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Lectinhistochemistry Evaluation of Rabbit's Tibia Implanted with Macroporous Biphasic Ceramic Implants

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Abstract. Many techniques are used to assess biomaterials implants, always intending to measure osseointegration success and tissue response facing the implanted material. Calcium phosphates are widely used as biomaterial and a major component of bone. Many processing methods have been used to achieve porous materials to allow bone ingrowth with an osteoconductive scaffold for bone. To obtain the macroporous BCP implant it was processed by direct consolidation using the protein-action technique, a globular protein based consolidation with ovalbumin. The samples were sintered at 1250ºC for 30 minutes, after sintering samples were cut in 4mm diameter cylinders, with 73% volume of porosity and mean pore size ranging about 100 μm. In the present work the macroporous BCP of HAp:β-TCP is assessed after bone implantation in rabbit's tibia by lectinhistochemistry (LHC) technique. Lectins are proteins from non-imune origin which binds with strong specificity carbohydrates, LHC is a technique which mark histologically carbohydrates present in glycoproteins of cells. The macroporous BCP cylindrical samples were implanted in male rabbits tibia to the evaluation of biocompatibility and osseointegration in a period of 2 weeks to 4 weeks. After euthanasia of rabbits, tibia samples from the surgery site were taken and fixed with formalin, decalcified, dehydrated and embedded with paraffin to perform histological slides for both morphological and molecular evaluation. The morphological evaluation were performed on histological slides stained with Haematoxilin and Eosin (HE), while for molecular evaluation LHC was performed on histological slides using the lectins PNA, UEA-1, WGA, sWGA and RCA-1 (Vector Labs). All samples osseointegrated well with the bone and the neoformed bone surrounding the implant took the shape of its surface. The implants also allowed bone ingrowth inside the pores towards the center of implant, characterized by islets of round bone present in the HE stained slides.

Introduction

Calcium phosphates (Ca-P) are crystalline ceramics widely used as bone fillers and scaffolds biomaterials, due to their similarities with natural bone mineral content and the induced healing response when directly interacting with bone tissue, since hydroxyapatite is one of major inorganic component of bone. The features of biocompatibility, osseoconduction, osseoinduction, resorption and stability are desirable to the success of implantation and are intrinsic of Ca-P ceramics. The combination of two Ca-P forming a biphasic ceramic (BCP), hydroxyapatite (HAp) and beta-tricalcium phosphate (β-TCP) intend to improve the biological response facing the biomaterial [1,2,3].

Porous biomaterials with interconnected porosity for bone implantation are aimed to act as scaffolds. They are ought to enable and provide a friendly environment to cell migration, angiogenesis and differentiation. Although no magic number exists, representing perfect pore size to allow these events, it is consensus that a pore size ranging between 50 and 500 μm is ideal to propitiate sustained growth and nourishment of newly formed bone tissue [3,4,5].

Besides pore size, the topography and surface specific area of the material are very important not only to allow the bone cells to anchorage appropriately, but also to induce the proper adsorption of negatively charged proteins, which will pad the biomaterial. As one of the first events after implantation, it propitiates the recognition of surface by cells and further matrix deposition [6].

Lectins are a heterogeneous group of proteins from non-immune source that bind with high specificity and sensitivity carbohydrates and molecules of carbohydrates linked to proteins or lipids, expressed as glycoconjugates [7,8,9]. The use of them in lectinhistochemistry (LHC) is a reliable laboratory method to perform the identification of specific sugars on the cell surface and in any sugar end containing molecule from sections of formalin-fixed paraffin-embedded tissues (FFPET). Although LHC has been long employed to characterize stored products in tissue samples mainly in lysosomal storage diseases due to their ability to locate, identify and distinguish carbohydrates in tissues sections, it recognizes glycoconjugates in any cell regarding the lectin type and presence of corresponding sugar [10]. Cells express glycoproteins in their membranes for many reasons, including intercellular communication and signal transduction, which pictures the state of the cell at that particular moment, concerning the surrounding environment and how they respond to stimulus, expressing their glycoproteins towards the microenvironment them are within [11].

Studies regarding the lectin bidding pattern (LBP) focus on how cells express themselves facing the alterations present at any particular moment. Tumor cells evaluated for their lectin bidding pattern, using LHC technique, has shown of great value not only to differentiate normal cells from cancer cells, but also to understand the cell communication within the tumoral microenvironment, with the same purpose a bone LHC evaluation for LBP of titanium based porous implants in rats has been performed [12,13,14]. The altered expression of membrane glycoproteins presents a particular LBP, which can be used to evaluate the cell behavior. The better understanding of what may be expressed by these resulted patterns is of great relevance, since the presence of a specific glycoprotein may relate to a specific cell function which can be desirable or not. The work has the aim to, with a molecular approach, recognize the glycoconjugates and patterns of cell expression of bone tissue when facing a macroporous biphasic implant. Thus evaluating the osseointegration morphology and also molecular behavior.

Materials and Methods

The obtaining of BCP macroporous implants was performed by direct consolidation using the protein-action technique, a globular protein based consolidation with ovalbumin. The BCP was compounded of hydroxyapatite - HAp (Strem Chemicals) and β-tricalcium phosphate, β-TCP (Fluka). The route that comprises foaming and setting through protein action, a globular protein, albumin of eggwhite (ovalbumin) was used [1]. The dispersion of HAp and β-TCP powder aqueous suspension was performed by an anionic dispersing agent (Dispex A40, ammonium polyacrylate - Ciba), the mixture was foamed, stirring with a double-blade mixer, aided by a nonionic surfactant (Genaminox, dimethyl alkyl amine oxide - Ciba) to stabilizes the foam, albumin was added to promote gelation by heat action. The resulting mixture was cast into petri dish like shaped moulds and put in kiln, desiccator and subsequently annealed to promote degradation of the protein. Finally specimens were sintered in oxidative atmosphere with dwell time at 1250˚C/30 minutes. Specimens with porosity in the range of 73% vol were used to cut the cylindrical samples with a diamond coated core drill of 4 mm. The mean pore size present on the samples was of 100 μm.

For care and use of laboratory animals, the guidelines and regulations of Center of Biomedical Sciences, CB-IPEN, was observed. Five adult albino New Zealand male rabbits (~2.5 kg) were anaesthetized by intravenous femoral injection of 1mg/kg xylazine chloride and 20mg/kg sodium pentobarbital 3%. The area of implantation was prepared and cortical proximal tibia bone defect was drilled in bone (4 mm) and filled with the BCP implant. The periosteum and skin were closed with simple uninterrupted sutures. Antibiotic therapy (0.5 ml of benzilpenicylin of 1.200.00 U.i.n.) was carried out during the period of 48 h. Two animals were kept alive for 2 weeks (15 days, early euthanasia) and three animals were kept alive for 4 weeks (30 days, late euthanasia) for this study, the subjects were enumerated from #1 to #5 (#1, #2 representing 2 weeks and #3, #4, #5 4 weeks).

After euthanasia, samples of the bone-implant were immersed in neutral solution of formalin 10% for at least 30 days. Fixed, the samples were decalcified in solution of formic acid 5%, and then processed in series of alcohol to dehydrate, followed by xylene the hydrophobic clearing agent and finally infiltration, embedding with histological paraffin to form the FFPET blocks. The blocks were cut in microtome to obtain thin slides sections of tissue (5 μm), which were used to perform conventional histological staining with Hematoxylin-Eosin (HE) and the LHC technique.

LHC was performed using five lectins (Vector Laboratories Inc. Burlingame, Ca, USA) with different specificity, they are listed on Table 1. After deparaffination of the tissue slides sections, they were hydrated in alcohol series following incubation in 3% hydrogen peroxide in methanol for 30 minutes at room temperature to block the endogenous peroxidase, afterward the slides were then rinsed 3 times with 0.01 M phosphate-buffered saline (PBS), pH 7.2, for 3 minutes each. Then the slides were treated with 0.1% bovine serum albumin in PBS for 30 minutes, prior to the incubation with lectins. The sections were then incubated with biotinylated lectins at room temperature for 2 hours, finished the incubation each lectin was washed separately, 3 times for 3 minutes each, with 0.01 M PBS. After the lectins were washed, followed the incubation with streptavidin-peroxidase complex (R.T.U.; Vector Laboratories Inc. Burlingame, Ca, USA), for 30 minutes at room temperature. The chromogen system with diaminobenzidine, Liquid DAB+® (Dako North America Inc. Carpinteria, Ca, USA) were used to stain the lectins in the tissue slide for 30 seconds, when the brownish color developed on the positively marked slides. All sections were counterstained with Mayer's hematoxylin. Each lectin was used at a dilution of 40 μg/ml with 1% bovine serum albumin in PBS, except for PNA, which was applied at a concentration of 20 μg/ml. The reactivity to lectins were estimated in a reactivity score and arranged in a scale of -(0) unreactive, +(1) discrete, ++(2) moderate, and +++(3) intense.

Table 1. Lectins used and their carbohydrate specificity. Adapted from Goldstein and Hayes [5]

Lectin	Acronym	Carbohydrate specificity
Ulex europaeus-I	UEA-1	α-L- Fucose
Triticum vulgaris	WGA	β -D- N-acetylglucosamine > N-acetylneuraminic acid
Succinil-WGA	sWGA	$(\beta$ -(1-4)-D- N-acetylglucosamine$)^2$
Arachis hypogaea	PNA	β -D- Galactose (1-3) N-acetylgalactosamine
Ricinus communis-I	RCA-1	β -D- Galactose > α -D- Galactose

Results and Discussion

Through the morphological evaluation of HE stained slides, subtle changes between 2 weeks and 4 weeks subjects could be perceived, and those were concerned to the duration of the healing process, which is expected, since healing, repair and regeneration of tissues develops through time. In all subjects was observed bone growth surrounding the implant towards the bone marrow compartment (Fig. 1A), 2 weeks subject #1, besides this outer growth, was present an overt bone ingrowth inside the pores, represented by rounded and crescent moon or banana shaped islets of bone tissue (Fig. 1B), 4 weeks subject #3, in several phases of differentiation depending on the deepness towards the implant center core. Another remarkable feature observed was the presence of newly formed blood vessels, organized with thin walls of endothelial cells, filled with blood cells and surrounded by different degrees of round cells or even eosinophilic substance in the same crescent moon or banana shaped form (Fig. 1C), 2 weeks subject #2, of what can be related to an initial bone cells migration and differentiation. The amount and location of new blood vessels and crescent moon or banana shaped forms cells inside the implant pores, as well as the amount and location of the rounded and crescent moon or banana shaped islets of bone tissue, give us a clue of how this process of new bone formation is taking place inside the implant. Presumably the first step is the establishment of blood vessels through the interconnected pores, thus allowing to the bone cells to populate its surroundings and differentiate towards the center of the pore. In a simplistic explanation it shows why rounded bone islets are located mostly in the outers portions of implant,

followed by the bold crescent moon or banana shaped bone islets with a reminiscent blood vessel inside them, while in the core of implant plumped blood vessels are present with the first draw of progenitor bone cells in the crescent moon or banana shape organization.

The LHC evaluation resulted in a homogenous LBP, despite one sample deviated a little from others, this difference took place in the subject #5 because when trimmed, the FFPET block remained with few implanted material differing from the analysed site of others subjects. The panel of LBP with the respective intensity score is represented on Table 2.

Table 2. Lectin binding pattern with their respective reactivity intensity for each subject

	2 weeks (#1)	2 weeks (#2)	4 weeks (#3)	4 weeks (#4)	4 weeks (#5)
Lectin					
PNA	++ (2)	+++ (3)	+++ (3)	+++ (3)	++ (2)
UEA-1	- (0)	- (0)	- (0)	- (0)	- (0)
RCA-1	++ (2)	++ (2)	++ (2)	++ (2)	+ (1)
WGA	++ (2)	++ (2)	++ (2)	++ (2)	++ (2)
sWGA	+ (1)	+ (1)	+ (1)	+ (1)	- (0)

Because the biomaterial implanted was the same for all the subjects the tissue response was similar for those, both morphologically and molecular. Without consider the function of the glycoproteins marked in this study, was observed some remarkable aspects of LBP, that does not relate with the intensity or positivity, but with the lectin selectiveness of which tissue and what cells are marked by them. One aspect relates to PNA lectin, which stained the new formed bone matrix in contrast to the old bone matrix without staining of the subjects euthanized in 2 weeks (Fig. 1D), nevertheless the 4 weeks (Fig. 1E) subjects had the new formed bone matrix losing its staining. This shows that one glycoprotein involved in the process of bone healing and growth is expressed during the early phase of the healing process, but diminish as it progress through time.

On the other hand for RCA-1 the same differentiation is present in the 2 weeks subjects (Fig. 1F), the lectin marked positively new formed bone, yet when 4 weeks subjects (Fig. 1G) were observed the lectin is marking old bone matrix and new with less distinction. RCA-1 has been related as an apoptotic marker [15], in this case it appears that the spread of staining may be related to the remodeling process taking place in the bone as the stimulus of the implant remains present.

For sWGA there was observed no difference between 2 weeks (Fig. 1H) and 4 weeks subjects (Fig. 1I), nevertheless it showed to be a good marker for mature osteocytes in the mineralized bone matrix (Fig. 1H), but also for the osseous progenitor cells in the differentiation process (Fig. 1J) inside the implant.

The decalcification of samples removed the inorganic material present on the tissue, thus it is assumed that the BCP implants were removed along, even though is evident an amorphous meshwork presence, poorly stained with HE (Fig 1A, 1B, 1C), still with LHC this amorphous meshwork markedly stained with PNA, RCA-1 and WGA lectin (Fig. 1D, 1E, 1F, 1G, 1K). This was very unexpected since its assumed that no material would be present, however as indicated by the LHC, glycoproteins are present because the sugars alone would be washed during the series of alcohol processing, thus their presence is from an ongoing adsorption process or even secreted from cells present in the healing process. The clear areas where the amorphous meshwork is not present or dislocated from the bone, is believed to be an artifact produced through the processing and staining methods of the tissue slides sections.

The UEA-1 lectin (Fig. 1L) did not stain positively in any of the samples.

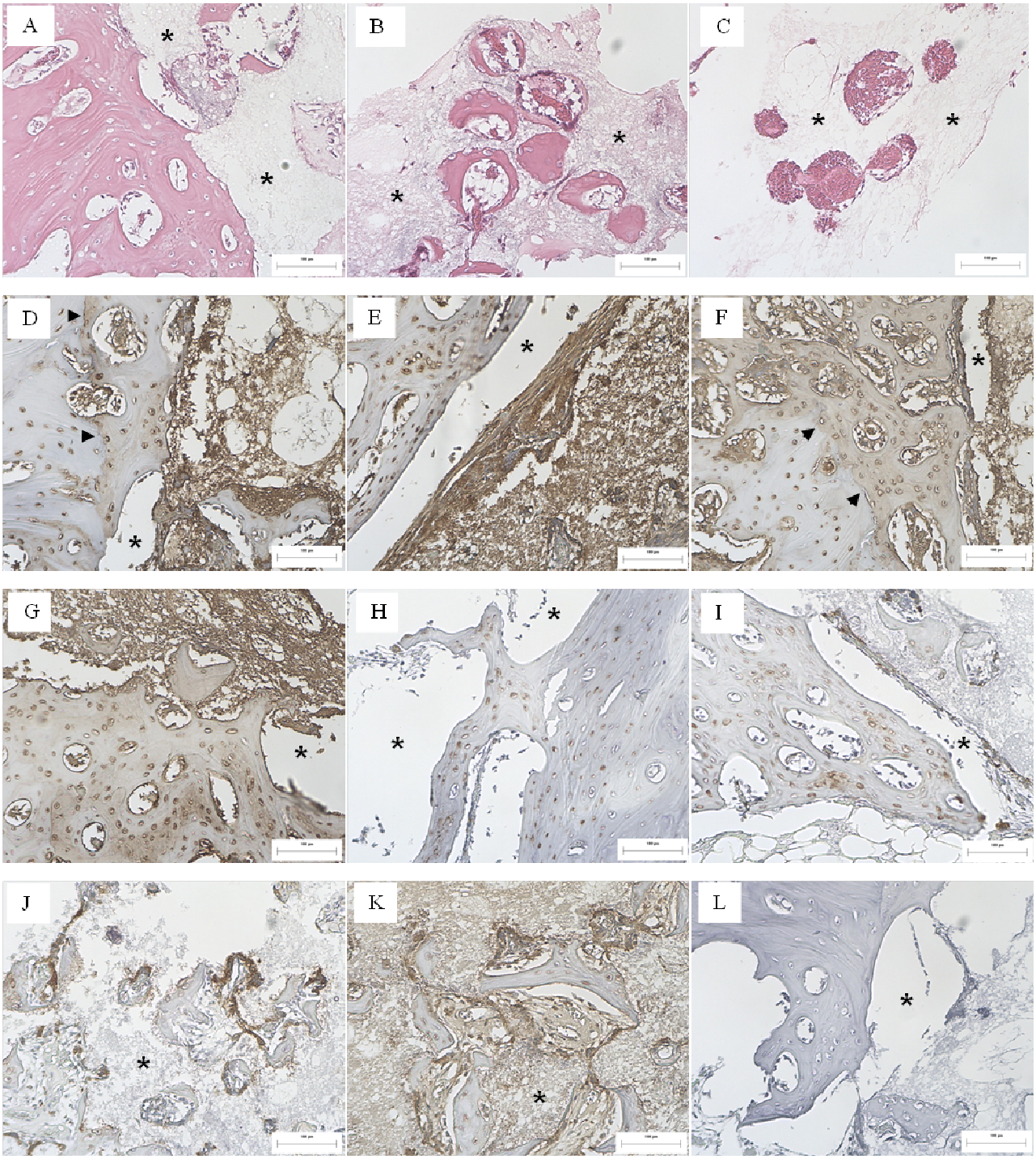

Figure 1. Bone tissue, rabbit, tibia. In all images the implant site is identified by *. A, HE staining; B, HE staining; C, HE staining; D, LHC staining, PNA ++(2), the arrow heads points to the new formed bone delimitation; E, LHC staining, PNA +++(3); F, LHC staining, RCA-1 ++(2), the arrow heads points to the new formed bone delimitation; G, LHC staining, RCA-1 ++(2); H, LHC staining, sWGA +(1); I, LHC staining, sWGA +(1); J, LHC staining, sWGA +(1); K, LHC staining, WGA ++(2); L, UEA-1 -(0) – Bar 100 µm.

Conclusions

The period of implantation did interfered in the lectin binding pattern of the bone tissue, for PNA and RCA-1, with selective binding of newly formed matrix against old bone matrix for both lectins in 2 weeks subjects. With 4 weeks subjects there was loss of matrix marking for PNA, and gain of matrix marking for RCA-1. The macroporous BCP presented good biological interaction as

for osseointegration, osseoconduction and osseoinduction, besides the angiogenic feature of the material. The lectin UEA-1 was negative for all samples and acted as an internal control for the LHC technique.

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