Enhancement of bone formation in rat calvarial bone defects using low-level laser therapy

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Objective. To evaluate the effect of low-level laser therapy (LLLT), using a GaAlAs diode laser device, on bone healing and growth in rat calvarial bone defects.

Study design. An animal trial of 4 weeks' duration was conducted using a randomized blind, placebo-controlled design. Standardized round osseous defects of 2.7 mm diameter were made in each parietal bone of 20 rats (n = 40 defects). The animals were randomly divided into an experimental and a control group of 10 animals each. In the experimental group, a GaAlAs diode laser was applied immediately after surgery and then daily for 6 consecutive days. The control group received the same handling and treatment, but with the laser turned off. Five rats from each group were killed on day 14 and the remainder on day 28 postoperatively. From each animal, tissue samples from one defect were prepared for histochemistry and samples from the contralateral defect for histology. Levels of calcium, phosphorus, and protein were determined by using atomic absorption spectrometry, colorimetry, and photometry, respectively. Student t-test and Mann-Whitney were used for statistical analyses.

Results. At both time points the tissue samples from the experimental animals contained significantly more calcium, phosphorus, and protein than the controls. Similarly, histological analyses disclosed more pronounced angiogenesis and connective tissue formation, and more advanced bone formation in the experimental group than in the controls.

Conclusion. LLLT may enhance bone formation in rat calvarial bone defects.


Defects in bone are not infrequent sequelae to pathological conditions such as tumors, cysts, or infections, or to trauma or surgery. The slow regeneration of bone in such defects can pose therapeutic problems.

Various strategies to stimulate healing of bone defects have been tested. These include autografting and allografting of cancellous bone, application of growth factors (mainly bone morphogenic proteins and OP-1), polymeric membranes, enamel matrix derivative, and others. 1-6 Autogenous bone grafts require lengthy surgery and are associated with significant donor site morbidity, including infection, pain, and hematoma formation. Furthermore, in large defects grafts are often resorbed before osteogenesis is completed. 7,8 Membrane application is also an expensive, lengthy process requiring surgical intervention. Although membranes like Emdogain have proved promising in healing of lesions in alveolar bone, its field of application to date is limited and it is indicated only for intrabony periodontal defects associated with periodontitis and trauma. Although the above methods are now established modes of treatment, failures still occur. The technical difficulties and unpredictable outcomes associated with surgical grafting techniques motivate further investigation of alternative, less technically demanding procedures to stimulate bone formation.

In this context, the concept of biostimulation of wounds by low-level laser therapy (LLLT) is attracting considerable attention. Although its effect on whole tissues has been studied quite extensively, the biological and cellular mechanisms underlying LLLT have not been clarified. It has been proposed that LLLT stimulates wound healing, 9-11 collagen synthesis, 12,13 and nerve regeneration. 14 In an experimental radius fracture in rabbits, Tang and Chai 15 reported that LLLT enhanced the activity of red blood cells, macrophages, fibroblasts, chondrocytes, and osteoclasts within the fracture area. LLLT may also increase DNA and RNA synthesis, 16,17 indicating an effect on cell growth and protein synthesis. It is suggested that low-level laser irradiation results in a small amount of singlet oxygen, which acts as a free radical that influences the formation of adenosine triphosphate (ATP), which has an important role in metabolism. 18

Furthermore, laser irradiation is assumed to intensify the formation of transmembrane electrochemical proton gradients in mitochondria. Thus, the efficiency of the proton-motive force is increased and more calcium is
released from the mitochondria by an “antiport” process. The additional calcium transported into the cytoplasm is believed to trigger mitosis and/or enhance cell proliferation.\(^\text{19}\)

A number of different lasers with different wavelengths, including helium-neon (632.8 nm), gallium-aluminum-arsenide (805 ± 25 nm), and gallium-arsenide (904 nm) have been used in different doses and treatment schedules.\(^\text{20,21}\) The use of gallium-aluminum-arsenide (GaAlAs) diode laser, as in the present study, has grown increasingly during the past 10 years. This kind of laser is known to have a high depth of penetration in comparison to other types and thus offers the clinician a penetrative tool of great efficiency.

Too low a dosage of LLLT has been shown to have no effect at all, and too high a dosage may result in little or no effect, while extreme dosage can lead to inhibitory effects.\(^\text{22,23}\)

Few studies have attempted quantitative assessment of the effect of LLLT on bone formation. The purpose of the present study was therefore to evaluate the effect of LLLT with a GaAlAs diode laser device on bone healing and growth in rat calvarial bone defects.

**MATERIAL AND METHODS**

**Animals**

Twenty 2-month-old Sprague-Dawley male rats weighing 250 g from (M&K) Ry, Denmark were used for the study. The rats were selected at this stage due to their rapid increase of skeletal size and providing a favorable model for studying normal bone healing. On arrival at the institution, the animals were housed in an environmentally controlled animal facility for 7 days for acclimatization. The animals were then subjected to a standardized surgical procedure using a stainless steel round bur to create a round defect in each parietal bone. All rats were fed a standard rat diet (RM1, Special Diets Services, Essex, England) and untreated water ad libitum. The rats were kept in standard cages, with B&K Universal Beekay Bedding (Nittendal, Norway) and living conditions (12 hours of light and 12 hours of darkness). Room temperature and humidity were maintained at 23°C and 55%, respectively.

The study was conducted as an animal trial of 4 weeks’ duration with randomized blind, placebo-controlled design. All animal procedures were conducted in accordance with Norwegian and European Union (EU) animal safety regulations. The experiments were performed in accordance with the Animal Welfare Act of December 20, 1974, No. 73, chapter VI, sections 20-22, and the Regulation on Animal Experimentation of January 15, 1996. Throughout the experimental period, care was taken to avoid unnecessary stress and discomfort to the animals.

**Surgical procedures**

Surgery was conducted under general anesthesia using Forene (Isoflurane, Abbot, Queenborough, England): oxygen flow rate: 1-2 L/min, Isoflurane: 5% initially and 2% maintenance for the duration of the surgery. The skull of the rat was shaved and disinfected with 70% ethanol. Lidocaine/epinephrine (Lidocaine/epinephrine, Astra, Södertälje Sweden) 1 mL, was administrated locally at the operation site in order to minimize bleeding. The surgery was conducted under aseptic conditions.

A 3-cm longitudinal midline incision was made along the sagittal suture, penetrating epidermis, dermis, and the fascial layers. These tissues were reflected laterally, exposing the underlying periosteum. A medial-anterior incision was made through the periosteum, which was then elevated and retained by a self-retaining retractor. In each parietal bone a standardized round defect, 2.7 mm in diameter, was created: the size of the defect had been predetermined in a pilot study showing that this defect did not ossify before 4 weeks of healing. Furthermore, the dimension of the laser beam limits the size of the defect. To be sure that the irradiation field is evenly distributed over the defect area, the defect should ideally not be larger than the beam width. A stainless steel round bur (Medicon, CMS, Tuttingen, Germany) equipped with a ring to control the depth of cutting, was used in a slow speed dental drill. To prevent overheating of the bone edges, the field was irrigated copiously with physiological saline solution. Care was taken to ensure that the underlying dura mater was left undamaged by the procedure. The fascial and superficial layers were repositioned and sutured with 4-0 polyglycolic acid sutures. The animals remained under observation until full recovery.

**Laser treatment**

To avoid bias from a potential systemic effect of LLLT, no internal controls were used. The rats were randomly divided into an experimental and a control group (A and B). The treatment regime for the control group was the same as for the experimental group, except that the sham treatments were performed with the laser device applied to the wounds of the control animals, but not switched on.

A photon-plus, gallium-aluminum-arsenide (GaAlAs) diode laser device (Rønvig Dental AS, Veile, Denmark), was used in this study. This system operates in the near-infrared spectrum at a continuous wavelength of 830 nm and a power output of 75 mw. A probe with a diameter of 18 mm delivered the laser beam. The probe was placed in light contact with the area to be treated. The spot size produced by the laser was approximately 0.13 cm\(^2\), with an incident power density of around 550
The treatment was initiated immediately after surgery and then carried out daily for 6 consecutive days. Each animal received 3J in each bone defect per session. The exposure time per defect was 40 seconds, giving an energy density of 23 J/cm². These conditions had been determined by previous experiments.²⁴

Specimen preparation
Five rats from each group were killed on the 14th day postoperatively and the remainder on the 28th day. The parietal bone specimens containing the defect area were dissected out and immediately fixed in 4% neutral buffered formaldehyde. The defects were excised using a calibrated round knife designed specially for this purpose. One defect from each animal was prepared for histochemical analysis and the contralateral defect for histological evaluation.

Histochemical analysis
The samples were dessicated at 105°C for 24 hours. Following cooling to room temperature the samples were weighed, dissolved in 0.2 mL HNO₃, and incubated at 95°C for 16 hours. The samples were then centrifuged at 13 000 (rps) for 39 minutes. Both the supernatant and sediment were collected. The former was used to assay calcium and phosphorus concentration, and the latter for acid insoluble protein levels (mostly collagens).

Calcium and phosphorus analysis. After the addition of 0.2 mL H₂O₂, the supernatant was heated at 110°C for 2 hours. The calcium content of the samples was determined by atomic absorption spectrophotometry (Perkin-Elmer 3300, Perkin-Elmer, Wellesley, MA), and the phosphorus content by colorimetry.

Protein assay. The sediment was solubilized by the addition of 100 μL sodium dodecyl sulphate (0.1%). The protein concentration was measured directly by photometric analysis at 310 nm, using a collagen-based standard curve for reference. To ensure reliable experimental data, 2 parallel measurements of calcium, phosphorus, and protein contents were made in separate runs. All measured values were within the range of the standard curve used, and the parallel test values obtained were close to identical in all but one case.

Histological analysis
The remaining 20 excised defects were prepared for histology. The specimens were fixed by immersion in 4% neutral buffered formaldehyde, and then decalcified in Decolc nr CT 1135 (salt acid 14% and polyvinylpyrrolidon 7%), dehydrated in a graded series of ethanol, placed in xylene, and embedded in paraffin. They were cut in transverse and vertical sections about 5 μm thick and then stained with hematoxylin-eosin. The sections were evaluated twice by 2 independent investigators in a blinded fashion. The interexaminer agreement was high, ranging from 92% to 96%. The sections were examined under a microscope (Nikon, Eclipse E 600, Tokyo, Japan) connected to an image analyser (Soft Imaging System GmbH, Heidelberg, Germany). Using a (×40) magnification objective and a fixed grid, the following parameters were measured: (1) numbers of newly formed vessels, (2) fibroblasts, (3) osteoid (bone matrix), and (4) bone.

The results were evaluated semiquantitatively, on a graded scale. The scoring was based on the degree of healing as: (0) absent, (1) mild, (2) moderate, and (3) pronounced, according to the criteria stipulated in Table I.

Statistical methods
All measurements and histological observations were made blindly without knowing if a treated or untreated specimen was evaluated.

Mean differences and standard deviations were calculated for the variables measured. Statistical analyses were performed by the statistical program SPSS 10.0 for Windows. The Student t test was used for the continuous variables (calcium, phosphorus, and protein) and a Mann-Whitney for graded variables. A P value below .05 was considered to indicate a statistically significant difference between the groups.

RESULTS
In all 20 rats, recovery from the anesthesia and the surgical interventions was uneventful, yielding a total of 40 bone defects for final analysis. Wound healing progressed without any signs of infection. Weight gain was normal in all animals. No side effects such as behavioral changes, paralysis, respiratory distress, or signs of pain were observed.

Calcium, phosphorus, and protein content
Histochemical analysis disclosed that the relative amounts of calcium and phosphorus in the healing wound tissue were significantly higher in the experimental group than in the sham-treated controls killed on both day 14 (P = .002 and P = .006, respectively) and day 28 (P = .002 and P = .039, respectively, Figs 1 and 2). The relative protein content was also higher in the irradiated animals killed on both day 14 and day 28 after surgery (P = .013 and P = .015, respectively, Fig 3). The results are summarized in Table II.

Histological findings
Histological examination showed ongoing normal healing processes in both groups. No pathological changes such as the presence of giant cells, bone resorption, or severe inflammation were observed.
The number of newly formed blood vessels and the amount of fibrous tissue were significantly greater in the laser-treated group on day 14 ($P = .01$ and $P = .04$, respectively), but not on day 28. At both time points, significantly greater amounts of bone were observed in the experimental groups ($P = .008$ and $P = .002$, respectively). In contrast, osteoid formation was similar in both treated and untreated groups on both day 14 and day 28 (Figs 4 and 5).

**DISCUSSION**

The present experiment was conducted to study the influence of LLLT on bone healing in rat calvarial bone defects. Healing was evaluated histochemically and histologically.

Histochemical analysis disclosed that LLLT significantly stimulated the deposition of calcium, phosphorus, and insoluble protein at both 14 and 28 days postoperatively. Notably, calcium and phosphorus deposits in the healing tissue in the experimental group were higher 14 days postoperatively than in the sham-treated group 28 days postoperatively. This strongly indicates that application of LLLT accelerates the maturation of new bone tissue. Histological examination disclosed that angiogenesis and growth of fibroblasts and bone were more pronounced in the irradiated group. Thus, both histochemical and histological evidence indicate that LLLT enhances metabolism and/or mineralization during early bone healing.
To avoid bias due to possible systemic effects of LLLT, the design of the present study specified external controls, i.e., separate groups of test and control animals. Systemic effects of LLLT have been confirmed in several investigations.25-29 LLLT can stimulate the release of substances such as growth factors and cytokines into the circulatory system, and so could affect the untreated side of an experimental animal or subject. This may explain why some studies using internal controls (e.g., the contralateral side of the same animal/patient) have failed to demonstrate beneficial effects of LLLT.

The results of the current investigation are in agreement with previous in vitro studies.32,33 Two principal mechanisms are proposed for the beneficial role of LLLT of a certain dosage in early bone healing; namely stimulation of osteoblast precursor cell proliferation and later stimulation of cell differentiation, increasing the number of osteoblastic cells, with a subsequent increase in bone formation.34

Because of its correlation with calcification, alkaline phosphatase activity is generally regarded as an impor-
tant marker of bone formation.\textsuperscript{35,36} Studies by Ueda and Shimizu\textsuperscript{37} and Barushka et al\textsuperscript{38} demonstrated a significant increase in alkaline phosphatase activity 12 and 15 days after laser irradiation.

Our results confirm the positive biostimulatory effect of LLLT, which depends mainly on the ability of the tissues to respond to light energy. The mechanisms underlying stimulation of bone healing are not well understood, but may be multifactorial and include promotion of angiogenesis,\textsuperscript{39} collagen production,\textsuperscript{12,13} osteogenic cell proliferation and differentiation,\textsuperscript{40} mitochondrial respiration, and ATP synthesis.\textsuperscript{41,42}

LLLT may also increase local blood flow, enhancing the supply of circulating cells, nutrition, oxygen, and inorganic salts to the bone defect.\textsuperscript{43} For example, Kobu Yoshihide\textsuperscript{44} demonstrated that in tissues treated with LLL irradiation, intraosseous blood flow increased by approximately 80\% and oxygen tension by approximately 15\%.

The healing of a bone defect depends primarily on the availability of osteogenic precursor cells in surrounding bone or soft tissue, and the ability of these cells to invade the defect and differentiate into osteoblasts. Our hypothesis for the mechanism by which LLLT can enhance bone healing is by stimulating the recruitment and/or maturation of osteoblasts.

The experimental model used in this study has numerous applications. For example, in craniomaxillofacial bone research, it would be appropriate for evaluation of the efficacy of osteopromotive materials in stimulating bone healing. A major advantage over intraoral sites such as alveolar defects is that potential contamination with oral flora is eliminated.

The rat calvarial defect is a convenient model because it is easy to manage and the risk of complications is minimal. Avoiding inclusion of the sagittal suture in the osseous defect by using such small bone defects minimizes the risk of damage to the mid-sagittal sinus. However, it also reduces healing potential because osteoblastic cells are not directly recruited from the suture tissues.

The results of the present study indicate several potential clinical applications for LLLT. It may significantly benefit the rate of fracture repair, the attachment

Fig 5. Histological images of calvarial bone defects. \textbf{A,} Irradiated bone defect 14 days postoperatively, showing moderate bone ingrowth into the defect and many blood vessels. \textbf{B,} Control bone defect 14 days postoperatively: bone ingrowth is negligible; fibrous ingrowth and angiogenesis are poor. \textbf{C,} Irradiated bone defect 28 days postoperatively, showing almost complete healing of the bone defect and pronounced bone formation. \textbf{D,} Control bone defect 28 days postoperatively: bone ingrowth is moderate, and accompanied by marked fibrous tissue formation and only a few blood vessels. (Hematoxylin-eosin, magnification $\times 4$). $B$ = Bone, $F$ = Fibroblasts. Scale bar = 1 mm.
of titanium implants to bone, and the treatment of large osseous defects. In turn, such 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.

To summarize, the effect of LLLT was more pronounced at day 14, suggesting that LLLT has a more favorable effect at early stages of bone healing. Further investigations are necessary to provide more detailed information on LLLT-improved early bone healing.

CONCLUSION

In this in vivo experiment on defects in the calvarial bone of rats, histochemical and histological analyses show that the application of LLLT with a GaAlAs diode laser device can promote bone healing and bone mineralization. These results suggest that LLLT may be clinically beneficial in promoting bone formation in skeletal defects. However, in order to disclose the underlying mechanisms by which this is achieved, further studies are needed to elucidate the effect of LLLT on growth factor, prostaglandin or cytokine expression, and matrix synthesis.

The authors would like to thank Ms Grazyna Jonski and Ms Soheilab Beygi for excellent laboratory assistance. We also wish to acknowledge the National Institute of Public Health for practical guidance.

REFERENCES


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