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Biocompatibility and osseointegration study of new prosthetic materials

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Summary

Objetive: Bone implants are increasingly used in clinical practice and, among the materials, Ti or its alloys are offer the best performance given their physicochemical properties. Alloys such as TiNbTa have been shown to improve the biomechanical characteristics of commercial pure Ti (c.p.), however, its osseointegration capacity needs to be evaluated. The objective of the present study was to assess the cytotoxicity and the adhesion, proliferation and differentiation capacity of osteoblastic cells in culture, influenced by discs of TiNbTa material versus Ti c.p.

Material and methods: At 4 and 7 days after culture, we analyzed the MC3T3 cell line, cell viability (AlamarBlue Cell Viability Reagent. Invitrogen, Spain), as well as cell proliferation and differentiation (alkaline phosphatase activity (ALP) and scanning electron microscopy (Fixation for SEM) Student's t test was performed to determine statistically significant differences between the two groups of study discs.

Results: The results obtained show very good cell viability during the study period, with no significant differences for both materials. Likewise, we detected a drop in ALP levels that was significant for both components between days 4 and 7 of the study ($p < 0.05$). Electron microscopy images revealed good adhesion capacity to the material, as well as cell differentiation against both types of discs.

Conclusions: The TiNbTa alloy as a material for bone implants offers good osseointegrative capacity, in addition to solving biomechanical problems that pure titanium presents as a component.

Key words: TiNbTa, cytotoxicity, biocompatibility, osteoblast cells, cell culture, cell adhesion, Young's modulus.

Introduction

The generation of functional tissue through tissue engineering has a high impact in various areas of regenerative medicine, among which is skeletal tissue. The first implants were used in the field of medicine, in 1909, when Kirschner wires and Steinman nails were developed for the fixation of bone fractures, where stainless steel was used. Over the years, steel has been improved, making it more resistant to corrosion and not causing harmful effects on the human body. In 1940, the study of titanium (Ti) began as a biomaterial for bone implantation[1].

The phase change determines the change in the crystalline structure of the material when subjected to temperature changes. Titanium's allotropic transformation occurs at 882ºC and goes from an α phase, which has a compact and hexagonal structure (HCP), little deformable and resistant at room temperature, to a β phase characterized by a cubic structure centered on the body (BCC), which is easily deformable and allows for carrying out heat treatments to optimize the material's properties[2].

Among the characteristics that make titanium one of the best materials for bone implants, its greater specific resistance (resistance/density), its high ductility and lower Young's modulus[3] compared to other elements such as, for example, stainless steel are noteworthy. At the same time, it is a non-ferromagnetic material, which does not present inconveniences when the patient with a Ti implant undergoes magnetic resonance imaging. Anchorage to bone tissue is possible because of the oxide layer formed in the material when passivated[4].

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Pure commercial Ti (c.p.) and other alloys such as Ti-6Al-4V are currently used mainly for bone implants[5]. However, both elements present a high elastic modulus (E: 100-112 GPa) compared to the elastic modulus of cortical bone (18.6-20.7 GPa) and trabecular (10.4-14.8 GPa) (Table 1) what produces the stress-shielding effect or stress-shielding[6]. This phenomenon is due to the stiffness of the bone implant material being greater than the stiffness of the bone, which places the entire load on the bone implant. Bone remodeling is largely regulated by the mechanical loads to which it is subjected, so that the presence of loads stimulates its bone formation, and the absence of them increases resorption. As a consequence of the bone's reduced load supported, it decreases its density in the area near the implant and, therefore, both premature fracture of the implant and loosening of the implant may occur[5].

At present, to eliminate the voltage shielding phenomenon, reducing the elastic modulus is sought, with several available solutions. One of them, reduce the density of the material used, through porosity. However, as the porosity increases, the mechanical resistance decreases. Another approach would be to search for face-centered cubic structure Ti alloys (BCC) or β-Ti alloys, which are low in elastic modulus and do not show a decrease in mechanical strength. For the stabilization of the β phase, β-stabilizing elements are required: Mo, V, Ta, Nb and Zr as β-isomorphic elements and Cr, Co, Cu, Fe and Ni as β-eutectoid elements. The advantage of β-isomorphic elements is their high amount of substitute solid solution and their inability to form intermetallic compounds of Ti, which have high E. Therefore, it has been shown that elements such as Nb and Ta have a high level of biocompatibility and ability to prevent the increase of particles, which would avoid a bad cohesion interface[7].

Niobium (Nb) and tantalum (Ta) are two transition metals widely used in alloys; in particular, Nb is used in the formation of steel. Until 1866, it was thought that both elements were the same, since they have very similar physicochemical characteristics. The TiNbTa alloy stands out for its high stabilization of behavior, due to the absence of β phase, which allows a decrease in the elastic modulus (49 + 3 GPa), its excellent elastic resistance (σy>1860 MPa) and its high biocompatibility[7,8]. Therefore, a material such as TiNbTa, with a good combination of high strength and low Young's modulus close to that of bone, could be used to prevent loosening of implants to avoid revision surgery (Table 1).

In recent years, a whole series of techniques have been developed with the aim of obtaining porous materials that present a Young's modulus closer to that of cortical bone. Specifically, Chicardi et al. manufactured a TiNbTa alloy with physicochemical properties more similar to those of bone[9]. However, the improvement in the design of materials, destined to be used as bone grafts, must consider their osseointegration capacity, and in this sense, evaluating the cytotoxicity and the adhesion, proliferation and differentiation capacity of osteoblastic cells influenced by the material is required. Our main objective was to evaluate the osseointegrative characteristics of the TiNbTa alloy, with a Young's modulus similar to that of trabecular bone, and to compare them with pure Ti.

Material and methods

1. Cell culture

The MC3T3 cell line (subclone 4) from ATTC (Manassas, Virginia, USA) was cultured in α-MEM medium (Gibco, Thermo Fischer Scientific, Spain) supplemented with 1% L-glutamine (200 mM) and 10 % fetal bovine serum. To induce differentiation, cells were treated with osteogenic culture medium supplemented with 50 g / ml ascorbic acid (Merck, Germany) and 10 nM β-glycerophosphate (StemCell Technologies, Canada). The cells were seeded in discs, 3 mm thick and 7 mm, of titanium c.p. and TiNbTa at a density of $5x10^3$, under conditions of 5% CO_2 at 37°C. Media changes were made every 48 h. Cultures were done in triplicate and readings for cell viability and alkaline phosphatase activity were done after 4 and 7 days.

2. Cell viability

The Alamar Blue assay (AlamarBlue Cell Viability Reagent. Invitrogen, Spain) was carried out according to the manufacturer's instructions. AlamarBlue is a non-toxic, cell-permeable compound, blue in color and non-fluorescent. Viable cells maintain a reducing environment within their cytoplasm. AlamarBlue reagent is an oxidized form of redox and is blue in color. When incubated with viable cells it changes from blue to red and becomes fluorescent. This change can be detected using absorbance methods.

At 4 and 7 days, the cells with cell growth are transferred to a new well and 80 µL of AlamarBlue are added on the disc and subsequently 720 µL of culture medium are added; This medium is incubated for 2 hours at 37 [deg.] C. and the absorbance is measured at the respective excitation and emission wavelengths of 570 and 600 nm (TECAN, Infinity 200 Pro). The results are presented as the percentage of reduction. The experiments were carried out in triplicate on each culture.

3. Alkaline phosphatase

We analyzed the activity of alkaline phosphatase (ALP) according to the manufacturer's protocol (Colorimetric Alkaline Phosphatase Assay Kit, Abcam ab83369, UK) in the culture supernatant. The test was carried out at 4 and 7 days, through the conversion of a colorless p-nitrophenyl phosphate into a colored p-nitrophenol. The absorbance at 405 nm of 4-nitrophenol (TECAN, Infinity 200 Pro) was measured and the ALP activity calculated from a standard curve. The experiments were carried out in triplicate on each culture.

4. Fixation for SEM

To visualize the cells in scanning electron microscopy (SEM), MC3T3 cells were grown on titanium discs c.p. and Ti NbTa for 4 and 7 days in triplicate. The samples were fixed with 10% formalin, followed by a dehydration step with ethanolic solutions and coated by gold plating using a sputum coating (Pelco 91000, Ted Pella, Redding, California, USA). All micrographs were obtained using a Jeol JSM-6330F scanning electron microscope and the acceleration voltage was 10 kV for SEM images.

5. Statistical analysis

All *in vitro* experiments were performed in triplicate for each condition studied. The variables were analyzed for the distribution of normality using the Kolmogorov-Smirnov test. Student's t test was performed to determine statistically significant differences between the two groups.

For the statistical handling of results, the SPSS version 22.0 package for Windows (IBM Corp., Armonk,

Table 1. Physical characteristics of pure titanium, the different phases and bone: elastic modulus and elastic resistance

Group	Compound	Young modulus (GPa)	Yield limit (MPa)
Pure titanium	Ti c.p.	100	650
Phase α+β	Ti-6Al-4V	112	1140
Phase β	TiNbTa	46-52	1860
Bone	Trabecular	0.5-2.0	40-60
	Cortical	20-25	150-180

New York, USA) was used. In all cases, the level of significance was considered to be $p<0.05$. Data are expressed as mean ± standard deviation (SD).

Results

The cell cultures on the Ti discs c.p. and in the alloy they had a similar cell growth. At 7 days of growth, the cells showed columnar and basophilic morphology under the light microscope (O.M.) compatible with the beginning of differentiation (Figure 1). The surface of the disc does not let light through, making it difficult to see cells clearly. However, in the highlighted parts, it could be observed that some cellular adhesion had occurred in the material, which would indicate that the alloy has good qualities to be used in implants.

Cell viability

Figure 2 represents the viability of the cell line as a function of the cell growth time (4 and 7 days) on the Ti discs c.p. and TiNbTa. At 4 days of culture, cell viability in the TiNbTa discs was similar to that of the Ti c.p. While at 7, we observed a slight increase in viability in the TiNbTa samples and a decrease in the Ti samples c.p., although without significant differences. In all cases, the percentage of viability was always higher than 150%. A toxic effect is considered when the viability is less than 75%, therefore, with the results obtained, it can be deduced that the cell culture is viable throughout the entire duration of the culture under all conditions.

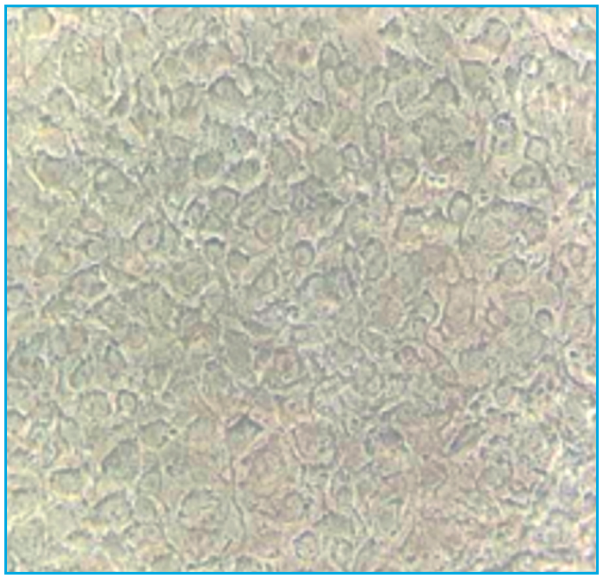
Alkaline phosphatase activity

We quantified the alkaline phosphatase (ALP) activity values in MC3T3 cell cultures at 4 and 7 days. Figure 3 represents the mean and standard deviation of the results obtained in both culture conditions. In the Ti discs c.p., the results obtained showed a decrease in the enzymatic activity, the difference found between days 4 and 7 being statistically significant ($p=0.001$). In the TiNbTa discs, a decrease in activity was also observed with culture time and there was a significant difference between days 4 and 7 of culture ($p=0.006$). In both conditions, at 4 days, the maximum enzymatic activity was obtained, since there is a greater proliferation and cell growth, as the viability values corroborated, and from day 7, the cells presented a more differentiated phenotype, as observed in the SEM images, and ALP activity decreased.

Cell morphology by scanning electron microscopy

A preliminary study was carried out to study the morphology and cell adhesion on the TiNbTa study material compared to the growth in Ti c.p. To do this, we visualized the samples of both types of disc at 4 and 7 days of culture. At 4 days, small cell clusters were observed on both types of surface and of similar density. At 7 days, the

Figure 1. Inverted optical microscope photograph (Olympus CKX53) with a 40X medium magnification objective, of cells grown at 7 days in the TiNbTa discs



images showed a monolayer growth over the entire surface of the disk, being similar in both materials. Cell-cell and cell-biomaterial junctions were also observed. The cells adhered through filopodia (thin cell projections, yellow arrow) and lamelopodia (broader extensions, yellow asterisk), thus demonstrating the connection of the cells with the biomaterial. We began to observe the presence of small vesicles with a hexagonal structure, suggesting a possible nucleation of hydroxyapatite, on the cell surface, suggesting the beginning of the mineralization process development (Figure 4).

Discussion

Inflammatory and degenerative problems of the bones and joints affect millions of people around the world. In fact, they represent half of the chronic diseases in people over 50 years of age in developed countries[10]. These diseases often require surgery, including replacement of the entire joint in cases of deterioration. This fact, accompanied by the increase in life expectancy and the aging of the population, entails a great demand for healthcare derivatives, among which the development of surgical implants and materials with a longer useful life period stand out.

Biomaterials constitute one of the most important advances in current medicine, improving the patient's quality of life and reducing the healing and convalescence time. In 2009, Bjursten highlighted the importance of increasing implant-bone bonding, given that the half-life of an implant is between 10 and 15 years[7,8], which implies an increase in the number of revision surgeries.

Figure 2. Cell viability of cells cultured in Ti discs c.p. or the TiNbTn alloy at 4 and 7 days of cell growth. Results presented as mean ± standard deviation

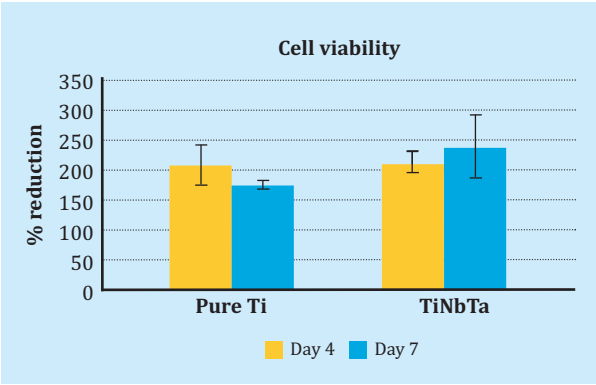


Figure 3. Activity of alkaline phosphatase in cell growth on Ti discs c.p. or TiNbTa for 4 and 7 days. *Ti c.p. 4 days vs. 7 days; ** TiNbTa 4 days vs. 7 days. P<0.05

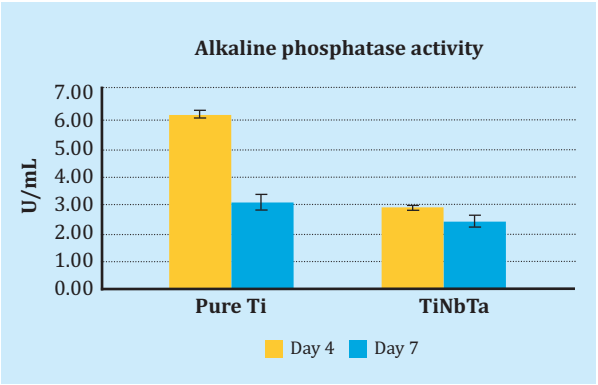
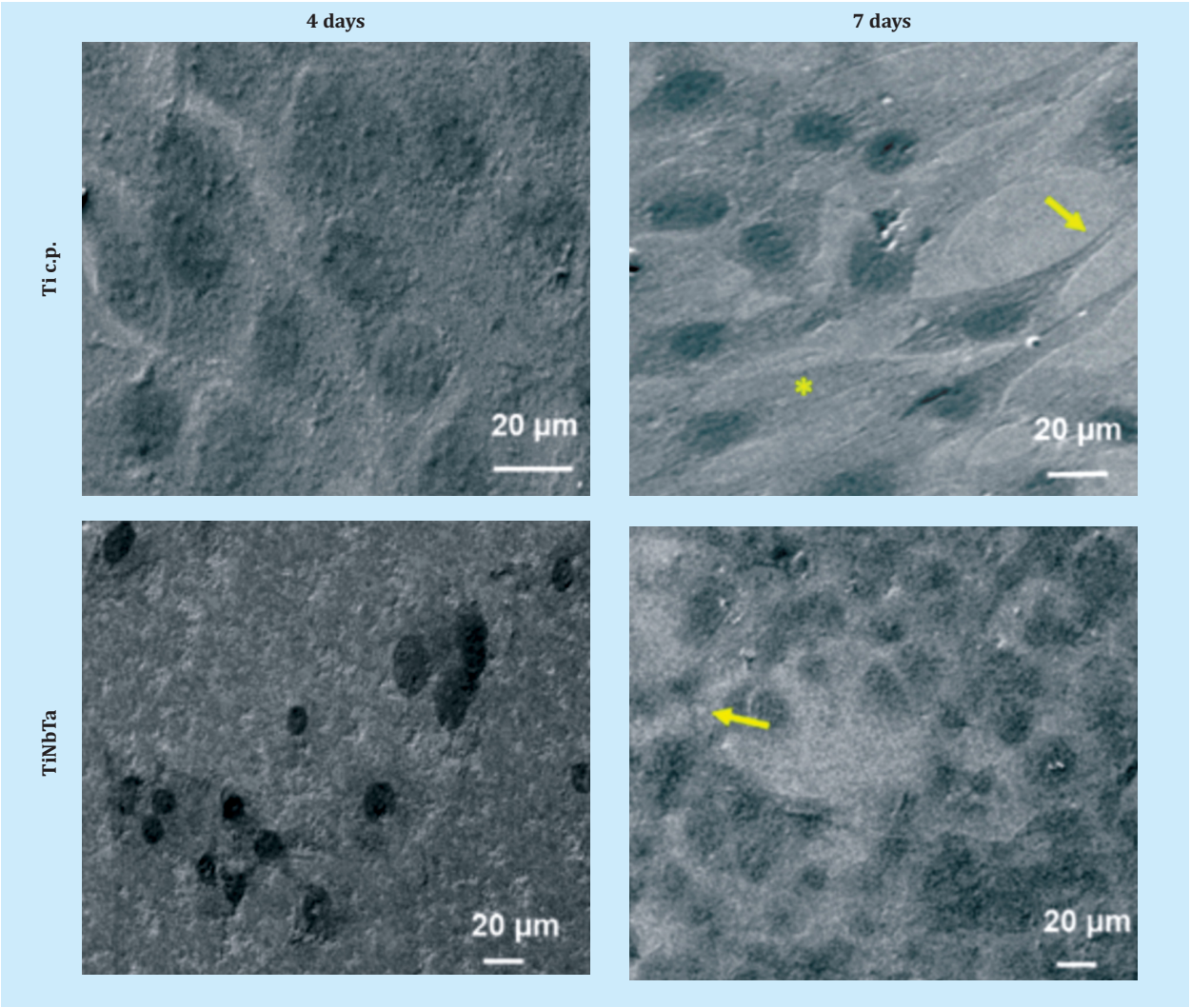


Figure 4. Scanning electron microscope micrographs at 4 and 7 days of osteoblast cultures on the surface of Ti c.p. or TiNbTa. Cell morphology and proliferation is shown on the two surfaces tested. Cell-cell interactions through filopodia (yellow arrows) and cell-surface interactions through lamellipodia (yellow asterisk)



In our study, we have carried out tests to assess a new material, TiNbTa alloy, with a Young's modulus similar to that of trabecular bone. Numerous studies have shown the decrease in elastic modulus in alloys with Nb and Ta, such as Ti35Nb5Ta7Zr, which shows an elastic modulus of 55GPa[11]. The advantage compared to the Ti c.p. or the Ti-6Al-4V alloy would be the lower Young's modulus of the alloy studied, which would considerably reduce the voltage shielding.

The percentage of viability is largely related to the biocompatible and cytotoxic properties of the material; various authors[12,13] have shown how high percentages of

viability are optimal for considering a candidate material to be used as a bone implant in humans. Our cultures, at different study times, presented a high degree of biocompatibility, as well as an absence of toxicity. Positive results were obtained between the cell line and the material. It could be suggested that if this material were implanted, it would tend to synthesize the bone matrix, adhere well to the bone, and be biocompatible[14-15].

The viability values indicate that there are no differences in cell growth between the TiNbTa and Ti c.p alloy, and in all conditions the values are above 75%. Therefore, both materials would be non-cytotoxic and therefore viable. On the other hand, the adequate activity of cellular metabolism confirms that the alloy does not release toxic residues for our cells[15-18]. Previous studies of animal implantation of materials such as Nb and Ta in soft and hard tissues of rats have shown the high biocompatibility of metals and osteogenesis[19,20].

We also quantify cellular activity by measuring ALP activity. Increased ALP activity is directly related to cell proliferation and is a marker of differentiation of the osteoblastic phenotype. In tissues such as bone and cartilage, ALP is expressed early in the calcification process and later in development, ALP expression is decreased. It has been shown that when ALP activity decreases, cell differentiation increases[14].

Our values confirm that the culture on the discs with the new material presents ALP activity that varies according to the study time. At the beginning of the culture, at 4 days, the MC3T3 cells showed a higher cell proliferation in Ti c.p. than those grown on the alloy material, indicating a slower growth in the TiNbTa discs during the first days of culture. At 7 days, the ALP values were similar in both samples and lower than the initial ones, which shows that the culture is behaving with similar characteristics in terms of proliferation and differentiation. In the images obtained by SEM, we observed that at 7 days the cells covered the entire surface of the discs, cell growth occurred equally in both samples and they reached cell confluence, and the lowest activity was consistent with the images cells that are starting the mineralization process. In addition, we began to observe the secretion of material that will form the extracellular matrix by the osteoblasts, and an increase in molecules with the appearance of hydroxyapatite was observed on the surface of the cells[21]. On the other hand, osteoblasts presented filopodia and lamelopodia, essential cellular structures during the cell adhesion process, which indicates that the union to other cells and to the material is direct; other authors describe these junctions in MC3T3 cells and Ti discs c.p.[22,23].

In conclusion, our *in vitro* study allows us to conclude that the novel alloy that combines elements such as Nb and Ta with Ti, in addition to improving the mechanical properties of the material, is, in the short term, biocompatible with osteoblastic cells, behaving with characteristics of viability, proliferation, differentiation and adhesion capacity of osteoblastic cell lines, in a very similar way to that of pure Ti.

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Conflict of interests: The authors declare no conflict of interest.

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