

Effects of low-level laser irradiation on proliferation and osteoblastic differentiation of human mesenchymal stem cells seeded on a three-dimensional biomatrix: in vitro pilot study

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Abstract Mesenchymal stem cells (MSCs) from bone marrow are a recent source for tissue engineering. Several studies have shown that low-level laser irradiation has numerous biostimulating effects. The purpose of this trial was to evaluate the effects of Nd:Yag laser irradiation on proliferation and differentiation of MSCs induced into the osteoblastic lineage. MSCs were collected from adult human bone marrow, isolated, and cultured in complete medium (α -MEM). Subsequently, they were treated with osteogenic medium, seeded in three-dimensional collagen scaffolds, and incubated. We used six scaffolds, equally divided into three groups: two of these were irradiated with Nd:Yag laser at different power levels (15 Hz, 100 mJ, 1.5 W, and one with a power level of 15 Hz, 150 mJ, 2.25 W), and one was left untreated (control group). Evaluations with specific staining were performed at 7 and 14 days. After 7 days, proliferation was significantly increased in scaffolds treated with laser, compared with the control scaffold. After 14 days, however, laser irradiation did not appear to have any further effect on cell proliferation. As concerns differentiation, an

exponential increase was observed after 14 days of laser irradiation, with respect to the control group. However, this was a pilot study with very limited sample size, we conclude, that low-level laser irradiation might lead to a reduction in healing times and potentially reduces risks of failure.

Keywords Mesenchymal stem cells · Tissue engineering · Low-level laser irradiation · Biomaterials

Introduction

Periodontal disease (PD) is an inflammatory condition with multifactorial etiology that has a high incidence in the population, depending on age, external behavioral habits such as food and smoking, and the concomitant presence of other chronic systemic diseases. PD is chronic and degenerative and leads to the destruction of the periodontal apparatus, with resorption of the alveolar bone, periodontal ligament, cementum, and gingiva. Eventually, PD leads to the loss of teeth, and bone atrophy, with severe consequences for the stomatognathic apparatus [1, 2].

Treatment of severe forms of the disease or bone atrophy for implant therapy require bone augmentation that can be conducted with different procedures, depending on the quantity of bone lost and on the quality of the remaining one. Different techniques can be used depending on the anatomical site that needs to be augmented: the alveolar ridge and/or the maxillary sinus [3]. These techniques allow to obtain a vertical and/or horizontal bone augmentation, but they present several problems. For example, in the use of autogenous bone grafts, obtained from the mandibular symphysis or ramus that are the primary donor sites for harvesting bone in the oral cavity, several concerns remain, such as donor site morbidity, nerve paresthesia, devitalization of

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natural teeth, and postoperative complications (e.g., swelling, discomfort, and pain) [4].

In order to overcome this kind of problems, new bone augmentation approaches in the field of tissue engineering have been investigated. Tissue engineering is a developing branch of medicine devoted to the study of molecular, cellular, and genetic techniques, which aim is the synthesis of new biomaterials, also for bone regeneration [5]. The last frontier in tissue engineering is represented by stem cells, which have demonstrated to be a promising biomaterial in regenerative medicine [6]. Stem cells have been described as immature or undifferentiated cells, able to generate daughter cells identical to themselves or to differentiate into different phenotypes [7]. These cells have a potentially unlimited mitotic activity, and are able to produce one or more highly differentiated cell lineages. Adult bone marrow-derived mesenchymal stem cells (MSCs) may be an important source for periodontal regeneration [8, 9]. Autologous MSCs are obtained easily and safely by means of percutaneous withdrawal from the patient's bone marrow, and due to their multilineage potential, they can be stimulated to generate non-hematopoietic tissue, including bone, cartilage, tendons, and ligaments [10]. Particularly, bone marrow-derived MSCs differentiate into the osteogenic lineage, if cultured in presence of dexamethasone, ascorbic acid, and β -glycerophosphate (osteogenic medium) [8, 11]. On the other hand, bone formation from MSCs requires a three-dimensional scaffold to guide cell growth and differentiation [12, 13]. An increasing number of biomaterials have been proposed as scaffolds for tissue regeneration, aiming to recreate the environment where the complex interaction between cells and their matrix occurs [14–16]. In addition, since bone marrow contains a low number of MSCs, cell expansion techniques are usually needed to obtain a sufficient quantity of cells for clinical trials [10, 17]. It is a great challenge to improve proliferation rate and differentiation success of transplanted cells for the future development of tissue engineering.

The aim of this study was to evaluate the effects of low-level laser irradiation (LLLI) on proliferation and differentiation of human MSCs seeded on a three-dimensional biomatrix. It has already been demonstrated that LLLI has different biostimulating effects, promoting wound healing process [18, 19], osteoblasts matrix production, DNA synthesis, and bone nodule formation [20, 21]. Several mechanisms of biological action have been proposed, although none are clearly established.

These include augmentation of cellular ATP levels [22]; manipulation of inducible nitric oxide synthase activity [23]; suppression of inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and IL-8 [24, 25]; upregulation of growth factor production such as PDGF, IGF-1, NGF, and FGF-2 [25]; alteration of mitochondrial membrane potential [22]

due to chromophores found in the mitochondrial respiratory chain [26] as reviewed in [27]; stimulation of protein kinase C activation [28]; manipulation of NF- κ B activation [29]; direct bacteriotoxic effect mediated by induction of reactive oxygen species [30]; modification of extracellular matrix components [31]; inhibition of apoptosis [22]; stimulation of mast cell degranulation [32]; and upregulation of heat shock proteins [33].

Materials and methods

Withdrawal and culture of MSCs

After the approval of the local Ethics Committee, MSCs were obtained from a patient selected for the study protocol: 15 ml of bone marrow were aspirated from the posterior iliac crest and transferred to the cell factory. The extracted bone marrow was centrifuged for 10 min at 1,000 RPM, then treated with 0.84% ammonium chloride for 5 min (to induce lysis of red blood cells), and centrifuged again. Cells were resuspended in complete medium, plated in a 75-cm² cell culture flask, and placed in a humidified incubator at 37°C with 5% CO₂ in air. The complete culture medium consisted of α -modified Eagle's medium (α -MEM; Bio Whittaker, Italy) supplemented with 20% ES cell screened fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 250 μ g/ml fungizone (Bio Whittaker, Italy). Two days after bone marrow extraction, suspended cells were removed and adherent cells were additionally cultured in complete medium for 2 weeks. Subsequently, they were plated in three new flasks to amplify their number. The culture medium was changed every 3–4 days.

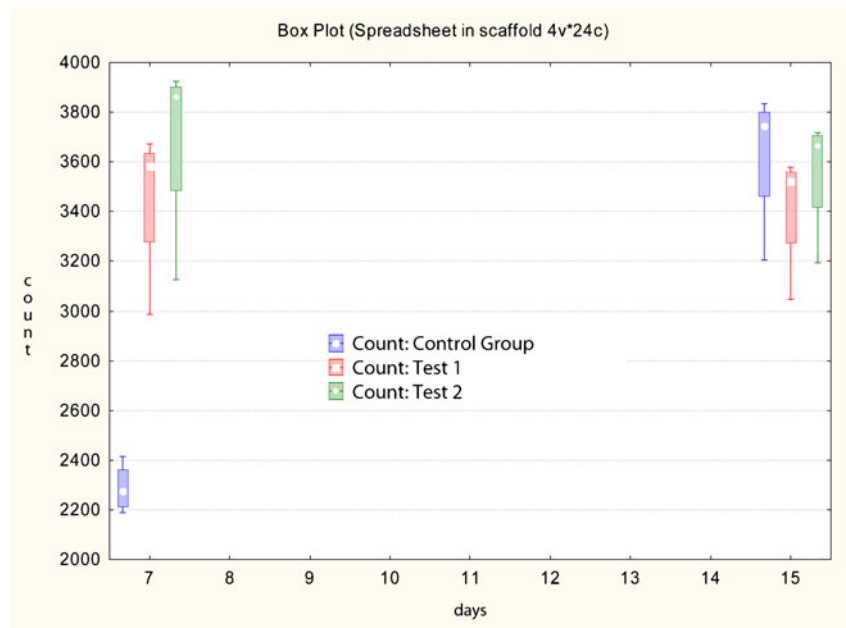
Osteogenic differentiation

At 90% of confluence, cells were detached with trypsin, counted, and plated in complete medium at a concentration of 3,500 cells/cm². After 4 days, cells were induced with osteogenic medium, consisting of complete medium supplemented with 100 nM dexamethasone, 10 mM glycerophosphate (Applichem GmbH, Darmstadt, Germany), and 0.05 mM ascorbic acid-2-phosphate (Sigma, St. Louis, MO, USA). In addition, 10% FBS (Hyclone, Logan, UT, USA) was added. The medium was changed every 3–4 days.

Cell seeding and differentiation on collagen scaffolds

Gingistat® (Vebas, Milan, Italy) was used as a scaffold in this study. Gingistat® is a sponge made of lyophilized collagen (type I). Collagen sponges were cut, under sterile conditions, into six 5×5×5 mm cubes. MSCs were suspended at a concentration of 5×10⁶ cells/ml and 10⁶ cells

Fig. 1 Statistical analysis performed using Kruskal–Wallis test, showing comparison between proliferation at 7 and 14 days



were poured onto each 125-mm³ scaffold through a 25-gauge needle. After 4 h at 37°C, the medium was replaced with fresh medium and the scaffolds were maintained in a humidified atmosphere at 37°C with 5% CO₂ in air. After 3 days, osteogenic medium was added and replaced every 3–4 days.

For each time point, a control cell sample was plated and induced with osteogenic medium, in order to adequately assess osteoblast differentiation.

Starting from day 21 until day 42, control flasks were stained with Alizarin red every week, in order to detect the

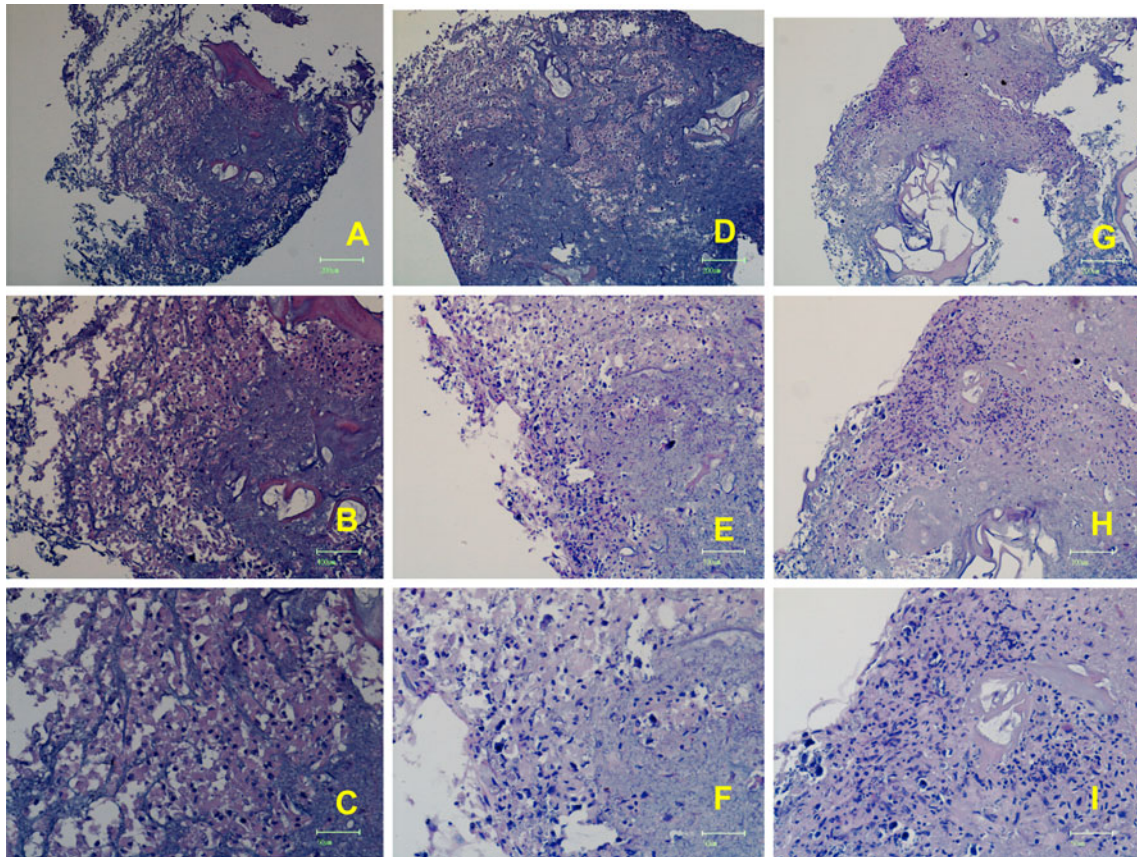


Fig. 2 Assessment of proliferation at 7 days: a–c control group; d–f group test 1; g–i group test 2

beginning of the mineralization process, which coincides with the initial positive staining with this agent. On the first day, when a reddish color appeared in the flask, a control scaffold was frozen and analyzed, for a more accurate and adequate assessment of the degree of cell differentiation. The preparation of MSC was made under good laboratory practice procedures and standard microbiologic analysis was performed to verify the product sterility.

Laser irradiation

In this study, six Gingostat® collagen scaffolds were used, each one containing 10^6 MSCs differentiated into the osteoblastic lineage. The scaffolds were divided into two samples: one was analyzed after 1 week, and the other after 2 weeks. Infrared laser irradiation was obtained using a Neodimium: yttrium-aluminum-garnet (Nd: YAG) pulsed laser (DEKA, Medical Electronics Laser Associated, Italy) emitting a 1,064-nm wavelength light, using 100 μ s pulsed time for each sample irradiated. The diameter of the fiber optic cable was 320 μ m. For each sample, one scaffold did not receive any laser treatment (control scaffold), one was irradiated with Nd:

Yag laser with a power level of 15 Hz, 100 mJ, 1.5 W (test 1), and one with a power level of 15 Hz, 150 mJ, 2.25 W (test 2); both tests 1 and 2 scaffolds received three irradiation cycles of 30 s each, separated by 30-s intervals. Distance between the laser head and scaffolds was 5 mm and laser divergency was 0.0042 rad. So if we consider 5 mm on defocalization, there is not important divergency to consider as variable. Furthermore, we used a plate with spiraling motion under the irradiated scaffolds in order to obtain a more homogeneous irradiation. For test 1, energy density (E.D.) was 0.84 J/cm^2 each cycle, with a total amount of irradiation of 2.52 J/cm^2 ; for test 2 E.D. was 1.27 J/cm^2 each cycle with a total amount of 3.81 J/cm^2 . Regarding power density, test 1 received 18.75 W/cm^2 and test 2 received 28.1 W/cm^2 . After the irradiation phase, the scaffolds were placed in complete medium and incubated in a humidified atmosphere at 37°C with 5% CO_2 in air. After 1 week, the three scaffolds of the first sample were fixed in paraformaldehyde, stained with hematoxylin and eosin (H&E) and Alizarin red, and analyzed with light microscopy. Two weeks after laser irradiation, the same procedure was performed for the second sample.

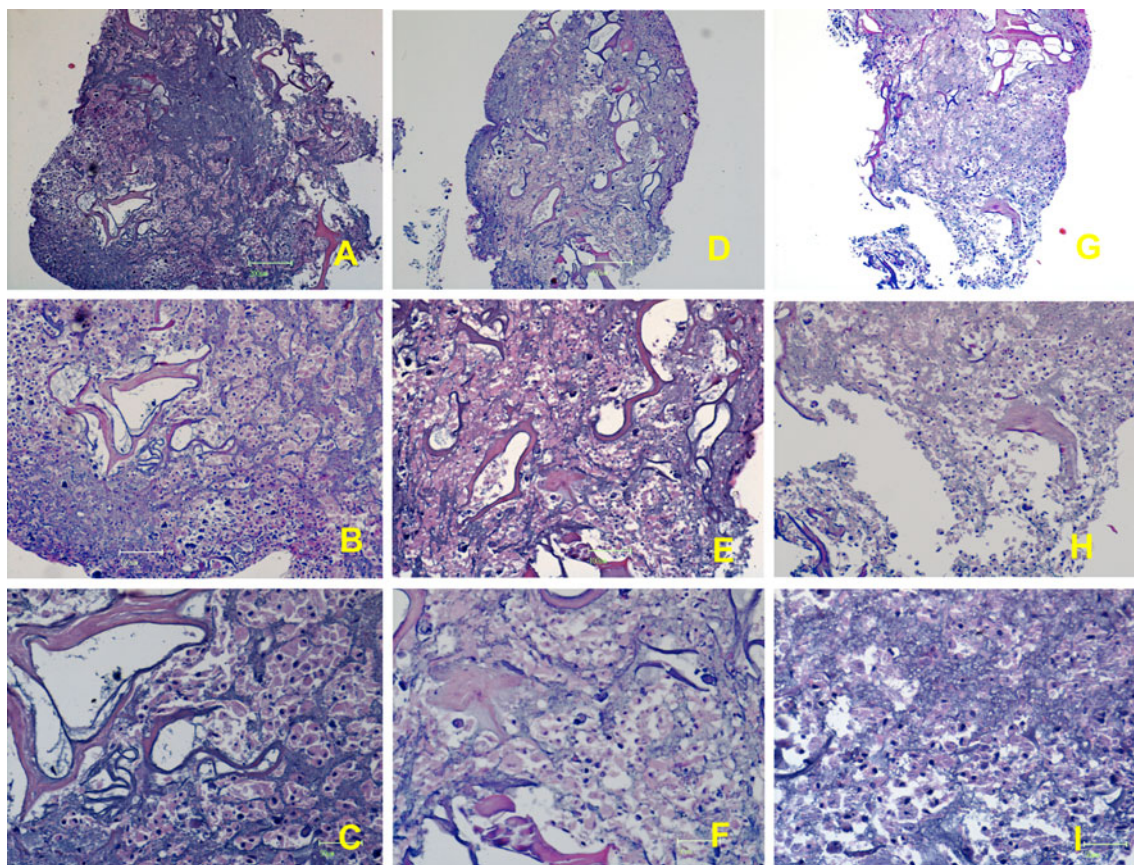


Fig. 3 Assessment of proliferation at 14 days: **a–c** control group; **d–f** group test 1; **g–i** group test 2

Paraffin inclusion and histological staining

At the fixed times, the scaffolds were washed in phosphate-buffered saline to remove residual medium, fixed in 4% paraformaldehyde for 45 min at room temperature, and included in paraffin by conventional techniques. Subsequently, scaffolds were dehydrated by immersion for 90 min in an ascending series of ethanol (50%, 70%, 90%, and 100%), 1:1 ethanol/xylene and pure xylene, incubated in 1:1 xylene/paraffin for 45 min, and immersed overnight in liquid paraffin at 70°C. On the following day, they were placed in appropriate molds and cooled in a refrigerator at 4°C.

In order to perform the analysis, scaffolds were cut into 7- μ m sections using a microtome, and placed on a gelatin-coated glass slide.

Before specific staining, paraffin was removed from the sections with xylene (5 min), and they were rehydrated by immersion for 10 min in a descending scale of ethanol (100%, 90%, 75%, and 50%) and distilled water.

Staining with hematoxylin and eosin

Sections were immersed in hematoxylin solution (Sigma) for 3 min, rinsed in running water, immersed in

eosin solution (Sigma) for 6 min, rinsed again in running water, and left to dry. Subsequently, they were mounted in entellan/xylene and examined under the microscope (Nikon Coolscope, applied magnification: 200, 100, and 50 μ m).

Staining with alizarin red solution

Sections were immersed in Alizarin red solution for 30 s, excess of dye was removed, and stained sections were fixed with acetone, 1:1 acetone/xylene, cleared with pure xylene and left to dry. Then, they were mounted in entellan/xylene and examined under the microscope.

Statistical analysis

Statistical analysis was performed using Kruskal–Wallis test. P value < 0.05 was considered statistically significant.

Results

At both time intervals, cell density and distribution in the three-dimensional scaffolds were examined with light

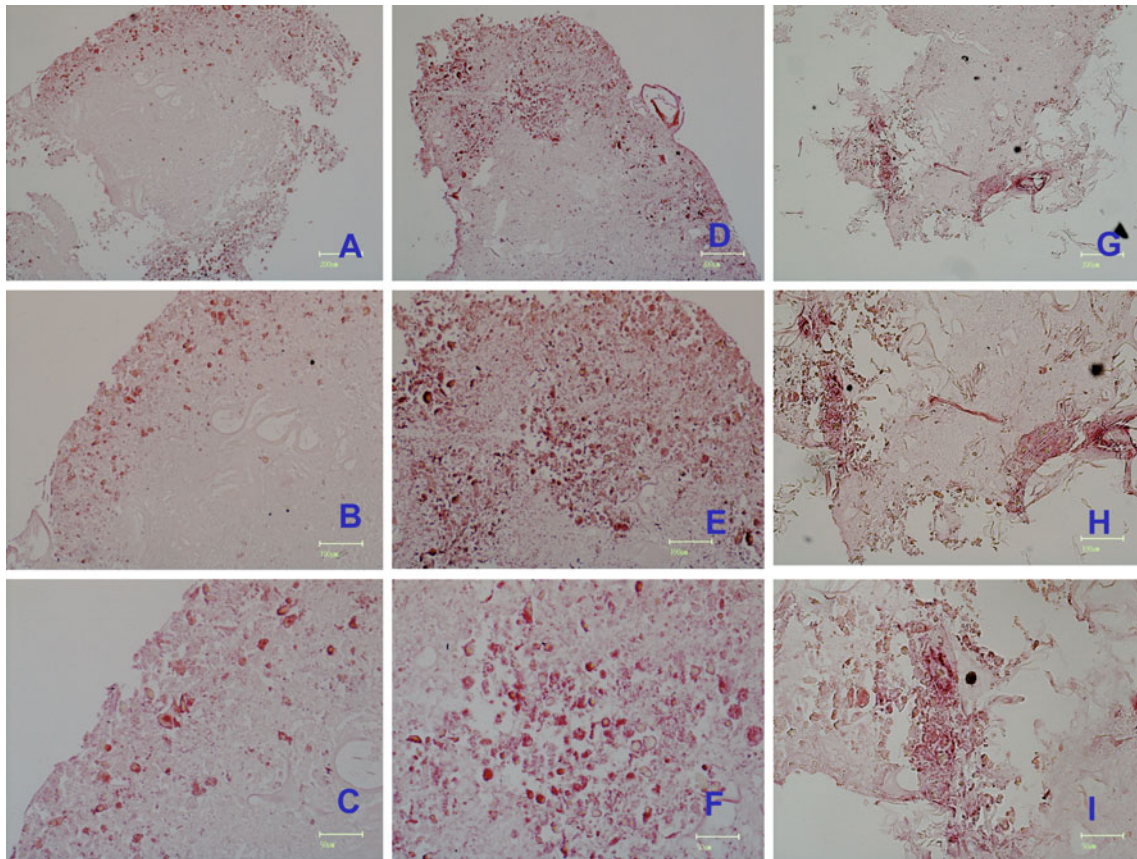


Fig. 4 Assessment of differentiation at 7 days: **a–c** control group; **d–f** group test 1; **g–i** group test 2

microscope, after H&E staining. The chart in Fig. 1 compares the number of cells, counted with H&E staining, present in the control, tests 1 and 2 scaffolds at 7 and 14 days, on the occasion of evaluations carried out. We observed that 1 week after laser irradiation, MSCs had proliferated and formed a tissue that covered the biomatrices, with a statistically significant increase of proliferation in the two irradiated scaffolds, compared to the control scaffold (Fig. 1). Moreover, three-dimensional cell distribution appeared more homogeneous in the test scaffolds. No signs of cell suffering were noticed, either in the test or control scaffolds (Fig. 2).

After 2 weeks, we did not record any increase of proliferation in test scaffolds, while we noticed a statistically significant increase of proliferation in control scaffold, which number of stem cells becomes comparable to that of test ones (Figs. 1 and 3). Irradiated cells showed the first signs of suffering: since these signs were present also in the control scaffold, they are probably not directly related to laser treatment, but associated with the physiological maturation of cell events; in fact, the process of cell proliferation decreases with simultaneous increase in the process of cell differentiation.

Since osteoblastic activities include extracellular matrix mineralization, Alizarin red staining was used to detect inclusions of calcium in the tissue and, consequently, to assess the degree of stem cell differentiation. One week after laser treatment, irradiated cells showed a small and not statistically significant increase in differentiation, compared to non-irradiated cells (Fig. 4). However, 2 weeks after laser irradiation, the situation was completely different: differentiation had increased exponentially in the irradiated biomatrices, while in the control scaffold the degree of differentiation was considerably lower (Fig. 5).

Discussion

Several studies that have investigated bone formation in vitro and the effect of LLLI on osteogenesis have focused their attention on 2D tissue cultures of bone-forming cells [34–36]. In this study, we introduced the use of a tridimensional structure containing stem cells. The commercial lyophilized collagen sponge Gingistat® is widely used as a scaffold for spontaneous bone regeneration in periodontal diseases. Its wide and long-

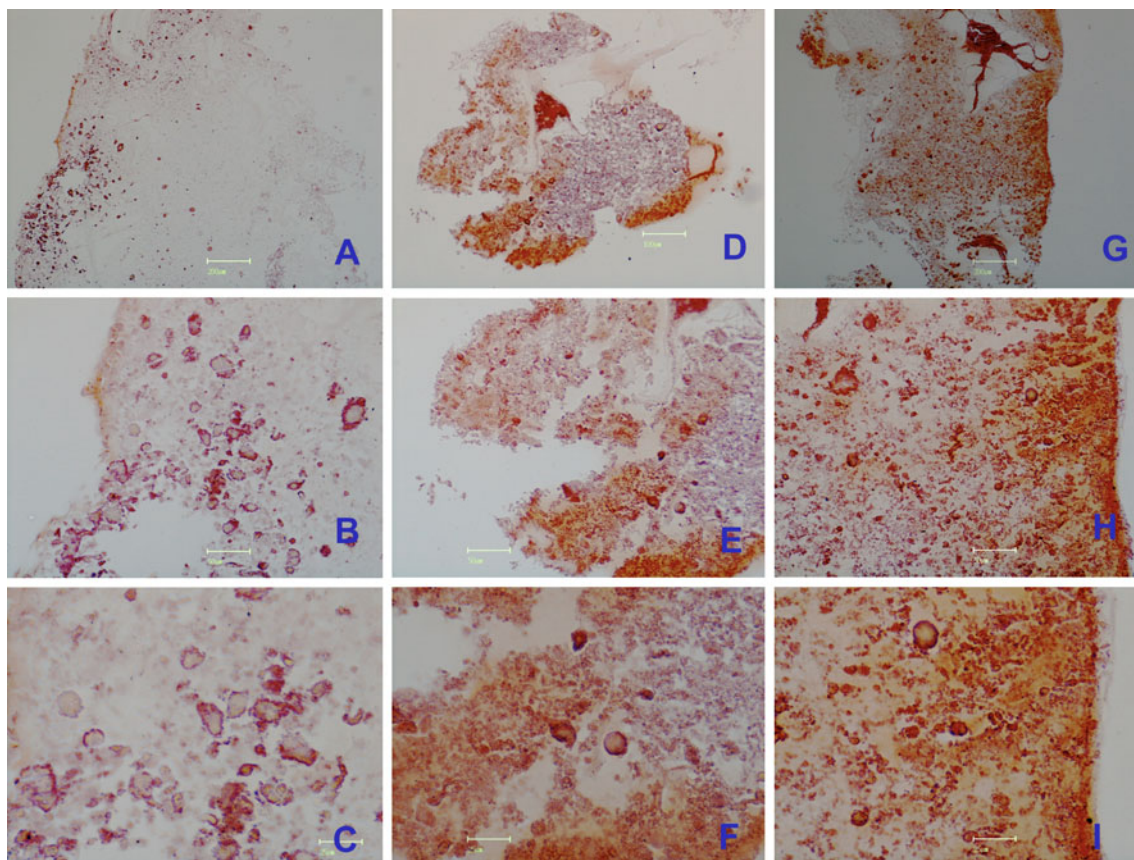


Fig. 5 Assessment of differentiation at 14 days: **a–c** control group; **d–f** group test 1; **g–i** group test 2

standing use reflects the good histo-compatibility of this kind of material. Due to the structural characteristics of the collagen sponge with large interconnected hollows, MSCs are easily adsorbed and distributed to the entire scaffold. MSCs seeded in the scaffold do not lose their capacity for differentiation.

Statistically significant cell proliferation was observed 7 days after irradiation: H&E staining demonstrated that LLLI had a biostimulating effect on bone marrow-derived MSCs. There was no statistically significant difference between different power levels of irradiation. After 14 days, the number of cells contained in both test and control scaffolds was similar, so we can say that LLLI is able to accelerate cell proliferation also in a tridimensional structure, obtaining 1 week earlier the same results of control scaffolds. The second relevant result is the exponential increase in differentiation of the irradiated biomatrices, which appears to coincide with the slowing down of the proliferative phase. In fact Alizarin red staining showed a great difference between test and control scaffolds after 14 days. Different energies of laser irradiation (100 or 150 mJ) seem to generate no differences between groups.

Wound healing process includes common steps, both if periodontal regenerative therapy is performed and if considerable amounts of bone are reconstructed to restore the correct bone volume after atrophy. These two types of surgery have different purposes: the first aims to restore periodontal health, saving natural teeth; the second aims to prepare bone for implant-supported prosthetic rehabilitation. In both cases, healing time plays an essential role in therapeutic success: shorter healing times reduce the risk of complications, such as infection, and the discomfort of the patients in the post-surgical period. Moreover, in case of prosthetic rehabilitation, the definitive prosthesis can be given earlier, improving not only the patient's oral conditions, but also his/her psychological status. Regarding periodontal and bone regeneration, although there are many different strategies, including cell injection, tissue culturing, porous and injectable scaffolds, and three-dimensional printing [37, 38], this field is most frequently linked to the paradigm of cell delivery within biocompatible scaffolds [39, 40]. In this context, a potential tissue-engineering approach to periodontal and bone regeneration involves the incorporation of progenitor cells and instructive messages in a prefabricated three-dimensional construct and subsequent implantation of the construct into the defect site [37]. Several studies have shown that autologous bone marrow mesenchymal stem cells can regenerate alveolar bone and periodontal ligament-like structures after transplantation in vivo [9, 41, 42]. The use of LLLI on these biomaterials before transplantation could lead to a faster and greater regeneration.

Considering all the limits of this pilot study that precludes trials on larger samples obtained from different subjects, we can say that laser stimulation has positive effects both on MSCs proliferation and differentiation, although at different

periods of time. Further studies in this direction are needed to confirm these data. Future protocols should provide:

1. Increase of the sample size, in order to obtain statistically significant results, comparable to the preliminary ones.
2. Standardization of laser incidence on the sample: in this study, the direction of the laser beam towards the sample could not be determined when analyzing the sample under the light microscope.
3. Evaluation of laser effects on the scaffold: we have no information about the interaction occurring between laser and collagen, and about laser propagation from the incidence point to the opposite side of the scaffold.
4. The thermal effects of laser on the collagen scaffold that could lead to the release of toxic substances in the human body.

In conclusion, the results of this study need to be confirmed by animal models and human clinical trials.

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