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To cite this article: M. Suárez, S. Pérez-López, C. Prado, A. Fernández, J. S. Moya, R. Torrecillas & L. A. Díaz (2021) Influence of roughness on initial in vitro response of cells to Al₂O₃/Ce-TZP nanocomposite, Journal of Asian Ceramic Societies, 9:1, 131-141, DOI: [10.1080/21570764.2020.1856311](https://doi.org/10.1080/21570764.2020.1856311)

To link to this article: <https://doi.org/10.1080/21570764.2020.1856311>

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Published online: 23 Dec 2020.

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Influence of roughness on initial in vitro response of cells to Al₂O₃/Ce-TZP nanocomposite

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ABSTRACT

Al₂O₃/Ce-tetragonal zirconia polycrystal (TZP) nanocomposite was synthesized by a colloidal processing route and sintered in air atmosphere. Sandblasting treatment was made to alumina toughened zirconia (ATZ) nanocomposite in order to evaluate the influence of surface roughness on the osteogenic differentiation performing in vitro growing a human osteoblast-like cell line, SaOs-2, and human adipose-derived mesenchymal stem cells (hADMSC) osteogenic differentiated. Smooth roughness values around $R_a = 0.5 \mu\text{m}$ were obtained when the abrasive material was below $90 \mu\text{m}$ increasing the expression of BGLAP and IBSP genes and $R_a = 1.5 \mu\text{m}$ was found with particles of sizes between 90 and $250 \mu\text{m}$ upregulating SPARC gene. The non-cytotoxicity and haemocompatibility of ATZ nanocomposite were proved. Alumina-ceria-stabilized zirconia nanocomposite presented in this work exhibits a high potential for application in the fabrication of dental implants due to their biological behavior and very promising mechanical properties.

ARTICLE HISTORY

Received 28 July 2020

Accepted 20 November 2020

KEYWORDS

Nanocomposite; roughness; biocompatibility; Al₂O₃/Ce-TZP

1. Introduction

For more than 40 years, commercially pure titanium and titanium alloys were widely used as dental implant materials due to their excellent biocompatibility, early osseointegration and high corrosion resistance [1]. Nevertheless, titanium may induce allergic reactions or sensitivities [2] and it possesses a dark color that could be exposed during peri-implant mucosa recession and ruin the entire esthetic result [3]. As a viable alternative to resolve these problems, some new ceramic materials were developed. Bioceramic materials offer excellent opportunities to combine the absence of metal ions, good bone ingrowth characteristics and improved esthetics due to the possibility of dyeing the product with pigments. In this context, alumina (Al₂O₃) was the first bioceramic used as an implant material [4], due to its low friction, wettability, wear resistance and biocompatibility. However, it showed insufficient physical properties. In the 80s, zirconia (ZrO₂) emerged as a ceramic material valid for implants because of its improved fracture toughness and mechanical strength with respect to alumina. Tetragonal zirconia polycrystals, specially 3 mol% yttria-stabilized zirconia (3Y-TZP), serves as a metal substitute in substrates and possesses

good physical characteristics; its bending strength doubles and its fracture toughness almost triples that of alumina [5]. Nevertheless, the big disadvantage of pure 3Y-TZP is its low temperature degradation (LTD) [6]. Actually, combining the positive properties of Al₂O₃ (wear resistance, hydrothermal stability and hardness) with those of ZrO₂ (strength and fracture toughness) it is possible to obtain alumina toughened zirconia (ATZ) and zirconia toughened alumina (ZTA) nanocomposites with a higher potential for application as dental implants [7]. Among them, composite materials with Ce-TZP and alumina have shown very promising mechanical properties to be used in the fabrication of implants [8]. Besides the mechanical properties of the bulk material, the characteristics of an implant's surface, such as composition, topography and roughness play an important role in cell-material integration and biocompatibility [9]. The interaction between cells and a biomaterial's surface is fundamentally relevant and essential in terms of the response of cells at the interface, affecting the growth and quality of newly formed bone tissue [10,11]. For this reason, cell culture models are routinely used to study the response of osteoblastic cells in contact with different substrates for

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This article has been corrected with minor changes. These changes do not impact the academic content of the article.

Supplemental data for this article can be accessed [here](#).

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implantation in bone tissue. Moreover, human adipose-derived mesenchymal stem cells (hADMSC) are considered to contain a group of pluripotent mesenchymal stem cells and manifest multilineage differentiation capacity, including osteogenesis, chondrogenesis and adipogenesis [12]. These last cells could differentiate into odontogenic lineage, expressing bone marker proteins, and might be used as suitable seeding cells for tooth regeneration [13]. Few data are available concerning the response of mesenchymal stem cells to ATZ. The purpose of the present study was to perform in vitro osteogenic differentiation assays growing a human osteoblast-like cell line, SaOs-2, and hADMSCs on different ATZ supports in order to determine the influence of the composition and surface roughness on the behavior of the cells in relation with these new implants. In this context, different tests were performed to study cytotoxicity, viability, hemolysis and differences in terms of osteogenic and apoptotic gene expression between the samples.

2. Materials and methods

The $\text{Al}_2\text{O}_3/\text{Ce-TZP}$ nanocomposite was made using the following materials: Ce-TZP (10 mol% CeO_2) from Daichi (Japan) with an average particle size of 35 nm (d_{50}) and a specific surface area of $15 \text{ m}^2.\text{gr}^{-1}$, $\alpha\text{-Al}_2\text{O}_3$ powder (TM DAR, Taimei Chemical Co., Japan) with a specific surface area of $14.6 \text{ m}^2.\text{gr}^{-1}$ and an average particle size (d_{50}) of 150 nm. In addition, the following chemical precursors were also used: i) aluminum chloride (Sigma-Aldrich, Spain), ii) zirconium IV-propoxide (70% solution in 1-propanol) (Sigma-Aldrich, Spain), iii) 2-Propanol (99.9% Panreac, Spain) and iv) absolute ethanol (99.97% Panreac, Spain). A colloidal processing route described in Rivera et al. [14] and L. A. Díaz et al. [15] was followed in order to obtain the nanocomposite. In this route Ce-TZP was coated by an alumina amorphous layer, using aluminum chloride as precursor and subsequently thermally treated in order to activate the formation of γ -alumina transition phase. After this, the alumina powders were also coating with zirconia nanoparticles using a zirconium propoxide as chemical precursor. Finally, both chemically modified raw materials (zirconia and alumina) were mixed using a ratio of 80/20 in volume, respectively, in a polypropylene container with zirconia balls for 72 h in order to ensure a good homogeneity of the mixture. After this, the material was dried at 120°C , grounded and sieved through <63 microns mesh.

2.1. Disk specimen preparation

The powders were cold isostatically pressed at 300 MPa into cylindrical rods of 50 mm in length and 9 mm in diameter. After surface machining and firing at 1475°C

for 1 h, disk-shaped specimens of 7 mm diameter and 1.3 mm thickness were prepared by cutting and polishing (applying microcrystalline diamonds of 9, 3 and 1 microns). In total, 36 disks were used for sandblasting tests and six disks more were used as target specimens.

2.2. Sandblasting process

Disks were sandblasted with white corundum and SiC powders (see Table 1). Laser diffraction (Beckman Coulter LS 13 320, USA) was used for the granulometric characterization of the selected fractions. The air pressure was applied perpendicular to the surface of the disk at 0.4 bars and at a distance of 10 mm using sandblaster equipment (Sandblaster I, Astursinter, Spain).

2.3. Surface roughness and morphology

The morphology of the samples and the raw materials used in the process of sandblasting was characterized by field emission scanning electron microscopy (FESEM) (FEI: Quanta FEG 650, USA). The surface roughness of the specimens was analyzed using a surface roughness tester (MicroTest: MT4002, Spain). Six measurements on each specimen according to ISO 4287–1997 [16] were performed. The assessed profile (R_a) as an arithmetical mean deviation was calculated. The ratio of monoclinic and tetragonal (X_m) and the amount of transformation (monoclinic volume content, v_m) induced by sandblasting were determined by X-ray diffraction (XRD) (Bruker D8 Advance, Germany) using the equations (1) and (2) described in Toraya et al. [17].

$$X_m = \frac{I_m(\bar{1}11) + I_m(111)}{I_m(\bar{1}11) + I_m(111) + I_t(111)} \quad (1)$$

Where

$I_m(\bar{1}11)$ is the peak height of monoclinic phase at around $2\theta = 28.2^\circ$

$I_m(111)$ is the peak height of monoclinic phase at around $2\theta = 31.3^\circ$

$I_t(111)$ is the peak height of tetragonal phase at around $2\theta = 30.2^\circ$

$$v_m = \frac{PX_m}{1 + (P - 1)X_m} \quad (2)$$

where P was a constant with value of 1.311.

This behavior was studied with a Tuttnauer Autoclave (2540EL, Tuttnauer, NY, USA) following ISO 13,356:2015 [18]. In this way, samples were placed in a suitable

Table 1. Sample references and materials used for surface treatment.

Samples	Sandblasting material
Sample NA	Non sandblasted sample (control)
Sample A	White Corundum $< 90 \mu\text{m}$, 60 s
Sample B	$90 \mu\text{m} < \text{SiC} < 250 \mu\text{m}$, 15 s
Sample C	$\text{SiC} < 90 \mu\text{m}$, 15 s

autoclave and exposed to steam at 134°C under a pressure of 0.2 MPa for a period of 5 h. After this period, cool the autoclave and remove and dry the test specimens.

2.4. In vitro studies methodology

Different tests were carried out in order to assess the biocompatibility, non-cytotoxicity and haemocompatibility of the Al₂O₃/Ce-TZP nanocomposite:

- (1) Human adipose-derived mesenchymal stem cells (hADMSCs) isolation and culture: hADMSCs were isolated from abdominal subcutaneous adipose tissue. Adipose tissue was submitted to mechanical digestion and then digested with collagenase I (Sigma-Aldrich, USA) DMEM solution (Lonza, Belgium) and the cell suspension was filtered and centrifuged. The obtained cell fraction was cultured in expansion medium to 80% confluence, at 5% CO₂ and 37°C. Finally, cells were harvested using Trypsin-EDTA 1X (Biowest, France).
- (2) Cytotoxicity tests using the neutral red uptake (NRU) assay and the MTS assay. The potential cytotoxic effect of materials on mammalian cells was determined following ISO 10,993-part 5 [19] for biomaterials and medical device testing. Samples were sterilized before use. SaOs-2 cells (human osteosarcoma cells, kindly provided by the SCT of the University of Oviedo, Spain) or human MSCs from adipose tissue were seeded onto 48-well plates at a density of approximately $4 \cdot 10^4$ cells/ml/cm² and cultured until achieving confluence. Then, samples were placed in contact with the cell monolayer. In the NRU test, the uptake of NR into the lysosomes/endosomes and vacuoles of living cells are used as a quantitative indication of viability. Cells were washed after 24 h and incubated with neutral red solution (Scharlab, Spain) for 3 h. After that, cells were washed again and NR was desorbed by adding a mixture of ethanol and acetic acid. The amount of NR extracted from the cells was measured at 540 nm and the percentage of viable cells was calculated (equation 3).

$$\% \text{ of viable cells} = 100 \times \frac{\text{Abs}_{540}(\text{extract})}{\text{Abs}_{540}(\text{blank})} \quad (3)$$

In the MTS assay, culture medium was removed after 24 h and fresh medium with MTS solution (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, USA) (5:1) was added to each well. After 3–4 additional hours in the presence of MTS, absorbance was determined at

490 nm and the percentage viability was calculated (equation 4).

$$\% \text{ of viable cells} = 100 \times \frac{\text{Abs}_{490}(\text{extract})}{\text{Abs}_{490}(\text{blank})} \quad (4)$$

- (3) Hemolysis index (ASTM F 756–08 [20]): Hb released into plasma when blood was exposed to the materials was measured: After a period of incubation, samples were removed and tubes were centrifuged. Each supernatant (100 µL) were mixed with Drabkin's reagent (100 µL) and cyanmethemoglobin was produced and detected by spectrophotometry at 540 nm. Total blood hemoglobin (TBH) was also measured. The hemolytic index was calculated (equation 5).

$$\text{Hemolytic Index} = \frac{\text{Hb released} \left(\frac{\text{mg}}{\text{mL}} \right)}{\text{TBH} \left(\frac{\text{mg}}{\text{mL}} \right)} \quad (5)$$

- (4) Osteogenic differentiation of SaOs-2 (human osteoblast-like cell line) and hADMSCs: Cells were seeded at a density of 50×10^3 cells on each of the four kinds of samples until confluence and cultured in differentiation medium supplemented with dexamethasone, ascorbic acid and β-glycerol phosphate (all reagents from Sigma-Aldrich, USA). Every 72 h, differentiation medium was changed, and in 3 weeks, the whole differentiation process was completed. In order to confirm the adequate osteogenic differentiation, alkaline phosphatase and alizarin red staining were performed.
- (5) Alkaline phosphatase staining: Differentiated osteogenic cells were fixed with formaldehyde (Merck, Germany), stained with a BCIP/NBT solution (SIGMA FAST™ BCIP/NBT, Sigma-Aldrich, USA) and observed by microscopy.
- (6) Alizarin red staining: Differentiated osteogenic cells were fixed with paraformaldehyde, stained with alizarin red solution (Merck, Germany) and observed by microscopy.
- (7) RNA extraction and cDNA synthesis: Total RNA was isolated using Tri reagent® solution (Ambion, USA). After evaluating the quality of the RNA through absorbance measurements, each RNA sample was reverse transcribed to obtain cDNA samples.
- (8) Real-Time quantitative polymerase chain reaction: Quantitative PCR was performed using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Fluorescence data during the primer extension step were acquired. The melting-curve analysis, to confirm product specificity, was performed immediately after amplification, following 1 min of denaturation at 95°C, 1 min of annealing at 65°C

and 60 cycles of 0.5°C increments (30 s each) beginning at 65°C while monitoring fluorescence. β -ACTIN (ACTB) was used as the endogenous control. This gene encodes one of six different actin proteins that are involved in cell motility, structure, integrity and intercellular signaling. Alkaline Phosphatase, Biomineralization Associated (ALPL) that may play a role in bone mineralization, Bone Gamma-Carboxyglutamate Protein or Osteocalcin (BGLAP) that contains Gla which functions in binding to calcium and hydroxyapatite, Collagen Type I (COL1) found in most connective tissues, Integrin Binding Sialoprotein (IBSP) that encoded the major structural protein of bone matrix and Secreted Protein Acidic and Cysteine Rich or Osteonectin (SPARC) required for the collagen in bone are used as bone differentiation genes. CASPASE 3 is used as an indicator of apoptotic damage.

- (9) Statistical analysis: Data using the Proc MIXED procedure of the SAS/STAT v8.2 software package (SAS Institute, Inc., Cary, NC, USA) were statistically analyzed. Ct values (number of cycles needed to generate a fluorescent signal above a predefined threshold) and Δ Ct values (difference between the Ct of the target gene and that of the housekeeping gene) for each gene and repetition were used as dependent variables. To quantify gene expression levels a relative standard method was used. The model fitted included the type of sample as the fixed effect and the sample as a random effect to account for the non-independency of the repetitions. For each sample level, least-square means and their corresponding standard errors were computed.

For descriptive purposes, a Student's *t* test on raw Ct means computed for samples using Proc GLM of SAS/STAT was also carried out, assuming that the sample type effect included two independent groups of normally distributed observations.

3. Results

3.1. FESEM

The representative microstructure of the sintered ATZ nanocomposite is shown in Figure 1 where two different phases can be observed. The lightest one corresponds to the Ce-TZP matrix with a particle size of 400–500 nm and the darkest phase corresponds to alumina with an average size of 250 nm. As it can be observed alumina grains are homogeneously distributed in the Ce-TZP matrix and no pores are observed. Grains with straight edges appeared in both phases indicating that the sintering process has been completed.

3.2. Surface roughness

Sandblasting is a commonly used surface treatment and involves impacting with hard particles at high velocities on a surface in order to erode it and leave a roughened surface with expected higher wettability. The FESEM micrographs of the modified ATZ surfaces by sandblasting with white corundum and silicon carbide for 60 s and 15 s are shown in Figure 2(a-c). Sandblasting with white corundum and SiC particles <90 microns (Figure 2(a and c), respectively), revealed a regular and

Figure 1. FESEM microstructure of polished surface of the ATZ nanocomposite.

Figure 2. FESEM images from the topographical appearance of sandblasted surfaces with white corundum and silicon carbide raw materials. (a) White corundum < 90 μm time: 60 s; (b) SiC < 250 μm and > 90 μm time: 15 s; and (c) SiC < 90 μm time: 15 s.

Table 2. Roughness values of the ATZ nanocomposite surfaces after sandblasting with corundum and SiC for 60 and 15 s (mean $\pm$ SD in μm). N.A (No Sandblasting).

Raw materials	$R_a < 0.5 \mu\text{m}$	$0.5 \mu\text{m} < R_a < 1.0 \mu\text{m}$	$1.0 \mu\text{m} < R_a < 2.0 \mu\text{m}$	$R_a > 2.0 \mu\text{m}$
W.C. < 90 μm , 60 s	Smooth	Minimally rough	Moderately rough	Rough
SiC 250–90 μm , 15 s	0.465 ± 0.06	0.580 ± 0.08	1.358 ± 0.11	
SiC < 90 μm , 15 s	0.031 ± 0.10			
N. A				

slightly waved structure. However, the surface of the sandblasted ATZ with SiC particles between 90 and 250 μm (Figure 2(b)) showed a more irregular structure with visibly larger voids and grooves, increasing the surface roughness according to the results shown in Table 2 for ATZ samples. The mean roughness index, R_a (arithmetical mean deviation of the profile), in case of samples sandblasted with SiC between 90 and 250 microns was significantly higher than the other

sandblasted samples. According to the Altbrektsson and Wennerberg classification [19], the samples sandblasted with white corundum present a “smooth” surface roughness ($R_a < 0.5 \mu\text{m}$), while the samples sandblasted with SiC show “minimally rough” and “moderately rough” surface roughness. Similar results have been found by Sato et al. [20] where sandblasting by SiC particles resulted in surface roughness values larger than those by alumina particles.

Figure 3. X-ray diffraction patterns for ATZ samples: as sintering; as sintering and sandblasting process; as sintering, sandblasting and aging process (see Table 3).

3.3. XRD diffraction

X-ray diffraction (XRD) patterns of characterized samples are shown in Figure 3. The materials do not present spontaneous phase transformation on their surface after sintering and any aging process. However, sandblasting processes lead to the transformation, under tension, of a part of the tetragonal zirconia to monoclinic zirconia since an increase of the intensity of monoclinic peak $(\bar{1}11)_m$ with respect to the tetragonal peak $(111)_t$ can be observed. The volumetric fraction (v_m) of the monoclinic phase is calculated according to equations (1) and (2) on (1) as-sintered surfaces, (2) as-sintered surfaces after sandblasting process, (3) as-sintered surfaces after sandblasting and its aging process and the results are shown in Table 3. The particle size and the kind of material used for the sandblasted process have an effect on the transformation phase of ATZ composite [21]. In this sense, the

effect of sandblasting on the monoclinic content was larger in case of SiC particles than alumina particles since it depends on the difference in the hardness of the material: e.g. the Vickers hardness of Al_2O_3 and SiC is 1800 and 2200, respectively. Furthermore, increasing the particle size increases the erosion of material and the transformation surface layer. This transformation process is reversible and, in every case, a posterior thermal treatment at 1200°C for 15 min succeeds in transforming the totality of the monoclinic zirconia back to its initial tetragonal state [22].

3.4. In vitro biological assays

ISO 10,993-5 [23] states that a material is considered non-cytotoxic when cell viability is above 70%. The potential cytotoxicity on SaOs-2 and hADMSCs was assessed by the MTS assay and the NRU method using tissue culture polystyrene (TCPS) as the blank. According to the results shown in Figure 4(a and b) all of the studied samples allowed for higher than 90% cell viability; therefore, none of the surface modification treatments can be considered cytotoxic.

Hemolysis is the alteration, dissolution or destruction of red blood cells that results in hemoglobin liberation into the surrounding medium. According to Stanley's classification criteria, a material is considered non-hemolytic for hemolytic indexes <2 while it is considered slightly hemolytic and hemolytic for hemolytic index values of 2–5 and >5, respectively. Different factors such as surface roughness, surface energy and surface tension and surface wettability can have an influence on the blood compatibility and it is shown that surface modification has a great

Table 3. Volumetric fractions of monoclinic phase zirconia present on different sandblasted surfaces.

Treatment	Monoclinic phase content ($V_{m\text{total}}$)
ATZ as-sintered	3,80% $\pm$ 3%
ATZ as-sintered and aging during 5 h	2,10% $\pm$ 3%
ATZ as-sintered sandblasting surface by white corundum < 90 μm , 60 s	24,73% $\pm$ 3%
ATZ as-sintered sandblasting surface by white corundum < 90 μm , 60 s aging during 5 h	27,00% $\pm$ 3%
ATZ as-sintered sandblasting surface by SiC 250–90 μm , 15 s	48,70% $\pm$ 3%
ATZ as-sintered sandblasting surface by SiC 250–90 μm , 15 s aging during 5 h	50,00% $\pm$ 3%
ATZ as-sintered sandblasting surface by SiC <90 μm , 15 s	31,70% $\pm$ 3%
ATZ as-sintered sandblasting surface by SiC <90 μm , 15 s aging during 5 h	36,00% $\pm$ 3%

Figure 4. Biological assessment: NRU and MTS assay. No reduction in cell viability was observed for either the SaOs-2 (a) or the hADMSC (b) cells for any of the surface modifications tested, since the percentage viability was always superior to 70%. Bars represent the means (SD) of triplicate measurements.

Figure 5. Alkaline phosphatase staining (a) and alizarin red staining (b) on substrate after hADMSCs incubation. White bar represents 500 microns.

potential for improving the hemocompatibility of biomedical materials and devices [24]. In this case, the studied ATZ nanocomposite showed a hemolytic index close to 0 (0.1–0.2) for all the surface modifications tested, which was <1% indicating non-hemolytic material.

ALP levels increase when active bone formation (osseous differentiation) occurs, as it is a by-product of this process. According to the results shown in Figure 5(a and b) a correct osteoblast differentiation of hADMSCs has been taken place since all of the differentiated cells used for gene expression studies were stained and, consequently, osteoblast differentiation confirmed.

Product identity was confirmed by electrophoresis on ethidium bromide-stained 2% agarose gels in 1X TBE buffer, which resulted in a single product of the desired length. In addition, an iCyclerIQ melting curve

analysis was performed, which rendered single product specific melting temperatures. No primer-dimers were generated during the 40 real-time PCR cycles conducted. All polymerase chain reaction efficiencies were above 90% and linearity was high, with correlation coefficients (R^2) above 0.989.

To quantify gene expression, the relative standard method (relative fold changes) was used and expression levels were determined for the ceramics and the control group (NA sample) by normalizing results with respect to β -ACTIN. For hADMSCs, a discreet increase was noticed in the relative expression of four of the studied genes for samples A, B and C when compared with the control sample. In Figure 6(a), these increases are plotted. In the problem group, the genes BGLAP, CASPASE 3, IBSP and SPARC were up-regulated 2.27-fold for sample A, 1.57-fold for sample C, 3.10-fold for

Figure 6. Relative quantification of the studied genes to day 21. mRNA levels for apoptotic and bone differentiation genes recorded in hADMSCs (upper part) and SaOs-2 cells relative to endogenous control gene β -ACTIN levels. Values are expressed as mean $\pm$ SEM. * indicates a significant difference with respect to the controls at p less than 0.05.

sample A, and 2.27-fold for sample B, respectively, with respect to the basal levels recorded for the endogenous control (β -ACTIN). However, these increases were only significant ($p < 0.05$) in the case of IBSP for sample A.

In the case of the SaOs cells, an even more discreet increase was observed in the relative expression of two of the studied genes for samples A, B and C when compared with the NA sample (control). These results are also shown in Figure 6(b). The IBSP gene is 1.55-fold up-regulated for sample B. The COL1A1 gene is up-regulated 2.95-fold for sample A, 2.85-fold for sample B and 1.74-fold for sample C with respect to the basal levels recorded for the endogenous control. In this case, differences between control and problem groups were not significant.

4. Discussion

The alumina toughened zirconia (ATZ) biomaterial studied in this work is a nanocomposite that combines

the properties of Al_2O_3 and Ce-ZrO_2 . Moreover, Ce-TZP does not suffer low temperature degradation. The good combination of mechanical properties of this nanocomposite has already been proven [14] and now it is necessary to confirm its good interaction with cells.

The ideal material to make implants should not be purely tolerated by the host but should interact with biological systems in a way that induces the appropriate host response for a specific application [25] controlled by the proteins that coat the surface of the biomaterial. The nature and activity of the proteins adsorbed on the surface depend on its physical and chemical properties. In fact, the biomaterial's surface properties such as topography and hydrophilicity will be determinant in terms of biocompatibility and other biological phenomena. Topography also can modify the shape and activity of mesenchymal stem cells leading to a higher differentiation rate of these cells into osteogenic lineage with the upregulation of osteoblastic genes [26].

Zhang Y et al. [27] showed how surface roughness affects osteoclastic differentiation as well as the stimulatory effects of osteoclasts on osteogenic differentiation of osteoprogenitor cells. Concerning osteoblast differentiation microscale surface roughness has been shown to enhance osseointegration of titanium implants through increased osteoblast differentiation while osteoblast proliferation remains greater on smooth titanium [28]. According to the literature, surfaces with $Ra \leq 1 \mu\text{m}$ are considered smooth and those with $Ra > 1 \mu\text{m}$ are described as rough. In general, the surface roughness range that favors osseous differentiation has been reported to be 1.0–1.5 μm [29].

Sandblasting is known to form surface roughness and irregularities on surface materials and, in the particular case of zirconia, induces a transformation from tetragonal to monoclinic phase due to the stress generated during the process [30]. Human cells were preferably attached to hydrophilic surfaces than hydrophobic ones. For this reason, the modification of the surface allows increasing the surface area and wettability of the ceramic surface, increasing protein adsorption, promoting the attachment, proliferation and differentiation of human cells. According to the literature [31], increasing surface areas would be more favorable for cell attachment due to mechanical interlocking at the initial cell attachment stage.

In the present study, SaOs-2 cells and hADMSC cells were used to investigate the influence of roughness on regulation of cell viability, hemolysis and osseous differentiation. According to the results obtained in the cytotoxicity and hemolysis experiments, the data obtained in our study revealed that there are no significant differences in the responses of osteogenic cells and hADMSCs toward the different surface modification treatments in terms of biocompatibility. In fact, none of the surface modification treatments induced significant cell death in osteoblasts, hADMSC or erythrocytes. Similar results regarding cell viability and hemolysis have been published over the years [32]. Given the bioinertness of ceramic materials, such as those studied in this work, treatments to modify the surface and provide a suitable environment are necessary in order to trigger a favorable biological response in terms of osseous differentiation.

Concerning gene expression, different osteogenic genes were studied in order to determine differences between samples. For hADMSCs, the increase was only lightly observed in the case of four genes BGLAP, CASPASE3, IBSP and SPARC. BGLAP was up-regulated 2.27-fold for sample A, CASPASE3 was up-regulated 1.57-fold for sample C, IBSP was up-regulated 3.10-fold for sample A and, finally, SPARC was up-regulated 2.27-fold for sample B with respect to the basal levels. However, the increase was only

significant ($p < 0.05$) in the case of IBSP in sample A. These findings, in terms of gene expression as well as histological results, showed that the roughness and the nature of the material are adequate to allow cells to achieve osteogenic differentiation, despite the fact that the surface roughness of the studied samples is clearly below the Ra values reported to favor osseous differentiation (1.0–1.5 μm). It is important to note that osteoblastic differentiation of MSCs into functional differentiated osteoblasts requires a series of steps involving the expression of different proteins at each stage. Alkaline phosphatase is regarded as a marker of early osteoblastic differentiation. In fact, it is the main signal that compromises cells to differentiate toward osteoblastic lineage; in a similar way, COL1 gene is over-expressed in the pre-osteoblastic phase coinciding with the beginning of the bone tissue-differentiating cascade, whereas secretion of Osteocalcin and IBSP as well as matrix mineralization is associated with the final differentiation phase. The observation of an increasing expression of IBSP with significant differences between groups for white corundum (sample A) showed that the differences in terms of osteogenic differentiation seem not to appear until the last phases, meaning that the gene expression pattern is similar for all samples during the entire process. Significantly, the main increase observed in IBSP is in agreement with the results obtained by Wang and cols [33] that pointed out the importance of the integrin-linked kinase/ β -catenin pathway in mediating signals from topographic cues to direct the osteogenic differentiation of cells. For SaOs-2 cells, the three samples (A, B and C) showed upregulation related to sample NA (control) for two of the studied genes, IBSP and COL1. These results are correlated with the different levels of roughness showed for the different samples, since NA shows the smoothest one. Moreover, these results also are according to the concept that the roughness affects the attachment and spreading of cells, showing that probably the number of osteoblastic cells is higher in the rough supports. However, this upregulation was not significant in any case. The expected pattern for SaOs-2 cells is clearly different from that of hADMSC because it is a human osteoblast-like cell line itself and it is supposed to express osteogenic genes such as COL1 at the beginning of the bone tissue differentiating cascade or IBSP that are over expressed at middle-to-late osteogenic differentiation. These observations are in agreement with Czekanska et al. [34], who described high levels of expression of osteocalcin, bone sialoprotein, decorin and procollagen-I. For both types of cells (hADMSCs and SaOs-2), the apoptotic gene studied, Caspase-3, did not show important differences between samples, meaning that neither material nor roughness had a clear influence on cell death. This circumstance is in agreement

with the absence of deleterious effects on cell viability.

5. Conclusions

The non-cytotoxicity and haemocompatibility of a nanocomposite ceramic material formed by alumina and ceria stabilized zirconia has been proved. The surface roughness of this nanocomposite can be adjusted depending on the particle size of the materials used for sandblasting. Smooth roughness values of around 0.5 μm are obtained when the abrasive material (white corundum or silicon carbide) is below 90 μm , while the use of silicon carbide particles of sizes between 90 and 250 μm leads to surface roughness values of around 1.5 μm . Moreover, the roughness and the nature of the material used have been proved adequate for cell osteogenic differentiation. An increase in the expression of BGLAP and IBSP genes was observed on samples sandblasted with white corundum below 90 μm , whereas SPARC gene was upregulated on samples sandblasted with SiC between 90 and 250 μm . Then, the studied nanocomposite is a very promising material for dental applications thanks to its good mechanical properties in comparison with conventional ceramics, the possibility of adjusting its surface roughness and the different in vitro results obtained.

Acknowledgments

The Spanish Ministry of Science, Innovation and Universities and the Spanish Higher Council for Scientific Research (CSIC) have supported this research under the Projects MAT2012-38645 and INPERIO, H2020-SMEInst-2018-2020-2 Ref CSIC-20185222, respectively. Catuxa Prado acknowledges financial support from the JAE-DOC program (CSIC).

Disclosure statement

This manuscript has a registered preprint (DOI: 10.21203/rs.3.rs-44,864/v1) with the following link <https://www.researchsquare.com/article/rs-44864/v1>. No potential conflict of interest was reported by the authors.

Funding

This work was supported by The Spanish Ministry of Science, Innovation and Universities and the Spanish Higher Council for Scientific Research (CSIC) under the Projects MAT2012-38645 and INPERIO, H2020-SMEInst-2018-2020-2 Ref CSIC-20185222, respectively. Catuxa Prado acknowledges financial support from the JAE-DOC program (CSIC).

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