UNIVERSIDADE DE SÃO PAULO

FACULDADE DE ODONTOLOGIA DE BAURU

PAULO HENRIQUE MARTINS FERNANDES

Effects of TiO_2 nanoparticles addition on dense bovine hydroxyapatite bioceramic on human odontoblasts

Efeitos da adição de nanopartículas de TiO_2 em biocerâmica densa de hidroxiapatita bovina em odontoblastos humanos

Bauru

2021

PAULO HENRIQUE MARTINS FERNANDES

Effects of TiO_2 nanoparticles addition on dense bovine hydroxyapatite bioceramic on human odontoblasts

Efeitos da adição de nanopartículas de TiO_2 em biocerâmica densa de hidroxiapatita bovina em odontoblastos humanos

Dissertação constituída por artigo apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo, como requisito para a obtenção de título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração em Dentística Operatória.

Orientadora: Profa. Dra. Ana Flávia Sanches Borges

Versão corrigida

Bauru

2021

Martins Fernandes, Paulo Henrique

Effects of TiO_2 nanoparticles addition on dense bovine hydroxyapatite bioceramic on humans odontoblasts / Paulo Henrique Martins Fernandes. – Bauru, 2021.

46 p. : il. ; 31 cm.

Dissertação (mestrado) -- Faculdade de Odontologia de Bauru, Universidade de São Paulo, 2021

Orientador: Profa. Dra. Ana Flávia Sanches Borges

Nota: A versão original desta dissertação/tese encontra-se disponível no Serviço de Biblioteca e Documentação da Faculdade de Odontologia de Bauru – FOB/USP.

Autorizo, exclusivamente para fins acadêmicos e científicos, a reprodução total ou parcial desta dissertação/tese, por processos fotocopiadores e outros meios eletrônicos.

Assinatura:

ERRATA

FOLHA DE APROVAÇÃO

DEDICATÓRIA

Dedico este trabalho à Ciência, criação mais importante de toda a história da humanidade, e sem a qual continuaríamos desorientados, apenas assistindo as sombras aparecerem no fundo da caverna. Também dedico à Faculdade de Odontologia de Bauru, ao Programa de Pós-Graduação e ao departamento de Dentística, Endodontia e Materiais Odontológicos, os quais me permitiram a inserção no mundo das ciências desde 2014, quando comecei minha Iniciação Científica. E agradeço imensamente a meus pais e familiares, que me proporcionaram este percurso, e sem os quais eu não seria nada.

AGRADECIMENTOS

À Faculdade de Odontologia de Bauru, da USP, em especial ao departamento de Dentística, Endodontia e Materiais Odontológicos, onde tive a oportunidade de vivenciar um excelente programa de Pós-Graduação.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pelo apoio financeiro, sem o qual não seria possível me dedicar exclusivamente às atividades do mestrado (processo 133552/2019-5).

À minha orientadora, Profa. Dra. Ana Flávia Sanches Borges, pelo acolhimento, orientação e apoio desde o período da minha graduação, durante a prática profissionalizante e o mestrado, e pelo desenvolvimento do nosso projeto e de todos os outros anteriores que deram fruto a este trabalho, sempre se mostrando ser um ser humano excepcional, correto, de ótimos ideais e de excelente parceria. Muito obrigado por toda a orientação e ajuda, principalmente nos momentos de dificuldade em meio a esta pandemia que surgiu durante o curso do meu mestrado e ainda continua.

À Profa. Dra. Diana Gabriela Soares, mais nova professora Dentística Operatória e que trouxe a fantástica linha de pesquisa de cultura celular e engenharia tecidual ao departamento, e toda sua equipe, em especial às pós-graduandas Ester Alves Ferreira Bordini e Fernanda Balestrero Cassiano, que nos permitiram a execução das análises biológicas.

À Profa. Dra. Maria Fidela de Lima Navarro, por todo o apoio, atenção, carinho e acolhimento desde meu primeiro ano na graduação como orientadora de iniciação científica e que após minha graduação continuou a me incluir em projetos e elaboração de artigos e me orientar na odontologia e nas ciências aplicadas como um todo, sendo minha maior referência nessas áreas.

Ao Prof. Dr. Paulo Noronha Lisboa Filho e sua equipe, que nos permitiram realizar as análises morfológicas e químicas de nosso trabalho.

A minha família, que sempre me estruturou, fundamentou e norteou para que eu pudesse me tornar o ser humano que sou e que me permitiu chegar onde estou e a minha namorada, Isabella, que sempre me apoiou, acolheu, ajudou nos momentos de maiores dificuldade desde que nos conhecemos.

A todos os meus amigos que me apoiaram durante toda a minha vida, mas principalmente durante a graduação e pós-graduação, em especial ao Pedro Henrique Magão, que se tornou um dos poucos amigos que tenho na vida e que me acompanhou, ajudou, fez companhia, foi meu parceiro de clínica e de seminários durante meu mestrado.

A todos os educadores que passaram por minha trajetória, construindo-a, desde o ensino fundamental até então, em especialmente a todos os professores e funcionários dos setores de Dentística e Materiais Odontológicos da FOB-USP, os quais muito me ensinaram tanto na graduação quanto na pós-graduação e com os quais vivenciei experiências magníficas.

“Nullius in Verba”

The Royal Society

ABSTRACT

This study evaluated the effect of TiO_2 nanoparticles on dense hydroxyapatite (HA) in human osteoblastic cells (SAOS-2). The natural source of HA was bovine bones. The experimental groups were performed from particulate HA powder with or without the addition of 5 or 8% TiO_2 (DPBHA, DPBHA/AnataseNp5% or DPBHA/AnataseNp5%), pressed into discs (Ø = 12.5 mm; thickness = 1.3 mm) uniaxially (100Mpa) and isostatic (200 MPa/1 min) and sintered at 1300 °C. Y-TZP discs had exact HA dimensions. The tests performed were: (1) Scanning Electron Microscopy and Dispersive Energy Spectroscopy (SEM / EDS) (2) Atomic Force Microscopy (AFM); (3) biological tests using the Alamar Blue (AB) and Alizarin Red (AR) methods. Obtained data were tabulated and submitted to 2-way ANOVA and Tukey tests for the results of AB and ANOVA and Tukey tests for AR. SEM revealed that the surface of DPBHA/AnataseNp5% resembles DPBHA surface but also contains smaller granules and that the character of DPBHA/AnataseNp8% resembles DPBHA/AnataseNp5% surface, but with irregular topography and Y-TZP showed a typical oxide ceramics surface pattern. EDS revealed Ca, O and P in all HA composed samples with Ti in the reinforced ones, and C, O and Zr in Y-TZP samples. AFM data corroborates with SEM analysis. AB test revealed excellent cellular viability for DPBHA/AnataseNp5% group and AR test revealed that all groups containing TiO_2np showed more mineralized matrix deposition than all other groups, with low statistical differences between reinforced groups and DPBHA cultivated in non-osteogenic medium and much bigger in osteogenic medium, with DPBHA/AnataseNp8% showing the best results. In conclusion, the addition of TiO_2np showed chemical, superficial and biological changes in the reinforced materials, with the DPBHA/AnataseNp5% group being the one that showed the best results in cell viability and DPBHA/AnataseNp5% mineralized matrix deposition.

Keywords: Durapatite, Biocompatible Materials, Ceramics

RESUMO

Este estudo avaliou o efeito de nanopartículas de TiO_2 em hidroxiapatita (HA) densa em células osteoblásticas humanas (SAOS-2). As fontes naturais de HA foram ossos bovinos. Os grupos experimentais foram confeccionados a partir de pó de HA com ou sem adição de 5 ou 8% de TiO_2 (DPBHA, DPBHA/AnataseNp5% ou DPBHA/AnataseNp5%), prensados em discos (Ø = 12,5 mm; espessura = 1,3 mm) de forma uniaxial (100Mpa) e isostática (200 MPa/1 min) e sinterizados a 1300 °C. Discos de Y-TZP continham as mesmas dimensões de HA. Os testes executados foram: (1) Microscopia Eletrônica de Varredura (MEV) e Espectroscopia por Energia Dispersiva (EDS) (2) Microscopia de Força Atômica (AFM); (3) testes biológicos utilizando os métodos de Alamar Blue (AB) e Alizarin Red (AR). Os dados obtidos foram tabelados e submetidos aos testes ANOVA 2 critérios e Tukey para os resultados de AB e ANOVA e Tukey para AR. A MEV revelou que a superfície de DPBHA/AnataseNp5% se assemelha à de DPBHA, mas também contém grãos menores e a caracterização de DPBHA/AnataseNp8% se assemelha à superfície de DPBHA/AnataseNp5%, mas com topografia irregular, e a Y-TZP mostrou um padrão superficial comum ao das cerâmicas oxidas. A EDS revelou Ca, O e P em todas as amostras compostas por HA com Ti nos grupos reforçados, e C, O e Zr nas amostras de Y-TZP. Os dados da MFA corroboram com análise de MEV. O teste de AB revelou excelente viabilidade celular para o grupo DPBHA/AnataseNp5% e o teste de AR revelou que os grupos contendo TiO_2np apresentaram maior deposição de matriz mineralizada que todos os outros grupos, com pouca diferença estatística entre os grupos reforçados e DPBHA, cultivados em meio não osteogênico, e deposição de matriz mineralizada muito maior em meio osteogênico, com DPBHA/AnataseNp8% apresentando os melhores resultados. Em conclusão, a adição de TiO_2np mostrou diferenças químicas, superficiais e biológicas nos materiais reforçados, sendo que o grupo DBPHA/AnataseNp5% mostrou os melhores resultados de viabilidade celular e DBPHA/AnataseNp8% de deposição de matriz mineralizada.

Palavras chave: Durapatita, Materiais Biocompatíveis, Cerâmica

SUMÁRIO

1. Introduction

1. INTRODUCTION

Restorative dentistry has ceramics as one of the oldest materials used for the aesthetic and functional restoration of teeth, mainly for its good mechanical properties, durability, and excellent optical properties, capable of simulating the characteristics of natural teeth [1–5]. Since the beginning of the use of the first dental ceramics of feldspathic composition, better properties have researched for this group, both looking for new ceramics and formulations and the reinforcement of these materials [1–9].

Searching for better ceramics for dental applications, aluminized ceramics, vitreous ceramics, and polycrystalline ceramics emerged. The last group depended on the advancement of CAD-CAM technologies, so these polycrystalline ceramics with high strength and no glass in their compositions could be used for restorations [1–6].

The evolutionary process of ceramics continues with the emergence of new ceramics and the reinforcement of this material with other materials [1]. An example of the application of mounts in ceramics is one of the most widely used ones, known as Y-TZP (yttria-stabilized tetragonal polycrystal zirconia). And composed of zirconia, it just has excellent mechanical properties when reinforced and stabilized by yttrium oxide, which allows the material to prevent the propagation of cracks, known as transformation toughening [8,10,11].

One of the ceramic groups that have been studied and used clinically is that of bioceramics. Bioceramics are non-metallic inorganic ceramic materials designed to interact with the biological system and fulfill a specific function and may be able to promote tissue regeneration [12–14].

They can be classified as natural, consisting of calcium carbonate, or synthetic, subdivided into bioinert such as Y-TZP and alumina, bioactive glasses such as borate and phosphate glasses, and calcium phosphate bioceramics, which occur naturally in the body [14], have greater similarity with mineralized tissues, excellent biocompatibility, integration with living tissues similar to the processes of natural bone remodeling, allows the apposition of osteoblasts on the surface of the material [12,14], but have limited mechanical properties [14].

A type of bioceramics is composed of hydroxyapatite nanoparticles, $Ca_{10}(PO4)_6(OH)_2$, which has the properties of being biocompatible, bioactive,

promoting osteoconduction and osteoinduction [14–16]. It is part of the natural composition of bones and teeth [17,18] And, bovine bones from meat production are renewable and an excellent source for obtaining hydroxyapatite nanoparticles [15].

HA nanoparticles are used in dental implants to improve osteogenesis, improve bone-implant contact, decrease microorganism's activity and bone grafts. It is also used to control dentinal hypersensitivity, readily penetrating the exposed dentinal tubules and reacting as a mineralizing agent, obliterating the tubules, being used in whitening gels as a remineralizing agent, and also in the remineralization of caries lesions [16].

The bioactivity present in bioceramics is significant in current dentistry. These materials can induce specific biological activities and stimulate a benevolent response in the host organism [19]. Bioactivity was first described in 1969 as the property of certain materials that cause specific biological responses at the material interface, resulting in bonds between tissues [20]. These materials can be bioactive glass, vitreous ceramics, phosphate-based ceramics. calcium, bioactive compounds, and bioactive coating materials [20], increasingly explored in the medical and dental fields.

One of the strategies adopted to obtain better material properties is the incorporation of nanoparticles in their compositions, acquiring better mechanical and biological properties, which has demonstrated in several studies [15,21–28]. One of the types of nanoparticles used in the reinforcement of materials is composed of titanium dioxide (TiO_2) [15].

TiO_2 nanoparticles are used in dentistry in whitening gels, reducing the time required for exposure to the gel. Thus, the treatment toxicity and as a dentin desensitizer in dentifrices, obliterating exposed tubules [29]. Studies show that the incorporation of these nanoparticles improves the mechanical and antibacterial properties of the materials [15,30–35], and that the application of these particles in the form of nanotubes on implant surfaces and other materials increased the properties favorable to the bone formation [36–40].

2. Article

2. ARTICLE

TIO_2 NANOPARTICLES ADDITION ON DENSE BOVINE HYDROXYAPATITE BIOCERAMICS INCREASES HUMAN OSTEOBLATS MINERALIZATION ACTIVITY

Fernandes, PHM[1]; Ferreira, EAB[1]; Cassiano, FB[1]; Lisboa-Filho, PN[2]; Fortulan, CA[3]; Soares, DG[1]; Borges, AFS[1];

1 – Department of Operative Dentistry, Endodontics and Dental Materials - Bauru Dental School, University of São Paulo

2 – Department of Physics, School of Sciences - São Paulo State University

3 – Department of Mechanical Engineering, São Carlos School of Engineering, University of São Paulo

ABSTRACT

This study evaluated the effect of TiO_2 nanoparticles on dense hydroxyapatite (HA) in human osteoblastic cells (SAOS-2). The natural source of HA was bovine bones. The experimental groups were performed from particulate HA powder with or without the addition of 5 or 8% TiO_2 (DPBHA, DPBHA/AnataseNp5% or DPBHA/AnataseNp5%), pressed into discs (Ø = 12.5 mm; thickness = 1.3 mm) uniaxially (100Mpa) and isostatic (200 MPa/1 min) and sintered at 1300 °C. Y-TZP discs had exact HA dimensions. The tests performed were: (1) Scanning Electron Microscopy and Dispersive Energy Spectroscopy (SEM / EDS) (2) Atomic Force Microscopy (AFM); (3) biological tests using the Alamar Blue (AB) and Alizarin Red (AR) methods. Obtained data were tabulated and submitted to 2-way ANOVA and Tukey tests for the results of AB and ANOVA and Tukey tests for AR. SEM revealed that the surface of DPBHA/AnataseNp5% resembles DPBHA surface but also contains smaller granules and that the character of DPBHA/AnataseNp8% resembles DPBHA/AnataseNp5% surface, but with irregular topography and Y-TZP showed a typical oxide ceramics surface pattern. EDS revealed Ca, O and P in all HA composed samples with Ti in the reinforced ones, and C, O and Zr in Y-TZP samples. AFM data corroborates with SEM analysis. AB test revealed excellent cellular viability for DPBHA/AnataseNp5% group and AR test revealed that all groups containing TiO_2np showed more mineralized matrix deposition than all other groups, with low statistical differences between reinforced groups and DPBHA cultivated in non-osteogenic medium and much bigger in osteogenic medium, with DPBHA/AnataseNp8% showing the best results. In conclusion, the addition of TiO_2np showed chemical, superficial and biological changes in the reinforced materials, with the DPBHA/AnataseNp5% group being the one that showed the best results in cell viability and DPBHA/AnataseNp5% mineralized matrix deposition.

Keywords: Durapatite, Biocompatible Materials, Ceramics

1 INTRODUCTION

Since the beginning of dental ceramics, better properties have researched for this material, seeking new ceramics and formulations and reinforcing these materials [1–9]. Hydroxyapatite bioceramics, has the properties of being biocompatible, bioactive, promoting osteoconduction and osteoinduction [14–16]. It is part of the natural composition of bones and teeth (13, 14), with bovine bones from meat production being a renewable and an excellent source for obtaining hydroxyapatite nanoparticles [15].

Bioceramics are non-metallic inorganic ceramic materials designed to interact with the biological system and fulfill a specific function and may be able to promote tissue regeneration [12–14]. It has remarkable similarity with hard tissues, excellent biocompatibility, interaction with living tissues similar to natural bone remodeling processes. It allows the apposition of osteoblasts on the surface of the material [12,14]. Hydroxyapatite (HA) shows bioactivity, which is very important in current dentistry. This material can induce specific biological activities and stimulate a benevolent response in the host organism [19]. However, it has limited mechanical properties [14].

One of the strategies adopted to obtain better material properties is incorporating nanoparticles in their compositions, improving mechanical and biological properties, as shown in several studies [15,21,22,24–28]. A type of nanoparticles widely used in the reinforcement of materials is composed of titanium dioxide (TiO_2) [15]. Studies showed that the incorporation of these nanoparticles improves the mechanical and antibacterial properties of the materials [15,30–32,35]. Applying TiO_2 nanotubes on implant surfaces and other materials increased the properties favorable to bone formation [36–40].

A preview study added TiO_2 nanoparticles (TiO_2np) to reinforce dense hydroxyapatite bioceramics made of bovine bone waste, which showed good mechanical properties by incorporating 5% TiO_2np [15]. This assay aims to investigate the biological properties of this material, evaluating the role of different percentages of titanium dioxide nanoparticles (5% or 8%) in HA microstructure by the surface and biological analyses. The null hypothesis is that TiO_2np don’t affect HA microstructure and natural potential.

2 MATERIAL AND METHODS

2.1. Specimen preparation

The study group compositions were dense polycrystalline bovine hydroxyapatite bioceramic (DPBHA); DPBHA with the addition of 5% anatase TiO_2np (DPBHA/AnataseNp5%); DPBHA with the addition of 8% anatase TiO_2np (DPBHA/AnataseNp8%); Y-TZP (commercial polycrystalline dental ceramic). All the steps to obtain the specimens are described below.

2.1.1. TiO_2 nanoparticles synthesis (adapted from Arruda, 2013)

Amorphous TiO_2np was weighted using a digital scale. Deionized water (100 ml) and nitric acid (10/15 ml) were added to a beaker on a digital magnetic stirrer at 300rpm and 90°C. TiO_2np was added to this liquid in small portions. Then, citric acid (5.50 g) was weighed on a digital scale and added in small amounts to 190 ml of deionized water in another beaker. The two liquid contents were mixed in a larger cup, followed by resting for 15 minutes. Ethylene glycol (10.5 ml) was added and kept stirring for a few hours to start pH measuring. As the pH was acid, Ethylene Diamine (ED) (about 2/3 ml) was added to the medium at approximately 290°C. The pH measurements were performed with a specific pH tape for reading with addition of 0.5 ml of ED. After reaching a pH around 3 and 4, the temperature was decreased to 180°C, with magnetic stirring maintained. The temperature was reduced to 70°C to evaporate the water from this solution overnight. Subsequently, the contents were taken to an oven to obtain a homogeneous powder.

2.1.2. Pure Hydroxyapatite

HA was obtained from bovine metatarsals of Canchim breed from animals tracked through the Brazilian Bovine and Buffalo Traceability System (BBBTS). HA from bone tissue was obtained through thermochemical processes to remove organic matter. The grinding of HA particles' size either decrease or increase the reactivity between them, reducing temperature and required time for sintering, and as a result, the final porosity of the ceramic.

2.1.3. Experimental dense bioceramics specimens

The experimental dense bioceramics were obtained using HA powder with or without TiO_2np addition. For this, the following materials were used:

- Polyvinyl butyral (PVB) (Butvar B98) as binding agent;
- Para-aminobenzoic acid (PABA) as a deflocculant of the alcoholic medium;
- Isopropyl alcohol as a solvent for the binder and liquid medium in the slip.

For grinding and obtaining a submicrometric powder, a polyethylene pitcher (85 mm height x 300 cm^3 volume) was used, filled with 45 vol% (500g) of 3Y zirconia spheres with 10 mm of diameter. The binder (PVB) provided plasticization and green resistance after shaping. The jar filled with slip in a concentration of 30% solid volume was placed in a ball mill with a speed of 104 rpm for 48 hours and in a vibrating mill for 72 hours. In the first grinding, the jar loaded with 30% vol of hydroxyapatite, 69.95% vol of isopropyl alcohol, and 0.05% weight of PABA was placed for 48 hours in a ball mill and 72 hours in a vibrating mill. After this period, 1.2% weight of PVB previously dissolved in isopropanol in 1:10 was added to HA weight and mixed in a vibrating ball mill for two hours. For nanomaterial samples (second grinding), we weighed TiO_2np in concentrations of 5% and 8% of valuable HA volume and added to HA with PVB in another smaller jar. The mixture was back to the vibrating mill for 10 minutes. After, the unloaded jar was dried with a thermal blower at approximately 80°C. All prepared powders were granulated and classified into #200 stainless steel mesh (≤75 μm sieves). Then, 0.5g was weighed, inserted in a metallic device previously lubricated with oleic acid PA ($C_{18}H_{34}O_2$, Labsynth, Diadema, Brazil), generating discs conformations of 15 mm in diameter and 1.4 mm in height, after uniaxial pressing of 100 MPa for 30 seconds. The specimens were vacuum packed and subjected to isostatic pressing with 200 MPa for 1 minute.

Sintering was carried out in a Lindberg Blue/M like oven in air atmosphere, from room temperature to 160°C with a heating rate of 2.7°C/min. At 4°C/min to 600°C, then at 5°C/min to 1100°C, and finally at 6°C/min to 1300°C (maximum temperature), with a 120-minute plateau followed by cooling the oven to room temperature. After sintering, the specimens reached 12 mm in diameter and 1.2 mm in height, considering a volumetric sintering shrink of approximately 21%.

2.1.4. Obtaining commercial specimens (emax ZirCAD, Ivoclar Vivadent)

Trademark Y-TZP blocks (ZirCAD, Ivoclar Vivadent) were cut into discs, 21% bigger in volume for final dimensions after sintering shrinking. All specimens were finished with #800 and #1200 silicon carbide sandpaper (Carbimet, Buehler) and polished with a 1mm diamond solution (water-based suspension – MetaDi, Buehler) with fine-grained felt discs.

All the specimens were cleaned in an ultrasonic bath, using deionized water (USC 750, Unique Group) for 5 minutes, and then they were sintered. After sintering, the disk-shaped specimens reached 12 mm in diameter and 1.2 mm in height, considering the volumetric sintering shrinkage of approximately 21%.

2.2 Surface Analyses

2.2.1. Scanning Electron Microscope / Energy Dispersive Spectroscopy (SEM/EDS)

The morphological analysis of the specimen's surfaces was carried out using a scanning electron microscope (JSM 5600LV 353, JOEOL, Tokyo, Japan). A sample of each group was evaluated with x500 x1000, x2000, x5000, and x10.000 magnification. The same microscope equipped with an X-radiation detector was used to perform EDS (Voyager, Noran Instruments) to analyze the chemical composition from different materials.

2.2.2. Atomic Force Microscopy (AFM) (n=3)

The measurements were performed using a colloidal fluid cell probe (Veeco Bioscope Catalyst, AFM), which determined the interaction forces of HA surfaces with or without TiO_2np. The atomic force microscope probe was pushed towards and pulled out of the surface of the specimens. The cantilever deflection was detected using the laser beam that reflected on the back of the cantilever. The cantilever spring constant was measured during the experiment to be 0.083 ± 0.010 N/nm using the thermal vibration method. This constant was used to convert raw AFM data from the displacement curve voltage scanner by force-distance using the commercially available AFM software. All measurements were performed at a constant temperature of 22.0 ± 0.5°C at a scanning rate of 0.1 Hz (200 nm/s).

Different positions of the specimen surfaces were repeated for more than ten cycles for each position. Typical force curves were shown. The AFM ScanAsyst image mode of the specimens was recorded. Electrokinetic measurements were performed using the ZetaPLUS Analyzer (Brookhaven Instrument Corp, USA). The data were

expressed as values of zeta potential, which were calculated from measurements of electrophoretic mobility using the Smoluchowski equation (Shrimali et al., 2016).

2.3. Biological Tests

2.3.1. Cellular Culture

SAOS-2 Cells of human osteoblastic lineage were grown in Petri dishes in Dulbecco´s Modified Eagle´s Medium (DMEM, SIGMA Chemical Co., St. Louis, MO, USA) containing 15% of fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA), 100 IU/mL of penicillin, 100 mg/mL of streptomycin, and 2 mmol/L of glutamine (GIBCO), which is considered a non-osteogenic medium (NOM). Cells were maintained in a cellular culture CO_2 incubator (atmosphere at 37ºC with 5% CO_2 and 95% air). Cells were subcultured until the required confluence for the experiment's execution. After this period, the disks of all experimental groups (n = 6) were placed inside cell culture plate wells and stabilized with silicon rings. A control group was made of glass coverslips as inert control (IC). 50.000 cells were seeded on each disk. Elapsed 30 minutes for cell adhesion the cells were maintained with 800µL of culture medium in each well, which was renewed at every 48 hours.

The same procedure was also performed with the same groups (n = 6) but maintained with 800µL of osteogenic medium (OM) consisted of DMEM + 15% FBS, 100 IU/mL of penicillin, 100 mg/mL of streptomycin, and 2 mmol/L of glutamine (GIBCO) supplemented with 50 mg / mL of ascorbic acid and 5 mM of β-glycerophosphate. The culture plates with the disks and cells were kept in a cell culture CO_2 incubator for 14 days.

2.3.2. Cell Viability

For this analysis, cells cultivated on disks (n=6) were incubated with DMEN supplemented with Alamar Blue dye (10:1, Thermo Fisher Scientific, Waltham, MA) at 37°C and 5% of CO2 for 3 hours, and the fluorescence was read at 570nm excitation and 585nm emission (Synergy H1, Biotek, Winoosky, VT). It was considered 100% of cell viability the mean value of the IC group (n=6) of glass coverslips on the first day of the test. Tests were performed at 1, 3, and 7 days.

2.3.3. Mineralized Matrix Deposition

After 14 days of incubation in either NOM or OM, samples (n=6) were fixed with 70% ethanol at 4°C for 1 hour, followed by washing with deionized water and incubation with Alizarin Red solution (40 mM, pH 4.2; Sigma Chemical) under 15 min agitation. After this period, cells were washed 3 times with deionized water. For quantitative analysis, cetylpyridinium chloride solution (10 mM, pH 7.0; Sigma Chemical) was applied for 15 min to dissolve the nodules, and the absorbance of the resulting solution was evaluated at 560nm.

2.4 Statistical Analysis

Cell viability data was submitted to two-way ANOVA followed by Tukey test for comparison between groups and mineralized matrix deposition data was submitted to ANOVA followed by Tukey test. All tests had statistical significance level of 5%.

3. RESULTS

3.1 - Superficial Analysis

3.1.1. SEM/EDS

SEM revealed that DPBHA surface (Figure 1) was composed of high densification regions with comparable size grains with irregular flattened polyhedral shape with rounded vertices and areas of a unflatten surfaces with substantial grooves.

DPBHA/AnataseNp5% surface had high densification areas similar to that of DPBHA but impregnated by smaller grains of regular size and irregular polyhedral shape that seems to be adhered to the surface (Figure 2).

DPBHA/AnataseNp8% surface was more irregular than DPBHA/AnataseNp5%, showing greater roughness also with high densification (Figure 3).

Y-TZP surface shows an oxide ceramic pattern different from all other ceramics evaluated (Figure 4).

EDS revealed that all samples composed of HA showed Ca, O, and P. Reinforced groups also showed Ti. Moreover, the two types of surface area in DPBHA had the same atomic distribution and chemical composition (figure 5). This also applied

to the two grain sizes of DPBHA/Anatase5% (figure 6) and to the higher irregular surface of DPBHA/Anatase8% (Figure 7). Y-TZP composition EDS showed C, O, and Zr with homogeneous distribution (Figure 8). All group compositions are shown in Tables 1-4 and Figures 9-12.

1.4. AFM

AFM analysis showed that the insertion of 5% TiO_2np in HA bioceramics increased its superficial roughness compared with DPBHA. 8% TiO_2np affected HA bioceramics even more, causing surface clusters and disorder and therefore, increased irregularity. However, all HA bioceramics samples with or without TiO_2np showed less surface irregularity than Y-TZP. (Figures 13-16)

3.2. Biological Tests

3.2.1. Cell Viability Test

Alamar Blue test revealed that between the periods of 1 and 3 days, all groups showed no statistically significant difference. At 1 and 3-day periods, all-ceramic groups showed cellular viability (CV) close to the IC group. The 7-day period analysis showed an increased CV in DPBHA/AnataseNp5% compared to IC group (with statistically significant differences) and significantly higher than all other ceramic groups (Figure 17).

3.2.2. Mineralized Matrix Deposition

In the non-osteogenic medium assay, CI, DPBHA, and DPBHA/AnataseNp5% showed statistical differences, and DPBHA/AnataseNp8% followed by DPBHA/AnataseNp5% had higher percentages of mineralized matrix deposition of all groups (Figure 18).

In the osteogenic medium assay, it was possible to observe a significantly higher deposition of mineralized matrix in TiO_2np reinforced groups. DPBHA/AnataseNp8% exhibited a mineralized matrix rate of almost 900% greater than IC group (Figure 19).

4. DISCUSSION

This study aimed to perform morphological, chemical and biological tests of the surface structure of dense hydroxyapatite bioceramics reinforced with TiO_2np (DPBHA/AnataseNp5%/DPBHA/AnataseNp8%).

Considering previous studies demonstrating that these reinforced bioceramics had better properties compared to unreinforced ones [15], the good biological properties of hydroxyapatite bioceramics [14–16] and TiO_2np [35–40], and the studies/applications of these materials in bone regeneration and implants [16,20,29,36–42], DPBHA/AnataseNp5% and DPBHA/AnataseNp8% performance of biological tests with human osteoblasts demonstrated part of the natural interactions of those cells with TiO_2np reinforced materials.

Morphological and chemical analyses were performed because osteoblasts behavior, bone formation, and repair mechanisms correlate with those properties. It was well demonstrated in studies and products involving chemical and topographic changes in implant surfaces [36,39,41,43,44]. Furthermore, the surface roughness morphology of the material is a significant factor for bone cell adhesion and fixation [45].

SEM revealed high densification on the surfaces of all evaluated bioceramics, which is probably due to the sintering processes of HA nanoparticulate powder with or without TiO_2np. Despite sintering causes the growth of the grains that composes the materials [46,47], what impairs mechanical properties of polycrystalline ceramics, also causes densification with the elimination of pores between particles by atomic diffusion caused by thermal energy, acting as a reinforcement mechanism [43]. It is possible to observe close contact between grains on the sample's surfaces and the absence of pores (Figures 1-3). This is a beneficial factor for the growth of osteoblasts, as demonstrated in a study that analyzed the surface differences of zirconia samples, in which osteoblastic cells showed better initial adhesion on more irregular surfaces, but over time, the cells proliferate better on more regular surfaces than on uneven and porous surfaces [45] and in a critical review showing that in the majority of the analyzed studies bone cells proliferation were better on smooth surface [47]. Indeed, in addition to SEM, AFM images (Figures 13-16) showed that DPBHA/AnataseNp8% had

considerably more irregular surface than DPBHA and DPBHA/AnataseNp5%, but the latter had the higher levels of cellular viability in 7-day analysis (figure 17).

In the DPBHA group, there were two superficial morphological types (Figure 1). However, the EDS analysis showed a homogeneous chemical composition across the sample, demonstrating no different chemical compositions between the two superficial morphological types (Figure 5).

DPBHA/Anatase Np5% SEM revealed a surface similar to that of DPBHA, but with a bimodal grain size distribution (Figure 2), with grains of similar size to those of DPBHA and smaller grains. EDS revealed a composition with a homogeneous distribution of Ca, P, O, and Ti, showing a similar chemical composition between the biggest and smallest grains (Figure 6). The presence of smaller grains may indicate that TiO_2np could prevent part of the growth of the grains, which would explain the fact of different sizes of grains of the same composition. Based on the concept that the grain growth driving force is contracted by the segregation energy of reinforcing materials, likely in metals, a significant incompatibility of atomic size between the additive and the host material limits grains movements and thus grain growth. In ceramic oxides, this effect is observed but cannot be explained [48].

In the DPBHA/AnataseNp8% group, SEM also revealed the bimodal distribution of grain size, but on an more irregular surface topography (Figure 3) with a homogeneous distribution of Ca, P, O, and Ti, demonstrating a similar design throughout the entire sample (Figure 7). Studies involving other materials reported that the concentration of dopants increases surface changes, affecting accumulation, aggregation, and irregularities [50,51], which may explain these effects in this group with higher reinforcement particles content.

Cell viability of SAOS-2 grew up on disks of the evaluated materials was analyzed by the Alamar Blue test that revealed higher cell viability in the DPBHA/AnataseNp5% group after one week of culture, even better than IC group (Figure 17). DPBHA/AnataseNp8% showed the second-best cell viability rate between ceramic groups, slightly higher than the DPBHA and Y-TZP groups and without statistical difference with IC and DPBHA/AnataseNp5% groups. These data show that the presence of TiO_2np promoted greater cell viability in the reinforced materials, which

proved to be excellent in DPBHA/AnataseNp5%. These results corroborate other studies, which indicated that the presence of TiO_2np increased cell viability of osteoblastic and pre-osteoblastic cells in materials containing this compound compared to groups without TiO_2np [52–54].

The mineralized matrix deposition test by Alizarin Red was performed to observe the effects of osteoblasts cultured in experimental materials compared to controls. In the assay with a non-osteogenic medium, bioceramics containing TiO_2np showed the best results of mineralized matrix deposition, with moderated discrepancies between all groups (Figure 18). DPBHA/AnataseNp8% showed a higher percentage of mineralized matrix deposition, almost two times higher than IC group. When the test was performed in an osteogenic medium, the groups containing TiO_2np showed much better results than the groups without TiO_2np (Figure 19). DPBHA/AnataseNp8% was the group with the highest percentage of mineralized matrix deposition, which was almost nine times higher than IC group and DPBHA/AnataseNp5% presented more than five times the amount of mineralized matrix deposition than IC group. In contrast, the control groups (IC, Y-TZP, and DPBHA) presented results similar to those of the assay without the osteogenic medium. These results demonstrate that TiO_2np reinforced HA bioceramics has high potential for mineralized matrix production by osteoblasts in osteogenic medium and considerable potential in the non-osteogenic medium.

The osteogenic medium contains ascorbic acid and β-glycerophosphate. Considering the mechanisms of bone neoformation, osteoblasts produce bone by synthesis and secretion of type I collagen, the main protein in a bone collagen matrix mineralized by osteoblasts. According to P. Katsimbri MB, 2017 “Mineralization is achieved by the local release of phosphates from osteoblast-derived matrix vesicles found within the osteoid”, which “together with the calcium from the extracellular fluid, hydroxyapatite crystals are formed” [55]. Therefore, there is calcium and phosphate in a living organism in the places where bone neoformation occurs. The β-glycerophosphate used in the culture medium serves as a source of phosphate for mineral production [56]. Ascorbic acid, present in the formulation of the osteogenic medium, is also present in human organisms and obtained through the diet, playing a

fundamental function role in the bone formation process and production of the bone collagen matrix [57] that will form the mineralized bone tissue [55].

In the analysis of osseointegrated implants, another factor to be considered is the three-dimensional gradient of Ca, P, and O that decreases from the bone to the implant surface, where an increase in Ti ions occurs [43]. The similarity between the chemical composition of the reinforced groups and bone-implant interface could contribute to the osseointegration process since these ceramics already have the required elements (Ca, P, O, and Ti). Therefore, different from conventional titanium implants, there would be no need for Ca, P, and O migration from bone tissue.

5. CONCLUSION

This study concluded that dense bioceramics composed of bovine hydroxyapatite reinforced with TiO_2np showed excellent cell viability in DPBHA/AnataseNp5%. In addition, the bioceramics that contained TiO_2np in their composition showed higher mineralized matrix deposition than other ceramic groups cultured in osteogenic medium. Thus, with good mechanic and excellent biological properties, this compound would be a promising material for bone healing and implants.

Acknowledgments

The authors thank the financial support by the São Paulo Research Foundation (FAPESP; process numbers: 2018/23639-0 and 2013/07296-2; CDMF/CEPID/FAPESP); the National Council for Scientific and Technological Development (CNPq; process number: 154705/2015-2), and the Coordination for the Improvement of Higher Education Personnel (CAPES) process number:001.

REFERENCES:

1. Anusavice KJ, Shen C, Rawls HR. Philips' Science of dental materials. 12th ed. Elsevier/Saunders, 2012.
2. Bayne SC, Ferracane JL, Marshall GW, Marshall SJ, van Noort R. The Evolution of Dental Materials over the Past Century: Silver and Gold to Tooth Color and Beyond. J Dent Res. 2019;98(3):257–65.
3. Robert Kelly J. Dental ceramics: current thinking and trends. Dental Clinics of North America. 2004;48(2):513–30.
4. Moshaverinia A. Review of the Modern Dental Ceramic Restorative Materials for Esthetic Dentistry in the Minimally Invasive Age. Dent Clin North Am. 2020;64(4):621–31.
5. Silva LH da, Lima E de, Miranda RB de P, Favero SS, Lohbauer U, Cesar PF. Dental ceramics: a review of new materials and processing methods. Braz Oral Res. 2017;31(suppl 1):e58.
6. Kelly JR, Nishimura I, Campbell SD. Ceramics in dentistry: Historical roots and current perspectives. The Journal of Prosthetic Dentistry. 1996;75(1):18–32.
7. Butt K, Thanabalan N, Ayub K, Bourne G. Demystifying Modern Dental Ceramics. Prim Dent J. 2019;8(3):28–33.
8. Guess PC, Schultheis S, Bonfante EA, Coelho PG, Ferencz JL, Silva NRFA. All-Ceramic Systems: Laboratory and Clinical Performance. Dental Clinics. 2011;55(2):333–52.
9. McLean JW. Evolution of dental ceramics in the twentieth century. Journal of Prosthetic Dentistry. 2001;85(1):61–6.
10. Bajraktarova-Valjakova E, Korunoska-Stevkovska V, Kapusevska B, Gigovski N, Bajraktarova-Misevska C, Grozdanov A. Contemporary Dental Ceramic Materials, A Review: Chemical Composition, Physical and Mechanical Properties, Indications for Use. Open Access Maced J Med Sci. 2018;6(9):1742–55.
11. Denry I, Kelly JR. State of the art of zirconia for dental applications. Dental Materials. 2008;24(3):299–307.
12. Ana ID, Satria GAP, Dewi AH, Ardhani R. Bioceramics for Clinical Application in Regenerative Dentistry. Adv Exp Med Biol. 2018;1077:309–16.
13. Sanz JL, Rodríguez-Lozano FJ, Llena C, Sauro S, Forner L. Bioactivity of Bioceramic Materials Used in the Dentin-Pulp Complex Therapy: A Systematic Review. Materials (Basel). 2019;12(7).
14. Pina S, Rebelo R, Correlo VM, Oliveira JM, Reis RL. Bioceramics for Osteochondral Tissue Engineering and Regeneration. Adv Exp Med Biol. 2018;1058:53–75.
15. Pires LA, de Azevedo Silva LJ, Ferrairo BM, Erbereli R, Lovo JFP, Ponce Gomes O, et al. Effects of ZnO/TiO2 nanoparticle and TiO2 nanotube additions to dense polycrystalline hydroxyapatite bioceramic from bovine bones. Dent Mater. 2020;36(2):e38–46.

16. Bordea IR, Candrea S, Alexescu GT, Bran S, Băciuț M, Băciuț G, et al. Nano-hydroxyapatite use in dentistry: a systematic review. Drug Metab Rev. 2020;52(2):319–32.

17. Traykova T, Aparicio C, Ginebra MP, Planell JA. Bioceramics as nanomaterials. Nanomedicine (Lond). 2006;1(1):91–106.

18. Kuttappan S, Mathew D, Nair MB. Biomimetic composite scaffolds containing bioceramics and collagen/gelatin for bone tissue engineering - A mini review. Int J Biol Macromol. 2016;93(Pt B):1390–401.

19. Mocquot C, Attik N, Pradelle-Plasse N, Grosgogeat B, Colon P. Bioactivity assessment of bioactive glasses for dental applications: A critical review. Dent Mater. 2020;36(9):1116–43.

20. Cao W, Hench LL. Bioactive materials. Ceramics International. 1996;22(6):493–507.

21. Menezes-Silva R, de Oliveira BMB, Fernandes PHM, Shimohara LY, Pereira FV, Borges AFS, et al. Effects of the reinforced cellulose nanocrystals on glass-ionomer cements. Dent Mater. 2019;35(4):564–73.

22. Dias HB, Bernardi MIB, Marangoni VS, de Abreu Bernardi AC, de Souza Rastelli AN, Hernandes AC. Synthesis, characterization and application of Ag doped ZnO nanoparticles in a composite resin. Mater Sci Eng C Mater Biol Appl. 2019;96:391–401.

23. Yin IX, Zhang J, Zhao IS, Mei ML, Li Q, Chu CH. The Antibacterial Mechanism of Silver Nanoparticles and Its Application in Dentistry. Int J Nanomedicine. 2020;15:2555–62.

24. Karthikeyan V, Chander NG, Reddy JR, Muthukumar B. Effects of incorporation of silver and titanium nanoparticles on feldspathic ceramic toughness. J Dent Res Dent Clin Dent Prospects. 2019;13(2):98–102.

25. Gad MM, Al-Thobity AM, Rahoma A, Abualsaud R, Al-Harbi FA, Akhtar S. Reinforcement of PMMA Denture Base Material with a Mixture of ZrO2 Nanoparticles and Glass Fibers. Int J Dent. 2019;2019:2489393.

26. Rezvani MB, Atai M, Hamze F, Hajrezai R. The effect of silica nanoparticles on the mechanical properties of fiber-reinforced composite resins. J Dent Res Dent Clin Dent Prospects. 2016;10(2):112–7.

27. Bai X, Lin C, Wang Y, Ma J, Wang X, Yao X, et al. Preparation of Zn doped mesoporous silica nanoparticles (Zn-MSNs) for the improvement of mechanical and antibacterial properties of dental resin composites. Dent Mater. 2020;36(6):794–807.

28. Vale AC, Pereira PR, Barbosa AM, Torrado E, Alves NM. Optimization of silver-containing bioglass nanoparticles envisaging biomedical applications. Mater Sci Eng C Mater Biol Appl. 2019;94:161–8.

29. Ziental D, Czarczynska-Goslinska B, Mlynarczyk DT, Glowacka-Sobotta A, Stanisz B, Goslinski T, et al. Titanium Dioxide Nanoparticles: Prospects and Applications in Medicine. Nanomaterials (Basel). 2020;10(2).

30. Shirkavand S, Moslehifard E. Effect of TiO2 Nanoparticles on Tensile Strength of Dental Acrylic Resins. J Dent Res Dent Clin Dent Prospects. 2014;8(4):197–203.

31. Ghahremani L, Shirkavand S, Akbari F, Sabzikari N. Tensile strength and impact strength of color modified acrylic resin reinforced with titanium dioxide nanoparticles. J Clin Exp Dent. 2017;9(5):e661–5.

32. Elsaka SE, Hamouda IM, Swain MV. Titanium dioxide nanoparticles addition to a conventional glass-ionomer restorative: influence on physical and antibacterial properties. J Dent. 2011;39(9):589–98.

33. Sodagar A, Akhoundi MSA, Bahador A, Jalali YF, Behzadi Z, Elhaminejad F, et al. Effect of TiO2 nanoparticles incorporation on antibacterial properties and shear bond strength of dental composite used in Orthodontics. Dental Press J Orthod. 2017;22(5):67–74.

34. Poosti M, Ramazanzadeh B, Zebarjad M, Javadzadeh P, Naderinasab M, Shakeri MT. Shear bond strength and antibacterial effects of orthodontic composite containing TiO2 nanoparticles. Eur J Orthod. 2013;35(5):676–9.

35. Ferrando-Magraner E, Bellot-Arcís C, Paredes-Gallardo V, Almerich-Silla JM, García-Sanz V, Fernández-Alonso M, et al. Antibacterial Properties of Nanoparticles in Dental Restorative Materials. A Systematic Review and Meta-Analysis. Medicina (Kaunas). 2020;56(2).

36. Wang N, Li H, Lü W, Li J, Wang J, Zhang Z, et al. Effects of TiO2 nanotubes with different diameters on gene expression and osseointegration of implants in minipigs. Biomaterials. 2011;32(29):6900–11.

37. Brammer KS, Oh S, Cobb CJ, Bjursten LM, van der Heyde H, Jin S. Improved bone-forming functionality on diameter-controlled TiO(2) nanotube surface. Acta Biomater. 2009;5(8):3215–23.

38. Brammer KS, Frandsen CJ, Jin S. TiO2 nanotubes for bone regeneration. Trends Biotechnol. 2012;30(6):315–22.

39. Bjursten LM, Rasmusson L, Oh S, Smith GC, Brammer KS, Jin S. Titanium dioxide nanotubes enhance bone bonding in vivo. J Biomed Mater Res A. 2010;92(3):1218–24.

40. Hashimoto M, Takadama H, Mizuno M, Kokubo T. Mechanical properties and apatite forming ability of TiO2 nanoparticles/high density polyethylene composite: Effect of filler content. J Mater Sci Mater Med. 2007;18(4):661–8.

41. Pajor K, Pajchel L, Kolmas J. Hydroxyapatite and Fluorapatite in Conservative Dentistry and Oral Implantology-A Review. Materials (Basel). 2019;12(17).

42. Habibah TU, Amlani DV, Brizuela M. Hydroxyapatite Dental Material. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 [cited 2021 may 28]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK513314/

43. Pellegrini G, Francetti L, Barbaro B, Del Fabbro M. Novel surfaces and osseointegration in implant dentistry. J Investig Clin Dent. 2018;9(4):e12349.

44. Jemat A, Ghazali MJ, Razali M, Otsuka Y. Surface Modifications and Their Effects on Titanium Dental Implants. Biomed Res Int. 2015;2015:791725.

45. Herath HMTU, Di Silvio L, Evans JRG. Osteoblast response to zirconia surfaces with different topographies. Mater Sci Eng C Mater Biol Appl. 2015;57:363–70.

46. Ji W, Rehman SS, Wang W, Wang H, Wang Y, Zhang J, et al. Sintering boron carbide ceramics without grain growth by plastic deformation as the dominant densification mechanism. Sci Rep. 2015;5:15827.

47. Champion E. Sintering of calcium phosphate bioceramics. Acta Biomater. 2013;9(4):5855–75.

48. Anselme K, Bigerelle M. Role of materials surface topography on mammalian cell response. International Materials Reviews. 2011;56(4):243–66.

49. Aidhy DS, Zhang Y, Weber WJ. Stabilizing nanocrystalline grains in ceramic-oxides. Phys Chem Chem Phys. 2013;15(43):18915–20.

50. Chrysafi I, Kontonasaki E, Anastasiou AD, Patsiaoura D, Papadopoulou L, Vourlias G, et al. Mechanical and thermal properties of PMMA resin composites for interim fixed prostheses reinforced with calcium β-pyrophosphate. J Mech Behav Biomed Mater. 2020;112:104094.

51. Saba N, Mohammad F, Pervaiz M, Jawaid M, Alothman OY, Sain M. Mechanical, morphological and structural properties of cellulose nanofibers reinforced epoxy composites. Int J Biol Macromol. 2017;97:190–200.

52. Cecchinato F, Xue Y, Karlsson J, He W, Wennerberg A, Mustafa K, et al. In vitro evaluation of human fetal osteoblast response to magnesium loaded mesoporous TiO2 coating. J Biomed Mater Res A. 2014;102(11):3862–71.

53. Smieszek A, Seweryn A, Marcinkowska K, Sikora M, Lawniczak-Jablonska K, Witkowski BS, et al. Titanium Dioxide Thin Films Obtained by Atomic Layer Deposition Promotes Osteoblasts' Viability and Differentiation Potential While Inhibiting Osteoclast Activity-Potential Application for Osteoporotic Bone Regeneration. Materials (Basel). 2020;13(21).

54. Abou Neel EA, Chrzanowski W, Knowles JC. Effect of increasing titanium dioxide content on bulk and surface properties of phosphate-based glasses. Acta Biomater. 2008;4(3):523–34.

55. Katsimbri P. The biology of normal bone remodelling. Eur J Cancer Care (Engl). 2017;26(6).

56. Langenbach F, Handschel J. Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res Ther. 2013;4(5):117.

57. Chin K-Y, Ima-Nirwana S. Vitamin C and Bone Health: Evidence from Cell, Animal and Human Studies. Curr Drug Targets. 2018;19(5):439–50.

APENDIX – FIGURES AND TABLES

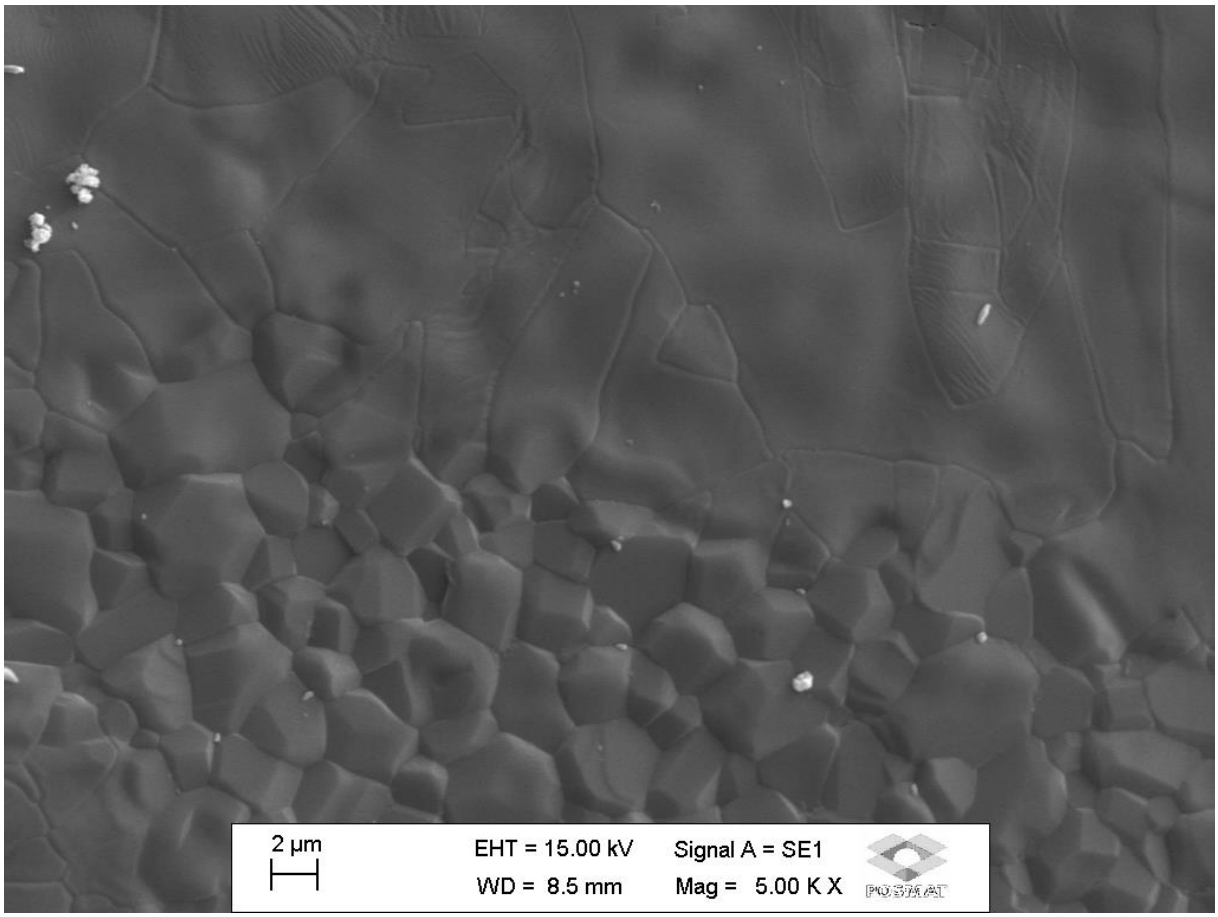

Figure 1 – MEV image from Pure hydroxyapatite bioceramics (DPBHA). It is possible to observe two morphological organizations

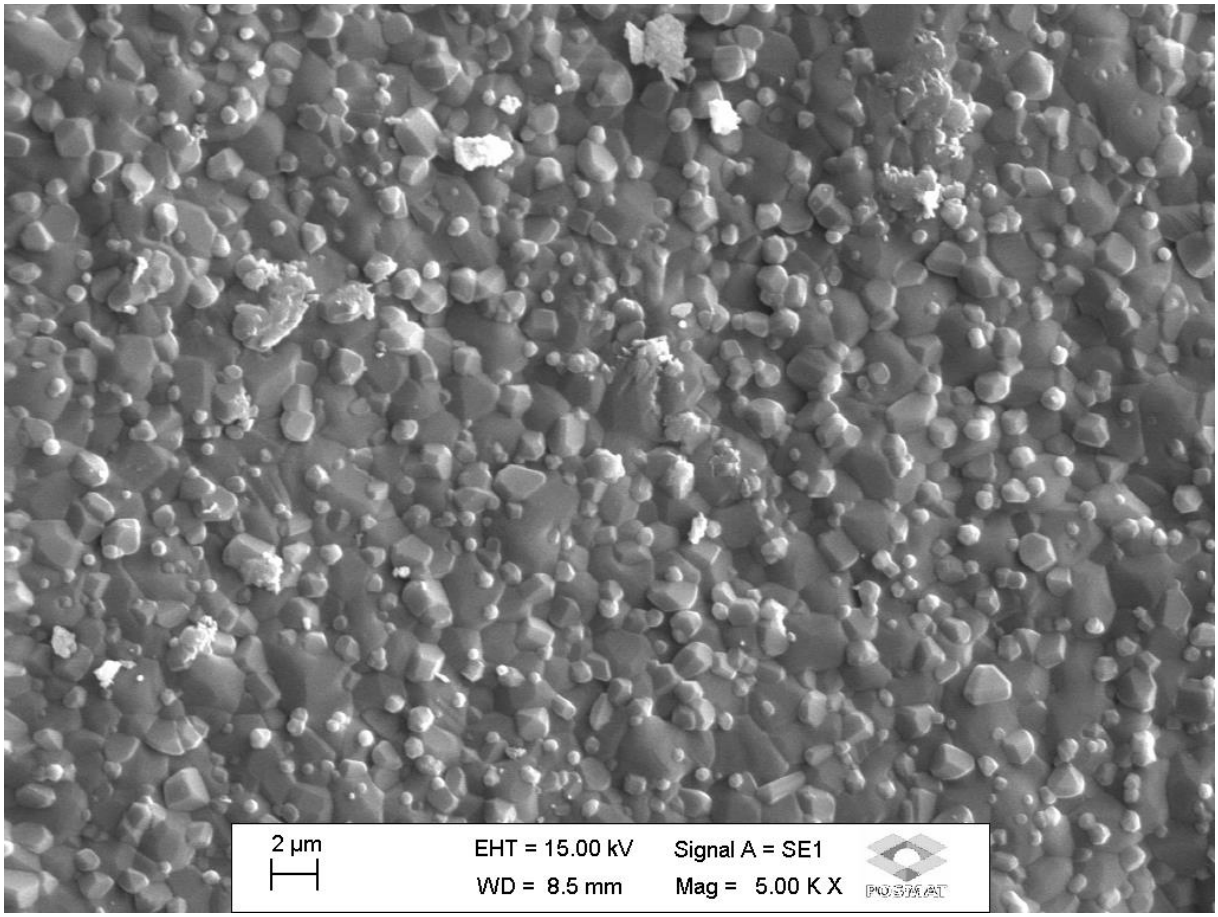

Figure 2 – MEV image from 5% TiO_2np reinforced hydroxyapatite bioceramics (DPBHA/AnataseNp5%). It is possible to note the bimodal grain size distribution

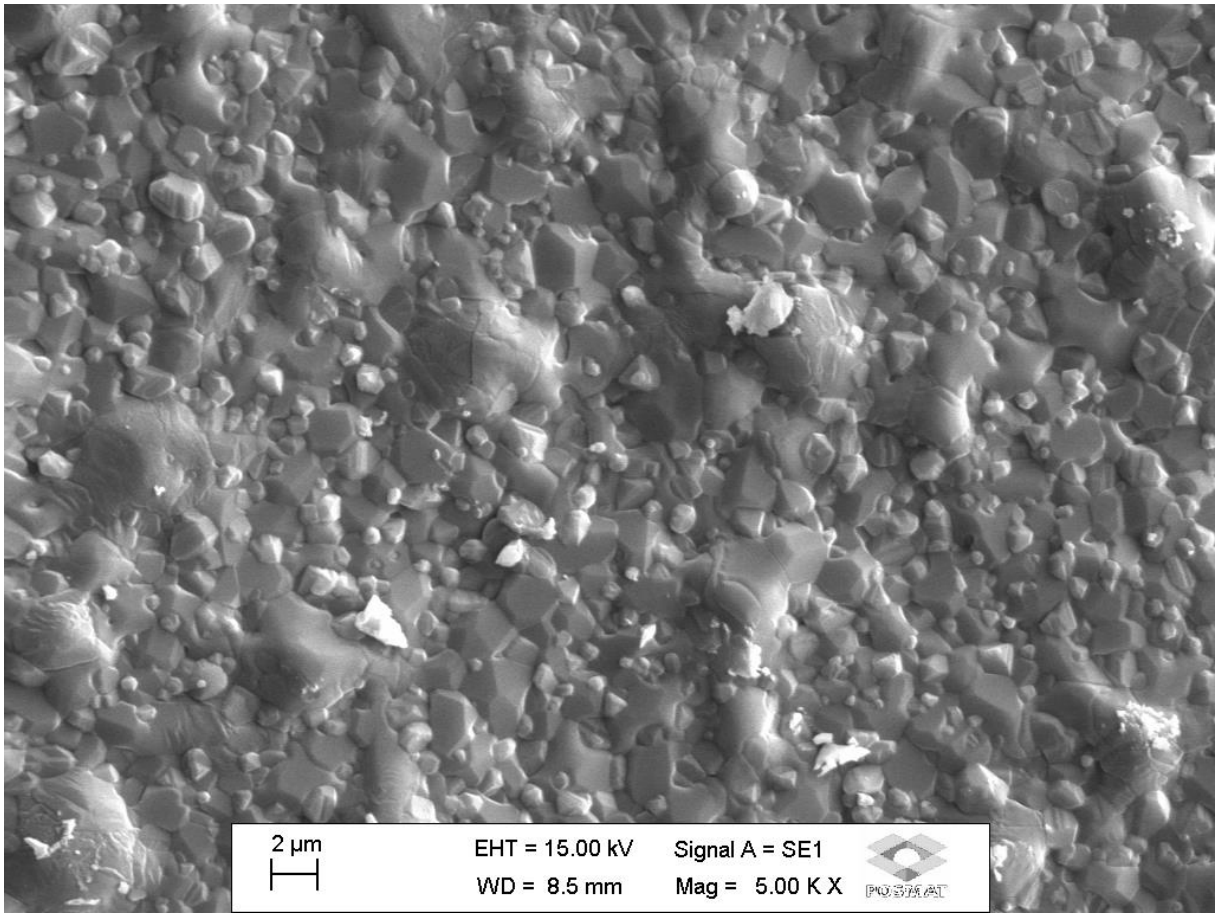

Figure 3 – MEV image from 8% TiO_2np reinforced hydroxyapatite bioceramics (DPBHA/AnataseNp8%). MEV analysis shows greater surface irregularity in this group.

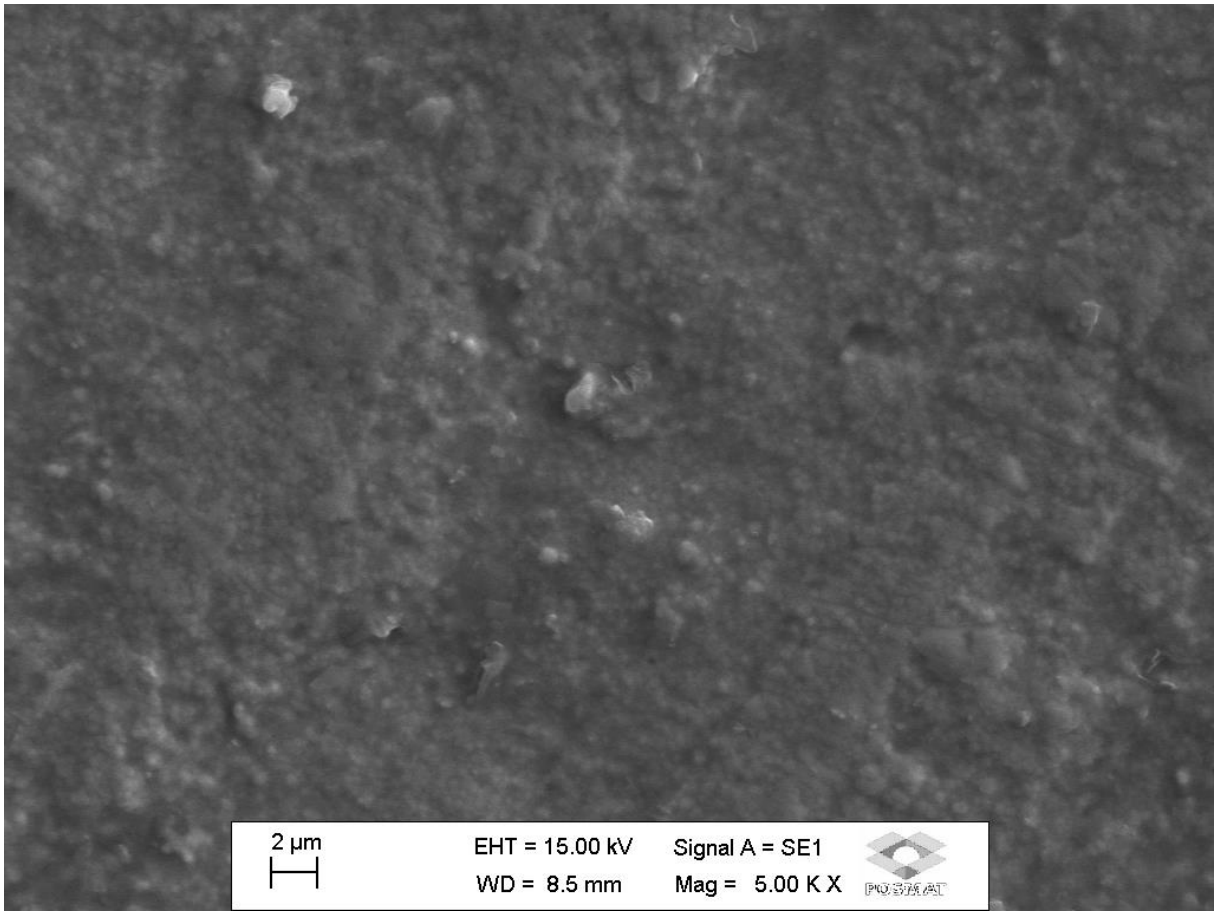

Figure 4 – Image from Y-TZP, showing polycrystalline ceramics pattern.

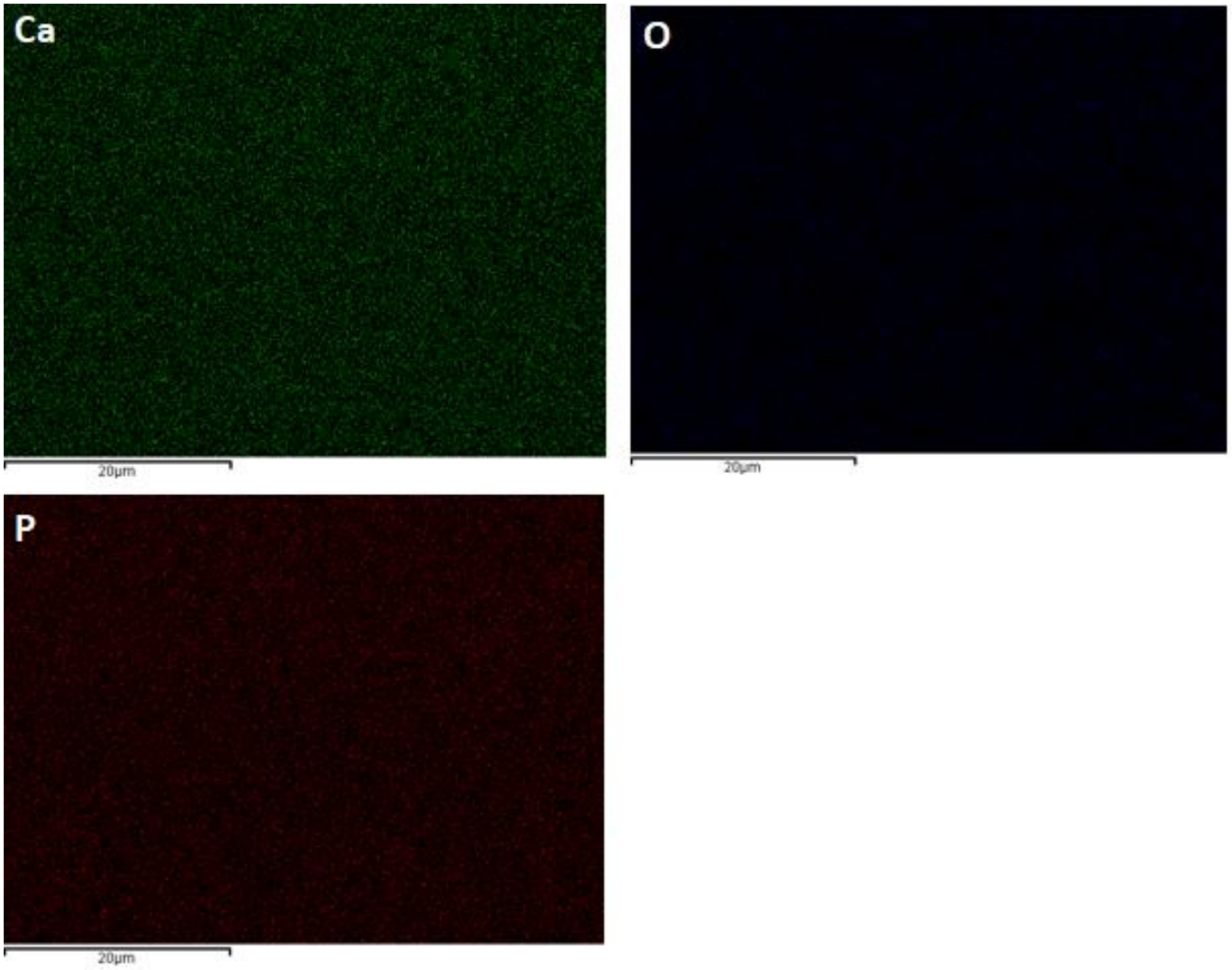

Figure 5 - DPBHA EDS chemical analysis. Ca, O and P atoms with homogeneous distribution.

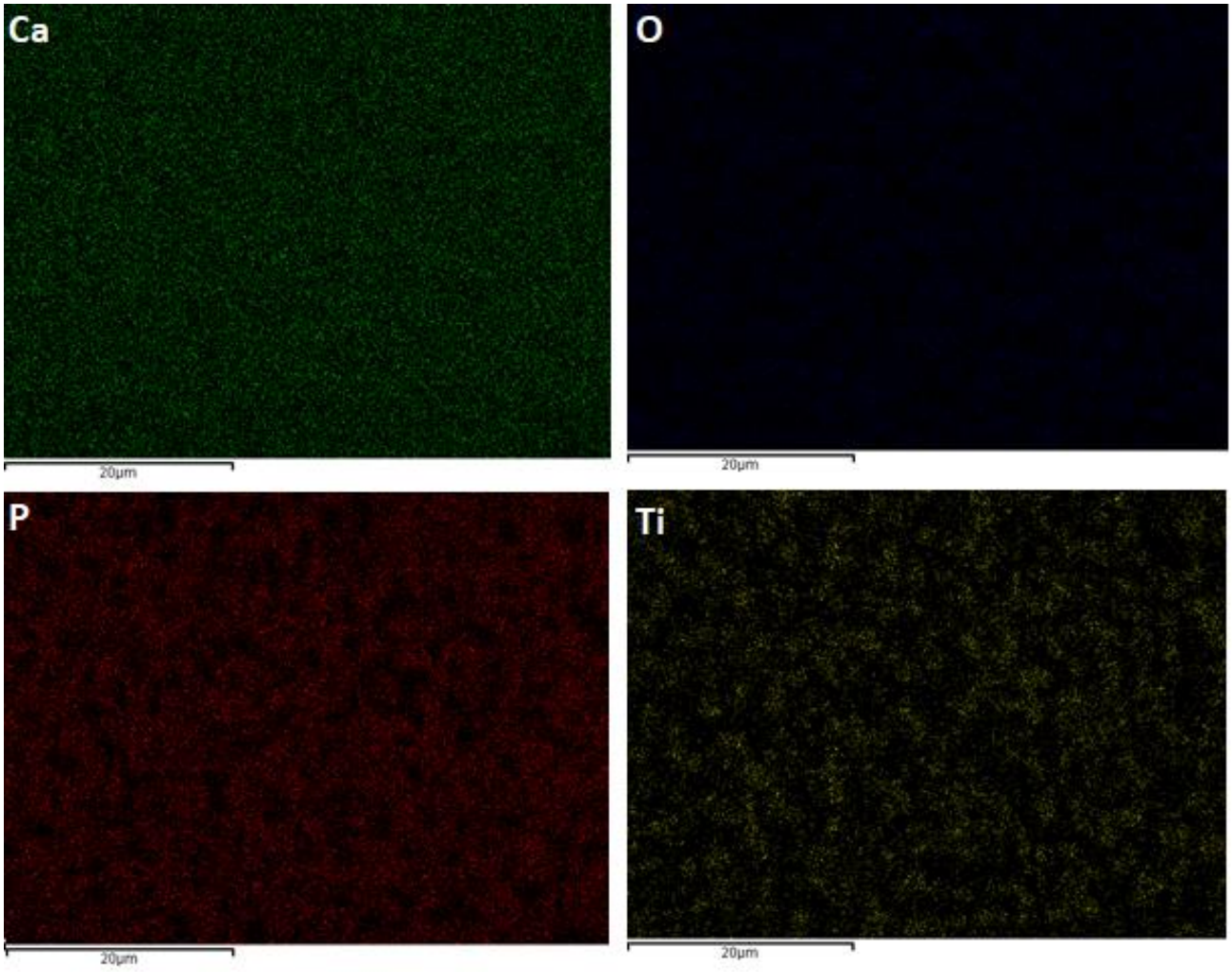

Figure 6 - DPBHA/AnataseNp5% EDS chemical analysis – Ca, O, P and Ti atoms with homogeneous distribution

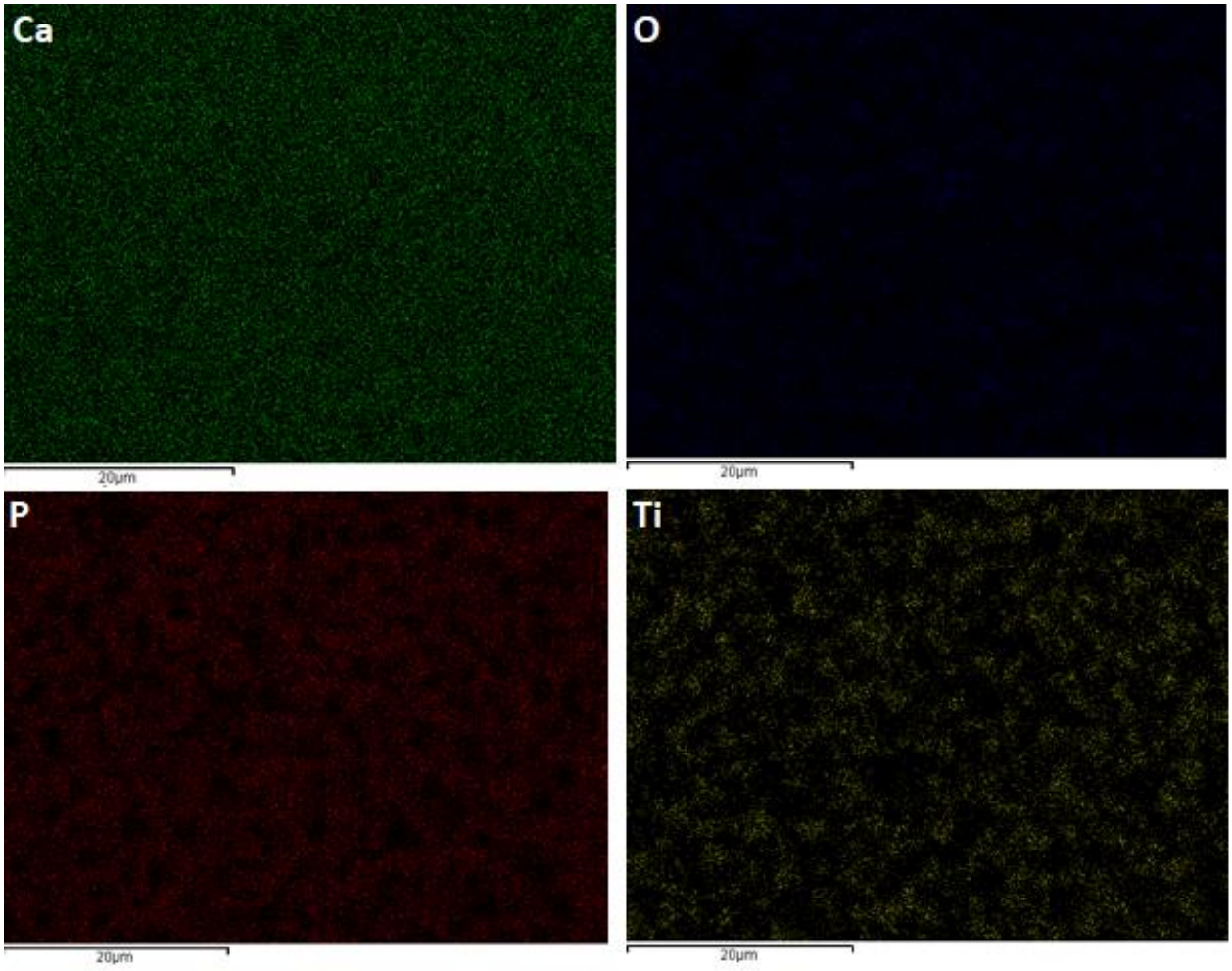

Figure 7 – DPBHA/AnataseNp8% EDS chemical analysis – Ca, O, P and Ti atoms with homogeneous distribution

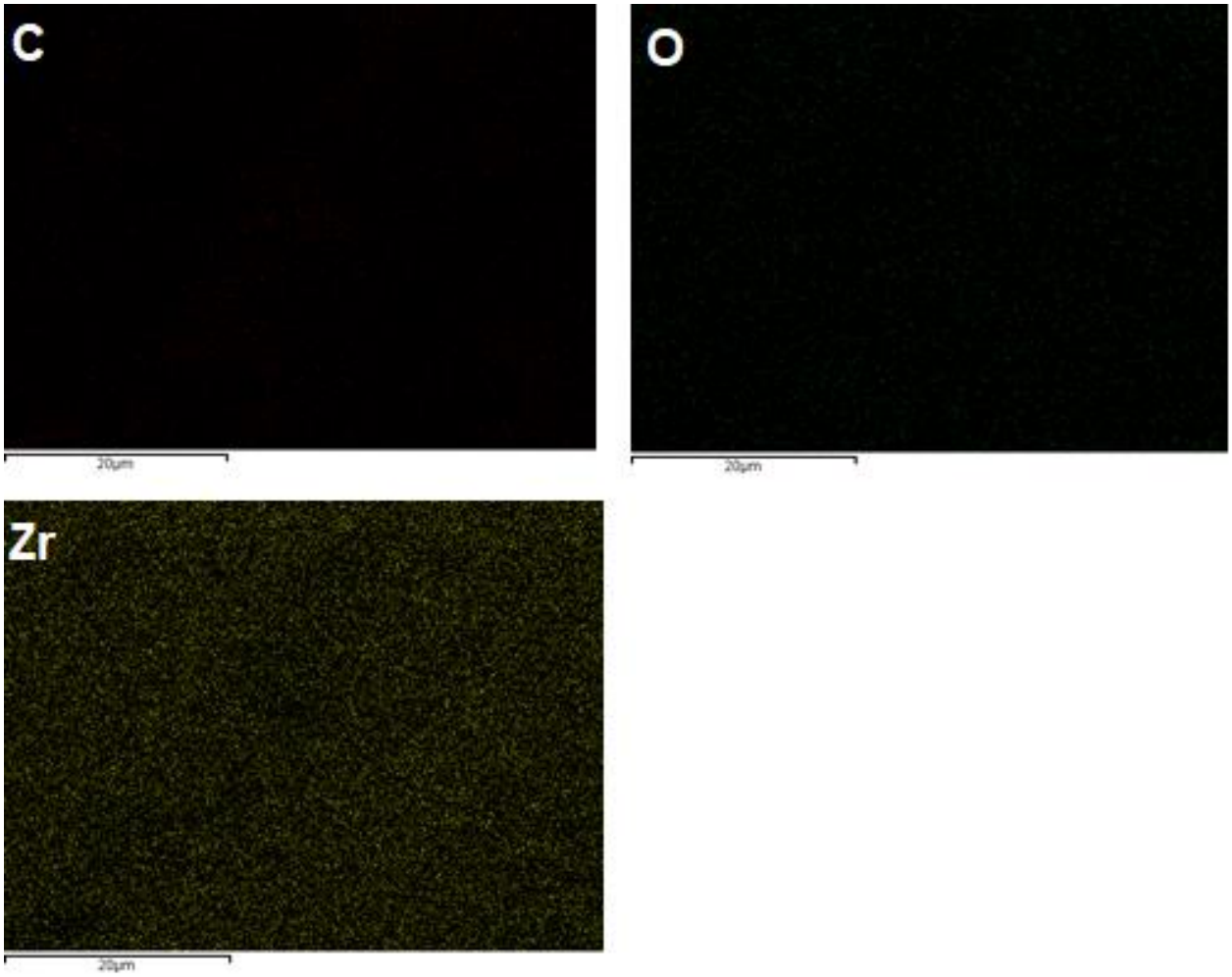

Figure 8 - YTZ-P EDS chemical analysis - C, O and Zr atoms with homogeneous distribuition

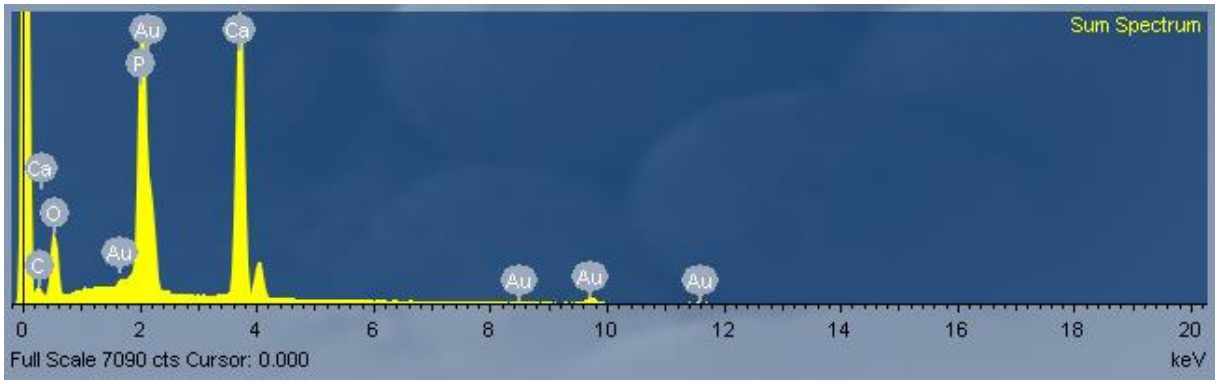

Figure 9 – EDS analysis of DPBHA showing predominance of Ca, O and P

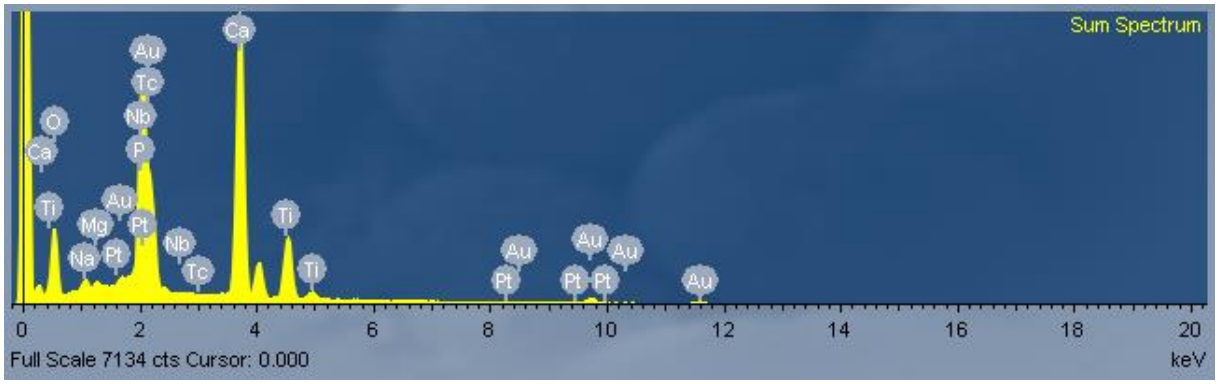

Figure 10 – EDS analysis of DPBHA/AnataseNp5% showing predominance of Ca, O, P and Ti

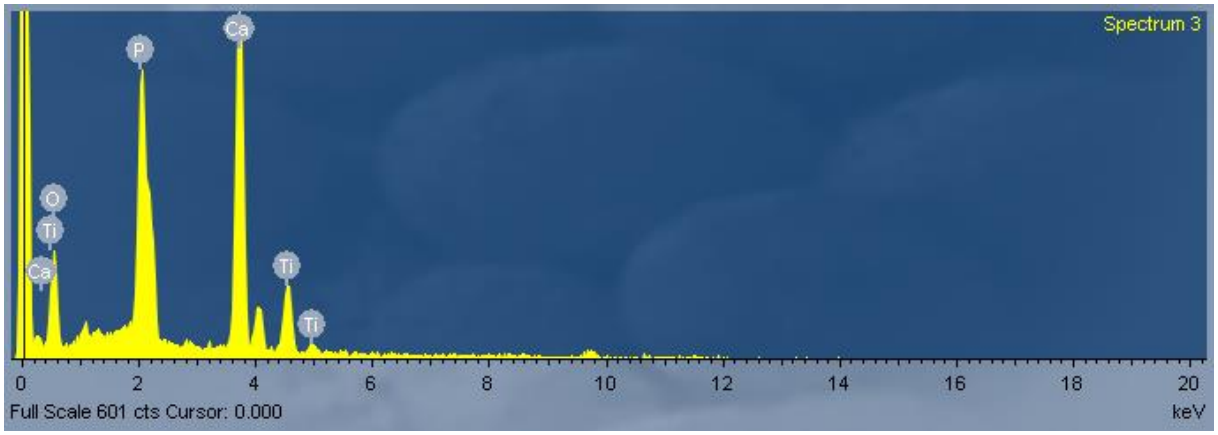

Figure 11 – EDS analysis of DPBHA/AnataseNp8% showing predominance of Ca, O, P and Ti

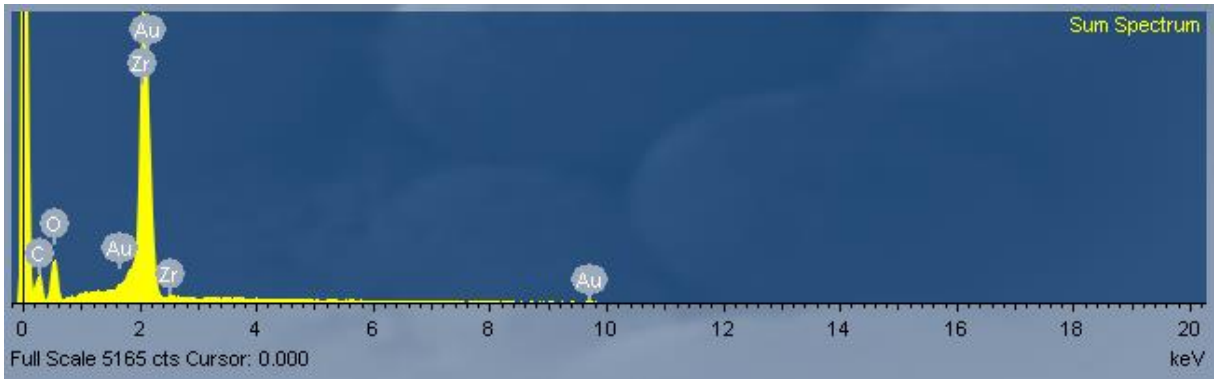

Figure 12 – EDS Analysis of Y-TZP showing predominance of C, O and Zr in its composition

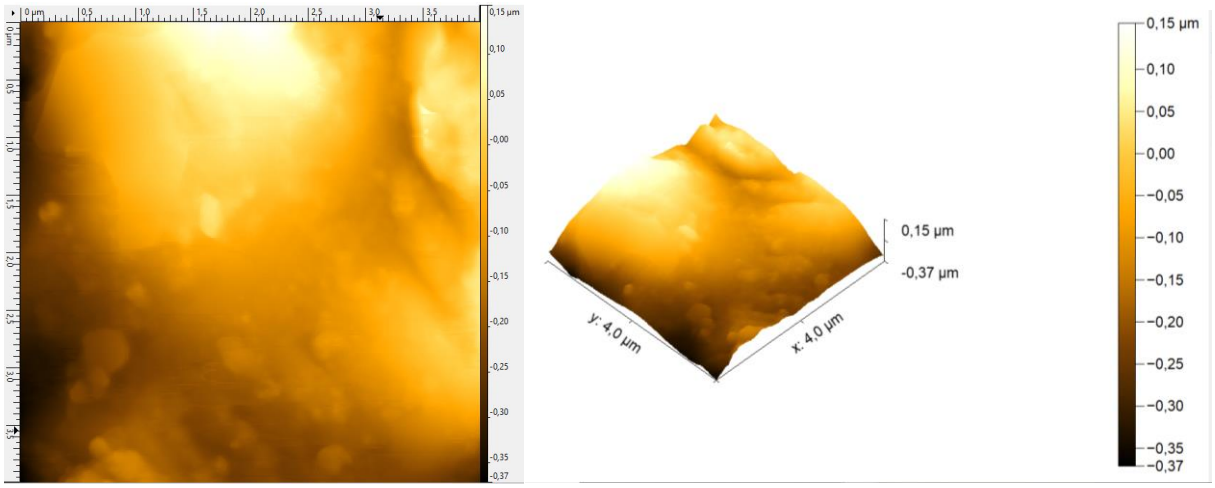

Figure 13 – AFM of DPBHA showing its surface roughness.

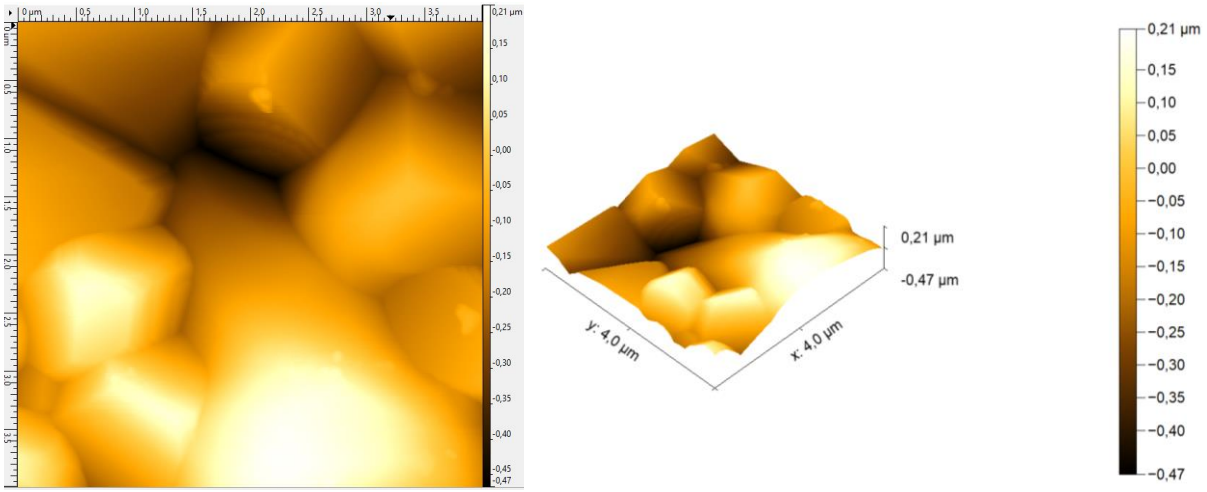

Figure 14 – AFM of DPBHA/AnataseNp5% showing an increase in the surface roughness with 5% TiO_2np addition.

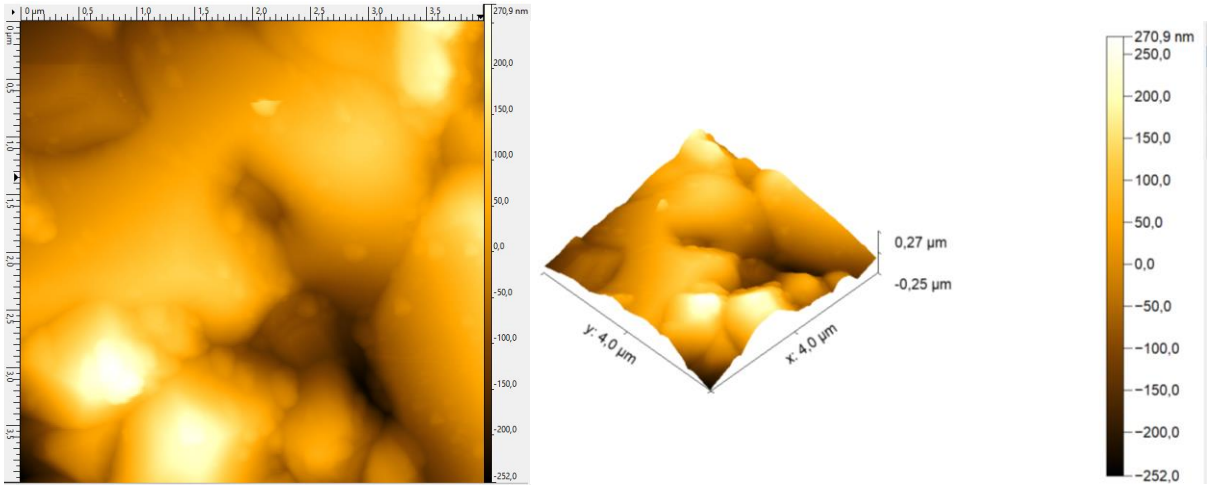

Figure 15 – AFM of DPBHA/AnataseNp8% showing even more surface irregularities with 8% TiO_2np addition.

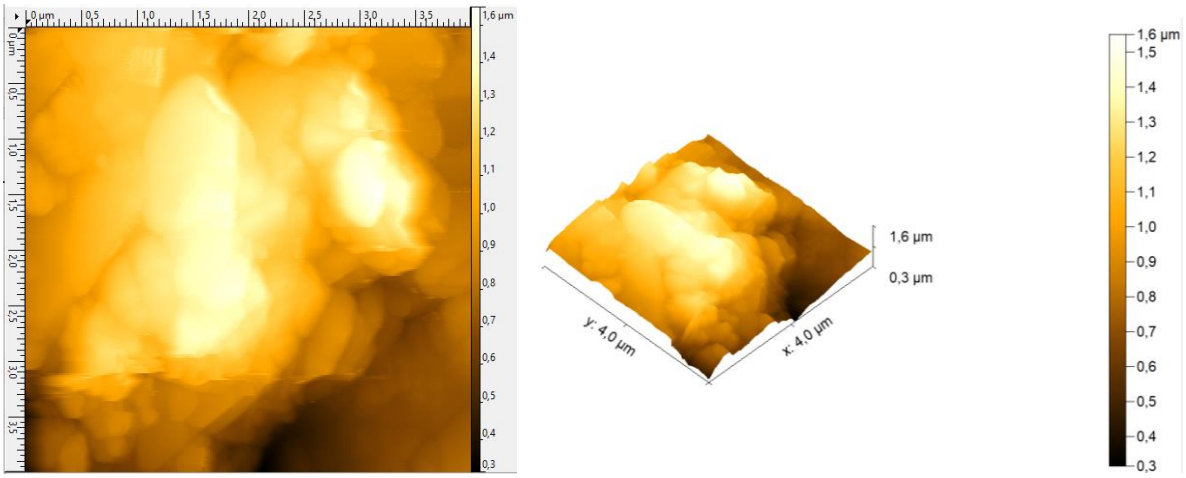

Figure 16 - AFM of YTZ-P showing the most irregular surface of all ceramic groups analyzed.

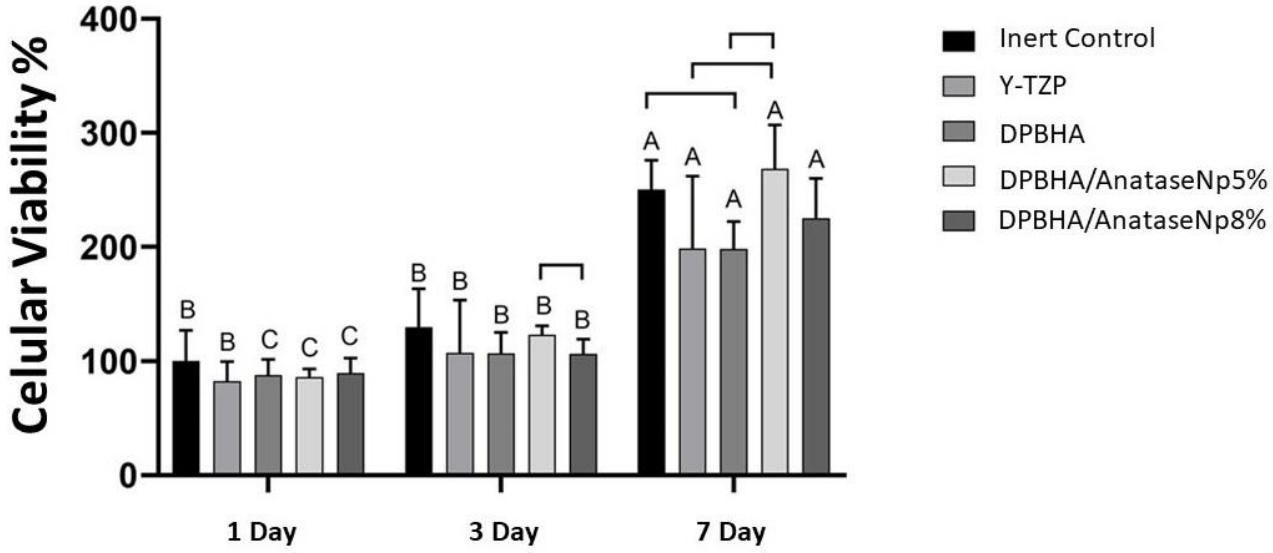

Figure 17 – Alamar Blue Test. Upper case letters mean intra-group comparisons over days and keys mean statistically significant differences among groups. It is possible to notice optimum cellular viability in DPBHA/AnataseNp5% at 7-days analysis.

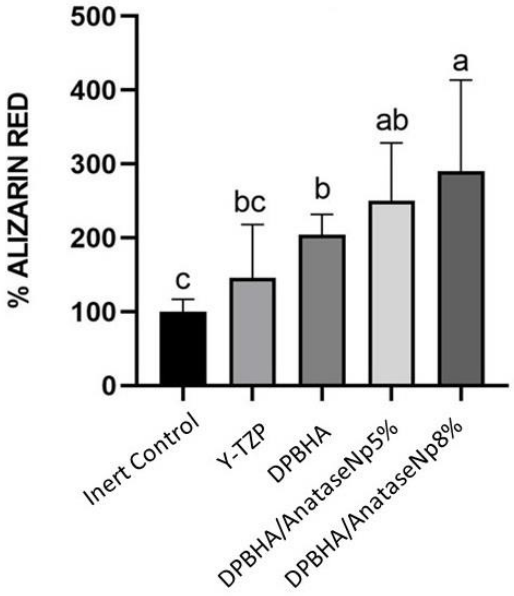

Figure 18 – Alizarin Red performed in Non-Osteogenic Medium showing that DPBHA/AnataseNp5% and DPBHA/AnataseNp8% presented the best mineralized matrix deposition results, with reasonable statistical differences.

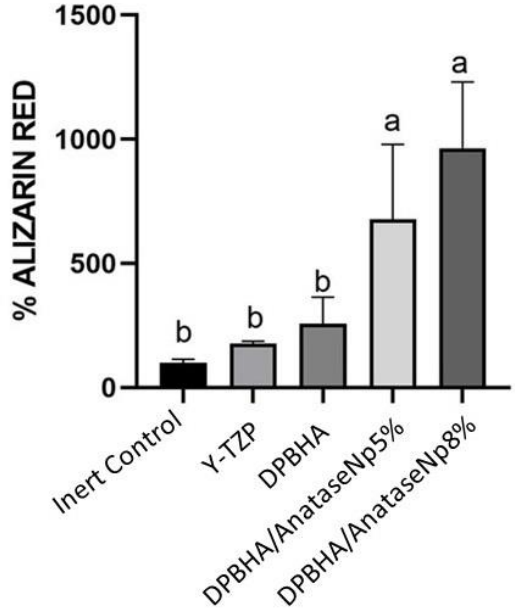

Figure 19 – Alizarin Red performed in Osteogenic Medium showing more mineralized matrix deposition by DPBHA/AnataseNp8% and DPBHA/AnataseNp5% than all other groups.

Table 1 – DPBHA chemical composition with higher percentages of Ca and O by weight

Element	Weight%	Atomic%
O K	25.01	44.32
P K	17.01	15.57
Ca K	35.38	25.02
Totals	100.00	

Table 2 - DPBHA/AnataseNp5% chemical composition with higher percentages of Ca and O by weight

Element	Weight%	Atomic%
O K	35.63	57.71
P K	11.50	9.62
Ca K	38.52	24.91
Ti K	14.36	7.77
Totals	100.00	

Table 3 - DPBHA/AnataseNp8% chemical composition with higher percentages of Ca and O by weight

Element	Weight%	Atomic%
O K	37.89	59.89
P K	11.87	9.69
Ca K	37.91	23.92
Ti K	12.33	6.51
Totals	100.00	

Table 4 – Y-TZP chemical composition showing higher percentage of Zr by weight

Element	Weight%	Atomic%
O K	21.98	37.19
C K	20.26	45.67
Zr L	57.76	17.14
Totals	100.00	