

Effect of Pulsed Wave Low-Level Laser Therapy on Tibial Complete Osteotomy Model of Fracture Healing With an Intramedullary Fixation

Atarodalsadat Mostafavinia,¹ Reza Masteri Farahani,¹ Mohammadreza Abbasian,² Mohammadmehdi Vasheghani Farahani,³ Mohammadjavad Fridoni,⁴ Sara Zandpazandi,⁵ Seyed Kamran Ghoreishi,⁶ Mohammad Amin Abdollahifar,¹ Ramin Pouriran,⁵ and Mohammad Bayat^{1,*}

¹Department of Anatomical Sciences and Biology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran

²Department of Orthopedic Surgery, Akhtar Hospital, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran

³Paramedical School, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran

⁴Department of Anatomy, Medical Faculty, Zanjan University of Medical Sciences, Zanjan, IR Iran

⁵Medical School, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran

⁶Department of Statistics, University of Qom, Qom, IR Iran

*Corresponding Author: Mohammad Bayat, Department of Anatomical Sciences and Biology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, IR Iran. Tel: +98-2122439976, Fax: +98-2122439976, E-mail: mohbayat@sbmu.ac.ir; bayatenator@gmail.com

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Abstract

Background: Fractures pose a major worldwide challenge to public health, causing tremendous disability for the society and families. According to recent studies, many in vivo and in vitro experiments have shown the positive effects of PW LLLT on osseous tissue.

Objectives: The aim of this study was to evaluate the outcome of infrared pulsed wave low-level laser therapy (PW LLLT) on the fracture healing process in a complete tibial osteotomy in a rat model, which was stabilized by an intramedullary pin.

Materials and Methods: This experimental study was conducted at Shahid Beheshti University of Medical Sciences in Tehran, Iran. We performed complete tibial osteotomies in the right tibiae for the population of 15 female rats. The rats were divided randomly into three different groups: I) Control rats with untreated bone defects; II) Rats irradiated by a 0.972 J/cm² PW LLLT; and III) Rats irradiated by a 1.5 J/cm² PW LLLT. The right tibiae were collected six weeks following the surgery and a three-point bending test was performed to gather results. Immediately after biomechanical examination, the fractured bones were prepared for histological examinations. Slides were examined using stereological method.

Results: PW LLLT significantly caused an increase in maximum force (N) of biomechanical repair properties for osteotomized tibiae in the first and second laser groups (30.0 ± 15.9 and 32.4 ± 13.8 respectively) compared to the control group (8.6 ± 4.5) (LSD test, P = 0.019, P = 0.011 respectively). There was a significant increase in the osteoblast count of the first and second laser groups (0.53 ± 0.06, 0.41 ± 0.06 respectively) compared to control group (0.31 ± 0.04) (LSD test, P = 0.001, P = 0.007 respectively).

Conclusions: This study confirmed the efficacy of PW LLLT on biomechanical strength, trabecular bone volume, callus volume, and osteoblast number of repairing callus in a complete tibial osteotomy animal model at a relatively late stage of the bone healing process.

Keywords: Low-Level Laser Therapy, Biomechanical Phenomena, Histology, Osteotomy, Tibial Fracture, Rats

1. Background

Fractures are the most common traumas in clinics, causing a lot of pain for patients (1). This injury is one of the main concerns for public health problems worldwide (2). In the United States, approximately six million fractures happen annually, in which 5% - 10% proceed to nonunion (3). Treatment that could potentially accelerate the healing process could help decrease the rate of this large problem.

The regeneration of bone formation in the healing process is a complex, organized, and orchestrated anatomical and physiological process (4). Fractured bone repair involves numerous molecular, cellular and tissue factors that have a direct effect on the repairing process. Throughout the reparative phase, a bone in the healing process might be exposed to re-fracture (5). There are complex clinical

conditions in which considerable bone regeneration quantity is required, such as for skeletal reconstruction of large bone defects caused by trauma, or skeletal abnormalities.

Presently, there are many different strategies to boost impaired or "insufficient" bone-regeneration, which include the gold-standard autologous bone graft (4). Improved local strategies such as tissue engineering, gene therapy, and low-level laser therapy (LLLT) are the main focus of researchers to overcome the limitations of current methods and techniques. The intent is to produce a bone-graft substitution using biomechanical properties identical to normal bone, accelerating the overall regeneration process (4-6).

According to recent studies, researchers have determined that continuous wave (CW) LLLT stimulates in vitro

mineralization through increased IGF-I and BMP production, Runx2 expression and ERK phosphorylation (7). There is evidence that CW LLLT has stimulated bone nodule formation in osteoblasts (8). Other researchers reported that CW LLLT promoted the acceleration of bone strength and consolidation after a fracture (9-11). In addition; it enhanced the callus development in the early stage of the healing process in healthy animals (12). Eventually, it increased the bone lamella meshwork density of compact bone, and increased bone strength in diabetic rats (13). As stated in other reports, CW LLLT increased mineralized bone tissue in fractured femurs (14). On the other hand, various reports provided conflicting results regarding the use of CW LLLT on the bone repair system (13-16). Furthermore, modulating outcomes for high bioactive glass-ceramic on consolidation of bones in rats (17).

The data proves that pulsed wave (PW) light has different effects than CW light in an experimental environment (18). The use of pulsed light is becoming more popular, and the literature includes three cellular studies on rat calvarial cells (19-21). The first in vivo study was on tooth movement acceleration in rat molars (22), the second in vivo study was on bone turnover in ovariectomized rats (23), and a final report was conducted on the healing of partial tibial osteotomy in streptozotocin-induced diabetic rats (24).

Currently, there is no published data on the effect of PW LLLT on the fracture healing of osteotomized tibia treated by an intramedullary pin in our rat models.

2. Objectives

We proposed that PW LLLT application immediately after fracture could accelerate the healing process, since concise cellular effects of PW LLLT demonstrated convincing results for in vitro and in vivo models. Specifically, the aim of our study was to evaluate the effects of two energy densities for PW LLLT at 890 nm on the fracture healing process of osteotomized tibia treated by an intramedullary pin in our rat models.

3. Materials and Methods

3.1. Animals and Study Design

This experimental study was conducted at Shahid Beheshti University of Medical Sciences, Tehran, Iran, from 2014 to 2015. In this investigation, we used 4-month-old adult female Wistar rats weighing roughly 190 g. They were purchased from the animal house at the Iranian branch in the Pasteur Institute, Tehran, Iran. Animals were housed in individual clean cages in standard conditions (Table 1) and received a standard diet and tap water *ad libitum*. We monitored rats' body weights (Sartorius TE214S A, Germany) every week. All procedures were approved by the Medical Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (protocol no. 1393-1-91-13237).

The animals were randomly (simple) divided into a control group and two laser-treated groups of five animals

each. Those who received laser treatment were divided according to the laser's energy density. Rats in these groups received either 0.972 J/cm² or 1.5 J/cm² PW LLLT just following the surgery (Table 2). The laser treatments were performed precisely after the surgery, three times per week for six weeks until the animals were sacrificed. There was one observer in the current study.

3.2. Tibial Diaphysis Fracture Model (Complete Osteotomy)

The following anesthetizing process was used: rats were anesthetized by injections of 50 mg/kg ketamine hydrochloride intramuscularly (Rotex Medica, Tritteu, Germany) along with 5 mg/kg diazepam (Caspian, Rasht, Iran). The first step was the preparation of aseptic povidone iodine (Tolidaru, Tehran, Iran) and an incision of 1 cm was made over the medial part of the crural region in the right hind limb, which exposed the tibia. The second step involved making three circular partial transversal standardized osteotomies deep in the central medullary canal on the mid-point of the tibia with a low speed drill (terminal: 1.0mm diameter; Delab; Dental Fabriktreffurt, Germany). The third step was to break the osteotomy site manually and divide the bone into two parts. During the osteotomy procedure, the bones were irrigated with saline solution to prevent burning. An intramedullary fixation was operated by using a stainless pin (diameter: 1.0 mm). The fragments in the fractures were contacted and stabilized. Furthermore, in the entire population, a 3 mm gap between the edges of the incision was conducted equally. Then, in the proximal end of the tibia, a pin was placed and cut into the femoral surface of the tibia, (Figure 1). Rats were allowed unrestricted activities and movements after regaining consciousness. The muscles were sutured using 04 catgut (Supa, Iran) and the skin was sutured using 04 nylon reversed cutting sutures. Rats received 50 mg/kg of ceftrax (Jaber ben Hayan, Tehran, Iran). The antibiotic therapy was performed immediately after the surgery, as well, while antibiotic treatment was conducted in 24 and 48 hours afterwards.

3.3. Biomechanical Examination

The rats were euthanized six weeks after the surgery by overdoses of ketamine, diazepam, and cervical dislocation. The right tibias were collected and weighed (Sartorius TE214S A, Germany) after the soft tissues, skin and muscles were removed from each tibia. The pins were extracted carefully. The measurement of total bone volume was performed using the immersion method (24). The biomechanical properties of five tibias from the groups were examined. Bones were subjected to three-point bending on a material testing device (Zwick/Roell Group, Z 2.5 H 15WN, Ulm, Germany) until fracture took place in the bone. The entire bones were oriented similarly in the testing machine. Two loading points, 19 mm apart, were used to mount each bone; a press head was subsequently activated to squeeze the center of shaft in bones until fracture occurred. The

compressive loading speed was 0.08 mm/s during the testing time. Data was automatically recorded by the material testing device, which received the data from the load-deformation curve. The following parameters were computed: bending stiffness (N/mm), energy absorption (N mm), and maximum force (N), and stress high load (N/mm²).

Bending stiffness is the slope on the linear portion of the load-deformation curve. Energy absorption is the amount of energy absorbed by the bone until breakage. Maximum force is the force needed to break the bone. The stress high load was calculated by dividing N by the surface area (mm²) of bone at the osteotomy site (25).

3.4. Histological and Stereological Examinations

The fractured bones were repositioned, fixed in formalin saline and decalcified in formic acid for three weeks, immediately following biomechanical examination (26). Sections 5- and 15- μ m thick were cut serially in the callus area from the specimens after they were embedded in paraffin. The sections were stained with hematoxylin and eosin, after which 15 sections were randomly selected for stereological examination.

3.4.1. Measurement of Bone Volumes

Using a light microscope connected to a camera, the trabecular bone volume (mm³), callus volume (mm³), and bone marrow volume (mm³) were calculated using the Cavalieri method. This method was considered as a product of the areas and measured tissue thicknesses between the saved sections. By using stereological software, we determined the total area of sections ($\sum A$) of the tibial bone fracture. The volume was estimated as (27, 28):

$$(1) \quad V_{(mPFC)} = k \times t \sum A_{(mPFC)}$$

3.4.2. Measurement of the Total Number of Bone Cells

The method used to estimate numerical density and Total number of osteocytes, osteoblasts and osteoclasts in 15 μ m sections was the optical dissector technique. The specimens were evaluated with a high numerical aperture, at 60 \times oil immersion magnification. The image analysis computer analyzed the picture that it automatically received. The focus plane was set at the surface of the specimen. Then, a set of three unbiased measurement frames was superimposed on the live image (Figure 2A). Simultaneously, the microcator that measured the optical distance through the specimen in the Z axis was reset to zero. To see the objects, the focus was gently moved down through the specimen. The focal plane was moved downwards in z-direction.

Bone cells in the measurement frames' permitted areas were counted as they came into focus until the microcator indicated that the focal plane had traveled 10 μ m through the specimen (Figure 2B). The numerical density

of cells was obtained by the following formula:

$$(2) \quad N_V \left(\frac{\text{Cells}}{mPFC} \right) = \left[\frac{\sum Q^-}{\sum P \times \frac{t}{f} \times b} \times \frac{t}{BA} \right]$$

We used the following formula to estimate the total number of bone cells (29, 30):

$$(3) \quad N(\text{bone cell}) = N_V \times V(\text{final})$$

3.5. Clinical Observations

Rats were observed daily. We measured the body weights of animals in all groups at the beginning and end of the study with a fine balance. At the time of sacrifice, the presence or absence of a solid union at the osteotomy site was confirmed by manual palpation performed by a reviewer blinded to the group assignments.

3.6. Statistical Analysis

The Mean \pm SD and their corresponding bootstrap intervals of resample size 1,000 was expressed for the data. Normal distribution of data was analyzed by using the one-sample Kolmogorov-Smirnov test. Analysis of variance (ANOVA) and Kruskal-Wallis tests were performed to compare the changes between groups with normal distribution of data. LSD's test was used to identify the differences.

The rats were divided into three groups of five rats each by a simple random technique. The sample size for comparing the groups was based on a simple linear monogram introduced by Day and Graham (31) with an effective and conservative size of 5, an approximate power of 0.9, and a significance level of 0.05. A P value less than 0.05 was considered statistically significant. Repeated measurement, median and IQR were added to statistical methods.

4. Results

4.1. General and Clinical Observations

We excluded 8 out of 23 rats from the experiment due to poor fracture healing (non-union) or death after surgery (Table 2). By using the ANOVA (and Kruskal-Wallis) test, we found there was not a notable difference in body weight from the beginning of experiment to the end (Table 3). The paired test showed final weights for the control group. The second group that received the laser did not have a significant increase in their weight throughout the study (P = 0.030, and P = 0.015, respectively). The tibia weights were as follows: 819.2 \pm 167.7 mg (control), 978 \pm 162.8 mg (first laser), and 895.4 \pm 287.4 mg (second laser). Total bone volume (mm³) was: 778.2 \pm 159.3 (control), 929.1 \pm 154.7 (first laser) and 850.6 \pm 273.1 (second laser). Table 4 shows the corresponding bootstrap intervals. The ANOVA (and Kruskal-Wallis) test showed no significant differences in weights or Total bone volume among the groups.

4.2. Biomechanical Results

As shown in Figures 3 and 4, PWLLT significantly increased the biomechanical properties for repair of osteotomized tibias compared to the control group. This effect was more significant in the second group, which received a higher energy density of laser.

4.2.1. Bending Stiffness (N/mm)

The ANOVA and Kruskal-Wallis for bending stiffness lead $p=0.04$ and $p=0.11$ respectively. Bending stiffness was significantly increased in the second group that received laser therapy compared to the control group (Figure 3, LSD test, $P = 0.014$).

4.2.2. Maximum Force (N)

The ANOVA and Kruskal-Wallis for maximum force variable produce $P = 0.02$ and $P = 0.03$ respectively. There was a significant increase in maximum force in the first ($P = 0.019$) and second laser ($P = 0.011$) groups compared to the control group (Figure 3, LSD test).

4.2.3. High Stress Load (N/mm²)

The ANOVA and Kruskal-Wallis for high stress load lead $P = 0.024$ and $P = 0.076$ respectively. There was a significant increase in high stress load in the second laser treated group compared to the control group (LSD test, $P = 0.008$). See Figure 4 for more details.

4.2.4. Energy Absorption (N mm)

According to the LSD test, there was a significant increase in energy absorption in the first laser group compared to the control ($P = 0.008$) and second laser groups ($P = 0.028$, Figure 3).

4.3. Stereological Results

PW LLLT significantly increased trabecular bone volume, bone callus, and osteoblast numbers in repairing osteotomized tibias compared to the control group (Figures 6 and 7).

4.3.1. Trabecular Bone, Callus, and Bone Marrow Volumes

Total bone volume (mm³) was calculated as: 778.2 ± 159.3 (the control group), 929.1 ± 154.7 (the first laser group) and 850.6 ± 273.1 (the second laser group). Bone marrow volume (mm³) in the control group was 25.0 ± 0.7 , for the first laser group, it was 25.3 ± 0.9 and 25.4 ± 0.9 for the second laser group. See Table 4 for the corresponding bootstrap intervals. ANOVA (and Kruskal-Wallis) showed no significant differences in bone marrow volumes in the groups. As seen in Figure 5, the PW LLLT administered in both laser groups significantly increased trabecular bone volume compared to the control group LSD test, $P = 0.001$ (the first laser group) and $P = 0.007$ (the second laser group). There were significant differences in callus volume between the first laser and second laser groups compared to the control group (LSD test, $P = 0.000$ and $P = 0.001$, respectively).

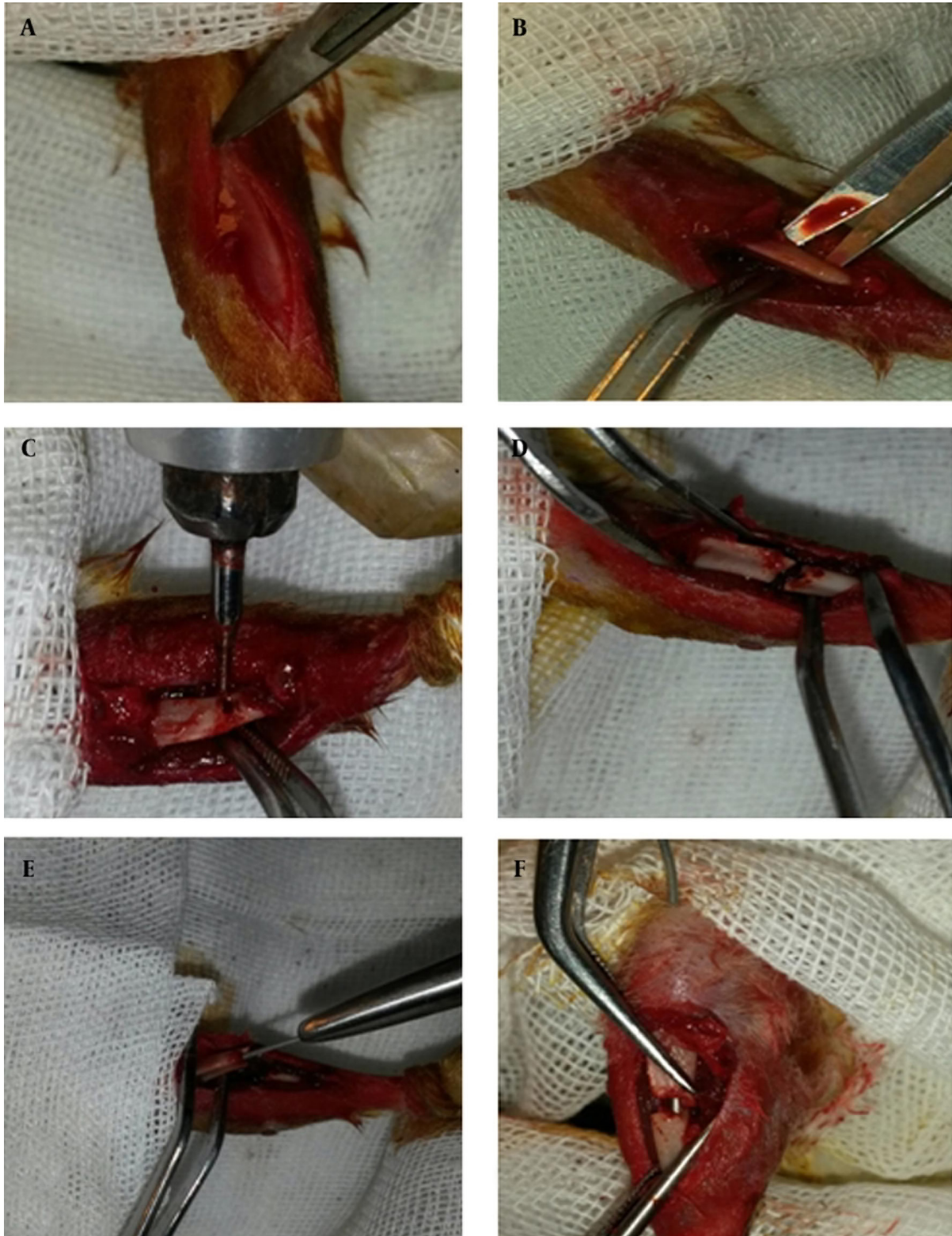
Table 1. Incidence of Death and Non-Union Among the Groups

Groups	Parameters			
	Death	Non-Union	Total Number of Mortality and Morbidity	Total Number of Rats
Control	0	4	4	9
First laser	1	2	3	8
Second laser	0	1	1	6

Table 2. Specifications of the Laser Used in This Study

Parameters	Dose and Unit
Peak power output	
First group	75 W
Second group	80 W
Average power	
First group	1.08 m W
Second group	1.15 m W
Power density	
First group	1.08 mW/cm ²
Second group	1.15 W/cm ²
Wave length	890 nm
Pulse frequency	80 Hz
Spot size	1 cm ²
Pulsed duration	180 μ s
Duration of exposure for each point	
First group	900 s
Second group	1300 s
Energy density	
First group	0.972 J/cm ²
Second group	1.5 J/cm ²

Figure 1. Steps in the Complete Osteotomy



A, the image shows the incision; B, exposed tibia mid-shaft; C, circular partial transversal standardized osteotomy procedure with low speed drill; E, complete bone fracture; insertion of a stainless wire; and F, maintenance of reduction with the stainless wire.

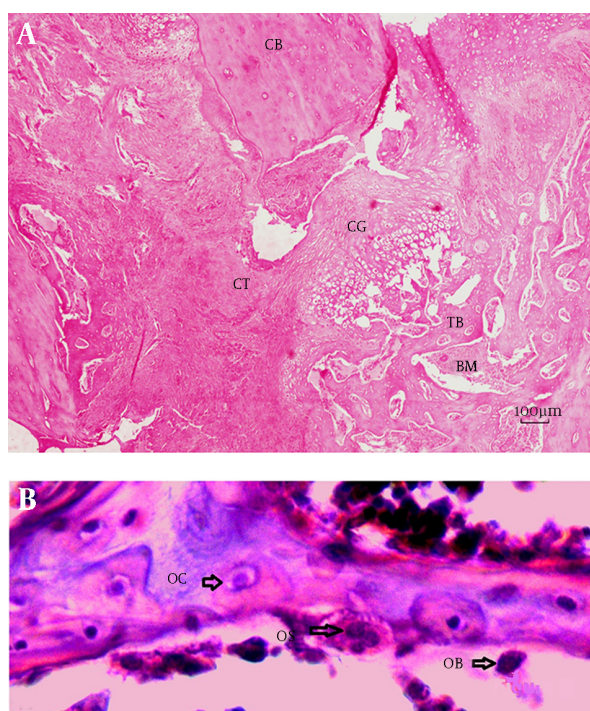


Figure 2. Light Micrograph of Stereological Examination, Bony Tissues and Cells, H and E.

4.3.2. Total Numbers of Osteocytes, Osteoblasts and Osteoclasts

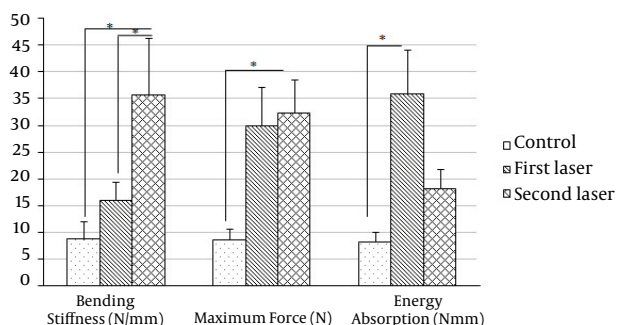
Total number of osteocytes (10^6) in the control group was 1.7 ± 0.1 . The first laser group had 1.7 ± 0.06 osteocytes and the second laser group had 1.7 ± 0.1 osteocytes. Total number of osteoclasts (10^3) were: 2.4 ± 0.3 (control), 2.2 ± 0.1 (the first laser group) and 2.4 ± 0.2 (the second laser group). Table 4 shows the corresponding bootstrap intervals. ANOVA (and Kruskal-Wallis) showed no significant differences in Total number of osteocytes and osteoclasts in the groups. The PW LLLT with 0.972 J/cm^2 significantly increased the total number of osteoblasts compared to the control group (Figure 7). To provide more details of our data for all variables considered in this research, Table 5 gives their median and IQR. Moreover, we applied the repeated measure analysis for tibia weights, Total bone volume (mm^3), bone marrow volume (mm^3), total number of osteocytes (10^6), and Total number of osteoclasts (10^3). The corresponding Wilks' Lambda statistics and their significant levels are given in Table 6. It is obvious that the results of repeated measure analysis support the previous results based on ANOVA and Kruskal-Wallis techniques. As seen in Figure 6, the PW LLLT administered in first laser group significantly increased total number of osteoblasts compared to the control group (LSD test, $p < 0.01$). Figure 7 shows estimated marginal means of studied groups for high stress load.

Table 3. Mean \pm SD (and Their Corresponding Bootstrap Intervals in the Parentheses) of First Weight (g) and Final Weight of Studied Groups

Groups	Weights, g	
	First	Final
Control	181.6 ± 15.6 (181.6 ± 3.3)	192.6 ± 15.7^a (192.6 ± 4.8)
First laser	176.8 ± 39.3 (176.8 ± 16.8)	193.0 ± 27.3 (193.0 ± 8.9)
Second laser	197.2 ± 21.56 (197.2 ± 5.9)	207.0 ± 18.2^a (207.0 ± 5.2)

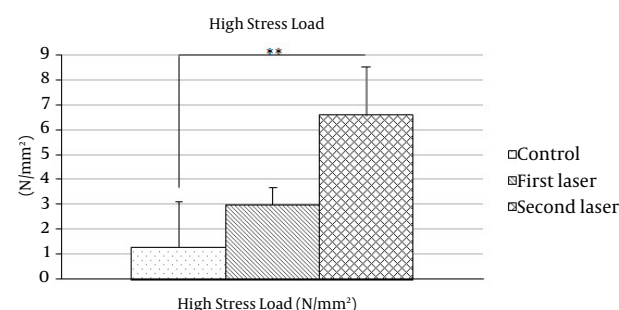
^aThe ANOVA test revealed no significant differences in body weight among all groups at the beginning and at the end of the study. The paired test showed that there were significant differences between the final weights of the control group and the second laser group, compared to their weights at the beginning of study ($P = 0.030$, and $P = 0.015$ respectively).

Figure 3. Mean \pm SEM of Bending Stiffness, Maximum Force And Energy Absorption of the Groups Compared by the LSD Test



* ($P < 0.05$).

Figure 4. Mean \pm SEM of High Stress Load in the Groups Compared by the LSD Test



* $P < 0.05$; ** $P < 0.01$.

Table 4. Mean ± SD and Their Corresponding Bootstrap Intervals of TIBIA Weights, Total Bone Volume (mm³), Bone Marrow Volume (mm³), Total Number of Osteocytes (10⁶), and Total Number of Osteoclasts (10³)

Variables	Group		
	Control	First Laser	Second Laser
Tibial weight, mg			
Mean ± SD	819.2 ± 167.7	978.0 ± 162.8	895.0 ± 287.4
Bootstrap Mean ± SEM	819.2 ± 71.3	978.0 ± 59.9	895.0 ± 84.4
Total bone volume, mm³			
Mean ± SD	778.2 ± 159.3	929.1 ± 154.7	850.6 ± 273.1
Bootstrap Mean ± SEM	778.2 ± 68.8	929. ± 55.7	850.6 ± 77.9
Bone marrow volume, mm³			
Mean ± SD	25.0 ± 0.7	25.3 ± 0.9	25.4 ± 0.9
Bootstrap Mean ± SEM	25.0 ± 0.2	25.3 ± 0.2	25.4 ± 0.2
Trabecular bone volume, mm³			
Mean ± SD	149.8 ± 3.9	164.3 ± 5.9	160.3 ± 5.4
Bootstrap Mean ± SEM	149.8 ± 1.0	164.3 ± 1.5	160.3 ± 1.3
Total number of osteoblast, 10⁶			
Mean ± SD	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
Bootstrap Mean ± SEM	0.3 ± 0.02	0.5 ± 0.03	0.4 ± 0.03
Total number of osteocytes, 10⁶			
Mean ± SD	1.7 ± 0.1	1.7 ± 0.06	1.7 ± 0.1
Bootstrap Mean ± SEM	1.7 ± 0.03	1.7 ± 0.03	1.7 ± 0.03
Total number of osteoclasts, 10³			
Mean ± SD	2.4 ± 0.3	2.2 ± 0.1	2.4 ± 0.2
Bootstrap Mean ± SEM	2.4 ± 0.07	2.2 ± 0.03	2.4 ± 0.04
Bending stiffness, N/mm			
Mean ± SD	8.7 ± 7.5	16.0 ± 7.9	35.8 ± 23.5
Bootstrap Mean ± SD	8.7 ± 2.1	16.0 ± 2.0	35.8 ± 5.6
Maximum force, N			
Mean ± SD	8.6 ± 4.5	30.0 ± 15.9	32.4 ± 13.8
Bootstrap Mean ± SEM	8.6 ± 1.1	30.0 ± 4.1	32.4 ± 3.3
Energy absorption, N mm			
Mean ± SD	8.2 ± 4.1	28.7 ± 22.9	18.2 ± 8.2
Bootstrap Mean ± SEM	8.2 ± 1.2	28.7 ± 6.0	18.2 ± 1.9
Stress high load, N/mm²			
Mean ± SD	1.3 ± 0.7	3.0 ± 1.6	6.6 ± 4.2
Bootstrap Mean ± SEM	1.3 ± 0.2	3.0 ± 0.4	6.6 ± 1.4

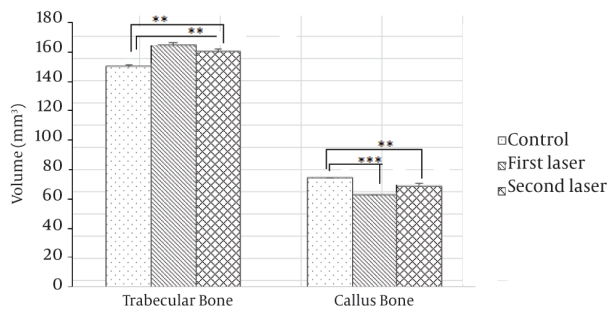
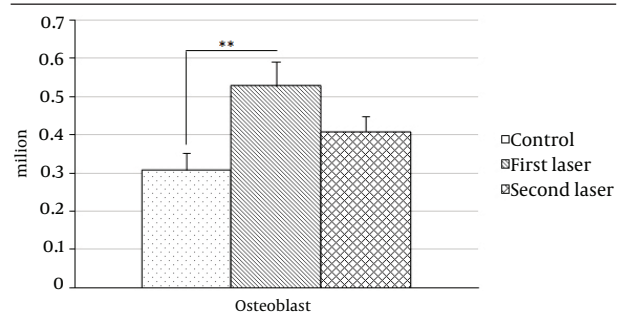


Figure 5. Mean ± SEM of Trabecular Bone and Callus Volumes in the Groups Compared by the LSD Test. ** P < 0.01; *** P < 0.001.

Figure 6. Mean ± SEM of Osteoblasts Number in the Groups Compared by the LSD Test



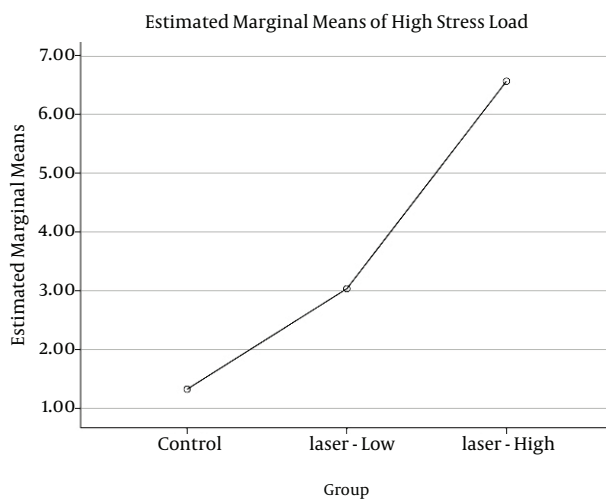
** P < 0.01.

Table 5. Median and IQR of Tibia Weights, Total Bone Volume (mm³), Bone Marrow Volume (mm³), Total Number of Osteocytes (10⁶), and Total Number of Osteoclasts (10³)

Variables	Group		
	Control	First Laser	Second Laser
Tibial weight, mg			
Median	883.00	926	703
IQR	56	114	409
Total bone volume, mm³			
Median	838.85	879.70	667.85
IQR	38.65	108.3	388.55
Bone marrow volume, mm³			
Median	25	25	25.10
IQR	0.7	1.10	1.4
Trabecular bone volume, mm³			
Median	150	162.5	158.13
IQR	42	7.15	8.82
Total number of osteoblast (10⁶)			
Median	0.36	0.51	0.38
IQR	0.13	0.22	0.07
Total number of osteocytes (10⁶)			
Median	1.68	1.70	1.68
IQR	0.12	0.02	0.14
Total number of osteoclasts (10³)			
Median	2.47	2.15	2.35
IQR	0.39	0.09	0.30
Bending stiffness, N/mm			
Median	8.23	13.56	30.05
IQR	6.73	9.23	27.14
Maximum force, N			
Median	6.83	32.79	24.83
IQR	6.18	18.6	22.83
Energy absorption, N mm			
Median	7.33	26.13	22.55
IQR	4.51	25.18	14.89
Stress high load, N/mm²			
Median	1.01	2.41	4.24
IQR	1.3	2.81	3.80

Table 6. Wilks' Lambda and the Corresponding Significant Levels of Repeated Measures Analysis for Tibia Weights, Total Bone Volume (mm³), Bone Marrow Volume (mm³), Total Number of Osteocytes (10⁶), and Total Number of Osteoclasts (10³)

Variables	Wilks' Lambda	Sig
Tibial weight, mg	0.063	0.01
Total bone volume, mm³	0.750	0.650
Bone marrow volume, mm³	0.772	0.678
Trabecular bone volume, (mm³)	0.168	0.069
Total number of osteoblast(10⁶)	0.045	0.010
Total number of osteocytes (10⁶)	0.884	0.831
Total number of osteoclasts (10³)	0.262	0.134
Bending stiffness, N/mm	0.063	0.016
Maximum force, N	0.19	0.083
Energy absorption, N mm	0.121	0.042
Stress high load, N/mm²	0.078	0.022

Figure 7. Estimated Marginal Means of High Stress Load of Studied Groups

** P < 0.01.

5. Discussion

This research assessed PWLLT effects on healing a surgically induced complete osteotomized tibia stabilized by an intramedullary pin in rat models. Few reports on the applications of PW LLLT using an infra-red diode laser for bone healing exist. Our findings have revealed statistically significant differences in biomechanical and stereological parameters between the control and laser-treated groups six weeks after surgery.

Exactly six weeks after surgery, maximum force was measured by a three-point bending test on bone pressure resistance to evaluate the biomedical properties of the bones. The bending test has been a valid method for evaluation of biomechanical strength in animal models (10, 13). The result for stereological analysis showed that PW LLLT significantly increased trabecular bone volume, bone callus volume, and the number of osteoblasts six weeks after the surgery. The increased amount of bone trabeculae (32) and number of osteoblasts in laser-treated rats could be explained by the fact LLLT induced cellular proliferation, which caused biostimulatory effects on multipotent cells, guiding them to differentiate into osteoblasts, which is the classic producer of bone matrix (33). This is a definite indication that PW LLLT increases new bone formation and accelerates the fracture healing process.

As of now, the PW LLLT biological effect on tissue healing has not been understood. However, it has been implied that by using low radiation doses of light energy, absorption by intracellular chromophores such as porphyrins and cytochromes could occur. The energy gets converted into metabolic energy, which involves the respiratory chain by making a transmembrane electrochemical proton gradient (34). Using LLLT can provide the following

benefits: cellular reactions acting as ATP synthesis promotion, the stimulation of electron transport chain, and reduction in cellular pH. The increase in the activity of macrophages, fibroblasts, lymphocytes, and other healing cells may happen because of the changes that occur in the cellular, and biochemical process, as well as in the plasma membrane as a consequence of using LLLT (34). LLLT can have many positive effects on the healing process, including: increase in the synthesis of collagen and DNA, rapid elimination of necrotic tissues, increase of Ca⁺ deposition, increase in the function of periosteum, increase in the function of osteoblast and osteocyte, new visualization, stimulation of enchondral ossification, faster differentiation of mesenchymal cells, increase in pre-osteogenic cells, and stimulation of callus formation (35). The metabolic process is also activated using this energy.

We used infrared laser light in this research because it enhanced the ability to penetrate subcutaneous and other tissue types (30) by using precise wavelengths, definitely positive results can be seen. Higher wavelengths make lower dispersion of waves than lower ones; in addition, it penetrates deeply into the skin. According to the reports, λ 632.8-nm laser light penetrates 0.5 - 1 mm before losing its intensity up to 37%. On the other hand, infrared wavelengths penetrate 2 mm before losing the same amount of energy. This is an obvious reason for using infrared laser light on bone tissue (36).

There is a quite convincing reason for using PW LLLT as a therapeutic tool, because the amount of energy needed to yield the formation of high quality tissue is not much (37). We evaluated two different energy densities (doses) of the 890 nm PW LLLT infra-red laser to define the best density for use on defective bones (38). Both 0.97 and 1.5 J/cm² energy densities used in the current study were lower than the densities used for CW LLLT (2.4 - 382 J/cm²) (9-15), which indicated that the use of a lower energy density for PW LLLT could yield the same outcomes.

CW LLLT and PW LLLT devices are currently available in clinics, providing wide range of therapeutic options to medical practitioners. However, the PW LLLT device is more potent than laser parameters when compared to the CW LLLT in peak and average power output, pulse frequency and pulse duration. It is assumed that by investigating different values of these parameters, researchers can select better protocols and achieve better outcomes by using PW LLLT devices (39).

Because there are 'quench periods' (pulse 'off' times) that follow pulse 'on' times, PW LLLT generates minor tissue heating. When it is necessary to use a laser on deep tissues, increased power is required to provide sufficient energy. This increased power can cause tissue heating in the surface layers. In this circumstance, pulsed light can be of benefit. Although CW LLLT causes the increase in temperature at the site of target tissues, PW LLLT has not caused a measurable change in the temperature of the irradiated area for the same delivered energy density (19). In this regard, Ilic et al. (40) have shown that pulsed light with peak power densities of 750 mW/cm² administered

for 120 seconds produced no neurological or tissue damage, whereas the same power density delivered by CW (for the same number of seconds) caused neurological deficits.

Our results showed that 1.5 J/cm² energy density provided more significant improvement in biomechanical parameters, compared to the 0.972 J/cm² and the control groups (13, 41). In contrast, Nissan et al. reported that low-power intensity (4 mW/cm² power density) LLLT was more effective on bone healing than higher power intensity (22.4 mW/cm²) LLLT (42).

Bossini et al. explored the effect of LLLT on bone repair in ovariectomized rats in a control group, animals irradiated with 60 J/cm² LLLT, and animals irradiated with 120 J/cm² LLLT (38). They observed higher amounts of newly formed bone and granulation tissue in both laser-treated groups than the control group. A picosirius probe demonstrated that animals with irradiation had higher deposition and more organized collagen fibers compared to the control group. However, there was not a huge difference in the biomechanical data (39).

According to our histological examination, 0.972 J/cm² PW LLLT significantly increased osteoblast counts compared to the control group. However, 1.5 J/cm² PW LLLT did not cause an increase in osteoblast counts compared to the control group. Rats treated with 1.5 J/cm² had more significant results compared to those treated by 0.972 J/cm².

According to the stereological parameters and biochemical markers in bone metabolism, the animal probe indicated a decrease in bone formation and minimal changes in bone re-sorption. On the other hand, these parameters are not quite relevant to osteoporotic fractures and projects researching orthopedic surgeries. Histological studies do not give direct information about the mechanical strength of bones. The most distinct reason for bone fractures following a least possible trauma is reduction in mechanical strength (43).

An intramedullary nail/rod pin is usually used to stabilize a long-bone fracture. It is the recognized treatment in long-bone diaphyseal and selected metaphyseal fractures (44). However, the use of intramedullary pins in some experimental groups may change the results, especially when the pins are removed prior to biomechanical assessment and reaming the fractures. This perhaps explains David's negative results (16). David et al. made bilateral open osteotomies in the tibias followed by an internal fixation with intramedullary wires. The right leg received He-Ne laser radiation for 0, 2, and 4 Joules every other day for 14 to 42 days, while the left leg served as the control. Radiological and histological examinations in the osteotomy sites failed to show any enhancing effect of He-Ne laser radiation on the bone-healing process. Biomechanically, the irradiated bones in two out of the six test groups were significantly weaker than the controls (16). In this current study, the wires were extracted delicately from osteotomized tibias and the bones were successfully submitted for biomechanical examination.

5.1. Weak and Strong Points of the Study

5.1.1. Weak Points and Limitations

There was a small sample size (n=5) for each studied group.

5.1.2. Strong Points

1) we used a biomechanical evaluation method, which gave direct information about the mechanical strength of the bone; 2) we tested two different doses of PW LLLT; 3) PW LLLT was used due to the different effects it had from CW LLLT, so we used a considerable lower energy density than CW LLLT. This has variety of benefits in clinical cases, such as the irradiated area does not produce heat or require the high amount of energy of the CW LLLT, meaning that patients can benefit more from the PW LLLT; 4) in this current study, we successfully reported the application of PW LLLT as an effective method for accomplishing a complete osteotomy model for fracture healing. The data can be used in compromised bone repair, such as in patients with OP or diabetes mellitus. These results should be verified in future studies. These findings and the methodologies of analysis are the strongest points in this study.

5.2. Conclusions

The findings in our study showed the efficacy of PW LLLT on biomechanical strength, trabecular bone volume, callus volume, and osteoblast number of repairing callus in an animal exemplary with a complete osteotomy of tibia during a relatively late stage of the bone healing process. Further studies need to be undertaken to determine the optimal parameters for PW LLLT in different bone repair circumstances in osteoporotic and diabetic animal models.

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Footnotes

Authors' Contribution: Atarodalsadat Mostafavinia, contributed to designing the project, the materials and methods section, and data collection. Reza Masteri Farahani, Mohammadreza Abbasian, Mohammadmehdi Vasheghani Farahani, Mohammadjavad Fridoni, and Sara Zandpazandi, contributed to the materials and methods section and data collection. Seyed Kamran Ghorishi, performed statistical analysis. Mohammad-Amin Abdollahifar, performed stereological examination. Ramin Pouriran, contributed editing the paper. Mohammad Bayat, designed and conducted the project, conducted data analyses, interpreted results, and wrote the manuscript.

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