EXPERIMENTAL ARTICLES

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THE COMPARATIVE STUDY OF THE EFFECT OF LOW-INTENSITY BROADBAND AND LOW-INTENSITY PULSED ULTRASOUND ON B16 MELANOMA CELLS *in vitro*

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Aim. To evaluate the effect of low-intensity broadband ultrasound (UMUS) and low-intensity pulsed ultrasound (LIPUS) on B16 melanoma cells *in vitro*.

Methods. The research was conducted on B16 melanoma cells under two modes of low-intensity ultrasonic sounding of cells, for which an SDG 2082 X Siglent generator (USA) with an ultrasonic emitter diameter of 20 mm was used with a frequency for UMUS of 1–7 MHz, intensity of 30 mW/cm, pulse duration of 50 μs, and for LIPUS with a frequency of 1.5 MHz, intensity of 30 mW/cm, pulse duration of 200 μs. Both modes of cell sounding were carried out with threefold irradiation, once a day after which the distribution of cells by cycle phases, the level of apoptosis and proliferative activity was determined by the method of ductal cytofluorimetry.

Results. Under the influence of UMUS ultrasonic sounding, the inhibition of the proliferation of melanoma cells by 1.6 times ($P < 0.05$), a 2-fold decrease in the content of aneuploid cells, compared to the control, was found. However, cytostatic and proapoptotic effects were not recorded. Under the influence of LIPUS sounding, an anti-proliferative effect, an increase in the content of dead and apoptotic cells, and decreased DI-index compared to the corresponding control were found.

Conclusions. Under the action of low-intensity broadband ultrasound (UMUS) and low-intensity pulsed ultrasound (LIPUS), a cytotoxic/cytostatic and pro-apoptotic effect, and a decrease in the content of the aneuploid cell population were revealed.

Key words: low-intensity broadband ultrasound (UMUS), low-intensity pulsed ultrasound (LIPUS), cytotoxic/cytostatic and pro-apoptotic effect, B16 melanoma cells.

Interest in the use of low-intensity ultrasound on biological objects has increased significantly in recent years [1]. This treatment is a promising therapeutic option because it is non-invasive, there is no risk of infection or tissue damage, and there are no adverse reactions. Low-intensity ultrasound (LIPUS) has many benefits, including promoting tissue healing, stimulating angiogenesis, and tissue regeneration, inhibiting inflammation and relieving pain, and stimulating cell proliferation and differentiation [2, 3]. The data available in the literature on the effect of LIPUS on tumor cells are scarce [5], and in relation to low-intensity broadband ultrasound (UMUS) they are isolated [4]. However,

the biological mechanisms of low-intensity ultrasound remain to be fully elucidated, and the side effects of using these methods for wound healing can be a stimulating effect on some forms of neoplasms, in particular melanoma, which is an aggressive tumor with a pronounced metastatic potential that originates from skin melanocytes and may be sufficiently sensitive to the therapeutic effects associated with ultrasound irradiation. That is why the purpose of this study was to comparatively study the effect of UMUS and LIPUS on the survival and proliferative parameters of B16 melanoma *in vitro*.

Materials and Methods

The study used cultured cells of the B16 line (transplantable melanoma of outbred white mice) [6, 7], obtained from the cell bank of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NASU. Cells were planted at a concentration of 15 thousand/well of a 24-well plate (Sardstedt, Germany) in RPMI-1640 medium (Sigma, USA) with 10% FBS (Sigma, USA) and 40 μg/ml gentamicin (Ukraine) under conditions of 5% CO_2, 37 °C, and 100% humidity were cultivated during an 18-hour adaptation period, the medium was replaced with a fresh one and 3-fold irradiation was carried out once a day in two modes (UMUS and LIPUS).

Methods of ultrasonic sounding

Exposure to ultrasonic waves was carried out through the bottom of the tablets using ultrasound gel Aqua Ultra Basic UBQ 5000 Ultragel (Hungary) as a contact medium in all groups. Physical characteristics of ultrasonic exposure by groups: group 1 — low-intensity broadband ultrasound (UMUS), frequency 1–7 MHz, intensity — 30 MW, pulse duration — 50 μs, group 2 — low-intensity pulsed ultrasound (LIPUS), frequency 1.5 MHz, intensity — 30 MW, pulse duration — 200 μs. Group 3 served as a control (without exposure to cells). To excite the ultrasonic transducer, an SDG 2082 X generator from Siglent was used as a source of electrical signals. Ultra-wideband conversion of generator signals into ultrasonic vibrations in the frequency range 1–7 a 15-mode piezoceramic transducer with vibration mode overlap and polarization normal to the radiating surface carried out MHz. The transducer diameter is 20 mm with a maximum thickness of 3 mm. After 3 days of irradiation, the cells were removed mechanically from 5 parallel wells, 3 groups from each exposure option and the corresponding control were combined, live and dead cells were counted and cells were analyzed by routine counting of the ratio of live and dead cells and cytofluorimetric analysis.

Determination of cell concentration and viability by counting cells in the Goryaev Chamber

Cells selected after cultivation were thoroughly mixed, aliquots of 20 μl were taken and 20 μl of 0.4% trypan blue dye solution was added. After staining for 5 min, the cells were thoroughly mixed and introduced with an automatic pipette into a glass coverslipped Goryaev Chamber. Excess solution was removed with filter paper. Cells were counted under a ×10 objective. The concentration of live and dead cells was calculated according to the following formula:

$$X = (a \times 4000 \times c)/b,$$

where X is the number of cells in 1 mm^3; a — the sum of cells counted in a certain volume of the chamber; b — the number of small squares counted; c — dilution of the cell suspension.

Determination of the cell cycle and apoptosis of cultured B16 melanoma cells

On the 5th day after the planting of cells and 3-fold irradiation with two modes (UMUS and LIPUS) of exposure to the cells, cell-biological studies were carried out. To determine the distribution of cells by cycle phases, the level of apoptosis, and proliferative activity, the method of ductal cytofluorimetry was used [8, 9]. At least 5×10^5 cells were used to prepare one sample. Cells were pelleted by centrifugation at 1000 g for 5 min. The supernatant was collected and the cells were washed with physiological or phosphate-buffered saline (PBS, pH 7.2). Cells were resuspended in 200 μl of PBS, 300 μl of citrate buffer (pH 6.8) containing 0.1% Triton X-100 was added. After 1 minute, 10 μl of ribonuclease and 10 μl of propidium iodide (Sigma, USA) were added to stain DNA. Incubate for 10 min at 37 °C in the dark and 30 min at room temperature. It was centrifuged at 1000 g for 10 min and the supernatant was removed. After that, the cells were fixed by adding 400 μl of PBS with 0.4% formalin and the DNA content in the samples was analyzed. To determine the number of cells in different phases of the cell cycle, cytofluorimetric analysis was performed using a flow cytofluorimeter (Becton

Dickinson, USA) equipped with an argon laser ($\lambda_{excitation}$ = 488 nm, $\lambda_{emission}$ = 585/40 nm). Samples were analyzed using the Mod Fit LT 3.0 program (BDIS, USA). The method of determining the level of apoptosis is based on the well-known fact that cells lose a part of deoxyribonucleic acid (DNA) due to its internucleosomal fragmentation in the process of programmed cell death. At the same time, with the help of flow cytometry, the percentage of cells contained in the hypodiploid zone of the histogram is determined (it appears in the form of a fraction located to the left of the main peak, which corresponds to diploid cells), in which cells that lose DNA, that is, those that have entered apoptosis.

Statistical analysis of the obtained research results was performed using Python 3.9.3 software (including the Scipy = 1.13.1 package) and Microsoft Excel. The Student's t-test was used to assess the reliability of the detected changes, the reliability of the values was accepted at $P < 0.05$. The obtained results are presented as $M \pm m$ (mean value ± standard error of the mean).

Results and Discussion

Ultrasonic sonication of B16 melanoma cells was performed in two modes under strictly controlled conditions with the sonication source placed under the bottom of the wells of a 24-well closed plate under laminar flow to maintain the sterility of cultured cells during the period of exposure to the cells. To maintain identical control conditions, the cells were also kept outside the CO_2 incubator for the duration of sounding in the experimental samples. The study of the biological characteristics of the cells was carried out on the 5th day of the general incubation of the cells and 3-fold once a day sounding of the cells.

Survival of B16 melanoma cells of regimes UMUS and LIPUS of "instrumental exposure"

According to the routine calculation of the ratio of live and dead cells after the end of the irradiation, the following was recorded: under mode UMUS, a cytostatic effect on melanoma cells was detected, which consisted in inhibiting cell proliferation by 1.6 times ($P < 0.05$), compared to the control (Fig. 1). The LIPUS regimen had a somewhat lower effect compared to the control by 1.4 times ($P < 0.05$), compared to the control, however, the percentage of dead cells under this regimen was 38.3% against the control, 24.3% for the incubation period, which was 1.5 times more significant than in the control (Fig. 2). As for the UMUS regimen, a tendency to increase the content of dead cells was noted, but due to a large deviation in parallel measurements, no significant difference was found against the control.

That is, when using both modes of sounding B16 melanoma cells, a somewhat higher cytotoxic and commensurate antiproliferative effect with UMUS was recorded for the LIPUS mode. A pro-apoptotic effect was also revealed for this regimen compared to the control (Fig. 3).

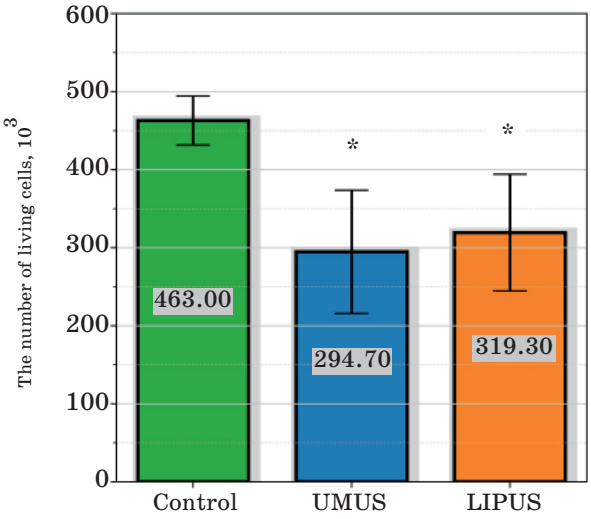

Fig. 1. **The number of living cells (thousands) of B16 melanoma under the influence of modes UMUS and LIPUS of irradiation**
***** — $P < 0.05$, vs control

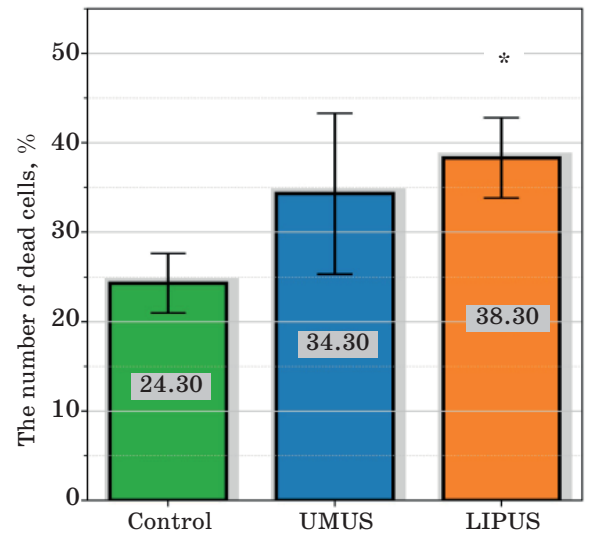

Fig. 2. **The percentage of dead B16 melanoma cells under the effects of modes UMUS and LIPUS of irradiation**
***** — $P < 0.05$, vs control

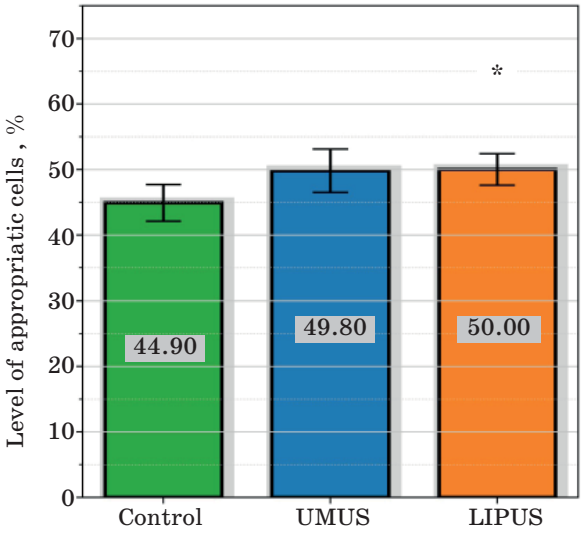

Fig. 3. **The level of apoptotic B16 melanoma cells under the influence of modes UMUS and LIPUS of irradiation**
* — $P < 0.05$, vs control

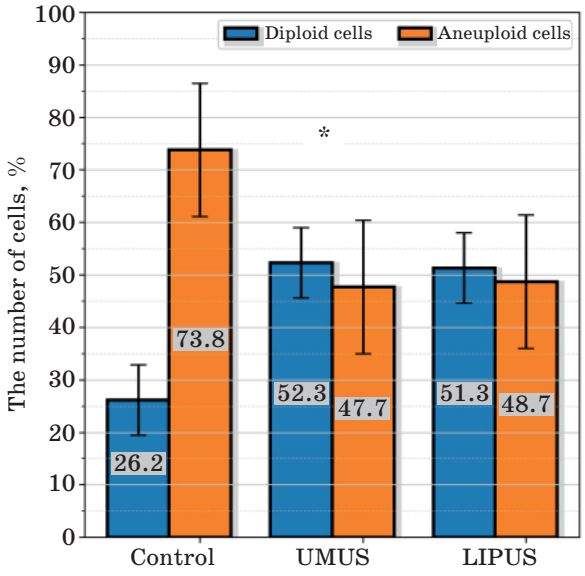

Fig. 4. **The number of diploid and aneuploidy B16 melanoma cells the influence of modes UMUS and LIPUS of irradiation**
* — $P < 0.05$, vs control

Table 1. **The content of diploid and aneuploidy cells in the phases of the cell cycle**

Cell population	Diploid cells			Aneuploidy cells		
Cell cycle phases	G0/G1	G2/M	S	G0/G1	G2/M	S
Control	80.9±16.8	4.5±4.5	14.6±12.3	51.9±2.6	17.4±1.4	30.7±1.3
UMUS	35.6±15.4*	12.9±6.2	51.5±16.2*	37.3 ±9.6*	9.2±5.0	53.5±13.6*
LIPUS	54.8±23.1	27.9±14.6*	17.3±13.8	36.3±23.4	27.7±10.5	36.1±13.0

* — $P < 0.05$, vs control.

It is known that B16 melanoma cells have two subpopulations of cells — diploid and aneuploid. Recently, great importance has been attributed to aneuploidy as a cause of genetic instability of neoplasia [10].

When cytofluorometric analyzing the ratio of aneuploid and diploid cells in the control, it was found to be approximately 3:1 (73.8% and 26.2%, respectively), while under both irradiation regimes, an increase in the content of diploid cells was found: a reliable increase in the content of diploid cells under modes UMUS — up to 52.3 % and unreliable in modes LIPUS due to a high deviation of 51.3±23.3% (Fig. 4, Tabl. 1).

The distribution by phases of the cell cycle for these subpopulations of cells, the following was recorded: the content of both aneuploidy and diploid cells in G0/G1, compared to the control, as well as in the phase of DNA synthesis — S (Table 1) changes during mode UMUS irradiation. Under mode LIPUS, the content of diploid cells in G2/M increases compared to the control.

Differences from the control mode LIPUS were also recorded by the DNA index (DI) - the ratio of the G1 aneuploid peak to the G1 diploid peak (Fig. 5, 6).

DNA ploidy, which reflects the content of DNA in the cell, is a well-known prognostic marker and also forms a part of the WHO 2008 classification as a separate clinical and prognostically distinct entity [11–14]. DNA index (DI), which is associated with DNA ploidy, is a reflection of the average amount of DNA in the tumor cells. DI is calculated by

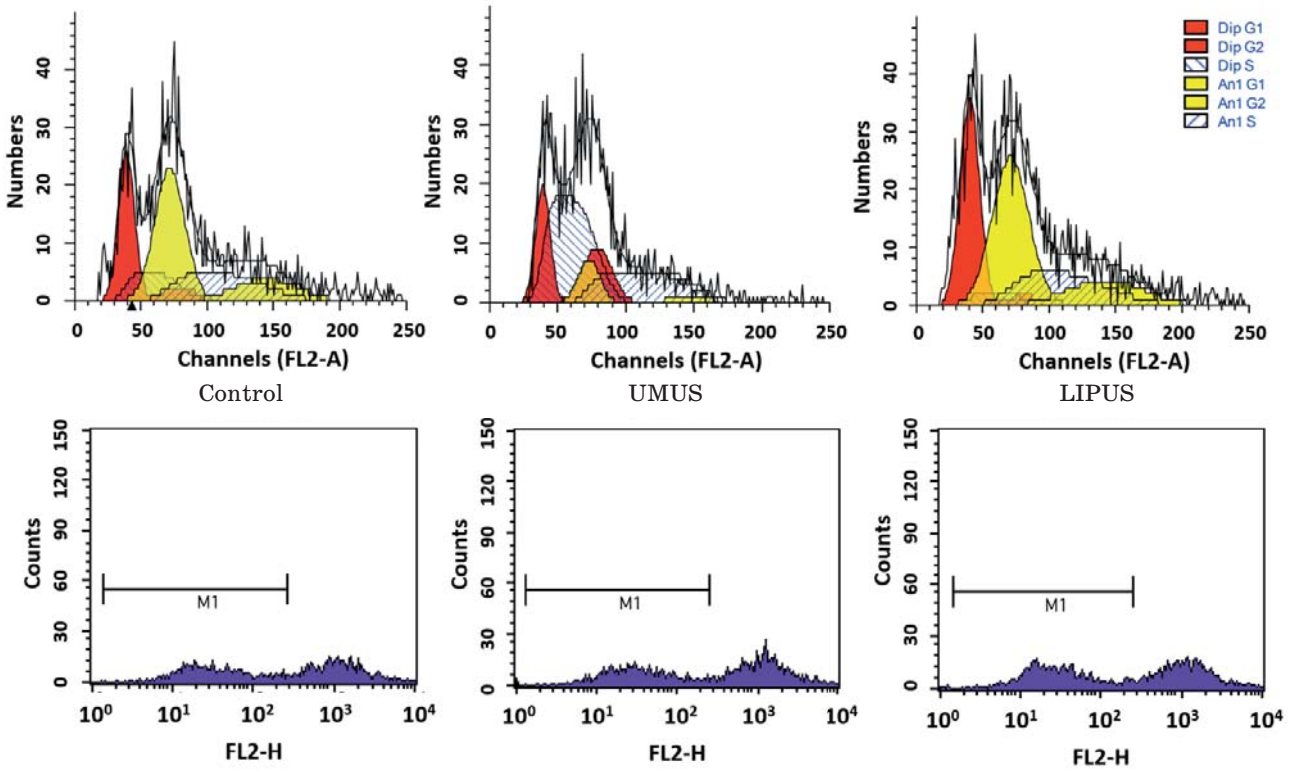

Fig. 5. **Typical histograms of the cell cycle and the level of apoptotic cells of melanoma B16 under the influence of modes UMUS and LIPUS of irradiation**

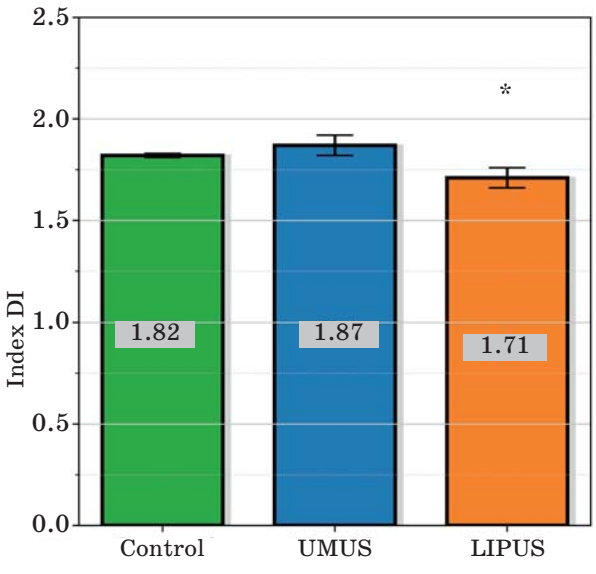

Fig. 6. **DI index (ratio of G1 aneuploid peak to G1 diploid peak) cells of melanoma B16 under the influence of modes UMUS and LIPUS of irradiation**
* — $P < 0.05$, vs control.

dividing the channel number of the G1 peak of the tumor cell by the channel number of the standard (diploid control) G1 peak. If the tumor cells are diploid, the DI is 1, and if the cells are aneuploid, the DI is greater or less than 1. For example, tetraploid cells have a DI of 2 because they contain twice as much DNA as normal diploid cells. cells, and hypodiploid tumor cells have DI < 1.

A decrease in DI in the two modes of ultrasound sounding was found only in the LIPUS mode compared to the control (Fig. 6).

A possible mechanism for the observed changes in the state of B16 melanoma cells under the influence of low-intensity ultrasound may be mechanotransduction. Mechanotransduction is a technique in which cells respond to mechanical stimuli within millisecond intervals necessary to propagate signals into the nucleus [15], where they are converted into autocrine, paracrine, or endocrine signals, which in turn trigger local or endocrine signals, which maintain homeostasis or control cell fate transformation [16].

Conclusions

Thus, as a result of the study conducted on cultured B16 melanoma cells under two modes of cell irradiation, no stimulation of cell growth was recorded, but on the contrary, a cytotoxic/cytostatic and pro-apoptotic effect and a decrease in the content of the population of aneuploid cells, as the main indicator of the progression of B16 melanoma cells, were revealed.

Authors' contribution

Conceptualization, L.G., O.M. and O.K.; resources, I.S., O.G., O.M. and V.H; writing—original draft preparation L.G. and O.K; writing—review and editing, L.G. and O.K.; visualization, I.S., O.G., and V.H.; supervision, L.G., K.V. and O.K. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

All authors have declared no conflicts of interest.

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ПОРІВНЯЛЬНІ ДОСЛІДЖЕННЯ ВПЛИВУ НИЗЬКОІНТЕНСИВНОГО ШИРОКОСМУГОВОГО ТА НИЗЬКОІНТЕНСИВНОГО ІМПУЛЬСНОГО УЛЬТРАЗВУКУ НА КЛІТИНИ МЕЛАНОМИ B16 *in vitro*

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Мета. Оцінити вплив низько інтенсивного широкосмугового ультразвуку (UMUS) та низько інтенсивного імпульсного ультразвуку (LIPUS) на клітини меланоми B16 *in vitro*.

Матеріали та методи. Дослідження проведено на клітинах меланоми B16 при двох режимах низькоінтенсивного ультразвукового озвучування клітин, для чого використовували генератор SDG 2082 X Siglent (США) з діаметром ультразвукового випромінювача 20 мм з частотою для UMUS 1–7 МГц, інтенсивністю 30 мВт/см, тривалістю імпульсу 50 μs, а для LIPUS з частотою 1,5 МГц, інтенсивністю 30 мВт/см, тривалістю імпульсу 200 μs. Обидва режими озвучування клітин проводили триразовим опроміненням, один раз на добу, після чого визначали розподіл клітин за фазами циклу, рівнем апоптозу та проліферативної активності методом протокової цитофлуориметрії.

Результати. За впливу UMUS ультразвукового озвучування виявлено пригнічення проліферації клітин меланоми в 1,6 рази ($P < 0,05$), дворазове зменшення вмісту анеуплоїдних клітин, порівняно з контролем. Однак цитостатичного та проапоптотичного ефектів не зафіксовано. За впливу LIPUS-озвучення виявлено антипроліферативний ефект, збільшення вмісту мертвих та апоптичних клітин та зменшення DI-індексу порівняно з відповідним контролем.

Висновки. За дії низько інтенсивного широкосмугового ультразвуку (UMUS) та низькоінтенсивного імпульсного ультразвуку (LIPUS) виявлено цитотоксичний/цитостатичний і проапоптотичний ефект та зниження вмісту популяції анеуплоїдних клітин.

Ключові слова: широкосмуговий ультразвук низької інтенсивності (UMUS), імпульсний ультразвук низької інтенсивності (LIPUS), цитотоксичний/цитостатичний і проапоптотичний ефект, клітини меланоми B16.