroeste, Unidad Nayarit (UNCIBNOR+), Calle Dos No. 23, Cd. del Conocimiento, Av. Emilio M. González, Cd. Industrial, C.P. 63173, Tepic, Nayarit, México.
E-mail: alecortes_1@hotmail.com

Cortés-Sánchez, A. D. J. (2018). Clostridium perfringens in foods and fish. Regulatory Mechanisms in Biosystems, 9(1), 112–117. doi: 10.15421/021816

Foodborne diseases are considered an important public health problem at a global level due to their levels of incidence and mortality as well as their negative consequences in economic and social aspects. Foodborne diseases are defined as those that are generated by the ingestion of food and water contaminated by chemical or biological agents commonly affecting health at the level of the gastrointestinal system. Among the risks and dangers to health from food are the diseases caused by *Clostridium perfringens*, a common bacterium which inhabits water, soil, vegetables, fish, the gastrointestinal system of human and animals and of course foods. The importance of this bacterium in health and food lies both in its cosmopolitan distribution, ability to generate heat-resistant spores and food poisoning, which makes control and prevention actions indispensable along the food chain. This article presents a general description of foodborne diseases, including those caused by consumption of food, such as fish, derived from contamination by *C. perfringens*; likewise, the actions and recommendations undertaken around the world for the prevention and control of these diseases are shown, including aspects related to the antimicrobial resistance phenomenon and its impact on public health.

Keywords: foodborne diseases; food safety; microorganisms; gastrointestinal symptoms

Foodborne diseases generalities

Foodborne diseases (FD) are those caused by the ingestion of food and water contaminated by microorganisms or chemical substances, the most common clinical manifestation being the appearance of gastrointestinal symptoms (WHO, 2018). These diseases are considered an important public health problem at a global level due to their incidence and mortality together with the negative economic and social repercussions (Palomino & Gonzalez, 2014; Lorenzo & Gálvez, 2015).

Estimates from the World Health Organization (WHO) indicate that annually around the world FD cause 420,000 deaths, of which one third are of children; while for the Americas region they indicate that around 77 million people fall ill and more than 9,000 die from these diseases (WHO, 2018a). In the United States alone, it is estimated that around 76 million people suffer from FD, 325,000 are hospitalized and 5,000 die each year, implying high health services costs (Olea et al., 2012). Factors such as the mobility of populations, growing urbanization, new forms and industries of production and sale of food, changes in eating habits, the globalization of the market and the lack of knowledge or perception of risk to health by all those involved in the chain of food production and service have contributed to a higher rate of contamination and incidence of diseases in consumers (Olea et al., 2012; Palomino & Gonzalez, 2014; Lorenzo & Gálvez, 2015).

Food due to its composition in water and nutrients is a favorable environment for microbial growth and in turn constitutes a vehicle for the transport of different diseases. Furthermore, if warm environmental conditions are present, microbial growth can be further favored by unhygienic practices during the handling of products; pathogenic microorganisms can be transferred to the surface of food from many sources (soil, water, insects, food handlers), which generates health risks for the consumer (Barbosa & Bermudez, 2010). Food contamination can be widespread and varied, with approximately 250 causal agents of diseases described; these contaminants can be of physical, chemical and biological origin, among which are included bacteria, viruses, fungi, parasites, prions, toxins and metals that can compromise the consumer's health; where contamination by biological agents (bacteria, fungi, viruses and parasites) represents the highest incidence and risk to health (Barreto et al., 2010; Olea et al., 2012; PAHO, 2016). Among the bacteria generally involved in the production of diseases through food are *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Clostridium perfringens*, *Campylobacter jejuni*, *Salmonella* spp., *Shigella* spp., among others (USDA, 2013).

This article presents a general description of foodborne diseases, including those caused by food consumption, such as fish deriving from contamination by microbial agents such as *C. perfringens*; likewise, the actions and recommendations for the prevention and control of these diseases are shown; in addition to showing the incidence of the phenolmenon of antimicrobial resistance by this pathogenic bacterium.

The genus *Clostridium*

The genus *Clostridium* is formed by around 150 species, which are both phylogenetically and phenotypically heterogeneous. Some species are of importance in various industrial areas, such as *C. acetobutylicum*, *C. beijerinckii* and *C. butirycum*, since they produce organic solvents (Castaño, 2008), *C. pasteurianum* are involved in deterioration in storage stability of food and juices that undergo inadequate thermal processes (Feng et al., 2010) while species such as *C. botulinum*, *C. perfringens*, *C. tetani* and *C. difficile* are of clinical importance and must be considered pathogenic to food, giving rise to diseases by action of extracellular toxins (Rhodehamel & Harmon, 2001; Morris & Fernandez, 2008; El-Shorbagy et al., 2012; García et al., 2016). The bacteria of

the genus *Clostridium*, are characterized by having a vegetative form in the form of bacilli. They are gram positive, anaerobic, do not form pili and fimbriae, have peritrichous flagella except for *C. perfringens* and form spores and toxins. They are located generally in nature and commonly as a constituent part in the gastrointestinal system of animals, spreading through food, thus generating food intoxication (Romero, 2007; Elika, 2013). The species associated with diverse pathologies in humans, including the alimentary toxi infections (*C. difficile*, *C. novyi*, *C. septicum*, *C. histolyticum*, *C. sordelli*, *C. fallax*, *C. botulinum* and *C. perfringens*); the toxins of the latter affect the nervous and gastrointestinal system respectively (Romero, 2007; Elika, 2013; OPS, 2016).

Clostridium perfringens

C. perfringens is a bacterium belonging to the family Bacillaceae in the form of a bacillus measuring: 4–8 μm x 0.3–1.5 μm, Gram positive, capped, non-mobile, sporulated, it is relatively aerotolerant, catalase negative, fermented carbohydrates (glucose, lactose, sucrose, fructose, maltose and galactose) with production of acid and carbon dioxide, produce H_2S and hydrogen, its optimal growth temperature is 37–45 °C and it tolerates NaCl concentrations of 2%, is anaerobic but able to grow at values of Eh of +350 mV and reduce its environment to less than –400 mV, it has a cosmopolitan distribution in the environment mainly in soil, wastewater, spices, vegetables, raw foods, processed foods and in the gastrointestinal system of man and animals (Pascual & Calderón, 2000; Morris & Fernández, 2008; Massoc, 2008; Santos & Heredia, 2011; Gamboa et al., 2011; El-Shorbagy et al., 2012; CDC, 2017). Being a microorganism sporulated under unfavorable conditions or growth stress in its vegetative forms, it generates heat-resistant spores as forms of survival, whose process is linked to the production of toxins (Pascual & Calderón, 2000; Morris & Fernandez, 2008; Massoc, 2008; El-Shorbagy et al., 2012). A high correlation has been established between the ability of *C. perfringens* strains to produce toxins and generate food poisoning and conditions such as necrotic enteritis and gas gangrene (Rhodehamel & Harmon, 2001; El-Shorbagy et al., 2012). The ability of *C. perfringens* to generate spores by sulfite-reducing clostridia, being inhabitants of the gastrointestinal system of mammals and resistant to different conditions of environmental stress, makes them useful as indicators of fecal contamination of water for human use and consumption which can put health at risk (Rios et al., 2017).

Food poisoning by *C. perfringens* tends to be self-limiting, symptoms appearing suddenly such as watery diarrhea, severe abdominal pain and cramping 8 to 16 hours after ingestion lasting approximately 24 hours. The vast majority of outbreaks of disease are related to inadequate handling, preparation and preservation of food, such as temperature control, especially in meat and meat products (Perdomo & Melendez, 2004; Massoc, 2008; CDC, 2017). It is estimated that *C. perfringens* is capable of generating toxinfection through ingestion in foods with inocula above 10^5 to 10^8 CFU/g (Pascual & Calderón, 2000; Santos & Heredia, 2011; PAHO, 2016). The population in general is susceptible to poisoning by this pathogen but children under 5 years of age, older adults, pregnant women and people with weakened immune systems are at higher risk of *C. perfringens* infection and experience more severe symptoms (CDC, 2017).

The virulence factors attributable to *C. perfringens* include the capacity to generate different toxins. Among those one usually finds the toxin alpha (α), beta (β), epsilon (ε) and iota (ι) that are used for the bacterial classification of the species into five toxinotypes from A to E according to the toxins produced where the type A produces (α), B produces (α), (β) and (ε), C produces (α) and (β), D produces (α) and (ε) and E produces (α) e (ι) (Morris & Fernandez, 2008; Gamboa et al., 2011; El-Shorbagy et al., 2012). This microorganism also generates different enzymes hydrolytics: lecithinases, hemolysins, hyaluronidases, collagenases, DNAase and amylases (Santos & Heredia, 2011). It also presents the synthesis of other protein toxins that also contribute to virulence but are not considered for their classification, such as CPE enterotoxin encoded by the cpe gene responsible for diarrhea in humans and animals, the NetB associated with necrotic enteritis in birds and β_2 toxin, related to enteritis cases (Morris & Fernandez, 2008; Gamboa et al., 2011). Foodborne diseases that have *C. perfringens* as a causative agent are commonly related to type A strains, while necrotic enteritis is caused by type C and some type A strains (Santos & Heredia, 2011). According to estimates from the Centers for Disease Control and Prevention of the United States of America (CDC), approximately one million cases of foodborne diseases caused by *C. perfringens* are recorded annually, it being one of the most common causative agents of food poisoning (CDC, 2017). Reporting food poisoning by *C. perfringens* is not mandatory in this country. However, the centers for disease control and prevention estimate 250,000 cases per year due to food poisoning due to *C. perfringens* type A (Santos & Heredia, 2011).

***C. perfringens* and fish**

Fish is considered an important source of food due to its easy digestibility, high nutritional value due to its composition of water, proteins, lipids, vitamins, minerals and carbohydrates, besides representing a source of economic and living income for millions of people around of the world (FAO, 1998, 2016; Sheyin & Solomon, 2017). However, these same characteristics make this food a highly susceptible source of deterioration and decomposition by enzymes of the fish itself (autolysis) and microorganisms, converting them into a vehicle of diseases when consumed (Avdalov, 2009). The microbiote present in fish is related to various factors such as: the nature of the water, temperature, food, season of year from which they are extracted, among others. Human activities have had a detrimental effect on water, so there may be a risk of contamination with pathogenic microorganisms from it, as well as by those activities involved in capture, cultivation (aquaculture), processing and conservation actions, such as for example the use of poor quality water, poor hygiene practices in handling, while fish processing facilities which are contaminated can be contaminated by chemical or biological agents (García & Calvario, 2008; Avdalov, 2009; Sheyin & Solomon, 2017). *C. perfringens* being a cosmopolitan microorganism can be located in diverse environments such as the soil, aquatic ecosystems, gastrointestinal system of animals and fish, as well as raw and processed foods (Matches et al., 1974; Kimura, 1996; Santos & Heredia, 2011; Sheyin & Solomon, 2017). This microorganism has been implicated in outbreaks of food-borne diseases that are related to the consumption of meat and derivatives, including fish; in the United States of North America in the period from 1998 to 2008, 2468 cases of foodborne diseases were reported, in which there were 461 cases of this pathogen being identified as the causative agent (Kimura, 1996; El-Shorbagy et al., 2012; Painter et al., 2013; CDC, 2017). The cases of foodborne diseases that have *C. perfringens* as a producing agent are related to inadequate storage, processing and food service operations, preferably in places that concentrate large groups of people such as hospitals, school canteens, prisons and old people's homes, or in events with food service (Kimura, 1996; El-Shorbagy et al., 2012; CDC, 2017). The ubiquitous nature of this microorganism and its spores make it a frequent problem and challenge for the food industry and establishments that produce large amounts of food (Santos & Heredia, 2009).

Isolation of *C. perfringens* in foods and fish

For the isolation and detection of *C. perfringens* in food, there are several standardized methods in the scientific collection, such as the one developed by the International Organization for Standardization (ISO) ISO 7937:2004 for the colony count of *C. perfringens* (Fig. 1), the method reported by Rhodehamel & Stanley (2001), presented in the Bacteriological Analytical Manual (BAM) of the Food and Drug Administration (FDA) of the United States of America (Fig. 2) is also the method reported by McNamara & Lattuada, (1998) presented in the microbiology laboratory guide of the United States Department of Agriculture (USDA) and the Food Safety and Inspection Service (FSIS) (Fig. 3). All these methods present some similarities in conditions and culture media used for isolation, quantification, and biochemical confirmation for the emission of results to *C. perfringens*. It should be noted that prior to the microbiological analysis, a core point of the method to be developed is actions that involve the collection and transport of the

sample, so it is recommended that the sample once collected for analysis should be transported immediately to the laboratory at temperatures 0–10 °C. The samples collected for analysis that cannot be analyzed immediately require special treatment; being sensitive, *C. perfringens* may lose viability in prolonged periods of refrigeration and freezing, so they should be treated with a buffered glycerin saline solution, stored or sent frozen to the laboratory (McNamara & Lattuada, 1998; Rhodehamel & Harmon, 2001).

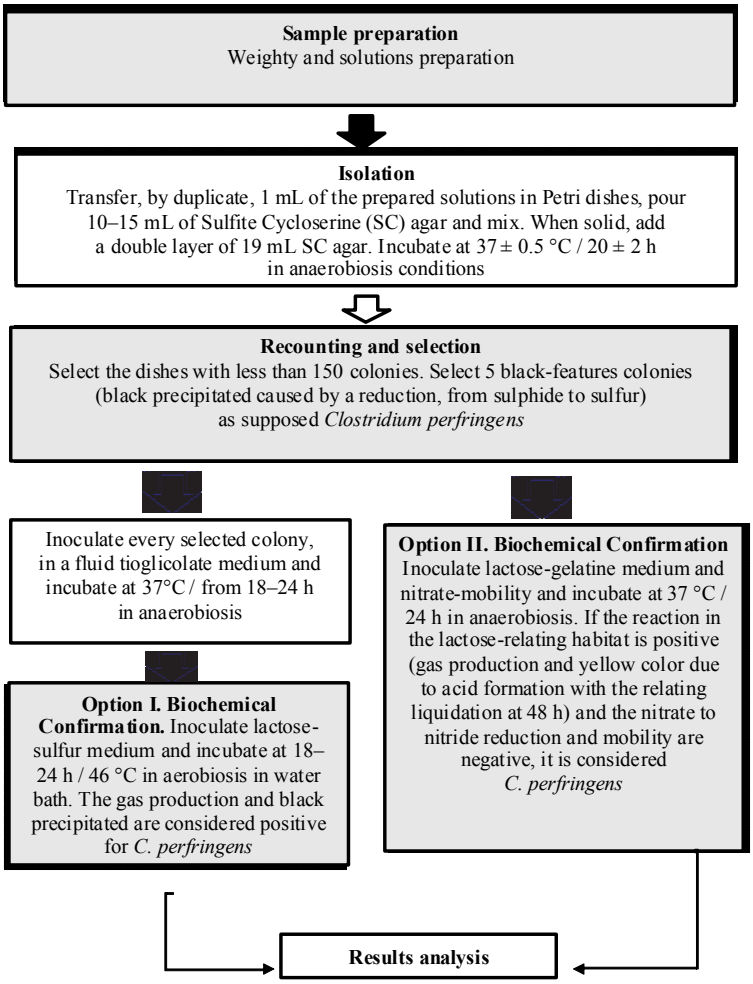

Fig. 1. Method for plate count of *Clostridium perfringens* in food samples (ISO 7937: 2004; ANMAT, 2013)

Control and prevention of foodborne diseases

Food poisoning by *C. perfringens* is commonly due to inadequate handling of the temperature in the processing, maintenance or reheating of food, as well as its preparation in advance of consumption (Santos & Heredia, 2011). Low levels of *C. perfringens* occur in raw meats, poultry, soups, dehydrated sauces, raw vegetables or spices during food preparation; this coupled with the heat resistance and oxygen that can be decreased during cooking processes favors the growth of bacteria; thus spores that survive cooking can germinate and grow rapidly in foods that do not undergo adequate refrigeration processes after the cooking treatment (Rhodehamel & Harmon, 2001; Santos & Heredia, 2011). On the other hand, *C. perfringens* generally loses its viability when food is frozen or kept in prolonged refrigeration; for analytical issues in the laboratory this characteristic can hinder isolation and detection in food as well as establish the causes of an outbreak of food poisoning (Rhodehamel & Harmon, 2001). The actions focused on the control and prevention of diseases by this pathogen in food are involved in the stages of production and storage of food in which the cooling of food must be below 10 °C, for two or three hours, and the preservation of hot foods above 60 °C prior to consumption. In the case of reheating cold or refrigerated food, these should reach a minimum internal temperature of 75 °C. Likewise, cross contamination with utensils and contaminated surfaces must be prevented (OPS, 2016).

Throughout the food chain, from primary production through industry to household manipulation prior to consumption, strategies and procedures have been developed, recommended and implemented in a global manner that contribute to minimize the health risks of the consumer, some of these are: good agricultural practices (GAP), good livestock or livestock practices, good fishing practices and aquaculture that contribute to reducing the contamination of food with soil and fecal animal matter, thus minimizing the bacterial load on the subject premium, as well as the application of good manufacturing practices (GMP) appropriate hygienic practices in the handling and processing, development and implementation of control systems based on Hazard Analysis and Critical Points (HACCP), compliance with the microbiological criteria of raw materials in order to reduce or prevent pollution by diverse microorganisms including strains of the genus *Clostridium* and finally, for home consumption, the World Health Organization (WHO) has developed informative handbooks aimed at the general population on hygiene conditions in the storage, handling and preparation of food in a healthy and safe way (WHO, 2007; García & Calvario, 2008; Ramírez & Ishihara, 2008; Avdalov, 2009; FAO, 2009; Elika, 2013; ANMAT, 2013; Gómez et al., 2015). On the other hand, other measures of control and prevention of contamination and deterioration of food include the use of chemical preservatives; in the food industry this is considered an alternative and common practice to reduce the pathogenic and deteriorating microbial population thus avoiding risks for the health and prolonging the life of the products; In different investigations around the world, effective results have been reported when combined with other barrier technologies against *C. prefringens* (Santos & Heredia, 2011).

At the international level, there are different limits or microbiological specifications established for the sanitary quality of various pro-

cesssed foods which are susceptible to contamination with *Clostridium* spp., Mexico has the official Mexican standard NOM-130-SSA1-1995 focused on packaged foods in hermetically sealed containers and subjected to heat treatment; indicating the sanitary dispositions and specifications in the microbiological character, such as the presence of anaerobic mesophiles that consist of bacteria of the genus *Clostridium*, among which are *C. sporogenes*, *C. putrificans*, *C. hystoliticum*, *C. bifermentans*, *C. perfringens* and *C. botulinum* of sanitary interest. For specific products such as formulas for infants, foods and non-alcoholic beverages for infants and young children, the Official Mexican Standard NOM-131-SSA1-2012, mentions the provisions, sanitary and nutritional specifications, as well as the labeling and testing methods including in the microbiological specifications the anaerobic mesophilic microorganisms. With respect to sanitary quality and safe consumption of food, products and fishing services, the Official Mexican Standard NOM-242-SSA1-2009 establishes sanitary specifications and test methods for fresh, chilled, frozen and processed fishery products, being the microbiological specification acceptable for *C. botulinum*, in fresh, chilled and frozen products as "absent" while the specification corresponding to the presence of sporulated anaerobic thermophiles and sporulated anaerobic mesophiles in commercially acceptable sterilized fishery products is "negative".

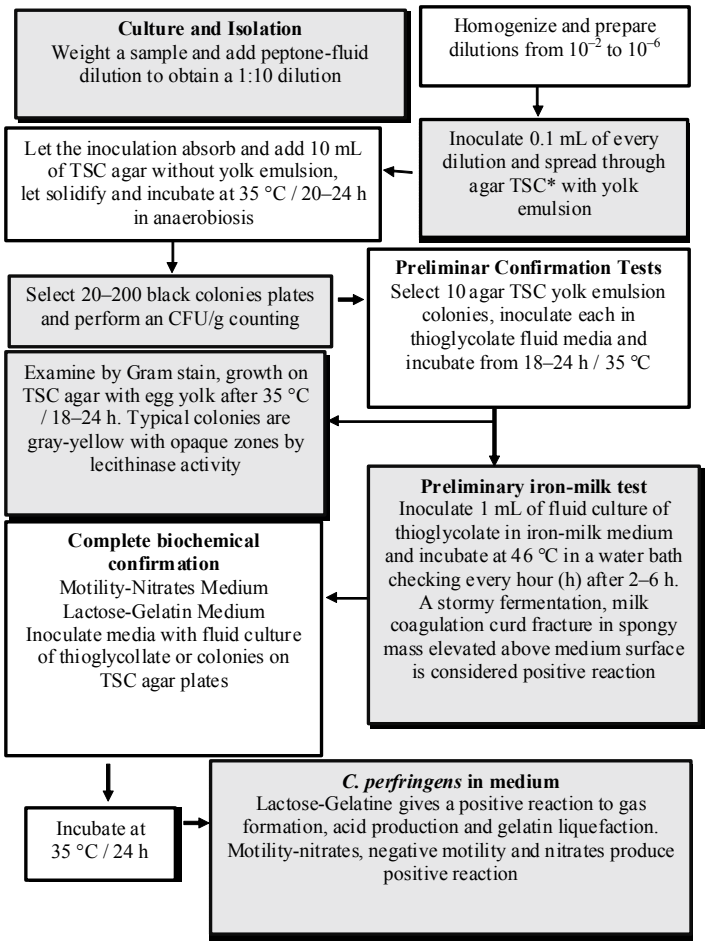

Fig. 2. Culture and isolation of *C. perfringens* from food (Rhodehamel & Harmon, 2001): * – Tryptose-Sulphite-Cycloserine Agar (TSC)

On the other hand, the Official Mexican Standard NOM-251-SSA1-2009, indicates the guidelines for hygiene practices for the process of food, beverages or food supplements; involving facilities, areas, equipment and utensils, services, storage, control of operations and raw materials, it also refers to the good practices of health and hygiene of the personnel, which also recommends the establishment of a system of hazard analysis and critical control points (HACCP) in order to prevent the generation of food that carries a risk to the health of the consumer. In Europe, on the part of the European Parliament and the Council of the European Union, Regulation (EC) No 178/2002 establishing the principles and general requirements of food legislation was established. European Food Safety Authority and procedures relating to safety to food safety are set; likewise by Commission Recommendation 2004/24/EC (DOCE 19/12/03) on the official control program for food products that recommends taking representative samples of spices at the level of import, production, packaging establishments, wholesale trade, establishments that use spices in food preparation and retail trade, in order to count *C. perfringens* with a microbiological limit of 100–1000 CFU/g (Elika, 2013).

Several studies have been carried out around the world that have reported the isolation and profile of resistance to different antimicrobials by pathogenic microorganisms (*C. perfringens*, *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*), among others which come from different natural environments such as soil, food, animals and human beings. These studies generate information for a more rational use of antibiotics in the areas of clinical, agriculture, livestock, fisheries and aquaculture, in addition to the detection of strains with antimicrobial resistance, which contributes to alert us about possible genetic transfer mechanisms in nature and generate strategies in control (Abraham et al., 2011; Gamboa et al., 2011; Granier et al., 2011; Puig et al., 2011; Slavic et al., 2011; Elhadi et al., 2014; Gharaibeh et al., 2014).

At global level, different actions have been developed and recommended in order to reduce the transmission of resistance to antimicrobials in the food chain and thus achieve food safety, among these actions are good manufacturing practices, sanitary control through of HACCP systems, good agricultural, livestock and aquaculture hygiene practices, in addition the Codex Alimentarius has issued guidelines such as CAC/GL 77-2011 for the risk analysis of antimicrobial resistance transmitted by food and the code of practice to minimize and contain antimicrobial resistance CAC/RCP 61-2005 (PAHO, 2015; FAO, 2017; FAO, 2018). The control of resistance to antimicrobials is a common task and, therefore, it is necessary that the participation of govern-

ments around the world with the support of the scientific community generate actions focused on control and surveillance in the agricultural and clinical field by implementing surveillance and control policies and systems for the use of antimicrobials and the phenomenon of resistance, all in favor of the availability to the world population of food of good in nutritional quality and safety (Padgett et al., 2011; Gestal et al., 2014).

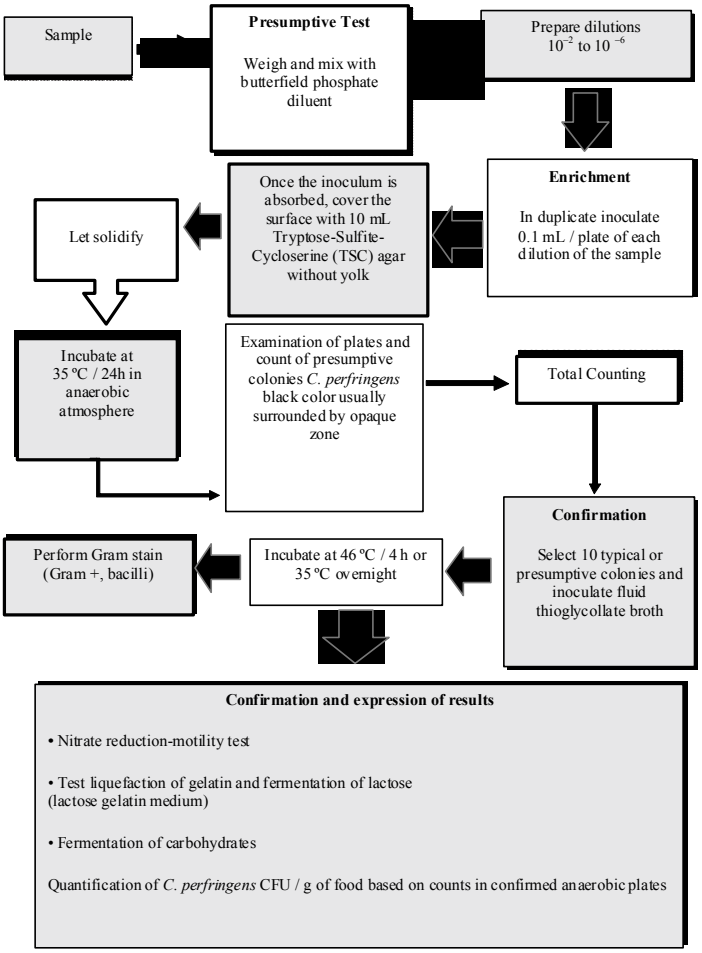

Fig. 3. Method of quantification of *C. perfringens* in foods (McNamara & Lattuada, 1998)

Conclusions

Food is a priority need for human beings to achieve survival, growth, development and for fulfillment of their daily activities. However, during the production of food and throughout the food chain there can be microbial contaminations and these can be vehicles of biological hazards to health, giving rise to different diseases. Foodborne diseases are considered to be of great relevance worldwide in public health aspects due to their incidence and mortality rate, together with the fact that several years ago the appearance and increase of resistance to antimicrobials was reported. These diseases have different causal agents thus potentiating their importance, given the negative repercussions on health, economy and society.

C. perfringens is considered a danger of biological origin along the food chain and is described as a threat to human and animal health, since through the contamination of food and subsequent consumption it generates negative effects on health. This is complicated by reports worldwide on the isolation of strains resistant to different antimicrobials. Different strategies, procedures and actions have been developed and implemented around the world through international organizations, the food industry, government and academia to control and prevent food contamination by food pathogens such as *C. perfringens*, in an effort to guarantee the supply of safe food to the general population in all stages that go from its primary production through its processing and transformation until its conservation, manipulation and preparation prior to its consumption.

Satisfying the growing demand for safe and nutritious food globally still requires a great common effort from the food industry, governments, and academia, including the general population, through their awareness of the different risks and dangers to health from food, handling and storage conditions for consumption in order to minimize the incidence of foodborne diseases and their impact on health.

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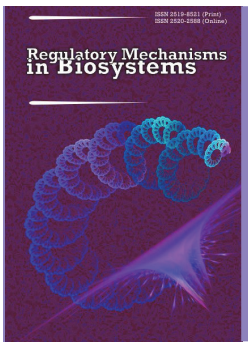


Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 118–123
doi: 10.15421/021817

The impact of certain flavourings and preservatives on the survivability of larvae of nematodes of Ruminantia

O. O. Boyko*, V. V. Brygadyrenko**

**Dnipro State Agrarian-Economic University, Dnipro, Ukraine*
***Oles Honchar Dnipro National University, Dnipro, Ukraine*

Article info

Received 03.01.2018
Received in revised form 29.01.2018
Accepted 31.01.2018

Dnipro State Agrarian and Economic University, Serhii Efremov st., 25, Dnipro, 49600, Ukraine.

Oles Honchar Dnipro National University, Gagarin Ave., 72, Dnipro, 49010, Ukraine. Tel. +38-050-939-07-88. E-mail: brigad@ua.fm

Boyko, O. O., & Brygadyrenko, V. V. (2018). The impact of certain flavourings and preservatives on the survivability of larvae of nematodes of Ruminantia. Regulatory Mechanisms in Biosystems, 9(1), 118–123. doi: 10.15421/021817

Annualy, helminthic diseases are one of the causes of economic losses in agriculture. Ruminantia are most often observed to be infected with nematodes of the gastrointestinal tract, including *Strongyloides papillosus* and the representatives of the Strongylida order. Identifying factors which could cause a decrease in the level of infection of agricultural animals with helminthiases would allow stock-raising facilities to regularly achieve high quality dairy and meat production in sufficient quantity. On the basis of this study, we determined the impact of flavourings and source materials approved for use in and on foods (isoamyl alcohol, isoamyl acetate, raspberry ketone, trilon B, methylparaben) on the survivability of larvae of *Strongyloides papillosus* and *Haemonchus contortus*, parasitic nematodes of Ruminantia animals. Among these substances, the lowest efficiency against the nematode larvae was found in isoamyl alcohol, isoamyl acetate and trilon B. A stronger effect was caused by methylparaben (for L_3 *S. papillosus* LD_{50} = 0.67 ± 0.04%, L_1 and L_2 *S. papillosus* – LD_{50} = 0.0038 ± 0.0008%, L_3 *H. contortus* – LD_{50} = 0.89 ± 0.15%). Minimum efficient dosage of the solutions was 10 g/l. Significant antihelminthic properties were manifested by raspberry ketone (for L_3 *S. papillosus* LD_{50} = 1.00 ± 0.72%, L_1 and L_2 *S. papillosus* – LD_{50} = 0.07 ± 0.06%, L_3 *H. contortus* – LD_{50} = 0.39 ± 0.26%). The results show that there is considerable potential for further studies on the antiparasitic properties of these substances against nematodes in the conditions of farming enterprises and agricultural complexes.

Keywords: *Strongyloides papillosus*; *Haemonchus contortus*; antiparasitic activity; flavouring agents; isoamyl alcohol; isoamyl acetate; raspberry ketone; Trilon B; methylparaben

Introduction

Throughout the world a daily struggle takes place against parasitic diseases of agricultural animals. These infestations cause significant economic damage to farming enterprises and large stock-raising facilities, which consequently fall short of their potential in meat and dairy production. The most common parasitic diseases are helminthiases (Vercruysse et al., 2001; Biffa et al., 2007; Van der Voorta et al., 2013; Boyko et al., 2016). Ruminantia in Ukraine and other countries of Europe are regularly observed to be infected with the following nematodes of gastrointestinal tract: Haemonchosis, Nematodirus, Trichostrongyloidosis, Chabertiosis, Bunostomosis, Protostrongylidoses, Strongyloides, etc. (Lindqvist et al., 2001; Bhutto et al., 2002). Some of the abovementioned parasites are related to the feeding of nematodes on the blood of mammal hosts. As a result, the biochemical indicators of the milk of cattle, sheep and goats change (Boyko et al., 2016). The quality of the meat products decreases as well. The helminths constantly release a significant number of toxins into the host's organism, causing intoxication (Faye et al., 2003; Cringoli et al., 2008).

In the struggle against helminthic diseases, therapeutic and preventive measures play a significant role. They are aimed at chemotherapy and chemoprophylaxis with the use of anthelminthic preparations. The most common (Ploeger et al., 1990; Kloosterman et al., 1996; Veneziano et al., 2004) preparations against helminths are broad-spectrum preparations on the basis of macrocyclic lactones (Ivermectin, Doramectin, Abamectin, etc), Benzimidazoles (Albendazole, Mebendazole, Fenbendazole). Modern veterinary medicine also applies alternative methods against helminths using herbaceous preparations. However, the extent of their impact is differently interpreted by various authors (Rahmann & Seip, 2006; Burke et al., 2009; Cheng et al., 2009; Lu et al., 2010; Chhetri et al., 2015; González-Coloma et al., 2017). For preventing helminthic infestations, it is recommended to cultivate the pastures, plough the land on their territories, provide a pen-system of grazing, conduct mechanical cleaning from bushes, rocks, drying, and taking measures against intermediate hosts and other methods. Our previous studies devoted to the impact of food additives on the survivability of the larvae of the nematode of pigs *Strongyloides ransomi* (Schwartz and Alicata, 1930), and eggs of *Ascaris suuum* Goeze, 1782 indicated positive results for certain flavourings, preservatives and other types of food additives used in the food industry. Therefore, a relevant issue today is determining their impact on other species of nematodes often found in agricultural animals (Boyko et al., 2017).

Materials and methods

In the summer of 2017, we collected faeces of Ruminantia on the territory of Dnipropetrovsk oblast of Ukraine to the amount of 100 g from every individual (n = 56). The material was transported in plastic containers at a temperature of 22–24 °C to the parasitological laboratory of Dnipro State Agrarian-Economic University. The samples with helminths for use in the experiment were

identified using the McMaster method. For the study, we selected third age larvae (L_3) of *Haemonchus contortus* (Rudolphi, 1803) from the Strongylida order and larvae of first, second and third age (L_1, L_2, L_3) of *Strongyloides papillosus* (Wedl, 1856) from the Rhabditida order (Van Wyk & Mayhew, 2013) (Fig. 1). For the experiment, the larvae were cultivated during 8 days at a temperature of 22–24 °C. The larvae material was collected using the Baermann test (Zajac & Conboy, 2011).

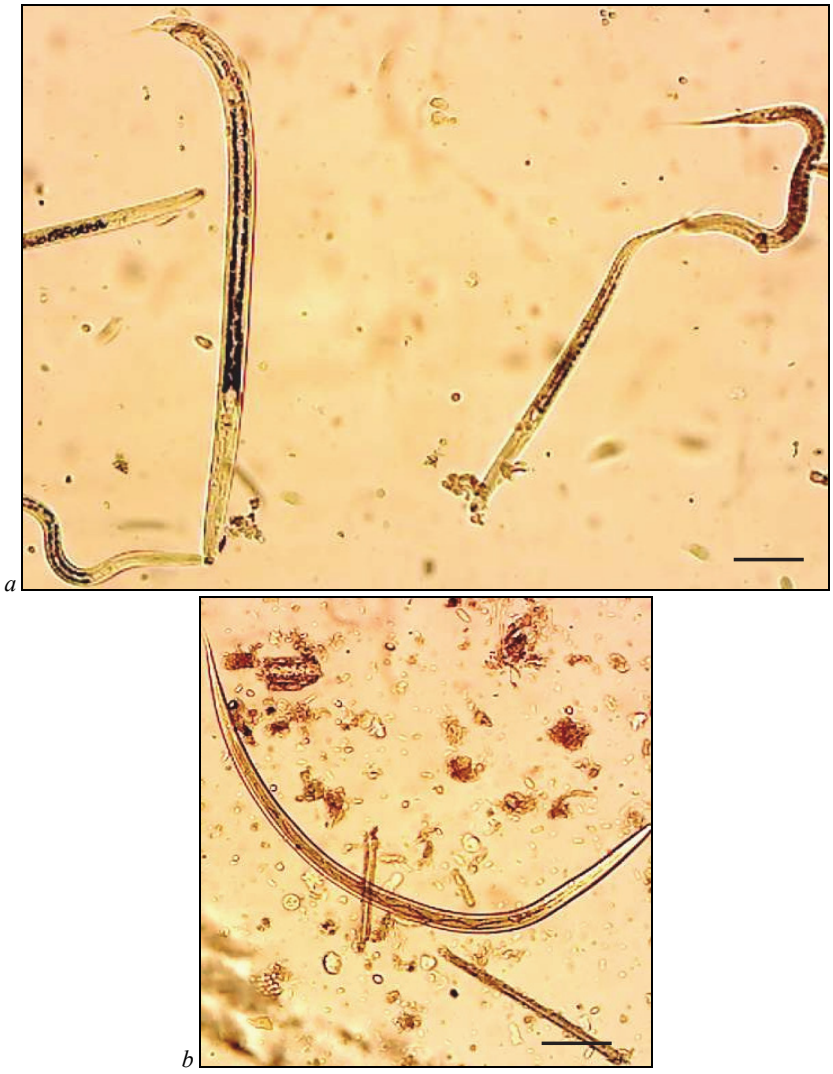
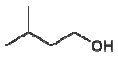
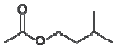
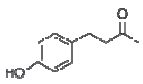


Fig. 1. *Strongyloides papillosus* (Wedl, 1856) of different ages (*a*) and *Haemonchus contortus* (Rudolphi, 1803) L_3 (*b*): bar – 10 μm

Table 1

Usage and properties of the flavourings and preservatives* used for determining the level of survivability of *Strongyloides papillosus* (Wedl, 1856) and *Haemonchus contortus* (Rudolphi, 1803) larvae

Substance name	Chemical formula	Structural formula	Properties	Content	Usage	
					in food industry	in medicine
Isoamyl alcohol (3-methylbu-tan-1-ol)	$C_5H_{12}O$		optically inactive colourless substance with unpleasant odour	fusel oils	used for preparing extractions with pleasant fruit odour	no data on usage
Isoamyl acetate (3-methyl-butyl acetate)	$C_7H_{14}O_2$		colourless substance with sharp pear odour	in some fruits	pear extraction for producing fruit water, caramel, etc.	no data on usage
Raspberry ketone (4-(4-hydroxyphenyl) butan-2-one)	$C_{10}H_{12}O_2$		colourless substance with citrus odour	in red raspberries	as a food additive with fruit flavour	used in cosmetology
Trilon B (2,2',2",2'"-(ethane-1,2-diyldinitrilo)tetra-acetic acid), E_{385}	$C_{10}H_{16}N_2Na_2O_8$		white crystalline powder or crystals of white colour	–	in food preservation, as antioxidant	in the production of medical preparations and in cases of heavy metal intoxication, in dentistry, as a preservative in eye preparations
Methylparaben, E_{218}	$C_8H_8O_3$		white crystalline substance with distinctive odour	in the roots of *Oxalis tuberose*	as a preservative	as an antiseptic

Note: * – properties of the substances are given according to Lide, 1980; Fahlbusch et al., 2002; Soni et al., 2002; Catalog of Organics and Fine Chemicals, 2004; Nomenclature of Organic Chemistry, 2014.

Sediment with larvae was obtained by centrifugation (4 minutes at 1500 circles per minute), which was put into 1.5 ml plastic test tubes in equal portions. Then 1 ml of 1.0% water solution of each tested substance was added to the larvae cultures (0.1 ml, 20–40 ind.). The exposure lasted 24 hours. The temperature regime in the thermostat was within 22–24 °C. The nematode larvae were affected by food additives from the group of flavourings, and also preservatives (the experiment used three concentrations of the tested substances: 1%, 0.01%, 0.0001%). Every variant of the experiment was repeated eight times. The laboratory studies were conducted using chemically pure isoamyl alcohol, isoamyl acetate, raspberry ketone, Trilon B, and methylparaben (Table 1).

The statistical analysis of the results was performed through a set of Statistica 8.0 (StatSoft Inc., USA), the figures is show the median, 25% and 75% quartiles, minimum and maximum values. LD_{50} (%) was calculated as average (x) ± standard deviation (SD).

Results

The results indicated a complete absence of anthelminthic properties in isoamyl alcohol (Fig. 2*a*) and isoamyl acetate. With exposure to isoamyl alcohol, we observed around 60% vital larvae of L_3 *S. papillosus* in 1% solution. Less resistant to the impact of isoamyl alcohol were L_1 and L_2 *S. papillosus*. The percentage of the surviving larvae of these two stages after 24 hours of exposure to 1% solution was 55%. With further solutions of isoamyl alcohol, 80% of *S. papillosus* larvae survived. Larvae of L_3 *H. contortus* were found to be the most resistant to different concentrations of this substance. At 0.0001–1% solution of this alcohol, 100% of them survived.

The next flavouring, isoamyl acetate, manifested the weakest influence on the mortality of nematode larvae of Ruminantia (Fig. 2*b*). In 1% solution, almost all larvae of *S. papillosus* and *H. contortus* survived. Similarly, at 0.01% and 0.0001% concentrations of this substance, most of the larvae of all studied nematode species survived.

1% solution of raspberry ketone caused 100% mortality only to L_1 and L_2 *S. papillosus*. 30–50% of L_3 *S. papillosus* and *H. contortus* survived in this concentration. In 0.01% solution of this substance, over 80% of the Ruminantia nematode larvae survived. 0.0001% concentration of raspberry ketone in 100% of cases did not affect the survivability of these parasites (Fig. 2*c*).

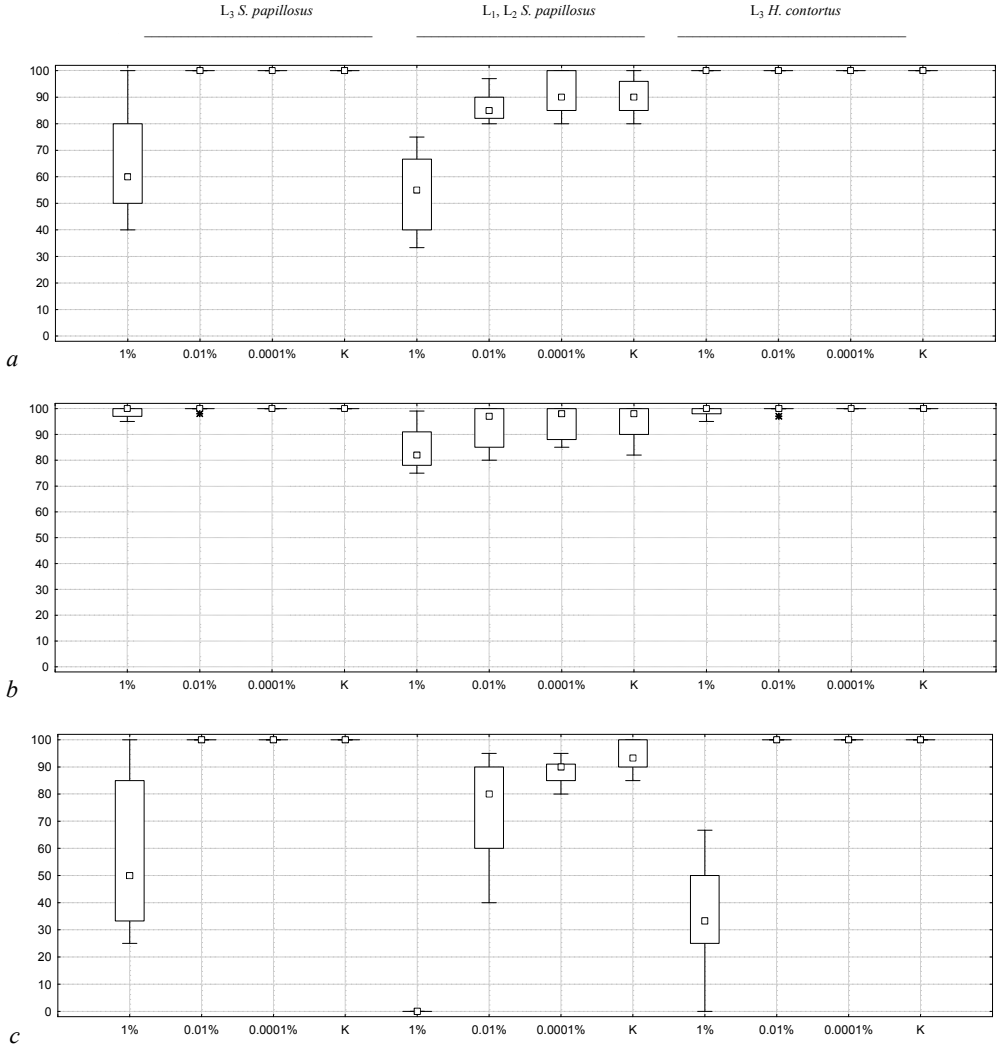


Fig. 2. The effect of flavourings on the survivability of Ruminantia nematodes: *a* – isoamyl alcohol, *b* – isoamyl acetate, *c* – raspberry ketone; the ordinate axis shows the percentage of nematode larvae which survived over the 24-hour experiment; the abscissa axis shows the concentration of the active substance in the solution (%), K – control, where the concentration of the active substance equals 0%; L_3 – invasive larvae of *S. papillosus* or *H. contortus*, L_1, L_2 – non-invasive larvae of *S. papillosus*; the small square in the center corresponds to the median, the lower and upper borders of the large rectangular correspond to the first and the third quartiles, respectively, vertical line segments, directed up and down from the rectangular, correspond to minimum and maximum values (n = 8)

The second stage of the experiment was determining the anthelminthic properties of some preservatives (trilon B and methylparaben). About 60% of L_1 and L_2 *S. papillosus* larvae died after 24 hours in 1% solution of trilon B. At the same concentration, all the rest of larvae survived in the percentage of 75–100%. The most resistant to 1% solution of trilon B were the larvae of *H. contortus*

(100% larvae survived). The next solutions of trilon B had no positive effects – all larvae survived (Fig. 3*a*). The solution of methylparaben in 1% and 0.01% concentrations caused death of non-invasive *S. papillosus* (Fig. 3*b*) larvae in 100% of cases. Invasive larvae of this species, similarly to *H. contortus* larvae are sensitive to methylparaben only at its maximum, 1% concentration of the active substance. The analysis of the study results indicated complete absence of anthelminthic properties in isoamyl alcohol and trilon B. Minimum LD_{50} (%) indicators for L_3 *S. papillosus* were registered for isoamyl acetate, raspberry ketone и methylparaben (Table 2).

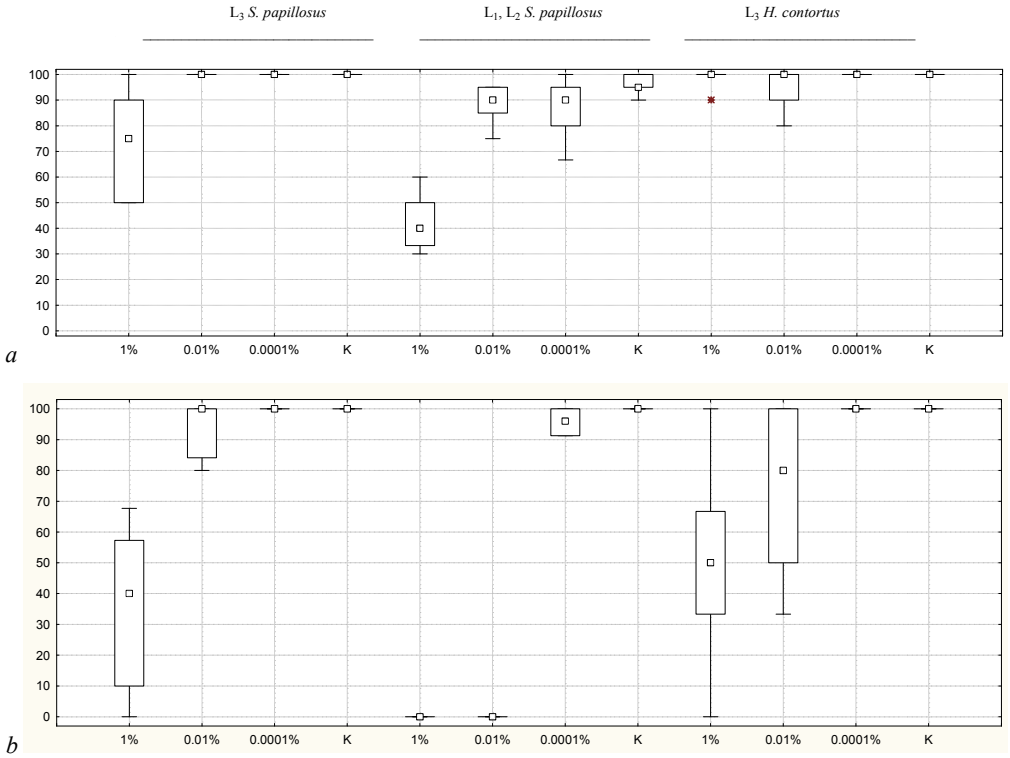


Fig. 3. The effect of trilon B (*a*) and methylparaben (*b*) on the survivability of Ruminantia nematode larvae: see notes to Fig. 1

Table 2
LD_{50} (%, x ± SD) for *S. papillosus* and *H. contortus* larvae in laboratory experiment during 24 hours

Substance	*S. papillosus*, L_3	*S. papillosus*, $L_1 + L_2$	*H. contortus*, L_3
isoamyl alcohol	–	–	–
isoamyl acetate	–	–	–
raspberry ketone	1.00 ± 0.72	0.07 ± 0.06	0.39 ± 0.26
trilon B	–	0.80 ± 0.46	–
methylparaben	0.67 ± 0.04	0.0038 ± 0.0008	0.89 ± 0.15

Discussion

Therefore, according to the results of our studies and analysis of the data from the literature, the additives used in the food industry can affect parasites, including nematode larvae of Ruminantia, in a certain concentration. Data on using food additives with the purpose of affecting the parasites are quite limited. Their impact on parasitic Acari and insects has been studied by Lee et al. (2008), Knoblauch and Fry (2011), Shen et al. (2012), Belkind et al. (2013) et al. Shen et al. (2012) indicate the significant effect of cinnamaldehyde against parasitic Acari *Psoroptes*. LD_{50} equals 107 mg/ml, (with 24 hours exposure) for Acari of this genus. Also, this food additive has been studied by Na et al. (2011) as an acaricide preparation against *Dermanyssus* of birds. LD_{50} for *Dermanyssus* sp. was 0.54 mg/ml (with 24-hour exposure). According to Lee (2004), p-anisaldehyde food additive is capable of acaricide properties. Other works are devoted to the impact of the food additive cinnamaldehyde on larvae of blood-sucking insects. LD_{50} for larvae of mosquitoes was 40.8 mg/ml. Taylor (2009) used benzyl alcohol against fleas and also indicated that this additive has insecticidal properties. Benzaldehyde has been proved to have an impact on insects. It was used against *Galleria mellonella* (Linnaeus, 1758). The authors of these studies, Ullah et al. (2015), recommended the additive for the compound of insecticidal preparations. Lee et al. (2008) have also used benzaldehyde (LD_{50} with 48-hour exposure – 0.004–0.200 mg/sm^2 against *Sitophilus oryzae* (Linnaeus, 1763) (Coleoptera, Curculionidae). Anthelminth properties of benzyl alcohol additive were proved by Chalquest (2002). Pedersen and Woldum (2011) recommend using it as solvent of preparations against parasites.

Food additives are often used as antimicrobial agents. Their impact on microorganisms has been studied by Chiang et al. (2005), Sato et al. (2006), Somolinos et al. (2008), Si et al. (2009), Belletti et al. (2010) and many other authors. Ribeiro et al. (2016) have studied antimicrobial, antifungi, and also insecticidal impact of 83 compounds from different tissues of *Ricinus communis*. Some of them are used as additives in the food industry. They include alkaloids, terpenoids, flavonoids, benzoic acid derivatives, coumarins, tocopherols, and fatty acids. The antimicrobial properties of cinnamaldehyde against *Escherichia coli* and *Salmonella enterica* were studied by Manu (2016). For obtaining antiseptic and fungicidal effect, E_{218}, a methylparaben preservative, is used (Shapiro et al., 2002; Posey et al., 2005; Kromidas et al., 2006; Rebbeck et al., 2006; Ishiwatari et al., 2007; Meyer et al., 2007; Gopalakrishnan et al., 2012). It is also used in the composition of insecticides (Bell, 1990). According to the results of our studies, this substance also affects other parasitic nematodes of Ruminantia.

The impact of ethylenediaminetetraacetic acid (EDTA or trilon B) on *Cryptococcus* has been studied by Lai et al. (2016). Currently, fungal diseases are difficult to treat, and such treatment is conducted using expensive preparations. Therefore, these authors' work was aimed at intensifying the effect of modern preparations by using them with ethylenediaminetetraacetic acid and other synergic agents for decreasing the therapeutic dose, increasing the efficiency and preventing development of *Cryptococcus* resistance. The results of our experiments indicated that usage of trilon B did not cause any death of parasitic nematodes. Our study also indicates a

high level of anthelminthic impact of methylparaben and raspberry ketone. These substances affect not only microorganisms and are used as a fungicide, but are also, according to the results of our tests, capable of having an effect on *S. papillosus* and *H. contortus*, parasitic nematodes of Ruminantia.

Conclusions

Additives used in the food industry are significant in the struggle against helminthiases of Ruminantia. Among the flavourings and source materials approved for use in and on foods, raspberry ketone and methylparaben are most efficient against nematode larvae. Minimum efficient dosage of solutions of these substances is 10 g/l or 1% solution.

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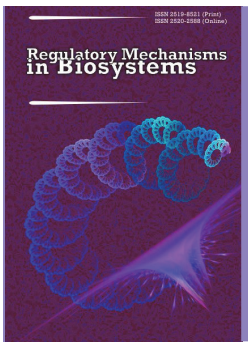

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1), 124–129
doi: 10.15421/021818

Violations of cell-molecular mechanisms of bone remodeling under influence of glucocorticoids

S. B. Pavlov, N. M. Babenko, M. V. Kumetchko, O. B. Litvinova

Kharkiv Medical Academy of Postgraduate Education, Kharkiv, Ukraine

Article info

Received 07.02.2018
Received in revised form 26.02.2018
Accepted 28.02.2018

Kharkiv Medical Academy of Postgraduate Education, Amosova st., 58, Kharkiv, 61176, Ukraine. Tel. + 38-057-711-80-48. E-mail: cndl@med.edu.ua

Pavlov, S. B., Babenko, N. M., Kumetchko, M. V., & Litvinova, O. B. (2018). Violations of cell-molecular mechanisms of bone remodeling under influence of glucocorticoids. Regulatory Mechanisms in Biosystems, 9(1), 124–129. doi: 10.15421/021818

The fact is disturbance of the processes of bone tissue remodeling leads to a change in the balance between synthesis and resorption of bone and the development of osteoporosis. The most common cause of secondary osteoporosis is the use of glucocorticoid therapy. The aim of this study is to investigate the cellular-molecular mechanisms of disturbance of the processes of bone remodeling regulation, reflected by hormones and intercellular mediators (for example parathyroid hormone, calcitonin, RANKL, osteoprotegerin, P-selectin, interleukin-17, transforming growth factor-β1, adiponectin and visfatin) on the background experimental glucocorticoid osteoporosis. The experimental study carried out in two groups of white female rats. Disturbance of bone tissue remodeling was verified by histological examination of the femoral head, vertebrae of the thoracic and lumbar spine of rats and the measurement of bone density. The study of the levels of hormones and intercellular mediators in the blood serum of animals was carried out by the method of enzyme immunoassay. The bone mineral density of the experimental group was reduced compared to the bone mineral density of the control group. The study of the histostructure of the femoral head and vertebrae in rats of the experimental group in comparison with the animals of the control group revealed changes in the structural organization of bone tissue, confirmed by histomorphometry, indicating inhibition of the processes of osteosynthesis. The article analyzes the nature of the involvement of hormones and cytokines in pathogenetic mechanisms of development of bone tissue disorders. The levels of cytokines RANKL, osteoprotegerin, interleukin-17 and calcitonin in the blood serum of animals of the group with the violation of bone tissue remodeling by glucocorticoids were higher than in intact animals. Serum levels of P-selectin, parathyroid hormone, transforming growth factor-β1, adiponectin and visfatin were lower than similar levels in animals from the control group. The use of glucocorticoids increases the expression of RANKL and inhibits the synthesis of osteoprotegerin, resulting in stimulation of bone resorption. The effect of glucocorticoids in the experimental model is realized by changing the production of the studied hormones, cytokines and adhesion molecules. These changes stimulate the apoptosis of osteoblasts and inhibit their proliferation and differentiation, which is another mechanism of bone loss. Correlations found during the study reflect the relationship in the system of regulation of bone tissue remodeling under the influence of glucocorticoids. A complex system for regulating bone remodeling, which includes many regulatory pathways and their interactions, requires further study.

Keywords: cytokines; hormones; glucocorticoids; remodeling of a bone tissue; adipokines

Introduction

When bone remodeling processes are disturbed, the balance between formation and resorption of bone tissue changes, controlled and coordinated by different types of bone cells. This often leads to the development and progression of osteoporosis, which, according to World Health Organization data, is the second most important health problem after cardiovascular disease (Klimova et al., 2014). Secondary osteoporosis refers to bone disorders, which are a secondary complication of various diseases. The most common cause of secondary osteoporosis is the consequences of glucocorticoid therapy. Glucocorticoids reduce bone density through several mechanisms: suppression of sex steroid hormones, inhibition of gastrointestinal absorption and renal calcium reabsorption, stimulation of parathyroid hormone secretion and inhibition of bone formation resulting from a change in the balance between osteoclasts and osteoblast activity (Kaneko et al., 2012). Glucocorticoids are capable of altering the proliferation and metabolism of bone cells (Brennan-Speranza et al., 2012). It is assumed that the development of glucocorticoid-induced osteoporosis occurs in two steps. In the initial, rapid steps of the use of glucocorticoids, bone loss occurs due to both a reduction in bone formation, and as a result of accelerated bone resorption. In the second, slower phase, the rate of osteoclast-mediated bone resorption slows down; the predominance is the suppression of bone formation (Canalis et al., 2007). Thus, with prolonged glucocorticoid therapy, the number of osteoclasts is usually maintained in the normal range, while the number of osteoblasts is significantly reduced, in contrast to postmenopausal osteoporosis, in which there is increased bone resorption (Weinstein, 2011). Apoptosis of osteoblasts, induced by the constant use of glucocorticoids, is identified as the main cause of osteoporosis, bone loss and fractures.

The process of bone remodeling is controlled by various local and systemic factors. Parathyroid hormone and calcitonin are some of the major hormonal regulators of bone resorption.

Parathyroid hormone is synthesized by parathyroid glands. Its main function is to maintain blood calcium homeostasis. The effect of parathyroid hormone is an increase in the concentration of calcium in the blood, a decrease in the calcium content in the bones (demineralization of the bone matrix) and a decrease in the phosphate content in the blood

plasma. Excess content of parathyroid hormone leads to a disorder of bone tissue, endocrine disease such as hyperparathyroidism, bone disease such as osteoporosis.

Calcitonin, which is a 32-amino acid hormone, is secreted by the cells of the thyroid gland. This hormone mainly acts, supplementing the function of parathyroid hormone, counteracting increased bone resorption caused by parathyroid hormone.

In addition to systemic hormonal regulation, a number of cytokines and growth factors are involved in bone remodeling. At the molecular level, bone resorption is regulated through the interaction of the receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) against the background of the permissive action of the macrophage colony-stimulating factor. RANKL functions as a key factor for osteoclastogenesis, since its binding to the RANK receptor (receptor activator of nuclear factor-κB) promotes the activation of osteoclasts and the resorption of bone tissue. Osteoprotegerin reduces RANKL-RANK interactions and thereby inhibits osteoclastogenesis (Remuzgo-Martínez et al., 2016). In this regard, the balance between RANKL and osteoprotegerin actually determines the amount of resorpted bone and the degree of change in bone mineral density.

It has been established that the RANKL/RANK/OPG-cytokine system that initiates osteoblasto- and osteoclastogenesis in bone tissue induces differentiation of osteoblasts and osteoclasts, as well as the process of mineralization of the vessel walls (Sage et al., 2010). RANKL and osteoprotegerin can be the molecular link between calcification of the arteries and bone resorption, which underlies the clinical combination of vascular disease and osteoporosis. In the regulation of bone remodeling, adipokines are also involved, affecting bone remodeling by suppressing intracellular osteogenic signals, while simultaneously promoting the secretion of adipogenic signaling molecules such as adiponectin and visfatin (Muruganandan & Sinal, 2014).

The aim of the study was to investigate the cellular-molecular mechanisms of disturbance of bone tissue remodeling regulation processes, as reflected by intercellular mediators (for example parathyroid hormone, calcitonin, RANKL, osteoprotegerin, P-selectin, interleukin-17, transforming growth factor-β1, adiponectin and visfatin) in experimental glucocorticoid osteoporosis.

Materials and methods

The experimental study was carried out in two groups of white female rats at the age of 9 months with a mass of 250 ± 30 g. First group is a group of animals with a violation of bone tissue remodeling caused by glucocorticoids – 18 rats, second group (control) – 10 rats. Creation of a model of experimental disturbance of bone tissue remodeling by glucocorticoids was carried out by administration of dexamethasone phosphate 6 mg/kg by weight intramuscularly twice a week for a month (Liu et al., 2011). Control group – intact animals.

The experiments were carried out in accordance with the principles of the "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and the "General ethical principles of animal experimentation" approved by the First National Congress on Bioethics (Kiev, 2001).

Disturbance of bone remodeling was monitored by direct measurement of bone density, which was calculated as the ratio of bone mass (grams) to its volume (centimeters cubic) (Podkovkin et al., 2008).

For histological examination, the femoral head, as well as the vertebrae of the thoracic and lumbar spine of the rats, were isolated. The material was fixed in 10% neutral formalin, decalcified in 5% nitric acid, encased in paraffin according to a conventional technique (Sarkisov & Perov, 1996). Sections 7–10 μm thick were stained with hematoxylin and eosin,van Gieson's picrofuchsin , analyzed in the field of view of the "Primo Star" microscope (Carl Zeiss). Photomicrographs of the preparations were performed using a Microocular digital camera.

Morphometric analysis of histopreparations was carried out using a light microscope LOMO (lens ×10, eyepiece ×8). The central sections were analyzed, in each individual case (at least three). In the area of the femoral head of animals with experimental osteoporosis, the severity of osteopenic manifestations was assessed for the following parameters: width of the cortex and thickness of the bone trabeculae (estimated with the help of an ocular screw micrometer MOV-1-16×), and also by the ratio of the volume of bone trabeculae to the total volume of spongy bones (conducted using the ocular-mesh insert G. G. Avtandilov (289 points of intersection)), guided by the method proposed by the author (Avtandilov, 1990). As a control, similar indices of intact animals were used.

The studies were carried out in the blood serum of animals by the method of enzyme immunoassay. The levels of parathyroid hormone, calcitonin and transforming growth factor-β1 were determined using DRG kits (Germany). To quantify the levels of RANKL, a set of reagents "ampli-sRANKL" Biomedica (Austria) was used. The levels of osteoprotegerin were determined using the Human Osteoprotegerin Instant eBioscience kit (Austria). P-selectin levels were determined using the Human sP-selectin Platinum ELISA kit eBioscience (Austria). A set of Vector-Best (Russia, Novosibirsk) was used to quantify interleukin-17 levels. Visfatin levels were determined using the RayBio kit (USA), adiponectin levels, using the BioVendor kit (Czech Republic).

The statistical processing of the results was carried out using the Statistica 6.0 analysis package using the Kruskal-Wallis non-parametric ANOVA criterion for independent samples and correlation analysis. The results are presented in the form x ± SE, where x is the arithmetic mean, SE is the standard error of the arithmetic mean. Statistically significant differences were considered at $P < 0.05$.

Results

The measured bone mineral density of the animal group with the violation of bone tissue remodeling with glucocorticoids was significantly decreased in comparison with the bone mineral density of the animals in the control group (1.37 ± 0.041 and 1.62 ± 0.059 g/cm³ respectively, $P < 0.05$). A review of the histological specimens of the vertebral bodies and the proximal femur in the rats of the control group showed a typical structure of bone tissue. The spongy bone was represented by wide anastomosing bone trabeculae, separated by intertrabecular spaces that contained red bone marrow. In the thickness of the beams, lacunae with osteocyte bodies were uniformly distributed; dark blue, slightly wavy cement lines were clearly contoured. The cortical layer, represented by a compact bone, was of sufficient width all along.

A study of the histostructure of the head of the femur and vertebrae of rats with experimental osteoporosis in comparison with the animals of the control group revealed changes in the structural organization of bone tissue. In the proximal part of the femur and the bodies of the vertebrae of the thoracic and lumbar regions of experimental animals, similar changes were found, which testify to the inhibition of bone formation processes.

In the spongy substance of bone tissue, these changes were associated with the thinning of the trabeculae and the dilution of the trabecular network. There was a decrease in the number of trabeculae and their contacts between themselves and the cortex (Fig. 1). Most of the trabeculae were thinned, had uneven edges and blind ends, which indicates the prevalence of bone resorption processes. Uneven staining of the matrix of bone tissue, uneven distribution of osteocytes, basophilia, thickening of cement lines in the areas was observed. Microcracks of trabeculae along the stratification of cement lines and single microfractures were noted (Fig. 2).

Cortex on the parts of the head of the femur and vertebral bodies was thinned and uneven in width. We detected expanded lacunae of osteocytes, vascular channels and single cavities filled with red bone marrow and reticulo-fibrous tissue, which reflects the process of rarity of compact bone. Osteocytes were of various sizes, their distribution in the regions was uneven. As a manifestation of a violation of calcification, sharply basophilic walls of a part of lacunae were revealed.

The results obtained are confirmed by histomorphometry – the method of objective evaluation of the state of bone tissue remodeling at the cellular and tissue levels. Morphometric examination of histopreparations of the femoral head recorded quantitative and qualitative changes in the parameters of bone tissue. It was found that the ratio of the volume of bone trabeculae to the volume of the spongy bone in the examined

area in animals with experimental osteoporosis was reduced by 32.9%, the width of the cortical layer and the thickness of the trabeculae were less (by 56.8% and 56.1% respectively, $P < 0.05$) in comparison with the parameters of the animals of the control group, which indicates a decrease in bone formation.

Thus, histological analysis and reduction of bone mineral density showed that the use of dexamethasone enabled us to obtain a model of bone tissue remodeling disturbance.

The levels of cytokines RANKL, osteoprotegerin, interleukin-17 and calcitonin in the blood serum of animals of the group with the disorder of bone tissue remodeling by glucocorticoids were higher than in intact animals, but the difference was statistically insignificant (Table 1). The most significant was an increase in the concentration of osteoprotegerin (by 55.5%) and RANKL (by 35.9%) in the serum of animals with experimental osteoporosis, which corresponds to the contribution of these cytokines to bone remodeling.

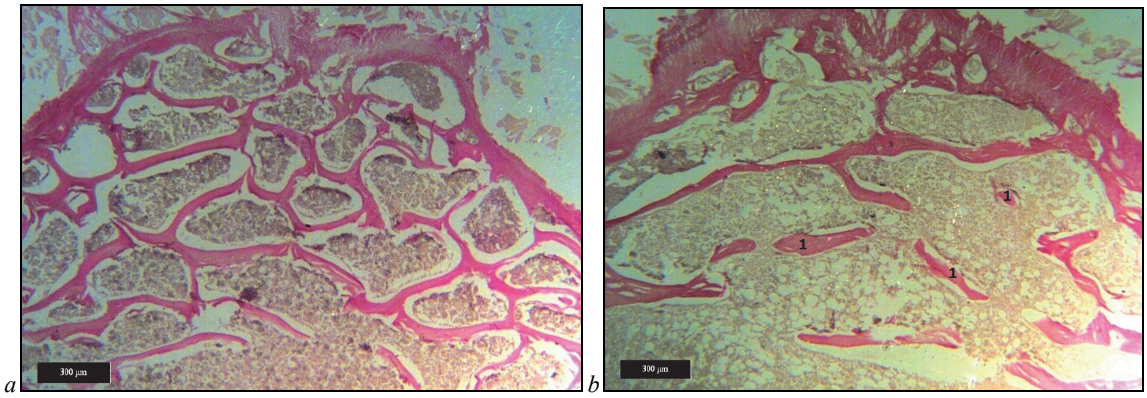

Fig. 1. Areas of the vertebrae of the lumbar spine of the rats:
a – the control group, *b* – with experimental osteoporosis; *1* – isolated trabeculae; van Gieson's picrofuchsin

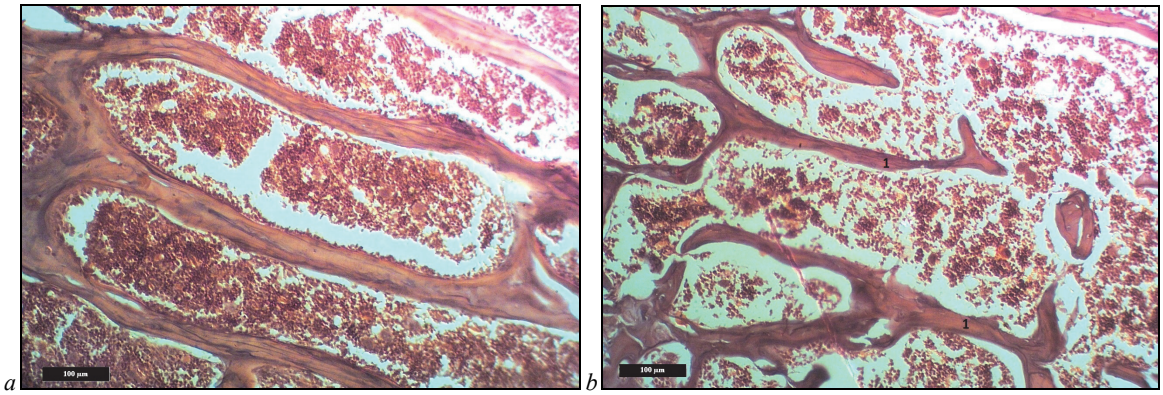

Fig. 2. Areas of the vertebrae of the thoracic spine of rats:
a – the control group, *b* – with experimental osteoporosis; *1* – thinned trabeculae, not forming a network; hematoxylin-eosin

Table 1
Levels of cytokines in the control and experimental groups (x ± SE)

Cytokine	Groups	
	control, n = 10	group with the violation of bone tissue remodeling by glucocorticoids, n = 18
Receptor activator of nuclear factor-κB ligand, pmol/l	0.131 ± 0.021	0.178 ± 0.046
Osteoprotegerin, pg/ml	22.7 ± 2.2	35.3 ± 9.5
P-selectin, ng/ml	2.23 ± 0.09	1.66 ± 0.07*
Interleukin-17, pg/ml	28.6 ± 1.3	29.0 ± 1.3
Transforming growth factor-β_1, ng/ml	26.5 ± 1.6	26.2 ± 1.5
Adiponectin, μg/ml	0.663 ± 0.021	0.631 ± 0.014
Visfatin, ng/ml	141.6 ± 26.7	133.3 ± 22.9
Parathyroid hormone, pg/ml	7.85 ± 1.03	5.24 ± 0.95
Calcitonin, pg/ml	8.81 ± 0.85	9.52 ± 1.37

Note: * – $P < 0.05$ compared with the control group.

At the same time, in the group of animals with disturbed bone remodeling of glucocorticoids, the serum P-selectin level was signifycantly lower (by 25.8%) in the same level in animals from the control group ($P < 0.05$). The concentration of parathyroid hormone decreased by 33.2% ($P > 0.05$), while the decrease in the levels of transforming growth factor-β_1, adiponectin and visfatin in the serum of animals in the experimental group was insignificant and statistically insignificant.

Research on intra group interrelations between indicators of cytokines revealed several correlations of different force In the control group, the presence of an inverse strong bond was established ($r = -0.899$, $P < 0.05$) between the content of visfatin and interleukin-17, medium strength feedback ($r = -0.695$, $P < 0.05$) between RANKL and transforming growth factor-β_1. In a group with experimental osteoporosis, these correlations became insignificant ($r = 0.133$ and $r = -0.006$ respectively, $P > 0.05$). In a group with a disturbance of bone tissue remodeling by glucocorticoids, a direct relationship of average force ($r = 0.626$, $P < 0.05$) between the levels of visfatin and P-selectin was revealed. In the control group, a similar relationship was not found ($r = -0.056$, $P > 0.05$). The presence of a connection between the studied parameters is explained by their mutual influence on the metabolism of bone tissue.

Discussion

The observed increase in serum RANKL level in animals in the experimental group may contribute to the loss of bone mass through the RANK/RANKL/OPG pathway, which corresponds to a decrease in bone mineral density in these animals. Binding of osteoprotegerin to RANKL prevents the destruction of bone tissue. The balance between the formation and destruction of bone determines the level of activation of osteoclasts (Luan et al., 2012). The administration of high doses of dexamethasone to rats inhibits the differentiation of osteoblasts and cell

proliferation. The use of glucocorticoids increases the expression of RANKL and inhibits the synthesis of osteoprotegerin, resulting in stimulation of bone resorption. In our study there is an increase in the concentration of osteoprotegerin in the blood serum of animals with experimental disturbance of bone tissue remodeling induced by glucocorticoids, which can be considered as a compensatory reaction to glucocorticoid-induced bone resorption. Osteoprotegerin is an important prognostic factor; in addition to regulating bone turnover, this cytokine inhibits vascular calcification and modulates inflammation in the vascular wall (Scialla et al., 2011). Osteoprotegerin is stimulated by numerous inflammatory mediators, such as interleukin-1, tumor necrosis factor-α, transforming growth factor-β and interferon-γ. Osteoprotegerin stimulates the expression of adhesion molecules and leukocyte infiltration in the walls of the vessels, which promotes the expression of RANKL.

In our study, there was a slight increase in the concentration of proinflammatory interleukin-17, produced by activated T-cells. Interleukin-17 supports osteoclastogenesis, depending on the signal path of RANKL-RANK. Cytokine increases the sensitivity of osteoclast precursors to RANKL by increasing RANK expression on osteoclastic progenitors (Adamopoulos et al., 2010). Moreover, interleukin-17 activates the expression of RANKL in osteoblasts, synovial and mesenchymal cells, thereby increasing the RANKL/OPG ratio and enhancing osteoclastogenesis. Thus, interleukin-17 through osteoblasts indirectly affects bone resorption, which ultimately contributes to the loss of bone tissue, reflected by a decrease in bone mineral density in our study. At the same time, interleukin-17 is an important mediator of the response to the treatment of glucocorticoids. According to the literature, dexamethasone reduces the expression of interleukin-17A in models of asthma in mice (Lu et al., 2013), or does not affect the production of interleukin-17 (Zeng et al., 2015), or Th17 cells (T-helpers 17) show limited sensitivity to dexamethasone (McKinley et al., 2008). Thus, the level of this intercellular mediator is apparently due to its ability to amplify the differentiation of osteoclasts and their functional activity by means of RANKL (direct and indirect pathway), and due to the effect of glucocorticoids on the T-cells producing this cytokine.

Suppression of the formation of bone tissue occurs as a result of the physiological effect of parathyroid hormone through the effect on the population of osteoblasts that secrete insulin-like growth factor 1 and cytokines that stimulate the metabolism of osteoclasts. Activated osteoclasts secrete alkaline phosphatase and collagenase, which leads to bone resorption. Reduction in the concentration of parathyroid hormone in our study, apparently, can be explained by a violation of the balance between the opposite effects that arise with the use of glucocorticoids. Glucocorticoids inhibit calcium absorption in the intestine and increase urinary calcium excretion (by inhibiting renal tubular reabsorption of calcium). This, as a rule, leads to a decrease in the levels of ionized calcium in the serum, which in turn can lead to an increase in the levels of parathyroid hormone. At the same time, an increase in bone resorption has an opposite effect on the secretion of parathyroid hormone by parathyroid glands, leading to a decrease in the levels of this hormone (Mazziotti et al., 2016).

In turn, calcitonin, being an antagonist of parathyroid hormone, reduces the concentration of calcium in the serum and slows the activity of osteoclasts, reducing the destruction of bone. The mechanisms of the effect of calcitonin on remodeling of bone tissue remain unclear until the end. Calcitonin has been shown to modify cell cultures of osteoblasts and osteocytes (Plotkin et al., 1999). The hypothesis based on the finding of calcitonin receptors on osteocytes is that calcitonin can potentially modify osteocyte products, such as fibroblast growth factor 23 or, more importantly, sclerostin, known as bone growth regulator (Davey & Findlay, 2013). Changes in the level of the hormone in the experimental group, apparently, are due to the effect of dexamethasone and are compensatory in nature, aimed at maintaining homeostasis, in particular, the level of ionized calcium.

Glucocorticoids also regulate many aspects of endothelial physiology, including the expression of adhesion molecules, the production of proinflammatory cytokines, and the maintenance of endothelial barrier integrity. The inhibitory effect of dexamethasone on the expression of P-selectin (Xiping et al., 2010), described as a biomarker of the development of atherosclerosis, is described. P-selectin is an adhesive transmembrane glycoprotein that is transferred to the cell surface from platelet α-granules and Weibel-Palade bodies of endothelial cells during activation of these cells, while the protein is partially released into the blood plasma, where it circulates in a soluble form. The binding of P-selectin to a specific glycoprotein ligand-1 allows interaction between leukocytes and endothelial cells, leukocytes and platelets, platelets and endothelium, thus involving cells in the emerging thrombus (Zubairova et al., 2013). Reduction of the concentration of P-selectin in the blood serum of animals of the group with experimental disturbance of bone tissue remodeling by glucocorticoids may be explained by the inhibitory effect of dexamethasone.

During the bone remodeling cycle, direct and indirect communications between bone cells are performed in a process called a binding mechanism. Transforming growth factor-β1, along with factors such as insulin-like growth factor, bone morphogenetic proteins, fibroblast growth factor, and platelet-derived growth factor appear to act as a coupling factor because it is stored in bone matrix and is released when bone resorption (Florencio-Silva et al., 2015). This cytokine is a growth factor that is involved in the control of proliferation, migration, differentiation and survival of many cell types. Transforming growth factor-β_1 can stimulate the proliferation of osteoblasts and regulate the functions of osteoclasts, thus being a regulator of bone remodeling. It affects the formation and resorption of bone and the production of some proinflammatory cytokines. This cytokine is an important modulator of vascular remodeling in atherosclerosis (Wan et al., 2012). Although transforming growth factor-β_1 enhances proliferation and early differentiation of osteoblasts, blocks their apoptosis, promoting the formation of bone tissue, this cytokine is able to inhibit osteoclastogenesis by activating the transcription factor Smad4 in osteoclasts (Morita et al., 2016). Transforming growth factor-β_1 has a double effect on osteoblasts: lower levels of this cytokine contribute to the differentiation of osteoclasts, while higher concentrations inhibit it (by increasing osteoprotegerin expression that inhibits RANKL-induced osteoclast differentiation) (Crane et al., 2016). These data confirm the correlation between the levels of RANKL and transforming growth factor-β_1 revealed by us. Glucocorticoids stimulate apoptosis of osteoblasts, inhibit their proliferation and differentiation depending on the dose, duration and stage of cellular differentiation (Yamashita et al., 2014). Apparently, the level of transforming growth factor-β_1 in the blood serum of animals of the group with the experimental violation of bone tissue remodeling by glucocorticoids is due both to its dysregulatory role in the stages of osteoblast and osteoclast differentiation and to the action of dexamethasone.

Regulation of bone remodeling includes not only osteoblastic and osteoclastic cell lines, but also other bone marrow cells. Adipocytes are obtained from the same precursor cells as osteoblasts, mesenchymal stem cells, and the balance between the two types of cells is important for bone remodeling. The number of adipocytes in the bone marrow varies in different pathophysiological conditions. Elevated levels of glucocorticoids also contribute to adipogenesis, limiting osteoblastogenesis (Li et al., 2013). The dexamethosone used in our experimental study serves as a potent adipogenic factor. Accelerated adipogenesis in the bone marrow is associated with the progression of osteoporosis (Sharma et al., 2014).

Adiponectin is the most common adipokine, the protein mediator, first described as a fatty tissue product secreted by adipocytes and regulating energy metabolism. Adiponectin plays a key role in various processes, such as energy metabolism, inflammation and cell proliferation, showing insulin-sensitive, anti-inflammatory, anti-atherosclerotic and antidiabetic properties (Padmalayam & Suto, 2013). It inhibits the production of adhesion molecules in endothelial cells. Adiponectin and its receptors are expressed in bone tissues and participate in bone metabolism. However, the literature presents contradictory results on the role of adiponectin in remodeling bone tissue. Adiponectin affects the differentiation of mesenchymal stem cells into preosteoblasts, as well as the proliferation and maturation of osteoblasts, which contributes to the regeneration of bone (Liu et al., 2013). In addition to positive modulation of osteoblasts, adiponectin also has a negative effect on os-

teoclasts. It increases the apoptosis of osteoclasts and reduces the proliferation of osteoclast precursor cells (Tu et al., 2011). Adiponectin inhibits the osteoclastic differentiation induced by macrophage colony-stimulating factor and RANKL, both mouse macrophages and human CD_{14}+ mononuclear cells and, consequently, inhibits bone resorption activity of osteoclasts. It is reported that in the animal model, adiponectin inhibits osteoclastogenesis, reduces bone resorption and increases bone mass (Oshima et al., 2005). At the same time, there is evidence that an increase in adiponectin activates RANKL and inhibits osteoprotegerin, resulting in a decrease in bone formation (Pacheco-Pantoja et al., 2014).

There is no consensus on the effects of glucocorticoids on the expression of adiponectin. Some sources describe that glucocorticoids reduce the serum adiponectin concentration (de Oliveira et al., 2011). While in the studies of Jang et al. (2008) an increase in the serum adiponectin level after therapy with dexamethasone has been observed. One of the main reasons for the contradictory results of glucocorticoids exposure to adiponectin expression is that its expression is regulated by a variety of mechanisms (various transcription factors, signaling cascades and hormones). For example, glucocorticoids increase the concentration of glucose in the blood, which subsequently increases the concentration of insulin, which can regulate the expression of adiponectin. Glucocorticoids affect adipogenesis and differentiation of adipocytes, as well as adipogenic transcription factors that can influence the expression of adiponectin. In addition, glucocorticoids are powerful anti-inflammatory agents and cause a decrease in pro-inflammatory cytokines, which can also indirectly regulate the expression of adiponectin. The observed decrease in the level of adiponectin in the serum of animals of the group with the violation of bone tissue remodeling with the help of glucocorticoids is associated with the direct influence of glucocorticoids on the overall energy metabolism, which complexly regulates the processes of bone remodeling, as well as the fact that the concentrations of adiponectin in the blood do not satisfactorily reflect its products by adipose tissue. An excess of glucocorticoids reduces the level of adiponectin and its activating effect on osteoblasts, reinforcing the processes of resorption in the balance of bone remodeling.

An important role in bone tissue remodeling is played by visfatin, which is a 52 kDa adipocytokine hormone secreted primarily by visceral fat. Visfatin is a mediator of inflammation and acts as a proinflammatory cytokine (Laiguillon et al., 2014), stimulating the production of interkeukin-1β, interkeukin-6, tumor necrosis factor-α, which confirms the correlation between the content of visfatin and proinflammatory interleukin-17. Visfatin has insulin-mimicking properties and has a hypoglycemic effect (Cinar & Gurlek, 2013). The role of visfatin in endothelial dysfunction has not been adequately studied, but it has been found that visfatin enhances the expression of adhesion molecules by activating the NF-κB signaling pathway (Lee et al., 2009). These data are confirmed by the positive correlation we found between the levels of visfatin and P-selectin.

Studies have shown that the synthesis and secretion of visfatin are modulated by glucocorticoids, tumor necrosis factor-α, interkeukin-6 and growth factors (Al-Suhaimi & Shehzad, 2013). There is evidence that visfatin may contribute to bone destruction with an increase in adipogenic differentiation in osteoporosis (Tsiklauri et al., 2016). On the other hand, this adipokine can stimulate the proliferation of osteoblasts and be a negative regulator of osteoclastogenesis, suppressing the differentiation of monocytes into osteoclasts (Liu et al., 2013). The reason for such discrepancies in the published data related to visfatin remains to be determined. The decrease in the level of visfatin in our experiment, apparently, is due to the depletion of the compensatory reserves of the bone tissue remodeling regulation system, caused by the long-term intake of glucocorticoids into the body.

Studies show that adiponectin and visfatin are oppositely related to the mineral density of bone tissue. Although the mechanisms underlying these correlations are unclear, modulation of bone metabolism by these adipokines can be proposed (Iacobellis et al., 2011). In our research the opposite situation is observed. In the study of intragroup relationships in the control group, direct correlations of average strength between the level of adiponectin, visfatin, and bone density were found ($r = 0.404$ and $r = 0.628$, respectively, $P > 0.05$), but these relationships were not significant. In the group with experimental osteoporosis, the correlation between the content of adiponectin and bone density changed direction ($r = –0.214$, $P > 0.05$), while the relationship between the content of visfatin and bone density remained straight ($r = 0.380$, $P > 0.05$). The data obtained may indicate the complexity and ambiguity of the role of adiponectin and visfatin in the regulation of bone metabolism. Thus, the use of glucocorticoids leads to disruption in the regulation of bone remodeling, reflected by hormones and intercellular mediators. The revealed correlations are an important indicator of changes in the system of regulation of bone tissue remodeling. The appearance of new connections and a change in their direction between pairs of intercellular mediators can be a manifestation of one of the mechanisms of bone tissue remodeling under the influence of glucocorticoids.

Conclusions

Reduction of bone mineral density and histological analysis of bone samples of a group of animals with a violation of bone tissue remodeling by glucocorticoids show that the use of dexamethasone has made it possible to obtain a model of bone remodeling disturbance. The studied changes in the concentrations of hormones and intercellular mediators indicate their important role in disturbances in the regulation of bone tissue remodeling under the influence of glucocorticoids. The imbalance between the RANKL and OPG levels, which results from the violation of the feedback mechanism, promotes bone resorption and, consequently, leads to a disturbance of bone remodeling. The degree of participation of adiponectin and visfatin in regulatory interactions of bone and energy metabolism was determined taking into account the interrelation of these adipokines. Correlations found during the study reflect the relationship in the system of regulation of bone tissue remodeling under the influence of glucocorticoids. A complex system of regulating bone remodeling, which includes many factors and their interactions, requires further study, which in the future can lead to the development of methods for treating patients with osteoporosis.

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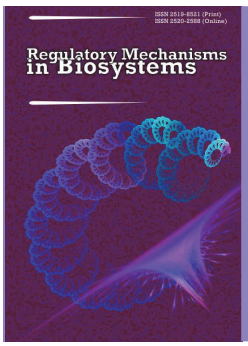

Regulatory Mechanisms in Biosystems

ISSN 2519-8521 (Print)
ISSN 2520-2588 (Online)
Regul. Mech. Biosyst., 9(1)

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