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Raman ratios on the repair of grafted surgical bone defects irradiated or not with laser (λ780 nm) or LED (λ850 nm)

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ABSTRACT

This work aimed to assess biochemical changes associated to mineralization and remodeling of bone defects filled with Hydroxyapatite + Beta-Beta-tricalcium phosphate irradiated or not with 2 light sources. Ratios of intensities, band position and bandwidth of selected Raman peaks of collagen and apatites were used. Sixty male Wistar rats were divided into 6 groups subdivided into 2 subgroups (15th and 30th days). A standard surgical defect was created on one femur of each animal. In 3 groups the defects were filled with blood clot (Clot, Clot + Laser and Clot + LED groups) and in the remaining 3 groups the defects were filled with biomaterial (Biomaterial, Biomaterial + Laser and Biomaterial + LED groups). When indicated, the defects were irradiated with either Laser (λ780 nm, 70 mW, $\Phi \sim 0.4$ cm^2) or LED (λ850 ± 10 nm, 150 mW, $\Phi \sim 0.5$ cm^2), 20 J/cm^2 each session, at 48 h intervals/2 weeks (140 J/cm^2 treatment). Following sacrifice, bone fragments were analyzed by Raman spectroscopy. Statistical analysis (ANOVA General Linear Model, $p < 0.05$) showed that both grafting and time were the variables that presented significance for the ratios of ∼1660/∼1670 cm^{-1} (collagen maturation), ∼1077/∼854 cm^{-1} (mineralization), ∼1077/∼1070 cm^{-1} (carbonate substitution) and the position of the ∼960 cm^{-1} (bone maturation). At 30th day, the ratios indicated an increased deposition of immature collagen for both Clot and Biomaterial groups. Biomaterial group showed increased collagen maturation. Only collagen deposition was significantly dependent upon irradiation independently of the light source, being the amount of collagen I increased in the Clot group at the end of the experimental time. On the other hand, collagen I deposition was reduced in biomaterial irradiated groups. Raman ratios of selected protein matrix and phosphate and carbonate HA indicated that the use of biphasic synthetic micro-granular HA + Beta-TCP graft improved the repair of bone defects, associated or not with Laser or LED light, because of the increasing deposition of HA.

1. Introduction

Bone is a dynamic, highly organized and specialized biological tissue composed of metabolically active cells (osteoblasts, osteocytes, osteoclasts) integrated into a rigid framework (extracellular matrix – EM). EM consists primarily of collagen fibrils, mineral phase (phosphate and carbonated hydroxyapatite [HA] crystals) and water [1]. The presence of apatite crystals in the bone increases both strength and rigidity of the collagenous matrix as well as serving as repository storage of Ca, Mg and inorganic phosphate ions. Bone mineral crystals possess a relatively narrow range

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of sizes and properties that differ with the site, age, health status, mineral composition, crystallite size, perfection and orientation [2].

The loss of bone fragments or the removal of necrotic or pathologic bone, or even some surgical procedures, creates bone defects, which may be too large for spontaneous and physiological repair. Autologous bone is the most common type of graft used in oral implantology, prosthetic surgery, on the treatment of congenital defects and reconstructive procedures of the bones [3,4].

Biomaterials are used for replacing autologous grafting being HA the most commonly used one. HA based biomaterials can be manufactured in different composition and shape [5,6]. It may be used isolated, associated to a membrane (guided bone regeneration), or mixed to an autologous bone graft [3]. Both autologous and xenografts have been used to provide a framework or to stimulate new bone formation. The integration of these grafts has been shown to be positively stimulated by irradiation with selected wavelengths [3,5,7–9].

Since the 1980s calcium phosphate materials (amorphous, dicalcium ($CaHPO_4{\cdot}2H_2O$), tricalcium phosphate ($Ca_3[PO_4]_2$), octacalcium ($Ca_4H[PO_4]_3 2.5H_2O$), and calcium hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) and composite grafts (combination of two or more calcium phosphates) have been clinically used [10]. Recently Beta-tricalcium phosphate (Beta-TCP) has been extensively studied for bone repair [11,12].

Differently from HA in its calcium-to-phosphorus ratio, TCP exists in two main allotropic forms: Alpha and Beta (according to the temperature at which they form). Beta-TCP also shows improved solubility and degradation properties when compared to HA. These important properties contribute for its use as a scaffold material for bone repair. Its relatively quick reabsorption ultimately allows its complete replacement by newly formed bone [11].

Several studies have demonstrated that near infrared (NIR) Laser Phototherapy (LPT) is the most suitable for bone repair due to its higher penetration depth in the bone tissue when compared to visible laser light [13]. Although the use of LPT on the bone healing has been growing steadily and several studies have demonstrated positive results on the healing of bone tissue, there are still a few reports on the association of LPT and biomaterials [3–5,7–9,13–22].

Our team has shown improved bone repair on different experimental models using LPT by evaluating the intensities of selected Raman spectral bands [5,7,13–16]. There are evidences that some LED wavelengths possesses similar photo-stimulatory effects to the observed when LPT is used [23,24]. Further investigative studies to establish an optimal protocol as well as studies to clarify the mechanisms of the effect of LED light on different tissues are important and desirable [25,26]. Despite the growth of successful reports of applications of different phototherapies in many areas, their use in bone repair associated with grafting with biomaterials needs to be further studied.

Recent studies have shown that LED Phototherapy (LED-PT) induces a quicker repair process as well as the presence of a newly formed bone of quality. These features were observed in a previous study in which similar parameters were used with LPT or LED-PT [27]. It seems likely that the effects of LED irradiation are similar to those of the Laser and that the mechanism involved being also related to the light absorption by the cytochrome-C-oxidase [28,29]. Our group has shown favorable results of the use of LED-PT or LPT on both stimulating and acceleration bone repair [27,30] as the mechanism seems similar [31,32]. These have been regarded to the stimulation of the proliferation of both osteoblasts and fibroblasts by both light sources. Fibroblasts have the capacity to synthesize collagen, main organic component observed during bone repair [14]. On the other hand, our team has also demonstrated, using Raman spectral analysis, that infrared LED-PT improved deposition of HA in healing bone grafted or not with a biomaterial [30].

Vibrational techniques such as Raman spectroscopy have been used to provide information on the metabolic status of bone grafted or not, including the mineral content and matrix composition of the tissue. This may provide early indication of the success or failure of the grafting [8,33]. Raman spectra bring numerical information of chemical bonds by inelastic scattering of light during the polarization of the electron cloud. The unique molecular information of the biochemical composition provided by a bio-tissues or changes on it may be assessed quickly and nondestructively by this technique [33–37].

Bone composition may be studied using the calculation of ratios between selected bands: mineral-to-matrix ratio, mineral crystallinity, carbonate-to-phosphate ratio, and collagen cross-linking (Table 1) [1,38–49]. Mineral-to-matrix ratio assesses the bone mineral content related to the organic phase and is calculated by the ratio of phosphate ν_1 band to that of one of the collagen bands (amide I, proline/hydroxyproline or CH_2) [1,43–46]. Mineral crystallinity has also been considered as an indicator of the size of mineral crystals and depends on both disorder and strain in the crystallites. It is calculated by either width or position of the phosphate ν_1 band [49]. Carbonate-to-phosphate ratio is calculated by calculating the intensity ratio of the carbonate ν_1 to phosphate ν_1 peaks. This ratio shows the carbonate substitution (content) of the phosphate HA in the bone [1,43,50,51]. Human bone mineral differs in composition from stoichiometric hydroxyapatite (HA) [$Ca_{10}(PO_4)_6(OH)_2$] in that it contains additional ions, of which carbonate is the most abundant species. It generally is agreed that the carbonate ion can substitute at two sites in the apatite structure, namely the hydroxyl and the phosphate ion positions, giving A and B-type CHA, respectively. A-type CHA can be formed by high-temperature reaction of HA with carbon dioxide gas, yielding a highly crystalline material. On the other hand, B-type CHA often is formed by precipitation, and carbonate in the B site has been found to reduce crystallinity and precipitate size. There are different models that has been used to describe carbonate substitution in precipitated apatites. Some of these models assume the occurrence of a coupled substitution whereby the negative charge caused by replacing PO_4^{3-} with CO_3^{2-} is balanced by the substitution of a monovalent ion (typically sodium) for calcium. Other models effect the charge balance required by the substitution of carbon ate for phosphate by calcium vacancy formation or by a mixture of these two mechanism [1,43,50,51]. These two indicate the bone composition and maturation. Collagen cross-linking is related to both quality and maturation of collagen and it is estimated by calculating the ratio of either the area or the intensity of amide I at $\sim$1670 cm^{-1} to the $\sim$1660 cm^{-1} [41–43].

As the used of biomaterials and phototherapies has been shown capable of improving the repair of different types of bone defects, it was hypothesized that the combination of both techniques would be clinically efficacious on quickening and improving the repair of bone defects. The aim of this study was to evaluate both mineralization and remodeling of bone defects grafted or not with micro-granular HA + Beta-TCP associated or not with two phototherapies (Laser and LED), through the assessment of the ratios of selected Raman peaks (Raman metrics): quality of collagen (QC): $\sim$854/$\sim$881 cm^{-1} (collagen type II/collagen type I, related to the quality of collagen and collagen cross-linking), mineral-to-matrix Ratio (MMR): $\sim$960/$\sim$854 cm^{-1} and $\sim$960/$\sim$1454 cm^{-1} (HA/collagen, related to the quantity of mineralization related to the collagen matrix), and bone composition (BC): $\sim$1070/$\sim$960 cm^{-1}, and $\sim$1070/$\sim$1077 cm^{-1} (carbonated HA/phosphate HA, related to B-type carbonate substitution in HA lattice), and positions and bandwidth of $\sim$960 cm^{-1} (bone maturation/HA crystallinity).