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Initial effects of low-level laser therapy on growth and differentiation of human osteoblast-like cells

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Initiale Effekte von Low Level Laser Therapie auf Wachstum und Differenzierung von humanen osteoblastären Zellen

Zusammenfassung. Der Einsatz von Soft-Lasern im Rahmen einer Low level-Laser Therapie ist mittlerweile eine klinisch etablierte Behandlungsmethode. *In vitro* Studien haben gezeigt, dass Soft-Laser auch eine biostimulatorische Wirkung auf unterschiedlichste Zelltypen haben.

Das Ziel dieser Untersuchung war die Effekte von Low level-Laser Therapie auf das initiale Wachstums- und Differenzierungsverhalten von *in vitro* kultivierten humanen osteoblastären Zellen zu untersuchen.

SaOS-2 Zellen wurden mit Laser-Dosen von 1 J/cm² und 2 J/cm² mit einem Dioden Laser mit 670 nm Wellenlänge und einer Ausgangsleistung von 400 mW bestrahlt. Unbehandelte Zellen dienten als Kontrollgruppe. 24 h, 48 h und 72 h nach der Bestrahlung wurden die Zellen geerntet und ihre Vitalität bestimmt. Zusätzlich wurde die Aktivität der Alkalischen Phosphatase ermittelt und die Expression von Osteopontin und Collagen Typ I mittels semiquantitativer RT-PCR untersucht.

Zellen, die mit 1 J/cm² bestrahlt worden waren wiesen, sowohl eine höhere Vitalität als auch eine höhere Aktivität der Alkalischen Phosphatase gegenüber den Kontrollen auf. Auch die Expression von Osteopontin und Collagen Typ I mRNA war gegenüber der Kontrollgruppe erhöht. Hingegen führte eine Verdopplung der Laserleistung zu einer Abnahme der Zellviabilität in den ersten 48 h und zu einer konstant niedrigeren Alkalischen Phosphataseaktivität. Während die Expression von Collagen Typ I und Osteopontin mRNA in unbehandelten und mit 1 J/cm² bestrahlten Zellen im Verlauf des Experiments leicht abnahm, konnte eine Zunahme ihrer Expression nach Bestrahlung mit 2 J/cm² beobachtet werden.

Unsere Beobachtungen deuten darauf hin, dass Low level-Laser Therapie eine biostimulatorische Wirkung auf SaOS-2 Zellen bereits in der inititalen Kulturphase hat. Diese Ergebnisse können dazu beitragen, neue Therapie-Konzepte in der Regeneration von Knochendefekten zu entwickeln. Weitere Untersuchungen über einen verlängerten Zeitraum wären hilfreich, dieses Potential genauer zu beurteilen.

Summary. Low-level laser therapy is a clinically well established tool for enhancement of wound healing. *In vitro* studies have also shown that low level laser therapy has a biostimulatory effect on cells of different origin.

The aim of this *in vitro* study was to investigate the initial effect of low-level laser therapy on growth and differentiation of human osteoblast-like cells.

SaOS-2 cells were irradiated with laser doses of 1 J/cm² and 2 J/cm² using a diode laser with 670 nm wave length and an output power of 400 mW. Untreated cells were used as controls. At 24 h, 48 h and 72 h post irradiation, cells were collected and assayed for viability of attached cells and alkaline phosphatase specific activity. In addition, mRNA expression levels of osteopontin and collagen type I were assessed using semi-quantitative RT-PCR.

Over the observation period, cell viability, alkaline phosphatase activity and the expression of osteopontin and collagen type I mRNA were slightly enhanced in cells irradiated with 1 J/cm² compared with untreated control cells. Increasing the laser dose to 2 J/cm² reduced cell viability during the first 48 h and resulted in persistently lower alkaline phosphatase activity compared with the other two groups. The expression of osteopontin and collagen type I mRNA slightly decreased with time in untreated controls and cells irradiated with 1 J/cm², but their expression was increased by treatment with 2 J/cm² after 72 h.

These results indicate that low-level laser therapy has a biostimulatory effect on human osteoblast-like cells during the first 72 h after irradiation. Further studies are

needed to determine the potential of low-level laser therapy as new treatment concept in bone regeneration.

Key words: LLLT, SaOS-2, osteoblast-like cells, diode laser.

Introduction

During recent decades, low-level laser therapy (LLLT) has become a clinically well accepted tool in regenerative medicine and dentistry, where it is used to enhance healing processes and to treat functional disorders [1]. A number of different types of laser, among them HeNe, GaAlA, argon, and diode lasers, have been used successfully for different applications [2].

Treatment with LLLT has been shown to have beneficial effects on a variety of pathological conditions, including pain relief [3], inflammation [4] and wound healing [5–8]. Although the biological mechanisms underlying the biostimulatory effects of LLLT are not fully understood, numerous experimental and clinical studies suggest that LLLT modulates cellular metabolic processes leading to an enhanced regenerative potential of the biological tissues [9–11]. Recently, it has been demonstrated that LLLT enhances the proliferation of mesenchymal and cardiac stem cells [12]; other reports correlate LLLT-induced enhanced wound healing to increased expression of procollagen type I and III mRNA [13] and to increased collagen synthesis [14].

The use of LLLT is also well established in dentistry, because of its anti-inflammatory, analgetic and regenerative effects and its conditioning effect on tooth enamel [15, 16]. Early *in vivo* studies on bone regeneration after LLLT treatment showed increased bone deposition after tooth extraction, suggesting enhancing effects of LLLT on ossification [17]. These findings are supported by the *in vitro* observation that LLLT significantly increased cellular proliferation, bone nodule formation and alkaline phosphatase (ALP) activity of rat calvarial cells [18], and enhanced the osteogenic differentiation of murine mesenchymal stem cells [19]. Furthermore, animal studies demonstrated that LLLT enhances the functional attachment of titanium implants to bone and promotes bone healing and mineralization [20].

As the regeneration of bony defects is a key problem in regenerative medicine, the biostimulatory effects of LLLT could prove a desirable co-treatment in the repair of bone defects. The aim of our study was therefore to investigate the *in vitro* response of osteoblast-like cells (SaOS-2) to LLLT of different dosages.

Materials and methods

Cell culture

The human clonal osteoblastic cell line SaOS-2 was obtained from the American Type Culture Collection (ATCC #: HTB-85, Rockville, MD, USA). SaOS-2 cells are derived from a human osteogenic sarcoma and posses an osteoblastic phenotype [21]. SaOS-2 cells were cultivated in DMEM/Ham's F-12 1:1 (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS Gold, PAA Laboratories, Cölbe, Germany), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (PAA Laboratories, Cölbe, Germany), and 1 mM HEPES (Gibco Life Technologies, Grand Island, NY, USA) at 37 °C, 95% humidity and 5% CO_2.

Experimental setup

SaOS-2 cells were seeded at a density of 1.5×10^5 cells/well in 6-well polystyrene tissue culture dishes (BD Biosciences, Bedford, MA, USA). After 48 h of cultivation the medium was changed and the cells were irradiated with a 670 nm diode laser (diode laser with directly mounted fiber optics, Helbo Photodynamic Systems, Austria) at an output power of 400 mW (continuous wave modus) for 30 s or 1 min corresponding to an energy density of 1 J/cm² or 2 J/cm² respectively. The laser beam was adjusted to exactly cover the bottom of one culture well (11.5 cm above the bottom of the culture plate). To avoid any influence of possible scattered irradiation, all other wells in the plate were covered leaving only one well uncovered for irradiation. Cells were analyzed at 24 h, 48 h and 72 h after laser irradiation, with non-irradiated cells serving as controls. The experiment was performed in duplicate.

Assay for viability of attached cells

Duplicates of six wells were used for each assay. After incubation for specified times, viability was measured using the metabolic XTT assay (Roche Diagnostics, Mannheim, Germany), in which XTT is converted by viable cells to a formazan, a colored product, and conversion is directly proportional to the number of viable cells. Cells were incubated with a final concentration of 200 µg/ml XTT and 5 µM reducing agent PMS for 4 h at 37 °C before the cell culture supernatants were transferred to 96-well plates and the absorbance from converted XTT solution was read at 450 nm in a plate reader (Anthos htll, type 12500, Anthos Labtec Instruments, Wals, Austria). Average absorbance values from blank wells were subtracted from seeded wells to obtain net absorbance values. Data are shown as mean ± SD of two independent experiments.

Total cellular protein synthesis

Cells were harvested by trypsinization (0.05% trypsin, 1 mM EDTA, Invitrogen, Carlsbad, CA, USA) and the cell pellets were homogenized in lysis buffer (containing 1% Nonidet P40, 0.1% SDS, 150 mM NaCl, 50 mM Tris/pH 7.4, 10 mM EDTA, 10 mM p-nitrophenol phosphate, 250 U/l aprotinin, 40 µg/ml leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride and 40 mM β-glycerophosphate, all from Sigma, St. Louis, MO, USA). Insoluble components were removed by centrifugation (rcf = 20,000), 4 °C for 20 min and the protein content of the cleared lysate was determined using the Micro BCA™ protein assay (Pierce Chemical, USA) according to the manufacturer's instructions. This method uses a reactive solution of bicinchoninic acid (BCA) and $CuSO_4$. Cu^{2+} ions are reduced by proteins to Cu^+ ions, which form a complex with BCA. The crimson coloration of this complex is directly proportional to the protein concentration and was quantified by measuring the absorbance at 562 nm (Hitachi U-2000 Spectrophotometer, Hitachi High Technologies, Krefeld, Germany).

Alkaline phosphatase (ALP) specific activity

ALP activity was quantified using a colorimetric endpoint assay (Diagnostic kit 104-LL, Sigma, St. Louis, MO, USA) to determine early osteoblastic differentiation. The assay measures the conversion of the colorless substrate *p*-nitrophenyl phosphate by the enzyme ALP to the yellow product *p*-nitrophenol, where the rate of color change corresponds to the

amount of enzyme present in solution. Standards of *p*-nitrophenol in concentrations ranging from 0 µM to 250 µM were prepared from dilutions of a 1000 µM stock solution and assayed in parallel. Cells were harvested by trypsinization (0.05% trypsin, 1 mM EDTA, Invitrogen, Carlsbad, CA, USA), lysed in 100 µl 1 M Tris pH 8.00 by ultrasonification for 4 min, and then 10 µl of the suspension were mixed with 100 µl 7.6 mM *p*-nitrophenyl phosphate solution and incubated at 37 °C for 15 min. Substrate solution was prepared by mixing an aqueous solution of 4 mg/ml 4-nitrophenyl phosphate disodium salt (Sigma, St. Louis, MO, USA) with an equal volume of 1.5 M alkaline buffer (Sigma, St. Louis, MO, USA). The reaction was stopped by adding 1 ml 0.05 N NaOH and the final absorbance was measured at 410 nm with a spectrophotometer (Hitachi U-2000 Spectrophotometer, Hitachi High-Technologies, Krefeld, Germany). All samples were run in triplicate and specific ALP activity was expressed in U/µg cellular protein as determined by the Micro BCA™ protein assay.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated according to the manufacturer's instructions using the RNeasy Mini kit (Qiagen, Hilden, Germany) and the yield was determined by spectrophotometry at 260 nm. The Qiagen One Step RT-PCR kit (Qiagen, Hilden, Germany) and primers for osteopontin (OPN), collagen type I (Coll I) and beta-actin were used in a one-step RT-PCR reaction. First, 1 µg of total RNA in aqueous solution was reverse-transcribed in a volume of 50 µl containing 400 µM of each NTP, 10 units of RNase inhibitor, 10 µl 5 x Qiagen buffer, 2 µl RT-PCR enzyme mix and 800 nM of each primer. Sequences of primers, product sizes and annealing temperatures are summarized in Table 1. Reverse transcription was carried out at 50 °C for 30 min. After an initial denaturation step at 95 °C for 15 min, 40 PCR cycles were run, each consisting of denaturation at 94 °C for 1 min, annealing for 1 min at the respective temperature given in Table 1, extension at 72 °C for 1 min, and finally elongation at 72 °C for 10 min. The PCR products were separated by electrophoresis of 20 µl of each reaction mixture in a 2% agarose gel at 100 V/cm in 1 × Tris acetate EDTA buffer. Following electrophoresis, the gels were stained with ethidium bromide, destained in distilled water and photographed with a charge-coupled device camera. To obtain a semi-quantitative assessment of gene expression, the integrated density values of the gene-specific bands were quantified using Quantity One® Software (Biorad, Hercules, CA, USA) and the expression was normalized to beta-actin. The size of the PCR products was determined by comparison with a standard 1 kb DNA ladder (Life Technologies Inc., Gaithersburg, MD, USA).

Confirmation of RT-PCR results

In order to confirm the semi-quantitative RT-PCR results, the amplified products of OPN and beta-actin were separated on an agarose gel, stained with ethidium bromide, excised from the gel and purified using the QIA quick gel extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted in Tris buffer (pH 8.5) and sequenced at the UCLA Sequencing & Genotyping Core facility on an ABI 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Statistics

All measurements were made in duplicate and are expressed as mean ± SD of three independent experiments (n = 6 for each individual group and assay). In addition, one-way ANOVA and Tukey's multiple comparison test were used to reveal significant differences between the different investigation groups. The level of significance was set at $P < 0.05$.

Results

Assay for viability of attached cells

In all samples the number of attached viable cells increased during the cultivation period. In control cultures the net absorbance increased from 1.56 ± 0.40 SD after 24 h to 2.08 ± 0.45 SD after 48 h and 2.49 ± 0.32 SD after 72 h of cultivation. Cell cultures irradiated with 1 J/cm² showed net absorbance of 1.99 ± 0.27 SD 24 h post-irradiation, 2.17 ± 0.13SD at 48 h and 2.50 ± 0.43 SD at 72 h. Cells irradiated with 2 J/cm² showed net absorbance of 0.85 ± 0.03 SD after 24 h, 1.66 ± 0.06 SD after 48 h and 2.48 ± 0.002 SD after 72 h (Fig. 1). After 24 h the viability of attached cells irradiated with 1 J/cm² was significantly higher ($P < 0.01$) than in those irradiated with 2 J/cm², but there were no significant differences compared with the control group. Cells irradiated with 1 J/cm² showed higher cell viability over the first 48 h. After 72 h of cultivation, all three groups showed fairly similar cell viability.

Alkaline phosphatase (ALP) specific activity

No significant alteration in ALP specific activity was detectable during the observation period in the control cultures (24 h: 3.49 ± 0.77 SD; 48 h: 3.09 ± 1.13 SD; 72 h: 3.75 ± 2.71 SD) or in cultures irradiated with 2 J/cm² (24 h: 1.56 ± 0.88 SD; 48 h: 1.78 ± 0.15 SD; 72 h: 1.56 ± 1.00 SD), whereas increased ALP activity was observed in cultures irradiated with 1 J/cm² (24 h: 2.98 ± 0.45 SD;

Table 1. Sequences of forward and reverse primmers of OPN, Coll Type I and β-actin

Gene	Primer sequence		Amplicon (bp) length	RT-PCR annealing temperature
Osteopontin (OPN)	5´ cca agt aag tcc aac gaa ag 3´	(forward primer)		
	5´ ggt gat gtc ctc gtc tgt a 3´	(reverse primer)	347	50
Collagen type I	5´ tga cct caa gat gtg cca act 3´	(forward primer)		
(Coll I)	5´ acc aga cat gcc tct tgt cc 3´	(reverse primer)	197	50
Beta-actin	5´ cca tca tga agt gtg acg tg 3´	(forward primer)		
	5´ aca tct gct gga agg tgg ac 3´	(reverse primer)	225	60

48 h: 5.15 ± 2.67SD; 72 h 4.77 ± 3.48 SD). Although the specific ALP activity was lower in cells irradiated with 2 J/cm² than in the two other groups, there was only a significant difference when comparing the 1 J/cm² and 2 J/cm² groups at 48 h and 72 h after irradiation ($P < 0.05$) (Fig. 2).

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

The relative expression of osteopontin (OPN) mRNA remained constant over time in the control group (24 h: 75.84 ± 19.09 SD; 48 h: 64.13 ± 12.20 SD; 72 h: 66.8 ± 15.25) and in the cultures treated with the lower laser dose (24 h: 75.84 ± 19.09 SD; 48 h: 64.13 ± 12.20 SD; 72 h: 66.8 ± 15.25). Although OPN mRNA expression was consistently higher in cells treated with 1 J/cm² than in untreated control cells, this difference was not statistically significant. Interestingly, the expression of OPN mRNA was lowest of all in cells treated with 2 J/cm² after 24 h but increased 2-fold with time to the highest expression observed (24 h: 47.53 ± 4.45 SD; 48 h: 60.60 ± 20.40 SD; 72 h: 92.33 ± 1.45) (Fig. 3a).

Collagen type I (Coll I) mRNA expression decreased over time in the control group (24 h: 111.40 ± 3.77 SD; 48 h: 105.16 ± 6.46 SD; 72 h: 61.00 ± 35.32 SD) and to a lesser extent in the cultures treated with a laser dose of 1 J/cm² (24 h: 133.27 ± 60.11 SD; 48 h: 144.25 ± 34.34 SD; 72 h: 77.73 ± 4.82 SD) with the latter showing a consistently higher expression of Coll I mRNA. In contrast to these groups, cells irradiated with 2 J/cm² showed a slight increase in Coll I expression over time (24 h: 107.58 ± 1.29SD; 48 h: 102.16 ± 1.75 SD; 72 h: 115.93 ± 1.53). It must be noted, however, that the differences were not statistically significant (Fig. 3b).

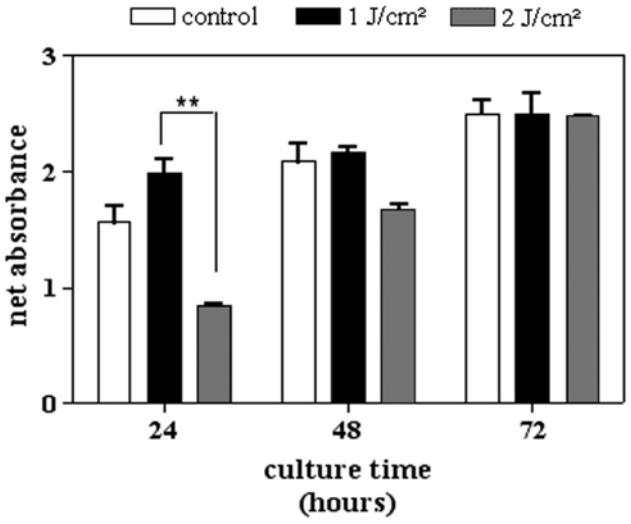

Fig. 1. Viability of attached cells. Proliferation of SaOS-2 cells treated with 1 J/cm², 2 J/cm² and controls assayed using XTT to measure formazan turnover. Cell proliferation/viability is expressed as absorbance in assay wells corrected for the absorbance of control wells

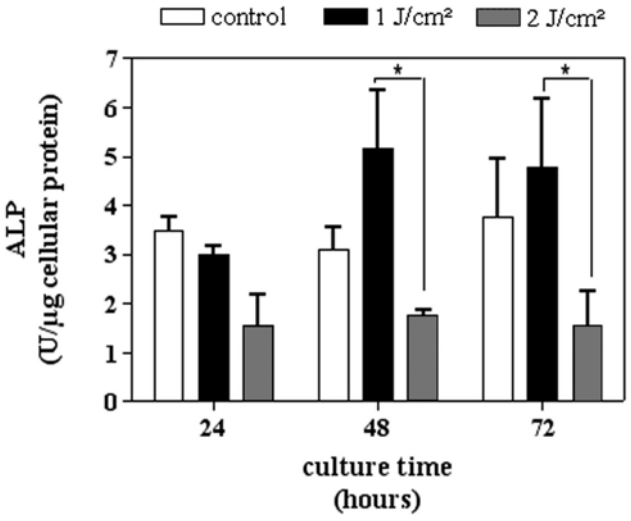

Fig. 2. ALP specific activity. ALP specific activity of SaOs-2 cells treated with 1 J/cm², 2 J/cm² and untreated control cells shown in U/µg total cellular protein

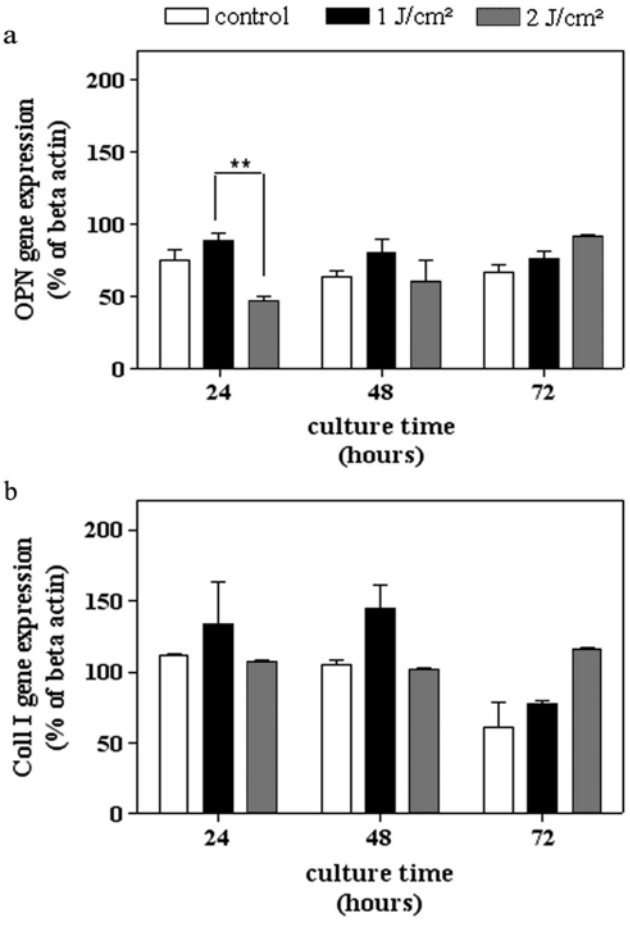

Fig. 3. Relative gene expression of OPN and Coll I mRNA. Relative expression of OPN (**a**) and Coll I (**b**) normalized to the expression of the housekeeping gene beta-actin. Results are expressed as % beta-actin

Discussion

The presented *in vitro* study evaluated the effects of LLLT on adherent human osteoblastic cells and showed that cell viability and ALP specific activity were higher in cells treated with 1 J/cm², although statistically significant differences from control cells were not detected.

LLLT has been shown to have various biostimulatory effects, such as promotion of wound healing [22] and enhancement of fibroblast [23] and chondral proliferation [24]. LLLT has also been reported to increase collagen synthesis [25], anti-inflammatory activity [26] and nerve regeneration [27]. However, reports on the effects of LLLT on osteoblastic cells are controversial: some studies have demonstrated positive results of LLLT on bone healing [28], whereas others did not observe any significant effects [29].

In a study by Ozawa et al. [30] analysis of the number of mitoses and the alkaline phosphatase levels in osteoblasts suggested a biostimulatory effect on the irradiated cells. In an experimental study in sheep, Jakse et al. [31] concluded that LLLT has no positive effect on bone regeneration within a cancellous sinus graft, but were able to show a positive effect on osseointegration of dental implants.

These inconsistent reports may be attributed to the wide variety of laser setups and types used in different studies. A study on the effect of laser irradiation on human fibroblast cultures (HeNe laser, wavelength 633 nm) showed significant effects at a laser output below 2.91 mW, whereas higher doses (5.98 mW) appeared to have no effect at all [32]. Dörtbudak et al. [28] reported higher bone matrix production of rat bone marrow cells after irradiating the cells three times with a laser dose of 1.6 J/cm² (diode laser, wavelength 690 nm). The laser doses used in our study apparently lie within the range that achieves biostimulatory effects on bone cells *in vitro*.

Our results showed that LLLT has a mild enhancing effect on growth and differentiation of SaOS-2 cells *in vitro*. We achieved the best results with a laser dose of 1 J/cm². Cells treated with this dose showed slightly increased proliferation and increased levels of ALP specific activity. This agrees with the findings of Ueda et al. [33] and Ozawa et al. [18], who also reported increased proliferation and ALP specific activity in cells of the osteoblastic lineage. Also, the osteoblastic makers Coll I and OPN were expressed at higher levels in these cultures.

Treatment with 2 J/cm² resulted in lower proliferation and OPN and Coll I mRNA expression during the first 48 h of cultivation; however, 72 h after irradiation the number of viable cells and the mRNA expression of OPN and Coll I were higher than in the two other groups, although the differences were not statistically significant. The experimental setup of our study was intended to investigate the initial effects of LLLT on human osteoblastic cells during the first 72 h after irradiation. A study on human fibroblasts investigated the effect of laser doses of 1.2 mW (HeNe laser), which resulted in higher proliferation in the irradiated group four days after the treatment [34], and Dörtbudak et al. [28] reported a marked increase in bone matrix production between day 12 and day 16 after irradiation. It is therefore probable that the small initial effects observed directly after LLLT would be enhanced after a prolonged cultivation period.

Tissue engineering of bone is an exciting new technology in regenerative medicine [35]. As the enhancement of osteoblast proliferation is one of the major aims of bone tissue engineering approaches, the use of LLLT during the tissue engineering process and in the treatment of patients directly after implantation may offer new perspectives in the development of a clinically established bone substitute. Increased proliferation and differentiation under laser treatment may contribute to shortening the time between collection of tissue samples and transplantation of the *in vitro*-generated bone neo-tissue. The biostimulatory effects of LLLT could also enhance bone healing around dental implants.

Conclusion

We have shown that LLLT has a mild biostimulatory effect on human osteoblast-like cells during the first 72 h after laser irradiation. Further studies will be needed to evaluate the effect of LLLT over more prolonged cultivation periods.

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