Activation of Bone Marrow Multipotent Stromal Cells by Laser and EHF Radiation and Their Combined Impacts

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The aim of the investigation was to study the effect of laser and extremely high frequency (EHF) radiation on the proliferative activity of bone marrow multipotent stromal cells (MSCs) in "normal" and "suppressed" states *in vitro*, as well as the ability of these factors to influence the content of MSCs in the bone marrow *in vivo* and *in vitro*.

Materials and Methods. Laser radiation of low and moderate intensity, acoustic pulses generated by laser radiation in biological tissue, and EHF radiation have been used for mono and combined (applied for the first time) impacts on MSCs *in vivo* and *in vitro*. Short-term fragmentary laser heating of rat shins *in vivo* has been used to stimulate the colony-forming efficiency of MSCs. Stimulation of proliferative activity and MSCs content were studied on the strains derived from human bone marrow, rabbits, guinea pigs and rats.

Irradiation of MSCs strains was performed in the "normal" state, as well as in the "suppressed" strains with the decreased proliferative activity induced by the reduction of fetal serum concentration in the nutrient medium of the cultivated cells. Exposure doses were varied by altering the power and time of irradiation.

Results. A twofold increase of colony number was observed when the bone marrow was heated by a laser irradiation, and a marked stimulation of colony-forming efficiency exceeding the reference values by 85% under EHF irradiation of bone marrow suspension with the dose of 8 J/cm^2 was also noted.

The effect of physical factors greatly depends on the MSCs state: there is a significant enhancement of proliferative activity of the cells being in the "suppressed" state. Acoustic pulses of laser-induced hydrodynamics cause a statistically significant ($p<0.01$) increase of proliferative activity of human MSCs (by 80% relative to the control). The proliferative activity of human MSCs was not enhanced under combined impacts compared to the exposure to mono acoustic pulses of laser-induced hydrodynamics.

Conclusion. The studied physical effects *in vivo* and *in vitro* increase the content of MSCs in the initial bone marrow, as well as their proliferative activity in the process of MSCs strains development *in vitro*. Application of these techniques in clinic will make it possible to obtain the necessary cell number at earlier passages for autologous MSCs transplantation preventing thereby chromosomal aberrations in MSCs cultures.

Key words: multipotent stromal cells; proliferation activity of stromal cells; colony-forming efficiency; laser radiation; EHF radiation; acoustic pulses of laser-induced hydrodynamics.

At the end of the last century a unique category of stromal precursor cells [1], which were described as the stem cells of the bone marrow stroma or multipotent stromal cells (MSCs) in subsequent investigations [2], was discovered at Federal Research Centre for Epidemiology and Microbiology named after the honorary academician N.F. Gamaleya. The stromal component of the bone marrow is presented by various types of cells. The main types of the bone marrow stroma are reticular cells, as well as endothelial cells,

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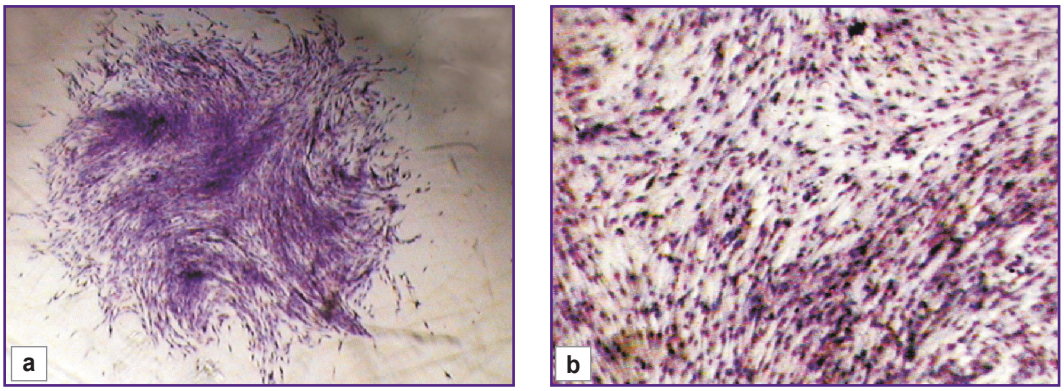

Figure 1. Micrographs of multipotent stromal cells: (a) a clone colony formed by multipotent stromal cells; ×10; (b) the structure of multipotent stromal cell colonies, ×50

adventitia of large vessels and so on. At an explantation of bone marrow into monolayer cultures, reticular cells, attaching to the culture flask, acquire morphology of fibroblasts and form clone colonies consisting of several thousand fibroblasts (Figure 1). The obtained evidence of colony clonal nature [1] allowed researchers to identify the content of these cells in the organs of hemopoiesis and immunity, to study the alterations in their number in various pathological body conditions or effects on the organism (trauma, radiation, etc.).

The interest to the study of the properties and potentialities of MSCs is determined by wide therapeutic capabilities of these cells opening great perspectives of their application in various fields of regenerative medicine [3, 4]. Presently, they are successfully used in traumatology and orthopedics, maxillofacial surgery, and stomatology to restore bone tissue, joint hyaline cartilage, tendon-ligamentous apparatus and other tissues [5, 6]. MSCs and their progeny possess a great proliferative potential [7, 8].

Some authors [9] have shown that no impairments of the chromosomal set in MSC preparations occur at the first passages, but they are found at higher passages — the cells with an altered karyotype, polyploidy and so on. Therefore, the possibilities of effective propagation of MSCs, derived from the donor bone marrow, and subsequent transplantation of these cells into the organism at early passages is of great interest. In this connection, the development of methods of activating proliferative and differentiating potentials of the cells to speed tissue formation and regeneration is one of the key tasks of tissue engineering. Different growth factors and cytokines accelerating cell proliferation and metabolism are traditionally used with this purpose. In recent time, some other approaches are being developed consisting in exposure of tissues and cell cultures to various physical factors capable of stimulating the cell functional activity. In particular, laser radiation of visible and near IR ranges [10–12], extremely high frequency (EHF) radiation [13–17], processes of acoustic pulses of laser-induced hydrodynamic (ALIH) [18, 19] are now being used.

Interest to the study of laser-induced hydrodynamics process effect on the biological objects is connected with the recent advent of new technologies of treating various diseases such as osteomyelitis [19, 20], osteochondrosis [21–23] and others, based on formation of channels in the biotissue by moving the optical fiber end, which is heated by laser radiation. This induces different hydrodynamic processes in the biotissue, in particular, generation of specific acoustic pulses in the range of 0.1–2.0 kHz takes place [19, 24, 25]. Therapeutic action is supposed to be associated with these acoustic pulses and is caused mainly by low frequency mechanical oscillations of the tissue (effects of mechanobiology) [26].

The effect of EHF radiation on various organisms is known to occur only at definite frequencies considered to be active, and the resultant effects may be cumulative [17]. EHF radiation with wavelength of 7.1 mm is commonly used in medical practice.

Combined effects of physical factors on cells and organisms can lead to synergetic or antagonistic results [27, 28]. It depends on the parameters of the exposures, their sequence, intervals between them and on the object condition. Combined effects on the stem cells have not been examined so far despite the interest shown in such works. In the present study the attempt was undertaken to investigate the combination of acoustic pulses and EHF radiation. Both these factors may be referred to the category of low-intensity non-thermal exposures.

Obviously, the explantation of bone marrow into the tissue culture impairs the MSCs microenvironment. Transfer of these cells from their natural niche maintaining their stem status to a new one, for example, to the tissue-engineered construct, can result in alteration of their status. As a consequence, “suppression” of MSCs, undesirable alteration of their proliferative and differentiating potentials may occur. This fact shows the necessity of investigating the effects of diverse physical factors on both “normal” and “suppressed” MSCs.

The aim of the investigation was to study the effects of laser radiation of low and moderate intensity,

laser-generated acoustic pulses, EHF radiation and combinations thereof on the alteration of proliferative activity of multipotent stromal cells *in vitro* in the states conventionally called "normal" and "suppressed", and to assess the ability of these factors to increase MSCs content in the bone marrow under the exposure *in vivo* and *in vitro*.

Materials and Methods. The objects of the investigation were bone marrow MSCs of humans, rabbits, guinea pigs and rats.

Ethical principles. All experimental operations were performed under anaesthesia using legal veterinary preparations (Zoletil and Rometarum) and observing all rules of asepsis and antisepsis. The work was performed in accordance with ethical principles established by European Convention for the Protection of Vertebrata used for Experimental and other Scientific Purposes (the Convention was passed in Strasburg, March 18, 1986, adopted in Strasburg, June 15, 2006) and approved by Ethics Committee of the Federal Research Centre for Epidemiology and Microbiology named after the honorary academician N.F. Gamaleya, Ministry of Health of the Russian Federation.

Animals were withdrawn from the experiment by narcosis overdose.

Obtaining MSC strains of the human bone marrow. Human bone marrow was received from the clinic of the Central Research Institute of Stomatology and Maxillofacial Surgery, Ministry of Health of the Russian Federation with the informed consent of the patient. The obtained human bone marrow trepanation was placed in the vial with a fresh nutrient medium, monocellular suspension was prepared with a syringe, filtered through a four-layer capron filter, and the total number of the cells was counted. The cells were explanted in the plastic vials (Nuc 80 cm^2; Nuc, Denmark) containing 15 ml of complete culture medium consisting of 80% alpha-MEM nutrient medium (Sigma-Aldrich, USA), 20% fetal bovine serum (FBS) (Hyclone, USA) and antibiotics calculated as $(3.5–4.0)\cdot10^4$ cells per 1 cm^2 of the vial bottom area. The cells were cultured at 37°C in 5% CO_2 atmosphere. On days 12–14, when discrete colonies of stromal fibroblasts were forming in the vials, the first passage was performed using standard methods [7]. Once a confluent layer was reached by the cells, the next passage was conducted.

Obtaining bone marrow strains from rabbits and guinea pigs. A pelvic bone wing was removed in a rabbit under local anesthesia by 0.5% novocaine solution in aseptic conditions and placed on a Petri plate. In the box, the wing was splitted by a scalpel, the bone marrow was scraped out and transferred to the vial with a nutrient media. Guinea pigs and rats were put to sleep, thigh bones were isolated under aseptic conditions, the bone ends were cut and the bone marrow was flushed away by a syringe into the nutrient medium. Subsequent manipulations were similar to the procedures done for obtaining MSCs strains of the human bone marrow.

Obtaining MSCs strains in the altered "suppressed" state. Fibroblast colonies, formed by day 12–14 after explantation of the human or rabbit bone marrow, were subject to passaging in the optimal culturing conditions — in the medium consisting of 80% alpha-MEM medium, 20% FBS and antibiotics. At the next passage, the removed cells were divided into two parts and explanted into cultural vials with a nutrient medium of various compositions. In the first case, the culture medium consisted of 80% of alpha-MEM medium and 20% FBS and was designated by us as "normal", in the second case, it consisted of 97% alpha-MEM and 3% FBS and was designated as "suppressed". Further, these cultures were managed concurrently up to IV–VI passages. Such significant reduction of FBS resulted in the stable decrease (about threefold) of cell proliferation. Prior to MSCs exposure to low-intensity fields, the cultures were treated by 0.25% trypsin and removed from the plastic. 3 ml of MSCs suspensions, containing $3.0\cdot10^5$ cells, were poured into 15-mm-diameter plastic test tubes. The cell suspension or precipitated cells were exposed to radiation.

Investigation of MSCs proliferation stimulation after exposure. MSCs from every tube with irradiated cells and the tubes with the control cells were explanted into three 25-cm^2 culture vial by $1\cdot10^5$ cells in each, then cultivated in the incubator at 37°C and 5% CO_2 for 5 days. After cultivation, the cells were removed from the plastic and the number of the grown cells was calculated.

Investigation of MSC content in the rat bone marrow after exposure. On day 3, the animals were withdrawn from the experiment, the exposed tibial bones and contralateral unexposed control shins were isolated. In each series of tests, bone marrow cell suspension common for the three rats was prepared. Cell suspension of the bone marrow from the contralateral shins of the same rats was prepared in a similar way. $5\cdot10^5$ of bone marrow cells were explanted into 25 cm^2 vials containing 5 ml complete culture medium with antibiotics and placed into the incubator. When cultivation was accomplished, (on days 12–14) the vials were washed twice with saline solution, fixed with 70° ethanol for 30–45 min, stained according to Giemsa method, and the number of the grown colonies was calculated using binocular magnifier.

Fragmentary thermal laser effect on the bone marrow cells in vivo. Investigations were carried out on Wistar male rats weighing 100–120 g. The tibia bone was aseptically exposed under ether narcosis. The bone marrow was locally irradiated through the bone in 10 points of its upper third. The points were located at a distance of 1 mm from each other in two parallel lines positioned along the axis of the upper bone part. Contact irradiation was done by means of optic fiber having 0.6 mm in diameter. A fiber laser (IRE-Polus, Russia) with 1.56 μm in wavelength was used for this purpose. The duration of laser pulse on each point was 0.5 s. In this way fragmentary exposure was performed — the intact tissue was left around the areas of laser heating.

The animals were divided into 4 groups according to the 4 irradiation doses: 3 rats for each dose. The laser irradiation power was: 0.1, 0.2, 0.6 and 1.2 W. Totally, 5 series of irradiations were conducted. The measured parameter was a colony-forming activity of MSCs, i.e. the ratio of the number of colonies, grown from the suspensions of irradiated and non-irradiated bone marrow, to the number of explanted cells.

The temperature field being formed in the bone marrow was measured by a thermocouple under the conditions modeling experiments *in vivo*.

Factors of low-intensity physical impact on the bone marrow MSCs

1. *He-Ne laser radiation.* A batch-produced LGN-213-1 laser (Russia) with radiation 0.63 μm in wavelength and 0.8 mW in power was used for irradiation. A test tube with 3 ml MSCs suspension was placed in the divergent laser beam with 0.4 mW/cm^2 in power density and exposed for 60 s. Laser radiation power was controlled with the help of FieldMaster power meter using LM-2VIS power meter head (Coherent Inc., USA).

2. *Acoustic pulses of laser-induced hydrodynamic processes* were generated by means of a specially developed ALIH apparatus. Initially, an acoustic signal generated during the moving of the optic fiber with laser radiation 0.97 μm in wavelength along the bone marrow canal of the calf shin bone was recorded. Radiation power was 5 W, and an absorbing coating was applied to the fiber end [19]. The tube with suspension or precipitated MSCs was placed in the apparatus cuvette filled with water. The signal, recorded into the apparatus memory unit and amplified, generated acoustic pulses in the water with the help of piezoceramic transducer. MSCs were exposed for 50 s.

3. *EHF radiation.* Apparatus (Akvastin; IRE-Polyus, Russia) with radiation 7.1 mm in wavelength and output power density of the radiator, horn and rod antenna of 5, 0.5 and 3 mW/cm^2, respectively, was used in the work. The tubes with precipitated MSCs were installed on the radiating end of the apparatus head and exposed for 30 s.

To study the effect of EHF on the MSCs colony-forming efficiency *in vivo*, the bone marrow suspension was prepared by a standard technique. The rod antenna of the EHF radiator was placed over the suspension, which was mixed by a magnetic stirrer. The exposure time was 15, 45 and 90 min. After 15 min of exposure, it was stopped, the necessary number of cells was selected for the explantation, and then the exposure was continued. The same procedure was done in 45 and 90 min. 5·10^5 cells were seeded into 25 cm^2 plastic vials with complete culture medium and cultivated in a standard mode for 12–14 days.

The effect of EHF radiation on the proliferative activity of bone marrow MSCs of guinea pigs was studied using horn antenna for cell exposure. Each well of 6-well plate was seeded with 5·10^4 cells of MSCs strains and allowed them to adhere to the plastic for 60 min prior to the exposure. The antenna of the EHF apparatus was placed either above, irradiating MSCs through the millimeter layer of the culture medium, or below, putting it directly to the plastic. In the first case, a power of EHF radiation was significantly reduced due to the absorption by the culture medium. In the second case, the radiation reached MSCs actually without any loss, as the plastic insignificantly absorbs EHF radiation. The horn antenna diameter fully matched the diameter of the well with the cells. The cells were exposed for 0.5, 1, 3 and 9 min. Wells with the same quantity of unexposed explanted cells served as the controls. The cultivation was discontinued on day 4 after the exposure. The grown cells were removed from the plastic and counted.

Combined effect. The experiments were carried out in different sequences: ALIH+EHF, EHF+ALIH. In the first case, the cells were exposed to acoustic pulses of ALIH apparatus for 50 s, and then, in a 60 s interval, to EHF radiation for 30 s. In the second case, the exposure was performed in reverse order.

Statistical data processing. Differences between separate samples were tested using nonparametric Mann–Whitney U-test (ANOVA). Mean values and standard deviations were calculated by the results of the measurements. In some cases, relative values were presented on histograms when mean results for the exposed cells were normalized to the mean values for the appropriate control experiments considering their mistakes.

Results and Discussion. The dynamics of the temperature growth in the bone marrow, presented for the two different laser powers on Figure 2 (a) demonstrates that the tissue is monotonically heated during the laser pulse action and is cooled up gradually

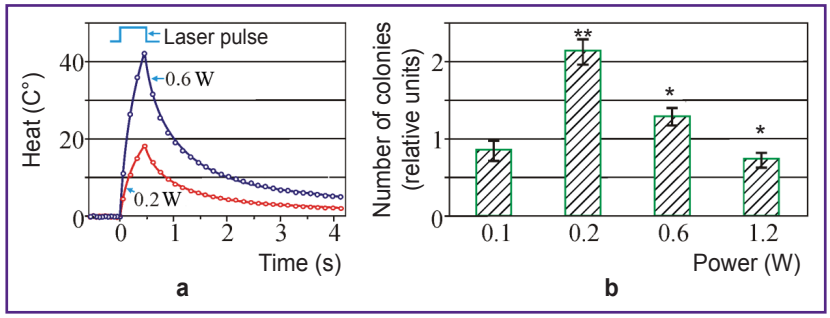

Figure 2. Influence of short-term thermal laser exposure on the colony-forming efficiency of multipotent stromal cells in the bone marrow of the rat shin: (a) dynamics of temperature alteration in the bone marrow under the action of 0.5-s laser pulse duration at various laser radiation powers; (b) a relative change of the number of multipotent stromal cell colonies depending on laser radiation power under fragmentary thermal exposure. Value *1* on y-axis corresponds to the control; statistically significant difference from the control: * p<0.2; ** p<0.01

to the initial temperature after the pulse has ended. When laser power increases from 0.2 to 0.6 W, maximal heating of the bone marrow tissue grows monotonically from 17 to 42°C, respectively.

Dependence of relative change of MSCs colony number in the bone marrow of the rat shin on the laser radiation power under fragmentary thermal laser exposure is presented on Figure 2 (b).

In the specimens exposed to 0.1 W power radiation, the number of colonies grown *in vitro* does not change. At 0.2 W power, a twofold increase of colony number ($p<0.01$) is observed, showing a significant rise of the colony-forming cell number compared to the control specimens. This effect reduces with the increase of the power to 0.6 W, whereas at the power of 1.2 W a slight suppression is noted ($p<0.2$).

The growth of MSCs in the bone marrow under a short-term fragmentary laser heating to ~60°C (at 0.6 W and ~2 s heating time) is likely to be caused by their stimulation directed to the restoration of the partly impaired homeostasis. When irradiated at 1 W for 5 s, the tissue temperature rises to ~100°C, and the volume of overheated tissue also increases. Such a significant growth of the temperature and overheated tissue volume seems to cause much greater damage to the bone marrow. In such conditions, cells left intact in case of local heating, are not already able to restore homeostasis and the quantity of MSCs diminishes. This is the way the mechanism of hormesis is displayed, and being sufficiently universal, is thought to be realized in our experiment.

The increase of MSCs colony-forming efficiency is also noted under the action of EHF radiation on the bone marrow cell suspension of the rats using a rod antenna (Figure 3). Irradiation by 8 J/cm^2 for 15 min did not stimulate the cell colony formation, a 45-min irradiation resulted in the marked stimulation of the colony-forming efficiency exceeding the control values by 85%, whereas a 90-min exposure caused its essential inhibition.

The results of studying the effect of low-intensity laser radiation, EHF radiation as well as ALIH processes on the proliferation of the human bone marrow MSCs in the "normal" and "suppressed" conditions (Figures 4, 5)

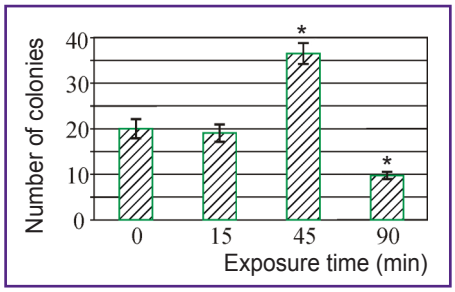

Figure 3. The number of multipotent stromal cell colonies in the rat bone cell suspension depending on the time of EHF radiation exposure; * statistically significant difference from the control, $p<0.01$

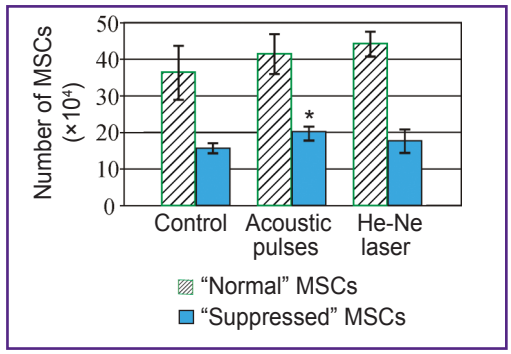

Figure 4. Proliferation of the "normal" and "suppressed" human multipotent stromal cells (MSCs) after the exposure to acoustic pulses and He-Ne laser radiation; * statistically significant difference from the control, $p<0.01$

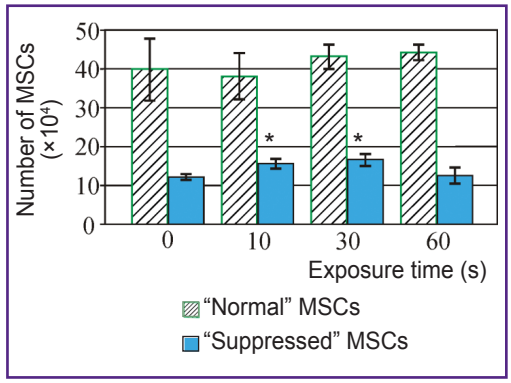

Figure 5. Proliferation of the "normal" and "suppressed" human multipotent stromal cells (MSCs) after the exposure to EHF radiation using various exposure time; * statistically significant difference from the control, $p<0.01$

show that mean values of MSC number after the action of various physical factors are higher than the control values almost for all experiments. Great statistically significant ($p<0.01$) differences from the controls are registered when "suppressed" MSCs were exposed to acoustic pulses and EHF radiation. Proliferative activity of the "suppressed" MSCs after the impact of acoustic pulses for 60 s (See Figure 4) and EHF radiation for 10 s (See Figure 5) grew by 30%. Increase of EHF radiation time to 30 s enhanced MSCs proliferative activity to 40% relative to the controls, and 60-s exposure leveled the positive effect.

The results of our investigations show that the effect of various low-intensity physical factors (low-intensity laser radiation, EHF radiation and ALIH processes) greatly depends on the state of the cells themselves. Physical factors did not practically influence MSCs in the "normal" state, but increase their proliferative activity in the "suppressed" state.

Exposure of rabbit MSCs to EHF radiation and acoustic pulses, including their combination, did not result in the enhancement of the cell proliferative activity

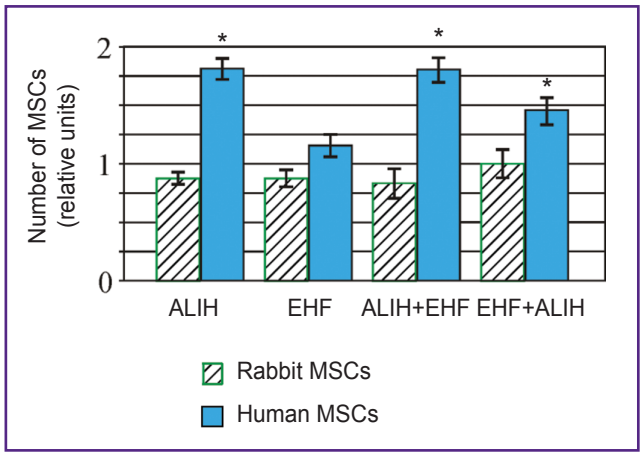

Figure 6. Proliferation of human and rabbit multipotent stromal cells (MSCs) after the impact of different physical factors: acoustic pulses and EHF radiation. Values normalized to the control are shown on y-axis; * statistically significant difference from the control, p<0.01

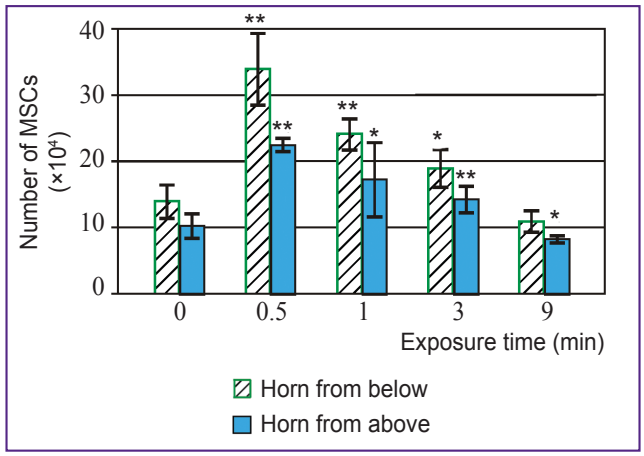

Figure 7. Proliferation of multipotent stromal cells (MSCs) of guinea pig bone marrow after the exposure to EHF radiation using various exposure time and horn position of the emitter antenna; statistically significant difference from the control: * p<0.05; ** p<0.01

at p<0.05 (Figure 6). Moreover, a tendency to inhibition of MSCs growth was observed under the influence of these physical factors, with the exception of the combined impact in the EHF radiation + sequence of acoustic pulses.

However, statistically significant (p<0.01) enhancement of the cells proliferative activity by 80% relative to the controls was registered for human MSCs in all cases of using acoustic pulses. The obtained result, associated with a positive effect of the specific ALIH processes on the human MSCs, has been an expected one for us. Laser-induced hydrodynamics processes are the main therapeutic factor when biotissues are exposed to moderate power laser radiation. It is acoustic pulses accompanying the laser-induced hydrodynamics processes that are supposed to trigger regeneration of the tissue according to the mechanism of mechanobiology.

The study of the combined effects of ALIH and EHF radiation showed that there was no increase of the human MSCs proliferative activity compared to the acoustic pulses mono impact (See Figure 6). Moreover, while the combined effect in the sequence of ALIH+EHF does not significantly change the MSCs proliferation rate, the inverse sequence (EHF+ALIH) reduces significantly (by 20%) the proliferation rate. The latter indicates to antagonicity of the dose of EHF radiation and acoustic signal.

The EHF irradiation of the adhered cells with the help of the horn antenna demonstrated, that their growth did not depend on the horn position. The greatest cell increase number (~2.3 times) was noted in the wells exposed during 0.5 min. The MSCs proliferative activity was lower and exceeded the control values by 1.7 and 1.3 times when irradiation lasted 1 and 3 min, while 9-min exposure resulted in the inhibition of the cell growth (Figure 7).

Conclusion. Application of the physical factors (the low and moderate intensity laser radiation, acoustic pulses generated by laser radiation, and EHF radiation) *in vivo* and *in vitro* increase the content of multipotent stromal cells in the initial bone marrow, and enhance their proliferative activity in the process of cell strain development *in vitro*. Employment of these techniques in clinics will make it possible to obtain the necessary cell number at earlier passages for autologous MSCs transplantation and prevent thereby chromosomal aberrations in the cell cultures.

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Conflicts of Interest. The authors have no conflicts of interest related to this study.

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