Histol Histopathol (2004) 19: 43-48

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

The effects of low laser irradiation on angiogenesis in injured rat tibiae

I. Garavello[1*], V. Baranauskas[1] and M.A. da Cruz-Höfling[2]

[1]Department of Semiconductors Instruments and [2]Department of Histology and Embryology, Institute of Biology, State University of Campinas (UNICAMP), Campinas (SP), Brazil. *Present address: Departamento de Fisioterapia, Faculdade de Ciências e Tecnologia, Universidade Estadual Paulista (UNESP), President Prudente, SP, Brasil

Summary. The influence of He-Ne 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. A small hole was surgically made with a dentistry burr in the tibia and the injured area received a 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 investigated. Light microscopic examination of histological sections of the injured area and quantification of the newly-formed blood vessels were undertaken. Low-level energy treatment accelerated the deposition of bone matrix and histological characteristics compatible with an active recovery of the injured tissue. He-Ne laser therapy significantly increased the number of blood vessels after 7 days 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. These effects were attributed to laser treatment, since no significant increase in blood vessel number was detected between 8 and 15 non-irradiated control tibiae. Molecular mechanisms involved in low-level laser therapy of angiogenesis in post-traumatic bone regeneration needs further investigation.

Key words: Angiogenesis, He-Ne laser, Bone wound healing, Tibia, Rat

Introduction

The formation of new blood vessels could be a determinant event both in health and disease. Physiological processes related to the female reproductive system, wound healing, the recovery of damaged tissues after infarct or burning, or pathological processes such as tumor growth, diabetic retinopathy, psoriasis, and rheumatoid arthritis, all involve angiogenesis, i.e., the sprouting of new capillaries from pre-existent vessels. In case of embryo development, *de novo* appearance and growth of new blood vessels occurs, an event known as vasculogenesis (Kalka et al., 2000; Nguyen and D'Amore, 2001). Both are complex processes involving the proliferation and migration of endothelial cells and require the assistance of the vascular endothelial growth factor (VEGF) (Mustonen and Alitalo, 1995; Shibuya, 1995), the most potent and specific growth factor, although other angiogenic factors also influence these processes. The understanding of the underlying mechanisms modulating blood vessel growth is potentially useful in controlling desirable or undesirable tissue viability. Hence progress towards introduction of novel therapeutic options in the treatment of a variety of diseases or disabilities should be encouraged. Recently, healing studies have been performed, demonstrating activation of the healing process by He-Ne laser irradiation of the cortical region of rat tibiae surgically-injured by a rotating burr drill (Freitas et al., 2000; Garavello-Freitas et al., 2003). In the course of the investigation we suspected that laser radiation could be stimulating the revascularization of the injured area and participating as an additional factor in the increased bone trabecular area produced during bone repair in tibiae submitted to laser therapy. The present work was designed to assess quantitatively whether low-power (He-Ne) laser radiation is able to stimulate vessel growth during tibia wound healing in animals with similar lesions.

Offprint requests to: Maria Alice da Cruz Höfling, Departamento de Histologia e Embriologia, Instituto de Biologia, Caixa Postal 6109, Universidade Estadual de Campinas (UNICAMP), CEP 13083-970 – Campinas, SP, Brasil. FAX: (55) (19) 32893124. e-mail: hofling@unicamp.br

Material and methods

Surgical procedure and laser radiation

The experiments were performed using 24 young adult male Wistar rats (250-280 g; 75-day-old). After intraperitoneal anesthesia with sodium pentobarbital (0.1 ml/100 g rat weight), a small incision was made in the skin of the rats to expose the antero medial surface of the

tibia. A 1.6 mm diameter hole was then made using a dentistry burr in the mid-region of the diaphysis; only one cortical tibia surface was perforated. One group of rats (n= 12) was irradiated with a 1 mW He-Ne laser (λ=633 nm, Gaussian beam diameter ω = 1.1 mm), without using a converging lens. The irradiation was targeted at the injury site through the skin and was aligned perpendicular to the longitudinal axis of the tibia. Irradiation was started 24 h after surgery and was applied daily for 5 min or 15 min to two groups of rats (n= 6 for each exposure time), corresponding to incident energy density dosages of 31.5 and 94.5 Jcm^{-2}, respectively. Each group of rats was sacrificed with an overdose of anaesthetic on the eighth (n= 3 for each dose) or fifteenth (n= 3 for each dose) day after injury. One group of 12 non-irradiated rats with injured tibiae served as a control (Table 1).

Histology and quantitative analysis

Both the irradiated and control tibiae were removed and processed for histological and morphometric studies in the following manner. The tibiae were dissected and fixed in phosphate-buffered 10% formalin solution (pH 7.3) for 24 h and decalcified (10% formalin in 10% acetic acid solution for a period of 20 days, followed by 10% formalin in 5% EDTA for 48 h, both solutions changed each day). The injured area and 1 to 1.5 mm of surrounding bone was cut from the whole tibia and the bone samples were then rinsed in phosphate-buffered saline (twice 15 min), dehydrated in a graded ethanol series, embedded in paraffin and sectioned (5 μm thick). All the sections obtained from injured tibiae, irradiated or unirradiated controls used to quantify the number of blood vessels, were taken parallel to the long axis of the tibiae. Prepared histological slides were stained with Masson's trichrome for analysis of the occurrence and quantification of newly-formed blood vessels under light microscopy. Vessel quantification was performed by counting microvessels, including sinusoid capillaries present in bone marrow interspersed among trabeculae of the neoforming primary bone. A "point count" grid with 100 mesh (1x1 mm each) or 9x9 configuration with 81 test points each representing the intersection of two orthogonal lines was employed. Vessels that fell within a two square (or greater) mesh were counted only for the mesh in which it predominantly fell. Counting always started at the superior left hand side and proceeded to the right and then in a zig-zag fashion (Aherne and Dunnill, 1988). Fifteen slides from each irradiation dose were analyzed. Five fields of the tissue from each slide were randomly chosen for blood vessel counting, with a total of 75 fields for each laser dosage in both periods of 7 and 14 consecutive days of irradiation. The corresponding control group received the same procedure.

Table 1. Number of blood vessels in unirradiated regenerating rat tibiae at 8- and 15-day post-injury and in tibiae irradiated with He-Ne during 7 or 14 days at the dose of 31.5 Jcm^{-2} and 94.5 Jcm^{-2}. The figures represent the mean ± standard deviation of 75 tissue fields taken at random in each experimental group; n= 6/control group; n= 3/each of the four irradiated group. Experimental groups with the same letter indicate that there was no treatment difference; conversely groups with different letters indicate that there was significant differences in relation to treatment.

PERIOD (days)	8	7		15	14	
Dose (Jcm^{-2})	(control)	31.5	94.5	(control)	31.5	94.5
mean	278.1^{a}	293.9^{a}	369.8^{b}	291.6^{a}	232.6^{c}	214.1^{c}
sd	17.7	81.4	80.6	42.4	39.8	37.7

P≤ 0.001

Statistical analysis

All data are expressed as means ± standard deviation (SD). Differences between groups were analyzed by the parametric Student-Newman-Keuls Method (Streiner and Norman, 1994). Blood vessel data was obtained by counting for two irradiation doses over two periods of irradiation (7 and 14 days, starting 24 h post-surgery) and equivalent controls (8 and 15 days). P≤0.0001 was considered statistically significant.

Results

Histology

Histological sections of bone samples were examined by light microscopy for comparison of the number of newly-formed blood vessels present in the bone marrow compartment of unirradiated and irradiated tibiae. Tissue damage zones were identified by the presence of primary structure of the trabecular bone. Comparison of 8-day untreated controls with 7-day laser-stimulated samples (31.5 Jcm^{-2}) showed a non significant increase in blood vessels in the latter (Figs. 1,2, Table 1). However, when comparing 8-day controls to 7-day tibiae irradiated with doses of 94.5 Jcm^{-2}, a significant increase in blood vessels in the bone marrow compartment (Figs. 1,3, Table 1) was found. A significant increase in the number of blood vessels was also found between the two groups irradiated with doses of 31.5 Jcm^{-2} and 94.5 Jcm^{-2} over 7 days (Figs. 2, 3, Table 1). A non significant increase in the number of blood vessels was observed in untreated controls at the 8th and 15th day post-surgery (Table 1). Conversely, a significant decrease in the number of vessels was observed by comparing 7-day irradiated tibiae receiving either 31.5 or 94.5 Jcm^{-2} with those irradiated for 14 days (Table 1). A similar significant decrease in the number of vessels was also found in the14-day irradiated group, no matter the dose applied when compared to 15-day non-irradiated control group (Table 1).

During the repair progress irregularly formed trabeculae of primary bone temporarily united the extremities of the injured tibia, forming a bone callus. A correlative change was also observed in the histological appearance of primary bone trabeculae in irradiated

tibiae. In the 7-day irradiated tibiae (31.5 Jcm^{-2} dose) the bone matrix was deposited throughout the intertrabecular area (Fig. 2). It appeared as blue-stained elongated shadows around active osteoblasts, a feature not observed in bone callus of laser-untreated controls (Fig. 1). Over the same 7-day period of irradiation, but with the dose of 94.5 Jcm^{-2}, this feature was not so conspicuous as in the former laser dose, but was improved in relation to controls (Figs. 1-3). From the beginning to the end of the second period of irradiation

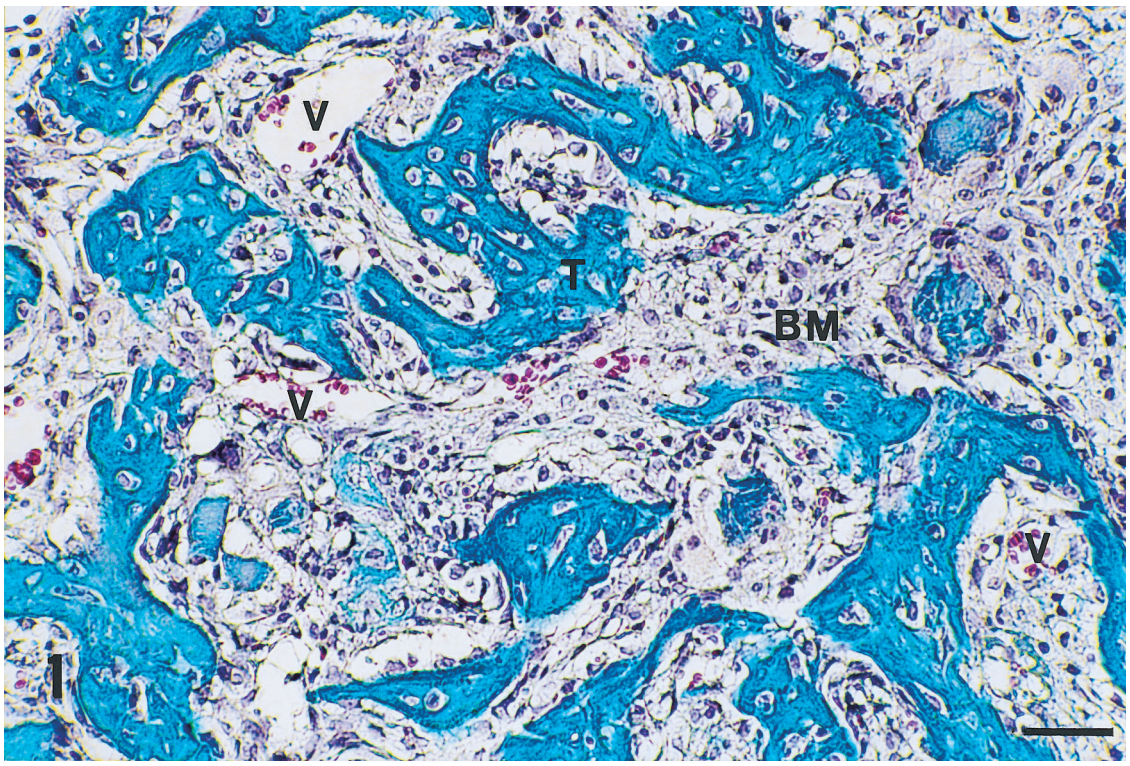

Fig. 1. Histological appearance of a rat tibia bone callus 8 days after surgical injury (controls non-irradiated). The area of healing is now occupied by primary bone trabeculae (T), which by this time is thin and whose profile occupies a lesser area than that occupied by the bone marrow compartment (BM). Note newly-formed blood vessels (V) scattered along this compartment. Masson's trichrome. Bar: 40 μm.

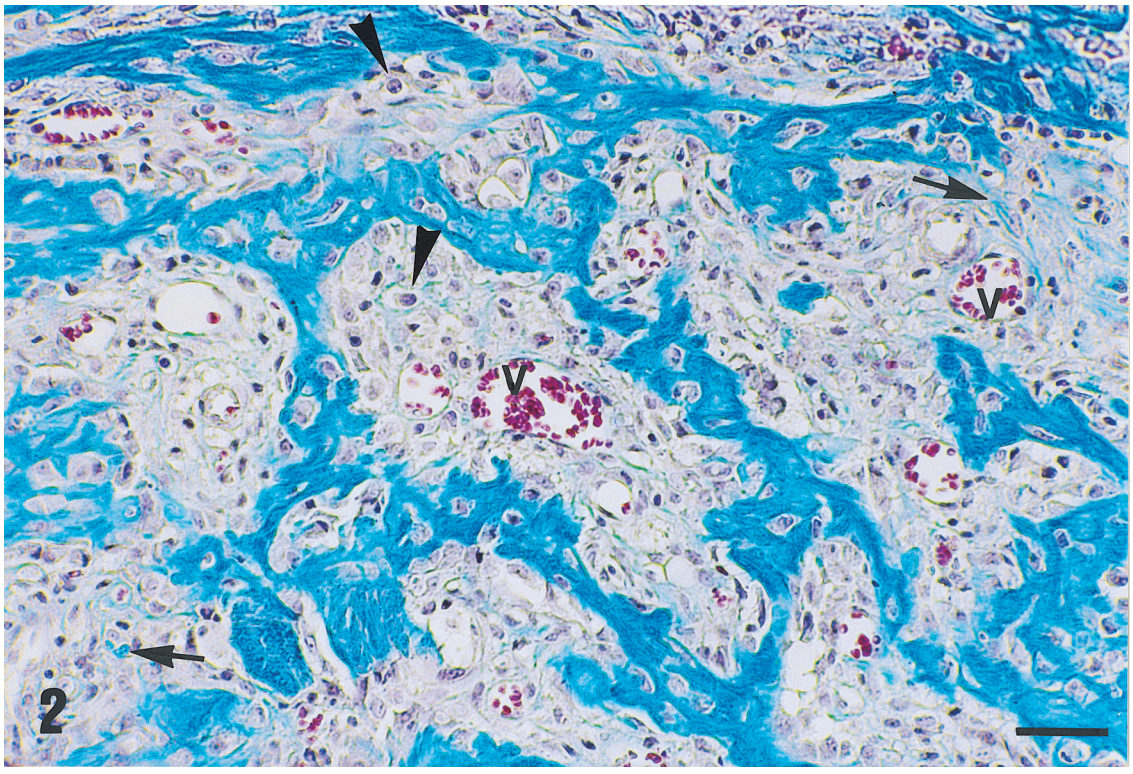

Fig. 2. Histological appearance of a rat tibia bone callus 8 days after surgical injury and irradiated for 7 days with He-Ne laser at a daily dose of 31.5 Jcm^{-2} (5 min each day). Regions as the above are used to quantify newly-formed blood vessels. Note that several blood vessels (V) appear in the bone marrow compartment. Note also that along with the forming trabeculae numerous points of bone matrix deposition appear as blue, irregular, thin fibril bundles (collagen) (arrows) around osteoblasts (arrowheads) throughout the bone marrow, indicating that active synthesis of bone matrix is in course. Masson's trichrome. Bar: 40 μm.

the histological appearance of the primary bone trabeculae gained an embodied consistency, with the bone matrix appearing more compact. Trabeculae were wider and consequently the bone marrow compartment was narrower than in control and irradiated tibiae at the end of the first week post-injury. A common finding by the end of the second week of daily irradiation was the incipient appearance of Havers systems, a characteristic

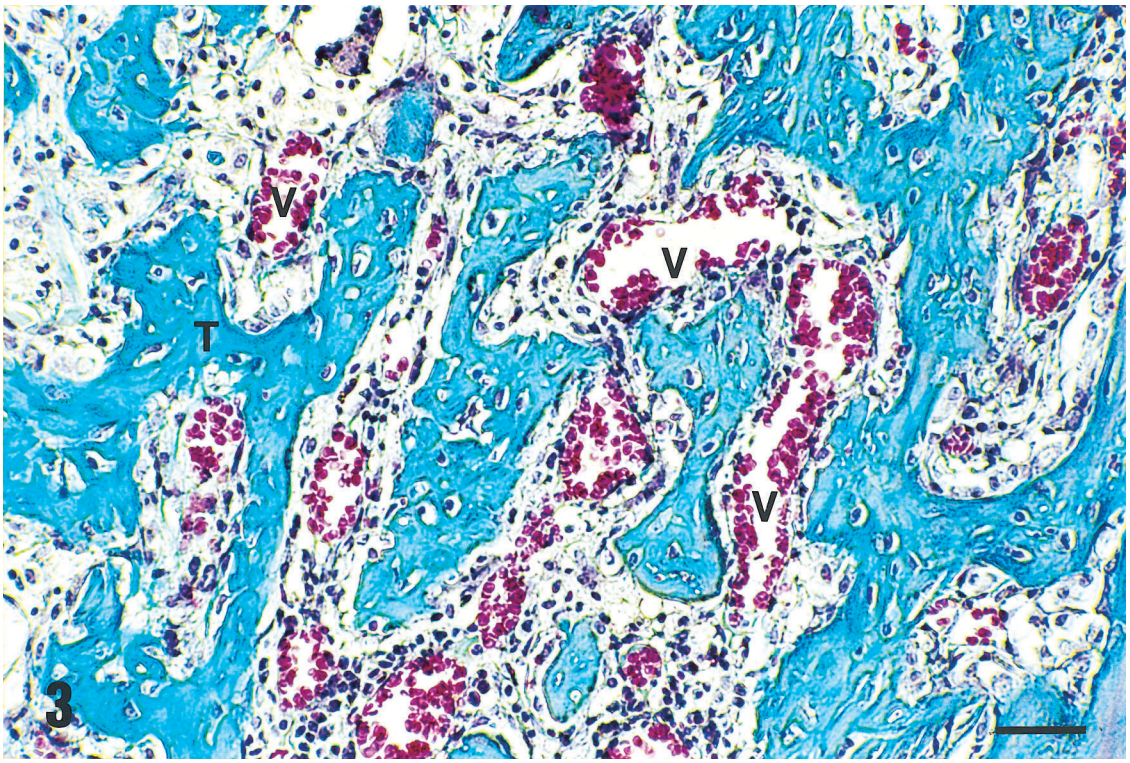

Fig. 3. Histological appearance of a rat tibia bone callus 8 days after surgical injury and irradiated for 7 days with He-Ne laser at a daily dose of 94.5 Jcm^{-2} (15 min each day). The number of newly-formed well-developed blood vessels (V) significantly increases by this period. Note that primary bone trabeculae (T) have a more compact look than in Figs. 1, 2. Arrow points at matrix being deposited. Masson's trichrome. Bar: 40 μm.

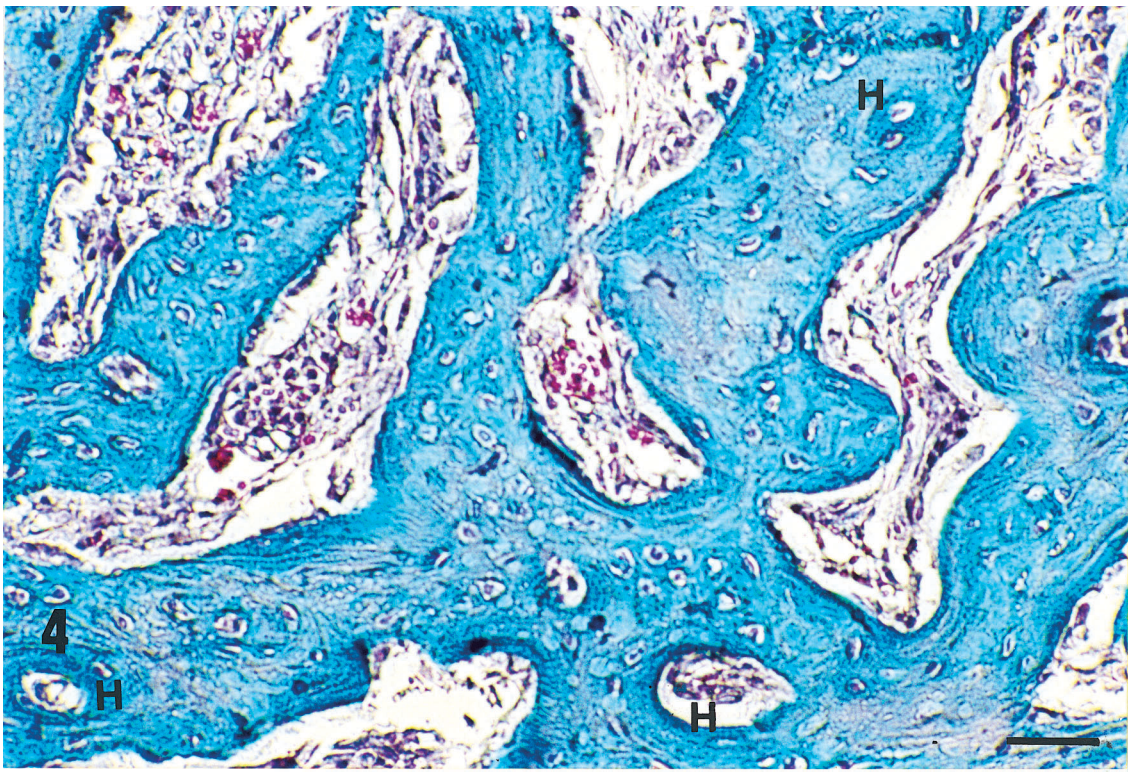

Fig. 4. Histological appearance of a rat tibia bone callus 15 days after surgical injury and irradiated for 14 days with He-Ne laser at a daily dose of 94.5 Jcm^{-2} (15 min each day). Blood vessels are not so conspicuous as in Fig. 3. Note that the wider the trabeculae area the narrower the compartment of bone marrow, a feature typical of this stage of bone repair. Note also that in some regions the bone matrix has already assumed an arrangement that resembles the aspect of a system of Havers, typical of the mature secondary bone (H). Masson's trichrome. Bar: 40 μm.

of mature lamellar bone (Fig. 4).

Discussion

The counting of newly-formed blood vessels in the marrow of tibiae during the wound healing process in rats clearly indicates that He-Ne irradiation transcutaneously applied to the injury site of rat tibiae had a dual modulatory effect on revascularization, a stimulatory and an inhibitory. This greatly resembles the results obtained in former experiments on osteogenesis using a similar model (Garavello-Freitas et al., 2003). Briefly, whilst a significant angiogenic stimulating effect had been observed at the end of the first week with a daily dose of 94.5 Jcm-^{2} (cumulative dose of 220.5 Jcm-^{2}), a significant inhibitory effect (dose-independent) was found at the end of the second week of laser therapy (cumulative doses of 441.0 Jcm-2 or 1,323 Jcm^{2}). The positive effect on angiogenesis during the first week of laser irradiation was attributed to the laser therapy, since it did not occur in non-irradiated controls. Similarly, there was a significant increase in the area of primary bone trabeculae; but then the effective dose was 31.5 Jcm^{-2} (Garavello-Freitas et al., 2003). Evidence confirming that an active osteogenesis was actually occurring with this dose was reinforced in this work by the presence of numerous shadows of matrix deposition at the marrow compartment (see Fig. 2). On the other hand, the number of vessels had decreased significantly after 14 days of irradiation (with both doses), which paralleled with the decrease of the trabecular area seen with the 94.5 Jcm^{-2} at the same period (Garavello-Freitas et al., 2003). The results strongly suggest that the depression of matrix synthesis and angiogenesis during the second week of treatment was elicited by an inhibitory light effect, and not related to inhibitory factors common during the bone remodelling phase, as the VEGF expression declined, since in non-irradiated control tibiae the number of blood vessels increased (non-significant, $P<0.001$) and there was a significant increase in the bone growth ($P\leq 0.05$). Osteogenesis depends extensively on blood supply, being therefore a process posterior to angiogenesis. Bone is a tissue remarkably vascularized, therefore the use of a controlled surgical procedure in rat tibiae to assess angiogenesis (and osteogeneis) is highly appropriate. Recently, it has been shown that human fracture hematoma contains the angiogenic cytokine vascular endothelial growth factor and an inherent capability of inducing angiogenesis and promoting revascularization (Street et al., 2000), and that angiogenic factors such as lipidomental factors and fibroblast growth factors can accelerate bone formation and fracture healing in a rodent femoral fracture model (Cornell and Lane, 1992). On the other hand, a positive effect of a low-power laser therapy LLLT (He-Ne) (31.2 Jcm^{-2} irradiation by 2- 9 days post-injury) on the neovascularization process during gastrocnemius regeneration in the *Bufo viridis* toad has been reported (Bibikova et al., 1994). The period for occurring stimulation of angiogenesis was similar to that found by us, but the dose was different. However the experimental models were different, i.e., toad/muscle (muscle tissue has a highly-ordered paracrystalline array of macromolecules) versus rat/bone (woven-fibered collagen fibrils of neoformed primary bone). The present study has shown that laser therapy increased the blood flow after the first 7 days of irradiation, which could account for the presence of angiogenic factors probably enhanced by light energy. All the data obtained permit us to suppose and suggest that LLLT is capable of affecting cellular processes, as demonstrated in numerous reports dealing with laser investigation on post-fracture bone regeneration (Trelles and Mayayo, 1987; Saito and Shimizu, 1997), post-injury skeletal muscle regeneration (Weiss and Oron, 1992; Bibikova and Oron, 1995), tendon healing (Enwemeka, 1992; Reddy et al., 1998), skin ulcer healing (Schindl et al., 1999), teeth treatment (Kawasaki and Shimizu, 2000) and crushed sciatic nerve recovery (Rochkind et al., 2001). However, the choice of an appropriate laser dose and period of treatment in conformity with the tissue characteristics is mandatory for a successful laser photomodulatory effect on repair processes. Periods longer than 7 days (at both doses) adversely influenced neovascularization and osteogenesis in the rat model. Numerous studies have shown that variation in the incident energy densities of LLLT induces significant differences in the proliferation of resting lymphocytes in vitro (Agaiby et al., 2000), modulates the immunological functions by significantly increasing or decreasing the levels of interleukin-1 alpha (IL-1 alpha), interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma) in cultured human peripheral blood mononuclear cells stimulated with different mitogens (Funk et al., 1992), promotes cell proliferation and the release of fibroblast growth factors from fibroblasts (Yu et al., 1994a,b), modulates fetal cardiomyocyte proliferation and significantly increases the expression of proangiogenic genes, transforming growth factor-beta (TGF-beta) and VEGF by cardiac myocytes (Khanna et al., 1999), increases production of VEGF by smooth muscle cells, fibroblasts, and cardiac myocytes and stimulates cultured endothelial cells growth (Kipshidze et al., 2001). Laser photostimulation also increases ATP synthesis, promotes nucleic acid production and cell division (Wedlock et al., 1994; Yaakobi et al., 1996; Yu et al., 1997). At appropriate irradiation conditions cytochrome-c oxidase is a specific target of HeNe laser light, being considered as a mitochondrial photo-acceptor (Pastore et al., 2000), besides being able to stimulate the synthesis of cytosolic and mitochondrial proteins (Greco et al., 1989; Vacca et al., 1996), and protein synthesis in hepatic cells (Vacca et al., 1996). Despite the fact that how specific interactions could be established between the incident light energy and the tissue light absorbing capacity in order to produce an adequate response by the tissue is unknown, it could be expected that general mechanisms related to a good physiological performance of the organism under

adverse conditions will contribute to the positive effects of the laser therapy.

We conclude that HeNe laser irradiation can interfere positively or negatively in the formation of new blood vessels (angiogenesis) and bone matrix during a regenerating process. Adequate manipulation of these properties could be of key importance in numerous biological situations where stimulation or inhibition of angiogenesis are required.

Acknowledgements. The authors are grateful to Dr. Ismael F. Freitas Jr. for statistical analysis, Dr. Ana Maria Osório (FCT-UNESP) for technical support, and to Dr. Steve F. Durrant (FEEC-UNICAMP) for critical suggestions. I.G. was a recipient of a scholarship from CAPES. We also acknowledge the anonymous referees which contributed to the improvement of the manuscript with their suggestions. This work was supported by FAPESP, CNPq and FAEP-UNICAMP.

References

Agaiby A.D., Ghali L.R., Wilson R. and Dayson M. (2000). Laser modulation of angiogenic factor production by T-lymphocytes. Lasers Surg. Med. 26, 357-263.

Aherne W.A. and Dunnil M.S. (1988) Morphometry. Edward Arnold Publ., London, 205p.

Bibikova A. and Oron U. (1995). Regeneration in denervated toad (*Bufo viridis*) gastrocnemius muscle and the promotion of the process by low energy laser irradiation. Anat. Rec. 241, 123-128.

Bibikova A., Belkin V. and Oron U. (1994). Enhancement of angiogenesis in regenerating gastrocnemius muscle of the toad (*Bufo viridis*) by low-energy irradiation. Anat. Embryol. 190, 597-602.

Cornell C.H. and Lane J.M. (1992). Newest factors in fracture healing. Clin. Orth. Relat. Res. 277, 297-311.

Enwemeka C.S. (1992). Ultrastructural morphometry of membrane-bound intracytoplasmic collagen fibrils in tendon fibroblasts exposed to He-Ne laser beam. Tissue Cell 42, 511-523.

Freitas I.G.F., Baranauskas V. and Cruz-Höfling M.A. (2000). Laser effects on osteogenesis. Appl. Surface Sci. 154-155, 548-554.

Funk J.O., Kruse A. and Kirchiner H. (1992) Cytokine prodution after helium-noen laser irradiation in cultures of human peripheral blood mononuclear cells. J. Photochem. Photobiol. B 16, 347-355.

Garavello-Freitas I., Baranauskas V., Joazeiro P.P., Padovani C.R., DalPai-Silva M. and Cruz-Höfling M.A. (2003). Low power laser irradiation improves histomorphometrical parameters and bone matrix organization during tibia wound healing in rats. J. Photochem. Photobiol. B: Biology 70, 81-89.

Greco M., Guida G., Perlino E., Marra E. and Quagliariello E. (1989). Increase in RNA and protein synthesis by mitochondria irradiated with helium-neon laser. Biochem. Biophys. Res. Commun. 163, 1428-1434.

Kalka C., Asahara T., Krone W. and Isner J.M. (2000). Angiogenesis and vasculogenesis. Therapeutic strategies for stimulation of postnatal neovascularization. Herz 25, 611-622.

Kawasaki K. and Shimizu N. (2000). Effects of low-energy laser irradiation on bone remodeling during experimental tooth movement in rats. Lasers Surg. Med. 26, 282-291.

Khanna A., Shankar L.R., Keelan M.H., Kornowski R., Leon M., Moses J. and Kipshidze N. (1999). Augmentation of the expression of proangiogenic genes in cardiomyocytes with low dose laser irradiation in vitro. Cardiovasc. Radiat. Med. 1, 256-259.

Kipshidze N., Nikolaychik V., Keelan M.H., Shankar L.R., Khanna A., Kornowski R., Leon M. and Moses J. (2001). Low-power helium-neon laser irradiation enhances production of vascular endothelial growth factor and promotes growth of endothelial cells in vitro. Lasers Surg. Med. 28, 355-364.

Mustonen T. and Alitalo K. (1995). Endothelial receptor tyrosine kinases involved in angiogenesis. J. Cell. Biol. 129, 895-898.

Nguyen L.L. and D'Amore A. (2001). Cellular interactions in vascular growth and differentiation. Int. Rev. Cytol. 204, 1-48.

Pastore D., Greco M. and Passarella S. (2000). Specific helium-neon laser sensitivity of the purified cytochome c oxidade. Int. Radiat. Biol. 76, 836-870.

Reddy G.K., Stehno-Bittel L. and Enwemeka C.S. (1998). Laser photostimulation of collagen production in healing rabbit Achilles tendons. Lasers Surg. Med. 22, 281-287.

Rochikind S., Nissan M., Alon M., Shamir M. and Salame K. (2001). Effects of laser irradiation on the spinal cord for the regeneration of crushed peripheral nerve in rats. Lasers Surg. Med. 28, 216-219.

Saito S. and Shimizu N. (1997). Stimulatory effects of low-power laser irradiation on bone regeneration in midpalatal suture during expansion in the rat. Am. J. Orthod. Dentofacial Orthop. 111, 525-532.

Schindl A., Schindl M., Schindl L., Jurecka W., Honigsmann H. and Breier F. (1999). Increased dermal angiogenesis after low-intensity laser therapy for a chronic radiation ulcer determined by a video measuring system. J. Am. Acad. Dermatol. 40, 481-484.

Shibuya M. (1995). Role of VEGF-flt receptor system in normal and tumor angiogenesis. Adv. Cancer Res. 67, 281-316.

Street J., Winter D., Wang J.H., Wakai A., McGuinnes A. and Redmond H.P. (2000). In human fracture hematoma inherently angiogenic? Clin. Orthop. 378, 224-237.

Streiner D.L. and Norman G.R. (1994). Biostatistics. The Bare Essential Mosby-Year Book, St. Louis, p 260.

Trelles M.A. and Mayayo E. (1987). Bone fracture consolidates faster with low-power laser. Lasers Surg. Med. 7, 36-45.

Vacca R.A., Marra E., Passarella S., Petragallo V.A. and Greco M. (1996). Increase in cytosolic and mitochondrial protein synthesis in rat hepatocytes irradiated in vitro by He-Ne. J. Photochem. Photobiol. B 34, 197-202.

Wedlock P., Shepard R.A., Little C. and McBurney F. (1994). Analgesic effects of cranial laser treatment in two rat nociception models. Physiol Behav. 59, 445-448.

Weiss N. and Oron U. (1992). Enhancement of muscle regeneration in the rat gastrocnemius muscle by low energy laser irradiation. Anat. Embryol. 186, 497-503.

Yaakobi T., Maltz L. and Oron U. (1996). Promotion of bone repair in the cortical bone of the tibia in rats by low energy laser (He-Ne) irradiation. Calc. Tiss. Intl. 59, 297-300.

Yu W., Naim J.O. and Lanzafame R.J. (1994a). The effect of laser irradiation on the release of bFGF from 3T3 fibroblasts. J. Photochem. Photobiol. B 59, 167-170.

Yu W., Naim J.O. and Lanzafame R.J. (1994b). The effects of photoirradiation on the secretion of TGF-ß PDGF and bFGF from fibroblasts in vitro. Lasers Surg. Med. 6, (Suppl), 8.

Yu W., Naim J.O. and Lanzafame R.J. (1997). Effects of photoirradiation on wound healing in diabetic mice. Lasers Surg. Med. 20, 56-63.

Accepted September 9, 2003