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# Combined Light and Thermal Stimulation of Bone Marrow Stem Cells

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**Abstract**

**Introduction:** The purpose of this study is to achieve a significant increase in the proliferative activity of mesenchymal stem cells (MSCs) of the bone marrow (BM) at early passages after laser exposure to a suspension of these cells and to estimate the effect of light and heat components of laser radiation on the proliferation of BM MSCs.

**Methods:** The studies were performed on rats BM MSCs. MSC suspension was placed into the wells and heated by using laser radiation (980 nm wavelength) or a water bath at 70 °C providing similar temperature dynamics. The studies were carried out in 3 comparison groups: (1) control suspension of MSCs, which was not subjected to heating in a water bath or laser exposure; (2) MSC suspension, which was heated for in a water bath; and (3) suspension of MSCs, which was subjected to laser exposure. The exposure times for the 2nd and 3rd experimental groups were 10- 50 seconds.

**Results:** Under optimal parameters of laser action on the suspension of BM MSCs, a six-fold increase in the number of BM MSCs colonies was registered compared to the control. The role of the light and heat components of laser exposure to MSCs was determined by comparable heating of a suspension of BM MSCs in a water bath, at which only a twofold increase in the number of colonies was maximally obtained.

**Conclusion:** The increase in the MSC proliferation activity occurs due to their Thermo-Photobiomodulation. The result obtained is important for practical use in cell transplantation in the treatment of traumatic injuries of bone, cartilage, and tendon tissues when a rapid and multiple increase in the initial number of autologous BM MSCs is required.

**Keywords:** Bone marrow mesenchymal stem cells; Laser heating; Thermo-photobiomodulation; Cell therapy.

## Introduction

Mesenchymal stem cells (MSCs) are currently widely used in medical practice[1] for the treatment of wounds,[2] regeneration of intervertebral discs,[3] bone and cartilage tissues,[4] diabetic retinopathy,[5,6] spinal cord injury,[7,8] acute respiratory distress syndrome,[9] and so on. The efficacy and safety of treatment for a specific disease depend on the choice of MSCs source (fat, bone marrow [BM], allogeneic umbilical cord, etc).[10] Thus, for the restoration of bone, cartilage, and tendon tissues, the best option is to use BM stromal cells, which demonstrate a higher ability to differentiate into chondrogenic and osteogenic cell types than adipose MSCs.[8,10,11] In this case, autologous MSCs are transplanted from the patient's BM. Unfortunately, the proportion of MSCs among BM cells is only ~$10^{-4}$, while for therapeutic use, it is necessary to transplant $10^8$-$10^9$ cells into the area of bone damage. This amount of BM MSCs is achieved by initial cultivation of the patient's native BM with further multiple passaging (15-20 passages). This increases the likelihood of cell polyploidy and other chromosomal aberrations. To exclude such undesirable changes, it is necessary to increase the number of MSCs sufficient for reverse transplantation in 3-5 passages.[12,13]

To implement this therapeutic task, researchers are developing various methods for activating the proliferative and differentiation potentials of cells. In this regard, many research teams have studied the effects of various physical effects on MSCs, such as ionizing radiation,[14] EHF radiation,[15] low-intensity laser radiation (LILR) in the visible and near-IR ranges,[16-22]

pulsed ultrasonic exposure,[23] thermal heating,[24] and other influences.[25]

Previously, we conducted studies demonstrating that the fragmental (fractional) thermal effect of laser radiation with a wavelength of $\lambda = 1.56$ μm on the BM of rats in vivo makes it possible to increase the content of MSCs by two times.[26] In the study conducted by Chailakhyan et al,[27] the change in the number of MSCs in the BM at different times was studied after local exposure

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to infrared laser radiation with λ = 980 nm of moderate power on the BM of rats in vivo. It was shown that the content of MSCs increased from the moment of exposure for approximately two days and then decreased to the initial value within approximately 8 days.

In cases of traumatic bone injury, cells must be transplanted into the injured area as quickly as possible. We believe that a further increase in the number of these cells after laser irradiation and removal of the patient's BM for reverse transplantation can be achieved at early passages with laser thermal exposure (λ = 980 nm) to a suspension of BM MSCs in vitro.

In our study, we aimed to achieve a significant increase in the proliferative activity of BM MSCs after short-term laser exposure to a cell suspension at a wavelength of 980 nm of moderate power and to assess the role of light and heat components in the mechanism of action of laser radiation.

## Materials and Methods

The studies were carried out on BM MSCs, obtained from three male Wistar rats weighing 300-350 g, from the Stolbovaya nursery (Moscow Region). All experiments were carried out in accordance with the European Convention (Strasbourg, 1986) and the Helsinki Declaration of the World Medical Association on the Humane Treatment of Animals (2000). All animals were kept under standard vivarium conditions and provided with granulated laboratory food and constant access to water.

### *Preparation of Cell Suspensions and Explantation of Bone Marrow Cells*

In the experiments, we used a suspension of BM MSCs from three rats, prepared as follows. The animals were euthanized by an overdose of anesthesia, and the femurs were isolated under aseptic conditions. Next, the epiphyses of the bones were cut off and the BM was washed into a nutrient medium with a syringe. A single-cell suspension was prepared by sequential passage through a syringe with a decreasing needle diameter, filtered through a 4-layer nylon filter, and the total number of cells was counted. All the culture experiments were performed under sterile conditions.

The cells were explanted into culture flasks (Nunc 80 $cm^2$) at a rate of $3.5–4.0 \times 10^4$ cells per 1 $cm^2$ of the bottom area of the flask. The vials contained 15 ml of complete culture medium, consisting of 80% alpha-MEM culture medium (Sigma), 20% fetal bovine serum (FBS) (Hy clone), and antibiotics. Cultivation was carried out at a temperature of 37 °C in an atmosphere of 5% $CO_2$. On days 12-14, when discrete colonies of MSCs formed in the flasks, the 1st passage was performed. The flasks were washed with Hanks' solution, treated with 0.25% trypsin solution for 30 seconds, turned over, and kept in trypsin vapor for 30 minutes at 37 °C. Trypsin was then drained and MEM was added, which was used to wash the cells from the surface of the flask. The resulting suspension was pipetted until a single-cell suspension was formed.

The number of cells was determined by using a Countess™ Automated Cell Counter (Invitrogen, USA), after which $5.0 \times 10^5$ cells were transferred into new flasks with a culture medium. When the cells reached confluence, the next passage was performed. The MSC suspension of the second passage was obtained by the method described above. The cells were counted, and a working suspension, which was subsequently used throughout the experiment, was prepared. At the end of the experiment, which lasted for 3 hours and 40 minutes, the number of MSCs in 1 mL of the suspension and the number of live and dead cells were recalculated. A comparison of the number of cells at the beginning and end of the experiment revealed their identity. Thus, the working suspension of the MSCs remained stable and did not introduce errors in the experimental results. During the experiments, the MSC suspension (0.5 mL of MSC suspension in a nutrient medium containing $5 \times 10^5$ cells) was placed into the wells of a 24-well plate. The thickness of the suspension layer in the wells was approximately 2.8 mm. Then the suspension was heated by using laser radiation or a water bath heated to 70 °C.

### *Laser Exposure*

A fiber laser (LS-0.97, IRE Polus, Russia) with a wavelength of 980 nm and maximum power of 60 W was used. Laser radiation was delivered by an optical fiber with a core diameter of 0.6 mm and a numerical aperture of 0.22. The output end of the optical fiber was located at a distance of 55 mm from the surface of the suspension. In this case, the diameter of the laser beam in the plane of the suspension surface was approximately 15 mm, which corresponded to the diameter of the well. The radiation power was controlled by using a PM10 meter (Coherent, USA), and the exposure time was controlled by using a stopwatch. In all the experiments, the output power of the laser radiation was set to 12 W, and the irradiation dose was determined by the time it took to reach the desired temperature. This choice of radiation power was due to the need to ensure uniform heating of the suspension in the well of the plate to a temperature of approximately 60 °C in approximately one minute. It is necessary to note that the attenuation of the intensity of radiation with a wavelength of 980 nm at a suspension thickness of ~2.8 mm was approximately 12.6%. This allows us to state that the suspension is heated by laser radiation, not uniformly over its thickness.

Laser irradiation of the MSCs suspensions was performed in a sterile box. Laser radiation was delivered inside the box by using a sterilized optical fiber. A closed plate prepared under sterile conditions, with wells filled

with a suspension of MSCs, was transferred to a box in order for us to implement laser exposure. During the irradiation, the lid of the plate was removed.

### *Water Bath Heating*

To elucidate the role of the thermal component of the action of laser radiation with λ = 980 nm on the proliferative abilities of BM MSCs in vitro, we performed a comparative study of their heating in a water bath. In this case, using a sterile box turned out to be problematic. Therefore, the water bath was placed in the laboratory room, and heating was performed on a plate closed with a standard plastic lid. Our estimates showed that the presence of a lid did not change the heating dynamics of the MSC suspension obtained at the first stage of our experiment

To heat the suspension in a water bath, a cylindrical metal vessel with a diameter of 25 cm, a volume of 10 L, 3/4 filled with water was used. The vessel was installed on a stove with adjustable power. By gradually increasing the power of the tile, we reached conditions when the temperature of the water surface stabilized at approximately 70 °C. The closed plate with the wells in which the MSC suspension was placed was partially immersed in heated water. The suspension was heated by transferring heat from water through the polymer bottom of the well with a thickness of 0.8 mm.

At the first stage of this work, we studied the conditions under which the temperature dynamics of the surface of the MSC suspension in the cases of heating with a laser and water bath coincided were determined. The temperature dynamics of the suspension surface layer (~20 μm) were monitored by using a FLIR 655sc infrared camera (Sweden) with a frequency of 25 Hz and an accuracy of 0.05 °C. The operating wavelength range of this camera is 7.5-14 μm.

Figure 1 shows the schemes of the preliminary experiments in which the modes of heating the suspension by laser radiation (A) and water bath (B) were selected. In both cases, the IR camera recorded the temperature field of the near-surface layer of the suspension over the entire heating period.

Figure 2a shows the fragments of infrared camera frames reflecting the temperature fields of plate wells filled with a nutrient medium in cases of heating with a laser (left) and a water bath (right) when the temperature of the nutrient medium reaches approximately 40 °C.

In Figure 2b, the increase in the temperature of the near-surface layer of the suspension with respect to room temperature (24 °C) in the cases of heating by laser radiation and a water bath heated to 70 °C is shown as a function of the time. One can see that during the first 50 seconds, the scenarios of temperature change in both cases were very close.

### *Study Groups*

The studies were carried out in three comparison groups: (1) control suspension of MSCs, which was not subjected to heating in a water bath or laser exposure; (2) MSC suspension, which was heated for a specified time in a water bath; and (3) suspension of MSCs, which was subjected to laser exposure for a specified time. The

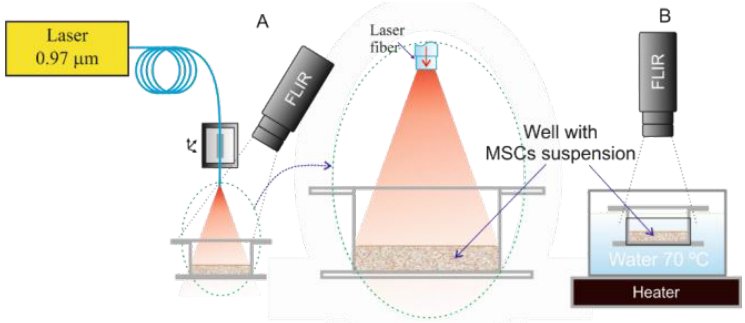


**Figure 1.** Schemes for Measuring the Heating Dynamics of an MSC Suspension Using Laser Radiation (a) and a Water Bath (b)

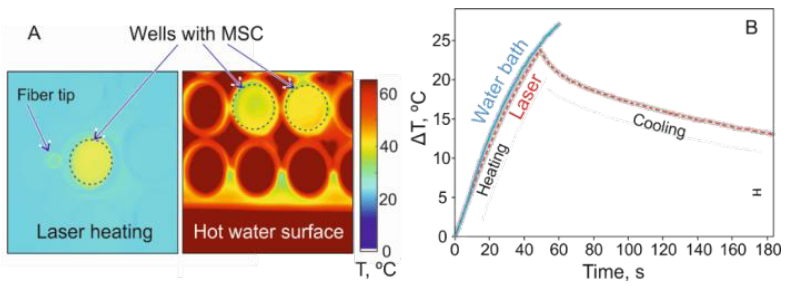


**Figure 2.** Heating of the MSC Suspension by Laser Radiation And Using a Water Bath. (A) thermographic images obtained during the heating of wells with MSC suspension. (B) Heating dynamics of the MSC suspension in the wells. The line widths correspond to the 95% confidence interval. The dynamics of cooling the MSC suspension after switching off the laser radiation is shown

exposure times for the 2nd and 3rd experimental groups were 10, 15, 20, 25, 30, 35, 40, 45, and 50 seconds.

### *MSC Explantation Into Flasks, Fixation, Staining and Colony Counting*

After physical exposure, 100 ± 10 cells were seeded in culture flasks of 25 $cm^2$ in three replicates (three flasks) for each exposure dose, as well as for the control group, to determine cell survival and proliferative potential.

We used a small number of cells per flask to ensure the discrete growth of colonies[28] in the flask and their reliable visual count. The standard deviation (variance) of the number of cells from the mean value was ± 10. On the 8th day, vials with colonies were fixed with 96% alcohol and stained with azure-eosin according to Romanovsky. The stained colonies were counted under an MS-1 ZOOM microscope (Biomed, St. Petersburg, Russia). Photographs of vials with fixed and stained colonies were taken by using a Canon EOS 650D digital camera (Japan).

Statistical processing of the experimental data for the control and experimental groups was performed by the GraphPad Prism 7.00 package for Windows (GraphPad Software Inc.). Quantitative data that did not pass the Shapiro-Wilk normality test were analyzed by using the Kruskal-Wallis test on ranks. Differences between the data from the control and both experimental groups were analyzed by means of two-way ANOVA followed by Tukey's multiple comparison test at the selected significance level of $P < 0.05$.

## Results and Discussion

Well-defined colonies were observed in flasks eight days after the explantation of intact and physically exposed cells. Figure 3 shows the photographs of stained MSC colonies that grew after 8 days in the control experiment and after heating the initial cell suspension in a water bath for 10 seconds and using laser radiation for 40 seconds when 100 ± 10 cells were seeded.

Studies of MSC colony formation showed that 98 ± 8 colonies were formed in control vials after 8 days. This corresponds to the number of seeded cells, considering the dispersion of their number in the sampling of the corresponding volume of suspension with a normal distribution. In general, the number of colonies in flasks with cells that were heated by laser radiation or in a water bath significantly exceeded the number of initial cells. Thus, for the case shown in Figure 3, the number after heating with a water bath for 10 seconds was approximately 200, and after laser heating for 40 seconds, it was approximately 600.


**Figure 3.** Photographs of vials with MSC colonies 8 days after explantation of 100 cells. Shown are a vial with intact cells (Control), a vial with cells heated in a water bath for 10 seconds (Water Bath), and a vial with cells heated by laser for 40 seconds (Laser).

It should be noted that colonies can differ significantly in terms of diameter and number of cells. Figure 4 shows the micrographs of individual colonies grown in control flasks, which can be conditionally characterized as small (a), medium (b), and large (c). The stained nuclei of the individual cells were clearly visible. Estimates of the number of cells in these colonies yielded the following approximate values: ~60, ~600, and ~4000, respectively. The density of MSCs in the colonies also increased with their size, especially in the central regions.

As shown in Figure 5, laser exposure of MSCs for 10, 20, and 25 seconds reduced the efficiency of colony formation. At this interval, the exception was laser exposure for 15 seconds, which led to a significant increase in the number of colonies compared to the control values by almost two times.

In the time interval of laser heating of 30–50 seconds, a powerful peak was distinguished, with a maximum at 40 seconds, at which a ~6-fold increase in the number of colonies compared to that of the control was recorded. On the right wing of this broad peak, a gradual decrease in the efficiency of colony formation was observed, and even with a 50 second laser exposure, the number of colonies exceeded the control values by approximately two times.

For heating the MSC suspension in a water bath, as shown in Figure 5, exposure for 10 seconds led to a 2-fold increase in the efficiency of colony formation. With a further increase in heating time, this parameter gradually decreased. Heating in a water bath for up to 40 seconds led to an increase in colony formation efficiency. At an exposure of 45 seconds, the number of colonies did not differ from that of the control. Maintaining the MSC suspension in a water bath for 50 seconds led to significant suppression of colony formation (the efficiency decreased to 75%).

Thus, experimental studies showed that both types of influence on the MSC suspension (laser irradiation and heating in a water bath) led to significant changes in the efficiency of colony formation (Figure 3). At the same time, the dependence of this parameter on time for these types of impacts differed significantly (Figure 5).

It should be mentioned that the laser exposure parameters were chosen in such a way that the temperature growth curves of the MSC suspension upon heating by laser radiation and in a water bath were the

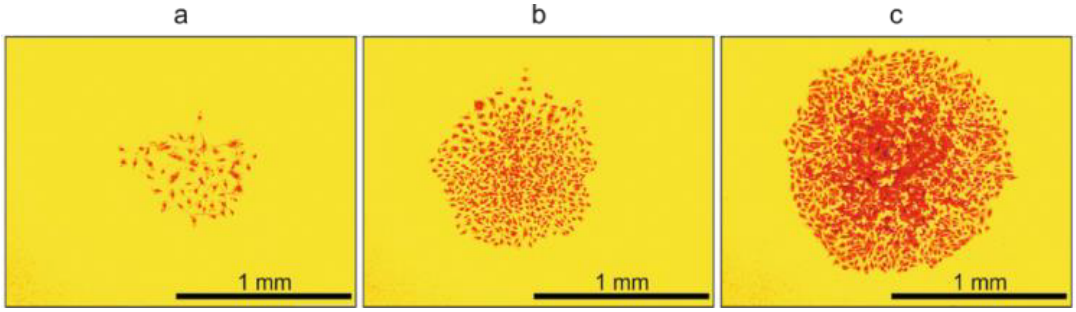


**Figure 4.** Examples of Micrographs of Colonies of Various Sizes Grown in the Control Vial (Control, Figure 3), Indicated in the Text as Small (a), Medium (b), and Large (c)

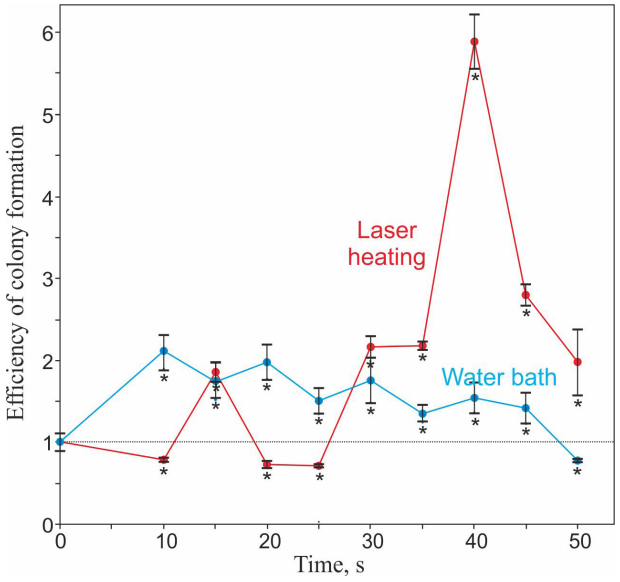


**Figure 5.** The Efficiency of Formation of MSC Colonies After Laser Irradiation of Cell Suspension and After Their Heating in a Water Bath With Respect to Control as a Function of Exposure Time

same (Figure 2b). Therefore, the significant difference between the curves in Figure 5 can be explained by the additional effect of laser light on cells, in addition to the purely thermal effect. A comparison of these curves shows that in the heating time range of 10–25 seconds, the additional effect of laser radiation on the efficiency of colony formation was inhibitory, and in the range of 30–50 seconds, it was stimulating. In this case, the stimulating effect of laser radiation was maximal at an exposure time of 40 seconds. It should be noted that at an exposure of 50 seconds, the additional laser stimulation of the cells led to an increase in the efficiency of colony formation, while as a result of heating the bath, it significantly decreased compared to the control (Figure 5).

Of course, there are physical differences between the states of the suspension when heated by a laser and a water bath. In the first case, heating is performed uniformly throughout the volume. In the second case, heat mainly comes from below by conduction through the bottom of the well, and heating of the suspension in the volume occurs owing to the emerging convective flows. In other words, when heated in a water bath, hydrodynamic flows arise, and noticeable temperature gradients can occur near the bottom of the well and throughout the entire thickness of the suspension. It is possible that these factors, in addition to the additional laser effect, played a role in the appearance of such significant differences in the efficiency of MSCs colony formation for the two physical factors (Figure 5).

The additional effect of laser light on cells is that, in addition to being purely thermal, it falls under the widely used term "photobiomodulation" (PBM). PBM using red or near-infrared (NIR) light has already been used to stimulate the proliferation of various cells, including stem cells.[29]

A number of studies have shown that the proliferation rate of MSCs grown in vitro after short-term irradiation with LILR increases – 2-4 times.[30,31] In our study,[32] it was shown that the physiological state of the cells is of great importance. If the cells are weakened, for example, when they are grown with a reduced (3%) serum content in the nutrient medium, then external physical influences can stimulate their recovery, proliferation, and metabolism. If the cells are in the physiological norm, then weak external influences do not have a significant effect.

The mechanisms of action of LILR on cells are mainly associated with changes at the molecular level in the energy states of enzymes and other cellular elements.[30,33] Currently, several hypotheses explain the mechanism of action of LILR. According to one study, the respiratory chain of the mitochondria is considered the main target.[34] The absorption of light by cytochrome c oxidase leads to an increase in membrane potential, an increase in the production of ATP, and a subsequent flux of protons and calcium ions.[35] According to another hypothesis, the action of LILR involves the production of a small amount of reactive oxygen species (ROS).[36] ROS can act as mediators in several cellular pathways, including kinase pathways that activate cell division.[37,38] However, there is no generally accepted understanding of this phenomenon. A similar situation developed with respect to other physical factors. Currently, there is an accumulation of experimental data that gradually clarifies the overall picture and forms the basis for new biomedical

technologies. Additional studies are needed to provide an unambiguous answer regarding the mechanisms of action of laser radiation in cells.

For the laser action on the MSC suspension, we chose a wavelength of 980 nm from the near-infrared range. This choice is due to the fact that, in order to influence cells located inside the tissue, for example, in the BM canal, preference should be given to near-infrared light, which has a maximum penetration depth into the biological tissue. It is important to note that even in this relatively narrow wavelength range, the efficiency and mechanism of laser action strongly depend on the wavelength used. For example, the maximum stimulation of MSCs by radiation with a wavelength of 810 nm occurs when they are irradiated with a dose of $\Phi = 3$ J/cm$^2$, and by radiation with a wavelength of 980 nm at $\Phi = 0.03\text{-}0.3$ J/cm$^2$.[39] This difference, in our opinion, is due to the fact that radiation with a wavelength of 980 nm mainly affects calcium ion channels, whereas radiation with a wavelength of 810 nm affects mitochondrial cytochrome c oxidase. Because ion channels are very sensitive to temperature, the observed result may be partly due to the thermal effect, which is much stronger at a wavelength of 980 nm due to the greater absorption coefficient of water.

The results obtained show that the method we developed for activating BM MSCs with a single laser irradiation in vitro can be used intraoperatively to create tissue-engineered constructs in accordance with the concept of minimally manipulated cells.[40] The idea is to use autologous cells to create scaffolds. These cells can be isolated with minimal manipulation intraoperatively from various tissues, seeded onto a scaffold, and implanted into the patient in a one-step procedure. We believe that to increase the proliferation activity of cells, they can be exposed to laser light with certain parameters before being placed in the scaffold or directly into the scaffold.[41,42] In the work,[43] the authors studied the dose effect of low intensity laser radiation (LILR) with a wavelength of 915 nm on the viability of Saos-2 human osteoblast-like cancer cells. They revealed the hormesis effect which claims the existence of a radiation dose in the range of 10 J/cm$^2$ with maximal cells proliferation. It should be noted that there was no noticeable heating. Similar effects were also detected when stem cells were thermostatically heated at a temperature of about 41 °C.[44]

In our study, the laser radiation with a wavelength of 980 nm had a significantly higher absorption coefficient in water. This caused heating of the cell suspension (see Figure 3B), the temperature of which reached 44 °C in 40 seconds, while the radiation dose was 272 J/cm$^2$. With these exposure parameters, we obtained a 6-fold increase in the number of MSCs compared with the control and about 4-fold increase compared with water bath heating. It allowed us to conclude that combined photo and moderate thermal action on stem cells could result in the synergic effect on cells proliferation. The question may arise whether such exposure will lead to accelerated proliferation of cancer cells. Indeed, a number of studies have shown that low doses which are typical of PBM can lead to an increase in the proliferation of cancer cells.[45] However, as shown in Andreeva et al,[45] high doses significantly suppress the proliferation of cancer cells, while normal cells remain viable.

It can be assumed that the synergistic effect of photo- and moderate thermal effects on stem cells can also explain the appearance of characteristic features in the range of 10-25 seconds in the curve in Figure 5 with laser heating. Throughout this entire interval, some suppression of the efficiency of MSC colony formation is observed. However, the exception is the 15-second exposure, which resulted in an almost twofold increase in the number of colonies compared to the control. During this interval, only a moderate thermal effect on the cell suspension led to a significant increase in MSC proliferation by approximately two times. Obviously, in order to explain all the mechanisms leading to the discovered effect of the complex dose dependence of the effect of laser heating on stem cells, an additional set of non-trivial studies is required.

Recently, considerable attention from researchers has been paid to improving the regenerative functions of MSCs depleted by age or disease by exposure to various physical factors. The problem is raised about the need to rejuvenate autologous MSCs from elderly patients used for cell therapy. In the review[46] strategies for solving this problem, such as influencing the cell culture using hypoxia, heat shock, starvation by reducing the percentage of nutrient serum and pre-condtioning cells with various factors have been discussed.

In a number of works, LILR is used for these purposes.[47] There are reports about the possibility of significantly increasing the number of passages when growing MSCs without genome aberrations by treating them in a nutrient medium with LILR.[48] The mechanisms of action of various physical factors have been widely studied and associated with their influence on the intra- and intercellular signaling systems, which, as recent studies show, can both accelerate and slow down the processes of aging or cell death.[47,48] In intercellular information exchange, an important function is performed by signaling proteins such as p21, p27, p56, short RNAs, and everything that makes up the so-called secretomes. The subject of research is also their carriers, which include lipid membrane microvesicles and exosomes. It is clear that all of them can be exposed to various physical factors, including light and heat. Since in intercellular information exchange it is MSCs that play a primary role,[49] then it is important to study changes in the state of the MSC secretome under physical influences and find ways to specifically influence it. Perhaps, in the near

future, scientists will be able to begin solving this complex but undoubtedly promising task.

The goal of this study was to achieve a significant increase in the proliferative activity of BM MSCs under the action of laser radiation in the near-infrared region. The optimal parameters at which the efficiency of in vitro proliferation increased six-fold compared to that of the control were determined (Figure 5). Such an increase, as shown by comparative experiments with a water bath, cannot be explained only by the short-term thermal heating of the MSC suspension. With laser exposure, in addition to heating, the cells were irradiated with infrared light. We note that the parameters of such irradiation used by us fall outside the generally accepted range related to photobiomodulation because the latter, by definition, assumes that no noticeable heating occurs. In this regard, we believe that in our case, we are discussing laser thermo-photobiomodulation, in which there is a mutual enhancement of the effects of photobiomodulation and moderate thermal heating.

## Conclusion

The efficiency of MSC colony formation was studied after short-term exposure of cells to laser radiation with a wavelength of 980 nm and doses leading to moderate heating of the cell suspension. It has been shown that with optimal parameters, the efficiency of colony formation can be increased by six times compared to the control. During thermal heating in a water bath with similar temperature dynamics, the efficiency of colony formation increased only twofold. We believe that in the case of moderate laser heating of a suspension of MSCs, the high efficiency of colony formation is ensured by the synergic action of two stimulating physical factors: thermal and light. Thus, it can be argued that thermo-photobiomodulation occurs with the moderate laser heating of cells. The results obtained have great practical potential in transplantology, restorative, and regenerative medicine since they allow a significant increase in the number of autologous BM stem cells at early passages.

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### Authors' Contribution

**Conceptualization:** Alexander Sviridov, Ruben Chailakhyan.
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**Formal analysis:** Vladimir Yusupov.
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**Supervision:** Ruben Chailakhyan
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**Writing–review & editing:** Vladimir Yusupov, Nataliya Vorobieva, Alla Grosheva.

### Competing Interests

None.

### Ethical Approval

The study was approved by the Ethics Committee of the National Research Center for Epidemiology and Microbiology. N.F. Gamaleya of the Ministry of Health of Russia (protocol No. 18 dated May 29, 2020).

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