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THE INFLUENCE OF LASER RADIATION ON HUMAN OSTEOBLASTS CULTURED ON NANOSTRUCTURED COMPOSITE SUBSTRATES

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Abstract

Background and aims. *Carbon-based nanomaterials such as carbon nanotubes, graphene oxide and graphene have been explored by researchers as well as the industry. Graphene is a new nanomaterial which has commercial and scientific advantages. Laser therapy has proven highly useful in biomedicine, with the use of different laser types and energies for distinct purposes. The low level laser therapy (LLLT) can have anti-inflammatory, analgesic and biostimulant effects. Recent research has shown that laser radiation has different effects on osteoblasts. The aim of this study was to identify the influence of laser radiation on human osteoblastic cells cultured on nanostructured composite substrates.*

Materials and methods. *Four types of substrates were created using colloidal suspensions of nanostructured composites in PBS at a concentration of 30 µg/ml. We used human osteoblasts isolated from patella bone pieces harvested during arthroplasty. Irradiation of osteoblasts cultured on nanostructured composite substrates was made with a semiconductor laser model BTL-10 having a wavelength of 830 nm. The proliferation activity of osteoblast cells was assessed using the MTT assay. After laser irradiation procedure the viability and proliferation of osteoblast cells were analyzed using fluorescein diacetate (FDA) staining.*

Results. *The osteoblast cells viability and proliferation were evaluated with MTT assay at 30 minutes, 24 hours, 5 days and 10 days after laser irradiation. In the first 30 minutes there were no significant differences between the irradiated and non-irradiated cells. At 24 hours after laser irradiation procedure a significant increase of MTT values in case of irradiated osteoblasts cultivated on nanostructured hydroxyapatite, nanostructured hydroxyapatite with gold nanoparticles and 1.6% and 3.15% graphenes composites substrates was observed. A more marked proliferation rate was observed after 10 days of irradiation for irradiated osteoblasts seeded on nanostructured hydroxyapatite with gold nanoparticles and graphenes containing substrate. Using FDA staining we obtained very similar results with MTT test.*

Conclusions. *The association between the 830 nm laser irradiation of osteoblasts and their long-term cultivation of the nanostructured composite substrates induces the cell proliferation and differentiation and therefore it will be a useful alternative for bone regeneration therapy.*

Keywords: laser radiation, osteoblasts, nanostructures, composites, substrates.

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Background and aims

Carbon-based nanomaterials such as carbon nanotubes, graphene oxide and graphene have been explored by researchers as well as the industry. Graphene is a new nanomaterial which has commercial and scientific advantages. The single layer and few-layer graphenes received great interest due to their exceptional characteristics and properties in various fields of biotechnologies and nanomedicine [1].

Several studies worldwide have reported the biocompatibility of graphene derivatives in the proximity of different types of cells. Biris et al. [2] has demonstrated that osteoblast cells (MC3T3-E1) have a high ability to grow on graphene film. Agarwal et al. [3] have reported that reduced graphene oxide (rGO) is more biocompatible than single-wall carbon nanotubes using different cell lines including neuroendocrine PC12 cells, oligodendroglia, or osteoblasts. Recently, Gurunathan and coworkers have reported that microbially reduced graphene oxide shows significant biocompatibility with primary mouse embryonic fibroblast (PMEF) cells [4].

Laser therapy has proven highly useful in biomedicine, with the use of different laser types and energies for distinct purposes. Thus, low level laser therapy (LLLT) can have anti-inflammatory, analgesic and biostimulant effects. Therefore, the laser is used clinically for wound healing and tissue regeneration. Various studies have demonstrated the biostimulatory effect of low-level laser energy on cell populations of various origins [5,6,7]. Numerous studies have suggested that low-level laser therapy (LLLT) increases the regenerative potential of biological tissues by modulating cellular metabolic processes [8].

Recent research has shown that laser radiation has different effects on osteoblasts. In their study Pyo et al. concluded that LLLT on hypoxic-cultured osteoblast stimulates osteoblast differentiation and proliferation through increased expression of BMP-2, osteocalcin, and TGF-ß1 [9]. According to this research, Medina-Huertas and co-workers indicate that that low-level diode laser irradiation may be useful in the treatment of bone regeneration through a biostimulatory effect on osteoblasts that favors their growth and maturation. This effect appears to be mediated by the autocrine action of growth factors released by the cells themselves in response to the laser treatment [10].

Various composites have been investigated for their mechanical properties, biocompatibility, and bone forming ability for application to scaffolds for use in bone regeneration. Biodegradable polymers have been extensively applied for preparing the composites with bioceramics, because they can contribute not only to good flexibility to suit that of natural bone, but also to an appropriate degradation speed to provide space for new bone formation in vivo [11,12].

Experimental in vitro studies conducted by our research team in recent years related to laser radiation effects on human fibroblasts involved in healing and tissue regeneration processes [13,14] enabled us to develop new protocols of irradiation on other human cell types such as osteoblasts.

The aim of this study was to identify the influence of laser radiation on human osteoblastic cells cultured on nanostructured composite substrates. At the same time, the parameters of specific laser wavelength of 830 nm that produce a stimulant effect on human osteoblastic cells in combination with nanostructured composites were identified. Thus, the laser radiation role of increasing the number of cells in regenerative processes was established.

Materials and methods

Substrate Preparation

Four types of substrates were created using colloidal suspensions of nanostructured composites in PBS (Dulbecco's Phosphate Buffered Saline from Sigma Aldrich) at a concentration of 30 µg/ml. The chemical composition of substrates are shown in Table I. The suspensions were sonicated for 30 minutes, and 100µl/well were added in Nunclon 96-well plates. We created plates for each point of time for viability testing (30 min, 24 hours, 5 days and 10 days), a plate for un-irradiated controls and another for irradiated samples, with triplicates for controls and substrates. The plates were dried under a sterile air laminar flow in a class II hood for 16 hours and sterilized by exposure to ethylene oxide. Before starting the cell culture experiments the obtained susbstrates coatings were rinsed with PBS.

Table I. Composition of substrates.

Substrates	S1	S2	S3	S4
Composition	Nanostructured Hydroxyapatite (HA)	Nanostructured Hydroxyapatite (HA) + Gold Nanoparticles (Au NPs 1%)	Nanostructured Hydroxyapatite + Gold Nanoparticles (Au NPs 1%) + Graphene (1.6%)	Nanostructured Hydroxyapatite + Gold Nanoparticles (Au NPs 1%) + Graphene (3.15%)

Cell Cultures

We used human osteoblasts isolated from patella bone pieces harvested during arthroplasty as described by Tomuleasa et al. [15] after obtaining the patient`s informed consent. Mechanical processing and enzymatic digestion was applied using an enzymatic cocktail: 0.1% collagenase IV (Gibco) + 0.25% trypsin EDTA-4 (Sigma). Isolated cells and bone explants were seeded in 25-cm^2 Cole flasks (Nunc) and cultivated in Dulbecco's modified Eagle's medium (DMEM)/F-12HAM (Sigma) containing 20% fetal calf serum (FCS), 2 mM L-Glutamine, 1% antibiotics, 1% non-essential aminoacids (NEA) (all reagents from Sigma), in a humidified 7% CO2 atmosphere. After 6–8 weeks the cell monolayer reached the confluence and cells were trypsinized and reseeded. The morphological aspect of the isolated cells was fibroblastoid-like in the first passages, then the cells became rounded with a polygonal shape as the differentiation process occurred (Fig. 1). Immunocytochemical staining showed cells positivity for bone markers osteopontin and osteonectin.

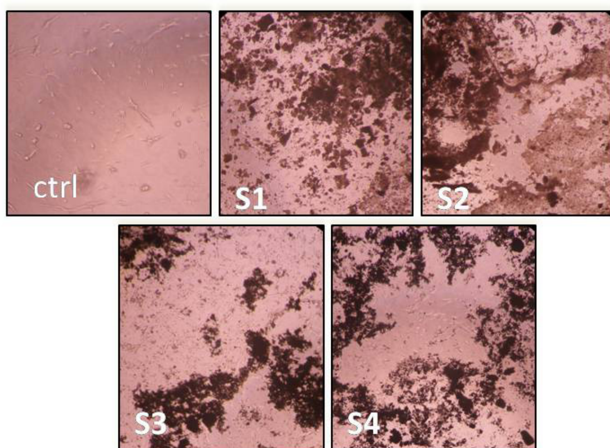

Figure 1. Microscopic aspect of osteoblasts seeded on the substrates (magnification x200).

In our experiments we used osteoblasts at the seventh passage: $2x10^5$ cells/well were seeded in 96-well plates (Nunclon, NUNC, Naperville, USA) in 200 µl complete medium in each well, using triplicate for each substrate and controls with or without laser therapy. At the level of each plate three rows of 10 wells each were seeded. Finally we obtained eight culture plates containing substrates and cells. The eight resulting culture plates were divided into two sets of four plates. For the first set of four plates the laser irradiation procedure was applied, while the second set of four plates remained non-irradiated and was maintained under the same experimental conditions.

Laser irradiation procedure of human osteoblasts cultured on substrates

Laser irradiation procedure was achieved in the first series of four Nunc 96-well plates. Irradiation of osteoblasts cultured on nanostructured composite substrates was made with a semiconductor laser model BTL-10 (Beautyline, Ltd, Prague, Czech Republic) having a wavelength of 830 nm. A handpiece with convergent emission of radiation was used for irradiation procedure of osteoblast cells on substrates.

Laser irradiation was performed in pulsed mode with a frequency of 50 Hz at an energy density of 3 J/cm^2 and the power output of the laser handpiece was 62 mW. Irradiated surface area was set to 0.50 cm^2, corresponding to the area of one well, and the irradiation distance was 1 cm equivalent to the height of the culture plate. The laser handpiece was set at a distance of 1 cm from the base of the well by a clamping device in contact with the edge of the well. The irradiation procedure was performed in a single session for all four culture plates, the parameters were set according to this protocol and on each culture plate there were 30 wells with substrates and osteoblasts.

All wells in each plate noted with C (Control-osteoblasts without substrate), S1 (osteoblasts on substrate S1), S2 (osteoblasts on substrate S2), S3 (osteoblasts on substrate S3) and S4 (osteoblasts on substrate S4) were irradiated using the laser parameters in accordance with the data in Table II. The time of irradiation for each well was 30 seconds and it was automatically set on the laser device.

The temperature throughout the irradiation was measured in each well using a digital multimeter UNI-T model UT33C (Uni-Trend Technology Limited, Dongguan City, China). In the laser irradiated areas throughout the procedure this temperature ranged between 27 and 31 degrees Celsius. The remaining four non-irradiated culture plates were kept under the same experimental conditions like the irradiated plates (Fig. 2).

Figure 2. Laser irradiation procedure and measuring the temperature in the wells.

Proliferation and cell viability tests

a. The MTT test (Thyazolyl Blue Tetrazolium Bromide)

The proliferation activity of osteoblast cells was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay. This method is based

Table II. The parameters used during laser irradiation procedure.

Laser parameters \ Substrate	C Control without substrate	S1 Osteoblasts on HA	S2 Osteoblasts on HA + 1% AuNPs	S3 Osteoblasts on HA + 1%AuNPs +1,6% graphene	S4 Osteoblasts on HA + 1% AuNPs +3,15% graphene
Irradiated area (A)	A= 0,50 cm^2	A= 0,50 cm^2	A= 0,50 cm^2	A= 0,50 cm^2	A= 0,50 cm^2
Irradiation distance (H)	H= 1 cm	H= 1 cm	H= 1 cm	H= 1 cm	H= 1 cm
Energy Density (D)	D= 3 J/cm^2	D= 3 J/cm^2	D= 3 J/cm^2	D= 3 J/cm^2	D= 3 J/cm^2
Power (P)	P= 62 mW	P= 62 mW	P= 62 mW	P= 62 mW	P= 62 mW
Frequency (F)	F= 50 Hz	F= 50 Hz	F= 50 Hz	F= 50 Hz	F= 50 Hz
Irradiation time (t)	t= 30 sec	t= 30 sec	t= 30 sec	t= 30 sec	t= 30 sec
Temperature (T)	T= 27-28°C	T= 28-30°C	T= 29-31°C	T= 29-30°C	T= 29-30°C

on the ability of mitochondrial dehydrogenases in living cells to reduce soluble tetrazolium salts to a blue formazan product whose amount is directly proportional to the number of living cells. One of four plates was measured at 30 minutes, 24 hours, 5 days and 10 days after laser irradiation procedure. The MTT assay procedure was as

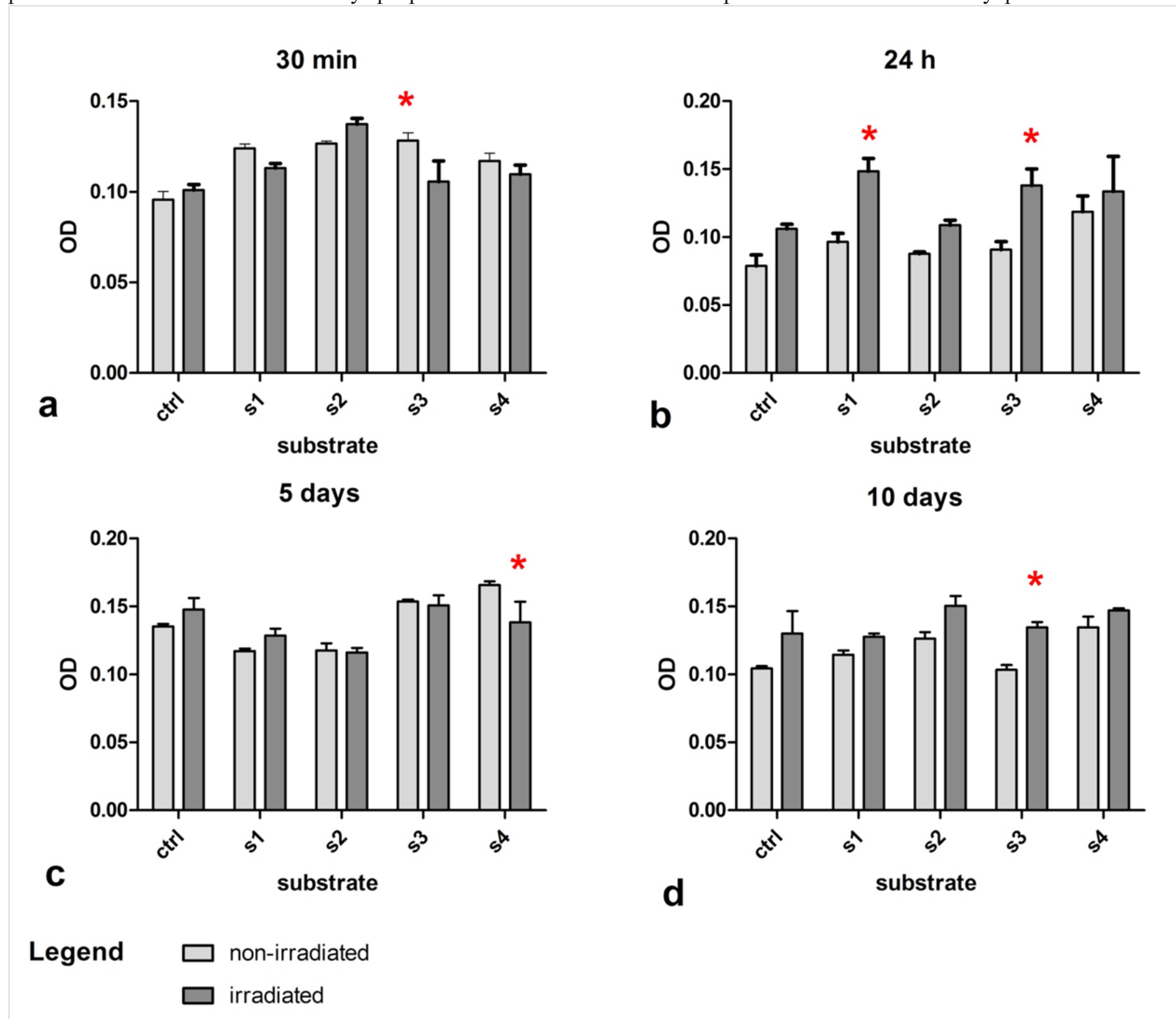

Figure 3. MTT viability test: at 30 minutes (a), 24 hours (b), 5 days (c), 10 days (d) after laser irradiation of osteoblasts cultured on nanostructured composite substrates (S1-S4). Statistical analysis was done with two-way ANOVA followed by Bonferroni posttest with comparison between non-irradiated and irradiated samples. (* indicates that $p<0.05$).

follows: a 100 μl of MTT (final concentration, 1 mg/ml) was added to each well, the plate was incubated for 1 hour at 37°C, the medium with MTT was replaced with 150 μl dimethyl sulphoxide (Sigma, USA) in order to solubilize the formazan produced. Each plate was directly scanned and measured in an ELISA microplate reader (TECAN-Sunrise, Program Magellan, Mannedorf, Switzerland), at 570 nm.

b. The Fluorescein Diacetate (FDA) test

After laser irradiation procedure the viability and proliferation of osteoblast cells were analyzed using fluorescein diacetate (FDA) staining. The principle of FDA test is the esterification of non-fluorescent FDA under the influence of enzymatic activity of the cells into a fluorescent compound (fluorescein). The intensity of the fluorescence is dependent on membrane integrity and metabolic activity of the cells, so the fluorescent signals are proportional with the number and size of viable cells. Osteoblast cell monolayers were washed twice with PBS supplemented with Ca^{2+} and Mg^{2+} and incubated 5 min in dark at 37°C with 100 µl/well with FDA solution (at a final concentration of 2.4 µM in PBS with Ca^{2+} and Mg^{2+}). After incubation the wells were washed twice with PBS and fluorescence intensity (FI) was measured at 488 nm using a BioTek Synergy 2 fluorescence microplate reader (Winooski, VT, USA). FDA assay was performed in this case after 30 minutes and 5 days of cultivation. Microscopic images were captured with a CCD camera (Axiocam MRM) adapted to a Zeiss Axio Observer D1 inverted fluorescence microscope using a 488nm filter. All images were analyzed using Axiovision Release 4.6.3. software.

Statistical analysis

Statistical analysis of the results was performed with a GraphPad Prism 5 software. Statistical significance was set at $p<0.05$. For analysis of MTT and FDA assay, we applied two–way ANOVA, Bonferroni posttest. Additionally we used for MTT test one-way ANOVA with Dunnet`s Multiple Comparison Test, for comparison of all probes with un-irradiated control.

Results

MTT viability tests

The osteoblast cells viability and proliferation were evaluated with MTT assay at 30 minutes, 24 hours, 5 days and 10 days after laser irradiation. The graphical aspects of MTT results are shown in Figure 3. In this case we analyzed the obtained results using two-way ANOVA followed by Bonferroni posttest with comparison between non-irradiated and irradiated samples. In the first 30 minutes there were no significant differences between the irradiated and non-irradiated cells, except S3 substrate where after irradiation a decrease of osteoblasts adhesion was observed. As a general tendency we observed an increased number of adhered cells on all substrates for unirradiated probes (Fig. 3 a). At 24 hours after laser irradiation procedure a significant increase of MTT values in case of irradiated osteoblasts cultivated on S1 and S3 substrates was observed. A similar behaviour was noticed for irradiated osteoblasts grown on S4 substrate, but without statistically significant differences (Fig. 3 b). After 5 days from irradiation there was a uniformity of test values at all samples. Only in the irradiated osteoblasts seeded on the substrate S4 a decrease in viability and cell proliferation was identified (Fig. 3 c). The results obtained after 10 days from laser irradiation revealed a significant increase in osteoblast cells proliferation in irradiated S3 sample, with the remark that there was a general trend of increase of absorption values in all samples, though there was no statistical difference between the irradiated and

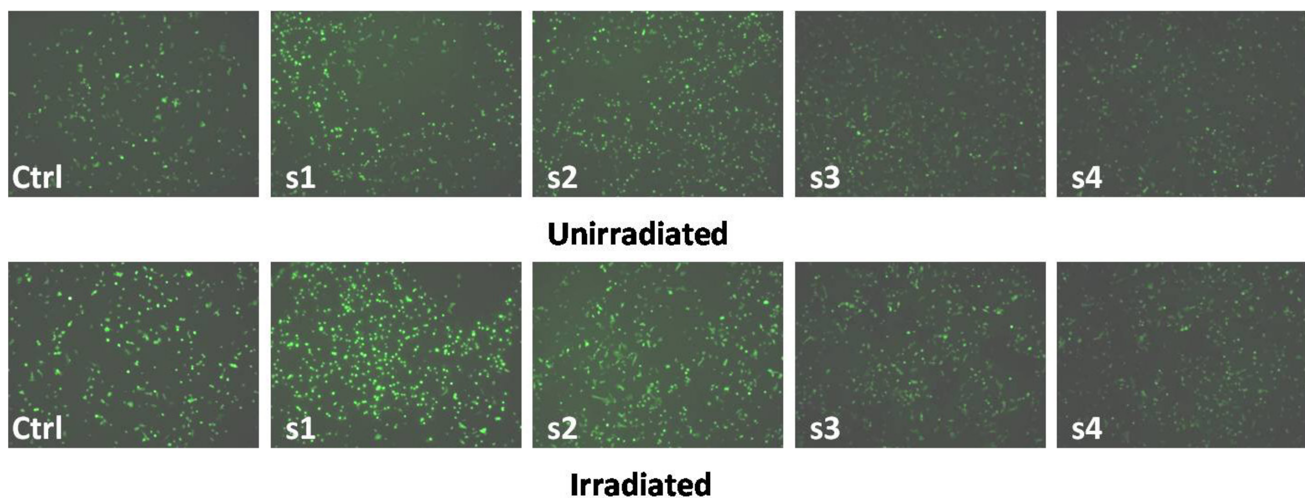

Figure 4. Fluorescence images of FDA stained osteoblasts after 30 min of irradiation. In the upper panel are images taken from unirradiated samples: control cells without substrate (Ctrl), cells cultivated on S1 substrate (s1), S2 substrate (s2), substrate S3 (s3), substrate S4 (s4). In the lower panel are images captured from irradiated samples. (magnification x100).

Table III. Statistical analysis of MTT values performed with one-way ANOVA followed by Dunnet`s Multiple Comparison Test using as as term of comparison the unirradiated cells without substrate. (* indicates that p<0.05, ** p< 0.001 to 0.01, *** p<< 0.001).

Dunnett's Multiple Comparison Test	P value summary "Significant? P < 0.05"			
	30 min	24 hours	5 days	10 days
ctrl vs s1	**	ns	ns	ns
ctrl vs s2	**	ns	ns	ns
ctrl vs s3	**	ns	ns	ns
ctrl vs s4	*	ns	*	*
ctrl vs ctrl-irrad	ns	ns	ns	ns
ctrl vs s1-irrad	ns	**	ns	ns
ctrl vs s2-irrad	***	ns	ns	***
ctrl vs s3-irrad	ns	**	ns	*
ctrl vs s4-irrad	ns	*	ns	**

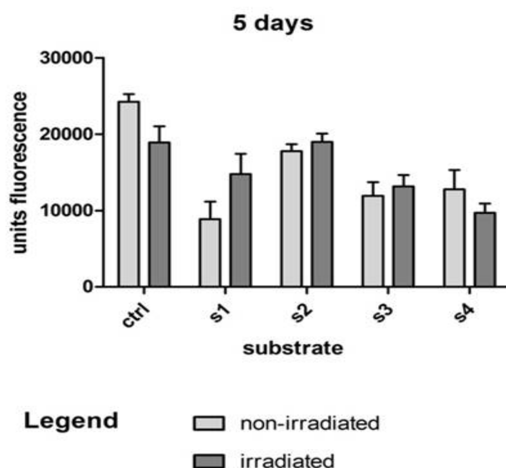

Figure 7. Graphical aspect of FDA values after 5 days of irradiation. Statistical analysis was done with two-way ANOVA followed by Bonferroni posttest with comparison between non-irradiated and irradiated samples.

Table IV. Statistical analysis of FDA values performed with one-way ANOVA followed by Dunnet`s Multiple Comparison Test using as as term of comparison the unirradiated cells without substrate (* indicates that p<0.05, ** p< 0.001 to 0.01, *** p<< 0.001).

Dunnett's Multiple Comparison Test	P value summary "Significant? P < 0.05"	
	30 min	5 days
ctrl vs s1	***	ns
ctrl vs s2	ns	*
ctrl vs s3	***	ns
ctrl vs s4	**	ns
ctrl vs ctrl-irrad	ns	ns
ctrl vs s1-irrad	*	***
ctrl vs s2-irrad	ns	***
ctrl vs s3-irrad	**	ns
ctrl vs s4-irrad	***	ns

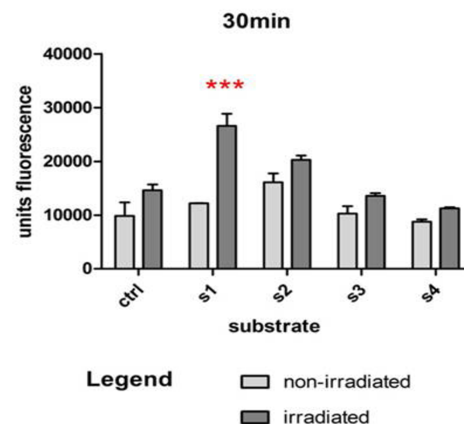

Figure 5. Graphical aspect of FDA values after 30 min of irradiation. Statistical analysis was done with two-way ANOVA followed by Bonferroni posttest with comparison between non-irradiated and irradiated samples (* indicates that p<0.05).

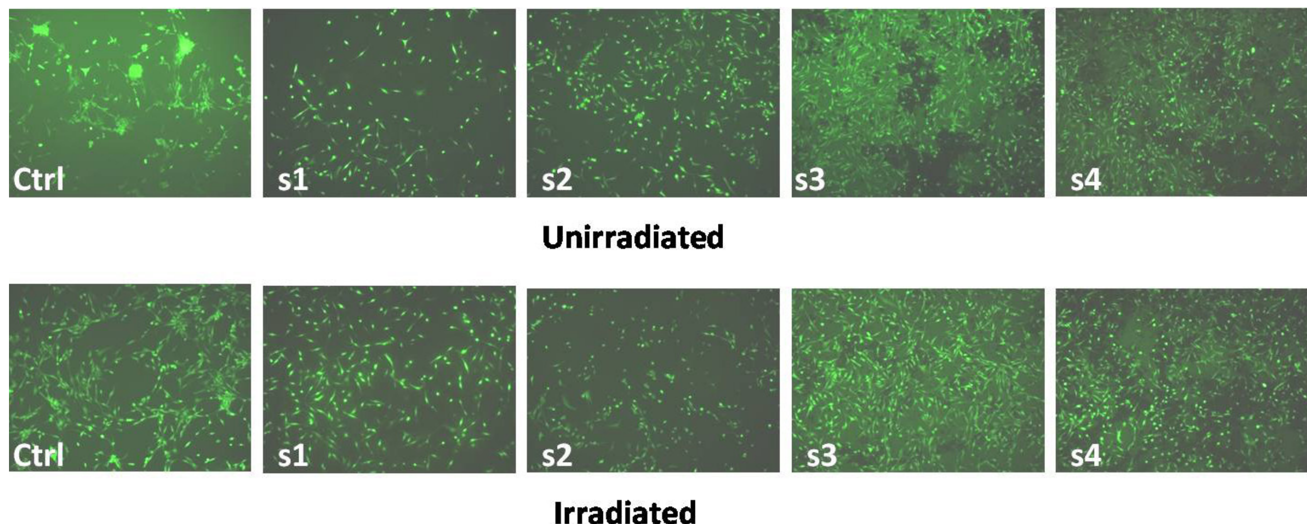

Figure 6. Fluorescence images of FDA stained osteoblasts after 5 days of irradiation. In the upper panel are images taken from unirradiated samples: control cells without substrate (Ctrl), cells cultivated on S1 substrate (s1), S2 substrate (s2), substrate S3 (s3), substrate S4 (s4). In the lower panel are images captured from irradiated samples. (magnification x100).

non-irradiated samples (Fig. 3 d).

In order to highlight the differences between all samples, we performed another statistical analysis taking as term of comparison the unirradiated cells without substrate. We used the one-way ANOVA analysis followed by Dunnet`s Multiple Comparison Test. The results are illustrated in Table III. For unirradiated osteoblasts we observed an increased number of adhered cells on all substrates after 30 min of irradiation, statistically significant when compared with uncoated plates. Comparing the unirradiated cells cultivated on plastic surfaces with irradiated cells grown on substrates, only S2 substrate showed to be more favorable for cell adhesion. At 24 hours after irradiation, the differences between control and unirradiates substrates had diminished and were maintained for irradiated cells on S1, S3 and S4 substrate. At 5 days after irradiation only the S4 unirradiated sample showed statistically significant increasing values. A more pronounced proliferation rate was observed after 10 days of irradiation for irradiated osteoblasts seeded on S2, S3 and S4 substrate as well as for unirradiated cell on S4 substrate.

FDA assay

Using FDA staining we obtained very similar results with the MTT test. In Figure 4 the fluorescence images of FDA stained osteoblasts after 30 min of irradiation are illustrated.

Culturing on substrates induced an increased adhesion and proliferation of irradiated samples, especially for S1 substrate, followed by S2 and S3 substrate. Measuring fluorescence intensity with a microplate reader we obtained graphical results showed in Figure 5. Two-way ANOVA with Bonferroni posttest showed a statistical significant difference only for S1 substrate.

Control unirradiated cells cultivated on plastic surface changed their shape and grouped in clusters as shown in images captured after 5 days of irradiation, as shown in Figure 6. S3 and S4 substrates sustained the most intense proliferation rate both for unirradiated and irradiated samples.

Fluorescence measurements are in contradiction with microscopy images and did not reflect the increased cell number observed in microscopy and did not demonstrate a statistical difference between samples (Fig. 7).

Using a different statistical analysis of FDA fluorescence measurements with one-way ANOVA followed by Dunnet`s Multiple Comparison Test, as term of comparison the unirradiated cells without substrate, we obtained significant differences between unirradiated control grown on uncoated plates and unirradiated or irradiated substrates S3 and S4 after 30 minutes of irradiation. After 5 days of irradiation significant differences were observed only for S1 and S2 irradiated samples. The results are illustrated in Table IV.

Discussion

The effect of low level laser therapy (LLLT) on bone regeneration has become a research topic increasingly addressed in the last years. LLLT is based on the principle of biostimulation tissue using monochromatic light. The precise mechanism of LLLT has not been completely explained. Some in vitro studies have shown that LLLT has stimulating effects on osteoblastic cells and accelerate bone repair process [16,17]. Another study reported delayed fracture healing or no effects after low level laser irradiation [18].

In our study LLLT irradiation of osteoblasts seeded on nanostructured composite substrates was performed in one session with an energy density of 3 J/cm^2. The laser parameters used in this study were similar to those in other studies from literature, which indicated that using an energy between 1 to 5 J/cm^2 is effective for inducing positive effects on tissues [19,20]. Khandra et al. showed that the irradiation on three consecutive days with a dose of 3 J/cm^2 enhanced production of osteocalcin and TGF-β [21]. Ozawa et al. and Saito et al. reported that the stimulatory effects of bone formation are achieved by repeated irradiation on three consecutive days, rather than one application using the same LLLT parameters [22,23].

Assessment of viability and proliferation of osteoblasts in our study was conducted by MTT assays and FDA test. At 30 minutes after irradiation with 830 nm laser, MTT assay showed a decrease of osteoblasts adhesion and proliferation seeded on the substrate S3, without significant differences between irradiated and non-irradiated cells. These results are similar to studies of Renno et al., who found that osteoblastic cell irradiation with 830 nm laser wavelength produced a slight inhibition of proliferation compared with unirradiated controls [18]. In anotherstudy, Bouvet-Gerbettaz et al. used a diode Laser (808 nm) to assess bone cell proliferation as well as osteoblastic and osteoclastic differentiation on murine bone marrow cells and found no significant change between the control (non-radiated) and LLLT groups [24].

Our results also showed a significant increase of the MTT test values in the case of irradiated osteoblasts seeded on the S1, S3 and S4 substrates at 24 hours after irradiation. These results are consistent with several studies in the literature and agree that irradiation of bone cells with LLLT causes a positive effect on bone formation. Bloise et al. used the laser diode with a wavelength of 659 nm on osteoblast cells which resulted in enhanced proliferation and cell differentiation [25]. Similar results were shown in the study carried out by Stein et al. using a helium-neon (He-Ne) laser (632 nm) on human osteoblast cell line, which also promoted cell proliferation and maturation of human osteoblasts [26]. In his study, Wu et al. reported that the low level laser irradiation significantly promoted hPDL cell proliferation at days 3 and 5 and at energy doses of 2 and 4 J/cm showed potential osteogenic capacity, as it stimulated

ALP activity, calcium deposition, and osteogenic gene expression [27]. In conclusion, Amid et al. in their study found that low level laser with low-energy density range appears to exert a biostimulatory effect on bone tissue, enhances osteoblastic proliferation and differentiation on cell lines used in in vitro studies [28].

Uniform MTT values at 5 days post irradiation are due to the initiation of the bone differentiation processes in our opinion. The general trend of increased absorption values in all samples at 10 days post irradiation would be explained by the presence of nanostructured composite substrates rather than by the achieved irradiation procedure. We believe that radiation effects are manifested mainly in the first 24 hours and from 5 to 10 days after the irradiation procedure the substrates have demonstrated the proliferative potential effect upon osteoblastic cells. The FDA test registered similar values to those obtained by the MTT assay, confirming the short-term effects of 830 nm laser radiation. Thus, at 30 minutes after irradiation an increased proliferation and adhesion in the irradiated samples, especially for S1 substrate, followed by S2 and S3 substrates was noted. At 5 days after the laser irradiation procedure, the most intense proliferation rate was supported by S3 and S4 substrates in both the irradiated and non-irradiated samples.

Our previous research has shown that the laser with a wavelength of 830 nm produced no structural changes in the irradiated substrates and there were no thermal deteriorations of the substrates identified. Therefore the wavelength of 830 nm used in this study manifests its effect mainly at the cellular level rather than at the level of the nanostructured composite substrates.

Conclusions

Irradiation of osteoblast cells cultured on nanostructured composite substrates with a 830 nm laser wavelength resulted in an increase of cell proliferation in the first 24 hours after the procedure. Nanostructured composite substrates on which osteoblastic cells were seeded showed their proliferative potential mainly at 5 days and respectively 10 days after exposure. The association between the 830 nm laser irradiation of osteoblasts and their long-term cultivation of the nanostructured composite substrates induces cell proliferation and differentiation and, therefore, it would potentially be a useful alternative for bone regeneration therapy.

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