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Photoengineering of Bone Repair Processes

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ABSTRACT

Objective: **This paper aims to report the state of the art with respect to photoengineering of bone repair using laser therapy.** ***Background Data:*** **Laser therapy has been reported as an important tool to positively stimulate bone both *in vivo* and *in vitro.* These results indicate that photophysical and photochemical properties of some wavelengths are primarily responsible for the tissue responses. The use of correct and appropriate parameters has been shown to be effective in the promotion of a positive biomodulative effect in healing bone.** ***Methods:*** **A series of papers reporting the effects of laser therapy on bone cells and tissue are presented, and new and promising protocols developed by our group are presented.** ***Results:*** **The results of our studies and others indicate that bone irradiated mostly with infrared (IR) wavelengths shows increased osteoblastic proliferation, collagen deposition, and bone neorformation when compared to nonirradiated bone. Further, the effect of laser therapy is more effective if the treatment is carried out at early stages when high cellular proliferation occurs. Vascular responses to laser therapy were also suggested as one of the possible mechanisms responsible for the positive clinical results observed following laser therapy. It still remains uncertain if bone stimulation by laser light is a general effect or if the isolate stimulation of osteoblasts is possible.** ***Conclusion:*** **It is possible that the laser therapy effect on bone regeneration depends not only on the total dose of irradiation, but also on the irradiation time and the irradiation mode. The threshold parameter energy density and intensity are biologically independent of one another. This independence accounts for the success and the failure of laser therapy achieved at low-energy density levels.**

INTRODUCTION

BONE LOSSES are major problems in many medical and dental specialties and may occur due to several physiologic and pathologic conditions. Physiologic bone loss occurs mainly due to aging. Bone tissue has an enormous regenerating capacity, and most of the time it is able to restore its usual architecture and mechanical properties. However, there are limits for this capacity, and complete recovery may not occur if there is deficient blood supply, mechanical instability, or competition with highly proliferating tissues. The loss of bone fragments or the removal of necrotic or pathologic bone, or even some surgical procedures may create bone defects. These defects may be too large for spontaneous and physiologic repair. There are several methods that can be used to ameliorate bone repair, and these include the use of grafts and lately the use of laser therapy.

Bone healing differs from the healing of soft tissues due to the morphology and composition being slower than in soft tissues, and bone healing requires consecutive phases, which differ depending upon the type and intensity of the trauma and the extent of the damage to the bone. The trauma to the bone is immediately followed by a sequence of reparative processes in which periostal osteogenic cells begin to proliferate and to differentiate in osteoblasts.

The effects of laser therapy on bone are still controversial, as previous reports show different or conflicting results. It is possible that the effect of laser therapy on bone regeneration depends not only on the total dose of irradiation, but also on the irradiation time and the irradiation mode. Most importantly, recent studies have suggested that the threshold parameters for energy density and intensity are biologically independent of one another. This independence accounts for both the success and failure of laser therapy at low-energy density levels. The

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possibility of ameliorating bone repair is an important step towards the application of photoengineering in living tissues.

LASER LIGHT ON BONE CELLS IN CULTURE

Although the use of laser therapy on the biomodulation of bone repair has been growing steadily, and several studies have demonstrated positive results on the healing of bone tissue. Laser therapy has been successfully used for improving bone healing in several conditions such as in alveolus of dental extraction, in bone fractures, during orthodontic treatments, and in dental implant post-operations.

A previous study reported the irradiation of osteoblasts in culture and found that calcium accumulation was enhanced by laser irradiation in a 46% increase over the controls.[1] Another group studied the effect of GaAlAs on bone formation *in vitro* and used doses of 10.8–108 J/cm^2 per day. It was reported that laser therapy significantly increased the number and the total area of bone nodules in a dose-dependent manner, and that cell proliferation and alkaline phosphatase (ALP) activity were higher in the early and middle culture periods, whereas the collagen content was higher in the middle and late periods as compared to controls. Calcium and phosphorus were both higher in the irradiated groups.[2] The same group also suggested that laser therapy stimulates cellular proliferation, especially proliferation of nodule-forming cells of osteoblast lineage, and cellular differentiation, especially to committed precursors, resulting in an increase in the number of more differentiated osteoblastic cells and an increase in bone formation.[3]

A previous report, using three groups of 10 cultures irradiated three times (days 3, 5, and 7) with a pulsed diode laser at wavelength of λ690 nm for 60 sec, and another three groups of 10 cultures each used as controls, found that all lased cultures demonstrated significantly more fluorescent bone deposits than the nonlased cultures. The difference was significant in the cultures examined after 16 days. Hence, it was concluded that irradiation with a pulsed diode soft laser has a biostimulating effect on osteoblasts *in vitro,* which might be used in Osseo integration of dental implants.[4]

A study was carried out to determine the effect of pulse frequencies of laser therapy on bone nodule formation in rat calvarial cells *in vitro.* The cultures were irradiated once with a GaAlAs laser (λ830 nm, 500 mW, 0.48–3.84 J/cm^2) in four different irradiation modes: continuous irradiation, and 1-, 2-, and 8-Hz pulsed irradiation (PI). Laser irradiation in all four groups significantly stimulated cellular proliferation, bone nodule formation, ALP activity, and ALP gene expression, as compared with the nonirradiation group. Notably, PI-1 and PI-2 irradiation markedly stimulated these factors, when compared with the CI and PI-8 groups, and PI-2 irradiation was the best approach for bone nodule formation in the present experimental conditions. It was concluded that low-frequency PI significantly stimulates bone formation *in vitro*; it is most likely that the pulse frequency of laser therapy is an important factor in the biological response of bone formation.[5]

A study from Israel investigated the effect of laser irradiation on proliferation and differentiation of a human osteoblast cell line. Cultured osteoblast cells were irradiated using HeNe laser irradiation (λ632 nm; 10-mW power output). On the second and third day after seeding, the osteoblasts were exposed to laser irradiation. The effect of irradiation on osteoblast proliferation was quantified by cell count and colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 24 and 48 h after second irradiation. It was found a significant 31–58% increase in cell survival (MTT assay) and higher cell count in the once-irradiated as compared to the nonirradiated cells was monitored. Differentiation and maturation of the cells was followed by osteogenic markers: ALP, osteopontin (OP), and bone sialoprotein (BSP). A twofold enhancement of ALP activity and expression of OP and BSP was much higher in the irradiated cells as compared to nonirradiated osteoblasts. It was concluded that laser therapy promoted proliferation and maturation of human osteoblasts *in vitro.*[6]

A team from Norway investigated the effects of laser therapy (λ830 nm, 84 mW, Ø ~ 35 mm, 1.5 or 3 J/cm^2) on osteoblast-like cells in culture on implant material. This study showed that laser therapy significantly enhanced cellular attachment. Also, cell proliferation was observed after 96 h, and levels of transforming growth factor–β_1 (TGFβ_1) production and osteocalcin synthesis were significantly greater on cultures irradiated with 3 J/cm^2. ALP levels were not significantly different between irradiated and nonirradiated groups.[7]

LASER LIGHT ON BONE TISSUE

An Italian group evaluated whether laser therapy stimulation could accelerate bone healing. Bone defects of a standard area were created in the distal epiphysis of 12 femora explanted from six rats, and they were cultured in BGJb medium for 21 days. Six defects were treated daily with GaAlAs (λ780 nm) for 10 consecutive days, whereas the remainder was sham-treated. Alkaline phosphatase/total protein (ALP/TP), calcium (Ca), and nitric oxide (NO) were tested on days 7, 14, and 21 to monitor the metabolism of cultured bone. The percentage of healing of the defect area was determined by histomorphometric analysis. After 21 days, significant increases were observed in ALP/TP in laser versus control, in NO in the laser versus control, and in Ca in control versus laser. The healing rate of the defect area in the laser group was higher than in the control group.[8]

A Spanish study analyzed the effects of a HeNe laser on bone fractures in an animal model, by using 2.4 J in one point, and found that the irradiated subjects exhibited better healing characteristics than the nonirradiated ones. The laser-treated group also showed increased vascularization and faster formation of bone tissue.[9]

The effect of laser therapy (HeNe) on bone repair in the tibia of the rat after a hole-type injury was investigated using biochemical and quantitative histomorphometrical methods. The histological evaluation revealed filling of the intramedullary canal with woven bone at the site of injury at 6 days after surgery, and progressive filling of the hole-injury gap in the cortical bone by membranous ossification. Direct irradiation of the hole injury with HeNe laser at 5 and 6 days after injury altered the osteoblast and osteoclast cell populations, as reflected by the significant 2.2-fold increase in ALP enzymatic activity over control, nonirradiated rats at 10 days post-injury, and a significant decrease of 40% in tatrate-resistant acid phosphatase (TRAP) activity at 11 days. Histomorphometrical

analysis revealed a more rapid accumulation of reparative new bone in the hole-type injury of the laser-irradiated rats.[10]

A previous study created bone defects in rats and found that, in irradiated animals, the calcium accumulation was increased compared to control and that the osteoblastic activity also increased.[11] Similar results were described by another study.[12] A Japanese study[13] expanded the midpalatal suture in a rat model and found that regeneration was accelerated and that neither irradiation in the late period nor the use of a single irradiation was effective.

A report from Japan investigated the effects of laser therapy (λ830 nm) on bone remodeling during orthodontic movement in rat model and found that laser therapy positively stimulated the movement of the tooth and that remodeling of the bone was accelerated, as evidenced by an increased number of osteoclasts. Proliferation of cells of the periodontal ligament and bone neoformation were more prominent on irradiated subjects when compared to controls.[14]

A Brazilian group studied activity in bone cells after irradiation with λ660-nm laser light using 10 J/cm^2 and found that activity was higher in the irradiated group than in the control group in regards to bone volume, osteoblast surface, mineral apposition, osteoclast, and eroded surface, and concluded that laser therapy increased the activity of bone cells (osteoblasts and osteoclasts) around the site of the repair without changing the bone structure.[15]

An *in vivo* model was used to evaluate whether λ780-nm laser light stimulation could improve biomaterial Osseo integration. After drilling holes, cylindrical implants of hydroxyapatite (HA) were placed into both distal femurs of rabbits. From postoperative day 1 and for 5 consecutive days, the left femurs of all rabbits were submitted to laser therapy treatment (300 J/cm^2, 1 W, 300 Hz, pulsating emission, 10 min). The right femurs were sham-treated (control group). At 3 and 6 weeks after implantation, histomorphometric and microhardness measurements were taken. A higher affinity index was observed at the HA–bone interface in the laser-tread group at 3 and 6 weeks; a significant difference in bone microhardness was seen in the laser group versus the control group.[16]

The influence of HeNe laser radiation on the formation of new blood vessels in the bone marrow compartment of a regenerating area of the mid-cortical diaphysis of the tibiae of young adult rats was studied by an Italian group. A small hole was surgically made with a dentistry burr in the tibia, and the injured area received daily laser therapy over 7 or 14 days transcutaneously starting 24 h from surgery. Incident energy density dosages of 31.5 and 94.5 Jcm^{-2} were applied during the period of the tibia wound healing. It was found that laser therapy accelerated the deposition of bone matrix and histological characteristics compatible with an active recovery of the injured tissue. HeNe laser therapy significantly increased the number of blood vessels after 7 days of irradiation at an energy density of 94.5 Jcm^{-2}, but significantly decreased the number of vessels in the 14-day irradiated tibiae, independent of the dosage.[17]

A study from an Israeli group investigated the therapeutic efficiency of laser irradiation and organic bovine bone graft, separately and together, on the post-traumatic regeneration of bone tissue in rats using infrared spectroscopy. When laser therapy was used (λ632.8 nm, 35 mW), the intensity of absorption of the inorganic component increased by 62%, compared to the control injured area, and decreased only 11.4% in the normal bone. The wavelength characteristics of the organic component remained unchanged; that is, the organic component was similar to that of normal bone. The Mineralization Index in the laser-treated group increased significantly to 1.86, compared to 0.63 in the control group and 2.04 in the normal bone.[18]

A Norwegian study used round osseous defects on the calvarias of rats irradiated with GaAlAs laser (λ830 nm, 75 mW, Ø ~ 18 mm, 23 J/cm^2). This study showed significantly increased levels of calcium, phosphorus, and proteins when compared to untreated controls. Pronounced angiogenesis and connective tissue formation, and more advanced bone formation were also seen on irradiated subjects when compared to their controls.[19]

SUCCESSFUL PROTOCOLS FOR PHOTOENGINEERING OF BONE REPAIR PROCESSES

A study carried out by our team evaluated morphometrically the amount of newly formed bone after the use of infrared (IR) laser light on surgical wounds created in the femur of rats. In this study, doses of 4.8 J/cm^2 per session, and 57.6 or 14.4 J/cm^2 treatment doses were used. A significant difference was found in the areas of mineralized bone between irradiated and nonirradiated subjects during early stages of healing, but not after 28 days.[20]

In another study, better bone healing around dental implants was observed in animals irradiated (λ830 nm, 40 mW, continuous wave [CW]) with a total dose per session of 4.8 J/cm^2 at 45 and 60 days after the placement of the implant compared with the control group. After 45 days, it was not possible to detect differences between irradiated and nonirradiated bone using scanning electron microscopy (SEM). At early stages, differences in bone organization and vascularization were detectable.[21] Later, we studied, through near-infrared Raman spectroscopy (NIRS), the incorporation of calcium hydroxyapatite (CHA) on the healing bone around dental implants submitted or not to low-level laser therapy (LLLT) using a rabbit model. Titanium implants were placed on the tibia and were irradiated with λ830-nm laser (seven sessions at 48-h intervals, 21.5 J/cm^2 per session, 10 mW, Ø ~ 0.0028 cm^2, 85 J/cm^2 treatment dose) The results showed significant differences in the concentration of CHA on irradiated and control specimens at both 30 and 45 days after surgery. It was concluded that LLLT did improve bone healing.[22]

We also assessed histologically the effect of laser therapy (λ830 nm) on the repair of standardized bone defects grafted with inorganic bovine bone on a rat model. The animals were irradiated every 48 h during 15 days, and the first irradiation was performed immediately after the procedure. The animals were irradiated transcutaneuosly at four points around the defect. At each point, a dose of 4 J/cm^2 was given (Ø ~ 0.6 mm, 40 mW), and the total dose per session was 16 J/cm^2. The results showed evidence of a more advanced repair in the irradiated group when compared to the nonirradiated groups. The repair of the irradiated group was characterized by both increased bone for-

mation and amount of collagen fibers around the graft within the cavity from the 15th day after surgery. Also considered was the Osseo conductive capacity of the bone graft.[23]

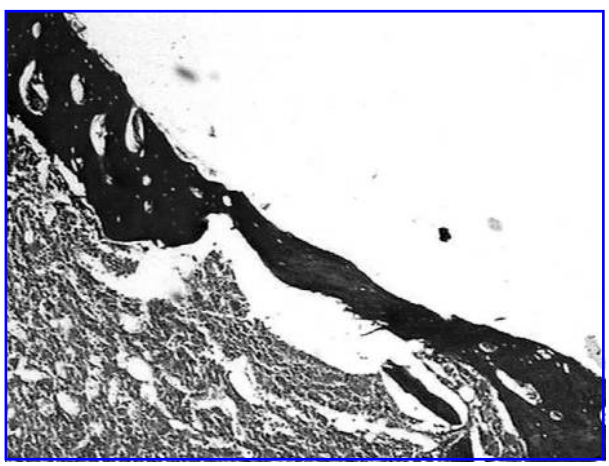
A study previously reported by our group examined the effect of laser therapy (λ830 nm, 40 mW, CW, Ø ~ 0.6 mm, 16 J/cm^2 per session) and inorganic bovine bone graft associated or not to decalcified bovine cortical bone membrane. The animals of the irradiated groups were irradiated every 48 h during 15 days; the first irradiation was performed immediately after the surgical procedure. The animals were irradiated transcutaneously in four points around the defect. At each point, a dose of 4 J/cm^2 was given (Ø ~ 0.6 mm, 40 mW), and the total dose per session was 16 J/cm^2. The results showed evidence of a more advanced repair on the irradiated groups when compared to nonirradiated ones. The repair of irradiated groups was characterized by both increased bone formation and amount of collagen fibers around the graft within the cavity from the 15th day after surgery, which was demonstrated through analysis of the Osseo conductive capacity of the bone graft and increments of cortical repair in specimens with membrane.[24]

We also examined the influence of laser therapy (λ830 nm) on the repair of bone defects submitted or not to synthetic microgranular hydroxyapatite implant (HA) and/or bovine bone membrane. The irradiated groups received seven irradiations every 48 h, with the first immediately after the surgical procedure. The dosimetry was 16 J/cm^2 per session, divided in four points of 4 J/cm^2 around the defect (Ø ~ 0.6 m, 40 mW). The results showed that all the experimental groups presented an improvement in repair of bone defects in all the observation periods when compared with the control group, mainly in the groups with membrane and/or groups that were irradiated. The repair of the defects submitted to laser therapy was more rapid in the periods of 15 and 21 days. By the 30th day, the level of repair of defects was similar in both the irradiated and nonirradiated groups. New bone formation was evidenced inside of the cavity by the Osseo conduction of the implant, and in the irradiated groups there was an increment of this new bone formation (Fig. 1a–e).[25]

In another study, we assessed histologically the effect of laser therapy on the repair of surgical defects treated or not with bone morphometric protein (BMPs), organic bovine bone graft, and guided bone regeneration (GBR) in a rat model. The irradiated groups received seven irradiations every 48 h, with the first immediately after the surgical procedure. Laser therapy (λ830 nm, 40 mW, CW, Ø ~ 0.6 mm) consisted of 16 J/cm^2 per session divided in four points (4 J/cm^2) around the defect. The results showed histological evidence of increased deposition of collagen fibers (15 and 21 days) as well as an increased amount of well-organized bone trabeculi at the end of the experimental period (30 days) in irradiated animals compared to nonirradiated controls. It was concluded that the association of laser therapy with BMPs, organic bovine bone grafts, and GBR increases the positive biomodulative effects of laser light (Fig. 2a–c).[26]

In another study, our group assessed histologically the effect of laser therapy (λ830 nm, 40 mW, CW, Ø ~ 0.6 mm, 16 J/cm^2 per session) on the repair of surgical defects on a rat model. The defects were filled with lyophilized bovine bone (organic matrix) associated or not with GBR. The animals in the irradiated group received 16 J/cm^2 per session, with the first irradiation immediately after surgery, and being repeated seven times every 48 h. The results of the study showed histological evidence of an improved amount of collagen fibers at early stages of the bone healing (15 days) and an increased amount of well organized bone trabeculae at the end of the experimental period (30 days) on irradiated animals compared to nonirradiated ones.[27]

A pioneer work by our team assessed histologically the effect of laser therapy on the healing of bone defects associated with autologous bone graft. In this study, laser therapy was applied to the surgical bed; to the graft; and to both the graft and the surgical bed. The dose per session was 10 J/cm^2. Laser therapy was carried out every other day for 15 days (λ830 nm, Ø ~ 0.5 cm^2, 50 mW, and 10 J/cm^2). In the groups in which



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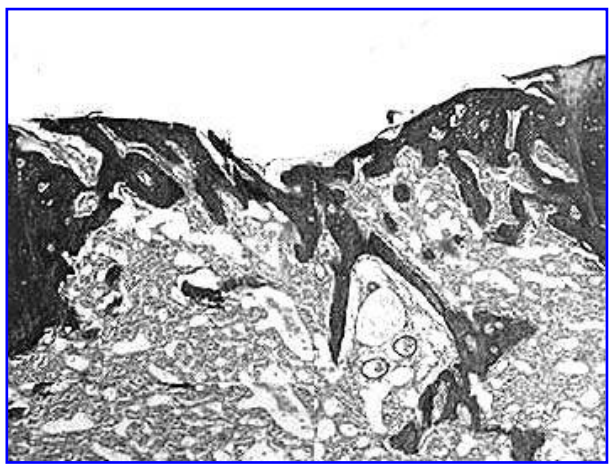

b

FIG. 1. (**a**) Photomicrography of control specimen 30 days after surgery showing advanced cortical repair. The cortical plate is restored, but it is thinner than the untreated area. The cavity shows only medullar tissue. Picrosirius; original magnification, ×40. (**b**) Photomicrography of specimen submitted to laser therapy 30 days after surgery showing complete cortical repair. The cortical plate is similar to untreated areas. The cavity shows medullar tissue and delicate trabecular bone. Picrosirus; original magnification, ×40. *Continued.*

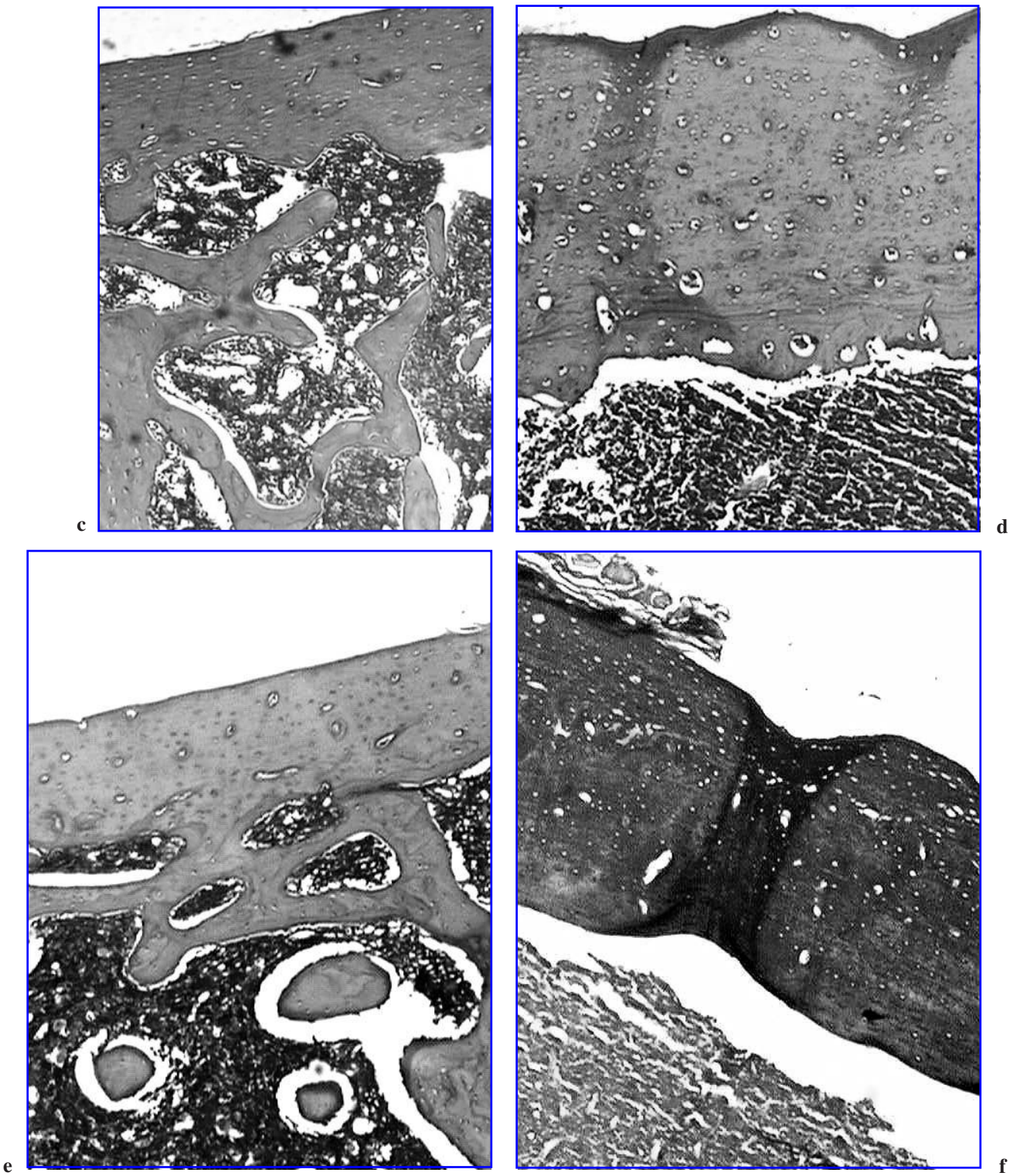

FIG. 1. *Continued.* (**c**) Photomicrography of grafted with hydroxyapatite (HA) 30 days after surgery showing partial repair of the cortical plate, which is thinner than untreated areas. Presence of remnants of particles of the graft within the defect were encircled by newly formed bone. Hematoxylin and eosin (HE); original magnification, × (**d**) Photomicrography of specimen grafted with HA and submitted to laser therapy 30 days after surgery showing complete cortical repair. The cortical plate is similar to untreated areas. HE; original magnification, ×40. (**e**) Photomicrography of specimen submitted to guided bone regeneration (GBR) 30 days after surgery showing complete repair of the defect with cortical plate similar to untreated area. HE; original magnification, ×40. (**f**) Photomicrography of specimen submitted to GBR and laser therapy 30 days after surgery showing complete repair of the cortical plate and typical medular tissue within the cavity. Picrosirius; original magnification, ×40.

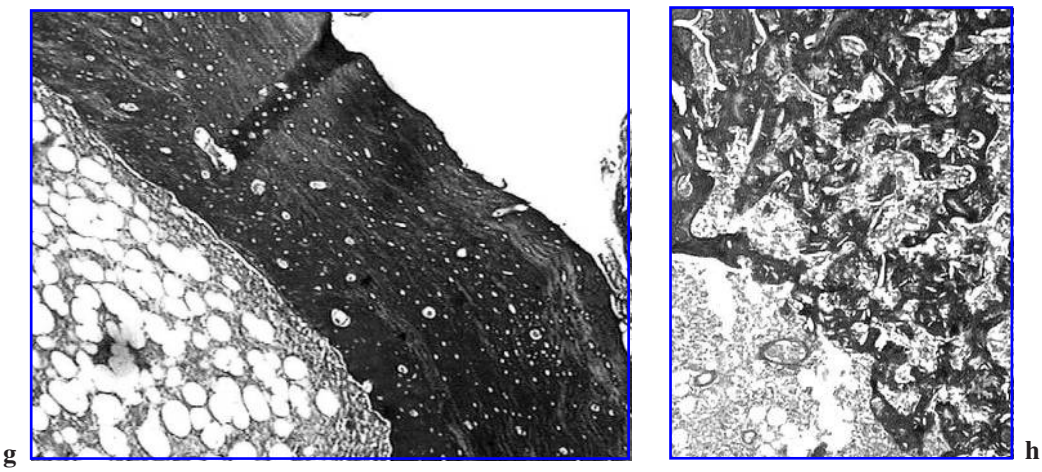

FIG 1. *Continued.* (**g**) Photomicrography of specimen grafted with HA and submitted to GBR 30 days after surgery showing complete cortical repair similar to untreated areas. Picrosirius; original magnification, ×40. (**h**) Photomicrocraphy of specimen grafted with HA, submitted to GBR and laser therapy 30 days after surgery showing complete cortical repair and the presence of delicate bone trabeculae within the cavity. Picrosirius; original magnification, ×40.

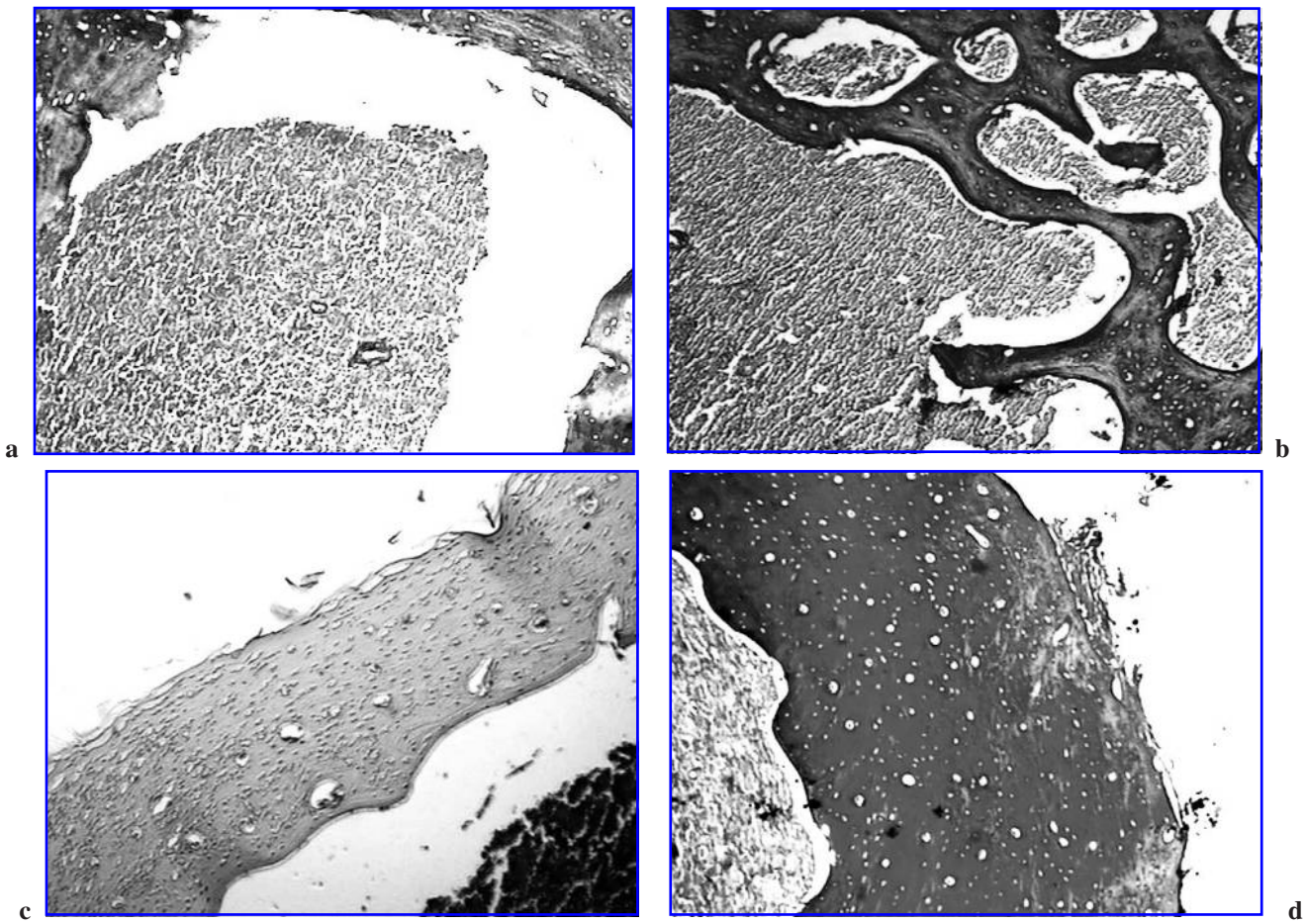

FIG. 2. (**a**) Photomicrography of control specimen 30 days after surgery showing the cavity filled by spongy bone. Picrosirius; original magnification, ×40. (**b**) Photomicrography of bone defect submitted to laser therapy 30 days after surgery showing newly formed trabecular bone originated from the cortical plate. Picrosirius; original magnification, ×100. (**c**) Photomicrography of specimen submitted to guided bone regeneration (GBR) 30 days after surgery showing complete cortical repair. The newly formed bone is thinner than untreated areas. HE; original magnification, ×40. (**d**) Photomicrography of specimen submitted to GBR and laser therapy 30 days after surgery showing complete cortical repair similar to untreated areas. Picrosirius; original magnification, ×40. *Continued.*

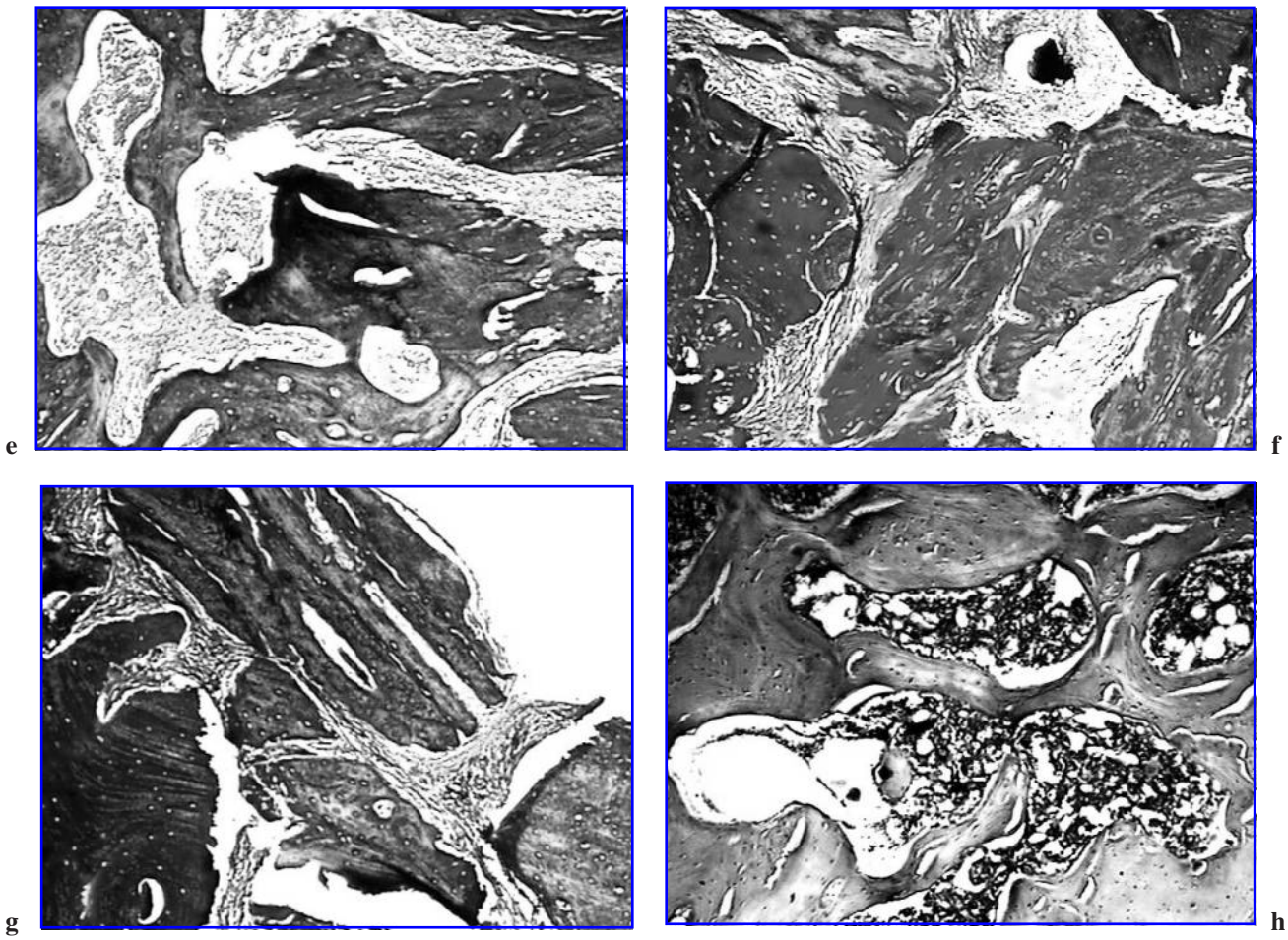

FIG. 2 *Continued.* (**e**) Photomicrography of specimen grafted with organic bovine bone and bone morphometric protein (BMPs) 30 days after surgery showing newly formed trabecular bone and remnants of the graft particles within the bone cavity. Picrosirius; original magnification, ×40. (**f**) Photomicrography of specimen grafted with organic bovine bone and BMPs, and submitted to laser therapy 30 days after surgery showing large amounts of collagen fibers and deposition of osteoid tissue, the presence of immature bone, and remnants of the graft. Picrosirius; original magnification, ×100. (**g**) Photomicrography of specimen grafted with organic bovine bone + BMPs, and submitted to GBR 30 days after surgery showing partial cortical repair and remnants of the graft within the cavity. Picrosirius; original magnification, ×40. (**h**) Photomicrography of specimen grafted with organic bovine bone + BMPs, and submitted to GBR and laser therapy 30 days after surgery showing the defect completely filled by mature trabecular bone and absence of particles of the graft. Picrosirirus; original magnification, ×40.

laser therapy was used transoperatively on the surgical bed, bone remodeling was both quantitatively and qualitatively more evident when compared to subjects of the other groups, indicating that the use of laser therapy transoperatively resulted in a positive biomodulative effect on the healing of bone defects associated with autologous bone grafts.[28]

CONCLUSION

Although bone tissue shows good regeneration that restores its structural and mechanical properties, this capacity for repair may be impaired by poor blood supply, mechanical instability, and the presence of other tissues with higher proliferative activity. Large bone losses result in large defects, which are too big for routine bone repair. As a means of improving the recovery of large bone defects, the use of several techniques, including photobioengennering, have been extensively studied.

Laser radiation possesses a wavelength-dependent capacity to alter cellular behavior in the absence of significant heating. The dispersion of laser light in the tissues is very complex, as tissue components influence the dispersion of the light. The results of our studies and others indicate that bone irradiated mostly with IR wavelengths shows increased osteoblastic proliferation, collagen deposition, and bone neorformation when compared to nonirradiated bone.

Mitochondrial changes have also been suggested as being responsible for the positive results of laser therapy. The photobiological response may be due to the absorption of a specific wavelength by some unknown molecular photoreceptor that participates in metabolic reactions on the cell, which cannot necessarily be directly linked to responses to the laser light it-

self. The absorption of a specific wavelength and the resulting excitation of primary molecular processes, which occurs on molecular receptors, may lead to the photobiological response.

The effects observed in irradiated subjects might be a result of positive effects of laser irradiation on the cell membrane and mitochondria. Positive effects on the synthesis of DNA and RNA, and on collagen synthesis and its precursors were also reported, as were positive effects on the levels of prostaglandin, phagocyte cytoplasmatic granules, neovascularization, and cell proliferation. Laser therapy influences the production of ATP.

The results of our studies indicate that the effect of laser therapy is more effective if the treatment is carried out at early stages when high cellular proliferation occurs. The mechanism that leads to a positive effect of laser light on different tissues remains not fully understood, as there are possibilities to be considered such as stimulation by the laser light of porphyrins and cytochromes to increase cellular activity, increasing the concentration of ATP and AlP and the release of Ca. Our experience also indicates that the magnitude of the biomodulative effect depends on the physiologic status of the cell at the irradiation time[29] or stimulant effect of the laser light during the initial phase of proliferation and initial differentiation of undifferentiated cells. However, this does not occur during more advanced stages.[28]

It is known that the stimulant effect of laser light on bone occurs during the initial phase of proliferation of both fibroblasts and osteoblasts as well as on initial differentiation of mesenchymal cells. Fibroblastic proliferation and its increased activity have been detected previously on irradiated subjects and cells cultures and these are responsible for great concentration of collagen fibers seen within irradiated bone.

Bone repair starts immediately after injury and damage to the local vasculature results on anoxia of the tissue. Blood vessels not fully impaired became enlarged due to vasodilatation and hemorrhage will flood the site. The coagulum will limit the site in which inflammation will start the repair. The fibrin on the coagulum will act as a framework for cell migration that will participate on the healing. It seems that the first cell type that actively participates in this process is the platelet. These cells will degranulate and, later, these granules will release growth factors such as PDGF, $TGF\beta_1$, $TGF\beta_2$, and others.

Angiogenesis may play a major role in bone repair. The production of growth factors and other angiogenic mediators influence the differentiation of osteoblasts. Local hypoxia leads to the regulation of the production of the angiogenic factors and on their receptors trying to restore the local blood supply at the wounded site. Blood vessels are important for both formation and maintenance of the bone tissue. Cortical bone has on the Harvesian systems its major source of nutrition. The Volkmann canals are responsible for the circulation of nutrients and cytokines and its signaling reach both the osteoblasts and osteocytes. Bone tissue is a major source of angiogenic and endothelial factors, and bone morphogenetic proteins, which are essential for both osteogenesis and angiogenesis.

It seems that laser effects were due to increased levels of growth factors such as fibroblast growth factor also found on healing bone tissue that acts on differentiated cells increasing the rate of proliferation and stimulating maturation and secretion of bone matrix. It is also accepted that acceleration of the repair may be a result of laser therapy on the synthesis of bone matrix due to increased vascularization and early onset of the inflammatory response.

Vascular responses to laser therapy were also suggested as one of the possible mechanism responsible for the positive clinical results observed following laser therapy. Therefore vascularization is an important and decisive factor for the healing of wounds and for the relief of the pain. The improvement of the vascularization following laser therapy is one of the possible mechanisms for the clinical effectiveness of this treatment that has been used on the control of the pain or to improve wound healing.

Our studies reflect the idea that nondifferentiated mesenchymal cells could be biomodulated positively to osteoblasts, which would more rapidly change to osteocytes. On the other hand, laser therapy seems ineffective when used on normal tissues. Biomodulating effects of laser therapy observed by other researchers demand some level of tissue deficiency. It is known that the osteogenic potential of mesencyhmal cells depends on several genetic factors and also on systemic and local inducer factors. Laser therapy may act as such an inducer factor. Laser therapy improves bone matrix production due to improved vascularization and antiinflammatory effects. These aspects would result in an increase of both the release of mediators and microvascularization, which would subsequently accelerate bone healing. It has been observed that PGE_2 activates wound healing, and increased levels of PGE_2 were observed by others.[30] There is evidence that PGE_2 is also produced by osteoblasts and that its effects may be therapeutic or adverse.[31]

The precursors of the bone cells are stem cells, and these possess enormous mitotic potential. These cells are numerous on all bone surfaces, possess receptors for growth factors, and originate osteoblasts. These cells accumulate on the bone surface and synthesize, transport and release proteins of the matrix initiating mineralization. Osteocytes are cells resulting from the imprisonment of the osteoblast by mineralized matrix. These cells are intimately linked to the bone matrix and interfere on the metabolism of both Ca^{2+} and P^{2+}. They are capable of communicating between themselves in order to maintain usual bone functions. The osteoclasts are multinucleated cells derived from the precursors of monocytic granulocytes of the bone marrow. They present lissomes and use the hydrogen pump to make the local environment acid and make the mineral structure soluble. They release photolytic enzymes which denaturates the proteins of the matrix. In summary, they are bone destroying cells. On the other hand, the osteoclasts are responsible for the early release of growth factors, conditioning the remodeling of the bone.

Improved bone maturation on irradiated subjects is due to increased deposition of calcium hydroxyapatite (CHA) as, during early stages of healing, the osteoblastic activity is chiefly proliferative and deposition starts later, which results in the formation of immature bone, still poor in CHA. This later maturation represents the improved ability of more mature osteoblasts to secrete CHA in irradiated subjects. Deposition of CHA represents bone maturation, and increased amount of CHA in bone is indicative of a more resistant bone. The observed differences in the rate of deposition of CHA between irradiated and control subjects is probably due to the choice of a wavelength with higher penetration and the ability to increase changes at cellular

levels, such as improved ATP synthesis, early osteoblastic differentiation,[32,33] and the release of growth factors.

It is known that HA crystals are found on collagen fibers, within them, and in the matrix around. To initiate mineralization, high local concentrations of Ca^{2+} and PO^{43-} ions must be reached in order to induce their precipitation into amorphous calcium phosphate, leading to HA crystal formation. This is achieved by membrane-bound matrix vesicles, which originate by budding from the cytoplasmic process of the chondrocyte or the osteoblast, and it is deposited within the matrix during its formation. It is known that laser therapy has the ability to stimulate cell proliferation, including of fibroblasts; these cells have the capacity to secrete collagen. In the matrix, HA crystals have been observed, and they will grow in clusters, which later coalesce to completely calcify the matrix, filling the spaces between and within the collagen fibers. It is known that, during the many stages of bone healing, several cytokines and growth factors regulate matrix production. Various factors such as bone morphogenetic proteins, TGFβ, and platelet-derived growth factor have been successfully used to augment healing in experimental models. Laser light also has positive effects on the release of several such mediators. Increased amounts of CHA may be positively correlated to bone mineral density, as higher intakes of calcium result in an increase in bone mineral density. However, is not known if higher amounts of CHA would interfere with attempts to strengthen bone, possibly by impairing magnesium absorption. This requires further clarification. The reason why the effect of laser therapy is not much detectable until 30 days after treatment is due to the fact that, during early stages of bone healing, the cellular component is more prominent and more prone to be affected by laser therapy. Later, bone matrix is the main component of the healing tissue. This is why the frequency of application of laser therapy is effective when carried out during the cellular phase when the number of osteoblasts is increasing. Later, the higher number of cells results in a larger deposition of bone matrix, which later incorporates CHA, characterizing maturation of the bone.

The treatment protocol used in our various studies is in agreement with our experience, as no existing parameters are universally accepted. A unique parameter able to produce by itself a photobiological response does not exist, but the conjugation of different parameters is in agreement with our experimental model.

It still remains uncertain if bone stimulation by laser light is a general effect or if the isolated stimulation of osteoblasts is possible. It is possible that laser therapy's effect on bone regeneration depends not only on the total dose of irradiation, but also on the duration and mode of irradiation. Most importantly, recent study has suggested that the threshold parameter energy density and intensity are biologically independent of one another. This independence accounts for both the success and failure of laser therapy achieved at low-energy density levels.[34]

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