Lasers Med Sci (2013) 28:513–518
DOI 10.1007/s10103-012-1096-1

ORIGINAL ARTICLE

The efficacy of the use of IR laser phototherapy associated to biphasic ceramic graft and guided bone regeneration on surgical fractures treated with miniplates: a Raman spectral study on rabbits

Antonio L. B. Pinheiro · Nicole Ribeiro Silva Santos · Priscila Chagas Oliveira · Gilberth Tadeu Santos Aciole · Thais Andrade Ramos · Tayná Assunção Gonzalez · Laís Nogueira da Silva · Artur Felipe Santos Barbosa · Landulfo Silveira Junior

Received: 4 October 2011 / Accepted: 9 April 2012 / Published online: 24 April 2012

Abstracts The aim of the present study was to assess, by Raman spectroscopy, the repair of surgical fractures fixed with internal rigid fixation (IRF) treated or not with IR laser (λ780 nm, 50 mW, 4×4 J/cm^2=16 J/cm^2, ϕ=0.5 cm^2, CW) associated or not to the use of hydroxyapatite and guided bone regeneration (GBR). Surgical tibial fractures were created under general anesthesia on 15 rabbits that were divided into five groups, maintained on individual cages, at day/night cycle, fed with solid laboratory pelted diet and had water ad libitum. The fractures in groups II, III, IV and V were fixed with miniplates. Animals in groups III and V were grafted with hydroxyapatite and GBR technique used. Animals in groups IV and V were irradiated at every other day during 2 weeks (4×4 J/cm^2, 16 J/cm^2=112 J/cm^2). Observation time was that of 30 days. After animal death, specimens were taken and kept in liquid nitrogen and used for Raman spectroscopy. Raman spectroscopy showed significant differences between groups (p<0.001). Basal readings showed mean value of *1,234±220.1*. Group internal rigid fixation + biomaterial + laser showed higher readings (*3,521±2,670*) and group internal rigid fixation + biomaterial the lowest (*212.2±119.8*). In conclusion, the results of the present investigation are important clinically as spectral analysis of bone component evidenced increased levels of CHA on fractured sites by using the association of laser light to a ceramic graft.

A. L. B. Pinheiro (✉) · N. R. S. Santos · P. C. Oliveira · G. T. S. Aciole · T. A. Ramos · T. A. Gonzalez · L. N. da Silva · A. F. S. Barbosa
Center of Biophotonics, School of Dentistry,
Federal University of Bahia,
Av. Araújo Pinho, 62, Canela,
Salvador, Bahia CEP:40110-150, Brazil
e-mail: albp@ufba.br

N. R. S. Santos
e-mail: ribeironicole@hotmail.com

P. C. Oliveira
e-mail: pchagas2005@yahoo.com.br

G. T. S. Aciole
e-mail: gilberthaciole@hotmail.com

T. A. Ramos
e-mail: thais_andraderamos@hotmail.com

T. A. Gonzalez
e-mail: tayna_gonzalez@yahoo.com.br

L. N. da Silva
e-mail: lay_lica@hotmail.com

A. F. S. Barbosa
e-mail: arturfelipes@gmail.com

A. L. B. Pinheiro · L. S. Junior
Universidade Camilo Castelo Branco,
São José dos Campos, São Paulo 12245-230, Brazil
e-mail: land-jr@uol.com.br

A. L. B. Pinheiro
Instituto Nacional de Ciência e Tecnologia de Óptica e Fotônica,
São Carlos, São Paulo 13560-970, Brazil

L. S. Junior
Universidade Camilo Castelo Branco Núcleo do Parque Tecnológico de São José dos Campos,
Rodovia Presidente Dutra, km. 138—Distrito de Eugenio de Melo,
São José dos Campos CEP 12247-004 São Paulo, Brazil

Keywords Biomaterials · Bone repair · Internal rigid fixation · Phototherapy

Introduction

A fracture is a loss of the integrity of the bone and its structure fails and occurs when bone cannot withstand an intense force. The healing of a fracture is an extremely interesting process, and in optimal conditions, injured bone may be reconstituted without scarring [1–4].

The repair of fractures, bony defects, periodontal pockets and alveolar socket are typical examples of processes involving bone remodeling. Despite being extensively studied over the past years, many studies have tried to develop techniques to improve the treatment of bone defects. These techniques include the use of different types of grafts, the use of membranes and bone morphogenetic proteins (BMPs) or the combinations of them [1–18].

Fractures have been treated with immobilization, traction, amputation, and internal fixation throughout history. Immobilization by casting, bracing, or splinting a joint above and below the fracture was used for most long bone fractures. The treatment of fractures consists of the reduction and fixation of dislocated segments. Internal fixation is used in the treatment of fractures as it provides sufficient stability for fracture healing without excessive rigidity. The choice of the type of internal fixation depends on the type of fracture, the condition of the soft tissues and bone, the size and position of the bone fragments, and the size of the bony defect [1–4, 19–21].

Many types of biomaterials have been used instead of using autologous grafts to minimize the morbidity of the procedures avoiding two simultaneous surgical procedures. One of the most commonly used biomaterial is the hydroxyapatite (HA; calcium HA—CHA). This type of graft may be produced using different composition and shape. It may be used isolated, associated to the use of a membrane (guided bone regeneration—GBR), or mixed to an autologous bone graft [22]. Recently, the use of phototherapy has been proposed as a method to improve bone repair under different protocols, including the association to biomaterials and GBR [1–9, 14, 16].

Raman spectroscopy is a vibrational spectroscopic technique that may be used to optically probe the molecular changes associated with diseased tissues. This vibrational spectroscopic technique is relatively simple, reproducible, nondestructive to the tissue, and only small amounts of material (micrograms to nanograms) with a minimum sample preparation are required. In addition, these techniques provide molecular-level information, allowing investigation of functional groups, bonding types and molecular conformations. Spectral bands in vibrational spectra are molecule specific and provide direct information about the biochemical composition. These bands are relatively narrow, easy to resolve and sensitive to molecular structure, conformation and environment [23].

Our group has strong evidences that the improvement of the maturation of irradiated bone is associated to an increased deposition of CHA during early stages of healing. This maturation may represent an increased secretion by osteoblasts on irradiated subjects. It is well accepted that deposition of CHA represents bone maturation being larger amounts of CHA on bone indicative of a more resistant and calcified bone [1–5, 11]. The aim of the present study was to assess, by Raman spectroscopy, the repair of fractures fixed with miniplates (internal rigid fixation—IRF) treated or not with a biphasic ceramic graft associated or not with GBR and irradiated or not with λ780 nm laser on an animal model.

Materials and methods

The Animal Ethics Committee of the School of Dentistry of the Federal University of Bahia has approved this research. Fifteen healthy adult male New Zealand rabbits (~8 months old, mean weight 2 kg) were kept under natural conditions of light, humidity, and temperature at the Laboratory of Animal Experimentation of the School of Dentistry of the Federal University of Bahia during the experimental period. The animals were fed with standard laboratory pelted diet and had water ad libitum. The animals were kept in individual metallic gages, kept at day/night light cycle and controlled temperature during the experimental period. The animals were randomly distributed into five groups (Table 1).

Prior to intramuscular general anesthesia, the animals received acepromazine (2 mg/kg Acepran® 0.2 %; Univet S.A, Cambuci, SP, Brazil,). The anesthesia was carried out 20 min later with ketamine (Ketalar®, 50 mg/ml 0.4 ml/kg; Lab. Parke Davis Ltda, São Paulo, São Paulo, Brazil) and 2 % xylazine (Rompum®, 20 mg/ml 0.2 ml/kg; Lab. Bayer Health Care S.A, São Paulo, São Paulo, Brazil). The animals had the right leg shaved, and a 3-cm-long incision was

Table 1 Description and distribution of the groups

Group	Procedure	Description
I	Basal bone	Control—no fracture
II	IRF	Fracture fixed with miniplate only
III	IRF + B	Fracture fixed with miniplate + Genphos® + Gen-derm®
IV	IRF + L	Fracture fixed with miniplate + laser
V	IRF + B + L	Fracture fixed with miniplate + Genphos® + Gen-derm® + laser

performed at the right tibia with a no. 15 scalpel blade. Skin and subcutaneous tissues were dissected down to the periosteum, which was gently sectioned exposing the bone. A complete surgical tibial fracture was created on animals in groups II, III, IV and V with a carborundum disk (Moyco Union Broach, York, PA, USA) under water refrigeration.

Animals in group II had the bone fragments fixed with miniplates only (Sistema 2.0 PROMM®; Comércio de Implantes Cirúrgicos Ltda. Porto Alegre, Rio Grande do Sul, Brazil). Animals in groups III and V were grafted with the 0.5-mm particle ceramic graft (GenPhos® HATCP. BAUMER®; Mogi Mirim, São Paulo, Brazil) and covered with a demineralized bovine bone membrane (Gen-derm®, BAUMER®; Mogi Mirim) prior similar fixation used on group I (Fig. 1). Animals in groups IV and V were further irradiated with laser light (λ780 nm, 50 mW, CW, *spot area of* 0.5 cm^2, TWIN FLEX®; MM Optics, São Carlos, São Paulo, Brazil).[1] The irradiation started immediately after treatment prior suturing (16 J/cm^2, 4×4 J/cm^2, 9 J) and was transcutaneously repeated at every other day during 2 weeks. After suturing (4-0 polyglactin, TRUSINTH®; Sutures India Pvt Ltda. Bangalore, Karnataka, India) and 4-0 nylon (TRUSINTH®, Sutures India Pvt Ltda.), the animals received intramuscular antibiotics (Pentabiótico®, penicillin, streptomycin, 20,000 UI 0.2 ml/kg IM; Lab. Forte Dogde Saúde Animal Ltda, Campinas, São Paulo, Brazil) and Banamine® (flunixin meglumine, 10 mg/ml, 0.1 ml/kg IM; Intervet Shering-Plough Animal Health, Cruzeiro, São Paulo, Brazil).

Following animal death 30 days after fracture, the samples were longitudinally cut under refrigeration (Bueler®, Isomet TM1000; Markham, ON, Canada) and stored in liquid nitrogen to minimize the growth of aerobics bacteria and because the chemical fixation is not advisable due to fluorescence emissions from the fixative substances [1–5, 11, 13].

Prior to Raman study, the samples were longitudinally cut and warmed gradually to room temperature and 100 ml of saline was added to the surface during spectroscopic measurements. For Raman measurements, a Raman system (P-1; Lambda Solutions, Inc., MA, USA) was used. Acquisition and storage of the Raman data were done in a PC (Dell Inspiron modelo 1501) and RamaSoft® *software* (Lambda Solutions, Inc.). The laser power used at the sample site was of 100 mW with spectral acquisition time 10 s. Three points were measured at the fractured site of each specimen. All spectra were collected at the same day to avoid optical misalignments and changes in laser power. The mean value of the intensity of the peak (~958 cm^{-1}, phosphate ν_1) was determined by the average of the peaks on this region. This intensity is related to the concentration of CHA of the bone. For calibration, the Raman spectrum of the solvent Indene with

[1] The manufacturer calibrated the equipment before the experimentation.

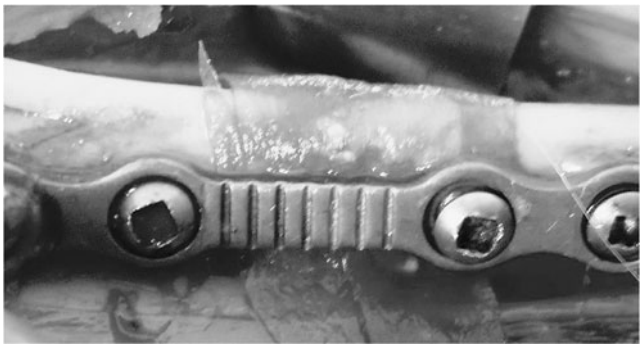

Fig. 1 Experimental design. The fracture was fixed with miniplates. When necessary, the biomaterial and membrane were used

known peaks was used due to its intense bands (800–1,800 cm^{-1}) in the fingerprint region [1–5, 11, 13].

The indene spectrum was also measured each time the sample was changed to be sure that the laser and collection optics were optimized. In order to remove the "fluorescence background" from the original spectrum, a fifth-order polynomial fitting was found to give better results facilitating the visualization of the peaks of CHA (~958 cm^{-1}) found on the bone. A baseline Raman spectrum of nontreated bone (*group I*) was also produced and acted as control (Fig. 2). The data were analyzed by the MatLab5.1® software (Newark, New Jersey, USA) for calibration and background subtraction of the spectra. Statistical analysis was performed using Minitab 15.0® software (Minitab, Belo Horizonte, Minas Gerais, Brazil).

Results

The Raman spectrum of bone shows prominent vibrational bands related to tissue composition (mineral and organic

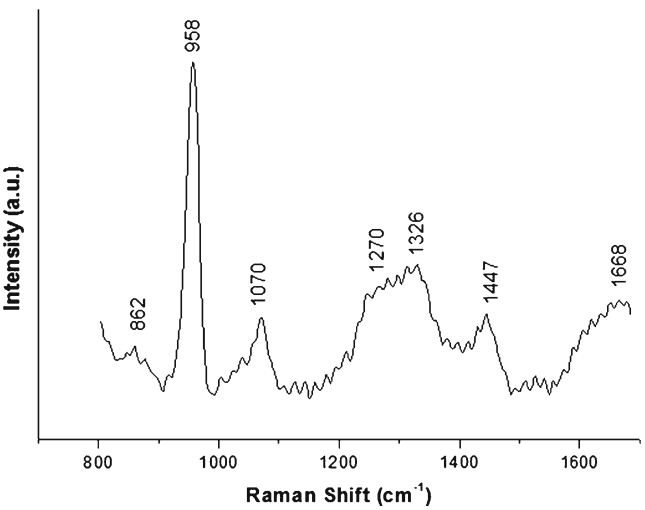

Fig. 2 Bone main Raman bands at 862, 958, 1,070, 1,270, and 1,326, 1,447 and 1,668 cm^{-1}

matrices). Figure 2 shows the bone main Raman bands at 862, 958, 1,070, 1,270, and 1,326, 1,447 and 1,668 cm^{-1}. The band at 1,668 cm^{-1} and the ones at 1,270 and 1,326 cm^{-1} are attributed to amide I and III stretching modes of lipids and proteins; the band at 1,447 cm^{-1} is attributed to the bending and stretching vibration modes of CH groups of lipids and proteins. The ones at 958 and 1,070 cm^{-1} are attributed to phosphate and carbonate hydroxyapatite from bone mineral, respectively; the band at 862 cm^{-1} may be attributed to the vibration bands of C–C stretch of collagen (tyrosine/proline ring) [1–5, 11, 13].

Figure 3 shows the mean spectra (dislocated) of CHA (~958 cm^{-1}) on control and treated animals. The intensity of the Raman shift is directly related to the concentration/incorporation of CHA by the bone. So, higher intensity represents higher concentration of CHA. Basal readings showed a mean value of 1,234±220.1. Groups IRF + biomaterial (B) + laser (L) showed higher readings (3,521± 2,670) and group IRF + B the lowest (212.2±119.8, Fig. 4). Table 2 shows a summary of the statistical analysis.

Discussion

The animal model used on the present investigation is usual as the responses observed during bone repair in rabbits are similar to the ones seen in humans [1–5, 11, 13]. Raman spectroscopy can be used to access the molecular constitution of a specific tissue and then classify it according to differences observed in the spectra [23–25]. Several studies found elsewhere on the literature has shown successful use of Raman spectroscopy as a diagnostic tool for healthy, diseased or healing bones [1–5, 11, 13, 23–26].

Of all of the biomaterials used to improve bone healing, HA is the most investigated one on both clinical and histological grounds. This biomaterial has been found to be effective in improving bone formation. Several techniques for the correction of bone defects have been proposed, amongst them the use of several types of grafts and membranes, and the combination of both techniques. It is accepted that although HA has osteoconductivity, the repair of the defect may be slow because of the need of the graft to be reabsorbed, slowing down the process. It is clear that the use of a graft prevents fibrosis of the lesion and also protects the cavity and acts as a framework for the deposition of newly formed bone [1, 6, 15, 16].

On the present study, the assessment of the CHA (~958 cm^{-1}) was chosen as it represents the peak of phosphatated hydroxyapatite, which is one of the major components of mineralized bones [1–5]. Our previous results

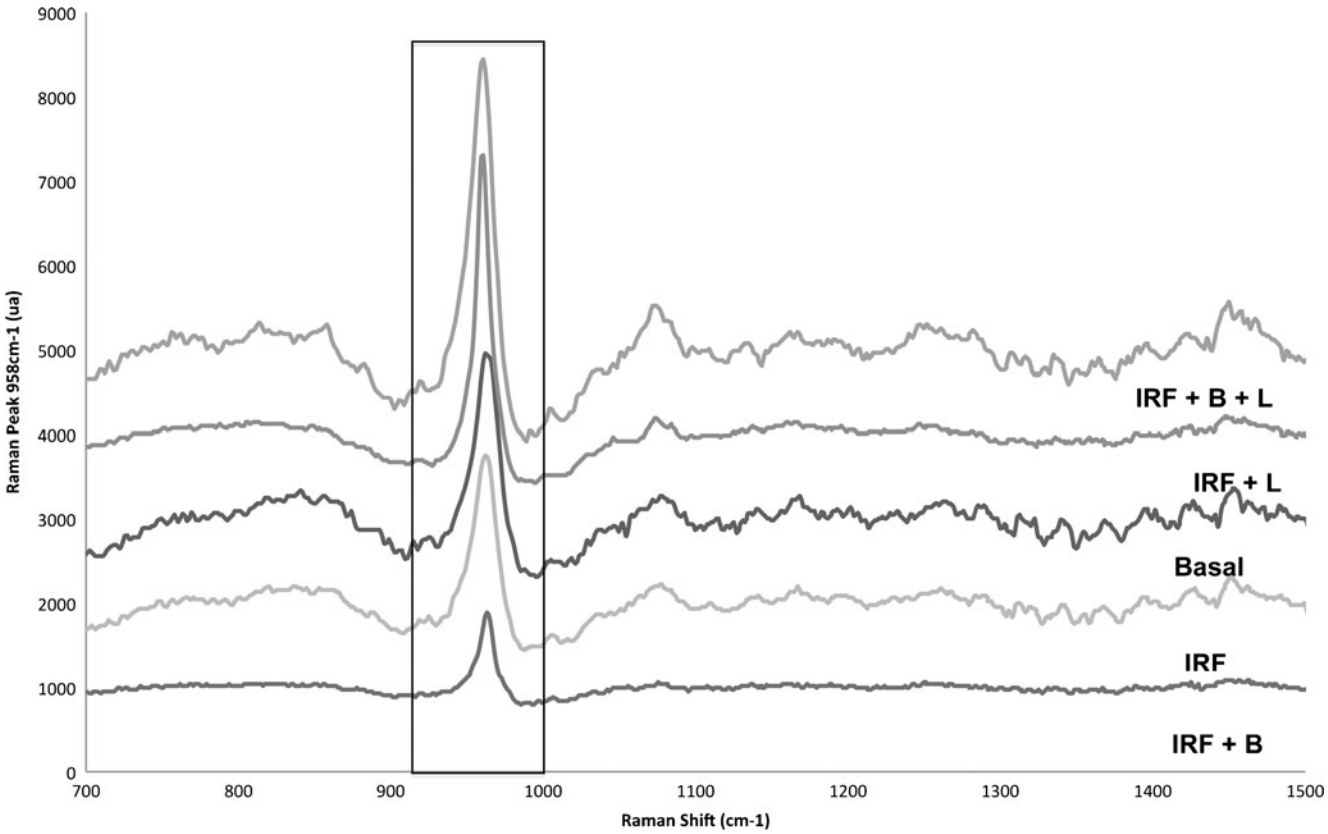

Fig. 3 Mean spectra of CHA (~958 cm^{-1}) on control and treated animals

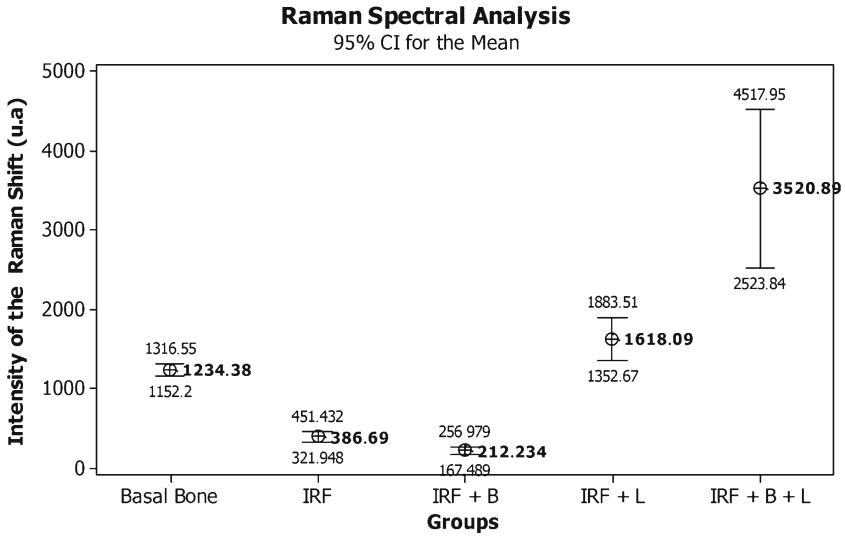

Fig. 4 Graphical representation of the Raman data. Basal readings showed a mean value of 1,234±220.1. Groups IRF + B + L showed higher readings (3,521±2,670) and group IRF + B the lowest (212.2±119.8)

indicate that NIR LPT is effective to improve bone repair mainly due to its higher penetration into bone when compared to visible laser light [1–18].

On this study we used a similar model previously reported by our team in which a different biomaterial was used (2). On the previous study we assessed the level of CHA (~958 cm^{-1}) on complete fractures animals treated with IRF treated or not with low-level laser therapy (LLLT) and associated or not to BMPs and GBR using Raman spectroscopic analysis, and concluded that the use of near-infrared (NIR) LLLT associated to BMPs and GBR was effective in improving bone healing on fractured bones due to increased levels of CHA as determined by Raman data [2].

On the present investigation, higher Raman peaks on animals treated with IRF + B + L were observed. This might be attributable to increased levels of CHA on the site. This finding is aligned with previous reports in which other type of biomaterial containing CHA associated to laser light was used [1–5, 11, 13].

It is important to make clear that the use of this experimental protocol was due to the fact that it is known that the isolated use of HA, GBR, and laser brings benefits on many clinical situations. However, the association used on the present study remains poorly explored elsewhere in the literature. We decided to assess the combination of different protocols in order to verify if the positive effect already described on the literature could be improved or if disadvantages could be ameliorated.

The results of the present study indicated that the level of CHA on nontreated bone differed significantly from the observed on animals of all experimental groups. This result was expected as the pattern and stage of repair differed among the different treatments used. It would be desirable that the intensities were close to the one observed on normal bone that would represent that the mineralization of the fractured bone. This was not observed when the IRF was used alone probably due to the fact that at the end of the experimental period, the gap of the fractured site was still on a more precocious stage of repair. The animals treated with IRF only showed significant lower intensity of CHA when compared to all experimental groups except the one seen on animals treated with the association to the biomaterial, when the intensity was lower. The use of the membrane also caused a decrease of the peak. This was probably due to an attenuation of the passage of light through the membrane reducing the amount of energy delivered to the tissue. The reason for this may be due to the fact that the insertion of the graft widened the gap and caused a delay of the mineralization as more time would be necessary to complete the repair of a larger area. However, when the biomaterial was associated to the laser, the intensity of the CHA peak increased in an impressive manner (Table 2).

The association of the IRF with the laser light caused a significant increase of the intensity of the peak of CHA when compared to all groups except when the laser was

Table 2 Mean and standard deviation of the peaks of the Raman peak of CHA (~958 cm^{-1}) on the groups

Group	Mean±SD
Basala	1,234.4±220.1bcde*
(IRF)b	386.7±73.4acde*
(IRF + B)c	212.2±119.8abde*
(IRF + L)d	1,618±711abce*
(IRF + B + L)e	3,521±2,670abcd*

Letters on right hand side column indicate the occurrence of significant differences between groups on the left hand side column

*$p \leq 0.05$

associated to the biomaterial and IRF that presented the highest intensity being also this difference significant. This finding is fully aligned with previous reports from our group that showed that irradiated bone, mostly with IR wavelengths, shows increased osteoblastic proliferation, collagen deposition, increased deposition of CHA and bone neoformation when compared to non-irradiated bone. The protocol used on the present study is similar to those used on previous reports from our team using different models [1–18]. In conclusion, the results of the present investigation are important clinically as spectral analysis of bone component evidenced increased levels of CHA on fractured sites by using the association of laser light to a ceramic graft.

References

1. Carvalho FB, Aciole GTS, Aciole JMS, Silveira-Junior L, Santos JN, Pinheiro ALB (2011) Assessment of bone healing on tibial fractures treated with wire osteosynthesis associated or not with infrared laser light and biphasic ceramic bone graft (HATCP) and guided bone regeneration (GBR): Raman spectroscopy study. Proceedings—SPIE 7887: 7887OT-1-7887OT-6
2. Lopes CB, Pacheco MTT, Silveira-Junior L, Cangussu MC, Pinheiro ALB (2010) The effect of the association of near infrared laser therapy, bone morphogenetic proteins, and guided bone regeneration on tibial fractures treated with internal rigid fixation: a Raman spectroscopic study. J Biomed Mat Res Part A 94:1257–1263
3. Pinheiro ALB, Lopes CB, Pacheco MTT, Brugnera A, Zanin FAA, Cangussú MCT, Silveira-Junior L (2010) Raman spectroscopy validation of DIAGNOdent-assisted fluorescence readings on tibial fractures treated with laser phototherapy, BMPs, guided bone regeneration, and miniplates. Photomed Laser Surg 28:89–97
4. Lopes CB, Pacheco MT, Silveira-Junior L, Duarte J, Cangussú MCT, Pinheiro ALB (2007) The effect of the association of NIR laser therapy BMPs, and guided bone regeneration on tibial fractures treated with wire osteosynthesis: Raman spectroscopy study. J Photochem Photobiol B 89:125–130
5. Pinheiro ALB, Aciole GTS, Cangussú MCT, Pacheco MTT, Silveira-Junior L (2010) Effects of Laser phototherapy on bone defects grafted with mineral trioxide aggregate, bone morphogenetic proteins, and guided bone regeneration: a Raman spectroscopic study. J Biomed Mater Res A 95:1041–1047
6. Pinheiro ALB, Gerbi MEMM, Limeira Junior FA et al (2009) Bone repair following bone grafting hydroxyapatite guided bone regeneration and infrared laser photobiomodulation: a histological study in a rodent model. Lasers Med Sci 24:234–240
7. Gerbi MEMM, Marques AMC, Ramalho LMP et al (2008) Infrared laser light further improves bone healing when associated with bone morphogenic proteins: an in vivo study in a rodent model. Photomed Laser Surg 26:55–60
8. Pinheiro ALB, Gerbi MEMM, Ponzi EAC et al (2008) Infrared laser light further improves bone healing when associated with bone morphogenetic proteins and guided bone regeneration: an in vivo study in a rodent model. Photomed Laser Surg 26:167–174
9. Torres CS, Santos JN, Monteiro JSC, Amorim PG, Pinheiro ALB (2008) Does the use of laser photobiomodulation, bone morphogenetic proteins, and guided bone regeneration improve the outcome of autologous bone grafts? An in vivo study in a rodent model. Photomed Laser Surg 26:371–377
10. Gerbi MEMM, Pinheiro ALB, Ramalho LMP (2008) Effect of IR laser photobiomodulation on the repair of bone defects grafted with organic bovine bone. Lasers Med Sci 23:313–317
11. Lopes CB, Pinheiro ALB, Sathaiah S, Da Silva NS, Salgado MA (2007) Infrared laser photobiomodulation (830 nm) on bone tissue around dental implants: a Raman spectroscopy and scanning electronic microscopy study in rabbits. Photomed Laser Surg 25:96–101
12. Weber JBB, Pinheiro ALB, Oliveira MG, Oliveira FAM, Ramalho LMP (2006) Laser therapy improves healing of bone defects submitted to autogenous bone graft. Photomed Laser Surg 24:38–44
13. Lopes CB, Pinheiro ALB, Sathaiah S, Ramalho LMP (2005) Infrared laser light reduces loading time of dental implants: a Raman spectroscopy study. Photomed Laser Surg 23:27–31
14. Gerbi MEMM, Pinheiro ALB, Ramalho LMP et al (2005) Assessment of bone repair associated with the use of organic bovine bone and membrane irradiated at 830 nm. Photomed Laser Surg 23:382–388
15. Pinheiro ALB, Limeira Júnior FA, Gerbi MEMM (2003) Effect of low level laser therapy on the repair of bone defects grafted with inorganic bovine bone. Braz Dent J 14:177–181
16. Pinheiro ALB, Limeira Júnior FA, Gerbi MEMM et al (2003) Effect of 830-nm laser light on the repair of bone defects grafted with inorganic bovine bone and decalcified cortical osseous membrane. J Clin Laser Med Surg 21:383–388
17. Pinheiro ALB, Oliveira MAM, Martins PPM (2001) Biomodulação da cicatrização óssea pós-implantar com o uso da laserterapia não-cirúrgica: estudo por microscopia eletrônica de varredura (Biomodulation of peri-implant bone repair with lasertherapy: SEM study). Rev FOUFBA 22:12–19
18. Silva Junior N, Pinheiro ALB, Oliveira MGA, Weismann R, Ramalho LMP, Nicolau RA (2002) Computerized morphometric assessment of the effect of low-level laser therapy on bone repair: an experimental animal study. J Clin Laser Med Surg 20:83–88
19. Alpha C, O'Ryan F, Alessandro S et al (2006) The incidence of postoperative wound healing problems following sagittal ramus osteotomies stabilized with miniplates and monocortical screws. J Oral Maxillofac Surg 64:659–668
20. Erdogan Ö, Esen E, Ustun Y et al (2006) Effects of low-intensity pulsed ultrasound on healing of mandibular fractures: an experimental study in rabbits. J Oral Maxillofac Surg 64:180–188
21. Chritah A, Lazow SK, Berger JR (2005) Transoral 2.0 mm locking miniplate fixation of mandibular fractures plus 1 week of maxillomandibular fixation: a prospective study. J Oral Maxillofac Surg 63:1737–1741
22. Le Guéhennec L, Layrolle P, Daculsi G (2007) A review of bioceramics and fibrin sealant. Eur Cell Mater 8:1–11
23. Movasaghi Z, Rehman S, Ihtesham U, Rehman IU (2007) Raman spectroscopy of biological tissues. Appl Spectros Rev 42:493–541
24. Nogueira GV, Silveira Júnior L, Martin AA A, Zângaro RA, Pacheco MTT, Chavantes MC, Pasqualucci CA (2005) Raman spectroscopy study of atherosclerosis in human carotid artery. J Biomed Opt 10:031117–1–031117–7
25. Penel G, Delfosse C, Descamps M, Leroy G (2005) Composition of bone and apatitic biomaterials as revealed by intravital Raman microspectroscopy. Bone 36:893–901
26. Carden A, Morris MD (2000) Application of vibrational spectroscopy to the study of mineralized tissues (review). J Biomed Opt 5:259–268