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# THERMAL CYCLING AS A METHOD FOR DISINTEGRATION OF BIFIDOBACTERIUM BIFIDUM

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The study compares the damaging effects of two thermal cycling methods on Bifidobacterium bifidum probiotic strain. The suspensions of freshly isolated bifidobacteria and of bacteria stored under hypothermic conditions for 24 hours were subject to the two methods of tenfold thermal cycling that involved slow cooling to (-23 ± 1) °C or rapid cooling to (-196 ± 1) °C followed by thawing at 37 °C. Cell survival was assessed by means of colony forming units (CFU) counting and by flow cytometry using carboxyfluorescein diacetate (cFDA) as fluorochrome. Thermal cycling with slow cooling to (-23 ± 1) °C had a more pronounced disintegrating effect on bifidobacteria. Preliminary storage of bifidobacteria under hypothermic conditions did not significantly increase their resistance to the disintegrating effect of thermal cycling.

**Key words:** bifidobacteria, thermal cycling, survival, flow cytometry, disintegration.

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

Studies of the low temperatures effects on bifidobacteria have been lasting for several decades. The main efforts were aimed at the development of technologies for long-term storage of bifidobacteria at low temperatures, ensuring their morphological, physiological and genetic stability. Development of long-term storage technologies is caused by their application in health care [11, 12, 14]. Today, the main methods for long-term storage of bifidobacteria are cryopreservation and lyophilization [11]. Optimal conditions that ensure the maximum survival and preservation of bifidobacteria functional activity during cryopreservation and lyophilization have been determined owing to the long-term studies of the of individual cryodamage factors influence on cell viability [3, 4, 5].

In vivo studies have shown that the therapeutic effects of cellular probiotics, especially their lyophilic forms, are obscure, short time or absent. First of all, the lack of probiotics efficacy is associated with their significant loss during their passage through the gastrointestinal tract and with low ability of probiotic cells to colonize the intestinal mucosa. Secondly, some patients also have a specific colonization resistance to introduced probiotics [15]. Survival of various strains of probiotic microorganisms in the gastrointestinal tract is about 20–40 % [1]. All the above mentioned necessitates the search for alternative approaches to the correction of microecological disturbances. One of these approaches is the use of probiotic derivatives: structural components and metabolites of probiotics.

Isolation of the bacterial structural components is quite difficult. One of the methods for obtaining bacterial structural components is freezing-thawing. Lactic acid bacteria tolerate repeated freeze-thaw cycles by immersion in liquid nitrogen [2, 10]. Each cycle results in loss of sensitive cells but more resistant ones remain. Therefore, cell disintegration by freezing-thawing is usually accompanied by other physical or mechanical methods [2].

Bacteria of the Bifidobacterium genus have significant biotechnological potential. For example, they can serve as a source of various biologically active structural components and metabolites [10]. Bifidobacteria belong to gram-positive bacteria. Their cell wall is stronger than that of many gram-negative bacteria and confers high mechanical stability. Bifidobacteria are also highly resistant to damaging factors that take place during freezing. High cryoresistance of bifidobacteria is explained by the presence of peptidoglycan and high content of phospholipids and unsaturated fatty acids in their cell membranes. However, there are differences in cryosensitivity among representatives of the same species. *B. bifidum* is more sensitive than other representatives that are protected by the polysaccharide capsule [3].

There is a number of factors, which may destroy cells during cooling. Rapid cooling increases the risk of cell damage due to intracellular crystallization. On the other hand, slow cooling leads to loss of water, which reduces the risk of intracellular crystallization but increases the negative impact of concentrated solutions on biological structures called "solution effects". Crystallization and recrystallization exacerbate the damage of microbial cells due to low-temperature, osmotic problems and oxidative stress. According to previously published data, the optimal freezing rate of bifidobacteria is 1 °C/min, while plunging in liquid nitrogen in saline solution has the greatest damaging effect on cells [6, 7]. The possibility of using the low-temperature as a destructive factor for probiotic cells necessitates the search for freezing parameters that can help to obtain the maximal yield of biologically active derivatives.

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**The purpose** of the study was to compare the damaging effects of the two thermal cycling methods on *B. bifidum* probiotic strain to determine the best approach to their disintegration.

**Materials and methods.** The probiotic strain B. bifidum (of medical product Bifidumbacterin, JSC Vivo-Actyv, Ukraine), stored in a lyophilized state at (6±2) °C, was rehydrated and cultured for 20–24 hours at temperature (37±1) °C in Thioglycol medium (Biolife, Італія). After checking the purity of the culture, the microbial mass was washed three times with 0.9% sodium chloride solution to remove components of the nutrient medium. Cell suspensions with an optical density (OD) of 10.0 units by McFarland scale in saline solution (~ $10^9$ CFU/ml) were prepared using a Densi-La-Meter device (Lachema, Czech Republic). The resulting suspensions were subjected to cooling immediately after preparation. Other suspensions were stored under hypothermic conditions (6 ± 2) °C before cooling for 24 hours. Cooling of the microbial suspensions was carried out in the following ways:

A – samples in 100 ml vials were placed into the cooling camera at t = (–23±1)°C. The volume of each sample comes to 50 ml;

B – 4 ml samples in 4.5 ml cryocontainers were cooled by direct plunging into liquid nitrogen up to the t = (–196±1)°C.

The frozen samples were warmed in a water bath at (37±1) °C until complete thawing. Such freeze-thaw cycles were repeated 10 times. Changes in the sample temperature during freezing-thawing were recorded using a copper-constantan thermocouple placed in the center of the sample.

Microscopic examination of native bifidobacteria suspensions preparations was carried out using the MPI-5 interference-polarization microscope (produced by PZO, Poland) equipped with digital camera with on-line access to a computer. The cell size and the concentration were determined with graduated ocular reticulum.

The number of colony forming units (CFU) represents the number of viable microorganisms. Serial dilutions method was used to determine the concentration of viable bacteria in samples prior and after the thermal cycling. 1 ml of test sample was dissolved in 9 ml of saline solution and vortexed until the sample was completely dissolved. From this first dilution, 10-fold serial dilutions ($10^{-2}$–$10^{-9}$) were prepared in saline solution. Appropriate dilutions were cultured in two parallel tubes with high columns of Bifidum medium (BM, "Pharmaktiv", Ukraine) to obtain a reasonable number of colonies for counting. The tubes were incubated at (37±1) °C for 48 hours. The number of CFU per ml of suspension was calculated by the formula: $[(A \cdot 10^x) + (B \cdot 10^y)]/2$, (where A is the average number of colonies in the dilution preceding the largest one ($10^{-x}$), B is the average number of colonies in the largest dilution ($10^{-y}$) and expressed as a decimal logarithm (lg CFU/ml).

The viability of bifidobacteria was determined by cytofluorimetry using carboxyfluorescein diacetate (cFDA) as fluorochrome. The stock solution (10 mM) of cFDA was prepared by dissolving 4.6 mg of cFDA/ml in acetone and stored at 20 °C in the dark. Next, the working solution was prepared by dissolving the stock solution in acetone to a concentration of 1 mM. Samples containing $10^3$ – $10^7$ cells/ml were incubated in phosphate-buffered saline supplemented with 10 μM cFDA for 30 min. Then the samples were kept on ice in the dark for no longer than 1 h before flow cytometry. After that these samples were analyzed by flow cytometer FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, USA).

All experiments were performed three times. The average values (M) and standard deviations (SD) were determined. The data were statistically processed using Excel 2010 software (Microsoft, USA). One-way analysis of variance (ANOVA) and subsequent multiple comparisons were performed using the Bonferroni correction. The differences between the obtained indices were considered statistically significant at $P < 0.05$.

**Results of the study and their discussion.** The mean cooling rate was low (~ 0.2 °C/min) when method A was used. After 4 hours of cooling the final temperature of (–23 ± 1) °C was achieved. Direct plunging in liquid nitrogen by method B allowed to cool the samples to (–196 ± 1) °C in 2 minutes. The mean cooling rate was ~ 109 °C/min.

Fig. 1 demonstrates bifidobacteria before (1a and 1b) and after (2a and 2b) disintegration by method A. In the samples that were not subjected to thermal cycling some cells had the shape of straight or slightly curved rods, 0.5-1.2 x 1.5-8 microns in size, with club-shaped bulges at their ends. These cells were located individually. Other cells had V- or Y-like shapes. They tended to form clusters occupying the entire field of view. As a result of thermal cycling, the shape, size and arrangement of these cells changed. Their mean size decreased. The cells acquired a grain-like shape typical for cocci form. They were located evenly across the field of view. The presence of different sizes cell aggregates was peculiar to thermally cycled bacteria.

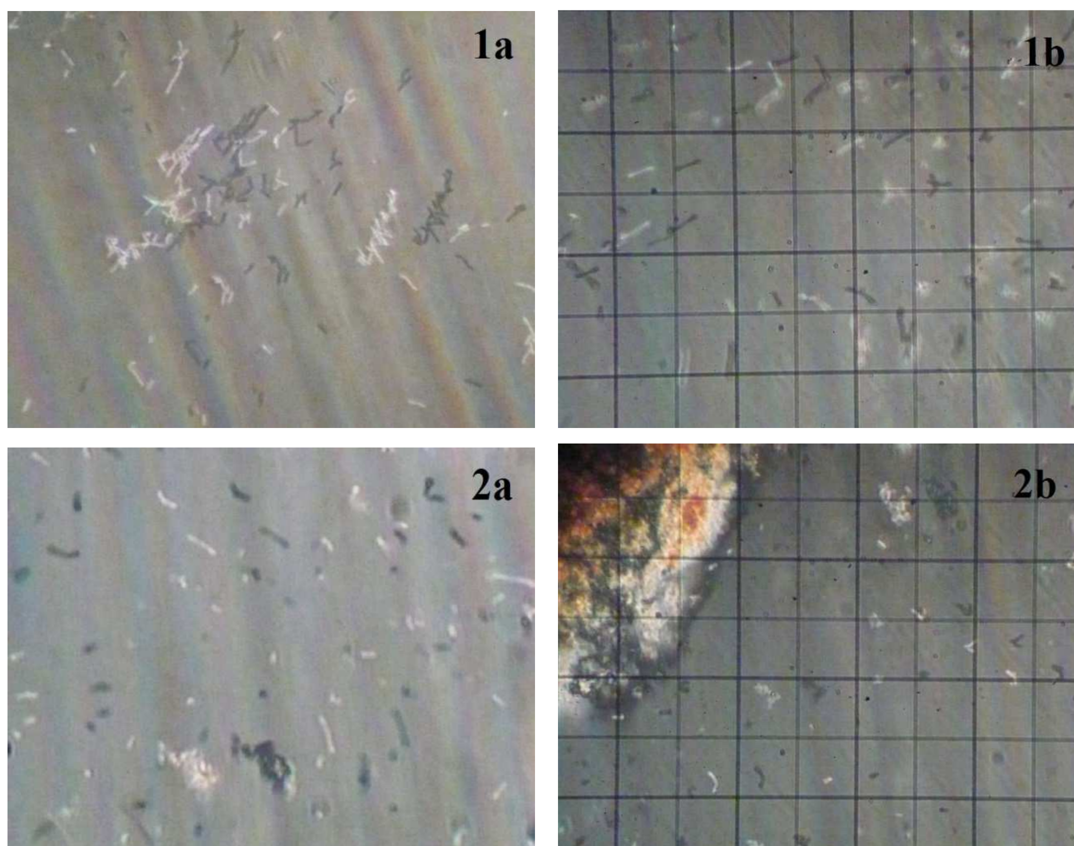


Fig. 1. Interference-polarization microscopy of bifidobacteria before (1a and 1b) and after (2a and 2b) disintegration by method A.

CFU counting allowed estimation of cell survival after the thermal cycling (table 1). Survival of bifidobacteria depended on the freeze-thaw regimen. The survival rate of bifidobacteria thermocycled by methods A and B came to 35 % and 53 %, respectively. Survival of bifidobacteria stored at hypothermic conditions before cooling did not significantly differ from that of freshly isolated bacteria. Thus, method A had a more pronounced damaging effect on bifidobacteria, so it was more suitable for disintegration of bifidobacteria.

Table 1

**Survival of B. bifidum after thermal cycling (TC)**

| Method of TC | Concentration of survived cells, lg CFU/ml, M±SD | |
|---|---|---|
| | F | H |
| A | 3.3±0.3 | 3.5±0.4 |
| B | 5.0±0.2* | 5.3±0.3* |

Notes: F – freshly isolated cells; H – cell stored in hypothermic conditions; * – the differences are statistically significant with respect to method A, $p < 0.05$.

Flow cytometry is a modern and efficient tool for viability of lactic acid bacteria [6]. Carboxyfluorescein diacetate (cFDA) is a non-fluorescent precursor, which upon entering the cell is converted into carboxyfluorescein (cF) by cell esterases [13]. It can fluoresce and is unable to penetrate membranes and leave cells unless they are damaged. Cells with intact membranes and functioning cytoplasmic enzymes are capable of holding the dye. Dead cells do not fluoresce because they lose enzymatic activity and cF through damaged membranes.

In order to compare the damaging effects of these two thermal cycling methods on bifidobacteria, flow cytometry with cFDA was applied. Additionally, freshly isolated bacteria were killed by heating to 70 °C for 30 min to be used as controls. Freshly isolated, killed and thermally cycled by methods A and B bacteria differed in their ability to be stained with fluorochrome (cF) and to scatter direct light. Within each experimental group the following cell subpopulations can be distinguished (fig. 2):

1) right top – undamaged cells of normal size with preserved esterase activity and membrane integrity, stained by cF;

2) left top – small undamaged cells stained by cF;

3) right bottom – cells of normal size that were not stained by cF. They are dead cells, damaged cells that have lost or inhibited esterase activity, impaired integrity or permeability of membrane;

4) left bottom – small dead or damaged cells and cellular debris (cell fragments, microparticles and microvesicles).

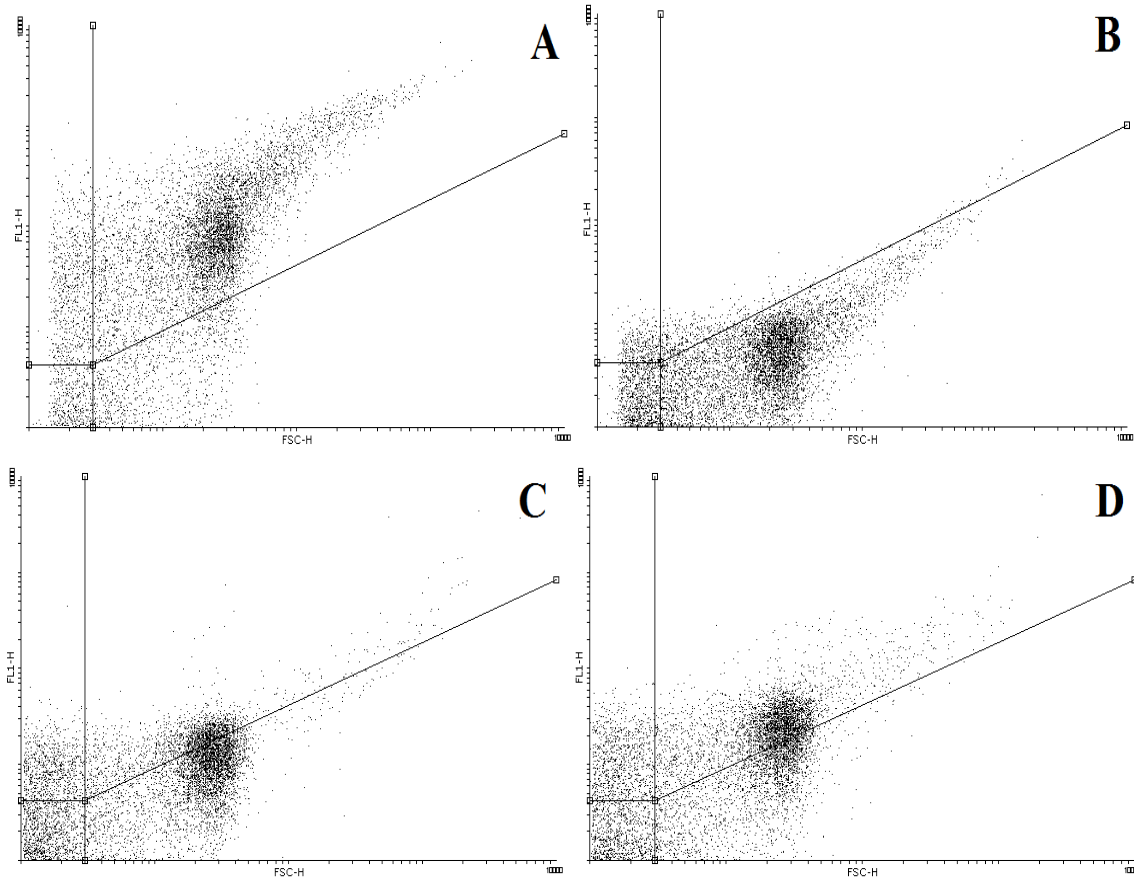


Fig 2. Dual-parameter dot plot of the forward scatter intensity versus cF-fluorescence of: A – freshly isolated; B – heat-killed; C – freshly isolated cells, thermally cycled by method A; D – freshly isolated cells, thermally cycled by method B.

Flow cytometry also showed that thermal cycling by method A caused more severe cell damage than by method B (table 2). The cells thermally cycled by method A had higher damaged /undamaged cells ratio comparing to method B. The data support the assumption put forward earlier that method A is more suitable for disintegration of bifidobacteria.

Flow cytometry revealed that samples previously stored under hypothermic conditions, after thermal cycling had a slightly lower damaged/undamaged cells ratio compared to freshly isolated samples. However, these differences in damaging effect were not significant.

Table 2

**Subpopulation composition of freshly isolated, killed, and thermally cycled bifidobacteria**

| | Quadrants | Subpopulation content (%) in cell suspensions | | | | |
|---|---|---|---|---|---|---|
| | | A | B | C | D | E |
| 1 | Right top | 60.22 | 3.94 | 18.11 | 36.38 | 20.09 |
| 2 | Left top | 7.55 | 3.20 | 8.82 | 9.74 | 9.27 |
| 3 | Right bottom | 17.65 | 73.09 | 46.39 | 29.86 | 44.76 |
| 4 | Left bottom | 14.58 | 19.77 | 26.68 | 24.02 | 25.88 |
| | | | | | | |
| Damaged /undamaged cells ratio | | 0.47 | 13.0 | 2.71 | 1.17 | 2.41 |

Notes: A —freshly isolated cells; B – heat-killed cells; C – freshly isolated cells, thermally cycled by method A; D – freshly isolated cells, thermally cycled by method B; E – stored in hypothermic conditions cells, thermally cycled by method A.

Previous studies showed that the use of saline solution as a cell medium during freezing of bifidobacteria led to more significant loss of cells than the use of cultural media or media with cryoprotectants. In the case of freezing in saline solution, the survival of bifidobacteria depended on the cooling rate. Cell survival was maximum when the cooling rate was 1 °C/min and decreased when the cooling rate became greater or less than 1 °C/min [3, 4]. That is why in the present study we used slow (~ 0.2 °C/min) and rapid (~ 109 °C/min) cooling rates.

The results of the study showed that ten-fold thermal cycling caused significant, but not complete loss of viable cell. The greatest loss of bifidobacteria and higher content of damaged and dead cells were observed after thermal cycling by method A. Thus, it can be stated that “solution effect” was the main factor that disintegrated bifidobacteria. Rapid cooling by method B did not have such a profound effect

on cell damage as in the case of method A. Therefore, the cells were better-tolerated higher cooling rates and intracellular crystallization that often accompanies such type of cooling. The results of the study are in good agreement with the data of other authors, who claim that "rapid" cooling or direct immersion in liquid nitrogen is accompanied by minimal loss of bacterial viability [2, 10]. Therefore, in the industrial production of probiotics, direct immersion of concentrated starter cultures in a cryoprotective medium is used [8]. Our data confirm that the damage to bacterial cells is due to osmotic imbalance rather than the formation of intracellular ice. It is known that the highest indicators of quantitative and functional losses of microorganisms are observed in the temperature range from -40 ºC to -60 ºC [7]. There is a certain critical limit of dehydration, the excess of which results in the death of bacterial cells due to inactivation and damage to critical sites in the cell - membrane lipids, proteins, nucleic acids, and some enzymes. Damage to the membranes and cell walls of bacteria as a result of thermal cycling leads to the release of cellular content into the surrounding. Consequently, the thermally cycled samples of bifidobacteria contain living cells, dead cells, cellular debris and material released from the destroyed cells after thermal cycling. In the present study, the cell disintegration efficiency was proved by determining the contents of living cells, dead cells, cellular debris in the samples. As far as the main purpose of disintegration is to obtain cellular derivatives, further identification of bifidobacteria derivatives in the extracellular media is necessary to confirm its efficacy. In addition, it is important to confirm that probiotic derivatives do not lose their biological activity during thermal cycling. These works are the next stage of the study on probiotic derivatives obtained by thermal cycling.

## Conclusions

1. Both methods of thermal cycling caused significant cell damage of *B. bifidum*. However, they did not lead to complete loss of viable cells.

2. Method A that involved slow cooling to (-23 ± 1) °C had a more pronounced disintegrating effect on bifidobacteria than method B that involved rapid cooling to (-196 ± 1) °C.

3. Preliminary storage of bifidobacteria under hypothermic conditions did not significantly raise their resistance to the disintegrating effect of thermal cycling.

*Prospects of further research: the data obtained in this work will be taken into account and used in subsequent studies of the biological activity of B. bifidum derivatives.*

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## Реферати


**ТЕРМОЦИКЛЮВАННЯ ЯК МЕТОД ДЕЗІНТЕГРАЦІЇ BIFIDOBACTERIUM BIFIDUM**
**Книш О.В., Пахомов О.В., Погоріла М.С., Савінова О.М., Балак О.К.**

У дослідженні порівнюється пошкоджуючий вплив двох методів термоциклювання на пробіотичний штам *B. bifidum*. Суспензії свіжовиділених біфідобактерій і бактерій, що зберігалися за гіпотермічних умов протягом 24 годин, піддавали десятикратному термоциклюванню двома способами, які передбачали повільне охолодження зразків до (-23 ± 1) °C або швидке охолодженням до (-196 ± 1) °C з подальшим відігріванням на водяній бані при 37 °C до повного відтавання. Виживання клітин оцінювали шляхом підрахунку колонієутворюючих одиниць і проточної цитометрії з використанням карбоксифлуоресцеїну діацетату (кФД) як флуорохрому. Термоциклювання з повільним охолодженням до (-23 ± 1) °C виявило більш виражену дезінтегруючу дію на біфідобактерії. Попереднє зберігання біфідобактерій за гіпотермічних умов не значно підвищувало їх стійкість до дезінтегруючої дії термоциклювання.

**Ключові слова:** біфідобактерії, термоциклювання, виживаність, проточна цитометрія, дезінтеграція.


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**ТЕРМОЦИКЛИРОВАНИЕ КАК МЕТОД ДЕЗИНТЕГРАЦИИ BIFIDOBACTERIUM BIFIDUM**
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В исследовании сравнивается повреждающее действие двух методов термоциклирования на пробиотический штамм *B. bifidum*. Суспензии свежевыделенных бифидобактерий и бактерий, хранившихся в гипотермических условиях в течение 24 часов, подвергали десятикратному термоциклированию двумя способами, которые предполагали медленное охлаждение образцов до (-23 ± 1) °C или быстрое охлаждением до (-196 ± 1) °C с последующим отогревом на водяной бане при 37 °C до полного оттаивания. Выживаемость клеток оценивали путем подсчета колониеобразующих единиц и проточной цитометрии с использованием карбоксифлуоресцеина диацетата (кФД) в качестве флуорохрома. Термоциклирование с медленным охлаждением до (-23 ± 1) °C оказывало более выраженное дезинтегрирующее действие на бифидобактерии. Предварительное хранение бифидобактерий в гипотермических условиях не значительно повышало их устойчивость к дезинтегрирующему действию термоциклирования.

**Ключевые слова**: бифидобактерии, термоциклирование, выживаемость, проточная цитометрия, дезинтеграция.


Рецензент Костенко В.О.

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# MORPHOLOGICAL FEATURES OF DOXORUBICIN-INDUCED LIVER DAMAGE ASSOCIATED WITH NONALCOHOLIC STEATOHEPATITIS

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The paper consideres the study of histological features of anthracycline-induced liver lesions concomitant with non-alcoholic steatohepatitis.The findings of the study have established the presence of moderate fatty degeneration of the liver with mild focal protein dystrophy of hepatocytes in the lobules of the animals with experimental non-alcoholic steatohepatitis. In the group of animals with anthracycline-induced liver damage, moderate periportal necrosis of hepatocytes along with a mild small-droplet fatty degeneration. Prominent total (centrilobular and periportal) subacute liver necrosis along with moderate fatty degeneration was found in animals with anthracycline-induced liver damage associated with experimental non-alcoholic steatohepatitis.

**Keywords:** non-alcoholic steatohepatitis, anthracycline-induced liver damage, rats.

*The work is a fragment of the research project “Development of methods for prevention and treatment of the drug-induced damages of the internal organs”, state registration No. 0115U001087.*

Doxorubicin belongs to anthracycline antibiotics that are considered as one of the most effective antitumor drugs widely used in oncological and oncohematological clinical practice [5, 6, 9-11]. Doxorubicin is the most important mainstay in the treatment of breast cancer, soft tissue sarcoma and aggressive lymphomas of high malignancy, acute lymphoblastic and myeloblastic leukemias [6, 9-11]. In some cases, high toxicity of doxorubicin may be restriction on its use [5, 6, 8-11]. Moreover, the toxic effect of doxorubicin on the tissues of the heart, kidneys, liver has been confirmed [5, 9, 12-14]. Notably, damaging effect of anthracycline antibiotics is due to its specific pharmacokinetics. Doxorubicin is able to accumulate intracellularly in concentrations 10-500 times higher than extracellularly [8]. Another important point in the development of toxic effects of all cytostatics, including anthracyclines, is the impact on all cells, both malignant and healthy [7, 8]. Doxorubicin is metabolized mainly in the liver with the formation of a highly toxic metabolite of doxorubicinol, which has a direct damaging effect on liver tissue [6]. Histological manifestations of liver lesions induced by anthracycline antibiotics are characterized by necrosis and degeneration of hepatocytes, sinus dilatation, vascular stagnation and hemorrhage [6, 11, 13, 14].

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