

Effect of Low Level Laser Therapy on Bone Histomorphometry in Rats

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Abstract: The aim of this study was to assess the histological effect of Low Level Laser Therapy (LLLT) (904 nm) on the repair of standardized bone defects on the femur of rats. Sixty male wistar rats were assigned into two equal groups. Group (A: laser group) and group (B: control group). A surgical fracture was done in middle third of femur of all rats. In group (A) a continuous wave 904 nm infrared laser was applied at dose 4 j/cm² at fracture site immediately post operative for 7 sessions, each session was 5 minutes. The animals were killed by over dose of general anesthesia on the 15th, 30th and 45th days after surgery, The specimens were processed and stained with Hematoxylin-eosin (H/E), special stain Mason trichrome and analyzed by light microscopy. The descriptive analysis of histological imaging showed greater degree of new bone formation, osteoblastic surface and collagen fiber in the irradiated group when compared with the control group. Based on the obtained results, this study concluded that LLLT was efficient in promoting bone healing, and increasing new bone formation in the process of surgically fractured femur in animal study.

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1. Introduction

Bone histomorphometry is the only procedure, which allows for assessment of bone turnover, static and dynamic tissue and cell activities of modeling and remodeling, and mechanisms underlying bone mass changes at the tissue and cellular levels ⁽¹⁾. Bone repair is a proliferative physiological process, in which the body facilitates healing of bone fractures. The healing potential of bone, whether in a fracture or fusion model, is influenced by a variety of biochemical, biomechanical, cellular, hormonal, and pathological mechanisms. A continuously occurring state of bone deposition, resorption, and remodeling facilitates the healing process. Long bone fractures heal by a slow process which may be associated with significant social, domestic and financial consequences. Delayed healing may exaggerate serious problems ⁽²⁾. In turn, enhanced bone healing should contribute to a reduction in health care costs in terms of earlier patient mobility and discharge from hospital and, not least, it should lessen the discomfort experienced by the patient following surgical treatment or trauma ⁽³⁾. Currently, bone increment stimulus has been achieved with the application of chemical stimuli, biomaterials, and bone morphogenetic proteins as well as the use of physical stimuli, such as ultrasound, electromagnetic fields and more recently low level laser therapy (LLLT) ⁽⁴⁾.

Laser therapy is a new approach applicable in different medical fields when bone loss occurs, including orthopedics and dentistry. It has also been used to induce soft-tissue healing, for pain relief, bone, and nerve regeneration. With regard to bone synthesis, laser exposure has been shown to increase osteoblast activity and decrease osteoclast number ⁽⁵⁾. Stimulation with LLLT enhances the healing environment, resulting in accelerated healing of bone defects in vivo and in vitro. The healing process has three phases: a substrate phase, a proliferative phase and a remodeling phase. Most accounts of laser biostimulation suggest that its greatest effects occur during the proliferative phase ⁽⁶⁾.

The use of LLLT in the biostimulation of bone repair has been growing steadily, and several studies have demonstrated positive results of LLLT on the healing of bone tissue ⁽²⁻⁴⁾. Also, data in the literature reveal that LLLT may stimulate osseointegration and can enhance bone ingrowths and functional recovery ^(5, 6). It is still difficult for one to compare studies about the action of LLLT on bone, because the experimental models and duration of treatments are very distinct ⁽⁷⁾.

Bone healing differs substantially from soft tissue healing because of its morphology and composition. Generally bone healing processes are slower than that of soft tissue. The natural course of

bone healing follows consecutive phases that differ from each other according to the type and intensity of trauma and the extent of bone damage. Several *in vivo* and *in vitro* studies have investigated the use of laser therapy in the biomodulation of bone repair through its photochemical and photobiologic properties⁽⁶⁻⁸⁾. The aim of this study was to investigate the histological effect of Low Level Laser Therapy (LLLT) (904 nm) on the repair of standardized bone defects on the femur to provide patients with a more comfortable postoperative recovery and faster healing.

2. Materials and Methods:

This study was designed to investigate the effect of LLLT on healing of bone fracture. Sixty young adult healthy male wistar rats were used in this study. Their weight ranged from 250g to 500g. Their ages were not less than 10 weeks and not more than 15 weeks. They were kept in animal experimental house of Kasr El-Aini hospital in separate plastic cages. This plastic cage measuring 50 x 24 x 16 cm and bedded on sterilized wood chips. They were maintained under controlled temperature (24 ± 2 C), light-dark periods of 12 h, and with unrestricted access to water and commercial diet.

The rats were divided into two equal groups, each group consisted of thirty rats the first group (A) (laser group) received LLLT while the rats in second group (B) (control group) were put under general medical observation. Each group (A) and (B) was subdivided into three subgroups (1, 2 and 3) according to their killing day (every fifteen days) at day 15, 30 and 45.

Laser unit

Laser Therapy (Phyaction 769) - Pasweg 6a - 3740 Bilzen - Belgium. Pulsed, infrared (IR) gallium arsenide, wave length 904nm. Mains voltage 110-240V (+/-10%), 50-60 HZ, Dimensions (W×d×h): 41×28×13 cm. Weight: 6kg. Clearly visible LCD display with digital display of all parameters. Convenient touch controls. Many laser probes can be supplied. Their Peak power was 16W, Pulse repetition frequency 2-30.000 HZ, and Max. average power 81,6 mW.

Computerized optical microscope

Two image processing and analysis system were used in this study: An image processing and analysis system; Leica imaging systems Ltd; Clifton road, Clifton Road, Cambridge, CB1 3QH England. This system was used as measuring tool for all histological parameters. The microscope allowed following magnifications: x40, x100, x200, x400, and x1000. It was linked to a Panasonic color CCTV

Camera; model WV-CP 210/G; Matsushita communication industrial Co., Ltd.;1-9-5,Otemachi, Chiyoda-ka, Tokyo, Japan. The images taken by camera were linked to computer with Qwin Windows's software for analysis of images.

Procedures:

Surgical procedure were done under general anesthesia by using an intra-peritoneal injection of a mixture of xylazine 10 mg/kg and ketamine HCL 90 mg/kg body weight⁽⁹⁾. The right leg of the animal was shaved and the femur exposed, then veterinarian made transverse fracture in mid shaft of femur and put two fragments close to each other, after that the skin was closed with nylon. The fractured leg was kept in elastic bandage for immobilization.

Group (A) were treated by LLLT (904nm). Laser irradiation was started immediately after the surgery procedure and it was performed on the 2nd, 4th, 6th, 8th, 10th and 12th day's post-operative. The irradiation was performed transcutaneously at dose of 4J/cm² was applied to 4 points around fracture site anterior, posterior, laterally and medially for 5 minutes every session. All rats were treated in the same way. The animals were positioned on a table in ventral decubitus, and manually immobilized. The laser was used on their hind limb, directly on the injury, at a 90° angle. The laser pen was covered by plastic film before each application to avoid contamination.

A dose of 4 J/cm² was applied to four points around the defect giving a total of 16 J/cm² per session and a total treatment dose of 112 J/cm². The protocol used on this study was based upon previous study carried out by Pinheiro et al, 2003⁽¹¹⁾ whom recommended doses ranging from 1.8 to 5.4 J/cm².

Morphometry

Animals were killed by over dose of general anesthesia on day15, 30 and 45. After the surgery procedures, the irradiated femur and control femur were immediately defleshed, dissected and fixed. Bone sample of mid shaft of femur was taken and prepared for histological examination. Measurements of bone histomorphometry include bone area, osteoblastic surface and collagen area.

The specimens were kept on 4% buffered paraformaldehyde solution for overnight. The samples were decalcified with 10% nitric acid and dehydration was followed by embedding in soft paraffin. Hard blocks were made with transverse section of 5µm thickness. The slices were stained with hematoxylin - eosin (H-E) stain which was widely used to evaluate bone changes. To evaluate

the data and compare variables in the 15th, 30th and 45th post operative day between laser group and control group morphometric analyses, images were digitized, and computer analysis was performed with a specific image processing and analysis program.

- Bone area: using midshaft transverse section, the bone area was measured at a magnification of x 40. of standard area $7286.78\mu\text{m}^2$; many fields were taken to cover the whole area. At each field bone tissue were detected, previously colored on the screen using special software (Qwin windows) through the camera. The bone area was expressed in mm^2 .
- Osteoblast surface: was measured with magnification of x100, and x400 in many fields of standard area $7286.78\mu\text{m}^2$, osteoblast surface was considered as the perimeter of trabeculae covered with osteoblastic cells. Measurement made by tracing the features of interest on screen viewed by the microscope through the camera. Osteoblast surface was expressed in μm .
- Bone collagen: The collagen fiber was detected by using maison trichrome. The collagen was measured with magnification of x100 in many fields of standard area $118476, 6\mu\text{m}^2$

Statistical design and data analysis

All analyses were conducted using the SPSS statistical package, version 10.0. Descriptive statistics were used for the means and standard deviations. The mean of the three trials is an estimate of the true value. The standard deviation is an indication of the progression of the single measurement. Paired t-test (for parametric data in one group), independent t-test (for parametric data between two groups). Repeated measurement analysis of variance ANOVA to record between and within groups differences. The level of significance was accepted as $p < 0.05$.

3. Results:

1- New bone formation surface area:

Table (1) represented the independent sample t-test results for new bone formation surface area at day 15, 30, and 45 between groups A and B. There was a significant difference between both groups in new bone formation surface area at day 15 where the t-value was (5.1) and p-value was (0.0007), there was a significant difference between both groups in new bone formation surface area at day 30 where the t-value was (3.97) and p-value was (0.001), and there was a significant difference between both groups in new bone formation surface area at day 45 where the t-value was (16.28) and p-value was (0.0003) (Figure 1).

Table (1): Independent sample t-test between groups A and B for new bone formation surface area at day 15, 30, and 45.

New bone formation surface area (μm^2)	day 15	day 30	days 45
Mean difference (μm^2)	1146.78	486.19	458.36
t-value	5.1	3.97	16.28
P-value	0.0007	0.001	0.0003
S	S	S	S

*SD: standard deviation, P: probability, S: significance.

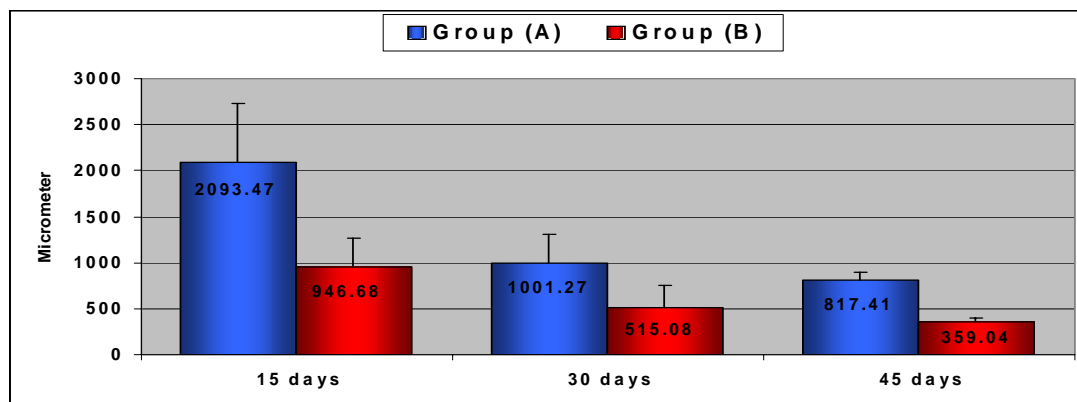


Figure (1): Mean and \pm SD of new bone formation surface area at 15, 30, and 45 days for group (A, B).

2. Osteoblast surface area:

Table (2) represented the independent sample t-test results for osteoblast surface area at day 15, 30, and 45 between groups A and B. There was a significant difference between both groups in osteoblast surface area at day 15 where the t-value was (13.32) and p-value was (0.0004), (Figure 2)

there was a significant difference between both groups in osteoblast surface area at day 30 where the t-value was (20.6) and p-value was (0.0001), and there was a significant difference between both groups in osteoblast surface area at days 45 where the t-value was (12.73) and p-value was (0.0005).

Table (2): Independent samples t-test between groups A and B for Osteoblast surface area at day 15, 30, and 45.

Osteoblast surface area (μm^2)	day15	day30	Day45
Mean difference (μm^2)	1117.59	628.23	315.26
t-value	13.32	20.6	12.73
P-value	0.0004	0.0001	0.0005
S	S	S	S

*SD: standard deviation, P: probability, S: significance.

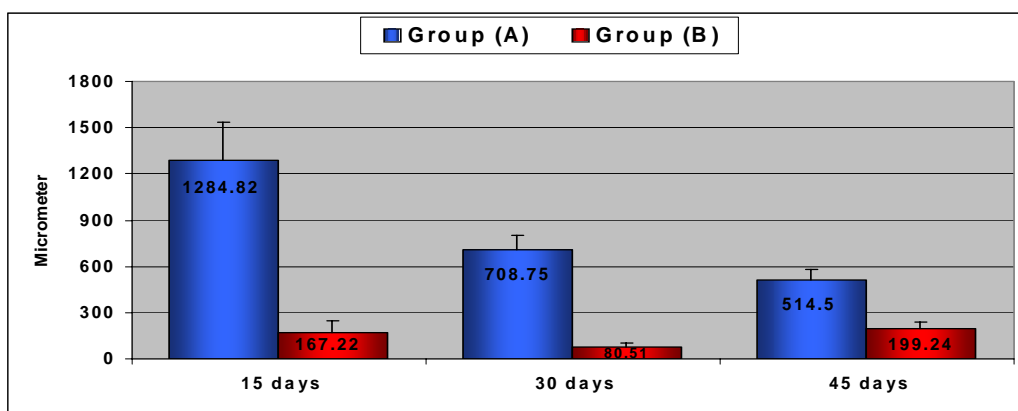


Figure (2): Mean and \pm SD of Osteoblast surface area at day 15, 30, and 45 days for group (A, B).

3. Collagen surface area:

Table (3) represented the independent sample t-test results for collagen surface area at day 15, 30, and 45 between groups A and B. There was a significant difference between both groups in collagen surface area at day 15 where the t-value was (6.61) and p-value was (0.0003), In (Figure 3) there

was a significant difference between both groups in collagen surface area at day 30 where the t-value was (6.72) and p-value was (0.0002), and there was a significant difference between both groups in collagen surface area at day 45 where the t-value was (2.63) and p-value was (0.01).

Table (3): Independent sample t-test between groups A and B for collagen surface area at day 15, 30, and 45.

collagen surface area (μm^2)	day15	Day30	Day45
Mean difference (μm^2)	14667.5	7679.1	1283.24
t-value	6.61	6.72	2.63
P-value	0.0003	0.0002	0.01
S	S	S	S

*SD: standard deviation, P: probability, S: significance.

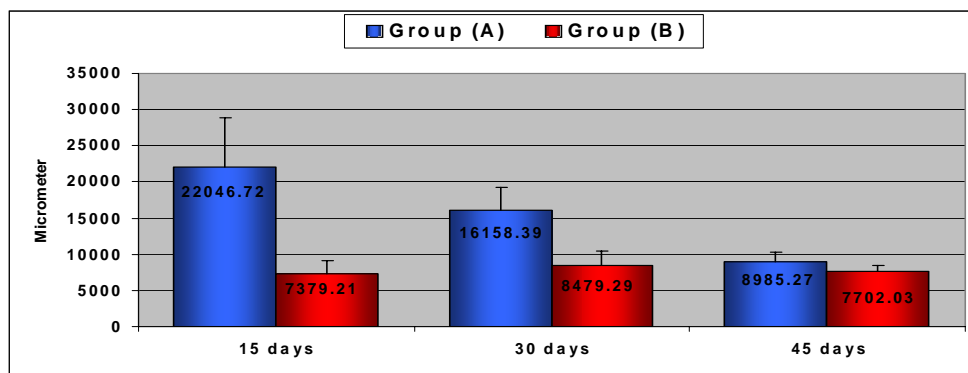


Figure (3): Mean and \pm SD of collagen surface area at day 15, 30, and 45 for group (A, B).

4. Discussion:

It has been postulated that LLLT has therapeutic efficacy on various clinical conditions. The aim of this study was to explore influence of LLLT on bone repair. Biostimulation provided by LLLT is an area of controversy. Many investigations have indicated a positive effect by the use of LLLT on bone reconstruction either in vivo^(8, 10) or in vitro^(11, 12). Contrarily; other researchers did not find any effect of LLLT on the healing of soft and hard tissues^(13, 14).

In current study infra red laser (IR) was chosen supported by previous several studies^(14,15) which have demonstrated that IR laser therapy is the most suitable method for bone repair due to its higher penetration depth in the bone tissue when compared to visible laser light⁽¹⁶⁾. The results of previous studies also concluded that the 4J/cm² energy density provided more significant results than the 8J/cm²⁽¹⁷⁾. Other experiments, have reported better tissue healing at laser exposure levels between 1 and 4 J/cm²⁽¹⁸⁾.

Nissan et al. 2006⁽¹⁹⁾ compared laser irradiation at 4 j/cm² and 10 j/cm² and concluded that a 4 j/cm² energy density significantly increased radio-calcium accumulation 2 weeks after surgery, whereas 10j/cm² had no effect. The results from **Garavello-Freitas et al. 2003**⁽²⁰⁾ showed that application of LLLT longer than 5 min (i.e. for 15 min) did not improve the bone healing process, and suggested that more future experiments were needed to be performed in order to find if stopping irradiation after the first 7 days would be more effective for bone healing.

In studies investigating the effects of laser on bone fracture consolidation, **Silva Junior et al. 2002**⁽¹⁰⁾ suggest that a total dose of 16 J/cm² per session is effective in increasing osteoblast proliferation in tibial fractures of rats. In addition, **Pinheiro et al 2003 and Renno et al 2006.**^(11, 21) using lower doses did not find any effect of laser on

bone consolidation. **Queiroga et al. 2008**⁽¹⁶⁾ concluded in their study a significant amount of newly formed bone within 15 days showed the biomodulated effect of laser therapy in the early stages of the repair process in which there was a large quantity of cells, mainly osteoblasts and undifferentiated cells. Some in vitro studies confirm the effects of laser therapy both in the visible and invisible spectra on cells from the osteoblast alignment⁽⁸⁾.

All of these results agree with study findings when the animals were examined on the fifteenth day after the creation of surgical fracture, it was observed that group (A) (treated with laser) was at a more advanced stage of repair than was the other group, presenting an area of new vessel formation, and newly formed bone tissue with a large concentration of osteoblasts and the absence of inflammatory reaction. On the other hand, group (B) was at an earlier stage of repair than group (A), with a cavity lesion, lower osteoblast concentration and more evident inflammatory infiltrate.

The same positive results about increased bone formation were suggested by **Lopes et al. 2007**⁽²²⁾ studies, who treated bone defect placing implants, and irradiating with LLLT. They found that infrared LLLT stimulated bone healing 15, 30 and 45 days after operation. They also found the presence of organizing connective tissue, numerous blood capillaries and fibroblasts at 15 days. However, the difference in collagen fiber maturation between the two groups at 30 days was not significant and finally, there were no difference at 45 days of the experiment. Nevertheless, bone formation was higher in the irradiated group at 30 and 45 days.

Additionally, the animal experimental model seems to be a useful technique for investigating the tissue reactions to LLLT, but the results reported from such experimental models cannot safely be put into practice to humans⁽²³⁾. Unfortunately, only a few studies based on humans

exist to support the positive effect of LLLT on alveolar bone regeneration ⁽²⁴⁾. This indicates the need for more human studies to help researchers securely support the positive or the negative action of LLLT. It can be supported that LLLT has the potential of beneficial effects on hard and soft regeneration under stable and no hurtful surgical conditions, irradiation with LLLT could reduce healing time and accelerate osseointegration ⁽²⁵⁾. Nevertheless, further investigations will be necessary to define stable protocols about LLLT use, such as type of laser, treatment duration, energy dose, optimum wavelength and distance from the irradiated tissues, in order to accomplish the best stimulatory action of LLLT.

The results of current study confirmed a positive effect of the soft laser in accelerating bone formation, resulting in a significant improvement in the quality of recovery and a decrease in recovery time. This is particularly important in presence of local and systemic conditions, which could retard the healing process, for example in patients with uncontrolled diabetes. In turn, such enhanced bone healing should contribute to a reduction in healthcare costs in terms of advancing patient mobility, timely discharge from hospital and reduction of the discomfort experienced by the patient following surgical treatment or trauma.

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