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Bone Regeneration with Wharton´s Jelly-Bioceramic-Bioglass Composite

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Abstract

The aim of this development is to optimize a bone substitute (BS) for use in tissue engineering. This is achieved through the combination of three phases in a biocomposite (BCO), in which each is reabsorbed in the site of implantation and replaced by autologous bone (patient's own). The inorganic phases are composed of irregular particles (150-300 microns) obtained by milling and sieving of a biphasic bioceramic (BC) of hydroxyapatite (HA of bovine origin) with 40 % (wt.) β-tricalcium phosphate (β-TCP, obtained by chemical synthesis) and Bioglass type 45S5 (45SiO_2 -24,5CaO - 24,5Na2O - 6P2O5, in % wt.). Instead, the organic phase consists of collagen extracted from Wharton's jelly (part of the human embryonic tissue) from physical and chemical self-developed process. The BC is produced by mixture of HA and β-TCP (< 45μm) and molding by gelcasting with albumin in aqueous solutions, drying and sintering at 1200°C for 2 hours. The BG is obtained from the mixture of the oxides, melting at 1350°C and cast onto metal. Each phase and BCO is subjected to studies by electron microscopy (SEM and EDS), X-ray diffraction (DRX) and infrared spectrometry (FT-IR). The biocompatibility is evaluated by in vivo studies using the laminar implant model in Wistar rats (n=40). Histological samples show high biocompatibility and ability to integrate with the bone tissue. 30 days after implantation, the material is completely reabsorbed and the bone regeneration process starts, the primary objective. The process developed allows the synthesis of a new BS with excellent biological properties for clinical use.

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Keywords: Biocomposite, Tissue engineering, Bioceramics, Bioglass, Wharton jelly.

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1. Introduction

Bone is a dynamic type of specialized and highly vascularized connective tissue, which consists of 70% mineral phase and 30% of organic phase. The first consists of hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), a crystalline calcium phosphate that provides high stiffness and compressive strength to bone tissue. In contrast, the second is mostly type I collagen, a fibrillar protein that gives tensile strength and flexural strength.

The bone can be injured in several clinical situations (cysts, tumors, hormonal diseases or trauma), generating bone defects, which can be repaired by the body, with the activity of bone-forming cells (osteoblasts). However, bone tissue engineering (BTE) must be required, when the defect exceeds a certain critical size and regeneration is affected. The clinical demand increases with population growth and BTE is applied in implant dentistry, craniofacial, plastic and orthopedic surgeries. Autologous bone graft remains the "gold standard", because of its biological properties: osteoconductive (provides the ideal environment for cell proliferation), osteoinductivity (promotes tissue formation), and osteogenesis. However, its limitation arises from the need for a donor site, which means prolonging patient rehabilitation, increases the risk of infection and morbidity. BTE is based on three pillars: the use of three-dimensional matrices or scaffolds, cells and biomolecular signals, with the ultimate goal of regenerating bone tissue by Martinez et al. (2012).

The scaffolds are based on BTE, and they must be able to be replaced by host tissue, be porous, to allow vascularization and not to cause immunogenic response in the patient. The synthesis of matrices has used various synthetic biodegradable polymers (polylactic acid, polyglycolic, polyvinyl alcohol or combination thereof, etc.), limited by its low mechanical strength and hydrolysis in the biological medium, leaving acid residues. Also, natural polymers are used (polysaccharides such as alginates, chitin, cellulose compounds, etc.) and proteins (collagen, silk, etc.), which are considered as the most important today. Collagen has excellent biocompatibility, providing structural support to the repair process. However, its mechanical behavior is poor, because it is often combined with reinforcing inorganic phases by Pek et al. (2008).

The above limitations have motivated this work, where it is proposed the use of bioceramic particles, BC, and bioactive glass, BG, scattered in a biological matrix. Inorganic phases chosen are known for their osteoconductive capacity (Martinez et al. (2012)), in the case of BC, HA + 40% β-TCP (β-tricalcium phosphate, $\beta\text{-}Ca_3(PO_4)_2$), with a degradation rate according to biological time. Instead, BG, with a composition close to *Bioglass*^{TM} (Pek et al. (2008)) exhibits osteoinductivity ability (Yuan et al. (2001)), related to the stimulation of certain genes, which increase the population of osteoblasts in the bone defect site. In addition, BG is capable of forming surfaces that can establish chemical bond with bone.

Moreover, the selected source of collagen is the Wharton's jelly, WJ, which is the extracellular matrix of the umbilical cord of mammals. In addition, WJ is a major candidate to enhance the regenerative capacity, because of it is rich in growth factors by Nekanti et al. (2010) and Taghizadeh et al. (2011).

The combination of above phases should produce a synergistic effect in relation to the biological and mechanical behavior, resulting in a bone tissue-like biocomposite, BCO. This concept is based on biomimetics (Pek et al. (2006)), in order to be adapted to the requirements of the organism and clinical application. This work is unprecedented in the known literature, and seeks to enhance the regenerative capacity of each phase.

2. Experimental Procedure

2.1 Synthesis and Characterization of Inorganic Phases

HA is obtained from fresh bovine bone with an identifiable traceability. The bone is cut into 5-20 cm^3 pieces, subjected to a chemical wash for removal of hemoglobin content and adipose tissue. This process is repeated by immersion in aqueous solutions at 40°C with ammonium bicarbonate (NH_4HCO_3, at 4% in wt.), sodium hydroxide

(NaOH at 5% in wt.), hydrogen peroxide (H_2O_2 at 1% wt.) and acetic acid ($C_2H_4O_2$ at 1% wt.), technical grade products (Química Oeste, Argentina), with periodic renewal of the solutions for 48 hs. The degreased and free of blood bone is dried under forced hot air flow (with centrifugal blower) within a vertical metal muffle provided with electrical heaters. There, the remaining collagen is subjected to pyrolysis at temperatures of 250-500°C for 3 hs., leaving a product covered with a carbon film, which is oxidized in an electric furnace at 900 °C for 3 hs. The result is spongy and cortical white bone, just constituted by HA according to phase analysis. This material is dried grounded in a ball mill (in 10 liters porcelain pot with 1 “diameter AISI 420 hardened steel balls) for 5 hs. The powder is manually screened to separate particle fractions of less than 45 μm size.

The grains of β-TCP phase are synthesized in Biomaterial Development and Innovation Center (Riga Technical University, Latvia) by means of modified chemical precipitation, by addition of an acid solution of H_3PO_4 to a suspension of $Ca(OH)_2$, both products of analytical grade (Merck, Germany), maintaining the pH 6 at 22°C under constant agitation. The precipitate is filtered, oven dried, sintered at 1100°C for 2 hs., and ground and screened to the same procedure of the HA.

Bioceramic, BC, is obtained by blending β-TCP and HA powders, with 40% (in wt.) of first phase, they are made in ethyl alcohol fluid bed, by means of ball mill for 3 hs, (in airtight PVC flasks with 1 "diameter porcelain balls). The extracted mud is dried in an oven and subjected to light grinding. This powder is dispersed in a 15 % (in wt.) aqueous solution of egg albumin, maintaining a fraction of 50% (in wt.) of solid, and homogenized by stirring at 300 r.p.m. for 10 min. The obtained slurry is cast into silicone rubber molds, which are wrapped in PVC film, and immersed in a thermostatic bath, maintained at 90°C for 1 h. The gelled pieces (10 x 10 x 50 mm^3) are oven dried, and finally taken to an electric furnace, where they are heated to 10°C min^{-1} up to the sintering temperature of 1200°C for 2 hs., followed by cooling at slower rate to room temperature.

Bioactive glass, BG, is obtained by mixing 45 g of SiO_2, 43.32 g of $CaCO_3$ of 38.01 g of Na_2CO_3, 30.27 g of $Na_2HPO_4.2H_2O$, analytical grade (Anedra, Argentina) in a ball mill. This formulation is chosen to obtain a composition similar to 45S5 *Bioglass*^{TM} ($45SiO_2$-24,5CaO-24,5Na_2O-6P_2O_5 in mol %). This mixture is subjected to thermal cycle in an electric furnace inside 500 cm^3 Pt crucible. The cycle is composed by heating at 10°C min^{-1} up to 1350°C (well above to glass melting point, close to 890°C), where it stays for 2 hs., and the molten material is cast onto a steel plate to produce rapid quenching. The BC and BG are grounded and screened in order to retain 150-350 μm grains, adequate for no generation of post implantation inflammatory reactions.

2.2 Preparation of Biological Matrix and Biocomposite, BCO

The biological matrix is extracted from the processing of human Wharton's jelly, WJ, following a series of physicochemical steps that are the result of own development, in the stage of a patent development. The placentas and umbilical cords are provided by the Carrillo Municipal Hospital, in agreement to a protocol approved by the Research and Ethics Committee. In general terms, the process includes steps of: hemoglobin washing (at -4°C), microbiological control, hydrolysis, inactivation, enrichment, esterification and de-ionization. The product is granulated from an aqueous solution into spray dry equipment, maintained at temperatures below 42°C. The entire process is conducted in sterile areas of a pharmacological laboratory to avoid contamination with pyrogens. The material is sterile packaged and stored in vacuum until its use.

BC particles are dry blended with 5% (by weight) of BG and are dispersed in an aqueous solution of the processed WJ at 10% (in wt.), so that the proportion of inorganic solid is 30% (in wt.) of dispersion. The viscous mass is molded by injection through a syringe into the rubber moulds which allow obtaining blocks (3x18x18 mm^3). Dehydration of the material is conducted by a forced flow of sterile filtered air for 24-48 hs., giving biocomposite stiff pieces, BCO. These parts are packaged in medical paper and polyethylene bags and sterilized with gamma irradiation dose of 10 kGy.

The tenacity of the pieces of BCO is suitable for cutting with manual saw and preparing 1.5 x 1.5 x 6 mm^3

micro-implants in the surgery room for implantation in rats.

2.3 Structural and Chemical Characterization

The BCO and its component phases are analyzed by means of the following techniques:

The microstructures of BC, BG and BCO were analyzed by a scanning electron microscope (SEM, *Zeiss Supra* model 40, Germany), including the local chemical analysis by energy dispersive spectroscopy on Ag plated samples (EDS, *Oxford Instruments*, UK). The identification of the crystalline phases was performed by X-ray diffraction (XRD, *Rigaku*, Japan) using $Cu_{K\alpha}$ radiation (λ = 0.1542 nm) with Ni filter, a vertical goniometer using a 2θ angle range of 20-70°, with a step of 0.2°.

The chemical nature of bonds were studied by infrared Fourier transform spectroscopy (FT-IR, *Perkin-Elmer*, model Frontier®, USA) in the range of 400-4000 cm^{-1} after 250 scans: Granular samples were mixed KBr crystals, transparent to infrared radiation, in ratios smaller than 1% (in wt.), for measuring the absorbance.

2.4 In vivo assays and histological processing

40 male Wistar rats (150 ± 5) g body weight were used for the tests, which were fed ad libitum, following guidelines established by the National Institutes of Health (USA) for the use and care of laboratory animals (NIH 1985) and the guidelines of the Faculty of Dentistry, Buenos Aires University (Res. 352/02 and 694/02 CD).

The animals were anesthetized with a solution of 8 mg of ketamine hydrochloride (*Ketalar ®, Parke-Davis*, USA) and 1.28 mg of Xylacina (*Rompun*, Bayer, Germany) per 100 mg of body weight. Both tibiae were shaved in the area of the tibial ridge with electric shaver, making an incision (approximately 1.5 cm. long) to the same level with a scalpel blade. The subcutaneous tissue, muscles and ligaments were dissected in order to expose the lateral aspect of the tibia in the diaphyseal area. A 1.5 mm diameter bore was made with a round bur by manual rotation, in order to prevent overheating and subsequent tissue necrosis. The implementation methodology employed is the "*laminar implant test*" described by Cabrini, where BCO micro-implants were introduced into both tibiae, parallel to the major axis of each, to be processed in two different ways. The procedure was finished with spaced point sutures without employing antibiotics.

The animals were sacrificed by an overdose of anesthetic, in groups of 10 to 7, 14 and 30 days post-implantation. The tibiae were resected and fixed in formalin solution 10%, and imaged. They were included in methyl methacrylate and paraffin. They were sectioned manually with a saw, at the level of BCO implantation area, making three slices (approximately 500 μm thick) perpendicular to the long axis of the tibia. Histological sections were obtained by grinding to obtain sheets of 50-70 μm thick, through a lens grinding machine (Silmar Optical Products, Argentina), and finished with water sandpaper AX-51 (Abrasives Argentinos SAIC) and glycerin, to a suitable surface finish. Three sections from each tibia were colored with 1% Toluidine blue - technical sodium borate solution and fixed with 5% ammonium molybdinate solution. The examination montage was made with analytical grade glycerin (Merck Chemical, Argentina), employing a light microscopy (*OM Carl Zeiss*, Axioskop 2 MOT model, Germany). The remaining cuts were vacuum metalized with 20 nm Ag film for study by SEM and EDS.

3. Results and discussion

3.1 Structural and Chemical Characterization

XRD analysis of BG and BC confirms the crystalline and amorphous nature, respectively (Fig.1). BC is composed of HA (file 34-0010) and β-TCP (file 55-0898) phases. Instead, BG has a single broad peak centered at 31.8° corresponding to SiO_2 (file 05-0492) (ICDD (2005)), with no evidence of other oxides, whose elements are detected by EDS.

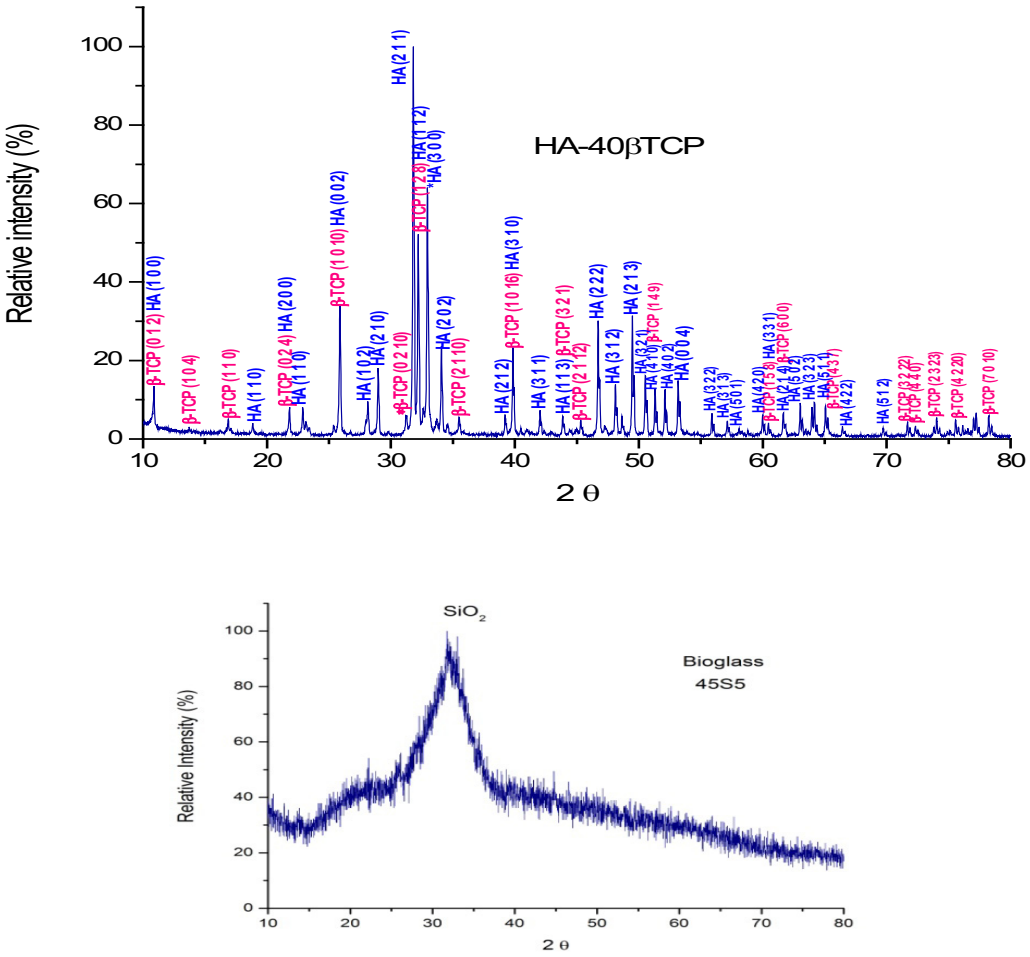

Fig. 1. Diffractograms of BC (up) and BG (beneath).

Analysis of FT-IR spectra of BC shows the predominant presence of the group PO_4^{3-} in the band 960-1109 cm^{-1}, and 602, 565 and 475 cm^{-1} (Guo et al. (2013) and Sha et al. (2011)); while the OH^-group .is in 3573 (Guo et al. (2013)) and Scalera et al. (2013)), 872 and 631 cm^{-1} (Guo et al. (2013)). In contrast, the spectrum of BG presents only some peaks that can be associated with the nominal composition of *Bioglass*, although the spectrum is rich in absorption peaks (Fig.2). The vibrations of the PO_2 group can be seen at 1416 and 1445 cm^{-1} (Qian et al. (2009)), and a trace PO vibration at 595 cm^{-1} (Ma et al. (2010)), while those associated with Si-O-Si amorphous silicate are visible at 1092 and 451 cm^{-1} (Qian et al. (2009) and Ma et al. (2010)).

The biological matrix exhibits the functional groups of the amides, characteristic of the macro-molecules of the poly-peptide's own collagen type I and II. Thus, amide I is present at 1656 and 1159 cm^{-1} (Boryskina et al. (2011)), II in 1539 cm^{-1} (Xu et al. (2010)), III in 1452 and 1244 cm^{-1} (Campos Vidal et al. (2011)), A at 3468 cm^{-1} and B at

2968 cm^{-1} (Campos Vidal et al. (2011)). Other functional groups (CH_3, CH_2, CH) can contribute to the absorption at 1339 cm^{-1} (Boryskina et al. (2011)), being the least clear association at 1090 cm^{-1}, further, may correspond to the vibrational states of C-C and C N (Boryskina et al. (2011)) (Fig.2). However, such information is not enough to say that the collagen matrix is present and therefore most likely corresponds to isolated poly-peptide chains resulting from the denaturation caused by chemical treatments of WJ.

The FT-IR analysis of BCO shows overlapping absorption peaks and bands of BC phases and biological matrix, which were individually detected before. The interaction between BC crystals and biological matrix, probably containing some amino acid residues of the poly-peptide chains, could be attributed to the phosphate band between 980 and 1150 cm^{-1} (Chang et al. (2003)) (Figs. 2-3). The presence of BG in BCO is not discernible in any case, because of its low concentration of BG (5% by weight of the mixture of inorganic particles). However, BG particles are individualized in the EDS spectra and in histological sections observed by SEM (Fig. 4). In addition, they exhibit BC particles partially reabsorbed in implanted BCO.

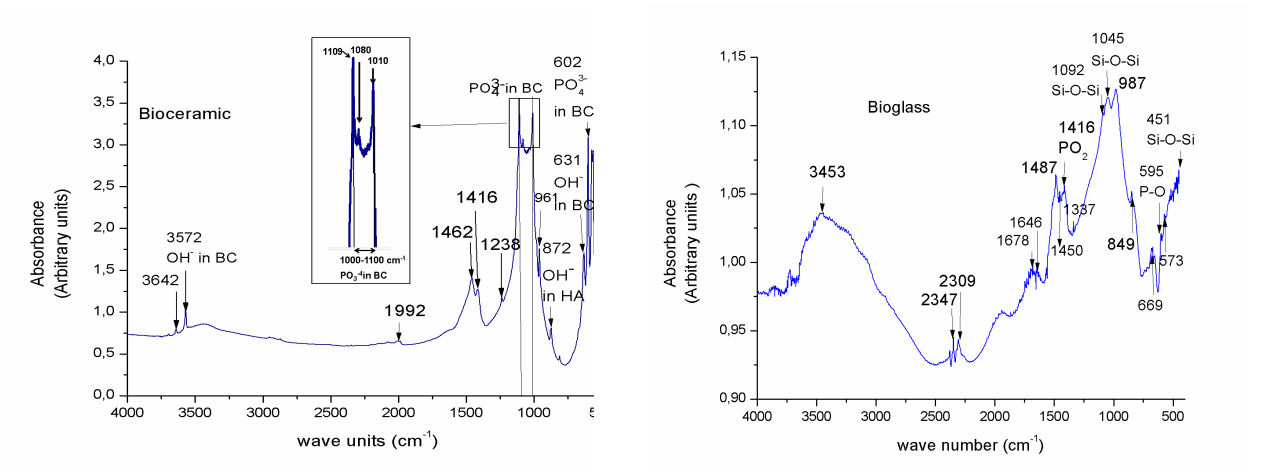

Fig. 2. FT-IR spectra of bioceramic (left) and bioglass (rigth).

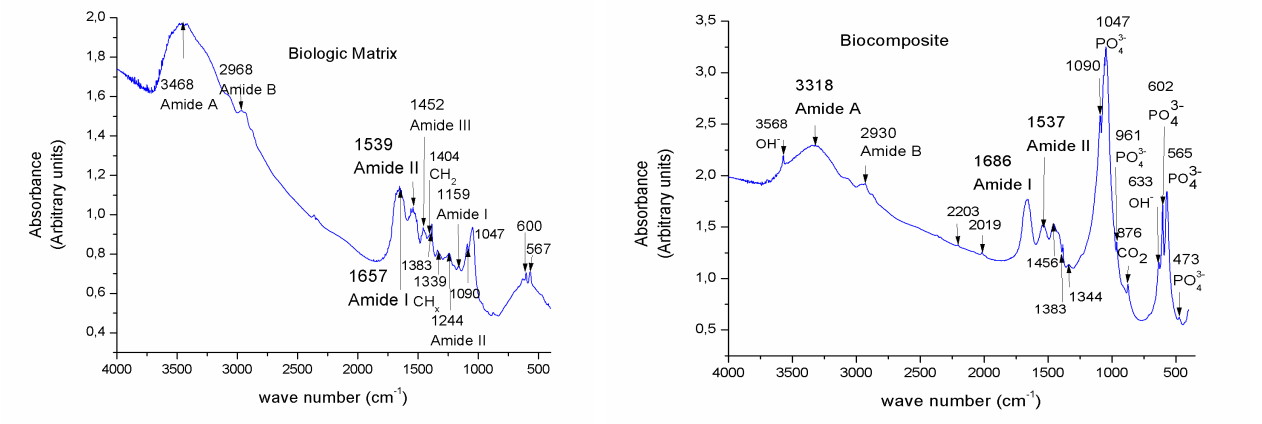

Fig. 3. FT-IR spectra of biological matrix (left) and biocomposite (rigth).

3.2 Biological Response

BCO has excellent biocompatibility, unremarkable in postoperative immediate and mediate stages (inflammatory reactions). Radiographic studies showed the presence of BCO at the site of implantation in all tibias, whose radiopacity decreases with implementation time, indicating the progressive resorption as the bone is regenerated, as shown in Fig. 5. In addition, it is found no trace of the original incision on the surface of tibia after 30 days of implantation.

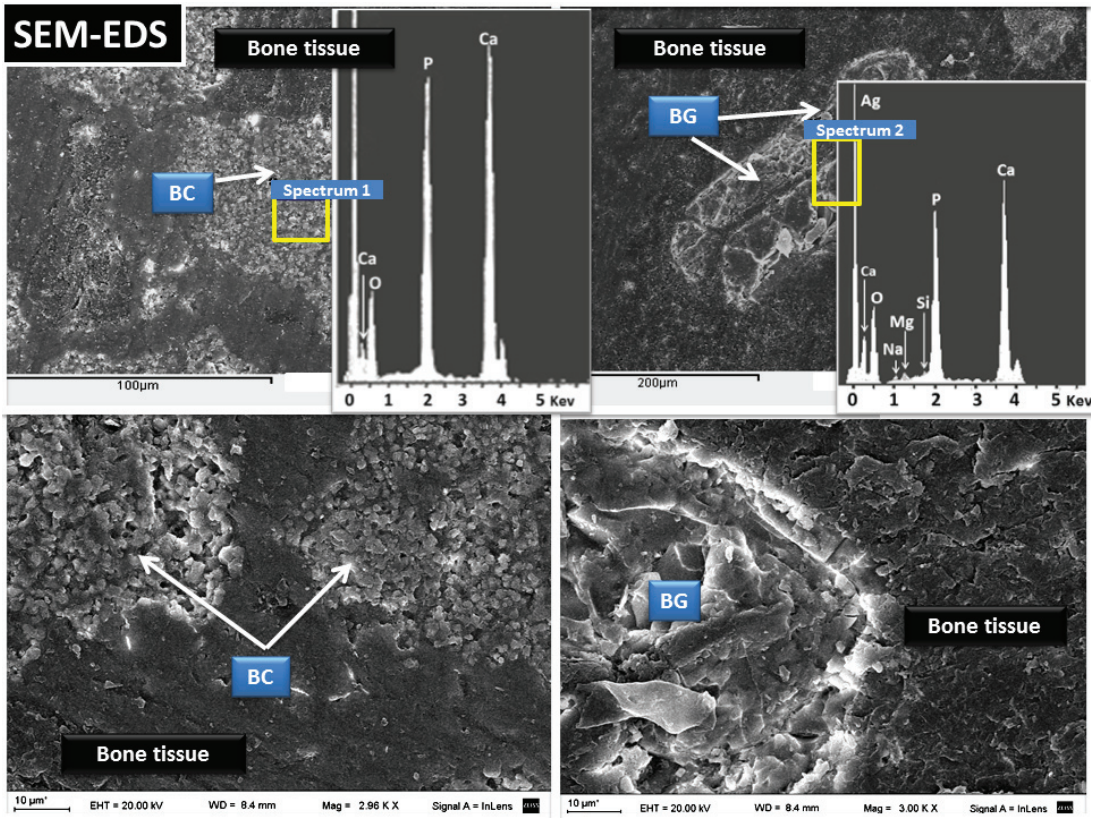

Fig. 4. BCO matrix with BC and BG particles

Histological sections did not detect the presence of inflammatory infiltrate in the evaluated experimental times (7, 14, 30 days post-implantation). The persistence of residual clot is observed in the implantation of BCO, and the presence of trabecular bone and reticular tissue, 7 days after implantation. Moreover, the persistence of particles of irregular morphology of BC and BG is visible, seeming that BC grains reabsorption occurs more rapidly, as shown in Fig.6. Laminar bone tissue is surrounding BG and BG particles, forming continuous and intimate adaptation morphology interface, from post-implantation 14th day. The volume of bone tissue grows over time in the BCO implantation.

3. Conclusions

The work demonstrates the technological feasibility of producing composites with high capacity for bone regeneration. These integrate Wharton's jelly biological matrix with bioceramics and bioglass particles, a

combination that is unprecedented in the literature. These results will be validated by clinical trials in humans, and allow the production of bone substitutes in the country in order to replace imported commercial products with comparable clinical response.

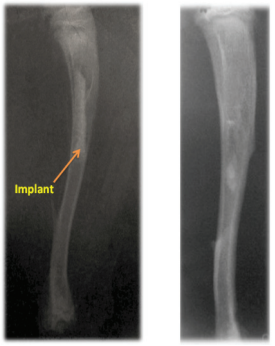

Fig. 5. Tibial radiographs of immediate and 30 days after implantation.

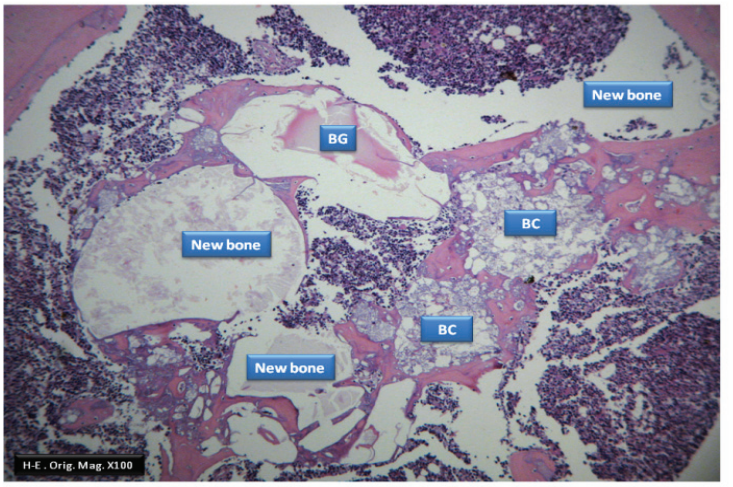

Fig. 6. Histological section showing the partially disaggregated BC particles, BG particles, and formed bone tissue at 30 days of implantation.

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