

Phenothiazine Chloride and Soft Laser Light Have a Biostimulatory Effect on Human Osteoblastic Cells

Elisabeth Stein, M.S.,¹ Jadranka Koehn, Ph.D.,² Walter Sutter, M.D.,¹ Constanze Schmidl,¹ Vesna Lezaic,¹ Gabriele Wendtlandt,² Franz Watzinger, M.D., Ph.D.,¹ and Dritan Turhani, M.D., Ph.D.¹

Abstract

Objective: Low-level laser therapy (LLLT) is a well accepted tool to accelerate wound healing and to reduce inflammation after oral implant insertion. Since there are no *in vitro* data on a combination of LLLT with prior photosensitization, it was the aim of this study to investigate if photosensitization with phenothiazine chloride results in an alteration of the biostimulatory effect of low-level laser irradiation. **Background Data:** LLLT and antimicrobial photodynamic therapy are well established for the treatment of peri-implantitis. *In vitro* studies have shown a biostimulatory effect of LLLT on various cell types, including osteogenic cells. **Materials and Methods:** SaOS-2 cells were treated with the photosensitizer phenothiazine chloride before irradiation with matched laser light. At 24-h intervals the viability and differentiation were analyzed in treated and untreated cells. **Results:** While the biostimulatory effect of the LLLT could be observed for the lower irradiation dose, the pretreatment with phenothiazine chloride did not significantly affect the growth and differentiation of the SaOS-2 cells. **Conclusion:** It can thus be concluded that combined treatment with phenothiazine chloride and LLLT does not result in a synergistic enhancement of the biostimulatory effect of LLLT, but there was also no evidence for antagonizing effects on growth and differentiation of human osteoblasts.

Introduction

LOW-LEVEL LASER THERAPY (LLLT) has become an accepted tool for several clinical applications.¹ LLLT has been successfully established in regenerative medicine and dentistry due to its anti-inflammatory,² analgesic,³ and regenerative effects.^{4–6} The exact biological mechanisms underlying the biostimulatory effect of LLLT are not yet fully understood, but experimental and clinical studies suggest that LLLT affects cellular metabolic processes, leading to an enhanced regeneration of biological tissues.^{7,8}

Photodynamic therapy (PDT) is a two-step therapeutic approach starting with the application of a photosensitizing agent and followed by irradiation with light energy that is spectrally matched to activate the drug.⁹ Although the chemical processes that cause the photodynamic effect are not yet fully understood, numerous experimental studies suggest that PDT initiates a photochemical process that results in the production of radical singlet oxygen molecules, strong oxidants that cause immediate and irreversible cytotoxic effects.^{10,11}

PDT is widely used today. Besides its application in the treatment of neoplasms,¹² PDT is also successfully used in dermatology¹³ and dentistry.¹⁴ In oncology, PDT is increasingly used as alternative treatment for superficial cancers (e.g., basal cell carcinomas and esophageal and endobronchial cancer).¹⁵ In dentistry, antimicrobial PDT (aPDT) is used for the treatment of aggressive periodontitis,¹⁴ the prevention of alveolar osteitis,¹⁶ and for the treatment of peri-implantitis.¹⁷ Experimental studies have revealed that He-Ne or Ga-Ar lasers in combination with the appropriate photosensitizing agents are able to significantly reduce the viability of bacteria.¹⁸

Among the topically used photosensitizers, 5-aminolevulinic acid (ALA) and phenothiazines are widely used. ALA is not a photosensitizer by itself, but it is metabolized to photosensitive protoporphyrin IX through the cellular heme biosynthetic pathway.¹⁹ In contrast, toluidine blue, also known as a potent photosensitizer, is a directly active drug that can be easily applied topically.¹¹

Since LLLT has been shown to have biostimulatory effects on bone cells *in vitro*^{20,21} and *in vivo*,^{22,23} and aPDT has great

¹Department of Cranio-Maxillofacial and Oral Surgery, and ²Department of Internal Medicine II, Department of Angiology, Medical University of Vienna, Vienna, Austria.

potential in the treatment of periodontal disease, the question arises whether the combination of LLLT with prior photosensitization with phenothiazine chloride might have synergistic biostimulatory effects. Therefore, the aim of this study was to investigate the *in vitro* effects of LLLT in combination with the prior application of phenothiazine chloride on the growth and differentiation of human osteoblastic cells.

Materials and Methods

Cell culture

The human osteosarcoma cell line SaOS-2 possesses an osteoblastic phenotype,²⁴ and was obtained from the American Type Culture Collection (ATCC #HTB-85; Rockville, MD, USA). SaOS-2 cells were cultivated in DMEM/Hamm'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, 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% CO₂.

Experimental set-up

For experimental treatment SaOS-2 cells were seeded at a density of 1.5×10^5 cells/well in 6-well tissue culture polystyrene dishes (BD Biosciences, Bedford, MA, USA). After 48 h of cultivation, the culture medium was removed and the cells were assigned to two groups. The samples in group 1 were washed twice with PBS, and osteogenic medium (DMEM/Hamm's F-12 medium 1:1 with 10% fetal calf serum [Cambrex Europe, Verviers, Belgium], 100 U/mL penicillin G, 100 µg/mL streptomycin sulfate, 0.285 mM ascorbic acid, 10 mM β-glycerolphosphate, and 0.1 µM dexamethasone) was added before laser irradiation.

The cells in group 2 were treated with the photosensitizing agent phenothiazine chloride (Helbo[®] Blue Photosensitizer; Helbo Photodynamic Systems, Grieskirchen, Austria). The photosensitizing agent (1 mL/well, concentration 1 µg/mL) was added to the culture wells. After incubation for 5 min, the cells were washed twice with PBS (Cambrex Europe) and osteogenic medium was added before laser irradiation.

The cells in groups 1 and 2 were irradiated with a 670-nm non-thermal diode laser unit (Helbo Photodynamic Systems) at an output power of 400 mW (continuous wave mode with no additional filters) for 30 sec or 1 min, corresponding to an energy density of 1 J/cm² or 2 J/cm², respectively. The laser unit was placed 11 cm above the bottom of the culture plate and the beam was adjusted to exactly cover the bottom of one culture well. Cells that were neither treated with phenothiazine chloride nor irradiated with laser light served as controls. The cells were maintained at 37°C, 95% humidity, and 5% CO₂, and were analyzed 24, 48, and 72 h after laser irradiation.

Assay for attached cell viability

Duplicates of six wells were used for each assay. After incubation for the specified times, viability was measured using the metabolic XTT-Assay (Roche Diagnostics, Mannheim, Germany). XTT is converted by viable cells to formazan, a colored product. The amount of conversion is directly pro-

portional to the number of viable cells. The cells were incubated with a final concentration of 200 µg/mL XTT and 5 µM of the reducing agent N-methyl dibenzopyrazine methyl sulfate for 4 h at 37°C before the cell culture supernatant was transferred to 96-well plates and the absorbance from converted XTT solution was read at 450 nm in a plate reader (Type 12500; Anthos Labtec Instruments, Wals, Austria). Average absorbance values from blank wells were subtracted from seeded wells to obtain net absorbance values.

Total cellular protein synthesis

The cells were harvested and lysed as previously described.²⁵ The protein content of the lysates was determined using the Micro BCA™ protein assay (Pierce Chemical, Rockford, IL) as previously described.²⁵

Alkaline phosphatase (ALP)-specific activity

ALP-specific activity was quantified using a colorimetric end-point assay (diagnostic kit 104-LL; Sigma, St. Louis, MO, USA) as previously described,²⁵ to determine early osteoblastic differentiation. All samples were run in triplicate and the ALP activity was expressed in units per microgram of cellular protein as determined by the Micro BCA™ protein assay.

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

Total RNA isolation, one-step RT-PCR reaction, and semi-quantitative assessment were performed using the RNeasy Mini kit (Qiagen, Hilden, Germany) and the Qiagen One Step RT-PCR kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and as previously described.²⁵

Sequences of primers, product sizes, and annealing temperatures are summarized in Table 1.

Confirmation of RT-PCR results

In order to confirm the semi-quantitative RT-PCR results, the amplified products were extracted from agarose gels and sequenced as previously described.²⁵

Statistics

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

Results

Assay for attached cell viability

In all samples the number of attached viable cells increased during the cultivation period (Fig. 1). In control cultures net absorbance increased from 1.17 ± 0.72 after 24 h, to 1.69 ± 0.95 after 48 h, and to 2.07 ± 1.00 after 72 h post-irradiation. The cells of group 1, which received only laser irradiation without prior photosensitization, showed net absorbances of 1.42 ± 0.99 at 24 h, 1.55 ± 0.98 at 48 h, and

TABLE 1. SEQUENCES OF FORWARD AND REVERSE PRIMERS, AMPLICON LENGTH, AND ANNEALING TEMPERATURES USED FOR SEMI-QUANTITATIVE RT-PCR

Gene	Primer sequence	Amplicon length (bp)	Annealing temperature (°C)
Osteopontin (OPN)	5' cca agt aag tcc aac gaa ag 3' (forward primer) 5' gg gat gtc ctc gtc tgt a 3' (reverse primer)	347	50
Collagen type I (Coll I)	5' tga cct caa gat gtg cca act 3' (forward primer) 5' acc aga cat gcc tct tgt cc 3' (reverse primer)	197	50
β -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

1.76 \pm 1.08 at 72 h post-irradiation with a laser dose of 1 J/cm². Cells treated with 2 J/cm² showed net absorbances of 0.859 \pm 0.03 after 24 h, 1.67 \pm 0.06 after 48 h, and 2.68 \pm 0.01 after 72 h of cultivation. Non-irradiated cells of group 2 treated with 1 μ g/mL photosensitizer showed net absorbances of 1.059 \pm 0.68 after 24 h, 1.32 \pm 0.78 after 48 h, and 1.80 \pm 0.89 after 72 h of cultivation. Cells of group 2 treated with a laser dose of 1 J/cm² showed net absorbances of 1.27 \pm 0.86 at 24 h, 1.61 \pm 1.12 at 48 h, and 1.59 \pm 1.09 at 72 h post-irradiation. Cells of group 2 irradiated with 2 J/cm² showed net absorbances of 0.73 \pm 0.05 at 24 h, 1.87 \pm 0.45 at 48 h, and 2.09 \pm 0.06 at 72 h post-irradiation. There were no statistically significant differences between the different study groups (Fig. 1).

Alkaline phosphatase (ALP)-specific activity

In control cultures ALP-specific activity (Fig. 2) decreased from 4.28 \pm 2.75 after 24 h to 3.41 \pm 2.48 after 48 h, and increased to 4.86 \pm 3.46 at 72 h post-irradiation. In cells of

group 1, which received only laser irradiation without prior photosensitization, ALP-specific activity increased from 3.15 \pm 2.27 at 24 h, to 3.89 \pm 3.20 at 48 h, and to 4.55 \pm 3.84 at 72 h post-irradiation with a laser dose of 1 J/cm². Samples irradiated with 2 J/cm² showed lower levels of ALP-specific activity over the entire cultivation period (at 24 h: 1.57 \pm 0.88; at 48 h: 1.78 \pm 0.15; and at 72 h: 1.56 \pm 1.00) in comparison to controls and cells treated with 1 J/cm².

Non-irradiated cells of group 2 treated with 1 μ g/mL photosensitizer showed increasing ALP-specific activity over time (at 24 h: 3.024 \pm 2.32; at 48 h: 3.35 \pm 2.12; and at 72 h: 4.85 \pm 4.03). Cells of group 2 treated with a laser dose of 1 J/cm² showed the highest levels of ALP-specific activity after 48 and 72 h (at 24 h: 2.90 \pm 1.86; at 48 h: 4.7 \pm 1.64; and at 72 h: 4.90 \pm 5.95) post-irradiation. Cells of group 2 irradiated with 2 J/cm² showed decreasing levels of ALP-specific activity (at 24 h: 3.41 \pm 0.50; at 48 h: 2.03 \pm 0.32; and at 72 h: 1.60 \pm 0.35), similarly to the corresponding group that received only laser irradiation without prior photosensitization (Fig. 2).

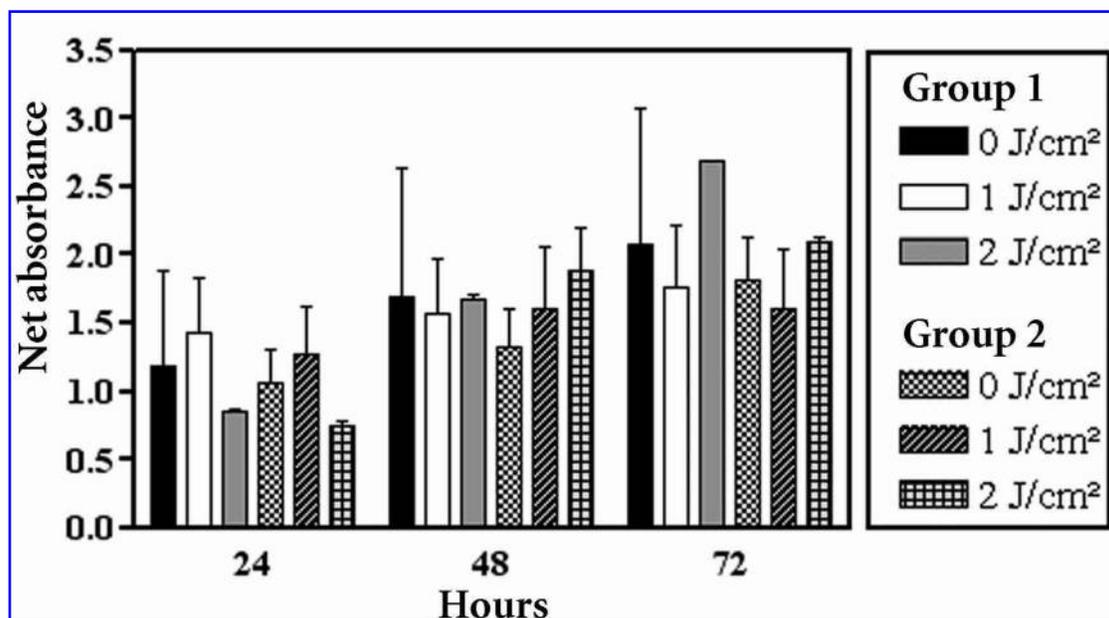


FIG. 1. Attached cell viability. Proliferation of SaOS-2 cells of group 1 (no pretreatment with phenothiazine chloride) and group 2 (pretreatment with phenothiazine chloride) as assayed by XTT-to-formazan turnover. Cell proliferation and viability are expressed as absorbance by assay wells corrected for the absorbance of control wells.

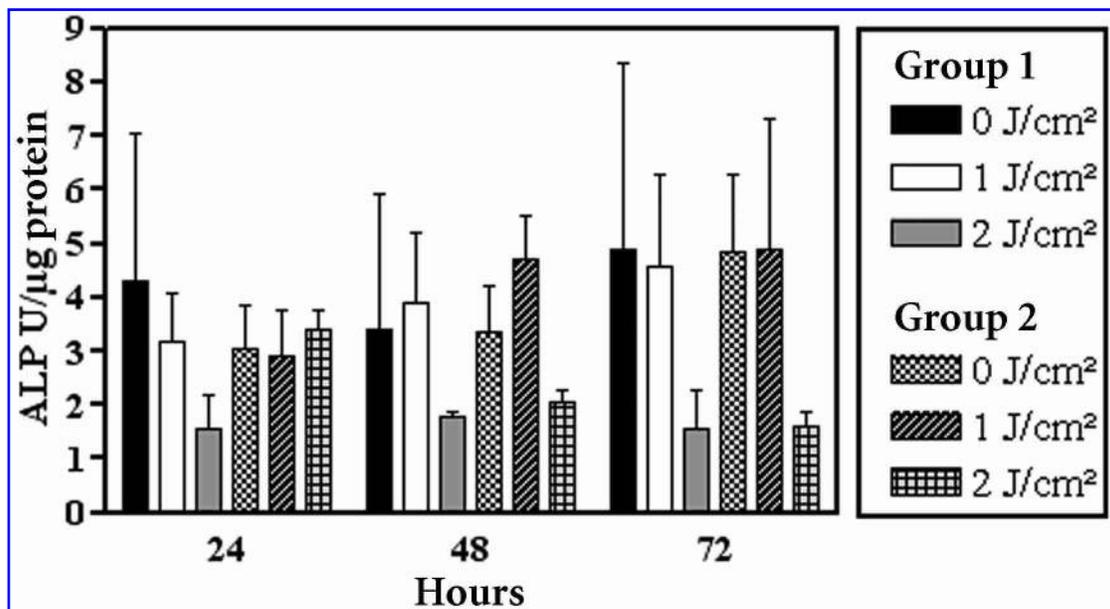


FIG. 2. ALP-specific activity. ALP-specific activity of SaOS-2 cells of group 1 (no pre-treatment with phenothiazine chloride) and group 2 (pre-treatment with phenothiazine chloride) expressed as units per microgram of total cellular protein.

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

The relative expression of collagen type I mRNA (Fig. 3a) decreased slightly over time in controls (at 24 h: 140.42 ± 45.06 ; at 48 h: 127.16 ± 49.50 ; and at 72 h: 82.08 ± 42.61). Cells of group 1 irradiated with 1 J/cm² showed a similar pattern of relative collagen I mRNA expression as that observed in controls (at 24 h: 133.27 ± 60.11 ; at 48 h: 144.26 ± 34.34 ; and at 72 h: 77.73 ± 4.82). In cells of group 1 treated with a laser dose of 2 J/cm², the relative expression of collagen I mRNA remained constant over time (at 24 h: 107.57 ± 1.29 ; at 48 h: 102.16 ± 1.74 ; and at 72 h: 115.93 ± 1.53) (Fig. 3a).

Non-irradiated cells and cells treated with 1 J/cm² showed a similar relative expression pattern of collagen I mRNA as that observed in controls (non-irradiated cells: at 24 h: 122.20 ± 49.66 ; at 48 h: 140.31 ± 65.47 ; and at 72 h: 92.17 ± 21.01) (irradiation at 1 J/cm²: at 24 h: 155.38 ± 60.61 ; at 48 h: 94.55 ± 13.09 ; and at 72 h: 64.76 ± 29.44). Similarly, group 1 cells treated with 2 J/cm² showed constant relative expression levels of collagen I mRNA over the entire cultivation period (at 24 h: 109.73 ± 0.74 ; at 48 h: 107.36 ± 2.60 ; and at 72 h: 114.03 ± 6.71). There were no statistically significant differences between the study groups (Fig. 3a).

The relative expression of OPN mRNA (Fig. 3b) remained constant in controls (at 24 h: 78.49 ± 21.93 ; at 48 h: 90.18 ± 54.01 ; and at 72 h: 82.06 ± 12.96), and in cells of group 1 treated with 1 J/cm² (at 24 h: 79.98 ± 21.94 ; at 48 h: 73.21 ± 27.34 ; and at 72 h: 73.30 ± 16.82). Interestingly, the expression of OPN mRNA was lowest with irradiation with 2 J/cm² after 24 h, but it had increased twofold at the end of the cultivation period (at 24 h: 47.53 ± 4.45 ; at 48 h: 60.60 ± 20.40 ; and at 72 h: 92.33 ± 1.45).

In non-irradiated cells of group 2 the expression of OPN mRNA was initially higher than in controls, but levels decreased over time (at 24 h: 100.35 ± 23.92 ; at 48 h: 86.25 ± 51.31 ; and at 72 h: 68.02 ± 13.87). A similar expression pat-

tern was also observed in cells of group 2 treated with 1 J/cm² (at 24 h: 97.75 ± 6.98 ; at 48 h: 79.00 ± 55.16 ; and at 72 h: 62.63 ± 29.21). Similarly to group 1, the cells of group 2 irradiated with 2 J/cm² showed initially lower expression of OPN mRNA (at 24 h: 48.66 ± 3.39 ; at 48 h: 67.65 ± 1.50 ; and at 72 h: 73.25 ± 6.04), although in this group expression levels increased to a lesser extent compared to group 1. There were no statistically significant differences in OPN mRNA expression between the study groups (Fig. 3b).

Discussion

Low-level laser therapy has been shown to induce various biostimulatory effects, such as enhancement of wound healing²⁶ and fibroblast²⁷ and chondral proliferation.²⁸ Reports on the effects of LLLT on osteoblastic cells are controversial. Some studies report a positive influence of LLLT on bone regeneration,^{23,29} while others found no significant effects at all.³⁰

Our previous work has shown that LLLT has a mild biostimulatory effect on human SaOS-2 cells during the first 72 h post-laser irradiation, as characterized by a slight enhancement of cell viability, ALP-specific activity, and the expression of osteopontin and collagen type I after treatment with 1 J/cm².²¹

PDT has been successfully used for endodontic disinfection.³¹ The principle by which bacteria are killed is based on a photochemical reaction that produces singlet oxygen radicals, which act as strong oxidants and cause irreversible lethal damage to the bacterial membrane, primarily by lipid oxidation.^{32,33} Healthy eukaryotic cells have cellular defense mechanisms against attack by oxygen radicals, mainly based on the activity of catalase and superoxide dismutase.

It was the aim of this study to investigate the combined effect of PDT and LLLT on growth and differentiation of human osteoblast-like cells, and to address the question whether pre-treatment with phenothiazine chloride en-

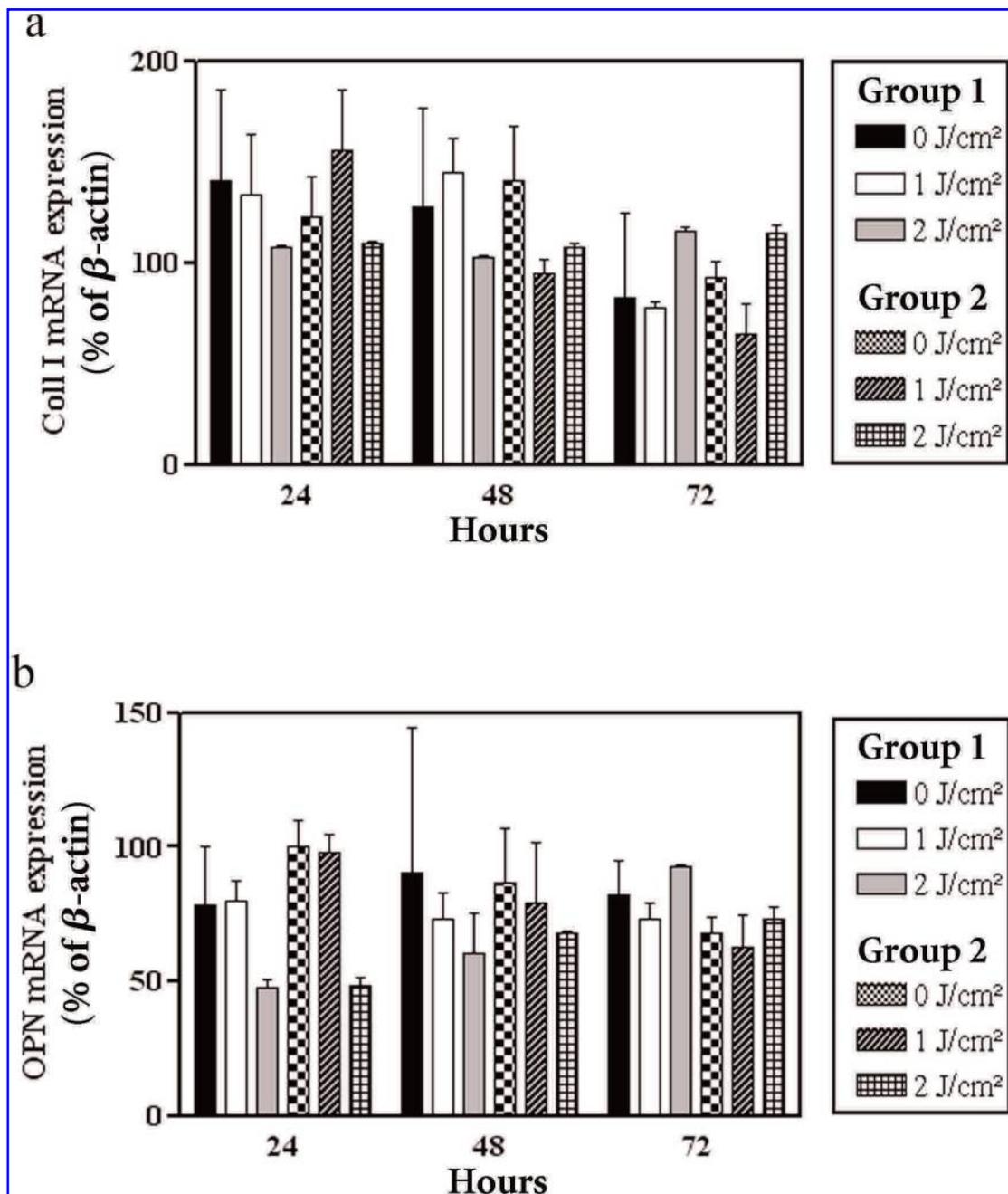


FIG. 3. Relative mRNA expression of OPN and Coll I mRNA. Relative expression of (a) Coll I and (b) OPN mRNA normalized to the expression of the housekeeping gene β -actin. Cells in group 1 were not pre-treated with phenothiazine chloride, while cells in group 2 were treated with phenothiazine chloride prior to irradiation. Results are expressed as percentage of β -actin mRNA level.

hances the biostimulatory effect of LLLT in a synergistic way or annihilates it.

We have shown that irradiation with a diode laser following photosensitization with phenothiazine chloride had no negative effect on the growth and differentiation of human osteoblastic cells, and did not counteract the biostimulatory effect induced by LLLT. There were no statistically significant differences in the growth and differentiation behavior between the two study groups, and we could not demonstrate any additional synergistic effect of PDT compared to LLLT alone.

Tissue engineering with human autologous cells is a nascent research subject that is gaining importance as an alternative to more traditional methods of bone augmentation.

In a study by Dortbudak et al.,²⁹ they reported a marked increase in bone matrix production between day 12 and day 16 post-irradiation, and Soudry et al.³⁴ reported markedly higher proliferation of fibroblasts 4 d after laser treatment. Therefore it seems probable that the slight effects observed in our studies of LLLT,²¹ and the combined treatment with phenothiazine chloride detailed here, could be enhanced by more prolonged cultivation.

We believe that PDT is a promising treatment for tissue engineering applications. The biostimulatory effect of LLLT combined with the antimicrobial action of PDT show great promise in the development of new methods of bone regeneration.

Conclusion

We found that the combined application of LLLT with phenothiazine chloride as a photosensitizer has neither a synergistic nor an adverse effect on growth and differentiation of human osteoblastic cells compared to the use of LLLT alone. Further studies over more prolonged cultivation periods are needed to evaluate the clinical potential of PDT as a treatment for accelerating wound healing, reducing inflammation after oral implantation, and for tissue engineering applications.

Disclosure Statement

No conflicting financial interests exist.

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Address reprint requests to:

Dr. Dritan Turhani, M.D., Ph.D.

Department of Cranio-Maxillofacial and Oral Surgery

AKH, General Hospital of Vienna

Währinger Gürtel 18-20

A-1090 Vienna, Austria

E-mail: dritan.turhani@meduniwien.ac.at