ORIGINAL PAPER

IN VITRO CHARACTERIZATION OF HYDROXYAPATITE-BASED BIOMATERIALS, USING MESENCHYMAL STEM CELL CULTURES FROM HUMAN BONE MARROW

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Abstract. *There is considerable interest in the discovery of biomimetic materials that improve cell adhesion and reduce the time of bone integration of implants. In vitro bone cells biocompatibility was performed for the two types of biocomposites hydroxyapatite-titanium (HApTi) and hydroxyapatite-titanium with added calcium fructoborate (HApTiCaFb). In order to investigate the potential cytotoxicity of biomaterials, cell adhesion, proliferation, and osteogenic differentiation, human mesenchymal stem cells (MSCs) were used. Regarding the in vitro biocompatibility, a slight improvement in the phenotype was observed for the samples to which CaFb was added.*

Keywords: *hydroxyapatite; calcium fructoborate; mesenchymal stem cell; human bone marrow.*

1. INTRODUCTION

The word "composite" refers to a heterogeneous, macroscopic combination of two or more materials (with different composition, morphology, and physical properties), being designed to produce specific physicochemical and mechanical properties. Therefore, the advantage of composites is that they have the best qualities of their constituents and often have some properties that the constituents alone do not have. Moreover, composite materials allow a flexible design, because their structure and properties can be optimized and adapted to specific applications [1-6].

Orthopedics is the medical area where the application of biomaterials is highly developed for both marketing and research purposes. The use of composites in orthopedics offers a variety of new implant designs and the ability to adapt the specific properties of the device to medical needs. Composite materials can reproduce both macroscopic and microscopic structures, as well as the most important mechanical properties of natural tissues. Composite materials are studied and tested to improve the performance and long-term stability of femoral implants and bone cement, to replace cartilage, to rebuild tendons and ligaments, and as bone grafts. Bone tissue combines a cellular component, osteocytes, and a matrix [7]. The matrix is a composite consisting of an organic portion, composed of collagen

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fibers, which gives strength and flexibility to the bones and an inorganic portion, composed of HAp microcrystals and mineral salts, giving hardness to the bone [8]. *In vitro* studies have shown that FbCa is a superoxide ion capture and an anti-inflammatory agent. It can influence the production of inflammatory mediators by macrophages, can be beneficial for the suppression of cytokine production and inhibits the progression of endotoxin-associated diseases, like boric acid and other sources of boron [9].

In the present study, *in vitro* biocompatibility tests were performed using MSCs seeded at constant density on biomaterials for biomedical applications that involve interaction with the respective bone cytotoxicity, cell adhesion, proliferation, and differentiation.

2. MATERIALS AND METHODS

2.1. DESIGN AND FABRICATION OF IMPLANTED CYLINDERS

The implanted biocomposite samples were prepared by powder metallurgy technology. The matrix material is represented by the HAp powder particles (average particle size, 200 nm; Merck, Darmstadt, Germany), which was reinforced by titanium hydride (TiH_2) powder particles (~100 μm; water atomized; Merck, Germany). The mixing content between the components was 75% weight HAp and 25% weight TiH_2.

The first step of preparing the biocomposite samples was HAp drying. One calcination cycle was completed in air at 900 °C for 1 hour. Then, the calcinated matrix and reinforcement powder particles were mixed in a planetary ball mill for 30 min. The ratio between the milling balls (stainless steel, 5 mm diameter) and the powder mixture was 2:1. The milling environment was ethanol (1 mL per 1 g powder mixture). The obtained mixture was then dried for 1 h at 200 °C in a conventional furnace, followed by the extraction of the milling balls from the powder mixture. The biocomposite samples were shaped using cold compaction operation at 120 MPa in a metallic die, with an inner diameter of 10 mm, on A009 electromechanical-computerized 100 kN testing machine (LBG testing company, Azzano, Italy), equipped with TCSoft2004Plus software. Next, compacts were submitted to two steps of sintering (TSS) heat treatment using a type L5/12 chamber furnace at a maximum temperature of 1200 °C. A protective gas atmosphere (pure argon 99.98%) was present during the entire treatment through a rear wall connection of the furnace [10]. During the first step of TSS, compact heating reached a peak temperature (T_1 = 900 °C) for a very short time (1 min) to achieve an intermediate density by the initiation of the diffusion process between HAp and TiH_2 powder particles. Next, the second step of TSS was completed, which involved rapid cooling to T_2 = 800 °C, and the dwell time was 10 hours. Thus, the final densification process occurred without grain growth of nanosized HAp particles [11]. A set of sintered samples was immersed into a CaFb-based solution (0.4 g/10 mL water), while another set was kept uncoated as control.

2.2. BIOCOMPATIBILITY EVALUATION TOWARD CELL CULTURE

Sterilization of materials

The materials were sterilized by incubation with a 1% penicillin-streptomycin solution in PBS for 2 h at room temperature. These materials were then used for cell seeding and subsequent experiments.

Culture of mesenchymal stem cells (MSCs)

MSCs were isolated from bone marrow harvested from patients undergoing prosthetic surgery at the Orthopedic Surgery Clinic of the Emergency Hospital in Craiova, Romania. It was obtained by informed, written consent from all subjects in the study in order to perform the research and publish the study's results. The research was conducted with the approval of the Hospital's Ethics Committee (No. 68/2016). The bone marrow was separated on Ficoll (Amersham) to isolate the MSC-containing fraction. Cells were cultured in LG DMEM (low-glucose content, 1 g/L), supplemented with 10% fetal serum (FBS), 1% Glutamax (Grand Island, New York), 50 U/mL penicillin, and 50 mg/mL streptomycin (Invitrogen). The medium was changed every 3 days, and the cells were expanded for approximately 10 days, aiming not to exceed 80% confluence, in a humidified atmosphere of 37°C and 5% CO_2. At passage 2, the cells were subjected to immunophenotyping by flow cytometry for validation of specific markers and then used in experiments or cryopreserved. For all experiments performed in the study, cells were seeded at a density of 5000 cells/cm^2 [12].

Immunofluorescence microscopy

The samples were tested by fluorescence microscopy using adhesion and proliferation markers. Five days after seeding, the samples were tested together with the standard and washed with PBS to remove unstained cells and serum proteins. The adhered cells were fixed with 4% PFA solution for 20 min at room temperature. After fixation, the cells were permeabilized with 0.2% Triton-X-100 for 3 min, and then samples were blocked for 1 h in 0.5% BSA. Cells were labeled with the primary antibody anti-Ki67 (NeoMarkers) for 30 min in blocking buffer. After removal of excess unbound antibodies, cells were incubated with secondary antibodies conjugated to Alexa Fluor 488 Phalloidin (1:100), which specifically binds to the actin filaments. After 30 min, the cells were washed and labeled for 1 min with Hoechst and then mounted on slides using Prolong Antifade solution (Invitrogen). Samples were fixed with Loctite glue to the slides to allow automatic scanning via the inverted TissueFAXS iPlus system, thereby enabling reconstruction of the image of the entire sample area and quantitative cytometric determinations, according to the procedure described below (20× objective). The cells were analyzed under a Zeiss Axio Imager Z1 microscope (Boston Microscopes, Massachusetts, USA) to highlight focal contacts (40× objective).

Image cytometric analysis

The TissueFAXSiPlus technology platform from TissueGnostics (Vienna, Austria) allows scanning and reconstitution of specimens mounted on microscope slides (TissueFAXS Slides module) using the PCO PixelFly camera. All images were acquired using the same settings. Quantitative analysis of the expression of the proteins of interest was performed using the TissueQuest software module, based on the detection of the cell nuclei stained with

4′,6-diamidino-2-phenylindole (DAPI). For the segmentation of the scanned images, the average nuclear size, the area of discrimination, the gray level of the pixels, and the threshold level of the background were specified. The intensities of each specific signal were determined on each fluorescence channel separately (FITC, TxRed, DAPI). The regions of interest (ROI) were defined so that only the outer edges of the samples were eliminated. The general set-ups (determining the gating strategy, *i.e.*, choosing the gates to delimit the population of interest, establishing the cutoffs between positive and negative cells for a specific marker) were made on a representative small size image. Following that, all the images were analyzed using the same settings after the necessary adjustments were made. For quality control of these settings, the forward and backward gating options of the software were used. The results were visualized as scattergrams and histograms. Cutoffs and gates were set on scattergrams. Statistics regarding the number of events analyzed and the percentage of Ki67-positive cells were exported from the software.

3. RESULTS AND DISCUSSION

3.1. IN VITRO CELL ADHESION AND PROLIFERATION

Our biocompatibility experiments aimed to determine the effects of HApTi and HApTiCaFb on the adhesion of human osteoprogenitor stem cells. Five days after seeding, cells grown on HApTi and HApTiCaFb substrates formed a semi-confluent monolayer on the surface of the biomaterials, whereas the cells reached confluence on the controls (standard cover slip material [CS] and non-coated titanium [Ti]) (Fig. 1A).

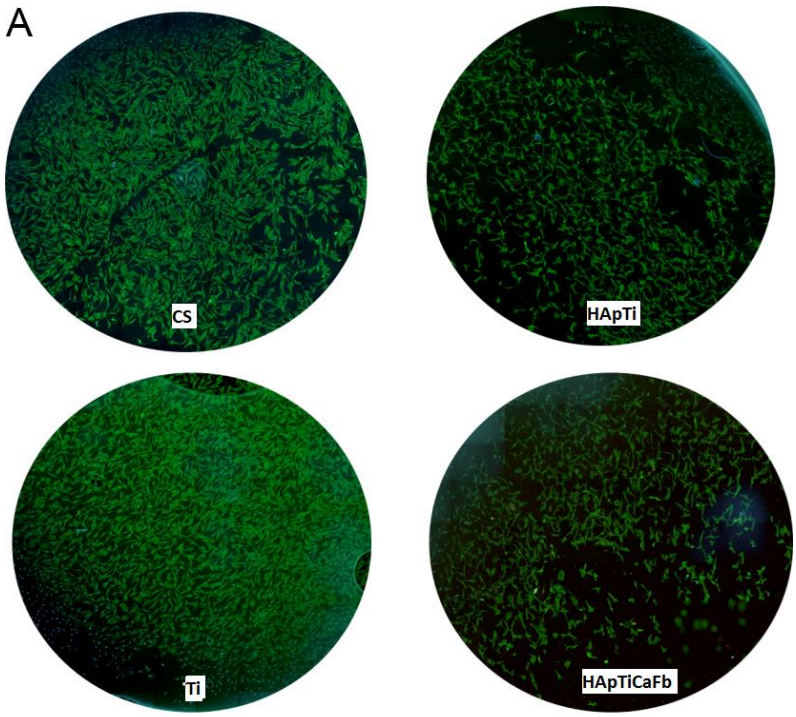

Figure 1A. MSC adhesion 5 days after cultivation on tested materials.

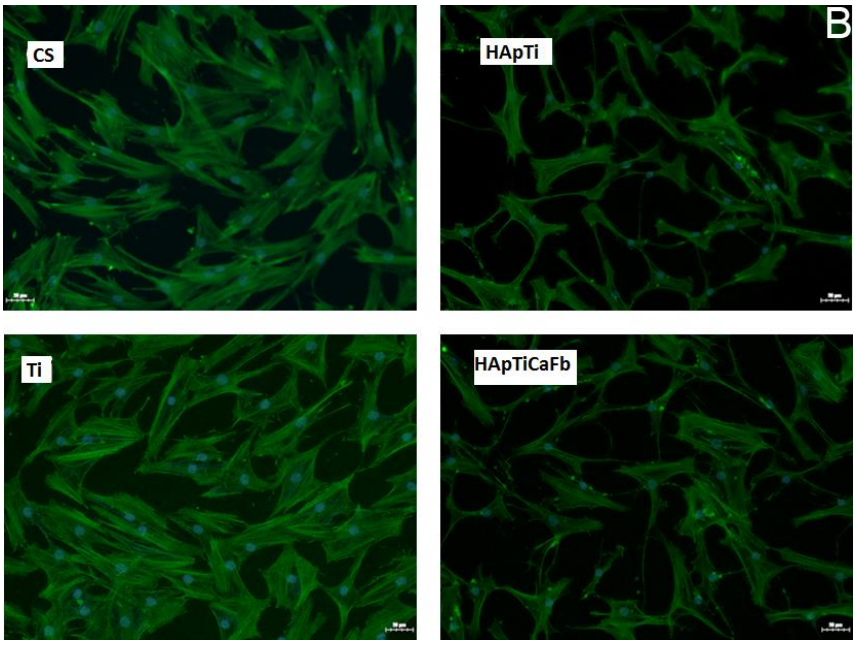

Figure 1B. Reconstitution of the entire sample was performed by scanning the samples with the TissueFAXSiPlus system

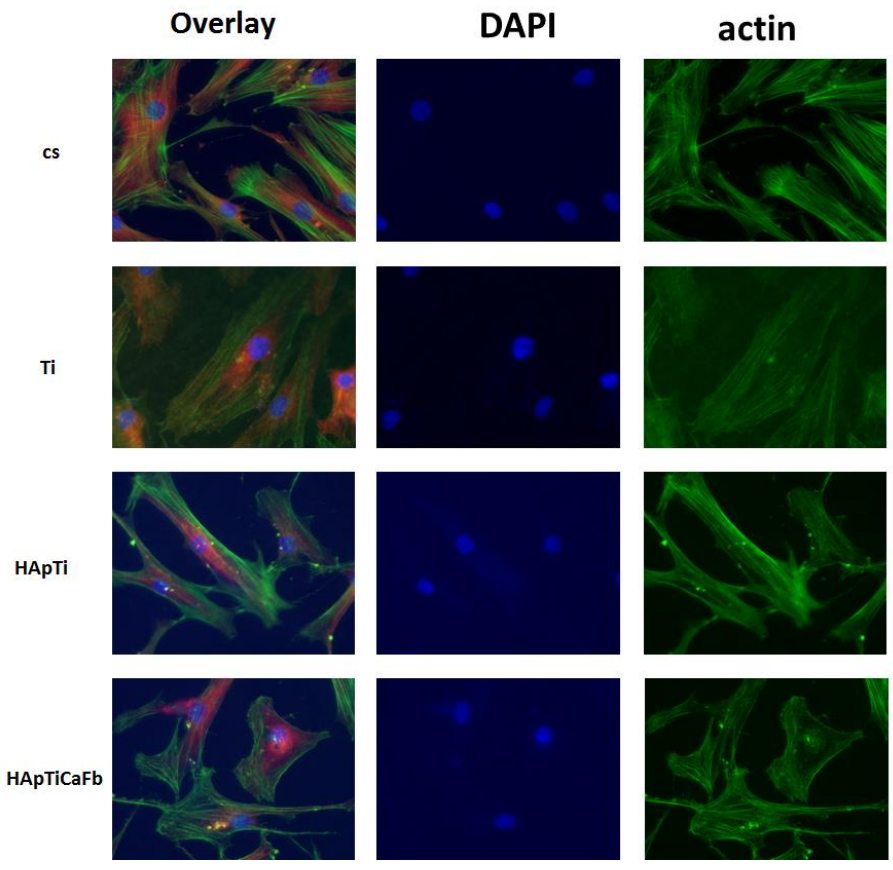

Figure 1C. Representative individual field of view obtained by acquisition with the ×20 microscope objective. Detailed images of the cells can be seen with the ×40 magnification obtained under the Zeiss Axio Imager microscope. Actin is labeled using Alexa Fluor 488 Phalloidin (green). Nuclei are stained with Hoechst (blue).

By comparing cell morphology in representative fields of view for each substrate, we found that cells grown on HAp disk had a thin cell body and developed long dendrites (Fig. 1B), compared to cells grown on glass or Ti. In another experiment, it was cultured the cells for 5 days on Ti disks, followed by fixation and fluorescence staining of the cells to visualize the actin cytoskeleton in focal contact points (Fig. 1C).

3.2. *QUANTIFICATION OF MESENCHYMAL OSTEOPROGENITOR STEM CELL PROLIFERATION ON HAPTI AND HAPTICAFB BIOMATERIALS BY IMAGE CYTOMETRY*

The proliferation of MSCs on the new polycyclic aromatic hydrocarbon surface was analyzed as well. It quantitatively analyzed the proportion of cells in proliferation by fluorescence labeling of the nuclear marker Ki67, which is expressed by all cells undergoing cell division. $Ki67^{+}$ cells were identified on both control samples (CS and Ti) as well as HApTi and HApTiCaFb samples (Fig. 2A). Quantitative analysis showed that the percentage of proliferative cells was higher on HApTi (23.91%) and HApTiCaFb (25.58%) disks than on Ti (16.26%) or glass (4.38%) (Fig. 2B).

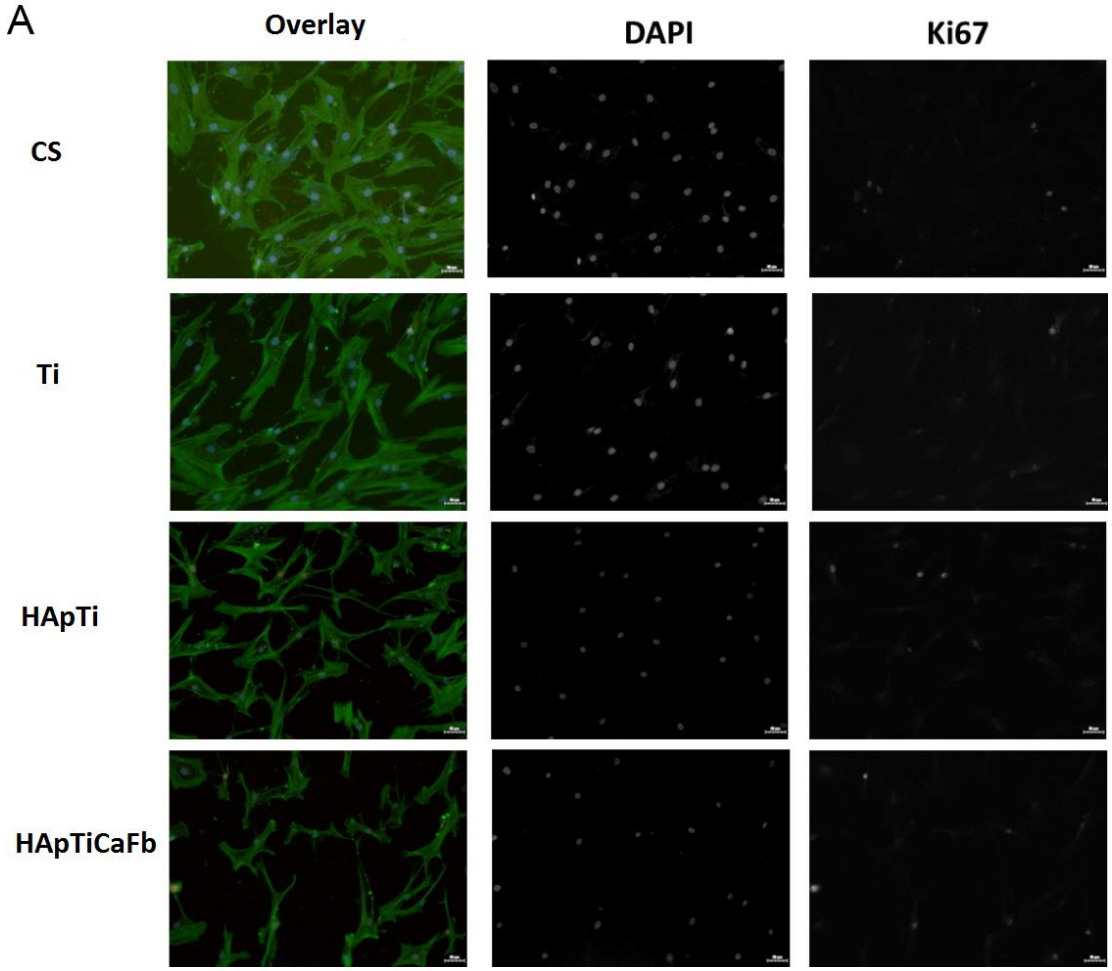

Figure 2A. Proliferation of MSC at 5 days after cultivation on tested samples.

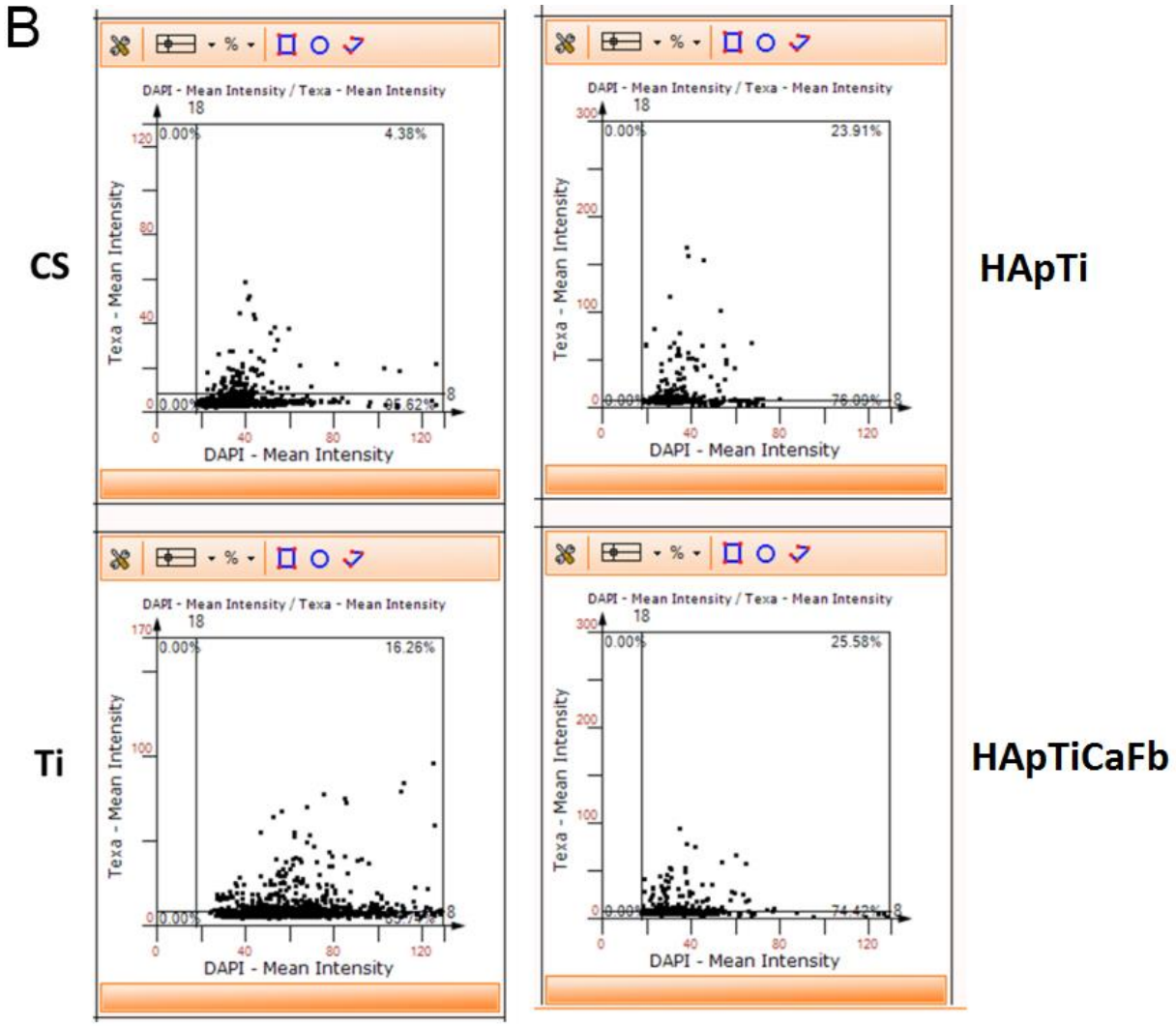

Figure 2B. Scattergram resulting from cytometric analysis of the images showing the percentage of Ki67$^+$ cells (upper right quadrant) for each sample type. Representative individual field of view obtained by acquisition with the ×20 objective of the TissueFAXS iPlus imaging system with the decomposition on fluorescence channel for nuclear signal (DAPI) and proliferation marker expression (Ki67).

4. CONCLUSIONS

The two sets of results indicate that HApTi and HApTiCaFb represent substrates with potential use in contact with osteoprogenitor cells. The modification of the cell morphology due to the reorganization of the cytoskeleton does not negatively affect their proliferation, which is even superior to that observed in the unmodified Ti substrate. Regarding this biocompatibility study using cell cultures, a slight improvement in the phenotype was observed for the samples with the addition of CaFb.

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