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Lectinhistochemistry evaluation of bone after implantation with macroporous titanium samples

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Abstract. Titanium and its alloys are widely used as biomaterials and interact well with bone tissue. In order, to evaluate more than just morphological osseointegration by histological slides the work aimed to approach a molecular evaluation of bone-implant using lectinhistochemistry (LHC), which binds with high specificity carbohydrates (sugar residues) presents in membrane glycoproteins with the use of lectins. The implanted samples were obtained by powder metallurgy, Ti-13Nb-13Zr alloy with and without gelatin. Pores were achieved by adding gellatin 5 wt% to the hydrogenated metallic powder, after near net shape processing, the samples were thermal treated in vacuum (300 ºC/90min) and sintered in high-vacuum (1150 ºC/14h). The samples were characterized for porosity (~30%), and subsequently were implanted in rat's femur bone. After 4 weeks of healing process, bone with implant were sampled to perform LHC in paraffin embedded tissue in histological slides using the lectins PNA, UEA-1, WGA, sWGA and RCA-1. All samples osseointegrated well with the bone, no fibrous capsule was present in the bone which was in contact with the implant. With the molecular approach of osseointegration, adjustments in the processing and structure of macroporous titanium based implants can be performed to achieve friendly structure.

Introduction

Titanium and its alloys are widely used as biomaterials, mainly to orthopedics and dentistry use. The employ of these metals on bone tissue, imply it to stay in direct contact with the corporal fluids and under constant load, challenged chemically, physically and mechanically [1]. Titanium and its alloys form a passivation layer (TiO_2) in their surface, which enhances its corrosion resistance when compared to other metals. Besides protection, this layer of oxide is considered to be responsible for the good interaction with cells, making viable to successfully osseointegrate the compound of bone tissue and metallic implant surface [2]. Despite the osseointegration dependence regarding the type of material (biocompatibility) this interaction of metallic implant and bone tissue also rely on the ability of absorb and transfer loads under mechanical stress (biofuncionality) [1]. To meet the needs so particular as of bone tissue, titanium alloy like the Ti-13Nb-13Zr had been developed with the purpose to approximate elastic modulus of bone (~10 to 30 GPa), since the alloy elastic modulus range around 78 GPa while titanium around 100 GPa. Implants with high elastic modulus promote tensions to concentrate at the adjacent bone tissue, thus enhancing the bone resorption process and consequently decreasing the lifespan of the implant [3].

Thus, clinically the success of a metallic implant relies on several factors. The topography is very important not only to allow the bone cells to proliferate appropriately regarding its shape, but also to induce the proper adsorption of negatively charged proteins, which will pad the biomaterial, in one of the first events after implantation, to propitiate the desirable recognition of padded surface by cells [4]. Although machined implants has had been used for many years, studies demonstrated that the modification of the topography pattern tends to also increase the direct contact of bone-implant and consequently the bond strength interface [5].

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Powder metallurgy provides a rough surface inherent of the process, but porosity can be added to improve the imbrication of bone to the implant, besides if the pores are interconnected there is possibility of bone ingrowth through it, increasing interaction of bone-implant. However, the pores and channels within the structure must have a proper size to allow not only the bone progenitor cell migration, but also their differentiation and nourishing through vasculature [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, also agglutinating and precipitating them. Lectins are involved in many cellular processes, such as a quality control for protein proper conformation, maturation and even transport, adhesion of infectious agents, recruitment of leucocytes and cell interactions in the immune system, as also malignancy and metastasis of tumor cells. They can be obtained and purified from an animal or vegetable origin, nowadays the number of available lectins is continuously increasing [7,8,9,10]. Lectinhistochemistry (LHC) is a reliable laboratory method to perform the identification of specific sugars on the cell surface and within the cytoplasm from sections of formalin-fixed paraffin-embedded tissues (FFPET) using purified lectins. LHC has been employed to characterize stored products in tissue samples due to their ability to locate, identify and distinguish carbohydrates in normal and pathological tissues [11]. Cells normally express glycoproteins in their outer membranes for purpose of intercellular communication and signal transduction, which is how they assimilate their surrounding environment and pears, how they respond to stimulus, express themselves towards each other and towards their microenvironment [12]. Although lectins are proteins of non-immune origin many times they also act to protect the body from infection or help the immune system to identify an aggressor, that is because its high specificity for sugars that are expressed by all normal cells, tumor cells and microorganisms like bacteria. One example is the mannose binding domain, in this case an animal lectin, expressed in the outer membrane of a phagocyte to help them to bind bacteria or other noxious organisms expressing membrane glycoproteins with mannose residue on their membrane, helping the opsonization [8].

Many studies of tumors cells evaluating their lectin bidding pattern, including LHC technique, is being performed by various groups. Lectin binding patterns in tumors was shown of great value not only to differentiate normal cells from cancer cells, but also to understand the cell communication within the tumoral microenvironment [13,14]. The altered expression of membrane glycoproteins presents a different set of cell to cell interactions and signals transduction, which, in fact represents the overt expression of an altered behavior inherent of tumor cells, who needs to evade, disrupt, invade and immortalize itself to sustain its corrupted existence [15,16,] The better understanding of these patterns helps to recognize the signs of biomaterial, implant, success based on the glycoproteins content of the respective tissue. Thus it is possible to evaluate the osseointegration not only by morphology, but also with a molecular approach.

Materials and Methods

The implants were obtained by powder metallurgy, from hydrides of titanium, niobium and zirconium, following being particulate and mixed in the stoichiometry of the alloy (Ti-13Nb-13Zr) to be processed with and without gelatin.

From the powder of the Ti-13Nb-13Zr alloy, were added 5 wt% gelatin powder (CAAL®), homogenized and dissolved in boiling water until high viscosity. The formed paste was cooled and put on kiln at 30 °C/24 h. After drying process, the material was broken into metal pestle and mortar coated with stainless steel and after this stage was selected by mesh sieve of 20.

The powders in its two formulations, with and without gelatin, were submitted to the cold isostatic pressing in silicone mold, with pressure of 140 MPa. Before sintering, the samples were heat treated in a vacuum furnace (10^{-2} mBar) at 300 °C/90 min, for the decomposition of gelatin and removal of carbon from it. Samples in crucible of alumina (Al2O3) and quartz tube were sintered to 1150 °C/14 h, in high-vacuum (10^{-5} mBar).

The samples were obtained in a cylindrical (5mmx2mm) desired size, near net shape, without machining. The implants were sterilized with a dose of 25 kGy through gamma radiation (Co60, Gammacell model 220 of the Nuclear and Energy Research Institute/IPEN).

For in vivo experiment, (approved by animal ethics committee under Project Nº 8/CEPA-IPEN/SP) nine rats *Rattus norvergicus*, male, Wistar, with approximate age of 10 weeks, were designated as animal model in this study, being allocated 3 animals per group, 2 experimental groups of implanted biomaterial and 1 control group, where only the surgery defect was performed with no material implanted. The period for the bone healing process was 4 weeks.

To the surgery, general anesthesia was performed with Ketamine (75mg/kg) + Xylazine (10mg/kg) administered via intraperitoneal. After surgery a single dose of morphine (10mg/kg) was applied to promote analgesia, reducing the stress of post-surgery pain and improving the welfare of animals. To avoid infectious bacterial contamination, a single dose of Pentabiotic (0.2ml/animal) intramuscular, was also injected. The surgery consisted of skin incision and muscle separation to access the periosteum and bone, in order, to provide access to the implant site. The implants' bed of insertion was drilled with spherical drill, driven by an electric motor (1,000 rpm and 30 N/cm), under constant irrigation (sterile saline solution). After the insertion of materials in both legs of experimental group, a simple interrupted stitch was done in the muscles to held them together and the skin was sutured with a simple continuous stitch both sutures used mono nylon (3-0 CR 3/8).

After 4 weeks, the euthanasia of animals was conducted in CO2 chamber. Soft tissues adjacent to the implanted area were dissected and a sample of 4 mm in the long axis of the femur (including the metallic implant) was removed with the aid of a cutting disk.

After sampling the bone-implant, they were immersed in neutral solution of formalin 10% for 21 days. Fixed, the samples were decalcified in solution of formic acid 5%, the metallic implant was displaced from the decalcified bone and after processing in series of alcohol and xylene the samples were embedded in 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 were employed and they are listed in 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 20 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 30 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. Types of lectin used and their carbohydrate specificity

Lectin	Acronym	Carbohydrate speficity†
Ulex europaeus-I	UEA-1	α-L-Fuc
Triticum vulgaris	WGA	β -D-GlcNAc > NeuNAc
Succinil-WGA	sWGA	(β -(1-4)-D- GlcNAc)^2
Arachis hypogaea	PNA	β -D- Gal(1-3) GalNAc
Ricinus communis-I	RCA-1	β -D- Gal > α -D-Gal

†Fuc = Fucose; Gal = Galactose; GalNAc = N-acetylgalactosamine; GlcNAc = N-acetylglucosamine; NeuNAc = N-acetylneuraminic acid. Adapted from Goldstein and Hayes [7].

Results and Discussion

Due to the removal of the implant to perform the FFPET slides necessary to LHC evaluation, the bone-implant interface could not be evaluated in these slides sections, but it is possible to observe with HE stained slides the convex irregular bone that was in contact with the implant without fibrous capsule (Fig. 1 A and B) and a concave smooth cortical bone which was not in contact with the implant on the opposite side (Fig. 1 A). The bone-implant interface, for the metallic implants presented here was evaluated previously and the data were published [6].

The scores representation are presented in the Figure 1 (C, D, E, F), which resulted on the Table 2. The intensities of reactivity are related to the amount of glycoprotein sugar residue present bond with the respective lectin.

Table 2. Types of lectin used and their respective reactivity score for each group

	Without Implant	Ti-13Nb-13Zr without Gelatin	Ti-13Nb-13Zr with Gelatin
Lectin			
PNA	++ (2)	++ (2)	++ (2)
UEA-1	- (0)	- (0)	- (0)
RCA-1	++ (2)	+++ (3)	+++ (3)
WGA	++ (2)	++ (2)	++ (2)
sWGA	- (0)	+ (1)	+ (1)

The porosity did not interfered in the lectin bidding pattern of the implanted samples, as observed on the Table 2. From both Ti-13Nb-13Zr alloy biomaterials samples, without gelatin and with gelatin, there wasn't difference in the lectin bidding pattern. Thus, although directly interacting with the bone, allowing its ingrowth toward the center of the implant [6], the pores did not interfered with the expression of membrane glycoproteins. However a different bidding pattern was observed in two lectins, RCA-1 and sWGA, differing the bone who undergone the surgical procedure only, from the bone which received the metallic biomaterial sample independently to the porosity content of the sample. This results leads to the presumed responsible for the altered expression of membrane glycoproteins, which is the Ti-13Nb-13Zr alloy itself.

The same way that glycoproteins and peptides added to biomaterials during processing, aid the integration of them with living tissues [4], in a reverse analysis if these recognition sites with interactive molecules are present and observed on the tissue that interacted with the biomaterial, it is a good prognosis regarding the biomaterial ability to interact with the living tissue.

With the molecular approach of osseointegration, adjustments in the processing, elements and structure of macroporous titanium based implants can be performed to achieve a friendly structure.

The larger reactivity for RCA-1 mends that the most abundant terminal sugar present in membrane glycoproteins of tested bone tissue was the β-D-Galactose, some authors found the expression of this sugar residue in cell membrane to be a cell marker related to signalling for apoptosis [17]. The β -D-Galactose abundance in the bone tissue does not relate to apoptosis, since the amount of osteocytes combined with the good fixation of the bone-implant previous to decalcifying samples prove against it, so this expression in bone tissue needs to be further evaluated, because it may involves the apoptosis signalling pathway, but inducing the bone remodelling by the biomaterial presence instead of apoptosis.

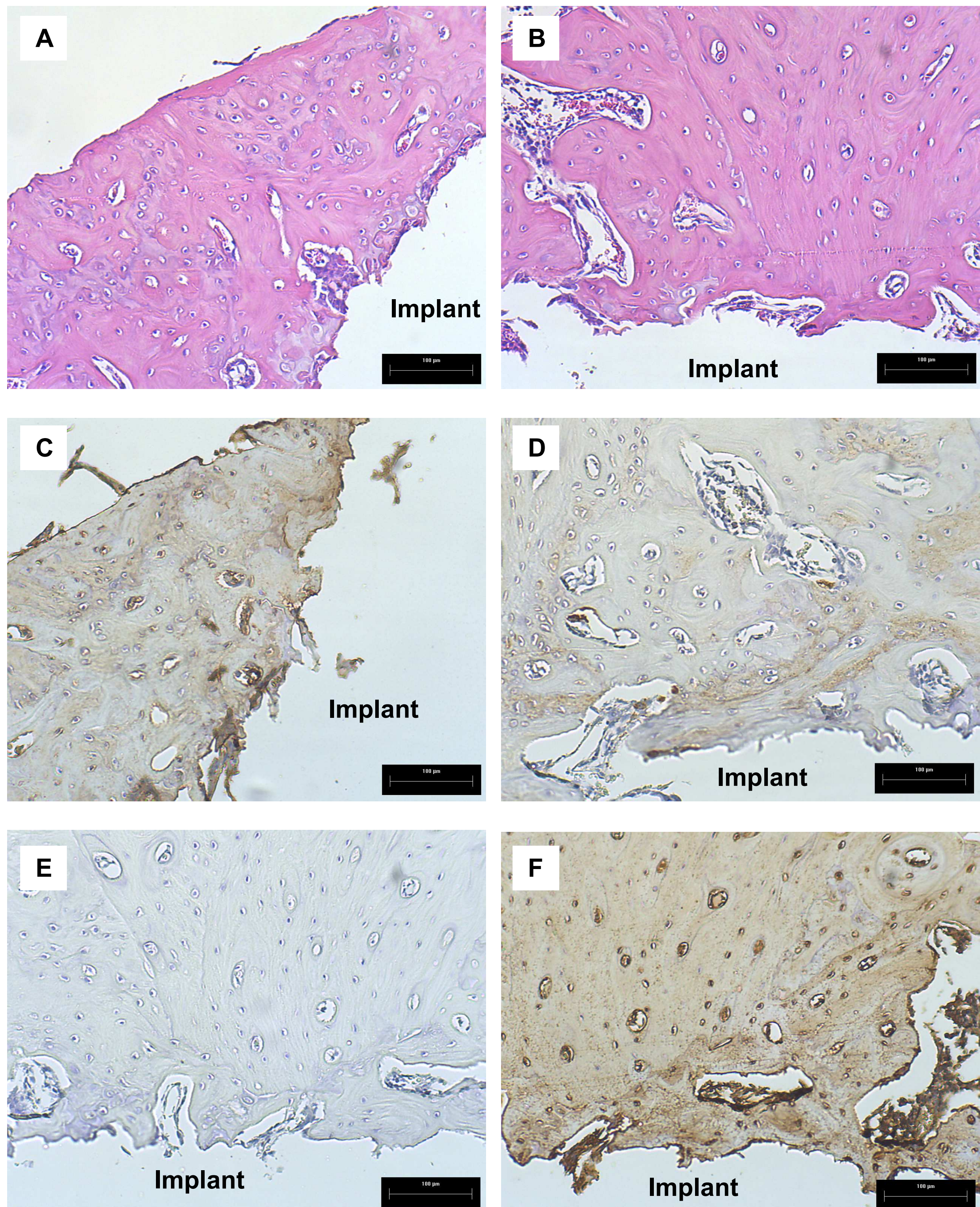

Figure 1. Bone tissue, rat, femur. In all images the implant site is identified. A, HE staining; **B,** HE staining; **C,** LHC staining, WGA ++(2); **D,** LHC staining, PNA +(1); **E,** LHC staining, UEA-1 -(0); **F,** LHC staining, RCA-1 +++(3) – Bar 100 µm.

Conclusions

The process of macropores obtaining with gelatin and the porosity itself did not interfered in the lectin binding pattern of the bone tissue, when compared both implanted Ti-13Nb-13-Zr materials.

There was difference in the lectin binding pattern of control group to both experimental groups for two lectins: sWGA and RCA-1. The lectin UEA-1 was negative for all groups and acted as an internal control for the LHC technique.

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