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The effect of 805 nm near-infrared photobiomodulation on proliferation and differentiation of bone marrow stem cells in murine rats

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Abstract. – OBJECTIVE: To evaluate the effect of near infra-red gallium-aluminium-arsenide (GaAlAs) diode laser (805 nm) irradiation on proliferation and differentiation of rat femoral bone marrow-derived mesenchymal stem cells (BMSCs) cultured in osteogenic medium.

MATERIALS AND METHODS: BMSCs were obtained from femurs of 60 Sprague Dawley rats (200 gm). The control group comprised isolated BMSCs supplemented with an osteogenic differentiation medium. On the other hand, in the experimental group, the BMSCs were irradiated with a near-infrared laser in addition to an osteogenic differentiation medium. The experimental group was irradiated with a soft tissue laser comprising of allium-aluminium-arsenic (Ga-Al-Ar) Diode at a near-infrared wavelength of 805 nm in continuous mode. The different output powers applied were 0.5 W, 1.0 W, 1.5 W and 2.0 W respectively. Various energy levels of 1, 4, 7 and 10 J were used for irradiation. Alkaline phosphatase (ALP) assay and Alizarin staining were performed to confirm osteogenic differentiation. Statistical analysis was done using a one-way ANOVA and a *p*-value of <0.05 was considered significant.

RESULTS: According to our findings, 1.27 J/cm2 was the optimal energy density value that significantly increased the BMSC proliferation at the output of 1.5 W with the power density of 1.27 W/cm^2. On 1.27 J/cm^2, there was a significant difference compared to the control group on the first day, and the osteogenic differentiation increased significantly on the 4th day compared to the 1st day.

CONCLUSIONS: According to our findings, 1.27 J/cm^2 was the optimal energy density value that significantly increased the BMSC proliferation at the output of 1.5 W with the power density of 1.27 W/cm^2.

Key Words:
Laser therapy, Mesenchymal stem cells, Cell proliferation, Cell differentiation, Rats, Sprague-Dawley rats.

Introduction

Since the invention of lasers decades ago, the field of medicine and dentistry has seen substantial scientific advances. The use of lasers has transfigured diagnosis, therapies and surgery. The distinctive properties of lasers have allowed for numerous applications in dentistry[1]. Principally, lasers employed are of two types: high-power lasers and low-level lasers. Photobiomodulation (PBM) is a low-level laser therapeutic modality that is widely accepted in treatment used to tackle a multitude of diseased conditions by inducing beneficial therapeutic changes[2]. The therapy in-

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volves irradiation of the affected tissue with a low-powered laser to accelerate healing, relieve inflammation and pain and subsequently restore function. Through lower energy densities and wavelengths of PBM, it possesses the tendency to effectively penetrate the tissues, resulting in biomodulatory effects on the targeted cells and tissues[3].

The capacity of PBM to induce a stimulatory effect on cell proliferation has spring boarded the success of regenerative dentistry. Subsequently, it has been utilized in numerous *in vitro* experiments and *in vivo* studies to stimulate the proliferation of cells[4]. Through its positive biomodulatory effect, PBM has not only proven to stimulate cell proliferation but also impart growth and differentiation in various types of cells including fibroblasts[5,6], cancer cells[7,8], endothelial cells[9] and osteoblasts[10] in culture. The mechanism through which PBM induces this cell proliferation and differentiation is still debatable. However, it has been theorized that intracellular chromophores are responsible for absorbing the irradiating energy. The following converted metabolic energy is utilized by the mitochondrial respiratory chain that synthesizes ATP, leading to the production of nucleic acids and proteins[11]. Near-infrared spectrum activates the cellular membrane transport mechanisms followed by the initiation of photochemical cascade in response to a photophysical stimulus that induces changes at cellular level[12].

The stem cells have multifarious applications and have extensively contributed towards the expansion of regenerative dentistry. Stem cells are primal cells with the potentiality of both self-renewal and multi-lineage differentiation[13]. Mesenchymal stem cells (MSCs) are an example of adult stem cells; these are progenitor cells that possess the ability to be reprogramed, therefore, giving rise to the generation of multiple cell types from a single cell unit[14]. The premier source for the isolation of MSCs is the bone marrow[15]. Evidence[16] suggests that the MSCs in the bone marrow cavity can further specialize into chondrocytes, osteoblasts and adipocytes. MSCs are rare in the bone marrow and this relative scarcity of cells, therefore, necessitates vast *in vitro* expansion, to achieve high numbers for the purpose of therapy or research[17].

In theory, PBM promises to rapidly increase the initial number of stem cells and later induce morphodifferentiation, thus making it more useful for tissue engineering[18]. However, the findings of studies pertaining to the effect of PBM on bone marrow stem cells (BMSCs) have shown variable results and remain inconclusive. The common feature in the available literature is the lack of standardization in irradiation parameters and protocols; as the variations in laser-related parameters such as wavelength, dose, power and time of irradiation can vitiate the achievement of the desired biological effects on BMSCs[11].

In this regard, the primary objective of the present study is to evaluate the effects of near-infrared gallium-aluminium-arsenide (GaAlAs) diode laser irradiation at the wavelength of 805 nm on the proliferation and differentiation of rat femoral BMSCs cultured in an osteogenic medium at various power outputs, power and energy densities. In addition, the study also focused on finding optimal energy density that induces significant BMSC proliferation and differentiation concerning the parameters of power output and power density.

Materials and Methods

Ethical Statement

The research protocol was reviewed and approved by the Research Ethical Committee of the Graduate School of Tokyo Dental College.

Cell Culture

The current experimental study was performed in compliance with the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments). BMSCs were obtained from Sprague Dawley rats (weighted at 200 g). The animals were sacrificed, both femurs were dissected, and later bone marrow cavities were washed with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 mg/L gentamicin sulfate, and 2 mg/L amphotericin, cultured in plates, and incubated in a humid atmosphere with 5% CO_2 at 37°C. The culture medium was changed at intervals of 3 to 4 days until the cells reached 80 to 95% confluency.

Study Groups

The control group was supplemented with an osteogenic differentiation medium while the experimental group was exposed to laser irradiation along with the supplementation with the osteogenic medium. The preparation of the osteogenic medium was performed through supplementing

Dulbecco's Modified Eagle's Medium with fetal bovine serum (10%) (Thermo Fisher Scientific, Waltham, MA, USA), β glycerophosphate (10 nM), ascorbate-2-phosphate (50 µg/ml), dexamethasone (100 nM) and streptomycin (0.05 µg/ml). Photosensitizer drugs were used in the culture medium of incubation; these included Methylene blue (1µM) and 5- aminolevulinic acid (1 mM). BMSCs were plated on plastic culture flasks and subsequently incubated in an osteogenic induction medium in a humidified atmosphere at 37°C (5% CO_2).

Irradiation Protocol

In the experimental group, after the fourth passage (P4) cell cultures were irradiated with a soft tissue laser comprising of allium-aluminium-arsenic (Ga-Al-Ar) Diode (Panasonic, Osaka, Japan) at a near-infrared wavelength of 805 nm in continuous mode for 5 seconds. The different output powers applied were 0.5 W, 1.0 W, 1.5 W and 2.0 W respectively. A maximal device output power of 2.5 W. The irradiation was carried out through the bottom of the culture plate. The distance from the tip of the laser probe to the bottom of the culture plate was maintained at 10 mm. Irradiation was performed 1 day after cell plating to 96-well. Subsequently, 200 µl of the osteogenic medium was replaced. Various energy levels of 1, 4, 7 and 10 J were used for irradiation. The culture was assessed at 1, 4, 7 and 14 days.

Alkaline Phosphatase (ALP) Assay

The activity of ALP indicates the presence of osteoblasts and the formation of new bone. ALP activity assay was carried out using ALP kit by following manufacturer's protocol after osteogenic induction. After rinsing with phosphate-buffered solution the cells were fixed in paraformaldehyde (4%) for half an hour. This was followed by washing by phosphate buffer solution thrice. The cell layer was then for 20 min in alkaline solution. The determination of total protein content was assessed in the sample using the bicinchoninic acid (BCA) method using a Pierce protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) (Figure 1).

Alizarin Staining

For the identification of calcium-containing osteocytes in the differentiated culture of BMSCs *in vitro*, alizarin red dye was used. The staining was performed after induction by the osteogenic medium. Once again, washing with fetal bovine serum (10%) and phosphate-buffered solution twice. Fixation was then done for a minute with isopropanol (60%). Alizarin Red (1%) was employed for staining after washing with distilled water for a duration of 3 min (Figure 2).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The expression of osteogenetic markers was assessed using reverse transcription-polymerase chain reaction (RT-PCR). RNA isolation was done using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) under the guidance of the manufacturer's instructions. cDNA was synthesized by the PrimeScript RT reagent kit (TaKaRa, Mississauga, ON, Canada). SYBR Premix Ex Taq II kit (TaKaRa, Mississauga, ON, Canada) was used for the process of RT-PCR. ABI Prism 7500 HT sequence detection system (Applied Biosystems, Thermo Fisher Scientific,

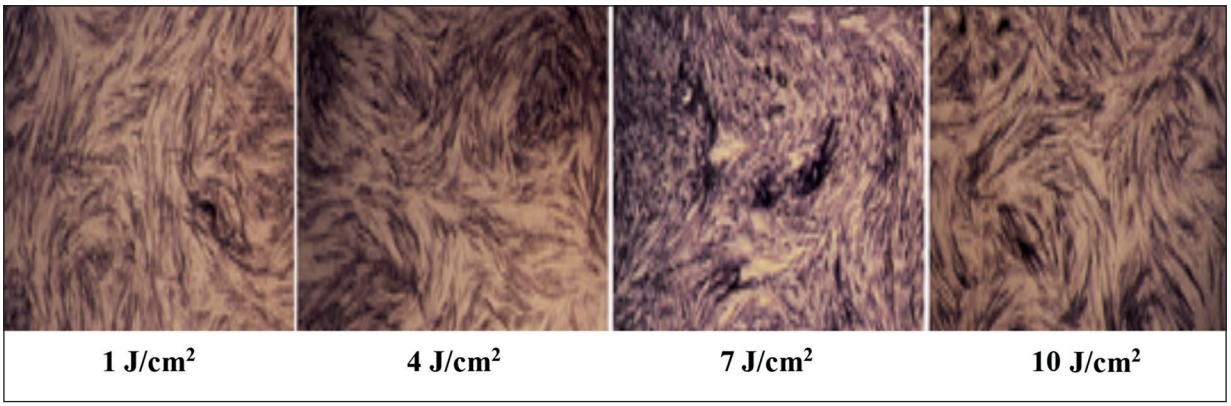

Figure 1. Osteogenic differentiation assessed by alkaline phosphatase assay at 4th day as observed under light microscope (magnification: 100×).

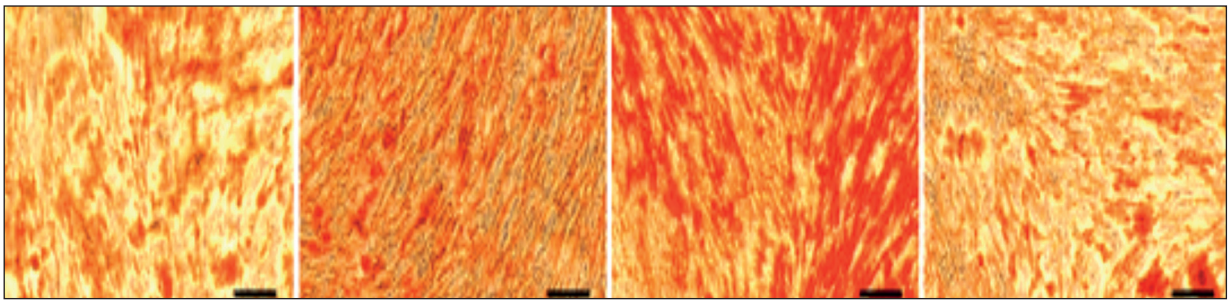

Figure 2. Osteogenic differentiation assessed by Alizarin staining at 14th day under light microscope (magnification: 100×).

Waltham, MA, USA) was used for detection. The primers used included Alkaline phosphatase (F: 5'-TCA GGG CAA TGA GGT CAC ATC-3', R: 5'-CAC AAT GCC CAC GGA CTT C-3'), Type 1 collagen (Type 1 collagen F: 50'-GCG AAG GCA ACA GTC GCT-3', R: 5'-CTT GGT GGT TTT GTA TTC GAT-3'), Osteocalcin (F: 5'-CCA CCC GGG AGC AGT GT-3', R: 5'-CTA AAT AGT GAT ACC GTA GAT GCG TTT G-3') and Bone sialoprotein 2 (F: 5'-CCA AGC ACA GAC TTT TGA GTT AGC-3', R: 5'-CTT TCT GCA TCT CCA GCC TTC T-3').

Statistical Analysis

Data were analyzed by using a statistical program for social sciences (SPSS- Version 22; IBM, Armonk, NY, USA) and a p-value ≤ of 0.05 was considered significant. Statistical analysis was done using a one-way ANOVA.

Results

Energy Density

The most significant results in BMSC proliferation and differentiation in the osteogenic medium were seen at the power output of 1.5 W at a power density of 1.27 W/cm^2 from day 1. A decrement was seen from days 4-7 after which cell proliferation tends to increase. The energy density value of 1.27 J/cm^2 caused a significant increment in BMSC proliferation. The energy density over this value could not significantly increase the proliferation of BMSCs in osteogenic medium. On 1.27 J/cm^2, there was a significant difference compared to the control group on the first day, and the osteogenic differentiation increased significantly on the 4th day compared to the 1st, but it increased significantly on the 7th day (Figure 3) (Table I).

Power Output

When the output energy is 0.5 W, after 1 day, the osteogenic differentiation increases in all laser irradiation compared to the control group. In both 1.5 W and 2.0 W, osteogenic differentiation increased slightly compared to the control group on day 1, but there was no significance, and similar to the control group on days 4 and 7 (Table I).

Days

In all groups, day 4 was cell count significantly increased compared to day 1. There was no significant difference between each group. It tends to decrease slightly after 4 days. Increase again after 7 days (Figure 1). The proliferation curve of the control group implies that cells that have been plated as 3.0x103 cells/well (about 1.0x104 cells/cm^2) proliferate at an early phase (1-4 days), differentiate at 4-7 days, and proliferate again after 7 days.

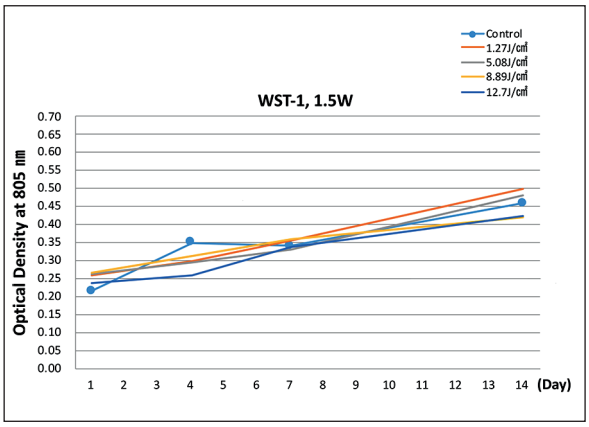

Figure 3. The figure shows BMSC proliferation at power output 1.5 W. The power density at 1.5 W was found to be 1.27 W/cm^2. The optimal energy density that resulted in significant cell proliferation was seen at 1.27 J/cm^2. Over 1.27 W/cm^2 of effective energy could not significantly increase the proliferation of BMSCs in osteogenic medium.

Table I. The numerical values of power output, power density with their respective energy densities at day 1, 4, 7 and 14 of the experiment.

Power output	0.5W	1W	1.5W	2W	*p*-value
Power density	0.25 W/cm^2	0.82 W/cm^2	1.27 W/cm^2	1.90 W/cm^2	0.05
Energy density (day 1)	1.25 J/cm^2	1.23 J/cm^2	1.27 J/cm^2	1.90 J/cm^2	0.05
Energy density (day 4)	5.00 J/cm^2	4.92 J/cm^2	5.08 J/cm^2	5.70 J/cm^2	0.05
Energy density (day 7)	8.75 J/cm^2	9.02 J/cm^2	8.89 J/cm^2	9.50 J/cm^2	0.05
Energy density (day 14)	12.50 J/cm^2	12.30 J/cm^2	12.70 J/cm^2	13.30 J/cm^2	0.05

Discussion

According to the existing literature, most studies[11,19,20] have used the visible light spectrum ranging from 600-700 nm to stimulate cell proliferation and differentiation. Stein et al[19] reported higher levels of proliferation, differentiation and maturation of osteoblasts when exposed to 632.8 and 670 nm respectively. Kim et al[20] concluded that the treatment of BMSCs in osteogenic differentiation medium with diode laser of 647 nm wavelength at energy values of 0.093 J, 0.279 J and 0.836 J respectively, modulated their differentiation into osteoblasts. The studies[11,21] conducted in the infrared red-light spectrum are rare but valuable with a mostly positive influence on the rate of cell proliferation.

The present study showed significant results in cell proliferation and differentiation at all power outputs utilized (0.5 W, 1.0, 1.5 W, 2.0 W). In 2013, Jawad et al[22] utilized a 940 nm wavelength diode laser in continuous mode at different power outputs to observe its effects on the human fetal osteoblast cell line. Significant cell differentiation was seen at 0.1 W and 0.2 W while 0.3 W of power increased the proliferation of osteoblasts. In contrast to the above studies, Bouvet-Gerbettaz et al[23] showed that PBM at a wavelength of 808 nm (energy density of 4 J/cm^2) working under continuous mode with thrice a week exposure to murine bone marrow, resulting in no alteration in BMSC proliferation and osteogenic differentiation. One explanation can be that the power output was maintained at 0.8 W which was considerably higher as compared to the present study (0.5 W) and this parameter can be the possible reason for influencing the results. In another study, Renno et al[24] used an 830 nm wavelength diode laser with an energy density of 10 J/cm^2 to irradiate osteoblastic (MC3T3) cell line grown on glass-ceramic bioscaffold. These settings caused reduced cell proliferation in the experimental group as compared to the controls. The reason for this decrement as according to the authors remains unclear but they speculated scaffold structure as one of the reasons. Decreased proliferation of BMSCs was also seen in the present study at similar energy density values.

The present study demonstrated that 1.27 J/cm^2 was the optimal energy density value that significantly increased the BMSC proliferation at the output of 1.5 W with the power density of 1.27 W/cm^2. It was also found that the range between J/cm^2 of energy density within the values of 1.0 W/cm^2 was effective on cell proliferation at an early phase. It was also observed that over 9 J/cm^2 of energy density was not effective in the proliferation and differentiation of BMSCs except for the group receiving 0.5W on the first day. According to a study by Soleimani et al[25] at 810 nm wavelength, the human BMSC cells exposed to PBM with energy densities of 2 J/cm^2 and 4 J/cm^2 reportedly had significantly higher BMSC proliferation and differentiation to osteoblasts as compared to their respective controls on the second, fifth, seventh, and tenth day of differentiation. The BMSCs were cultured in a medium supplemented with neurogenic and osteogenic inducing factors similar to the present study. No significant increment was observed in control and experimental groups receiving a dose of 6 J/cm^2. In another *in vitro* experimentation, Tuby et al[4] irradiated murine BMSCs and cardiac stem cells with diode (Ga-As) laser wavelength 804 nm at an output of 0.4 W and evaluated them after every seven days for four weeks. Similar to the results of the current study, the authors reported that the proliferation rate of cells was significantly higher in irradiated cells at all intervals for doses 1 and 3 J/cm^2.

The important contrasting feature was the power density of PBM used, which was exponentially higher in the present experimentation as compared to previous studies in the near-infrared spectrum. The present experiments used vary of power density values that included 0.25, 0.82,

1.27 and 1.90 W/cm^2 respectively. It was observed that over 1.27W/cm^2 of power density could not significantly increase the proliferation of BMSCs in osteogenic medium. In the previous studies, the power density of 50 mW/cm^2 (0.5 W/cm^2) was kept constant in all experiments except Soleimani et al[25], where it was valued at 167 mW/cm^2 (1.7 W)[4,22-25]. Recently, Fekrazad et al[26] performed a comprehensive study to evaluate the proliferating and differentiating effects induced by single and combination of different wavelengths of PBM on rabbit bone marrow. The authors concluded that while the stimulatory effects of combination lasers were unclear, wavelengths 810 nm (power density of 1.33 W/cm^2) and 660 nm (power density of 0.17 W/cm^2) with a uniform dose of energy (4 J/cm^2) not only caused BMSC proliferation but also induced osteogenic and cartilaginous differentiation.

The choice of laser type, probe's distance from the target and time of irradiation should be given special consideration for attaining more favorable results[27]. The limitations of the present study include the time duration of irradiation that was fixated at a period of 5 seconds. The variation in an irradiation period and its effect on cell proliferation can be studied in future studies. Similarly, in the present study distance from the tip of the laser probe to the bottom of the culture plate was maintained at 10 mm. The results may differ if the distance between the tip and culture plate is altered. This is one possibility on which future experiments can explore upon.

Conclusions

The present study was aimed at finding the optimum settings for imparting a significant level of proliferation and osteogenic differentiation in BMSCs induced in osteogenic medium, within the ambit of the near-infrared spectrum. According to our findings, 1.27 J/cm^2 was the optimal energy density value that significantly increased the BMSC proliferation at the output of 1.5 W with the power density of 1.27 W/cm^2. Other noteworthy findings were the prominent effect of power output on the energy density and power density that caused a significant increase in cell proliferation and differentiation.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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