

Bone Healing Effects of Diode Laser (808 nm) on a Rat Tibial Fracture Model

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Abstract. Low level laser therapies (LLLT) have analgesic, vasodilatory and anti-inflammatory effects. The present study investigated the effects of LLLT with a diode laser (808 nm) device on the healing of the rat tibial fracture. Forty eight, 8-week-old, male Sprague-Dawley rats were used for this study. After creating the tibial fracture model, the animals were randomly divided into laser and control groups. The animals were euthanized for histopathological and radiological evaluation. The biomechanical strength of the fractures was evaluated using a bending test. The histopathological and radiological evaluations suggested that the laser group developed new bone formations much earlier than those of the control group. The maximum tolerance force of the laser group was significantly higher than that of the control group ($p < 0.05$). These findings suggest positive effects of LLLT in accelerating the bone healing process, especially in the early stage of bone formation.

Many incidents of fracture occur every year world-wide and 5-10% of fractures experience delay in healing, even though treatment methods have improved over the past few decades (1). Healing of bone fractures is an important homeostatic process that depends on specialized cell activation and proliferation during the period of injury repair (2). Reduction in fractures and fixation are vital to healing but a variety of additional factors modulate the bone healing process. Fracture healing may be modified by several factors such as hormones, minerals, weight bearing, vitamins, proteins from the dietary perspective, and ultrasound and electrical stimuli (1, 3, 4). Recently, another kind of potential stimulating

factors has been suggested for veterinary medicine. Many studies have reported the effects of several drugs, fixation methods, and surgical techniques. However, potential effects of adjuvant modalities are important in accelerating the bone healing process (2).

Nowadays, low level laser therapy (LLLT), which is one of the relatively new therapeutic options, has been introduced and causes low or imperceptible temperature changes (5). Normalization of cell functions and homeostasis was promoted by LLLT in the healing of non-healed wounds (6). At the same time, LLLT has been used to treat hard tissue injuries by promoting wound healing and alleviation of pain (7-9). The biostimulatory effect of LLLT was pioneered in Budapest in the late 1960s by Endre Mester, who showed increased collagen synthesis in skin wounds (10). The concept of biostimulation of wounds by LLLT is attracting growing attention. Although its effect on various tissues has been studied quite extensively, the underlying mechanisms have yet to be clarified. Although much of the action of lasers acting on the skin is mediated *via* photothermal effects, LLLT typically causes low or imperceptible temperature changes (11, 12). It is suggested that low-level laser irradiation results in the production of a small amount of singlet oxygen, which acts as a free radical that influences the formation of adenosine triphosphate (10).

A number of different lasers with diverse wavelengths, including helium (632.8 nm), gallium-aluminium-arsenide (805±25 nm), and gallium-arsenide (904 nm), have been used in different doses and treatment schedules (13). Initial research used lasers based on inserted gases, including helium-neon, ruby, argon, and krypton. Subsequent studies have used semiconductor laser diodes, including gallium-arsenide and gallium-aluminium-arsenide devices. The use of indium-gallium-arsenide (In-Ga-As) diode laser, as in the present study, has increasingly grown during the past few years. In addition, there have been very few trials of LLLT with In-Ga-As diode laser in medicine.

One of the most important issues regarding LLLT as a therapeutic modality is how much energy is necessary to

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yield significant tissue formation of high quality within the shortest time span imaginable (14). LLLT has frequently been used in several health fields. However, whether LLLT can be useful as a treatment modality in hard-tissue healing has not yet been determined. At the same time, how it may be beneficial and effective is yet to be investigated (14, 15). Few studies have attempted quantitative assessment of the effects of LLLT on bone regeneration. The purpose of the present study was to evaluate the effects of LLLT with an In-Ga-As diode laser (808 nm) device on the healing of surgically created cortical bone defects in rat tibial fractures.

Materials and Methods

Animals and experimental design. A total of 48 male rats (Sprague-Dawley), 8 weeks old and weighing approximately 250-300 g, were randomly divided into two groups containing 24 animals each, and killed 1, 3, 4 and 8 weeks after surgery. The groups included: group 1 (control) – sham control, animals not exposed to laser; and group 2 (laser group) – animals treated with low level laser. All control animals were submitted to the same procedures of handling as were the laser-irradiated animals. Upon arrival at the Laboratory Animal Research Center of Chungbuk National University, the animals were housed in an environmentally controlled animal facility for 7 days for acclimatization. The rats were kept in standard cages, 2-3 in each, with 12-hour light-dark intervals. The room temperature and the humidity were maintained at $20\pm 2^\circ\text{C}$ and $50\pm 20\%$, respectively. All rats were fed pellet-type of commercial diet (Purina®, St. Louis, MO, USA) and tap water was available *ad libitum* from bottles. Care was taken to avoid unnecessary stress and discomfort of the animals throughout the experimental period. The animals were treated in accordance with the “Guide for Care and Use of Laboratory Animals” of Chungbuk National University. The study was conducted as an animal trial of 8 weeks duration with a blind controlled design.

Surgical procedures. At the beginning of the experiment, all animals underwent surgery in order to produce cortical bone defects and fractures of the right tibia. General anesthesia was obtained by intraperitoneal administration using a mixture of 2% xylazine hydrochloride (Rompun®; Bayer, Pittsburgh, PA, USA) 13 mg/kg and 125 mg of zolazepam hydrochloride with 125 mg of tiletamine hydrochloride (Zoletil®; Virbac Lab., Carros, France) in the recommended dose of 20 mg/kg. Under general anesthesia, the right leg of the animals was shaved, thereby exposing the tibia. The surgery was performed under an aseptic condition.

A 2.5-cm incision was made in the anterolateral direction of the right tibia. The incision penetrated the epidermis, dermis, and fascial layers. Lateral reflection of these tissues exposed the underlying periosteum. An additional medial-anterior incision was made through the periosteum. The periosteum was elevated and was retained by Debaquey forceps. A cortical bone defect was made on the lateral aspect at one-third along the tibia which at the level of the tibial tuberosity using a 3-mm in diameter round burr. A transverse fracture was created at the center of the round-shaped cortical bone defect with an orthopedic saw. A Kirschner wire (1.0 mm in diameter) was inserted into the medullary canal with a small Jacobs handchuck using a retrograde method. The fascial and

superficial tissue layers were repositioned and were sutured with 4-0 polyglycolic acid (Surgifit; AILEE, Busan, Korea) and 4-0 mono non-absorbable suture (Nylon; AILEE). As antibiotics and analgesics, 10 mg/kg of enrofloxacin (Baytril®; Bayer, Pittsburgh, PA, USA) and 0.2 mg/kg of meloxicam (Metacam®; Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA), respectively, were injected daily through a subcutaneous route for three days.

Laser treatment. The animals were randomly divided into two equal groups (1 and 2). Groups 1 and 2 were treated in a sham and experimental setting, respectively, differing only in the use of a diode laser device. A diode laser device of 808-nm wavelength (DVL-20 Diode Laser System®; Asuka Medical Inc., Kyoto, Japan) was used in this study. This system operates in the near-infrared spectrum at a continuous wavelength of 808 nm and with an output power of 1 W. Laser irradiation was performed under general anesthesia using isoflurane (3%) chamber induction. A light probe with a diameter of 0.4 mm delivered the laser beam, and the irradiation was administered by placing the probe in light contact with the area to be treated. Treatment started after three days and was carried out every other day. The treatment points were administered as follows: anterior, posterior, medial, and lateral aspect of the surgical site. A total of four points were irradiated. The treatment time per point was 0.02 seconds, giving an energy density of 15.38 J/cm^2 .

Body weights. Under general anesthesia, the body weight of each animal in each group was measured using an automatic electronic balance (Precisa Balance Series XB®; Precisa Instrument Ltd., Dietikon, Switzerland). In order to reduce errors originating from feeding, all animals were fasted with water allowed for 18 hours before sacrifice.

Radiological evaluation. The animals were radiographed at the end of the experimental periods (1, 3 and 4 weeks, respectively). The rats were euthanized by an intracardiac injection of 2 ml potassium chloride after general anesthesia using intraperitoneal administration of 13 mg/kg xylazine (Rompun®; Bayer, Pittsburgh, PA, USA) and 20 mg/kg of tiletamine/zolazepam (Zoletil®; Virbac Lab.). Immediately after the euthanasia, radiographs (KODAK DirectView CR500 system; Eastman Kodak Company Health Group, New York, NY, USA) in two planes, were captured from every tibia that had been operated on. Thereafter, differences between the control and the laser groups were quantified by applying a scoring system for bone defect healing evaluation (Table I). The scoring system was previously reported in the study of Santic *et al.* (16).

Histological evaluation. Histological evaluation was performed at 1, 3 and 4 weeks after surgery, respectively. Every right tibia was removed and soft tissues including skins and muscles were eliminated from the tibia. The Kirschner wire was removed from the tibia. Tissue samples (proximal half of each right tibia including the fractured and defected areas) were fixed in 10% formalin for 48 hours, decalcified, embedded in paraffin blocks, and cut longitudinally into 5- μm thick sections with a microtome (from medial aspect to lateral aspect of the tibia). For a microscopic descriptive analysis of each group, slides were prepared by using hematoxylin and eosin (H&E) dyes. Bone healing evaluation was performed using a microscope connected to an image analyzer. All measurements were performed using a magnifying objective ($\times 40$).



Figure 1. Representative radiological images of tibial bones after artificial fracture. The laser-treated group exhibited earlier new bone formation than did the control. The presented figures are representative images of each group. A, C, E, G, I, and K: Right lateral view of each tibia, B, D, F, H, J, and L: The Craniocaudal view of each tibia.

Table I. Scoring system for bone-defect healing evaluation on radiographs (16).

Radiographic appearance of the bone healing	Grade
No change from immediate postoperative appearance	0
Trace of radiodense material in defect	1
Flocculate radiodensity with flecks of calcification and no defect bridging	2
Defect bridged in at least one point with material of nonuniform radiodensity	3
Defect bridged on median and lateral sides with material of uniform radiodensity, cut ends of cortex remain visible	4
Same as grade 3, at least one of four ends of cortex remain visible	5
Defect bridged by uniform new bone, cut ends of cortex not seen	6

For the comparison of the differences between the two groups, objectively, a semiquantitative scoring system, which was modified from that of Santics *et al.* (16), was applied (Table II). The evaluation was carried out by three independent observers. The maximum quality of bone healing was evaluated at eight points.

Blood chemistry and complete blood count. At 4 weeks post surgery, complete blood count (CBC) (Cell-Dyn® 3700; Abbott Diagnostics, North Chicago, IL, USA), and blood chemistry (HITACHI 7020

Autonomic Analyzer; Hitachi High-Technologies Corporation, Tokyo, Japan) including alkaline phosphatase (ALP), calcium, and phosphate, were performed.

Bending test. A three-point bending test was performed at 8 weeks, for the measurement of the mechanical strength of regenerated bone tissue. The bending test was performed with a computer-controlled tensile test system (MultiTest1-j®; Mecmesin Limited, West Sussex, UK) fitted with a calibrated load-cell of 1000 N. The cross-speed

Table II. Bone healing scoring system based on histological criteria.

Histological parameters		Grade
Cortex	No sign of cellular activity	0
	Resorptive activity	1
	New bone formation	2
	Bridging to surrounding tissue	3
Callus	Fibrous	0
	Cartilaginous	1
	Bony	2
Bridging of the bone defect	No bridging of bone defect	0
	Bridging with fibrous tissue	1
	Bridging with fibrous and cartilaginous tissue	2
	Bone defect closure	3

range was set to 5 mm/min. The callus area of each tibia was tested and the maximum bone tolerance force before the point of fracture was measured.

Statistical analysis. All statistical analyses were performed with the SPSS software (Windows version 12.0, SPSS, Chicago, IL, USA). Statistical analyses were performed by using the Student's *t*-test to compare the data from the laser treated and control groups. Measurement of all the data was made blindly with no clue to whether the animal being evaluated was treated or untreated. All data were presented as the mean±standard deviation (SD). The results were considered to be significant when the level of probability (*p*-value) was 0.05 or less.

Results

All animals recovered well from the anesthesia and the surgical intervention. The wound healed with no sign of infection and the animals from both groups gained weight at a similar pace. Two animals were excluded from the study, one animal from each group, because of loosening of the Kirschner wire during the postoperative period. The remaining animals did not show any sign of loosening. Therefore, in each group, 23 rats, of 46 animals in total, were included in the study.

Change in body weights. Body weights of each group gradually increased and did not show any significant differences between the two groups (data not shown).

Radiological findings. The efficiency of bone healing of experimentally created bone defects and fracture was evaluated by three independent observers on the basis of radiological findings. The bone healing of the tibial fracture and the cortical defects gave different results between the control and the laser groups after 1, 3 and 4 weeks of surgery (Figure 1). The difference in bone defect healing between the

Table III. The degree of healing in radiological semi-quantitative evaluation. Measurements were made using a two-plane Computed Radiography (CR) image and the results are presented as the mean±SD.

Time post surgery	Control group		Laser-treated group	
	Number of animals	Grade	Number of animals	Grade
1 Week	6	1.67±1.11	6	3.13±0.37*
3 Weeks	5	3.17±0.37	6	4.80±0.75*
4 Weeks	4	4.30±0.47	3	5.00±0.71

**p*<0.05, *t*-test compared with each control group.

Table IV. The histological findings of semi-quantitative evaluation of bone healing. All measurements were calculated using a magnifying objective (×40). The parameters measured include cortex, callus, and bridging of the bone tissue. Maximum score was 8. The results are presented as the mean±SD.

Time post surgery	Control group		Laser-treated group	
	Number of animals	Grade	Number of animals	Grade
1 Week	6	4.33±0.82	6	6.00±0.63*
3 Weeks	5	5.33±2.16	6	7.60±0.55*
4 Weeks	4	6.67±1.53	3	8.00±0.00

**p*<0.05, *t*-test compared with each control group.

Table V. The results of bending test of healed bone with a tensile test system. The results are presented as the mean±SD.

	Control group (n=6)	Laser-treated group (n=6)
Maximum force (Newtons)	67.08±11.74	83.28±14.11*

**p*<0.05, *t*-test compared with the control group.

two groups was statistically significant after 1 and 3 weeks (*p*<0.05). At 1 week, the mean score in the control group was 1.67±1.11, while the score in the laser-treated group was 3.13±0.37. After 3 weeks, the mean score rose to 3.17±0.37 in the control group and 4.80±0.75 in the laser-treated group, respectively (Table III).

Histological findings. The histological evaluation demonstrated that new bone was formed in the laser-treated group earlier than expected (Figure 2). The bone-healing after 1 week was evaluated with a mean score of 4.33±0.82 in the control group

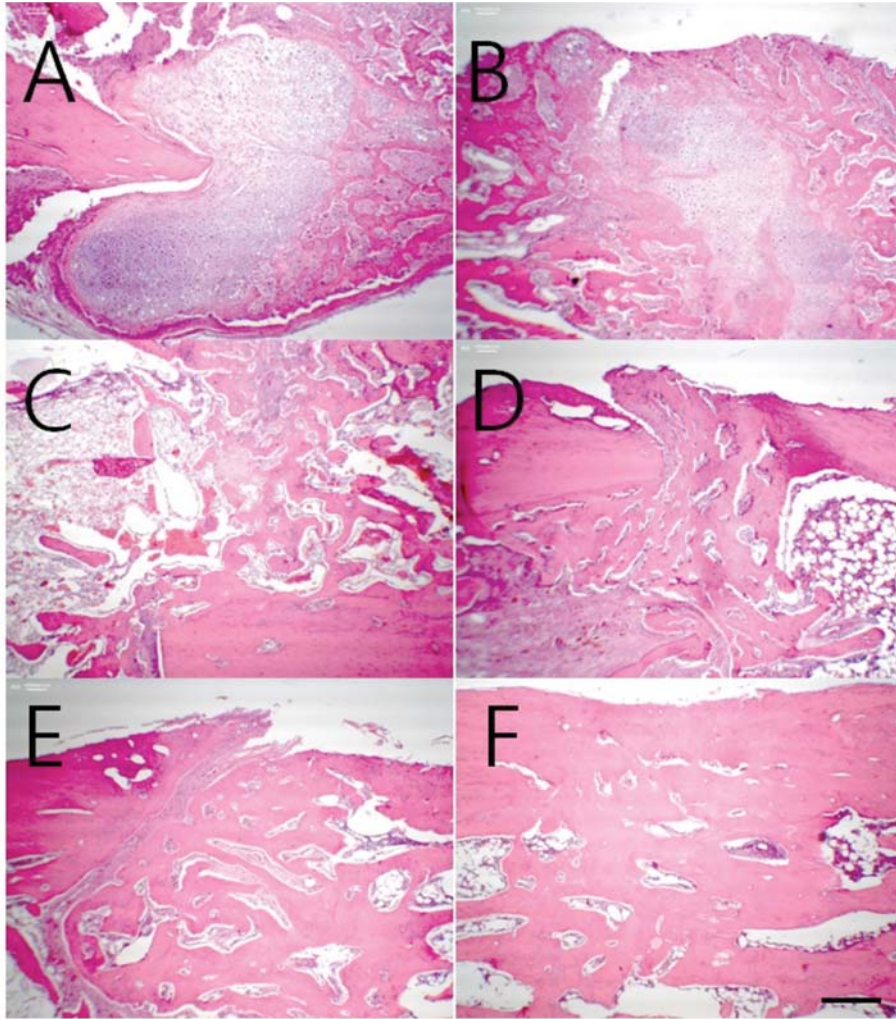


Figure 2. Representative histological images of tibial bone defect ($\times 40$). A: Control bone defect and, B: Laser irradiated bone defect 1 week postoperatively, showing moderate bony tissue growth in the laser-treated group compared with the control group which was composed mainly of cartilaginous tissues. C: Control bone defect and, D: Laser irradiated defect 3 weeks postoperatively, showing new bone formation with more trabecular bone in the laser-treated group compared with the control group. E: Control defect and F: Laser-irradiated defect 4 weeks postoperatively, bone regeneration was observed in both groups. Incomplete closing of defect was observed in the control group compared with complete closing of the cortical bone defect in laser group. Scale bar=300 μm .

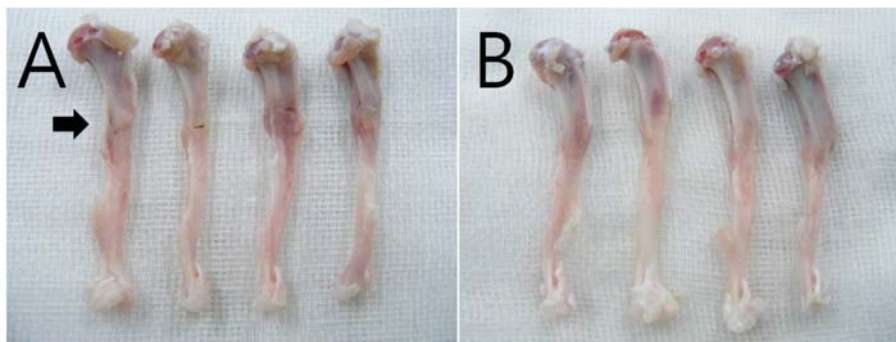


Figure 3. Representative macroscopic evaluation of the fracture and cortical bone defect at 8 weeks post surgery. Evaluation of bone healing was performed in tibial fracture region (arrow). All soft tissues, including the periosteum, were removed. A: Irregular cortical bone margins at the fracture can be seen in the control group. B: Smooth cortical bone margin and new bone formation can be seen in the laser-treated group.

and 6.00 ± 0.63 in the laser-treated group. After 3 weeks, the bone-healing was evaluated with mean scores higher than those after 1 week in both groups, and there were still significant differences in the mean scores between the two groups of animals. The mean score of the control group was 5.33 ± 2.16 , while that of the laser group was 7.60 ± 0.55 . After 4 weeks, the mean score was 6.67 ± 1.53 in the control group and 8.00 ± 0.00 in the laser group, respectively (Table IV). The results of the histological evaluation at 1 and 3 weeks were statistically significantly different between the two groups ($p < 0.05$).

Blood chemistry and complete blood counts. CBC and serum profile performed at 4 weeks post surgery were within the normal reference ranges and showed no significant differences between the two groups (data not shown).

Biomechanical strength. The value of the maximum bone force tolerance (maximum force) which was measured by three-point bending test at 8 weeks postoperatively demonstrated that more mechanical strength was found in the laser group (Table V). The mean of the maximum force was 67.08 ± 11.74 in the control group and 83.28 ± 14.11 in the laser group. This result revealed significant differences between the two groups ($p < 0.05$). Macroscopic evaluation of the tibial bone defect at 8 weeks post surgery showed that values correlated with the bending test results (Figure 3).

Discussion

This study was conducted in order to assess the effect of low level laser on the healing of surgically created tibial defects and fracture in rats, since there are very few reports on the applications of LLLT using In-Ga-As diode laser for bone healing. Our findings revealed statistically significant differences between the control group and the laser-treated group at 1 and 3 weeks regarding new bone formation. However, after 4 weeks post surgery, no statistically significant differences were observed in the radiological and histological parameters investigated between the groups. The findings of this study confirm the efficacy of laser irradiation during the early stage of bone healing and correspond with these of a previous study (15). Some studies showed that irradiation of low intensity in the early stage had a beneficial effect by stimulating mineralization in the process of new bone formation in surgically created bony defects (8, 17). Another study revealed that laser irradiation leads to more production of mineralized bone trabeculae (18). In order to eliminate any systemic alteration that may interfere in the bone metabolism, the serum chemistry and CBC were examined, and the results were within the standards of normality. No significant effect of low intensity laser irradiation was found on the activity of ALP at 4 weeks post surgery in the laser group, compared to

that of the control group, which suggests no influence on the osteoblastic activity in the process of new bone formation. This result was similar to previous studies using biochemical evaluation of irradiated rat bones (8). However, the study indicated that activity of ALP at 1 week in the laser group showed significant effect of LLLT, supported by histological and clinical observations reported previously (19, 20).

In the present study, the increased maximum tolerance force that the bone could resist was measured for the evaluation of their biomechanical properties using a three-point bending test at 8 weeks post surgery. The bending test was proven to be a valid method for the evaluation of the biomechanical strength in animal models (1, 2, 7). The results demonstrated significantly increased maximum tolerance force before fracture in the laser group, compared with the control group. This result suggests that LLLT on surgically created bony defects can improve the biomechanical strength of new bone during the healing process in a rat model. Laser irradiation is a form of electromagnetic field that can elevate the structural stiffness of bone callus. The biological mechanism by which LLLT affects tissue healing is not clearly known, but it was recently suggested that at low radiation doses, the light energy is absorbed by intracellular chromophores, such as porphyrins and cytochromes, and is converted to metabolic energy, involving the respiratory chain, *via* production of a transmembrane electrochemical proton gradient (7, 21, 22). The metabolic process was activated using this energy. Some studies showed that LLLT can stimulate bone formation by increasing osteoblastic activity, vascularization, organization of collagen fibers and adenosine triphosphate levels (23-25). However, one study showed that LLLT using gallium-aluminum-arsenide (Ga-Al-As) laser on rat tibial fracture models can facilitate fracture healing in the early stage of fracture with weak biomechanical properties (1). Wavelength is an important factor, which relates to the penetration of laser light through biological tissues (1, 26). This study revealed that diode laser irradiation increases bone formation in the initial stages of the healing process, and significantly enhances the biomechanical strength of newly formed bone tissues. He-Ne laser irradiation enhances the maximum force in evaluation of failure and structural stiffness (7). Several other studies also support our findings with respect to positive effects of laser on bone formation (7, 23, 25, 27).

In conclusion, the study suggests the positive effects of LLLT in accelerating the bone healing process in the early stage of bone formation.

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