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Infrared Laser Photobiomodulation (λ 830 nm) on Bone Tissue Around Dental Implants: A Raman Spectroscopy and Scanning Electronic Microscopy Study in Rabbits

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ABSTRACT

Objective: **The aim of this study was to assess, through Raman spectroscopy, the incorporation of calcium hydroxyapatite (CHA; ~960 cm^{-1}), and scanning electron microscopy (SEM), the bone quality on the healing bone around dental implants after laser photobiomodulation (λ830 nm).** ***Background Data:*** **Laser photobiomodulation has been successfully used to improve bone quality around dental implants, allowing early wearing of prostheses.** ***Methods:*** **Fourteen rabbits received a titanium implant on the tibia; eight of them were irradiated with λ830 nm laser (seven sessions at 48-h intervals, 21.5 J/cm^2 per point, 10 mW, ϕ~0.0028 cm^2, 86 J per session), and six acted as control. The animals were sacrificed 15, 30, and 45 days after surgery. Specimens were routinely prepared for Raman spectroscopy and SEM. Eight readings were taken on the bone around the implant.** ***Results:*** **The results showed significant differences on the concentration of CHA on irradiated and control specimens at both 30 and 45 days after surgery ($p < 0.001$).** ***Conclusion:*** **It is concluded that infrared laser photobiomodulation does improve bone healing, and this may be safely assessed by Raman spectroscopy or SEM.**

INTRODUCTION

Several oral pathologies, trauma, and surgery may cause the reduction of alveolar bone mass, quality, or both—and may lead to dental loss.[1] The use of dental implants is an effective technique of prosthetic rehabilitation as it restores the capacity of mastication, phonation, and esthetics.[2] It is known that the success of dental implants depends on the close contact between bone and the implant surface. Usually, there is a waiting time of about 4–6 months for loading.[2] Different techniques—such as the use of grafts, biomaterials,[3–5] bone transplants,[3] and the application of biostimulators such as ultrasound[6]—have been used in dentistry with the ultimate aim of improving the quality of bone around dental implants. Most recently, the use of laser photobiomodulation has been successfully used to improve bone quality.

Laser photobiomodulation has been successfully used in inflammation, acceleration of the cellular proliferation, and bone repair.[3,5,7,8] *In vivo* studies have resulted in better bone healing in laser-irradiated animals after the surgery when compared to controls.[3,9,10] Several studies have demonstrated that near IR (infrared) laser photobiomodulation is the most suitable for bone repair, due to its higher penetration depth on the bone tissue when compared to the visible laser.[2–5,11]

Although microscopic examination and image examinations are the most frequently used methods for assessment of bone healing around dental implants, these methods are unable to provide information at the molecular level.[12,13] An alternative method is near infrared Raman spectroscopy (NIRS), which has been used in several noninvasive diagnostic applications of biological samples such as several types of cancers,[14] human coronary arteries,[15,16] blood analysis,[17] biocompatible implants,[2,18] cell

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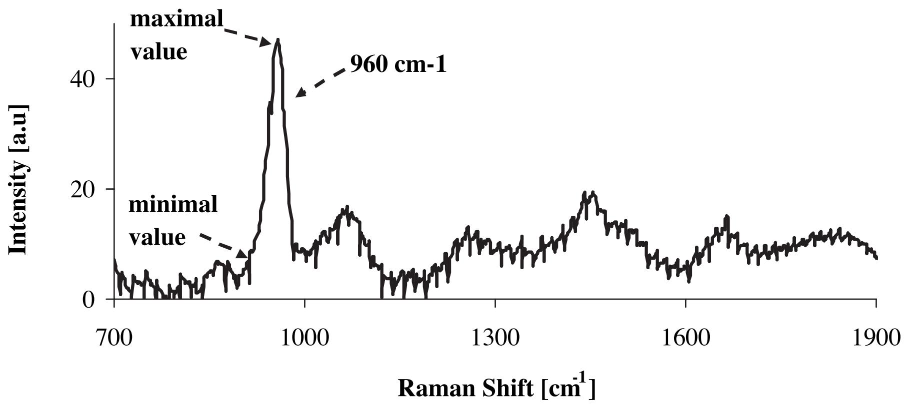

FIG. 1. Raman spectrum of maximum and minimal intensities of the 960 cm^{-1} peak.

culture,[19] bone diseases[12] and mineralization,[19] composite resin,[20,21] teeth,[22] to evaluate the microstructure of human cortical bone (osteon),[23] and to investigate the long-term effects on the surface microstructure of hydroxyapatite disks implanted inside articular capsules of mice.[18] In a recent study,[24] Raman spectroscopy was used to investigate the effects of laser photobiomodulation (λ660 nm, 10 J/cm^2) on the healing of fractured bone of rats by monitoring the level of calcium hydroxyapatite (CHA).

The aim of this study was to assess, by both Raman spectroscopy and scanning electron microscopy (SEM), the incorporation of CHA and quality of the healing bone around dental implants treated or not with IR laser photobiomodulation (λ830 nm).

METHODS

Fourteen healthy young male New Zealand rabbits (average weight, 2 kg) were used in this study and were kept on individual cages under controlled temperature and humidity in a day/night light cycle. The animals were fed with standard laboratory diet and had water *ad libitum* during the experimental time. Under general anesthesia (0.2% Acepran®, 1 mg/kg [Univet S.A, São Paulo, Brazil] and Butorfanol® 0.02 mL/kg [Fort Dodge Ltd., Campinas, Brazil]), and Zoletil® (50 mg, 15 mg/kg; VIRBAC S.A, Carro Cedex, France) a 4-cm-long incision was performed at the right tibia of each animal with a no. 15 scalpel blade. Skin and subcutaneous tissues were dissected down to the periosteum, which was gently sectioned exposing the bone. Under refrigeration and using a low-speed drill (1200 rpm), a cavity was prepared at the tibia for the insertion of the implant. Cylindrical titanium implants (ϕ2.6 mm, 6 mm long; DentFix®, Cambuí, Brazil) were used. After the insertion of the implant, the periosteum was repositioned, and suturing was performed up to the skin (Catgut® 3.0 and mononylon 3.0). All the animals received a single dose of Pentabiotico® (penicillin, streptomycin, 20,000 UI; Fort Dodge) immediately after surgery. Laser photobiomodulation (λ830 nm, 10 mW, ϕ~0.0028 cm^2; Thera Lase, DMC, São Carlos, Brazil) was carried out transcutaneously on nine animals in four points around the implants at 48-h intervals (21.5 J/cm^2, per point), with the first session carried out immediately after surgery and repeated at every 48-h for 15 days (86 J per session). Control subjects ($n = 6$) were submitted to a sham treatment following the same routine. The animals were humanely killed 15, 30, and 45 days after the surgery with an overdose of general anesthetics.

The samples were longitudinally cut under refrigeration (Bueler®, Isomet TM1000; Markham, Ontario, Canada) and divided into two halves. One half of each specimen was kept on 2.5% buffered glutaraldehyde solution and routinely prepared for SEM (Laboratório de Patologia Bucal, Faculdade de Odontologia, UNICAMP, Campus de Piracicaba, Brazil). The slides were analyzed by SEM (Jeol®, JSM, Brookvale, Pittwater, Australia). The other half of each specimen was stored in liquid nitrogen to minimize the growth of aerobic bacteria[2,15,16] and because the chemical fixation is not advisable due to fluorescence emissions from the fixative substances.[13] Prior to Raman study, the samples were warmed up gradually to room temperature, and 100 mL of saline was added during spectroscopic measurements. For Raman measurements, an λ830 nm Ti-Sapphire laser (model 3900S; Spectra Physics, Mountain View, CA) pumped by Argon laser (Spectra Physics, model 2017S) provided near-IR excitation. A spectrograph (model 250 IS; Bruker Optics, Chromex, Billerica, MA) dispersed the Raman scattered light from the sample, and a liquid-nitrogen cooled deep depletion CCD (model LN/CCD-1024-EHR1; Princeton Instruments, Tucson, AZ)

TABLE 1. CONCENTRATION OF CALCIUM HYDROXYAPATITE (CHA) AROUND IRRADIATED AND CONTROL DENTAL IMPLANTS

	15 days		*30 days*		*45 days*	
Group	*Medium*	*Inferior*	*Medium*	*Inferior*	*Medium*	*Inferior*
Irradiated	33	28	75	63	65	69
Control	30	25	47	27	36	39

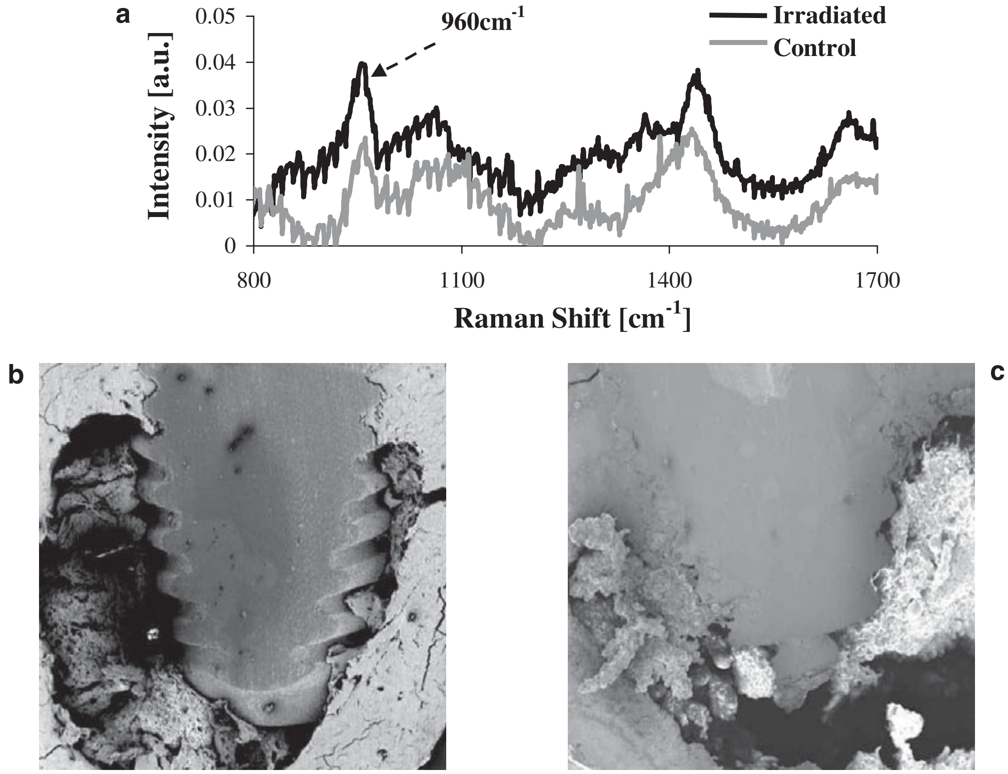

FIG. 2. (**a**) Means of all Raman spectrum of phosphate v_1 (960 cm^{-1}) at 15 days after surgery. (**b**) Scanning electron microscope (SEM) photomicrography of a control specimen 15 days after the surgery, ×35. (**c**) SEM photomicrography of an irradiated specimen 15 days after the surgery, ×35.

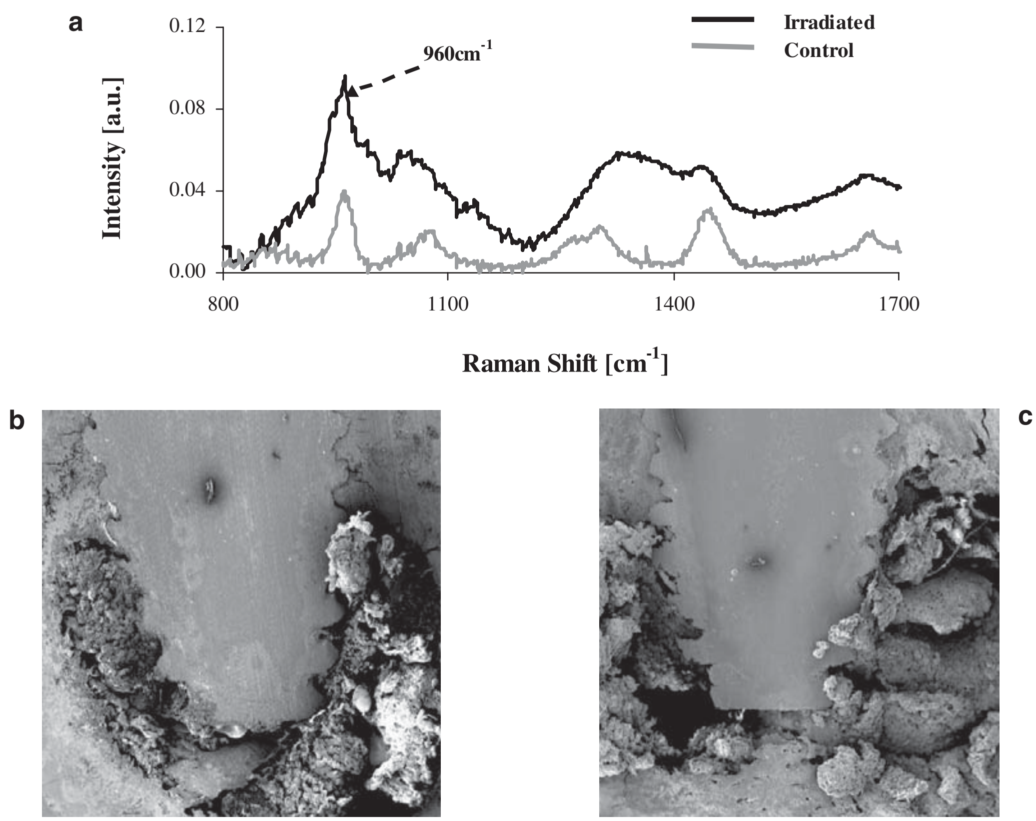

FIG. 3. (**a**) Means of all Raman spectrum of phosphate v_1 (960 cm^{-1}) at 30 days after surgery. (**b**) Scanning electron microscope (SEM) photomicrography of a control specimen 30 days after the surgery, ×35. (**c**) SEM photomicrography of an irradiated specimen 30 days after the surgery, ×35.

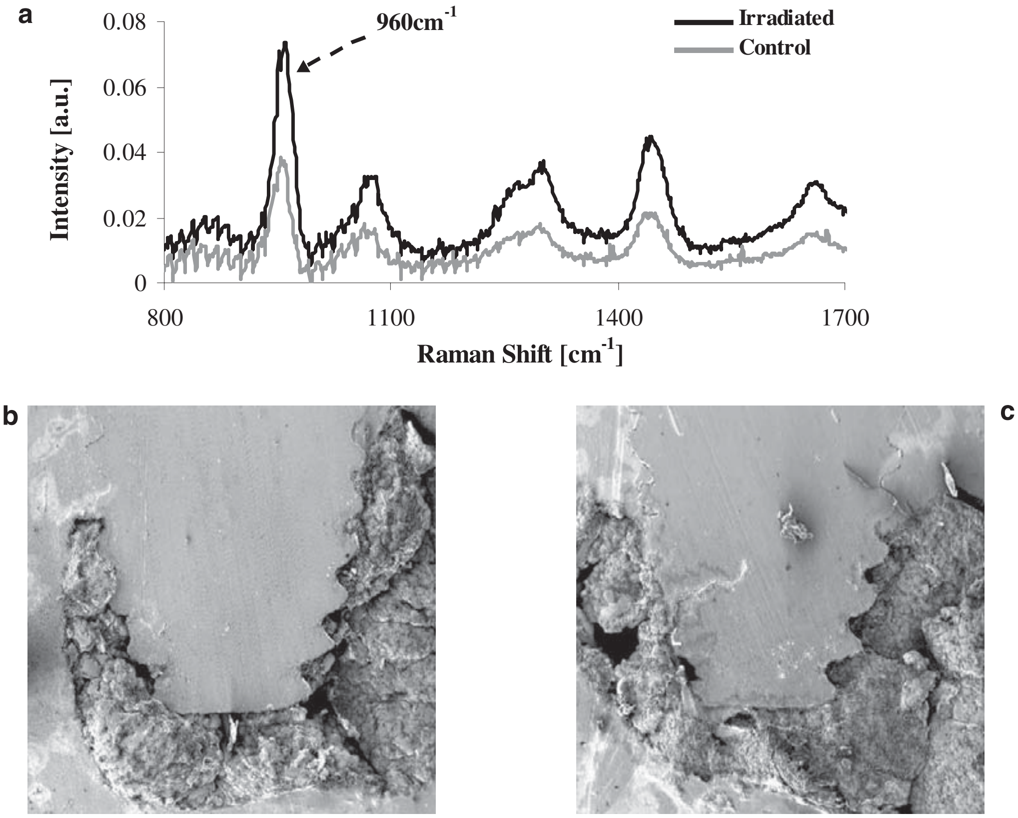

FIG. 4. (**a**) Means of all Raman spectrum of phosphate v_1 (960 cm^{-1}) at 45 days after surgery. (**b**) Scanning electron microscope (SEM) photomicrography of a control specimen 45 days after the surgery, ×35. (**c**) SEM photomicrography of an irradiated specimen 45 days after the surgery, ×35.

detected the Raman spectra. The system was controlled by a microcomputer, which stored and processed the data.[15–17] The laser power used at the sample was 80 mW, and spectral acquisition time was 100 sec. Four points for measurement around the implants at the medium and inferior thirds resulted in eight readings of each implant and 112 total spectra. The data was treated by *MatLab5.1*® software (Newark, NJ) in order to calibrate and subtract background of the spectra. For calibration, the Raman spectrum of a solvent, Indine, with known peaks was used[15,24] due to its intense bands in the region of our interest (700–1800 cm^{-1}). In order to remove the "fluorescence background" from the original spectra, a fifth order polynomial fitting was found to give better results, thus facilitating the visualization of the peaks of CHA (~960 cm^{-1}) found in the bone (Fig. 1).

Statistical analysis

Statistical analysis was performed using *Instat*® software (Aurora, CO). The Kolmorogov and Smirnov test, the t = test, analysis of variance (ANOVA), the Turkey-Kramer test, or the Mann-Whitney test was used for analysis.

RESULTS

The results of the Raman spectroscopy carried out in the implants of irradiated and control bone tissue can be seen on Table 1. The mean spectrum of the 960 cm^{-1} (phosphate v_1) peak of CHA and SEM of the healing bone of irradiated and control subjects during the experimental period of 15, 30, and 45 days can be seen in Figures 2–4. Statistical analysis showed no significant difference at day 15 between irradiated and non-irradiated subjects ($p > 0.05$). Up to day 45 after surgery, the amount of CHA was significantly higher on irradiated subjects, and a significant difference was found between the groups ($p < 0.001$; Fig. 5).

DISCUSSION

The successful use of Raman spectroscopy to assess the amount of CHA on bone was previously reported under different conditions.[18,24] Bone healing of rabbits takes about 42 days, and in humans a bone healing requires 4–6 months for the bone to become mature and resistant, and capable of receiving loading without compromising the stability of the implant.[25] The results of the present study indicate early bone maturation on irradiated subjects due to increased deposition of CHA from day 30 after laser photobiomodulation. Up to 15 days after surgery, there were no significant differences between irradiated and control subjects regarding the concentration of CHA; this may be due to the fact that, during early stages of healing, the osteoblastic activity was chiefly proliferative and deposition started later, which resulted in the formation of immature bone still poor on CHA.[25] SEM images showed that at this stage there were many spaces at the bone–implant interface. From day 15

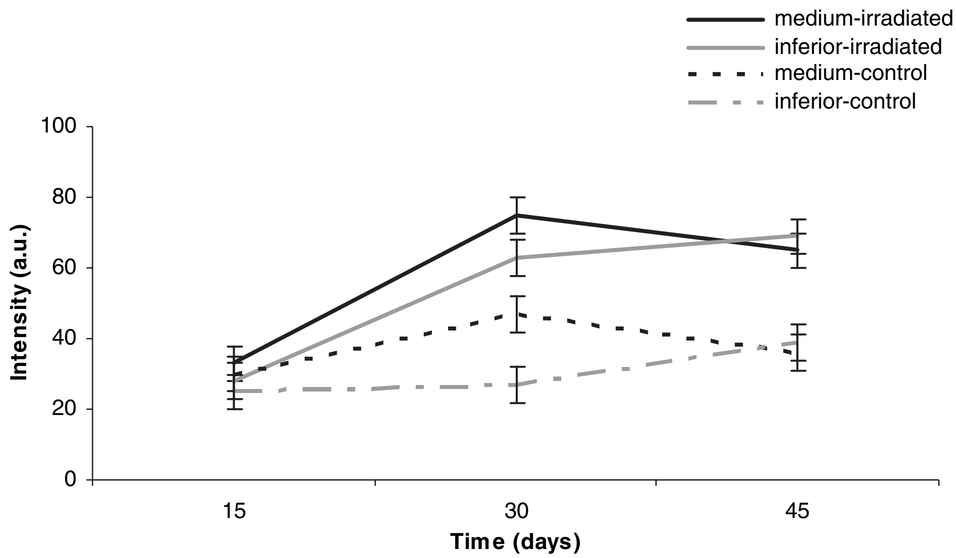

FIG. 5. Means of the intensities (phosphate v_1; ~960 cm^{-1}) of calcium hydroxyapatite (CHA) in healing bone around the dental implants observed during the experimental time.

onwards, the increased deposition of CHA was detectable in both groups and started being significantly higher in irradiated subjects from day 30. This represents the improved ability of more mature osteoblasts to secrete CHA in irradiated subjects, while in control subjects cell proliferation was still occurring. Deposition of CHA represents bone maturation. At day 30, significant differences were observed as fewer empty spaces were seen, and the distribution of the bone tissue was better and was more organized around the implants that were irradiated when compared to the control. At day 45, an increased amount of newly formed bone and an absence of spaces around the irradiated implants were observed, suggesting better bone healing.

The observed differences in the rate of deposition of CHA between irradiated and control subjects was probably due to correct choice of wavelength with higher penetration if the tissues (λ830 nm) and thus increased changes at cellular levels—such as improved ATP synthesis,[3,10] early osteoblastic differentiation,[11,26] release of growth factors,[30] increased levels of calcium, phosphorus, proteins, pronounced angiogenesis, and connective tissue formation.[3,11]

The reason that the effect of laser photobiomodulation was detectable only at 30 days after surgery was probably 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 photobiomodulation. Later, bone matrix becomes the main component of the healing tissue. This is why the frequency of application of laser photobiomodulation was important, as the laser irradiation was carried out during the cellular phase of healing, when the number of osteoblasts was increasing. Later, the higher number of cells resulted in a larger deposition of bone matrix, which later incorporated CHA, characterizing maturation of the bone around the implant.[23]

The present investigation evidenced a reduction of about 30% in healing time of the bone as the concentration of CHA at day 30 was similar to that observed at day 45 on both irradiated and control subjects. Normal healing of bone defects and the placement of implants on rabbits is recommended at 42 days after surgery.[25] It is possible to reduce the loading time of implants in the mandible of humans from 4 months, to approximately 2 months 24 days, and from 6 months, to 4 months 6 days on the maxillae. Session and treatment doses were also effective as previously described by our team[1,3,5] and by other groups using IR laser radiation on bone healing.[11,27–29]

In conclusion, the use of laser photobiomodulation was effective in improving bone healing as a result of increased deposition of CHA as measured by Raman spectroscopy and SEM analysis.

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