Lasers Med Sci 2002, 17:216–220

Laser Stimulation on Bone Defect Healing: An In Vitro Study

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Abstract. The aim of this in vitro study was to evaluate whether low-power laser (LPL) stimulation can accelerate bone healing. Bone defects of a standard area were created in the distal epiphysis of 12 femora explanted from six rats, and they were cultured in BGJb medium for 21 days. Six defects were treated daily with Ga-Al-As, 780 nm LPL for 10 consecutive days (lased group, LG), while the remainder were sham-treated (control group, CG). Alkaline phosphatase/total protein (ALP/TP), calcium (Ca), and nitric oxide (NO) were tested on days 7, 14 and 21 to monitor the metabolism of cultured bone. The percentage of healing of the defect area was determined by histomorphometric analysis. After 21 days significant increases were observed in ALP/TP in LG versus CG ($p<0.001$), in NO in the LG versus CG ($p<0.0005$) and in Ca in CG versus LG ($p<0.001$). The healing rate of the defect area in the LG was higher than in the CG ($p=0.007$). These in vitro results suggest that Ga-Al-As LPL treatment may play a positive role in bone defect healing.

Keywords: Bone healing; Experiment; Low-power laser; Organ culture

INTRODUCTION

The stimulatory effects of low-power laser (LPL) irradiation have been investigated in several medical fields, such as cultured cell response, wound healing, hormonal or neural stimulation, antiphlogistic reaction, pain relief and others [1–7]. Some authors have focused their attention on LPL as a potential stimulator of bone healing [8–11], and experimental results have shown the laser stimulation effects on bone. However, controversy still exists regarding the role of LPL when used as a therapeutic device [12,13], although published experimental studies have provided evidence of laser–tissue interactions.

The present study group have already reported positive results using a gallium–aluminium–arsenide (Ga-Al-As, 780 nm) LPL for stimulation on animal and human cartilage cells [14,15]. The positive findings obtained, encouraged the authors to conduct a study using the simple and reliable in vitro model of organ culture system proposed by Sun et al. [16]. The aim was to assess the effects of the same laser device on bone defects and determine whether laser stimulation can accelerate bone defect healing.

No reference to similar studies was found in the international literature.

METHODS

The Ga-Al-As semiconductor laser [M3000, SIMED srl, Mogliano Veneto (TV), Italy] was used as a LPL source, with wavenlength of 780 nm, output power of 2500 mW, and continuous or modulated output. The device was in the isolated class 1, type B, and in the laser class III B. All the instructions for the safe use of the device were strictly followed.

Six Sprague–Dawley female rats, aged 6 months and weighing 360 ± 20 g, were euthanised under general anaesthesia in compliance with the European and Italian laws on animal experimentation and the Animal Welfare Assurance No. A5424-01 by the National Institute of Health (NIH-Rockville, Maryland, USA).

Immediately after euthanisation, under aseptic surgical conditions, 12 femurs were

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dissected. After removal of soft tissues, the femurs were washed in 37°C phosphate-buffered saline solution. An identical bone defect (area 3.80 mm^2; depth 3 mm) was drilled into all of the distal femoral condyles to obtain a standard bone defect. The femurs were then maintained in BGJb culture medium (Fitton-Jackson modification, Sigma, St Louis, MO, USA) supplemented with 20% fetal calf serum (FCS, Boehringer Mannheim, I), penicillin 100 U/ml (Sigma), streptomycin 100 mg/ml (Sigma), β-glycerophosphate 10 mM (Sigma), and ascorbic acid 50 μg/ml (Sigma). The femurs were cultured in 25 cm^2 flasks and incubated at 37°C in air supplemented with 5% CO_2.

On the following day, laser stimulation was conducted on six femora (Lased Group-LG) cultured in the above-mentioned medium for ten consecutive days under a laminar flow hood at the following parameters: 300 J/cm^2, 1 W, 300 Hz, pulsating emission, 10 min. The spot size of laser irradiation was 0.5 cm^2. The laser parameters selected, had been used for a previous experiment and had ensured the best results [14]. Under the laminar flow hood, laser stimulation was performed on the target tissue after bringing the laser point to a perpendicular position 1 cm from the femoral defect and waiting until the selected density of energy for stimulation had been reached. The temperature of the culture medium was registered before and after laser stimulation by immersion of a thermometer, and it did not change during laser treatment. The remaining six femurs were sham-treated (control group-CG) with the laser off. Half of the culture medium was changed twice a week and no contamination was found during the experiment.

The supernatant from all cultures was collected on days 7, 14 and 21, to monitor the metabolism of cultured bone in the lased and control groups. The following parameters were tested: phosphatase alkaline activity (ALP; Sigma), total proteins (TP; Sigma), nitric oxide (NO colorimetric assay; Sigma) and calcium (Ca; Sigma).

At the last experimental time scheduled, lased and control femurs were fixed in 4% paraformaldehyde, dehydrated in a graded series of alcohols, infiltrated in methylmethacrylate and embedded in polymethylmethacrylate. Samples were cut (40 μm thickness) longitudinally on a diamond saw rotating microtome (Leica 1600, Ernst Leitz, Wetzlar, Germany), and stained with Fast Green. By means of a computerised system (Kontron S300, v.2, Kontron Elektronik, Munchen, Germany), the percentage of healing of the total area of the defect was calculated according to the following formula [17]:

$$\text{Area healed} = \frac{\text{Area reconstituted}}{\text{Area of the original defect}} \times 100$$

The space filled by the reparative, newly formed bone was calculated as a percentage of the total area (volume fraction) of the femoral gap. All measurements were taken by an experienced and blinded investigator.

Statistical analysis was performed using the software package SPSS/10.1 (SPSS Inc., Chicago, Illinois, USA). The Student's *t*-test was done to compare data, and the level of significance was set at $p<0.05$.

RESULTS

Results of the biochemical measurements (ALP/TP, Ca and NO) taken in the supernatant on days 7, 14, and 21 are reported in Table 1. ALP/TP measurements in the CG revealed a steady decrease in activity level over time. The LG data also showed a decreasing trend, but the metabolic activity was higher if compared to the corresponding CG values recorded on day 21 with the level of significance set at $p<0.001$. Measurements of Ca in the supernatant revealed its progressive accumulation in the culture medium of the CG. On day 21, the LG showed a significant decrease in calcium of the supernatant (CG versus LG at 21 days: $p<0.001$). NO dosage values in the CG were lower than in the LG, where a progressive increase was observed with significance reached on days 14 and 21 (LG versus CG at 14 and 21 days: $p=0.0004$ and $p<0.0005$, respectively).

Figures 1 and 2 show the histological appearance of unlased (CG) and lased (LG) femoral defects after 21 days: trabecular bone regeneration in the defect is more evident in the LG. The histomorphometric measurements in the CG (Table 2) revealed a mean percentage of bone defect healing equal to 7.9%, and an area without reparative, newly formed bone of 3.5 ± 0.11 mm^2, whereas the same values were 21.3% and 2.99 ± 0.49 mm^2 in the LG. The difference was statistically significant ($p=0.007$).

Table 1. Mean ± SD values of alkaline phosphatase/total protein (ALP/TP), calcium (Ca) and nitric oxide (NO) in six flasks of culture medium from the control and lased groups, on days 7, 14, and 21

	7 days		14 days		21 days	
	Control group	Lased group	Control group	Lased group	Control group	Lased group
ALP/TP (IU/ml/min/mg protein)	0.83 ± 0.09	0.77 ± 0.09	0.58 ± 0.05	0.64 ± 0.06	0.53 ± 0.08	0.66 ± 0.07
Ca (mg/dl)	3.83 ± 0.38	4.33 ± 0.57	4.36 ± 1.45	4.49 ± 0.67	4.51 ± 0.44	3.85 ± 0.37
NO (mM)	2.78 ± 0.53	3.04 ± 1.10	2.65 ± 0.22	3.91 ± 0.44	2.90 ± 0.40	5.89 ± 0.38

ALP/TP, lased group versus control group at 21 days: $p<0.001$.
Ca, control group versus lased group at 21 days: $p<0.001$.
NO, lased group versus control group at 14 and 21 days: $p=0.0004$ and $p<0.0005$.

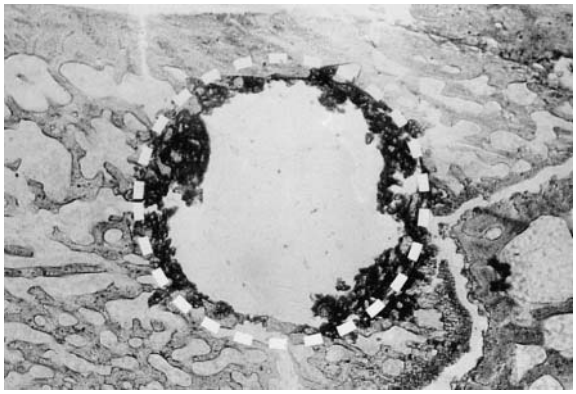

Fig. 1. Histological aspect of the bone defect without laser stimulation on day 21 (control group). The white dotted line shows the area of the original defect. The defect shows no evidence of bone regeneration. Fast green stained, magnification ×2.5.

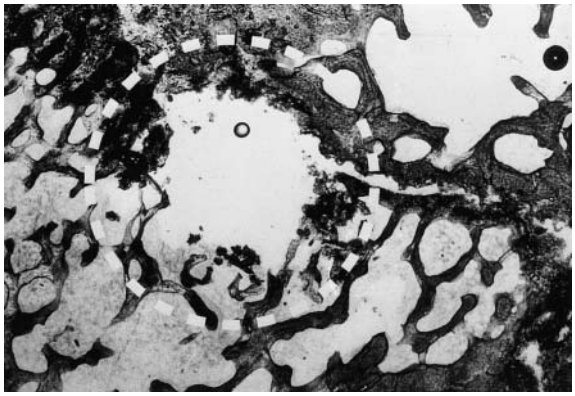

Fig. 2. Histological aspect of the bone defect treated with laser stimulation on day 21 (laser group). The white dotted line shows the area of the original defect. Bone regeneration highlights a higher percentage of healing in the lased group than in the control group (lased group versus control group: $p=0.007$). Enhanced regeneration of trabecular bone can be observed in the lased defect. Fast green stained, magnification ×2.5.

DISCUSSION

Results of this in vitro study confirm the positive effect of LPL stimulation on bone tissue. In fact, all the parameters investigated in the culture medium on day 21 are consistent with the higher percentage of bone healing in the lased group.

The ALP/TP activity suggests that laser exposition may stimulate osteoblasts; ALP activity is considered to be a good marker of active osteoblasts [10], is correlated with DNA and protein stimulation in osteoblasts [18], and directly enhances recovery of bone mass. The current ALP results are comparable with those achieved by Barushka et al. [10] using an experimental in vivo model. They have observed a higher ALP activity in the control group at 6 days, whereas their subsequent measurements demonstrated a higher activity in the lased group, as confirmed by the present in vitro study.

NO production values confirm that the positive effect of LPL on bone healing could be mediated by the increase in NO, and in the present study the NO value reached statistical significance from day 14. This short-life free radical plays an important regulatory role in in vitro osteoblast growth [19] and is accompanied by the inhibition of bone resorption, which implies a general influence on bone remodelling [20].

The lower calcium level observed in the lased group versus the control group is also due to the mineralisation process of the new bone tissue formed during defect healing. An in vivo study by Yaakobi et al. [21] on the effect of low-energy laser has shown the same trend for Ca and ALP values as observed in the present in vitro study. Moreover, they have described a reduced activity of ALP and a decreased incorporation of Ca in the

Table 2. Mean ± SD area of bone healing within the area of the femoral defect after 21 days in the control (six samples) and lased (six samples) groups

	Basal defect	After 21 days	% healing
Control group	3.80 mm^2	3.50 ± 0.11 mm^2	7.90
Lased group	3.80 mm^2	2.99 ± 0.49 mm^2	21.30

Lased group versus control group: $p=0.007$.

control group versus the lased group as of postoperative day 13.

In the present study accelerated defect healing was observed in vitro after only 3 weeks, although complete bone defect healing was not achieved. The current experiment demonstrates that osteoblast activity enhances osteosynthesis more actively, even without those favourable environmental conditions accelerating recovery of bone mass after in vivo LPL treatment [9,10].

At this time there is no clinical study on bone fracture demonstrating a direct therapeutic benefit solely due to laser treatment. Experimental studies on LPL treatment have become more and more reliable over the years. However, results are 'sometimes' difficult to compare, although laboratory findings seem trustworthy [13]. Consequently, further experimental studies are needed to prove such positive effects in humans. In vivo animal experiments suggest that there may be some effects due to LPL treatment. However, in vitro studies may only partially reflect the clinical situation. The in vitro model of tissue culture can highlight the sequence of events and parameters affecting tissue healing, whereas the same data cannot be extrapolated from in vivo studies because of the numerous cell populations and biochemical factors involved. In vitro cell experiments are much more controlled in the absence of the environmental extracellular matrix, but the intermediate organ culture system may be the most compromised [22], and therefore the most useful in the evaluation of the effects of laser radiation [5]. The organ culture system could be classified between in vitro cells and in vivo experiments. To the authors' knowledge, the present in vitro model has never been used to test laser stimulation in bone defect healing.

The laser parameters set, which had already been used for previous experiments and had ensured the best results [14], supported the authors' initial assumptions. Laser radiation has a wide range of effects on tissues [23]. As several laser systems are being currently used for stimulation, the properties of each device has to be carefully evaluated before selecting one for experimental or clinical application [24]. Through the regulation of heat distribution on tissues, the laser can produce controlled physical effects. 'Underexposure' or 'overexposure' to laser radiation can significantly change clinical or experimental results [25], and healing after laser treatment can benefit from an optimal energy density of laser radiation [5]. For all these reasons the present authors believe that these findings can be ascribed to the energy transferred to the irradiated tissues, since the absorption ability of laser radiation correlates with the energy density.

ACKNOWLEDGEMENTS

Financial support for this research was partially given by Fondazione Cassa di Risparmio of Bologna, the Rizzoli Orthopaedic Institute and the University of Bologna, Bologna – Italy.

We thank Patrizia Nini, Franca Rambaldi, Patrizio Di Denia and Claudio Dal Fiume (Dept of Experimental Surgery) for their technical assistance.

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Paper received 17 November 2001;
accepted after revision 7 February 2002.