IN VITRO BIOCOMPATIBILITY TESTING OF THREE NEWLY DEVELOPED TITANIUM ALLOYS FOR ORAL IMPLANTOLOGY

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The aim of the study was to evaluate the in vitro *biocompatibility of three newly developed titanium alloys: Ti-31.7Nb-6.21Zr-1.4Fe-0.16O, Ti-36.5Nb-4.5Zr-3Ta-0.16O and Ti-20Nb-5Ta, in order to establish whether the use of these alloys can have any toxic or injurious effects on biological systems and how they promote the osseointegration process. Ti-6Al-4V was used as a reference material. The key factors in the osseointegration of implants are related to the increased colonization of osteogenic cells on the implant surface, as well as to their differentiation into mature osteoblasts. Osseointegration of metallic implants involves increased osteogenic activity of bone cells, the biological response of osteoblasts in vitro being a good parameter for assessing the performance of metallic implant surface.*

The purpose of the study was achieved by studying the behavior of MC3T3-E1 murine pre-osteoblastic cell line on the new alloys, including adhesion, proliferation and differentiation. The results obtained showed that the newly-developed alloys elicit a good cellular response in terms of cellular survival, adhesion, morphology and proliferative potential, while the cell differentiation tests lead to the conclusion that the analyzed alloys support the osteoblastic differentiation of MC3T3-E1 cells to a similar level as the reference material. Therefore, these alloys could represent good candidates for dental implants.

Keywords: biocompatibility, *in vitro* tests, osseointegration, cytotoxicity, cell proliferation, cell differentiation, dental implants, titanium alloys.

1. Introduction

Integration of a dental implant into the host tissue determines its clinical success and longevity. There are four factors that may be correlated with implant material, which influence wound healing produced by inserting a dental implant: the mechanical properties of the material; the physico-chemical properties of the material; the topography of the surface material; the shape and design of the implant. One of the most important objectives in the biomaterials field is to prove the capability of progenitor cells to develop themselves into functional tissues. On the long term, metallic implants develop a characteristic interface and a specific bone matrix, with adequate biomechanical properties. *In vivo*, the development of such a matrix during implants osseointegration involves the recruitment of mesenchymal stem cells and their progressive differentiation in osteoblasts [1].

The deposition of a collagen matrix [2] and its mineralization [3] are considered functional stages that reflect *in vitro* cell differentiation.

In the biomaterials research field, *in vitro* tests are used as a screening method, being followed by *in vivo* tests. The current researches tend to develop *in vitro* systems using only primary human cell cultures or together with other cells types and biomaterials in a 3D system, trying to simulate the biologic systems, for a better understanding of the molecular mechanisms during the cell-cell and cell-matrix interactions [4]. The complexity of the interactions can be better understood by correlating the information obtained from simple tests, evaluating the cell morphology and viability, with modern molecular biological tests and microscopic tests. In dental implant prosthetics, cellular and molecular biology researches are necessary, in order to evaluate possible adverse biological effects after implantation, immune, non-immune and inflammatory host responses.

Titanium and titanium alloys have been widely used as biomaterials for dental implants, due to their superior mechanical properties, biocompatibility and lack of induction of allergic reactions. Previous research proved that Ti-based alloys with low Young's modulus are effective for inhibiting bone atrophy and enhancing bone formation [5-7]. Ti-6Al-4V alloy, used as a reference material in the present study, is a classic alloy used for dental implants, having the disadvantage of a toxic effect on the human body, due to the aluminium and vanadium content [8,9]. This is the reason why alloys containing Nb, Zr, Ta, as the titanium alloys studied in the paper, have been introduced later for implant applications [8].

In this context, the aim of this work was to investigate the biological performance of recently new-developed Ti-31.7Nb-6.21Zr-1.4Fe-0.16O, Ti-36.5Nb-4.5Zr-3Ta-0.16O and Ti-20Nb-5Ta alloys by evaluating osteoblast response in terms of viability, cell attachment and spreading, cell morphology, cell proliferation and differentiation. For comparative purposes, a commercial Ti-6Al-4V alloy was also investigated. We show that on all three tested alloys the cells displayed high survival rates, good cell adhesion and spreading, increasing cell proliferation rates over the incubation time and an osteogenic activity similar with that of control samples.

2. Materials and methods

2.1. Chemical and structural composition of the alloys

The chemical compositions of the newly developed alloys are illustrated in table 1. Titanium alloys were analyzed in the as-cast state. In the study, the classical Ti-6Al-4V (wt%) alloy was used as a reference material.

The qualitative structural composition was determined by XRD (X-Ray Diffractometry), using a PANalytical X'Pert PRO MRD diffractometer with the wavelength = 1.544 Å. The diffraction patterns were obtained for a diffraction

angle in the range of 2θ = 30-75° and were indexed using Crystal Impact Match v.2.4.3 software package. For indexing diffraction spectra the Crystallography Open Database COD 20150107 was used.

Table 1

Chemical composition of the as-studied alloys

Alloys	Elements [wt%]					
	Nb	**Ta**	**Zr**	**Fe**	**O**	**Ti**
Ti-20Nb-5Ta	20	5	-	-	-	rest
Ti-36.5Nb-4.5Zr-3Ta-0.16O	36,5	3	4,5	-	0,16	rest
Ti-31.7Nb-6.21Zr-1.4Fe-0.16O	31,7	-	6,21	1,4	0,16	rest

2.2. In vitro biocompatibility testing

2.2.1. Cell cultures

The cell culture model used in biocompatibility studies was represented by mouse pre-osteoblast MC3T3-E1 cell line, subclone 4 (CRL-2593™, American Type Culture Collection). The cells were seeded onto metallic specimens at a density of 5,000 cells/cm2 and maintained in a humidified atmosphere of 5% CO2 at 37 °C in Dulbecco's Minimal Essential Medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin/streptomycin (10,000 units/mL penicillin and 10 mg/mL streptomycin). Previous to *in vitro* tests, the Ti-based samples were sterilized by immersion in 70% ethanol, washed with sterile-filtered Milli-Q water and maintained under ultraviolet light in a sterile tissue culture hood for 1 hour on each side.

2.2.2. Fluorescence staining of actin and cell nuclei

After 2 hours and 48 hours cultivation, the cells that grew on test samples were fixed with 4% paraformaldehyde prepared in phosphate buffered saline (PBS) solution, permeabilized with 0.1% Triton X-100/2% bovine serum albumin for 15 minutes and washed with PBS. Afterwards, a sequential treatment with phalloidin-TRITC (tetramethylrhodamine B isothiocyanate) (10 μg/ml; Sigma-Aldrich Co.) and 2 μg/ml DAPI (4′6-diamidino-2- phenylindole) was performed. Labeled samples were washed with PBS and examined under an inverted microscope equipped with epifluorescence (Olympus IX71). The images were captured by means of Cell F image acquiring system.

2.2.3. The citotoxicity study

The potential toxic effects of the analyzed specimens on MC3T3-E1 cells were evaluated by detecting lactate dehydrogenase (LDH) activity released into the media, as a marker of plasma membrane lysis and cell death. This study was performed on aliquots from the cell culture media maintained in contact with the

analyzed specimens up to 5 days, by using a cytotoxicity detection kit (TOX-7, Sigma–Aldrich Co.) according to the manufacturer's protocol. High OD 490 nm values are indicative of a reduction in the cell viability.

2.2.4. Cell proliferation assay

MC3T3-E1 pre-osteoblasts growth on the samples was analyzed by MTT colorimetric assay. Cell cultures from days 1, 3 and 5 after plating were incubated with MTT solution (1 mg/ml in serum free culture medium) for 3 hours at 37 °C. Then, the MTT solution was decanted and formazan crystals were solubilized with 1 ml of dimethyl sulfoxide. Absorbance of the dye was measured at a wavelength of 550 nm and recorded using a microplate reader (Thermo Scientific Appliskan).

2.2.5. Cell differentiation assay

For the differentiation assay, the pre-osteoblastic cells were seeded onto samples at a density of 5 × 104 cells/cm2. At 24 hours post-seeding, differentiation was induced by maintaining the cells in culture media supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich) and 5 mM beta-glycerophosphate (Sigma-Aldrich). The medium was changed every two days during the culture period. Cell differentiation was evaluated after 4 weeks of culture, by Sirius Red staining for secreted collagen and Alizarin Red staining for matrix mineralization.

Briefly, after specified period of time, cell layers were washed three times with PBS and fixed in 10% paraformaldehyde.

Following three rinses in deionized water, cells were placed in a 0.1% solution of Sirius Red (Sigma) in saturated picric acid or in 1 mg/ml Alizarin Red S (Sigma-Aldrich) solution for 30 minutes, for collagen secretion staining and matrix mineralization assay, respectively. After washing the samples with deionized water, cells were air-dried for 24 hours. Subsequently, Sirius Red or Alizarin Red stain on specimens was dissolved in 0.2 M NaOH/methanol (1:1) or 5% v/v perchloric acid in order to measure the optical density at 540 nm or 405 nm, respectively.

3. Results and discussions

3.1. Determination of qualitative structural composition

Diffraction patterns obtained for the three titanium alloys are shown in figures 1-3.

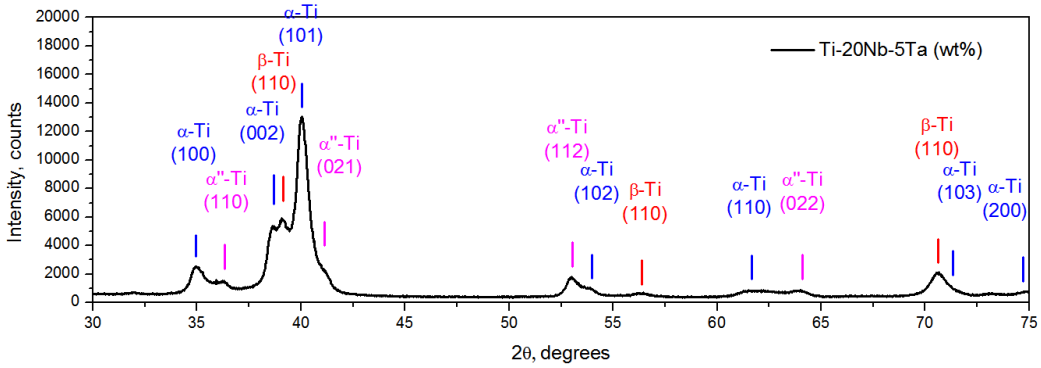

Fig. 1. Diffractogram of Ti-205Nb-5Ta alloy in as-cast state.

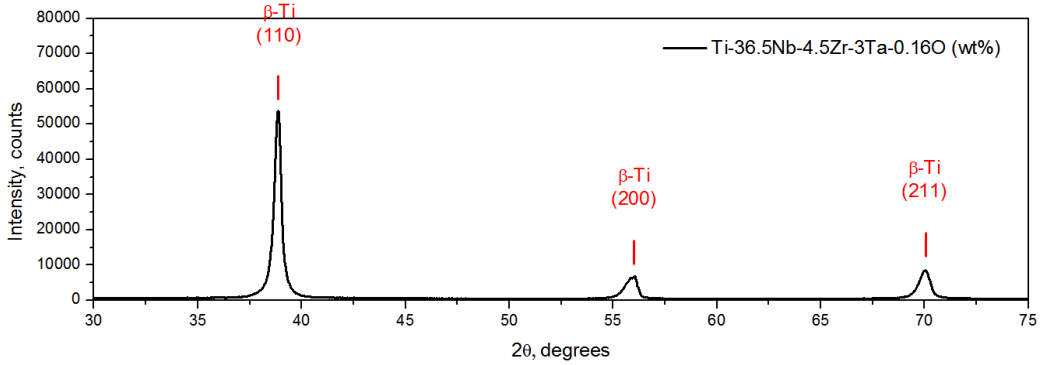

Fig.2. Diffractogram of Ti-36.5Nb-4.5Zr-3Ta-0.16O alloy in as-cast state.

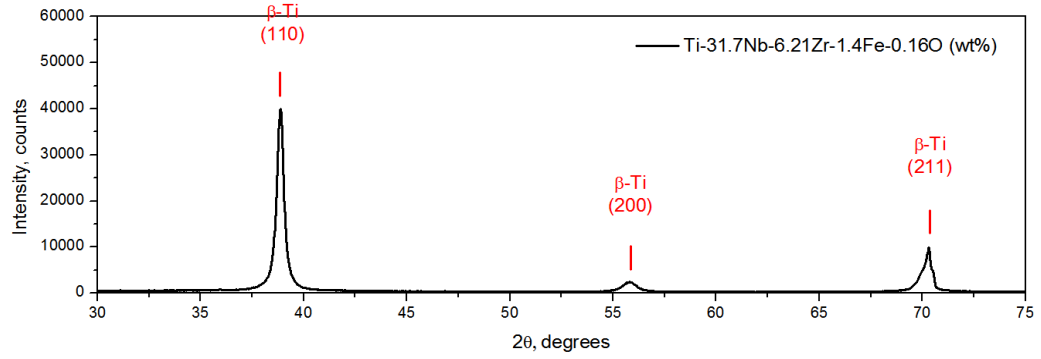

Fig.3. Diffractogram of Ti-31.7Nb-6.21Zr-1.4Fe-0.16O alloy in as-cast state.

The diffractograms were indexed to identify structural phase components, showing that Ti-205Nb-5Ta alloy is composed of α, β and α" phases, while Ti-36.5Nb-4.5Zr-3Ta-0.16O and Ti-31.7Nb-6.21Zr-1.4Fe-0.16O are β - phase alloys.

3.2. Morphology and adhesion of the pre-osteoblasts grown in contact with titanium alloy samples

An important objective of the present study was the analysis of the impact that the new alloys have on osteoblast adhesion and morphology. Fluorescence microscope images depicting actin cytoskeleton organization in osteoblasts cultured on test samples are shown in Fig.4. At 2 hours post-seeding the number of cells attached to the surface of the analyzed materials was approximately similar for all samples with no differences in terms of cell spreading. Moreover, after 24 hours of culture, all cells have acquired typical polygonal osteoblasts morphology.

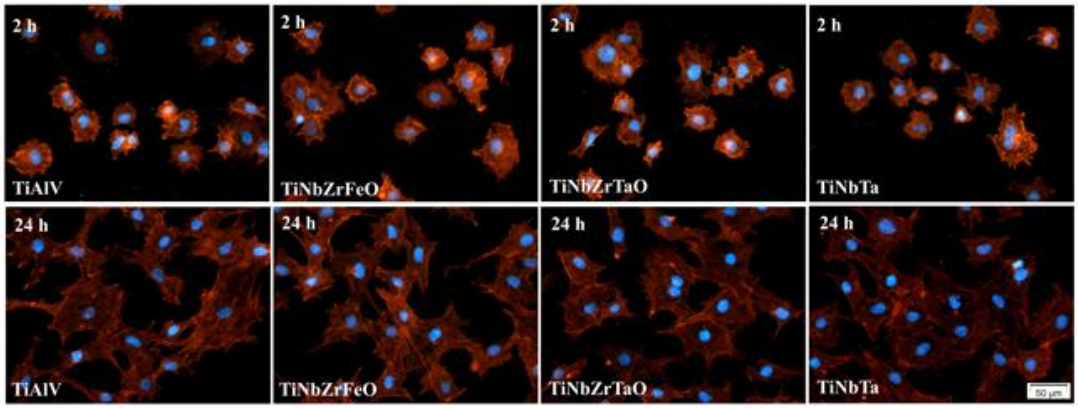

Fig. 4. Cellular morphologies at 2h and 24h after cell seeding, on the studied alloy samples – the actin filaments are stained with TRITC (red) and the nucleus with DAPI (blue).

3.3. The cytotoxic potential of the three titanium alloys

In order to determine if the three alloys may have possible detrimental effects on cell survival, the release of LDH into the culture medium at 1, 3 and 5 days after cell seeding was assayed (Fig. 5).

LDH is a cytosolic enzyme, present in all cell types and rapidly released into the culture medium, through destruction of the cellular membrane and consecutive cell death. The osteoblasts grown on the samples presented low LDH values, comparable with the reference support, suggesting that none of the as-studied alloys had considerable cytotoxic effects on the cultured cells.

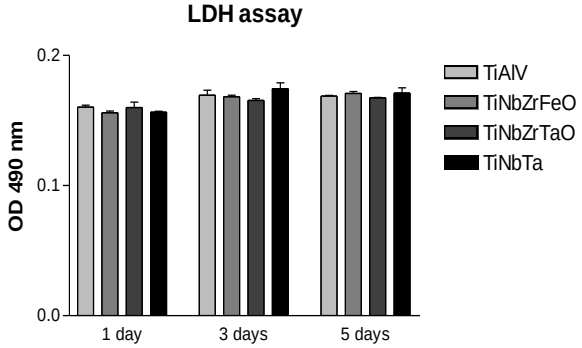

Fig.5. Evaluation of the LDH release into the culture medium by MC3T3-E1 pre-osteoblasts grown in contact with titanium alloy samples

3.4. Proliferation of osteoblasts grown in contact with the studied samples

The proliferation status of the cells maintained in contact with test samples for 1, 3 and 5 days was assessed by MTT assay. The results showed that the number of viable metabolically active pre-osteoblasts increased with the incubation time, without significant differences between samples at any point in time (Fig. 6). Thus, all three titanium alloys proved to sustain cell proliferation, which implies increased biocompatibility.

3.5. Evaluation of the differentiation process

The cultures were tested for collagen secretion, an essential constituent of the extracellular matrix protein synthesized by osteoblasts and for the matrix mineralization that takes place during the advanced phases of osteogenesis, being considered to be a complete osteogenic differentiation marker.

Figure 7 shows that there are no important differences between the analyzed samples after the evaluation of the collagen synthesis and the mineralization process of the extracellular matrix.

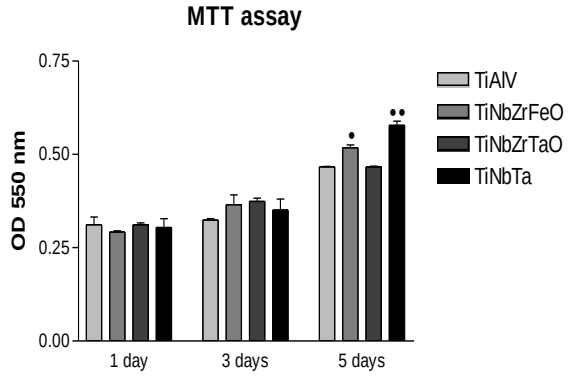

Fig.6. Cell proliferation rates of MC3T3-E1 cells grown in contact with newly developed alloys and with the reference material, at 1, 3 and 5 days after cell seeding

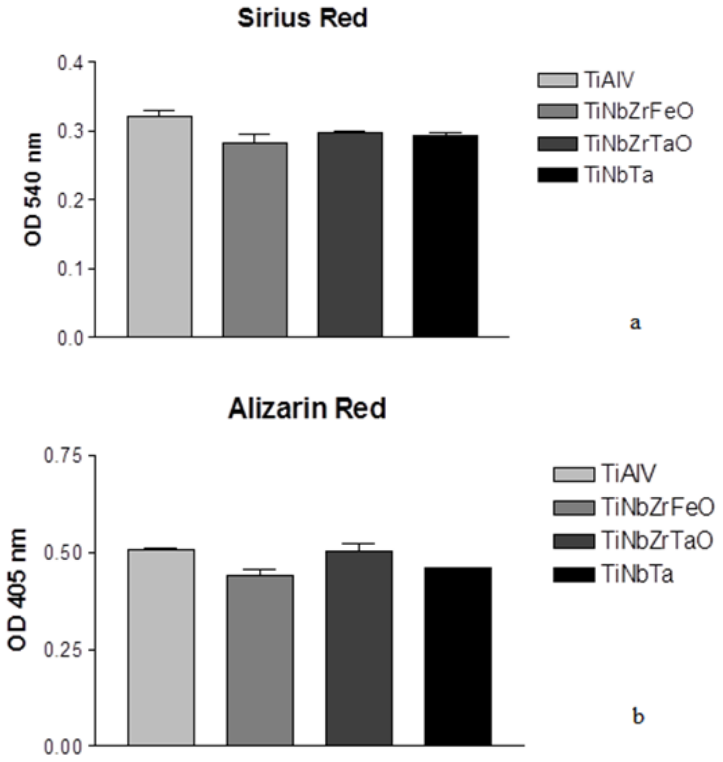

Fig.7**.** Differentiation of MC3T3-E1 pre-osteoblasts on the studied samples evaluated through the analysis of the collagen secretion and extracellular matrix mineralization 4 weeks after inoculation (a - Sirius Red staining, b - Alizarin Red staining).

6. Conclusions

The present study addresses the potential of the following titanium alloys: Ti-31.7Nb-6.21Zr-1.4Fe-0.16O, Ti-36.5Nb-4.5Zr-3Ta-0.16O and Ti-20Nb-5Ta (wt%), for their future use as dental implant alloys.

Fluorescence microscopy reveals that all metallic substrates are equally colonized by healthy osteoblasts with a typical polygonal morphology. The low LDH value, comparable with the reference support, suggests that none of the as-studied alloys had considerable cytotoxic effects on the cultured cells. Moreover, the MTT assay demonstrated that all three titanium alloys sustained cell proliferation. Thus, the results of the research work show that the as-studied alloys elicit a positive cellular response in terms of cells survival, cell adhesion and morphology, as well as in terms of the proliferation potential.

The evaluation of the pre-osteoblasts differentiating process of the as-studied alloys showed no important differences between the analyzed samples and proved that newly developed titanium alloys promote the pre-osteoblasts differentiation process similarly with the reference material.

Taken together, these findings prove that the newly developed Ti-alloys hold great promise for application in biomedical field, including oral implantology.

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R E F E R E N C E S

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