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The efficacy of low-level 940 nm laser therapy with different energy intensities on bone healing

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Abstract: The aim of this study was to evaluate the efficacy of low-level 940 nm laser therapy with energy intensities of 5, 10 and 20 J/cm^2 on bone healing in an animal model. A total of 48 female adult Wistar rats underwent surgery to create bone defects in the right tibias. Low-level laser therapy (LLLT) was applied immediately after surgery and on post-operative days 2, 4, 6, 8, 10 and 12 in three study groups with energy intensities of 5 J/cm^2, 10 J/cm^2 and 20 J/cm^2 using a 940 nm Gallium-Aluminium-Arsenide (Ga-Al-As) laser, while one control group underwent only the tibia defect surgery. All animals were sacrificed 4 or 8 weeks post-surgery. Fibroblasts, osteoblasts, osteocytes, osteoclasts and newly formed vessels were evaluated by a histological examination. No significant change was observed in the number of osteocytes, osteoblasts, osteoclasts and newly formed vessels at either time period across all laser groups. Although LLLT with the 10 J/cm^2 energy density increased fibroblast activity at the 4th week in comparison with the 5 and 20 J/cm^2 groups, no significant change was observed between the laser groups and the control group. These results indicate that low-level 940 nm laser with different energy intensities may not have marked effects on the bone healing process in both phases of bone formation.

Keywords: Bone and Bones; Low-Level Therapy; Lasers.

Introduction

Many innovative modalities, including low-level laser therapy (LLLT), are being developed to enhance bone metabolism and accelerate the repair process of the bone.[1] LLLT has been shown to be effective for biostimulating bone cells in both in vivo[2,3] and in vitro[4,5] studies. The widely accepted hypothesis regarding the mechanism of LLLT is that the laser energy activates cytochromes in the cell, resulting in the acceleration of cellular activity, increased ATP and ALP concentrations and the release of calcium.[6]

Different laser types with different wavelengths including Helium-Neon (He-Ne) and Diode (gallium-aluminium-arsenide, gallium-arsenide) have been used at different doses and different treatment schedules for LLLT.[7,8] In recent studies, the Ga-Al-As type of diode lasers has been shown to be more effective in biostimulation than He-Ne lasers due to the higher penetration ability into the deep tissues.[9] The dose-dependent

nature of LLLT results in a biostimulative effects at low doses, while higher doses lead instead to bioinhibition.[10,11] Bioinhibition is currently used as a physical therapy method in achieving pain control; however, it is an undesirable phenomenon in cases that require acceleration of tissue healing. Although it has been indicated that low-level laser doses are more effective than higher doses, the exact maximum biostimulative dose has not yet been clarified. There are some promising study results on bone healing related to LLLT application with higher doses such as 112 J/cm^2 [1], 300 J/cm.[2,12] However, some studies have indicated that low-dose applications including 4.8 J/cm^2 and 16 J/cm^2 energy intensities are effective for the biostimulation of bone tissue.[13,14] Some other studies claim that laser biostimulation occurs between 0.05 and 10 J/cm^2, while LLLT doses over 10 J/cm^2 have bioinhibitory effects.[11,15]

The aim of this study was to evaluate the effects of LLLT (940 nm) with 5 J/cm^2, 10 J/cm^2 and 20 J/cm^2 energy intensities on the healing of surgically created tibia defects in rats. This study was based on the hypothesis that the effectiveness of LLLT with a wavelength of 940 nm on bone tissue would increase in direct proportion to increased energy density up to a certain dose.

Methodology

A total of 48 female adult Wistar rats (aged 12 weeks and weighing 250–300 g) were used in this study. All animals were housed at the Animal Research Center of Karadeniz Technical University and kept under constant laboratory conditions at room temperatures of 20°C to 22 °C in a 12-hour light-dark cycle and were allowed free access to food and water. The experimental protocol was approved by the Animal Care and Ethics Committee of the Faculty of Medicine, Karadeniz Technical University (approval number: 2013/53). The surgical procedures described below were performed in accordance with the National Institutes of Health Guide for the care and use of laboratory animals. The treatment methods and time periods were numerically coded on slips of paper by a surgeon who was not associated with the study. The numbers were selected by the surgeon, which allowed the subjects to be randomly assigned into the 4 treatment groups as follows:

a. L5 group (12 rats): rats were subjected to LLLT with 5 J/cm^2 energy intensity;
b. L10 group (12 rats): rats were subjected to LLLT with 10 J/cm^2 energy intensity;
c. L20 group (12 rats): rats were subjected to LLLT with 20 J/cm^2 energy intensity;
d. Control group (12 rats): no LLLT was applied.

Surgery

For general anaesthesia, ketamine (50 mg/kg, Ketasol® %10, Richter Pharma, Austria) and xylazine (10 mg/kg, Rompun® % 2., Bayer, Germany) were intraperitoneally injected. After shaving and cleansing the skin with povidone iodine solution (Betadine®, Kansuk, Turkey) on the right tibia of each rat, local anaesthesia of 1:200.000 epinephrine (Ultracain ® DS, Sanofi-Aventis, Frankfurt/Main, Germany) was locally injected into the surgical area for bleeding control. A dermoperiosteal incision was performed to expose the tibia. After reflection of the dermoperiosteal flap, a standardized rectangular 5x2 mm bone defect was created using a motorized drill under copious irrigation with saline solution. The cutaneous flap was replaced and sutured with resorbable polyglycolide suture material, and the skin was disinfected with povidone iodine. The health status of the rats was monitored daily[16].

Low-level laser therapy

A 940 nm Ga-Al-As laser (EzLase 940, Biolase Technology, Inc. 4 Cromwell Irvine, USA) was used to apply LLLT transcutaneously at one point with the hand piece positioned perpendicular to the wound. The wounds and the marginal zones were illuminated with a spot size of 30 mm diameter. Irradiation was performed in continuous wave mode for 10 seconds with an optical output power of 1.5 Watts in the L5 group, 3 Watts in the L10 group and 6 Watts in the L20 group, and the energy densities applied were 5 J/cm^2, 10 J/cm^2 and 20 J/cm^2, respectively. The first dose was applied immediately after suturing of the skin wound, and the subsequent irradiations were performed 2, 4, 6, 8, 10, and 12 days postoperatively.

Histopathological analysis

Six animals from each group were sacrificed by high-dose xylazine and ketamine injection 4 and 8 weeks after the surgery. The right tibias of the animals were removed for histopathological analysis. After removal, the tibias were fixed in 10% buffer formalin (Merck, Darmstadt, Germany) and decalcified in Decal (Decal Chemical Corporation, New York, USA). Five micrometre slices were obtained in a serially sectioned (longitudinally) pattern and stained with haematoxylin and eosin (H.E stain, Merck). Histopathological analysis was performed by a single examiner (blinded to the treatment) under a light microscope (Olympus, Tokyo, Japan). Osteocyte, osteoclast, osteoblast, fibroblast and newly formed vessel counts were calculated by Analysis 5 Research (Olympus Soft Imaging Solution, Münster, Germany) software. Each analysis was repeated twice, and the average of the measurements was calculated. The Kappa test was performed to verify the level of intraexaminer agreement.

Statistical analysis

The statistical analysis of the parameters was performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The Kruskal Wallis and Mann-Whitney U tests, along with the Bonferroni correction when necessary, were used for intergroup comparisons. The significance level was $p < 0.01$. Intragroup comparisons were performed using the Wilcoxon Signed-Rank Test. The significance level was $p < 0.05$.

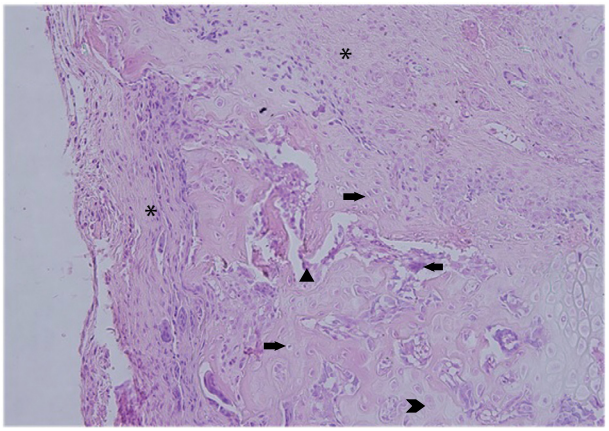
Results

Qualitative histological analysis

The Kappa test verified substantial intraexaminer agreement. Calcified cartilage and immature bone surrounded by fibrous tissue were observed at the 4th week in all groups as follows: control, L5, L10 and L20 (Figure 1). More intensely calcified cartilage was observed in the laser groups than the control group at the 4th week. Some indicators of progression from immature to mature bone structure such as distinct calcification borders and lamellation areas in the bone matrix were detected in all the groups at the 8th week evaluation (Figure 2). In addition, a non-homogenous bone matrix, which indicates on-going bone healing, was evident in all groups at the same week. The mean osteocyte, osteoblast, osteoclast, fibroblast and newly formed vessel counts were considered to evaluate the cellular changes in the defect area.

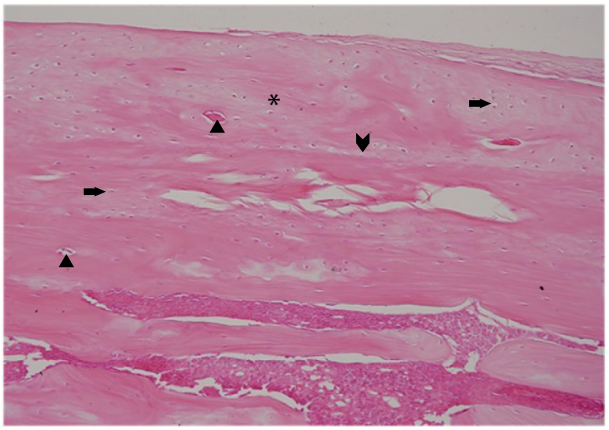
LLLT effects on osteocytes

Osteocyte number increased significantly in both the control and laser groups at the 8th week compared with the 4th week ($p < 0.05$). In addition, the number



Asterix: Fibrous tissue; Right arrow: Osteocyte; Left arrow: Osteoclast; Triangle: Osteoblast; Arrowhead: Calcified cartilage.

Figure 1. Fibrous tissue, osteocyte, osteoblast and calcified cartilage at 4th week evaluation.



Asterix: Non-homogeonus bone matrix; Right arrow: Osteocyte; Triangle: Vascular canal; Arrowhead: Calcification line.

Figure 2. Non-homogenous bone matrix, osteocytes, and calcification line at 8th week evaluations.

of osteocytes in the control group was greater than in the laser groups at the 4th and 8th weeks. However, no significant difference was observed among all groups at either time point ($p < 0.05$) (Table 1).

LLLT effects on osteoblasts

The osteoblast number decreased significantly at the 8th week compared with the 4th week in the control ($p < 0.05$), L20 ($p < 0.05$) and L5 ($p < 0.01$) groups. Although the osteoblast numbers in the L20 group at the 4th week and in the L10 group at the 8th week were greater than in the other groups, there was no significant difference among the control and all laser groups at the 4th and 8th weeks ($p > 0.05$) (Table 2).

LLLT effects on osteoclasts

The osteoclast number decreased significantly at the 8th week compared with the 4th week in all groups ($p < 0.01$). Although the osteoclast number in the L5 group was greater than in the other groups at the 4th week, there was no significant difference in osteoclast numbers among the control and all laser groups at the 4th and 8th weeks ($p > 0.05$) (Table 3).

Table 1. Analysis of osteocyte number among groups and dependent on time. Data presented as mean ± standard deviation (minimum–maximum).

Group	4th week	8th week	p-value (d)
Control	926 ± 277.58	1340.8 ± 297.61	0.037[A]
	(576–1266)	(958–1742)	-
L5	640.83 ± 99.76	1004.83 ± 278.14	-
	(504–767)	(695–1391)	0.037[A]
L10	685.67 ± 315.15	1127.33 ± 236.05	0.01[A]
	(342–1170)	(840–1435)	-
L20	780 ± 162.40	1231.83 ± 195.18	-
	(597–994)	(911–1401)	-
p-value (e)	0.191[z]	0.212[z]	-

For intragroup comparison, capital shifts were used. For inter group comparison, lower case shifts were used. A: $p < 0.05$; z: $p > 0.05$; d: Time dependent comparison of groups; e: Comparison of all groups among themselves in same time period.

LLLT effects on fibroblasts

The fibroblast number decreased in the L10 and L20 groups at the 8th week compared with the 4th week ($p < 0.01$), while the control and L5 groups exhibited no significant change between the 4th

Table 2. Analysis of osteoblast number among groups and dependent on time. Data presented as mean ± standard deviation (minimum–maximum).

Group	4th week	8th week	p-value (d)
Control	349.67 ± 240.03	97.67 ± 144.53	0.037[A]
	(102–788)	(0–375)	0.006[B]
L5	305 ± 106.89	63 ± 65.12	-
	(504–767)	(0–170)	0.262
L10	213 ± 169.50	153.5 ± 136.49	0.04[A]
	(102–788)	(24–339)	-
L20	354.5 ± 160.71	36.33 ± 45.75	-
	(144–417)	(0–120)	-
p-value (e)	0.401[z]	0.258[z]	-

For intragroup comparison, capital shifts were used; For inter group comparison, lower case shifts were used; A: $p < 0.05$, B: $p < 0.01$; z: $p > 0.05$; d: Time dependent comparison of groups; e: Comparison of all groups among themselves in same time period.

Table 3. Analysis of osteoclast number among groups and dependent on time. Data presented as mean ± standard deviation (minimum–maximum).

Group	4th week	8th week	p-value (d)
Control	3.5 ± 2.34	0	0.007[B]
	(0–6)	0	0.002[B]
L5	7.5 ± 3.93	-	-
	(4–15)	0	0.002[B]
L10	5.67 ± 3.88	0	0.002[B]
	(2–12)	-	-
L20	4.33 ± 2.16	-	-
	(1–7)	-	-
p-value (e)	0.203[z]	1[z]	-

For intragroup comparison, capital shifts were used; For inter group comparison, lower case shifts were used; B: $p < 0.01$; z: $p > 0.05$; d: Time dependent comparison of groups; e: Comparison of all groups among themselves in same time period.

and 8th weeks ($p > 0.05$). The L10 group exhibited significantly higher fibroblast numbers than the L5 and L20 groups at the 4th week ($p < 0.05$). The fibroblast numbers in the L10 group at the 4th week and in the control group at the 8th week were greater than in the other groups; however, the 8th week evaluation indicated no significant difference among all groups ($p > 0.05$) (Table 4).

LLLT effects on newly formed vessels

The newly formed vessel count decreased significantly at the 8th week compared to the 4th week in the L5 and L20 groups ($p < 0.01$). However, no difference was observed in the control and L10 groups between the two time points ($p > 0.05$). Although the newly formed vessel number in the L10 group was greater than in the other groups at the 4th and 8th weeks, no significant difference was observed among all groups at either time point ($p > 0.05$) (Table 5).

Table 4. Analysis of fibroblast number among groups and dependent on time; Data presented as mean ± standard deviation (minimum–maximum).

Group	4th week	8th week	p-value (d)
Control	547.17 ± 519.23	225 ± 297.61	0.171^{Z}
	(0–1280)	(0–730)	0.107^{Z}
L5	287.17 ± 120.92	125 ± 278.14	-
	(137–466)	(0–360)	0.004^{B}
L10	1043.83 ± 185.43	133.67 ± 236.05	0.002^{B}
	(760–1250)	(0–384)	-
L20	327.17 ± 218.64	0	-
	(123–703)	-	-
p-value (e)	0.014^{a}	0.3^{z}	-
p-value (f)	0.004^{a}	-	-
p-value (g)	0.004^{a}	-	-

For intragroup comparison, capital shifts were used; For inter group comparison, lower case shifts were used: a: $p < 0.05$, z: $p > 0.05$; B: $p < 0.01$, Z: $p > 0.05$; d: Time dependent comparison of groups; e: Comparison of all groups among themselves in same time period; f: Comparison of L5 and L10 groups at 4th week; g: Comparison of L10 and L20 groups at 4th week.

Table 5. Analysis of newly formed vessel number among groups and dependent on time; Data presented as mean ± standard deviation (minimum–maximum).

Group	4th week	8th week	p-value (d)
Control	4.33 ± 7.09	2 ± 3.09	0.589^{Z}
	(0–18)	(0–6)	0.005^{B}
L5	15.17 ± 15.99	0.5 ± 1.22	-
	(2–47)	(0–3)	0.121^{Z}
L10	23.67 ± 31.22	4.33 ± 8.26	0.007^{B}
	(0–86)	(0–21)	-
L20	5.17 ± 8.84	0	-
	(0–23)	-	-
p-value (e)	0.115^{z}	0.242^{z}	-

For intragroup comparison, capital shifts were used; For inter group comparison, lower case shifts were used; z: $p > 0.05$; B: $p < 0.01$, Z: $p > 0.05$; d: Time dependent Comparison of groups; e: Comparison of all groups among themselves in same time period.

Discussion

This study aimed to investigate the action of LLLT (940 nm) with different energy intensities on bone healing. It is hypothesized that the greater the energy intensity is increased in LLLT, a greater biostimulative effect can be gained during the bone healing process. The main findings of this study showed that LLLT with a wavelength of 940 nm Ga-Al-As laser may not have an inductive effect during the bone repair process of created bone defects of rats according to the histological analysis.

The bone healing mechanism can be hindered by local factors such as trauma, infection, and radio-osteonecrosis or systemic factors such as Paget Disease, Fibrous Dysplasia, Diabetes Mellitus, the use of corticosteroids or hormonal disturbances.[17,18] To date, several methods such as bone grafts, growth factors, platelet-rich fibrin, low-level pulsed ultrasound and low-level laser therapy have been proposed to promote bone healing.[19,20,21] Among all these methods, LLLT outshines other modalities due to the advantage of being non-invasive and the ease of application in clinical use. However, there is still no standard protocol for LLLT application to promote bone healing in the

literature. LLLT also has various effects in bone tissue according to the application parameters, such as the type of laser used, energy density (J/cm^2), time and frequency of irradiation, and wavelength, which can contribute effectively to the stimulation of bone cells by increasing the osteoblastic activity at the defect area.[2,14,22] Thus, the biomodulatory effects of laser irradiation seem to be dose dependent.[2] In addition, the method of application can also influence the biostimulatory effect of the laser on bone cells. However, all these effects have been subjected to extensive clinical research.[23,24]

Infrared lasers have better tissue penetration due to poor absorption by water and skin pigments.[25] Various energy densities have been experimentally used for accelerating bone healing in the literature.[1,16] Some authors have suggested that energy densities in the range of 1-5 J/cm^2 exert a stimulatory effect on bone tissue.[26] Another study's results state that a total energy density of 16 J/cm^2 is more effective on bone metabolism.[27] Additionally, some studies have suggested higher doses for stimulation of bone healing.[1,16] Because of this conflict, there is no accepted ideal energy density for promoting bone healing. In this study, the effectiveness of LLLT with different energy densities on bone healing was evaluated by application of the Ga-Al-As laser at 5, 10 and 20 J/cm^2 with an irradiation time of 10 seconds. The same surgical procedures were applied to all animals, and laser irradiation was administered from one point to a 3 cm^2 area of irradiation that included the whole defect area using a laser hand piece. However, in our study, no significant differences were found among the various energy densities of LLLT on bone healing process in histological analysis. Such differences could probably be explained by the variations in the study designs.

The control groups were independent rather than using the opposite tibia for control to eliminate possible systemic or local effects of the laser irradiation. One possible mechanism for systemic effects of LLLT on distant sites would be the ability of the laser to stimulate the release of growth factors or cytokines and the effects of these mediators at non-radiated sites.[28]

The histological analysis revealed that none of the energy densities of LLLT presented a significant change in any of the bone healing parameters compared to the controls. Osteoblast, osteoclast and fibroblast counts were significantly higher at the 4th week than the 8th week, in accordance with the early stage of bone healing, while the osteocyte number increased significantly at the 8th week due to the late stage bone remodelling mechanism. Khadra et al.[3] reported in their study that the newly formed vessel number was higher during the early stage of bone healing. Similarly, our study results showed that the newly formed vessel count was significantly higher at the 4th than the 8th week in the L5 and L20 groups, while no significant intergroup difference was present either the 4th or the 8th week. Although the number of fibroblasts did not change between the control and LLLT groups at either time point, the L10 group had a significantly higher number of fibroblasts than the L5 and L20 groups, which allowed the interpretation that LLLT with an energy density of 10 J/cm^2 might be effective in increasing fibroblastic activity. An in vitro study by Ozawa et al.[29] indicated that LLLT increased osteoblast proliferation during the early period of bone healing, which is consistent with our study results indicating higher osteoblast and osteoclast numbers at the 4th week.

LLLT has been proposed as an effective method for stimulating osteogenesis and accelerating bone healing.[20,24] Conversely, no significant effect of LLLT has been reported in cells.[30] The effect of LLLT has been investigated in bone tissue with wavelengths ranging from 670 to 1064 nm.[14,24] However, there is a scarcity of studies on the use of the longer 940 nm laser wavelength on the bone healing process in the field of photodynamic therapy. It has been demonstrated that laser biostimulation at low doses activates cellular activity, whilst higher doses inhibit cell functions, as described by a dose response curve.[10,13,30] However, it is reported that Ga-Al-As diode lasers with wavelengths of 780–950 nm can penetrate to deeper tissues.[31] Moreover, some authors have demonstrated positive biostimulatory effects of LLLT with longer wavelengths (904–980 nm) on the bone regeneration process.[14,24,32] In this study, a Ga-Al-As diode laser operating at a wavelength of 940 nm was used due to its improved tissue penetration profile. We thought that the low-intensity

irradiation from a 940 nm GaAlAs diode laser used in this study would be able to promote bone healing at a deeper level in healthy rats. In addition, to derive greater biostimulatory effects, we chose LLLT with a longer wavelength. However, our study results were not consistent with the previous results indicating the accelerating and promoting effect of LLLT on bone healing. There are a few factors that might be associated with the different results of our study. The longer wavelength of LLLT (940 nm) used in this study could be a relevant factor for the ineffectiveness of the laser biostimulation, as the positive biostimulatory effects of wavelength of 940 nm on bone healing have not been clearly reported in the literature. The irradiation time has also been one of the non-standardized parameters of LLLT in the literature. In a study evaluating the effect of LLLT irradiation time on extraction socket healing in rats, different irradiations were compared during 1, 2 or 5 minutes. Of these irradiation times, an irradiation of 5 minutes produced the highest gene expression rate.[33] However, many different irradiation times have been used to increase the biostimulatory effect of LLLT on the bone healing pattern in previous studies.[3,4,14]

Furthermore, most studies concerning the effects of LLLT on bone healing present a shorter irradiation period, varying from 10 seconds to 30 seconds, which is the most commonly used irradiation method in studies on the effect of LLLT on bone healing.[22,32] In our study, although the different energy densities were achieved by altering the power applied during a constant irradiation time of 10 seconds, LLLT showed no substantial promoting effect on the repair process of bone defects. It may be suggested that the time of irradiation applied to the tissue in our protocol was not sufficient to stimulate bone metabolism.

The power applied is another parameter potentially related to the unexpected results of our study. According to the Food and Drug Administration (FDA) clarification, therapeutic lasers offer a power of 5 mW to 10000 mW (10 W). It has also been reported in many studies that the powers applied for bone biostimulation ranged from 10 milliwatts to 8 Watts.[4,22,34] The majority of these studies have proposed the use of power at the milliwatt level. However, there are other promising study results for bone biostimulation with power at the Watt level.[4,35] Park et al.[24] suggest that low-intensity diode laser irradiation is beneficial for alveolar bone healing in both normal and diabetic rats when applied at a dose of 13.95 J/cm^2. Despite promising results with the use of power in that range, our study results did not comprise with the previous studies. This finding may be related to various factors, including the number of treatment applications. In our study, irradiation was performed postoperatively and on days 2, 4, 6, 8, 10, and 12 thereafter. The irradiation frequency was based on similar studies in which bone healing after LLLT was evaluated.[16,36] Most of the studies were performed for five sessions per week. However, different treatment application protocols were reported for LLLT at different powers for bone healing.[2,4,24] It may be speculated that different biostimulatory effects on bone healing patterns may occur by using LLLT with different energy densities/wavelengths and varying powers.

Conclusion

This study showed that the application of 940 nm LLLT with a diode laser at different energy densities (5 J/cm^2, 10 J/cm^2, 20 J/cm^2) may not accelerate the bone repair process in both the initial and the late phases of healing in created defects compared to the control. Further comprehensive studies of low-level 940 nm laser therapy for biostimulating bone tissue are required to identify the possible response mechanisms that may explain the contrasting results and to evaluate the effects of different powers, energy densities, irradiation times and frequencies in the analysis of laser irradiation.

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