

TESTAREA BIOCOMPATIBILITĂȚII IN VITRO A MATERIALELOR CU COMPOZIȚIE CHIMICĂ CONTROLATĂ

IN VITRO TESTING OF MATERIALS BIOCOMPATIBILITY WITH CONTROLLED CHEMICAL COMPOSITION

VLAD GABRIEL VASILESCU¹, MIRUNA SILVIA STAN², ION PĂTRAȘCU¹, ANCA DINISCHIOTU²,
ELISABETA VASILESCU^{3*}

¹ "Carol Davila" Medicine and Pharmacy University, Faculty of Dentistry, Calea Plevnei nr.19, 010221 Bucharest, Romania

² University of Bucharest, Faculty of Biology, Splaiul Independenței nr. 91-95, 050095 Bucharest, Romania

³ "Dunărea de Jos" University, Faculty of Engineering of Galați, Str. Domnească nr.111, 800201, Romania

In vitro testing of materials biocompatibility provides information on cell behavior in relation to chemical composition and surface topography of materials. The studied biomaterial is a new one proposed to be used in oral implantology and therefore requires studying its biocompatibility compared to conventional materials commonly used in oral implantology.

The in vitro response induced by this bioalloy with surface characteristics conferred by casting, corrosion and anodizing was evaluated on G292 human osteoblasts. The experimental obtained surface after anodizing presented a stable and consistent oxide "gel-looking" layer well highlighted in scanning electron microscopy images. The results demonstrated the ability of the biomaterial to support attachment, growth and proliferation of human osteoblasts. As compared to the other materials tested and demonstrates the improved properties that facilitated rapid cellular adaptation to these surfaces.

Testarea in vitro a biocompatibilității materialelor oferă informații privitoare la comportamentul celular în raport cu compoziția chimică dar și cu topografia suprafeței acestuia. Biomaterialul studiat este unul nou propus pentru a fi utilizat în implantologia orală și de aceea se impune studiul biocompatibilității acestuia în raport cu materialele convenționale, frecvent utilizate în implantologia orală. Răspunsul in vitro indus de acest bioaliaj cu caracteristici de suprafață conferite de turnare, coroziune și anodizare a fost evaluată pe osteoblaste umane G292. Suprafața obținută experimental după anodizare prezintă un strat stabil și consistent de oxizi cu aspect „geliform”, bine evidențiată în imagini de microscopie cu scanare de electroni. Rezultatele demonstrează abilitatea biomaterialului de a susține atașarea, creșterea și proliferarea osteoblastelor umane comparativ cu alte materiale testate și demonstrează proprietăți îmbunătățite care au facilitat adaptarea celulară rapidă la aceste suprafețe

Keywords: biomaterial, biocompatibility, cell adhesion, osteoblasts

1. Introduction

The basic criteria in selecting a material for making oral implants is its biocompatibility. Through an appropriate interaction between biomaterial and tissue, the implant - tissue assembly becomes an integrated, stable and durable system. The overall response of the body, considered as a measure of biocompatibility, is conditioned by the following factors: material (volume and surface chemical properties, surface roughness, surface energy, surface porosity, surface load, physic - chemical stability, chemical properties of the degradation products and physical characteristics of degradation products), implant (size, shape, modulus of elasticity, rigidity), host organism (species, in animal experiments, tissue type and location, age, sex, drug therapy, general health), and method of implantation (surgical technique, implant-tissue fixation) [2,3].

Among the classes of materials (metal,

ceramic, polymeric, composite), metals and alloys are mostly used for oral implants. Initially selected based on ease of manufacture and whose main characteristic was strength, in time biocompatibility and durability of metallic materials have become important criteria later on [4]. The influence of metallic biomaterial on the biological environment is shown by the release of metal ions throughout corrosion and by oxides or metal particles emission caused by mechanical actions [5,6]. If the implant material is not toxic for the body and does not degrade under the action of oral biological environment, it can be considered that the normal healing process will not be affected. But, if the material is subjected to a degradation process (chemical, electrochemical or mechanical), the resulting products can be released into the host tissue that may also influence cellular and extracellular activity [7]. When the degradation process speeds in extreme cases are high and the released products are biologically active or toxic,

* Autor corespondent/Corresponding author,
E-mail: elisabeta.vasilescu@yahoo.com

the local response can cause local tissue death, thus producing a zone of necrotic tissue.

The most commonly used metallic materials are stainless steel, alloys of cobalt chrome molybdenum, titanium and its alloys, as root, subperiosteal and transosseous implants. Titanium was originally used as pure metal being considered an almost ideal material in endosseous dental implantology [8]. It is considered an inert material because the contact with the tissue is rapidly inactivated by the formation of a thin layer of oxide (monoxide dioxide, trioxide). The thin film of titanium oxide (tenacious and protective) formed in less than one second ensures corrosion resistance (400 times higher than stainless steel) and allows the bone to grow into the implant. In fact, between titanium implant and surrounding bone, a solid bond is established with the bone growth on the rough surface of the metal and binding to it, thus making a mechanical, rigid anchoring that stabilizes the endosseous implant [9-13]. The surface of oxides consisting of TiO_2 , TiO_3 , Ti_2O_3 , Ti_3O_4 , attracts and binds biomolecules [14]. This oxide surface is contaminated not only with metals, but also with proteins or lipids. The contaminated surfaces change the composition of the oxides, favoring inflammation which is followed by the formation of granulation tissue [15]. Titanium alloys are even better tolerated than pure titanium because of the oxide layer that forms is higher (approx. 10 to 20 μm), very stable and regenerates every nanosecond. The corrosion resistance of these alloys can be improved by alloying with molybdenum, zirconium, rhenium, niobium, chromium, manganese (e.g. Ti-Al-V, Ti-Al-Mo, Ti-Al-Cr, Ti-Al-Cr, Co) [16]. The frequent selection of the Ti-6Al-4V alloy for implants is determined by a combination of favorable characteristics including corrosion resistance, durability, low modulus of elasticity and the ability to join with bone and other tissues (osseointegration) [17-19]. However, there are a number of problems related to the effects generated by alloy components [20-22]. The analysis of possible reactions to prolonged contact of living tissue with alloying elements of titanium alloys showed that the use of large amounts of

vanadium, cobalt and nickel is not recommended. The release of vanadium ions can cause serious damage to respiratory system and bone marrow [23-25]. There was an increased release of prostaglandin E2 in response to Ti-6Al-4V particles contact, and an important release of other inflammatory cytokines compared with Ti-6Al-7Nb particles [5,17]. Exposure of bone marrow cells to Ti-6Al-4V particles induced a significant increase in pro-inflammatory and osteolytic mediators' release which was responsible for the loss of prosthesis [26]. The strategy of developing highly biocompatible alloys was the main target of the present study in which we present a new titanium based alloy (Ti10Zr) of a chemical composition lacking in harmful chemicals such as vanadium or aluminum, and whose biocompatibility has been confirmed by the resistance to corrosion inside the mouth [27,28].

In order to investigate the *in vitro* biocompatibility was performed the degree of toxicity and the cell morphology, adhesion and spreading on these surfaces were evaluated. Also, the intracellular glutathione level of the osteoblasts adhered to the surface of the bioalloy was analyzed, being the major endogenous antioxidant in mammalian tissues responsible for reactive oxygen species (ROS) neutralization.

2. Materials and Methods

2.1. Materials

Materials used in the *in vitro* biocompatibility research are samples of new alloy cut from the cast blank and samples subjected to corrosion and anodizing process, having the chemical composition and physical-mechanical properties as shown in Table 1.

The metallographic samples (metallographic attack: 68 ml + 16 ml HNO_3 + glycerin 16 ml HF – 15 seconds) were subjected to microstructure analysis using optical microscopy.

The cast samples ($\varnothing 18mm$ slices) cut from cylinders (denoted Ti10Zr) were subjected to surface treatment by acid attack (denoted Ti10Zr corroded) and anodic oxidation (denoted Ti10Zr anodized) under the following conditions:

Table 1

Chemical composition and physical-mechanical properties of the alloy [27,28]
Compoziția chimică și proprietățile fizico-mecanice ale aliajului Ti10Zr [28, 29]

Chemical composition/ <i>Compoziție chimică</i>						
Elements <i>Elemente chimice</i>	Ti	Zr	Fe	Si	Cu	Ni
weight [%] <i>procente de greutate [%]</i>	88.8	9.906	0.608	0.392	0.032	0.010
Physically-Mechanical Properties/ <i>Proprietăți fizico-mecanice</i>						
Density/ <i>Densitate</i> (g/cm ³)	Hardness HV ₁₀₀ / <i>Duritatea</i> HV ₁₀₀ (daN/mm ²)		Elasticity modulus <i>Modul de elasticitate</i> (MPa)			
4.7	212 – cast; 290-320 – extruded and cold rolled		148,000			

- *sample Ti10Zr corroded*: polished metallographic paper 400 + degreased + washed + dried + acid attack + rinsed + dried;

- *sample Ti10Zr anodized*: polished metallographic paper 400 + degreased + washed + dried + acid attack + rinsed + dried + *anodized*.

Acid attack was carried out in a mixture of acids: HCl 37% + H₂SO₄ 98%, at a temperature higher than 80°C. Anodic oxidation process was conducted in a laboratory facility within IMNR Bucharest with the following components: anodizing tank of Teflon, mobile anodic aluminum bar, a fixed sample anode bar (titanium wire with square section) a circular cathode (Ti, Pt), heating and thermoregulation stirring block, temperature control sensor. Use was made of a suitable anodizing electrolyte consisting of: H₂SO₄ – 200 g/l, NaCl – 30 g/l, ethylene glycol – 200 ml/l. The anodizing cell worked at a constant voltage of 30 V and the electrolyte temperature was maintained at 35°C for 60 minutes. The analysis of surface structure was investigated with atomic force microscopy (AFM/EasyScan2 Model) and scanning electron microscopy (SEM). As control sample was used commercially pure (cp) Ti grade 4 in order to obtain a more accurate comparative analysis and interpretation of results. The chemical composition and mechanical properties of the Cp Ti grade 4 is shown in Table 2.

2.2. Cell culture

The human osteoblastic osteosarcoma G292 cell line (ATCC CRL-1423) was grown in McCoy's 5a (Gibco, USA) supplemented with 1.5 mM L-glutamine, sodium bicarbonate 2.2 g/L, penicillin (100 units/mL), streptomycin (100 mg/mL) and 10% fetal bovine serum (Gibco, USA), at a temperature of 37°C in a humidified atmosphere with 5% CO₂. The cells were detached from the culture dishes' surface using a solution of 0.25% trypsin - 0.53 mM EDTA.

2.3. Osteoblasts exposure to Ti10Zr surfaces

G292 osteoblasts were seeded on Ti10Zr or pure titanium samples in 6-well plates at a density of 5×10^4 cells/cm². The materials were previously sterilized by autoclaving at 180°C for one hour.

In parallel, cells were cultivated directly on the tissue culture plastic surface (TCPS) in the absence of any material, being considered as control cells. After 24 and 48 hours of incubation, culture medium was collected from each well in order to assess the lactate dehydrogenase (LDH) release and the fluorescence staining of cytoskeletal actin filaments and intracellular glutathione (GSH) was performed.

2.4. LDH cytotoxicity assay

LDH release into the culture medium was measured using a cytotoxicity detection kit (TOX7; Sigma-Aldrich, USA) according to the manufacturer's instructions. Briefly, the medium in which the cells were cultured (50 μl) was incubated at 25°C for 30 min in the dark with a mixture (100 μl) containing LDH substrate, cofactor (NAD) and dye (tetrazolium salt). The reaction was quenched with 1N HCl (15 μl) and the absorbance was read at 490 nm. The amount of formazan produced is directly proportional to the amount of enzyme released from the cells into the culture medium, and therefore with the number of viable cells.

2.5. Fluorescence labeling of actin filaments

After removing culture medium, cells were fixed with 4% paraformaldehyde for 20 minutes and permeabilized with a solution containing 0.5% Triton X-100 and 2% BSA (freshly prepared in PBS) for one hour at room temperature. After 3 washes with PBS (of 5 minutes each), the cells were incubated for one hour in the dark with 20 μg/ml phalloidin conjugated with FITC (fluorescein isothiocyanate) to stain actin filaments. The nuclei were label with 2 μg/ml DAPI (4'-6-diamino-3-phenylindole) for 15 min in the dark. The cells adhered to the surface of the wells or on the surface of the tested materials were visualized with Olympus IX71 inverted fluorescence microscope.

2.6. Fluorescence labeling of intracellular GSH

To establish the intracellular GSH distribution and content, GSH staining was performed as previously described [30] using

Table 2

Chemical composition and mechanical properties of the cp Ti grade 4
Compoziția chimică și proprietățile fizico-mecanice ale Cp-Ti grad4 [27]

Chemical composition/Compoziție chimică						
Elements Elemente chimice	Fe	C	N	O	H	Ti
weight [%] Procente de greutate [%]	0.3	0.01	0.04	0.35	0.01	>99
Physically-Mechanical Properties/Proprietăți fizico-mecanice						
Density/Densitate (g/cm ³)	Hardness/Duritate (HV5/30)		Elasticity modulus/Modul de elasticitate (MPa)			
4.5	280		106.000			

CellTracker Green CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes, Invitrogen) which penetrates freely through cell membranes and react with thiol group of GSH. At the end of incubation time, the cells were first labeled with 10 μM CMFDA which was added to the culture medium without fetal bovine serum for 30 minutes at 37°C in incubator humidified atmosphere with 5% CO_2 . After removing the dye and washing the cells with pre-heated medium, the cells were left in growth medium without serum for 30 minutes at 37°C and 5% CO_2 . After washing with PBS, the nuclei were labeled with 2 $\mu\text{g}/\text{ml}$ Hoechst 33342 for 15 min in the dark. The cells were visualized using Olympus IX71 inverted fluorescence microscope.

2.7. Quantification of fluorescence area

Fluorescence quantification of the entire cell was based on outlining the cell perimeter of green fluorescence provided by GSH-CMF (GSH-chloromethylfluorescein) products. The GSH nuclear fluorescence was measured by outlining nucleus perimeter based on the blue fluorescence

given by Hoechst 33342 and transposing to the green field. The level of fluorescence in the cytoplasm was calculated as the difference between the fluorescence of GSH-CMF registered in the cell and the one recorded in the nucleus. For each experimental group it was quantified the fluorescence of 50 cells taken from different fields and from 3 independent experiments using ImageJ 1.48 software.

2.8. Statistical analysis

All results were expressed as mean values \pm SD ($n = 3$) and were statistically analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons using GraphPad Prism6. A p value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Characterization of tested materials

The micro-topography analysis of the sample surface by atomic force microscopy (AFM) has provided useful information on the roughness profile and parameter values characterizing the 3D roughness profile groups (Figure 1). Roughness

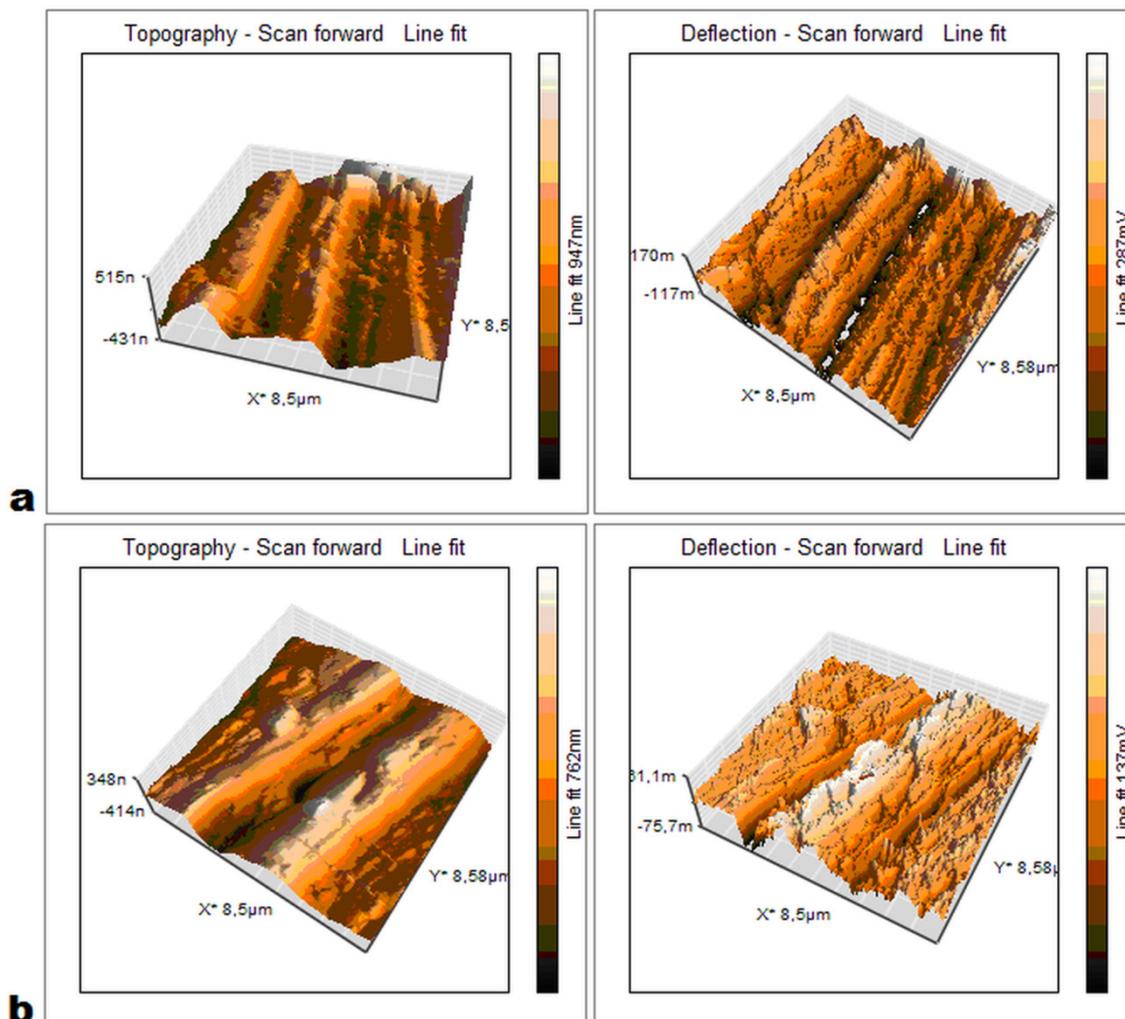


Fig. 1 - 3D AFM images of sample's surface: (a) Microscopic appearance of the surface of the sample cast, (b) Microscopic appearance of the surface of the sample cast + polishing / *Imagini 3D (AFM) ale suprafeței probei: (a) aspecte microscopice ale suprafeței probei din aliaj Ti10Zr în starea turnat; (b) aspecte microscopice ale suprafeței probei din aliaj Ti10Zr în starea turnat +prelucrare mecanică și lustruire la luciu oglindă.*

Table 3

Parameters which characterize the profile of surface roughness
 Parametrii valorici ai profilului de rugozitate ai suprafeței probei din aliaj Ti10Zr

Samples Proba	Parameters values/ Valori parametri						
	Area μm ²	S _a nm	S _q nm	S _z nm	S _p nm	S _v nm	S _m μm
a	73.45	107	133.56	1737	848.55	- 888.59	- 3.371
b	73.45	101.16	121.78	732.76	340.94	- 391.82	- 2.8066

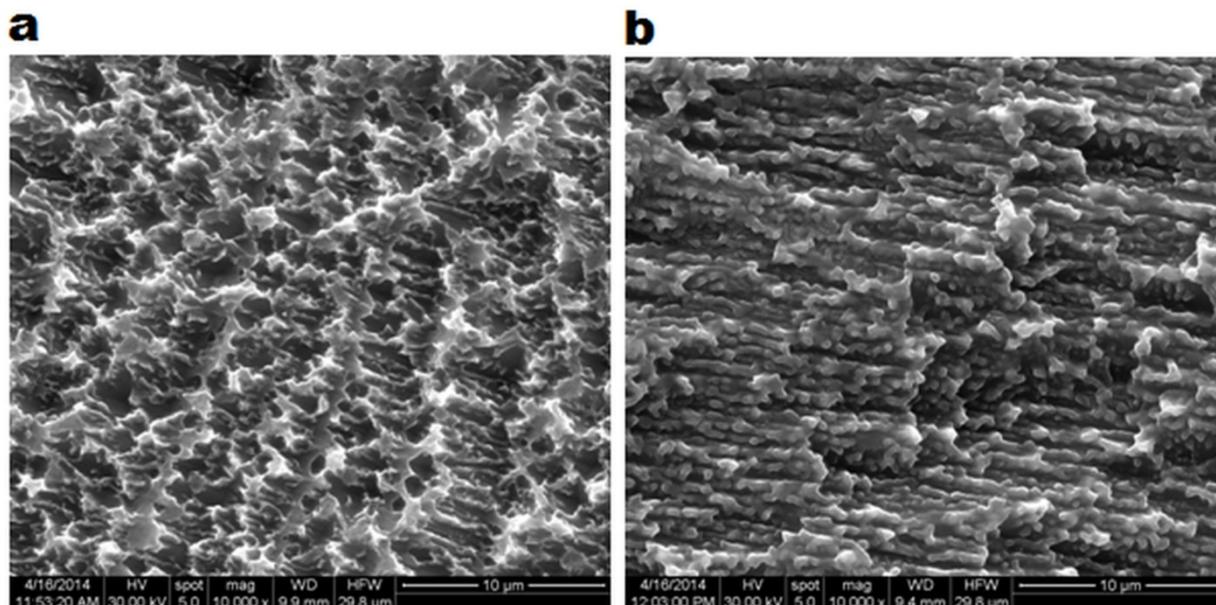


Fig. 2 - SEM surface morphology of sample corroded (a) and sample anodized (b) / Aspecte microscopice (SEM) ale morfologiei suprafeței probei corodate (a) și anodizată /oxidare anodică (b).

corresponds to a very fine grinding surface (Table 3). There are isolated peaks of high roughness having rounded shape, which is a characteristic of material processing.

The anodic oxidation method allows for the development of an oxide film on the surface of the material with a role in improving the adhesion and fixation properties. Anodic oxide films of titanium and its alloys show complex surface microstructures and morphologies. The Ti10Zr alloy samples processed in this way provides a special configuration of the surface, as shown in the scanning electron microscopy images (Figure 2a and 2b). The surface obtained has outstanding chemical stability and properties of biocompatibility and bioactivity in contact with the biological environment.

The oxide film is a basis for the formation of the osteoinductive matrix where osteogenic cell mitosis can occur with further osteoblasts and osteocytes activity.

3.2. Evaluation of cytotoxicity

The cytotoxicity caused by osteoblasts exposure to Ti10Zr or pure titanium was evaluated by quantifying the LDH release into the extracellular medium which is an indicator of cell membrane integrity. As Figure 3 shows, none of the tested materials exerted toxic effects on

osteoblasts membrane integrity, the values being comparable to those of TCPS (cells grown in the absence of any material).

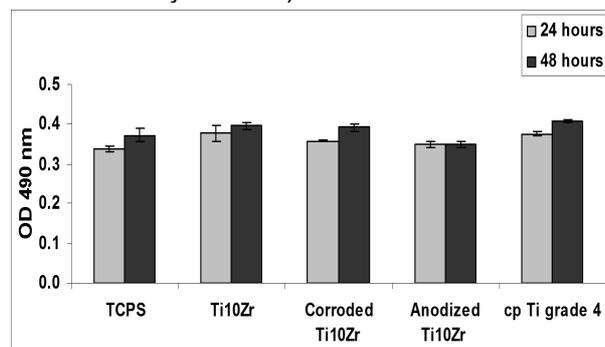


Fig. 3 - Comparative evaluation of LDH release in culture media by G292 osteoblasts after 24 and 48 hours of culture on TCPS, Ti10Zr or pure Ti samples. Data are expressed as mean ± SD / Evaluarea comparativă a nivelului LDH eliberat de osteoblastele G292 în mediu după 24 și 48 ore de cultivare a celulelor pe TCPS (Control) sau în prezența materialelor Ti10Zr și Cp-Ti. Rezultatele experimentale prelucrate statistic sunt exprimate ca valori medii ± SD (n=3)

3.3 Evaluation of osteoblasts morphology, adhesion and spreading

The organization of actin filaments was examined by fluorescence microscopy after 24 and 48 h of cell culture on the Ti10Zr surface (Figure 4).

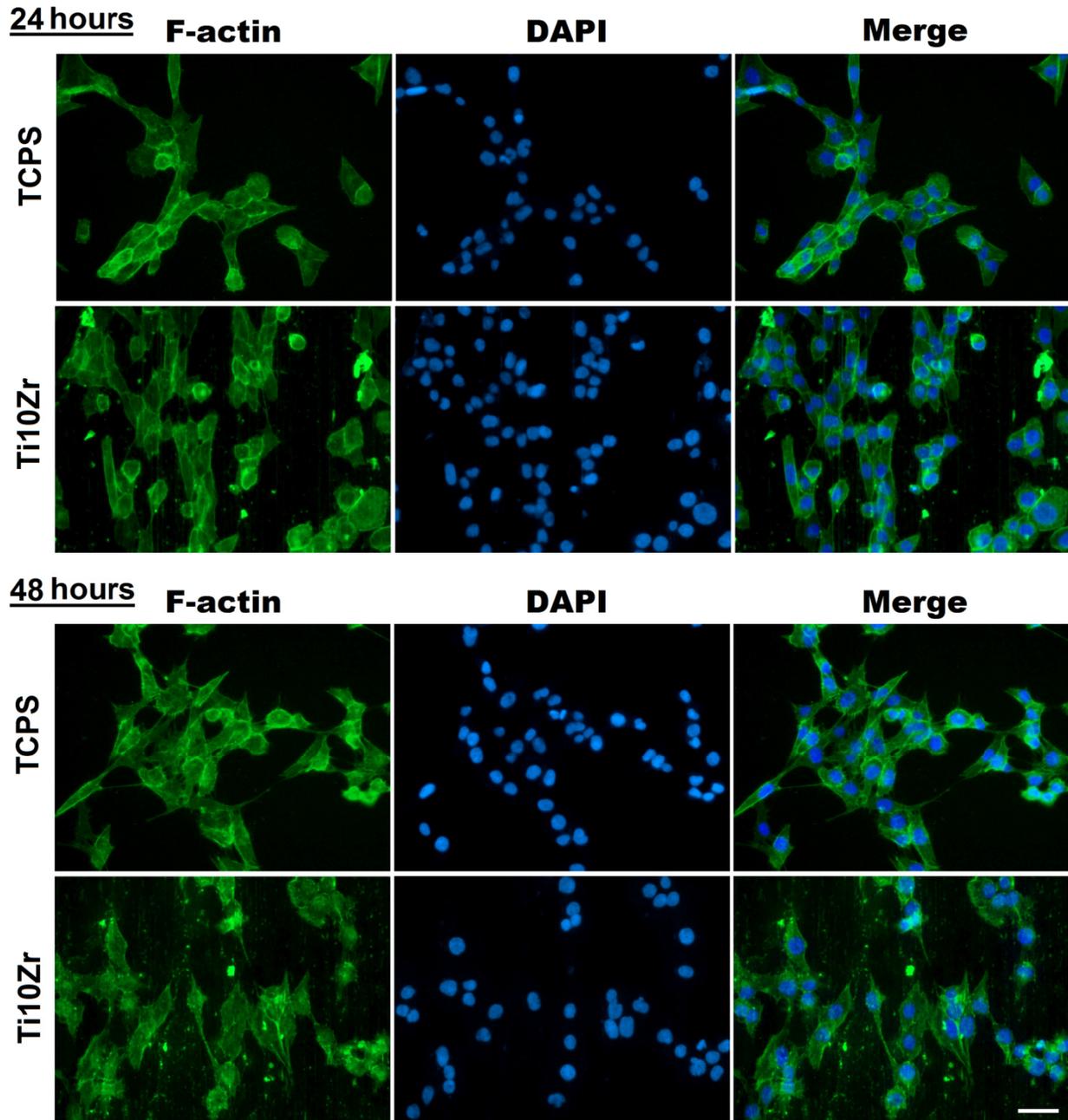


Fig. 4 - Fluorescence images showing the actin filaments labeled with phalloidin-FITC (nuclei counterstained with DAPI) in osteoblasts grown for 24 and 48 hours on TCPS or on the surface of Ti10Zr alloy. Scale bar = 50 μm / Evidențierea citoscheletului de actină prin marcarea fluorescentă a F-actinei cu faloidina-FITC (contracolorare nucleii cu DAPI) în osteoblastele crescute timp de 24 și 48 ore pe suprafața TCPS (Control) sau pe suprafața materialului Ti10Zr. Scala de măsură= 50 μm .

It was observed that cells grew in monolayer and displayed an osteoblast-like phenotype, with no major differences regarding the F-actin organization between these cells and the control ones. Also, round cells were noticed on the surface of Ti10Zr indicating osteoblasts undergoing mitosis. Good adhesion and spreading of osteoblasts on this type of surface were remarked after 24 and 48 hours, the cells showing a well organized actin cytoskeleton, cytoplasmic extensions interconnecting the neighboring cells. For both time intervals the cell densities were comparable or even higher than those observed for TCPS.

The morphological features of the cells adhered on the surface of corroded Ti10Zr, anodized Ti10Zr and cpTi grade 4 are shown in Figure 5. The number of osteoblasts attached on these surfaces was lower compared with Ti10Zr or TCPS, the cells being disposed at long distances one from other. Actin cytoskeleton was poorly organized, especially in the case of cpTi grade 4. In addition, it can be highlighted that the orientation of cells adhered on Ti10Zr was towards the direction of polishing process which was previously performed on the surface of this material further tested for its biocompatibility. This characteristic was also observed for the other

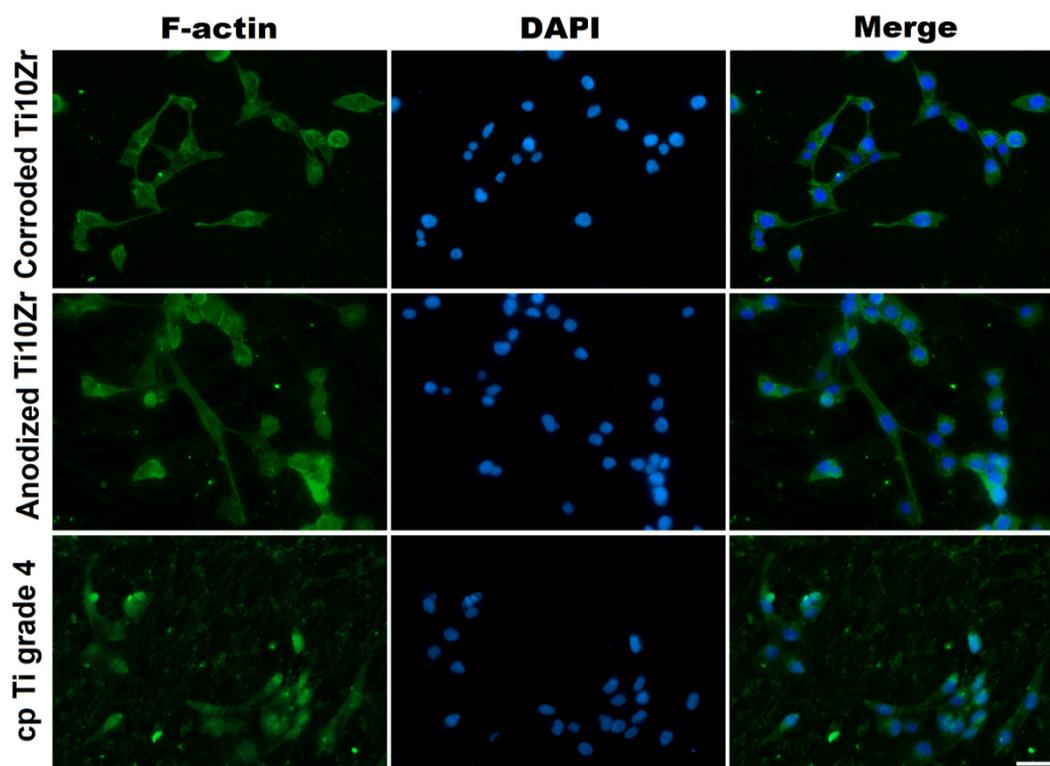


Fig. 5 - Fluorescence images showing the actin filaments labeled with phalloidin-FITC (nuclei counterstained with DAPI) in osteoblasts grown for 48 hours on the surface of corroded Ti10Zr, anodized Ti10Zr or pure Ti. Scale bar = 50 μ m / Evidențierea citoscheletului de actină prin marcarea fluorescentă a F-actinei cu faloidina-FITC (contracolorare nucleii cu DAPI) în osteoblastele crescute timp de 48 ore pe suprafața materialelor Ti10Zr, (corodat acid, oxidat anodic) și Cp-Ti grad4. Scala de măsură= 50 μ m.

polished material (i.e. cpTi grade 4) and is in agreement with previous findings [30]. However, it was not noticed for corroded or anodized TiZr, suggesting that additional processes have changed the properties of these surfaces, and thus have decisively influenced cell orientation and adherence. These differences detected between materials could have a major influence on how osteoblasts succeeded to adapt to surfaces with various features, Ti10Zr being the best option for dental and orthopedic implants.

3.4. Analysis of intracellular GSH distribution and content

Intracellular GSH level can be a valuable indicator of cellular proliferative capacity due to its role in redox regulation of nuclear proteins. Fluorescence labeling of GSH using CMFDA showed a distribution of this molecule quite similar in the cells cultivated on the surface of Ti10Zr and those attached on TCPS (Figure 6a).

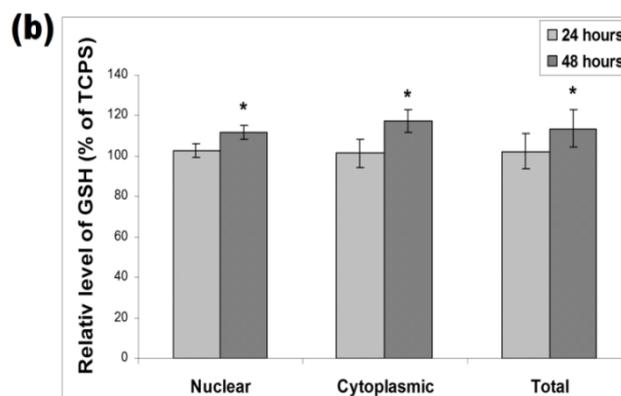
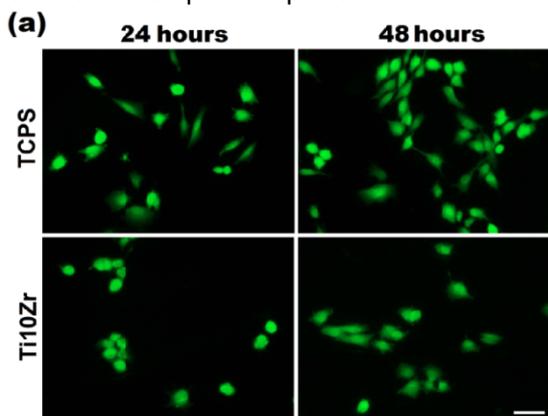


Fig. 6 - GSH distribution (a) and level (b) established after CMFDA staining of osteoblasts grown for 24 and 48 hours on TCPS or on the surface of Ti10Zr materials. Scale bar = 50 μ m. Data are calculated as mean \pm SD (n=3) and expressed relative to TCPS. * p <0.05 compared with TCPS / Distribuția (a) și nivelul GSH (b) determinată prin marcarea fluorescentă cu CMFDA la nivelul osteoblastelor crescute timp de 24 și 48 ore pe TCPS (Control) sau pe suprafața materialelor Ti10Zr. Scala de măsură= 50 μ m. Rezultatele experimentale prelucrate statistic sunt exprimate ca valori medii \pm SD (n=3) raportate la TCPS. * p <0.05.

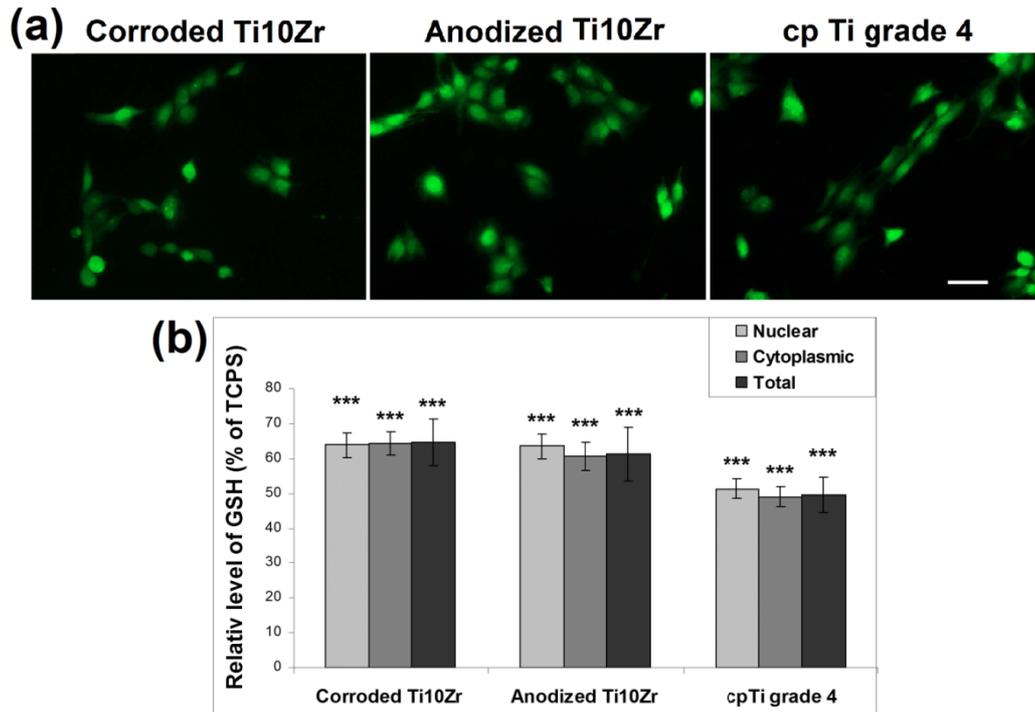


Fig. 7- GSH distribution (a) and level (b) established after CMFDA staining of osteoblasts grown for 48 hours on the surface of Ti10Zr or pure Ti materials. Scale bar = 50 μ m. Data are calculated as mean \pm SD (n=3) and expressed relative to TCPS. *** p <0.001 compared with TCPS / Distribuția (a) și nivelul GSH (b) crescute timp de 48 ore pe suprafața materialelor de Ti10Zr (acid corodat, oxidat anodic) sau Cp-Ti. Scala de măsură = 50 μ m. Rezultatele experimentale prelucrate statistic sunt exprimate ca valori medii \pm SD (n=3) raportate la TCPS. *** p <0.001.

Quantification of GSH-CMF fluorescence in the cells adhered on the surface of Ti10Zr material (Figure 6b) revealed that nuclear and cytoplasmic GSH levels were similar to TCOS after 24 hours, increasing by 12% and 17%, respectively, after 48 hours. This increase proves that this material stimulates cell proliferation after 48 h of cultivation due to its intrinsic properties which provide the best conditions necessary for a proper cellular growth.

GSH fluorescence labeling in cells adhered on the surface of corroded Ti10Zr, anodized Ti10Zr or simple Ti (Figure 7a) confirmed a significantly lower adhesion and cell growth compared with TCPS and Ti10Zr, which is in agreement with the results of F-actin staining. Fluorescence quantification indicated a decrease in GSH levels by up to half of the control in case of cpTi grade 4 after 48 hours of cultivation (Figure 7b). Thus, the biocompatibility of Ti10Zr was proven by this *in vitro* assay which validates the ability of this material to promote cell proliferation.

4. Conclusions

The results of this *in vitro* study demonstrate the biocompatibility of Ti10Zr alloy and confirm its efficiency to induce human osteoblasts attachment and proliferation. The absence of cytotoxicity, a well organized actin cytoskeleton and a stimulated cell proliferation compared with pure titanium underline the enhancement of its biological properties,

recommending Ti10Zr alloy for medical devices manufacturing such as dental implants.

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