Thermo-photobiomodulation of stem cells

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Abstract. The most important task of cell transplantology is to activate the proliferative potential of mesenchymal stem cells (MSCs) before receiving bone marrow cells from a donor. This is necessary to increase a sufficient number of MSCs in early passages, when the probability of chromosomal mutations is still low. The proliferative activity of cells can be activated using photobiomodulation (PBM) by exposure to low-intensity laser radiation in the visible and near-infrared ranges. Recently, it was shown in vitro that the combination of PBM and moderate laser-induced heating can lead to a significant increase in the efficiency of MSC colony formation. The main objectives of the study are to find the optimal parameters for such a combined effect and answer the question about the possibility of a synergistic effect of thermal heating and laser radiation. MSCs isolated from rat bone marrow were used for the experiments. MSCs were exposed to short-term laser radiation of moderate power with a wavelength of 980 nm and an energy density of 68-340 J/cm^2, accompanied by moderate heating of the cell suspension. Vials with grown colonies were photographed, then their number, size and number of cells in individual colonies were determined using special digital image processing methods. It was found that under optimal parameters, exposure to laser radiation of moderate power leads to an increase in the number of colonies by 4.1±0.5 times, and the total number of cells by 3.3±0.4 times compared to the control. It has been shown that this increase in cell number occurs as a result of the synergistic effect of photobiomodulation and moderate heating. Activation of colony formation after laser stimulation of MSCs occurs due to the migration of cells from the initially formed colonies with the subsequent formation of additional colonies by separated cells.

1 Introduction

Modern biotechnologies and medicine are focused on improving existing and developing new methods of treating various diseases, including previously incurable ones. A number of such approaches are in dire need of creating tools and methods that provide the ability to control the metabolism and proliferative activity of various cell cultures. Thus, mesenchymal stem cells (MSCs) are currently widely used in medical practice, usually

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these are autologous MSCs from the patient's bone marrow (Stoltz et al. 2015). The most important task of cell transplantology is the activation of the proliferative potential of MSCs before obtaining bone marrow cells from a donor. This is necessary in order to increase a sufficient number of MSCs at early (2–3) passages, when the probability of chromosomal mutations is still low.

To stimulate the proliferative activity of MSCs, many researchers working in this direction offer various physical factors: photobiomodulation (PBM) with low-intensity laser radiation in the visible and near-IR range, pulsed ultrasound, laser-induced hydrodynamics, extremely high frequency radiation and other [1-25].

We believe that, from the point of view of creating a technology, laser methods are the most preferable, since they use reliable, stable, and safe sources of laser radiation that are approved for medical use and demonstrate high efficiency in experimental studies. Thus, it was shown in (Bikmulina et al. 2020) that according to the results of *in vitro* cultivation for a week, the rate of MSC proliferation after short-term PBM (wavelength λ=804 nm, intensity I=50 mW/cm^2, energy density or fluence F=1 J/cm^2) increases by 2.4±0.3 times. It was demonstrated in (Chailakhyan et al.2022) that after local exposure to infrared laser radiation *in vivo* on the femur of rats (λ=970 nm, power P=0.3 W, exposure t=5 s per point, a total of 10 points), the efficiency of colony formation of MSCs taken from the bone marrow increased by 2.3 ± 0.3 times as early as two days after laser exposure.

Thus, many studies have been carried out on the photobiomodulation effect of laser light on MSC, including the effect on their proliferation (Stoltz et al. 2015; Shojaei et al. 2019; Karu 2007). In this case, such parameters of laser exposure were used that did not lead to a noticeable increase in the temperature of the biomaterial as a whole, which is the essence of PBM. Recently, in our article, based on *in vitro* experiments with exposure of MSC suspension to moderate power laser radiation with λ=970 nm, it was shown that the combination of PBM and moderate laser-induced heating can lead to a significant increase in the efficiency of MSC colony formation (Chailakhyan et al. 2023). To further study the obtained interesting effect, optimize the modes used, and clarify the mechanism of action of moderate-power laser radiation, detailed studies are required to obtain data not only on the number of colonies, but also on their size and the number of cells in the colonies. Such studies, taking into account the large amount of data obtained, can only be performed using the method of special digital processing (Bär et al. 2020).

In this article, we study the proliferation and efficiency of colony formation of rat bone marrow MSCs after short-term exposure to laser radiation of moderate power with a wavelength of λ=980 nm and various energy densities in the range of 68 - 340 J/cm^2 on a suspension of these cells using methods of special digital processing. The main difference between this approach and the well-known PBM is that in our case, the effect of laser radiation on biological material occurs against the background of its moderate heating. In this case it is appropriate to speak about the combined effect of moderate heating and PBM or in other words about thermo-photobiomodulation (TPBM) of MSC. The main objectives of this study are to find the optimal parameters of TPBM in terms of cell proliferation, and to answer the question of the possible existence of a synergistic effect between thermal heating and laser radiation.

2 Materials and Methods

The studies were carried out on male Wistar rats weighing 300-350 g, obtained from the Stolbovaya nursery, Moscow region. The experiments were 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) and 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, were provided with granulated laboratory food and access to water.

A schematic diagram of the various stages in the experiment is shown in fig. 1. The sequence of steps for each stage (fig. 1) is detailed below.

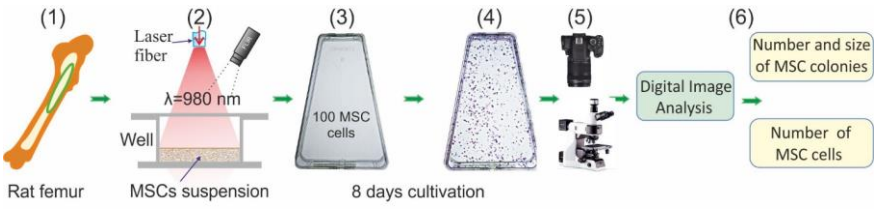

Fig. 1. Schematic diagram of the stages of the experiment (1) - (6).

(1) - Preparation of MSCs suspension from rat bone marrow. (2) – Exposure of the MSCs suspension in the well of the plate by laser radiation λ=980 nm for 10–50 s with temperature control using a thermal imaging camera. (3) - Planting 100±10 cells in culture flasks with a nutrient medium. (4) Cultivation of MSCs for 8 days at 37°C, fixation and staining. (5) - Photographing and microphotography of colonies. (6) - Digital Image Analysis with the determination of the number and size of colonies, as well as the number of cells in each of them.

2.1 Preparation of MSC suspension (stage 1)

All culture work was carried out under sterile conditions. A suspension of rat bone marrow MSCs was used in the experiments, obtained by the standard method described by us in the articles (Friedenstein et al. 1987; Lagar'kova et al. 2006). With the standard method of obtaining bone marrow MSCs, there is no need to test them for markers.

Animals were euthanized by an overdose of anesthesia, and with observance of asepsis, the femur bones were isolated, their ends were cut off, and the bone marrow was washed into the nutrient medium with a syringe. Then a single-cell suspension was prepared, filtered through a 4-layer nylon filter, and the total number of cells was counted. The cells were explanted into culture vials (Nunc 80 cm^2) at a seeding density of $3.5\text{-}4.0x10^4$ cells per 1 cm^2 of the flask bottom area. The vials contained 5 ml of complete culture medium, consisting of 80% alpha-MEM nutrient 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 were formed in the vials, the 1st passage was performed. To do this, the vials were washed with Hanks' solution, treated with 0.25% trypsin solution, kept for 30 minutes in trypsin vapor at a temperature of 37 °C, then the trypsin was drained and the MEM medium was added, which was used to wash the cells from the surface of the vials. The resulting suspension was pipetted until a unicellular medium was formed, when individual cells were isolated from each other. The number of cells was determined using a Countess TM Automated cell counter (Invitrogen, USA), after which volumes containing $5.0x10^5$ cells were taken and transferred to new vials with culture medium. When the cells reached the confluent layer, the next passage was made.

The MSC suspension of the second passage was obtained by the method described above, the cells were counted, and a working suspension was prepared, which was subsequently used throughout the experiment. 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 content

of live and dead cells in it were recalculated. Comparison of the number and viability of cells in the working suspension at the beginning and at the end of the experiment showed that these parameters did not change during the experiment. Thus, the working suspension of MSCs remained stable and the changes occurring in it did not introduce an error into the obtained experimental results.

2.2 Laser irradiation of MSCs suspension (stage 2)

A fiber laser (LS-0.97, IRE Polyus, Russia) with λ= 980 nm was used. The output tip of the laser optical fiber (core diameter 0.6 mm, numerical aperture 0.22) was placed at a distance of 55 mm from the surface of the suspension. The laser spot diameter in the suspension surface plane was approximately 15 mm, which corresponded to the well diameter. The output power of laser radiation was 12 W. Power control was carried out by a PM10 meter (Coherent, USA). The time of exposure to the MSC suspension was t=10, 15, 20, 25, 30, 35, 40, 45 and 50 s and was controlled by a stopwatch. With these parameters, the laser fluence varied from 68 J/cm^2 to 340 J/cm^2.

Immediately before laser irradiation, 0.5 ml of a suspension of MSCs in a nutrient medium (without FBS) containing 5x10^5 cells was placed into the wells of a 24-well plate. The thickness of the suspension layer in the well was approximately 2.8 mm. Laser irradiation of the MSC suspension was carried out in a sterile box, into which laser radiation was delivered via a sterilized optical fiber.

The temperature dynamics of the surface layer of the suspension (~20 μm) was monitored using a FLIR 655sc infrared camera (Sweden) with a frequency of 25 Hz, an accuracy of 0.05°C, and an operating wavelength range of 7.5–14 μm.

2.3 Planting MSCs in culture vials (stage 3)

After laser irradiation, MSCs were planted in 25 cm^2 culture flasks in 3 replications for each exposure dose and the control group. Each vial contained 5 ml of complete culture medium, consisting of 80% alpha-MEM nutrient medium (Sigma), 20% fetal bovine serum (FBS) (Hy clone) and antibiotics. 100 ± 10 MSC were placed in each vial.

2.4 Cultivation of MSCs (stage 4)

Cultivation was carried out at a temperature of 37 °C in an atmosphere of 5% CO_2 for 8 days. On the 8th day, vials with colonies were fixed with 96% alcohol and stained with azure-eosin according to Romanovsky.

2.5 Photographing and microphotography of colonies (stage 5)

Photos of vials with fixed and stained colonies were taken using a Canon EOS 650D digital camera (Japan). For a detailed study of MSC colonies and cell counting, microphotography was performed using a 3D microscope HRM-300 Series (Huvitz, Korea).

2.6 Digital Image Analysis (stage 6)

2.6.1 Pre-processing of digital vial images.

To determine the number of cells and colonies of MSCs, digital images of vials from Canon EOS 650D camera were pre-processed. At the first stage, a rectangle 23.5 × 48.3 mm in

size was distinguished in all images in the inner part of the vial, occupying 48% of the surface area of the side wall of the vial. Then, grayscale images were formed from digital photographs in jpg format (only the "green" palette was used for maximum contrast of MSCs). Next, the images were inverted, in which the darkest tones became the lightest, and vice versa. Then, the image of an empty vial was subtracted from the obtained images of vials with cells, which made it possible to completely remove the background. As a result, in the obtained images, cell colonies were clearly distinguished by light tones against a black background.

2.6.2 Determination of the MSCs number.

For the image with MSC colonies obtained as described above, the sum of all pixel values $S=k \cdot N$ was calculated, proportional to the total number of stem cells located there N, where k is the proportionality coefficient. To determine the coefficient k on the processed image, a rectangular area was analyzed, which included a colony of MSCs with a sufficiently large number of cells in the range $200<N_0<500$, where N_0 is the number of cells in the colony. For this area, the sum of all pixel values S_0 of the selected colony was determined. Then microphotography of this MSC colony was carried out, according to which the number of cells $\cdot N0$ located in it was determined. The required coefficient of proportionality was determined by the relation: $k=\cdot S_0/N_0$.

2.6.3 Determination of the number of MSCs colonies.

To determine the number of MSC colonies obtained as described above, the images were subjected to special digital processing. These pre-processed images are transformed with a Global thresholding method to create binary images. The default Global thresholding method uses Otsu's method (Otsu 1979) to derive a global threshold from which a user-defined constant is subtracted to prevent biasing towards background (Bär et al. 2020). Then, for the obtained binary images, in accordance with (https://habr.com/ru/post/114335), the properties of regions in the image were calculated, their centers and radii of the circles were determined. On the initial image of the selected rectangular area, corresponding circles were sequentially applied in certain places, next to which the value of their radius in mm was indicated.

2.7 Statistical analysis

Statistical analysis was performed using the standard software package GraphPad Prism version 7.00 for Windows (GraphPad Software, Inc., USA). Quantitative data were compared using the non-parametric Kruskal-Wallis test. Differences between groups were analyzed by two-way ANOVA at the chosen significance level $p<0.05$. All results are presented as mean and standard deviation.

3 Results

3.1 Heating of MSC suspension at stage 2 (Fig. 1).

Figure 2 shows curves reflecting the average surface temperature of the MSC suspension in the well of the plate, when it is heated by laser radiation for 50 s (Fig. 1, stage 2). During this time, the temperature of the suspension gradually increased and by 50 s it increased by 23.5±0.3°C, reaching 47.5±0.3°C (taking into account the room temperature of 24°C).

After turning off the laser, the temperature gradually decreases as a result of cooling (Fig. 2).

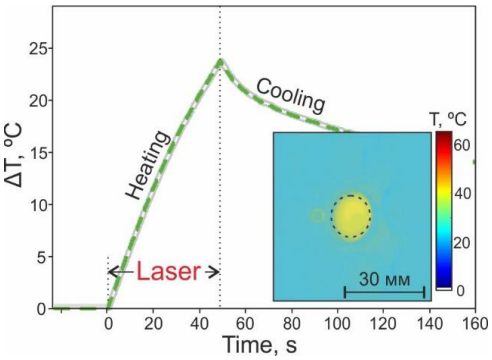

Fig. 2. Dynamics of heating of the MSC suspension in the plate well by laser radiation at stage 2

The line widths correspond to the 95% confidence interval. The inset shows a thermographic image of the well surface when heated for 40 s.

3.2 Cultivation of MSC colonies at stage (4).

Eight days after the suspension was irradiated and MSCs were cultivated, well-defined colonies were seen in all flasks. Figure 3 shows photographs of the vials without preheating the MSCs suspension (control) and after heating with laser radiation for 40 s.

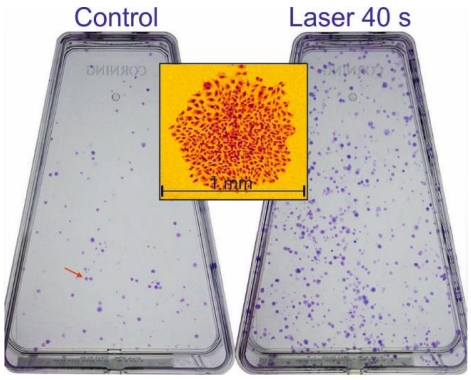

Fig. 3. Photographs of flasks with MSC colonies 8 days after cultivation of 100±10 planted cells: for a suspension without laser exposure (Control), and a suspension heated by laser radiation for 40 s (Laser 40 s).

The inset shows a micrograph of a colony marked with a red arrow in the photo of the control vial, with a total number of MSCs 480 pcs.

3.3 Digital Image Analysis with colony isolation at stage (6)

Figure 4 shows the sequence of images obtained from digital image processing of vials with MSC colonies. It can be seen from the figure that the proposed image processing algorithm copes well with the task, highlighting individual colonies and determining their effective radius. In some cases, very large cell colonies are formed, which can be formed from several closely spaced MSCs (region 2 in Fig. 4, shown by black arrows). With a very

dense merger of individual colonies into one large one, it is not possible to determine the true sizes of these colonies formed by individual cells.

3.4 Dependence of the efficiency of MSC colony formation on the time of laser exposure

Digital Image Analysis showed that in the control in the selected rectangular area on the image (blue dotted line in Fig. 4) the number of colonies is 104±10 pcs., and at the maximum (under laser exposure with a duration of t=40 s) it reaches 425±50 pcs. At the same time, the total number of cells in the formed colonies in the control was $(2.1\pm0.2)\cdot10^4$ pcs., and at the maximum (at t =40 s) - $(6.9\pm0.7)\cdot10^4$ pcs.

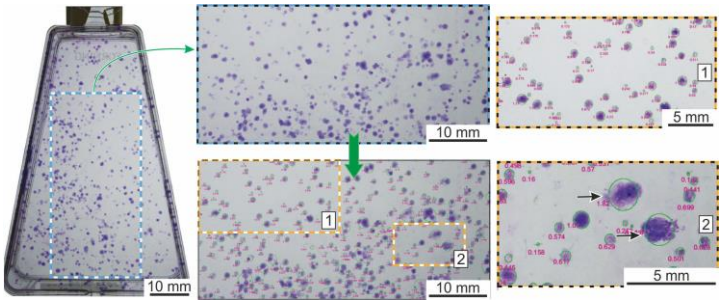

Fig. 4. Sequence of images obtained by digital image processing of vials for isolation of MSC colonies

A rectangular area (blue dotted line) is selected from the original image of the vial, which is further used for digital processing. As a result of processing, green circles are applied to the photographs of MSC colonies along their perimeter, next to which their radii are displayed in red numbers. Two areas are highlighted on the processed image, which are shown separately in an enlarged view. In area 1, almost all colonies are located separately from each other. In area 2, two large colonies (shown by black arrows) probably formed from several MSCs.

Taking into account the obtained ratio of areas (2.1), the number of colonies in vials is: in the control 218±21 pcs., and at the maximum (at t=40 s) it reaches 893±105 pcs. In this case, the total number of cells in the control vials in the control was $(4.4\pm0.4)\cdot10^4$ pcs., and under laser exposure at the maximum (t =40 s) - $(14.5\pm1.5)\cdot10^4$ pcs.

Figure 5 shows the dependences of the size and number of formed MSC colonies, as well as the total number of cells, on the time of laser exposure to the cell suspension. As for the size of MSC colonies, it did not differ significantly from the control for all experimental points (Fig. 5a) and averaged R=0.6±0.3 mm.

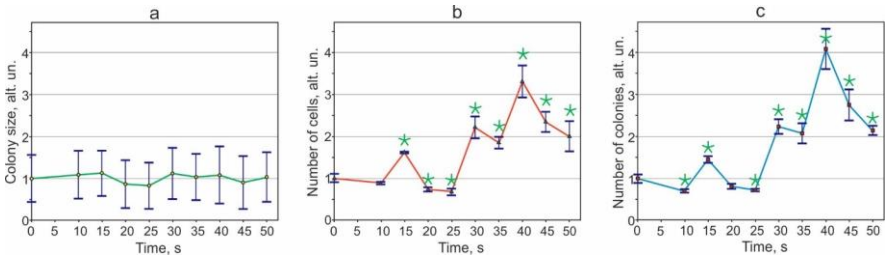

Fig. 5. Dependences of the size (a) and number of formed MSC (b), as well as the total number of colonies (c) on the time of laser exposure to the MSC suspension.

All values are normalized to control. The symbol * marks the experimental points that significantly differ from the control.

The other two parameters studied (number of cells and colonies) strongly depend on the laser irradiation time t (Figs. 5b and 5c). It should be noted that these two curves are very similar to each other. They show a local maximum at t=15 s, a local minimum at t=20-25 s and a large rise in the range t=30-50 s with a maximum at t=40 s. At t=15 s, the number of cells increased by 1.63±0.05 times, and that of colonies by 1.45±0.08 times. At t=25 s, the number of cells was 0.69±0.07 of the control, and the number of colonies was 0.72±0.05. At the maximum efficiency of laser exposure (t=40 s), the number of cells increased by 3.3±0.4 times, and colonies - by 4.1±0.5 times.

Fig. 6. shows some characteristic histograms of the size distribution of the formed MSC colonies. The results for the control and for the times of laser irradiation t=15, 40 and 45 s are shown. Histogram bars in the range of colony radii 0.3-1.4 mm for t=15 s are the highest, and for t=45 s they are the lowest.

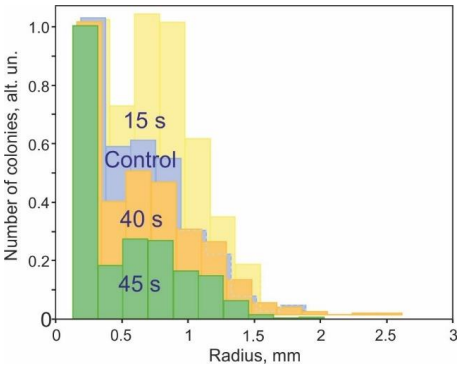

Fig. 6. Histograms of size distribution (radius) of formed MSC colonies for different times of laser exposure to cell suspension.

The results for the control and for the times of laser irradiation t=15, 40 and 45 s are shown. All histograms are normalized to their maximum values. Histogram bars in the range of colony radii of 0.3-1.4 mm for t=15 s are the highest, and for t=45 s they are the lowest. For better perception, all diagrams, except for the one presented in the foreground (t=45 s), were sequentially shifted relative to the previous one to the right and up by the same amount.

On all normalized histograms, the first column is allocated, corresponding to radii of 0.1-0.3 mm, the height of which for all histograms, except for that obtained at t=15 s, is the maximum. Note that for the histogram at t=15 s, the height of this column practically does not differ from 1 and is 0.98.

In the case of control, the maximum sizes of colonies fall within the range of 1.6-1.8 mm. The maximum sizes of colonies formed after laser exposure to the MSC suspension for t=15 s fall in the range of 1.3-1.5 mm, for t=45 s - in the range of 1.8-2.0 mm, and for t=40 s - in the range of 2.2-2.6 mm.

Figure 7 shows the dependence of the average number of MSCs per colony on the time of laser exposure to the cell suspension. It can be seen that for all cases, except for t=10 s, there are no significant differences from the control. As for irradiation with time t=10 s, such exposure led to a significant increase in the average number of cells in the colony by 25±6%.

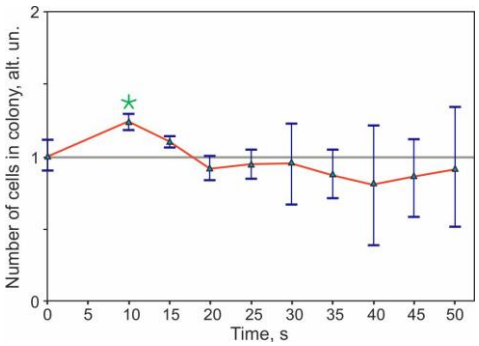

Fig. 7. Dependence of the average number of MSCs per one colony on the time of laser exposure to the cell suspension

All values are normalized to control. The symbol * marks the experimental points that significantly differ from the control.

4 Discussion

Experimental studies have shown that laser irradiation of a suspension of bone marrow MSCs with selected parameters (λ=980 nm, exposure time t=10 - 50 s, fluence F= 68 - 340 J/cm^2) leads to an increase in the temperature of the suspension to t =50 s at 23.5±0.3 °C (Fig. 2). Therefore, the laser action used cannot be attributed to the low-level, the absorption of which, by definition, does not lead to a noticeable rise in temperature. In this sense, the complex of biochemical processes triggered in a biological tissue under the action of photons of light, in our case, is not photobiomodulation in its pure form (Karu 2007; de Freitas and Hamblin 2016).

However, such a strong effect in terms of photobiomodulation did not lead in our case to the loss of the ability of MSCs to proliferate and form colonies. Moreover, at certain exposure times, the efficiency of their proliferation and colony formation significantly increased (see Fig. 3 and Fig. 5b,c). Thus, at t = 15 s, the number of cells during cultivation for 8 days increased by 1.63 ± 0.05 times compared with the control, and the number of colonies by 1.45 ± 0.08 times. And laser heating for t=40 s led to an increase in the number of cells by 3.3±0.4 times, and the number of colonies, by 4.1±0.5 times (Fig. 5b,c).

Interestingly, with such significant changes in the number of cells and colonies of MSCs, laser exposure to the suspension did not lead to a change in the average size of colonies with R=0.6±0.3 mm (Fig. 5). The laser exposures used also did not lead (Fig. 7) to a change in the average number of MSCs per colony (202 ± 41). The exception here is exposure with exposure t=10 s, at which the average number of cells in the colony significantly increased by 25±6%.

As for the size distribution of the formed MSC colonies, laser irradiation led to significant changes in the shape of the distribution (Fig. 6). So, at t=15 s, the relative number of colonies with effective radii R=1.3-1.5 mm exceeds the relative number in the control by 73%. With an increase in the time of laser exposure to t=40 s and t=45 s, the relative number of colonies in this range of radii compared with the control decreases to 83% and 46%, respectively.

The maximum sizes of formed colonies also differ significantly depending on the radiation dose (Fig. 6). If for control they correspond to the range R=1.6-1.8 mm, then for t=15 s the maximum colony radii do not exceed 1.6 mm, and for t=40 s they reach 2.6 mm. A detailed analysis of the obtained images of areas with large colonies (Fig. 4 area 2) shows that such very large colonies can be formed from the fusion of several colonies of MSCs. In

this case, the values of the effective radius obtained after digital processing can significantly exceed the value of the radius of a colony formed by one cell.

It should be noted that the first column with R=0.1-0.3 mm stands out on all normalized histograms in height (Fig. 6). This means that both for the control and in the case of laser exposure, there are a significant number of small colonies in the culture flasks. Their share of the total number of colonies found in the vials for control is 29% (total 63 colonies). The minimum proportion of small colonies is observed for the time of laser exposure t=15 s - 23% (72 colonies), and the maximum for t=45 s - 54% (284 colonies).

According to the Digital Image Analysis, in the control, the number of colonies is 218±21, which is approximately twice the number of seeded cells (100±10). Taking into account this circumstance and the shape of the size distribution diagram for the control (Fig. 6), we can draw the following conclusions:

1) the seeded cells initially significantly differ in their proliferation potential: ~30% of the cells created colonies with R<0.3 mm, ~50% - with 0.3 mm<R<0.9 mm, and only ~9% of the cells created the largest colonies with an effective radius of 1.6- 1.8mm;

2) during the formation of colonies, at least some of them separated cells, which subsequently formed their own colonies.

Note that the minimum number of colonies is 153±11 pcs. and 158±6 pcs. was found in vials at times of laser exposure t=15 s and t=25 s, respectively (Fig. 5). These values are less than in the control (218±21 pcs.), but still significantly higher than the number of cells seeded in flasks (100±10).

Thus, the experimental results showed that laser exposure under certain parameters leads to a significant increase in both the number of MSC colonies and the total number of these cells. Since the average number of cells in the colonies practically does not differ from the control, it can be concluded that the increase in the total number of cells occurs due to an increase in the number of colonies.

Obviously, in order to increase the number of colonies, it is necessary that, at some stage, one or more MSCs separate from the cells formed in the colony. These cells must move away at some distance from the parent (mother) colony and subsequently form new colonies. As we have shown, this process is also observed in the control, when 218±21 colonies formed after eight days of cultivation of 100±10 planted MSCs.

An increase in the number of registered colonies in comparison with the number of cells planted in the flask also occurs for all times of laser exposure. At the same time, in many cases, this increase is significantly greater than in the control. For example, at t=40 s, the number of colonies formed was four times greater than the number of planted cells (see Fig. 3 and Fig. 5c). However, how could this happen? We believe that in order to increase the number of colonies some cells must at some time separate, move away from the mother colonies for a certain distance, and then initiate the formation of new colonies. Based on this, we can conclude that such separation of a part of the cells from the growing maternal colony with the formation of new colonies, which we spoke about above, is stimulated by laser exposure. That is, our experiments showed that the laser exposure used, leading to moderate heating of the biomaterial, contributed to an increase in the migration ability of cells. This interesting result can be considered quite reasonable, since it is fully consistent with the data on an increase in the migration ability of MSCs as the result of photobiomodulation (Ahrabi et al. 2019; Yin et al. 2017).

As for the mechanism that leads to an increase in the rate of MSC proliferation, it may be due to the fact that laser exposure activates various chains of intracellular processes (Fekrazad et al. 2016) and stimulates the production of ATP, which is not only an energy currency inside cells, but it is also a critical signaling molecule that allows cells and tissues throughout the body to communicate with one another (Karu 2010). In this case, it should be taken into account that photobiomodulation with different wavelengths can act according

to different mechanisms of action. Thus, it was shown in (Wang et al. 2017) that photobiomodulation with λ=980 nm, although it has some effect on mitochondrial cytochrome c oxidase, mainly affects calcium ion channels. Based on this, it can be assumed that since ion channels are very sensitive to temperature, the result of an increase in the proliferation rate observed in our work is associated with the combined effect of both the laser radiation itself on MSCs (photobiomodulation itself) and increased temperature. In this regard, the mechanism by which the increase in proliferation and migration activity, as well as colony formation of MSCs, observed in the experiments carried out, can be called thermo-photobiomodulation (TPBM).

In our experiments, the shape of the curves reflecting the efficiency of colony formation and proliferation of MSCs in TPBM (Fig. 5 b, c) resembles the biphasic dose response well known for PBM or low level light therapy (Huang et al. 2009), when the maximum of the curve lies within the dose range, and at the edges, both at relatively small and at relatively large doses, the effectiveness of the impact decreases. When using high doses in PBM, an inhibitory effect can be observed first, when the proliferation rate becomes lower than in the control, and then, at very high doses, cell death (Andreeva et al. 2018).

In modern medical technology, photodynamic therapy is widely used - a method specially designed to suppress the growth of unwanted cells and using doses of laser exposure greater than for photobiomodulation. The essence of the method is that a photosensitizer is introduced into the body, and the absorption of laser radiation leads to a controlled rise in temperature and increased production of reactive oxygen species, which leads to cell death (Kwiatkowski et al. 2018). It turned out that the use of photodynamic therapy with low photosensitizer concentration and low doses of laser energy density may lead not to suppression, but to activation of cellular processes of mesenchymal stem cells (Fekrazad et al. 2020). We believe that in this case, the observed stimulation of MSCs under laser irradiation occurs in the same way as in our experiments via the TPBM mechanism.

It is clear that the conducted studies could answer only a part of the questions concerning the mechanism of laser-induced cell proliferation. So, according to previous studies, a change in the behavior of cell proliferation may be related to the reorganization of cytoskeleton [de Freitas and Hamblin, 2016; Amaroli et al. 2021]. Thus, further studies to detect cytoskeletal reorganization with changes in proliferative behavior after near-infrared irradiation would be useful to study the mechanism of action of TPBM.

5 Conclusion

In this work, the proliferation and efficiency of colony formation of rat bone marrow MSCs were studied after short-term exposure to laser radiation with a wavelength of λ=980 nm and an energy density in the range of 68-340 J/cm^2 on a suspension of these cells. It has been shown that such a laser effect, which leads to moderate heating of the suspension, can lead to a significant increase in the rate of MSC proliferation. With the help of special digital processing methods, it has been shown that the increase in cell number compared to control is due to an increase in the number of colonies formed, associated with a laser-stimulated increase in cell migration activity. It was concluded that an increase in the rate of MSC proliferation occurs as a result of the combined action of laser radiation and thermal heating via the thermo-photobiomodulation (TPBM) mechanism. The main difference between this approach and the well-known photobiomodulation (PBM) is that in our case, the impact of laser radiation on biological material occurs against the background of its moderate heating. The record increase in the number of colonies and the total number of cells compared to the control was due to this synergistic effect. The obtained effect of TPBM can be used to solve the most important task of cell transplantation - the activation

of the proliferative potential of MSCs to grow a sufficient number of these cells without chromosomal mutations.

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