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IN VIVO EVALUATION OF SAFETY AND TOXICITY OF CELL-FREE EXTRACTS CONTAINING BIFIDOBACTERIUM BIFIDUM AND LACTOBACILLUS REUTERI DERIVATIVES

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Evaluation of the toxicity and safety of cell-free extracts, containing biologically active derivatives of Bifidobacterium bifidum and Lactobacillus reuteri, was based on observations of animal behavior, clinical manifestations of toxicity, data of body mass dynamics and lethality. It was shown that all investigated cell-free extracts are non-toxic and are suitable for long-term use if they contain derivatives in a dose corresponding to the daily therapeutic dose of cellular probiotics. The introduction to mice of extracts containing derivatives of probiotics in a dose ten times exceeding the maximum daily human, led to a significant decrease in the mass gain index. The same effect had intraperitoneal and repeated introductions of extracts, especially obtained from bifidobacteria. None of the tested samples exerted a local irritant effect upon intradermal administration.

Key words: toxicity, cell-free extracts, probiotics derivatives, Bifidobacterium bifidum, Lactobacillus reuteri.

The work is a fragment of the research project: "Microbiological characteristic of new structural and metabolic complexes of lacto- and bifido- probiotics", state registration No. 0119U100686.

Probiotics are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [8]. Despite the fact that probiotics are the most recognized biocorrectors of biocenoses, the effectiveness of probiotics in correcting microecological disorders is not always sufficient. Insufficient therapeutic efficacy of classical probiotics containing live microorganisms is due to low survival of probiotic bacteria in the gastrointestinal tract of patients [13]. In addition, cellular probiotics can cause undesirable side effects [15]. It is known that the beneficial effects of probiotics are due to their biologically active metabolites and structural components. Therefore, the development of medicines based on derivatives of probiotics is considered promising. Current industrial strains should be starting strains in such situation [15]. The most widely used probiotics in the majority of countries include lactic acid bacteria (LAB) from the genera Lactobacillus and Bifdobacteria [1, 11]. All industrial probiotic strains have status GRAS (Generally regarded as safe) [5]. This means that neither whole probiotic bacteria used in an adequate dosage, nor the natural metabolites produced by them, have a toxic effect on the human body. However, there is no evidence that biotechnology-derived product: disintegrate of probiotic bacteria and metabolites obtained by changing cultivation conditions are also non-toxic. It is known that the morphological, biochemical properties of bacteria and the nature of their metabolism, and hence the composition of the metabolites they produce, depend on the environment and conditions of cultivation. We have developed a new method for obtaining biologically active derivatives of probiotic bacteria. It allows combining disjointed procedures for obtaining structural components of bacterial cells and their metabolites in a two-stage process. The method involves the cultivation of probiotics in their own disintegrates without the use of traditional nutrient media [2]. The derivatives thus obtained can be used in the development of new metabiotics. Therefore, there is a need for verification of the harmlessness of probiotic derivatives obtained by the above-mentioned method.

An important stage of preclinical new drug development is the study of their safety and toxicity. Currently, preclinical safety assessment is carried out primarily in studies using laboratory animals and in other laboratory tests before the administration to normal human subjects [10]. In accordance with enacted in many countries legislations experiments on laboratory animals should be planned and performed taking into account the principles of 3Rs (Replacement, Reduction and Refinement). This implies avoiding the use of animals or replacing them by other test systems, minimization the number of animals used per experiment, minimizing the suffering of animals and improving their welfare [12]. The

current in vitro and in vivo toxicology and related research for the preclinical program include the study of safety pharmacology; acute toxicity; subacute or subchronic toxicity; chronic toxicity; genotoxicity; carcinogenicity; developmental and reproductive toxicity; studies in juvenile animals [10]. Conventional approaches to assessing the toxicity of pharmaceuticals may not be appropriate for biopharmaceuticals. The latter have unique and diverse structural and biological properties: species specificity, immunogenicity and unpredicted pleiotropic activity. For biotechnology-derived products, the preclinical safety studies should be performed as described in ICH S6 (Guidance for industry S6 – Preclinical safety evaluation of biotechnology-derived pharmaceuticals) and S6 Addendum to Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals [3, 6]. These guides provide the basic framework for a preclinical safety assessment of biopharmaceuticals derived from characterized cells, using various expression systems, including bacteria, yeast, insect, plant and mammalian cells. Preclinical safety evaluation of biotechnology-derived products, in addition to studying safety pharmacology, exposure assessment, single and repeated dose toxicity studies, genotoxicity, carcinogenicity, reproductive performance and developmental toxicity, includes immunotoxicity and local tolerance studies. Biopharmaceuticals, structurally and pharmacologically similar to those commonly used in clinical practice, may need less extensive toxicity testing. A flexible approach should be used in each case to determine the safety studies of biotechnology products. For example, the duration of repeated introductions may vary depending on the particular biotechnological product and clinical indications [3]. Definition of LD_{50} for biotechnology-derived products is usually impossible due to their low toxicity. Therefore, carrying out of this test is inexpedient [4]. Regulatory standards for biotechnology-derived pharmaceuticals are generally similar in the European Union, Japan, United States, Ukraine and Russia [3, 4].

The purpose of the study was to evaluate the safety and toxicity of cell-free extracts containing derivatives of Bifidobacterium bifidum and Lactobacillus reuteri obtained by disintegration of probiotics and subsequent cultivation of them in their own disintegrates.

Materials and methods. Safety and toxicity of four experimental specimens was studied in this work. Two types of specimens were obtained from microbial cells of the industrial strain Bifidobacterium bifidum №1 (probiotic «Bifidumbacterinum-Biopharma», PC Biofarma, Ukraine). One of them contained structural components of bifidobacteria, obtained by physical method of disintegration – multiple freezing-thawing of the microbial mass of the probiotic (B), and the second specimen contained structural components and metabolic products, obtained by cultivating of probiotic on its own disintegrates (BM) [2]. Two other types of experimental specimens (L and ML) were obtained similarly from microbial cells of the industrial strain Lactobacillus reuteri DSM 17938 (probiotic «BioGaia», BioGaia AB, Sweden). Disintegrates and cultures grown in disintegrates were centrifuged at 1100 g for 15 minutes to remove whole cells and cellular debris. Then the supernatant was passed through sterile membrane filters with a pore diameter of 0,2 μm (Vladipor, Russia).

Experiments with the use of laboratory animals were carried out in accordance with the Law of Ukraine “On Protection of Animals from Cruel Treatment” (№. 3447-IV of February 21, 2006), following the requirements of the Bioethics Committee of the Institute, agreed with the provisions of the “European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes” (Strasbourg, 1986). Animals involved in experiments, received a standard diet and were kept under the conditions of vivarium, established by regulatory documents. In total, 810 outbred white mice and 25 albino guinea pigs of both genders, the same mass (± 10%) and age were used in the experiments. They were healthy, quarantined for 10-14 days, were not previously used in experiments. The route, frequency, duration of introduction and doses of test specimens were selected in accordance with the guidelines for assessing the safety and toxicity of drugs and biotechnology-derived products and taking into account the generally accepted doses and methods of administration for probiotic products in medical practice [1, 3, 4, 6]. The volume of the introduced experimental samples did not exceed the permissible values for mice [1]. All studies were accompanied by controls – groups of intact animals with similar physical parameters.

Single dose toxicity study (acute toxicity). 180 outbred white mice weighing 15±1,5g were involved in the experiment. Animals were not fed for 12 hours before testing. Mice were weighed to determine the initial group weight immediately before the introduction of the test specimens. Each group consisted of 10 mice. The test samples were administered to mice in two doses. The first dose contained derivatives of such a number of cellular probiotics, which is a daily therapeutic mouse dose (TD). Calculation of the daily dose of probiotics for animals was made based on the values of the maximum

daily human dose according to the formula [1]. The second dose contained derivatives of such a number of cellular probiotics, which exceeded the maximum daily human dose tenfold (MaxD). Thus, the principle of using high doses, which provides the necessary margin for the reliability of toxicology assessment, was observed [4]. The tested specimens in a volume of 0,5 ml were introduced orally through a metal atraumatic probe, slowly immersing it into the stomach of each mouse or were injected intraperitoneally. Control group of mice received a physiological solution of sodium chloride in the same way and volume. Animals were fed not earlier than after 4 hours after oral administration of the test samples. Access to water was free. The evaluation of the toxic effect of the test sample was based on observations of animal behavior, clinical manifestations of toxicity and lethality within 7 days of introduction of the test specimens. At the end of this term, the group weight of experimental and control mice was determined.

Repeated dose toxicity study was carried out on 90 outbred white mice weighing 15±1,5g, which were formed into groups of 10 animals. Test samples contained probiotic derivatives in two doses, corresponding to the doses of cellular probiotics: the daily therapeutic mouse dose (TD) and dose 10 times exceeding the daily maximum for human (MaxD). Derivative-containing filtrates were administered to mice intragastrically in a volume of 0.5 ml daily for two weeks. The control group of mice received orally 0.5 ml of physiological saline sodium chloride over a specified period. The evaluation of the toxicity of the test samples was based on observations of animal behavior, clinical manifestations of toxicity, lethality and data of body mass dynamics within 14 days of introduction of test specimens. Results of the single and repeated dose toxicity study are presented as weight gain indexes (WGI), calculated by the formula: WGI = (FW – IW) ÷ IW × 100%, where FW – final group weight of mice; IW – initial group weight of mice.

Local tolerance study was performed using the technique for detecting the dermonecrotic properties of probiotic strains [1]. For this purpose albino guinea pigs weighing 350±32 g were used. The skin at the place of introduction of the test sample was previously freed from the wool and treated with 70° alcohol. Undiluted and diluted 1:10 and 1:100 extracts, containing probiotic derivatives and physiological saline sodium chloride were injected intradermally in a volume of 0.2 ml. The results were taken into account after 30 minutes, 4 hours and then daily for 3-4 days.

All experiments were performed three times. Obtained data was processed using the standard Microsoft Excel 2010 software package. The average values (M), standard deviations (SD) were determined. The probability of differences (p) between groups was calculated on the basis of the Mann-Whitney U test. Differences were considered statistically significant at $p < 0.05$.

Results of the study and their discussion. A single administration of all four types of derivative-containing test specimens in both doses with the use of both methods of introduction did not lead to the death of mice. Symptoms of acute intoxication were absent. Throughout the observation period, the animals were active, with normal appearance, behavior and coordination of movements. Loss of appetite and violation of other physiological functions were not observed. Experimental animals did not differ from the control ones. Analysis of the results of the single dose toxicity showed differences in the dynamics of changes in animal's body weight of different experimental groups compared with control. There was no statistically significant difference between the increase in body weight of the experimental and control animal groups after a single intragastric introduction of all test samples at a dose that corresponds to a daily dose of cellular probiotics for mice (TD) (fig. 1). A single intragastric introduction of samples containing derivatives of lactobacilli at the MaxD did not have a significant effect on the increase in body weight of experimental animals compared to the control. However, after the introduction of test samples containing derivatives of bifidobacteria at the MaxD the final group body weight of the animals was significantly smaller than the control values. While the group weight of control animals increased by 10±0.9% during the monitoring period, the group weight of experimental animals, receiving extracts contained bifidobacterial derivatives, increased by only 3.7±0.78% (B) and 3.15±0.86% (MB).

There was no statistically significant difference between the increase in body weight of the experimental and control animal groups after a single intraperitoneal injection of test samples containing derivatives of lactobacilli and bifidobacteria at a dose that corresponds to the daily dose of cellular probiotics (TD) for mice (fig. 2). The final group weights of experimental animals receiving single intraperitoneal injections of filtrates, containing derivatives of both probiotics at the MaxD, were significantly lower than those of the control group. This is evidenced by calculated weight gain indexes

(WGI) for the control and experimental groups of animals: 7±0.5% (C); 2.5±0.39% (L); 2±0.47% (ML); -2.9±2% (B); -3.3±1% (MB).

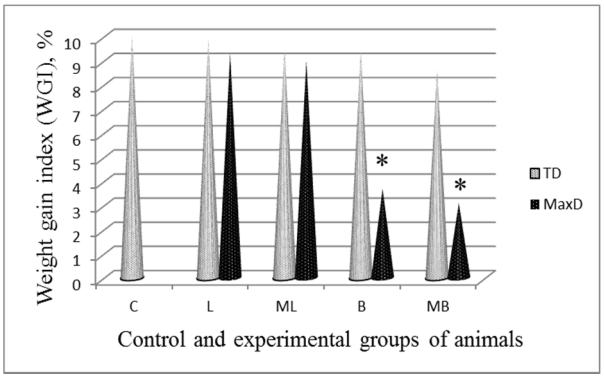

Fig. 1. – WGI after a single intragastric introduction of cell-free extracts containing probiotics' derivatives at TD and MaxD to mice (acute toxicity study). Notes: groups of animals administered: C – physiological saline sodium chloride; L – filtrate of L. reuteri disintegrate; LM – filtrate of L. reuteri culture grown in its own disintegrate; B – filtrate of B. bifidum disintegrate; BM – filtrate of B. bifidum culture grown in its own disintegrate; * – the differences are significant compared to the control group (C) of animals ($p<0.05$).

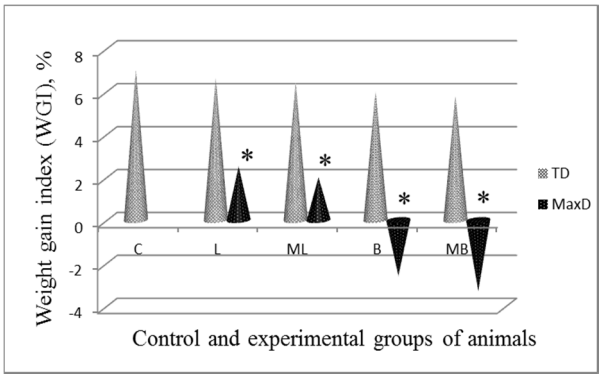

Fig. 2. – WGI after a single intraperitoneal injection of cell-free extracts containing probiotics' derivatives at TD and MaxD to mice (acute toxicity study). Notes: groups of animals administered: *C* – physiological saline sodium chloride; L – filtrate of L. reuteri disintegrate; LM – filtrate of L. reuteri culture grown in its own disintegrate; B – filtrate of B. bifidum disintegrate; BM – filtrate of B. bifidum culture grown in its own disintegrate; * – the differences are significant compared to the control group (C) of animals ($p<0.05$).

Daily intragastral administration of cell-free extracts containing probiotic's derivatives in both doses for two weeks did not cause death of mice. Pronounced symptoms of intoxication were absent. Animals that received lactobacilli derivatives at a dose corresponding to a daily therapeutic dose of cellular probiotic for mice had the same dynamics of body weight gain as the animals in the control group (fig. 3). Repeated introduction of cell-free extracts containing lactobacilli derivatives at the maximum dose resulted in a significant reduction of weight gain index. The introduction of filtrate of L. reuteri disintegrate was accompanied by an increase in the body weight of the animals for four days (WGI on the fourth day was 4.96±0.24%). From the fifth day the animals not only stopped gaining weight, but there was also a slight decrease in the body weight of the animals. At the fourteenth day WGI was 0.6±0.2%. The animals receiving ML filtrates slowly gained weight for 7 days (WGI on the seventh day was 3.71±1.96%). and then they stopped gaining weight and over the next seven days the body weight of the mice gradually returned to the initial values. On the fourteenth day WGI was 0.48±0.2%.

Comparison of the dynamics of the increase in the body weight of the control and experimental groups of mice, receiving the cell-free extracts containing B. bifidum derivatives at the therapeutic dose (TD), showed no significant differences (fig. 4). Repeated administration of filtrates containing bifidobacterial derivatives at the MaxD was accompanied first by a significant retardation of growth, and then, from the third day, by a gradual decrease in the weight of the experimental animals compared to the baseline values. After two weeks of oral administration, the WGI of the animals receiving the cell-free extracts containing the B. bifidum derivatives were - 6.14±0.39% (B) and -6.12±0.02% (*MB*), while the WGI of the control group of animals was 20±3% (C).

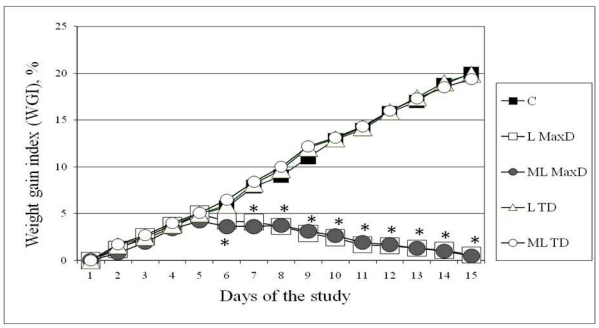

Fig. 3. – Dynamics of changes of the WGI during the repeated dose toxicity study of cell-free extracts containing L. reuteri derivatives at TD and MaxD. Notes: groups of animals administered: C – physiological saline sodium chloride; L – filtrate of L. reuteri disintegrate; LM – filtrate of L. reuteri culture grown in its own disintegrate; * – the differences are significant (for MaxD) compared to the control group (C) of animals ($p<0.05$).

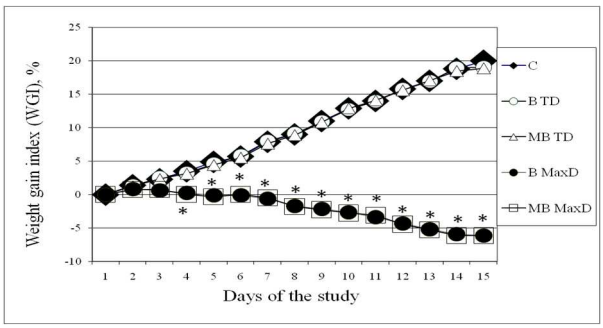

Fig. 4. – Dynamics of changes of the WGI during the repeated dose toxicity study of cell-free extracts containing B. bifidum derivatives at TD and MaxD. Notes: groups of animals administered: C – physiological saline sodium chloride; B – filtrate of B. bifidum disintegrate; BM – filtrate of B. bifidum culture grown in its own disintegrate; * – the differences are significant (for MaxD) compared to the control group (C) of animals ($p<0.05$).

After intradermal injections to albino guinea pigs of diluted and undiluted cell-free extracts (0.2 ml) containing derivatives of both probiotics, symptoms of inflammation and skin necrosis were not observed. This indicates that there is no local irritant effect of the test samples.

The results of this study are partially consistent with the data obtained by other researchers in studying the safety and toxicity of probiotic derivatives in vivo. Toxicity of lyophilized probiotic extract and new biodegradable nanoparticles obtained from metabolic cell-free supernatant of L. casei ATCC 39392 was investigated by Saadatzadeh et al. [14]. No significant toxicity or unexpected side effects or unusual effects were observed in animals. In vivo biological screening was shown that kefiran polysaccharide produced by L. kefiranofaciens did not induce any developmental toxicity in zebrafish embryos, at sub lethal concentrations [7]. Local application of B. longum lysate showed not only the absence of local irritating effect, but also a significant decrease in sensitivity and increased skin resistance to physical and chemical aggression [9].

Conclusions

1. The obtained results of single and repeated dose toxicity study show the absence of any toxic effect of the investigated cell-free extracts, containing B. bifidum and L. reuteri derivatives at a dose that corresponds to the daily therapeutic dose of cellular probiotics and indicate the possibility of their long-term use.

2. Significant decrease of the weight gain index after administration of cell-free extracts containing probiotic derivatives at a dose, exceeding the therapeutic one by several orders of magnitude, indicates that the probiotics' derivatives are not absolutely safe and require strict dosing.

3. Intraperitoneal introduction causes a more pronounced decrease of the weight gain index than intragastral administration of the cell-free extracts at the same dose.

4. At the same methods of administration and doses, cell-free extracts containing B. bifidum derivatives cause a more pronounced decrease of the weight gain index than cell-free extracts, containing L. reuteri derivatives.

5. Daily administration of cell-free extracts leads to a more significant decrease of the weight gain index than a single administration at the same dose, which confirms the presence of the accumulation effect associated with repeated administrations.

***Prospects of further studies**: the data obtained in the work will be used in the planning and implementation of further studies on the development of new metabiotics based on B. bifidum and L. reuteri derivatives.*

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Реферати

IN VIVO ОЦІНКА БЕЗПЕКИ І ТОКСИЧНОСТІ ДЕРИВАТІВ BIFIDOBACTERIUM BIFIDUM І LACTOBACILLUS REUTERI

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Оцінка токсичності та безпеки безклітинних екстрактів, що містять біологічно активні деривати Bifidobacterium bifidum і Lactobacillus reuteri, грунтувалася на спостереженнях за поведінкою тварин, клінічними проявами токсичності, даних динаміки маси тіла і летальності. Було показано, що всі досліджені безклітинні екстракти нетоксичні і придатні для тривалого застосування, якщо вони містять деривати в дозі, що відповідає добовій терапевтичній дозі клітинних прибутків. Введення мишам екстрактів, що містять деривати прибутків в дозі, що десятикратно перевищує максимальну добову для людини, призводило до значного зниження індексу приросту маси. Такий же ефект справляло внутрішньочеревне і повторне введення екстрактів, особливо отриманих з біфідобактерій. Жоден з досліджених зразків не чинив місцевої дратівної дії за внутрішньошкірного введення.

Ключові слова: токсичність, безклітинні екстракти, деривати прибутків, Bifidobacterium bifidum, Lactobacillus reuteri.

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IN VIVO ОЦЕНКА БЕЗОПАСНОСТИ И ТОКСИЧНОСТИ ДЕРИВАТОВ IFIDOBACTERIUM BIFIDUM И LACTOBACILLUS REUTERI

Кныш О.В., Исаенко О.Ю., Бабич Е.М., Рымша Е.В., Погорелая М.С., Балак А.К.

Оценка токсичности и безопасности бесклеточных экстрактов, содержащих биологически активные дериваты *Bifidobacterium bifidum* и *Lactobacillus reuteri*, основывалась на наблюдениях за поведением животных, клиническими проявлениями токсичности, данных динамики массы тела и летальности. Было показано, что все исследованные бесклеточные экстракты нетоксичны и пригодны для длительного применения, если они содержат дериваты в дозе, соответствующей суточной терапевтической дозе клеточных пробиотиков. Введение мышам экстрактов, содержащих дериваты пробиотиков в дозе, десятикратно превышающей максимальную суточную для человека, приводило к значительному снижению индекса прироста массы. Такой же эффект оказывало внутрибрюшинное и повторное введение экстрактов, особенно полученных из бифидобактерий. Ни один из исследованных образцов не оказывал местного раздражающего действия при внутрикожном введении.

Ключевые слова: токсичность, бесклеточные экстракты, дериваты пробиотиков, Bifidobacterium bifidum, Lactobacillus reuteri.

Рецензент Єрошенко Г.А.