Mechanical performance and osteoblast-like cell responses of fluorine-substituted hydroxyapatite and zirconia dense composite

Hae-Won Kim,1,2 Jonathan C. Knowles,2 Long-Hao Li,1 Hyoun-Ee Kim1
1School of Materials Science and Engineering, Seoul National University, Seoul, 151-742, Korea
2Division of Biomaterials and Tissue Engineering, Eastman Dental Institute, University College London, 256 Gray’s Inn Road, London WC1X 8LD, United Kingdom

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Abstract: A fluorine-substituted hydroxyapatite (FHA) and zirconia (ZrO2) dense composite (50:50 by volume) was fabricated, and its feasibility for hard tissue applications was investigated in terms of its mechanical properties and osteoblast-like cell (MG63) responses in vitro. The incorporation of fluorine into the hydroxyapatite (HA) structure was highly effective in producing a completely dense apatite–ZrO2 composite through a pressureless sintering route, by preventing the thermal degradation of the apatite and ZrO2. The resultant FHA–ZrO2 dense composite had excellent mechanical properties, such as flexural strength (310 MPa), fracture toughness (3.4 MPam1/2), hardness (10 GPa), and elastic modulus (160 GPa). The flexural strength and fracture toughness of the composite showed a noticeable improvement by a factor of ~4 with respect to the pure apatites (HA and FHA). The MG63 cellular responses to the composite were assessed in terms of the cell proliferation (cell number and [3H]-thymidine incorporation) and differentiation (alkaline phosphatase activity, osteocalcin, and collagen production). The cells on the FHA–ZrO2 composite spread and grew well, and proliferated actively during the culture period. The expression of alkaline phosphatase, osteocalcin, and collagen by the cells on the composite showed a similar trend to that on the pure apatites, although slight down-regulations were observed, implying that the FHA–ZrO2 50:50 composite retains the osteoblastic functionality and traits of the pure HA ceramics to a high degree. This finding, in conjunction with the considerable improvements in mechanical properties, supports the extended use of this composite for hard tissue applications. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 72A: 258–268, 2005

Key words: fluorine-substituted hydroxyapatite (FHA); zirconia (ZrO2); dense composites; load-bearing implants; mechanical properties; osteoblast-like cell responses

INTRODUCTION

Hydroxyapatite [HA, Ca10(PO4)6(OH)2] composites with ceramic oxides, such as zirconia (ZrO2) and alumina (Al2O3), have been studied, with a view to combining the biological activity of HA and the mechanical properties of the ceramic reinforcements.1–5 The poor strength and brittleness of HA restrict its use only to non-load-bearing parts in small sizes or to fillers in powder and granular form. On the other hand, ZrO2 and Al2O3 ceramics are attractive for use as dental implants and femoral heads, in which they bear high mechanical loads, by virtue of their excellent mechanical properties.9,10 However, they are nearly bioinert and mostly do not form bony tissues directly on their surface.11 This differing efficacy of the HA and ZrO2/Al2O3 ceramics has hampered their widespread utilization in hard tissue applications. Therefore, an appropriate combination of the two systems can extend their usage to biological applications that require bioactivity as well as high levels of mechanical integrity.

However, it has proven difficult to fabricate the dense composites of HA with ZrO2/Al2O3, because of the reaction between HA and the ceramic oxides, which results in phase degradation and poor mechanical properties.3–8 Many attempts have been made to overcome these problems, through such techniques as introducing external pressure (hot pressing and hot-isostatic pressing) and water atmosphere, and applying particle coatings.7,8 However, these methods met with limited success or were either technically deficient or not cost-effective. Recently, fluorine-substi-
tuted \( \text{FA} \) [\( \text{FA}, \text{Ca}_{10}(\text{PO}_4)_6(\text{OH},\text{F})_2 \) was observed to be effective in producing apatite–ceramic oxide composites.\(^3\)–\(^6\) Because of the high thermal stability of \( \text{FA} \), the reaction between \( \text{FA} \) and \( \text{ZrO}_2 \) was suppressed and, ultimately, dense composites were obtained. The resultant composites exhibited improved mechanical properties and favorable cell viability and functionality according to the preliminary tests.\(^3\)

Based on these previous results, in this report, we fabricated an FHA–\( \text{ZrO}_2 \) composite containing equivalent amounts of FHA and \( \text{ZrO}_2 \) (50:50 v/v), and addressed its mechanical and biological feasibility for hard tissue applications. The composition 50:50 chosen from a preliminary study was aimed at producing a composite possessing high mechanical properties, whereas using an amount of \( \text{ZrO}_2 \) phase that did not exceed that of apatite. The mechanical properties, including the flexural strength, fracture toughness, hardness, and elastic modulus were examined. For the biological tests, a human osteoblast-like cell line, MG63, was used. Despite its being a transformed cell line, the MG63 cells exhibit human osteoblastic traits, such as cell proliferation, alkaline phosphatase (ALP) expression, and the production of bone-associated proteins.\(^{12–14} \) The mechanical and \textit{in vitro} biological properties of the FHA–\( \text{ZrO}_2 \) composite were compared with those of pure apatite ceramics (HA and FHA).

**MATERIALS AND METHODS**

**FHA and FHA–\( \text{ZrO}_2 \) composite fabrication**

The fabrication of FHA–\( \text{ZrO}_2 \) composite was slightly modified from the method described previously.\(^4\) First, pure fluorapatite (FA) was made from high-purity \( \beta \)-tricalcium phosphate (TCP) (BDH, UK) and CaF\(_2\) (Sigma, UK) powders by their thermal reaction at 900°C for 3 h, as follows:

\[
3\text{Ca}_3(\text{PO}_4)_2\text{[\( \beta \)-TCP]}(s) + \text{CaF}_2(s) = \text{Ca}_{10}(\text{PO}_4)_6\text{F}_2[\text{FA}](s)
\]

The FA so obtained was mixed with pure HA (Sintering grade; Biotal, UK) at a ratio of \( \text{FA}/\text{HA} = 1 \) by ball-milling, followed by drying and heat treating at 1000°C for 3 h, to form an FHA solid solution as follows:

\[
1/2\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2[\text{HA}](s) + 1/2\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2[\text{FA}](s) = \text{Ca}_{10}(\text{PO}_4)_6\text{OH-F}[\text{FA}](s)
\]

The FHA powder was mixed with 50 vol % \( \text{ZrO}_2 \) powder (3Y-TZP; Tosoh Co., Japan), and the powder mixture was ball-milled, dried, and sieved to 25 \( \mu \)m. After molding in a metal die, the sample was pressureless sintered at 1400°C for 3 h in air, resulting in a dense FHA–\( \text{ZrO}_2 \) composite. For the purpose of comparison, pure HA and FHA ceramics were prepared after pressureless sintering at 1300°C for 2 h, and pure HA-50% \( \text{ZrO}_2 \) composite without the fluorine incorporation was also fabricated by pressureless sintering under the same conditions as the FHA–\( \text{ZrO}_2 \) composite.

**Characterization and mechanical tests**

The porosity of the sintered specimens was measured by the Archimedes method, and the microstructure was observed using scanning electron microscopy (SEM; Cambridge Ltd., UK) after polishing with diamond slurries to 1 \( \mu \)m. The phase of the specimens was characterized by X-ray diffraction (XRD; Philips, Holland) using a scanning rate of 0.5° 20/min. The formation of FHA solid solution was observed from the XRD patterns using silicon powder (Si >99.999%; Sigma) as an internal standard, and the lattice parameters were calculated to determine the amount of fluorine substitution.

For the mechanical tests, the surface of the samples was polished with diamond slurries to 1 \( \mu \)m. The biaxial flexural strength (\( \text{S} \)) was measured for each disc type sample with dimensions of about 25 \( \times \) 1.5 mm (diameter \( \times \) thickness), using a flat-on-sphere on three-ball support,\(^{15,16} \) by applying a load cell at a crosshead speed of 2 mm/min using an Instron machine (Instron 4505, UK). The elastic modulus (\( \text{E} \)) of the specimens was measured by an ultrasonic pulse method. The fracture toughness (\( \text{T} \)) of the specimens was determined by the indentation fracture method\(^{17} \) using a Vickers indenter at loads of 4.9–19.6 N. The Vickers hardness (\( \text{H} \)) was measured by applying indentation loads at 0.98–4.9 N.\(^{17} \) Five specimens were used for the measurement of the flexural strength and elastic modulus, and the data on 10 different indentations were averaged to determine the fracture toughness and hardness. The fractured and indented surfaces were observed with SEM and optical microscopy.

**Cell proliferation**

The human osteoblast-like MG63 cells were used after subculturing, as described previously.\(^3\) The cells were washed with phosphate-buffered saline (PBS), detached with trypsin/ethylenediaminetetraacetic acid solution (0.25% trypsin) at 37°C for 10 min, and then centrifuged and resuspended for further reseeding and growth tests. The specimens (FHA–\( \text{ZrO}_2 \), HA, and FHA) used for the cell tests were prepared in disc shapes (\( \sim 12 \times 1 \) or 25 \( \times 1 \) mm) after polishing to 1 \( \mu \)m and sterilizing at 121°C for 20 min.

To measure the effect of each material on cell growth, all of the specimens were placed in individual wells of 24-well plates. Cells were seeded at a density of 2 \( \times 10^4 \) cells/well in 0.1 mL of medium and incubated for 6 h. Then, 1.5 mL of medium was added and the cells were cultured for up to 7 days in an incubator humidified with 5% \( \text{CO}_2/95\% \) air at 37°C. At each culture period (1, 3, 5, and 7 days), the cells were trypsinized and stained with trypan blue, and the live cells were counted using a hemocytometer (Superior Co.,
removing the media and rinsing with PBS, 400 µL was added to each well and the cells were left for 4 h. After
counting per minute (cpm) of incorporated [3H]-thymidine per counter (LKB Wallac, Finland). The results are shown as

At each time period, 50 µL of [3H]-thymidine solution was added to each well and the cells were left for 4 h. After
removing the media and rinsing with PBS, 400 µL of papain solution [1 µL/mL papain (type III; Sigma), 5 mM cysteine, and 5 mM ethylenediaminetetraacetic acid in PBS at pH 5.7] was added to each well containing the labeled cells, which were then incubated for 24 h at 60°C. After this, 150 µL of cell digest was added to 5 mL of scintillation fluid (Unisolv; NBS Biologicals, NJ) and the amount of isotope incorporated was measured using a liquid-scintillation counter (LKB Wallac, Finland). The results are shown as counts per minute (cpm) of incorporated [3H]-thymidine per cell.

The cell morphology was observed using SEM (JSM5600; JEOL, Japan). The cells were fixed in 2.5% glutaraldehyde for 2 h at room temperature, dehydrated through a series of graded ethanol (70, 90, and 100%), and critical point dried with CO₂. After gold coating for the sake of conduction, the cells were observed at an accelerating voltage of 15 kV.

Cell differentiation

The intracellular ALP activity was measured to observe the cell functionality. ALP is recognized as a marker for osteogenic and osteoblastic cells, which undergo sequential process, eventually in bone formation. The cells were plated at a density of 2 × 10^3 cells/mL in six-well plates containing each material (~25 × 1 mm) and then cultured for up to 10 days. The cell lysates were prepared as follows. At each time period (5 and 10 days), the culture medium was removed and the cell layers were washed with PBS and then removed with a cell scraper (Nunclon; NUNC). After centrifugation, the cell pellets were washed with PBS and resuspended by vortexing them in 0.5 mL of deionized water containing 0.1% Triton X-100. The cell pellets were disrupted via a cyclic freezing/thawing process. A portion of the prepared cell lysates were used to determine the ALP activity, and the remainder was kept for a future osteocalcin (OC) production assay. The ALP activity of the cells was measured colorimetrically using p-nitrophenyl phosphate as a substrate (ALP kit 104-LL; Sigma, USA). The substrate is hydrolyzed to p-nitrophenol and inorganic phosphate by the enzyme ALP. Under alkaline conditions, the p-nitrophenol was converted to a yellow product and its absorbance was subsequently measured at 410 nm using a spectrophotometer (Titertek, Finland). The ALP activity was calculated from a standard curve, after normalizing to the total protein content, which was obtained using bovine serum albumin as a standard.

The OC produced by the cells was measured by enzyme immunoassay (Biomedical Technologies, Inc., MA). The cell layers removed from each sample were added to enzyme immunoassay sample buffer and homogenized by sonification. A 100-µL aliquot of cell lysate was put into a 96-well plate, and incubated at 4°C for 24 h. After washing with PBS twice, 100 µL of OC antiserum was added to each well, and then 100 µL of donkey anti-goat immunoglobulin G peroxidase was added and the cells were incubated for 2 h at room temperature. After washing with PBS twice, 100 µL of substrate solution was added to the wells and incubation continued for 30 min at room temperature, followed by the addition of 100 µL of stop solution (2M H₂SO₄). The absorbance was measured at 450 nm, and the OC expression of the cells was determined based on an OC standard prepared in the range of 0.1–2 ng/mL.

The collagen present in the cell extracts was quantified by staining the triple helical collagen fibrils with red dye (Sirius red F3BA; BDH). At each culture period (5 and 10 days), after detecting the cells, 50 µL of cell lysates from each well were transferred into a 96-well plate, and incubated at 37°C for 16 h in a humidified atmosphere, followed by 24 h in a dry atmosphere. One hundred microliters of 0.1% Sirius red F3BA in saturated aqueous picric acid was added to each well, and the cells were incubated for 1 h at room temperature. The plates were washed with 200 µL of 10 mM HCl three times, each time for 30 s, and eluted with 200 µL of 100 mM NaOH for 10 min. The absorbance was read at 540 nm using a microplate reader. The optical density was compared with a standard curve of collagen samples (Sigma).

Statistical analysis

Cellular tests were performed in three or six replicate samples: n = 3 for cell proliferation and DNA synthesis, and n = 6 for ALP, OC, and collagen production tests. The data are represented as means ± 1 standard deviation (SD). For the statistical analysis, the data were compared using the Student t test and one-way analysis of variance. Statistical significance was considered at p < 0.05.

RESULTS

Fabrication of FHA and FHA–ZrO₂ composites

The XRD patterns of the apatite powders (HA, FHA, and FA) are shown in Figure 1(A–C). All powders exhibited typical apatite peaks without much difference among them. However, a closer examination revealed peak shifts among the apatite phases (noted as dotted lines with respect to the HA peaks). The peaks shifted steadily with increasing fluorine concentration, and the shifts were more obvious at certain crystal planes. These peak shifts reflected the change in lattice parameters of the apatite structure resulting from the fluorine incorporation. The lattice parameters of the HA powder were 9.4170 (a axis) and 6.8830 (c axis), whereas those of FA were 9.3690 (a axis) and 6.8850 (c axis), which in both cases were quite similar to the theoretical values: 9.4176 (a axis) and 6.8814 (c axis) for HA, and 9.3671 (a axis) and 6.8840 (c axis) for FA.
Moreover, the lattice parameters of the FHA powder were 9.3920 (a axis) and 6.8820 (c axis), with the a axis value being about halfway between the HA and FA values. In the case of fluorine substitution for the hydroxyl ion, the apatite structure contracts along the a axis without much change in the c axis, and the a axis decreases almost linearly with increasing fluorine content.\textsuperscript{3,22,23} It was deduced from these results that the FHA powder that we obtained formed a nearly complete solid solution through the solid-state reaction of HA and FA powders at an equivalent ratio, as suggested in Eq. (2).

Using the FHA powder, an FHA–ZrO\textsubscript{2} composite was produced at a 50:50 volume ratio by pressureless sintering at 1400°C for 3 h in air, and its typical SEM morphology is shown in Figure 2(A). The composite had a highly dense structure (porosity <2%). The FHA (dark area) and ZrO\textsubscript{2} (bright area) phases, which were well contrasted when observed by a normal scanning mode, featured a homogeneous and highly intermixed composite. The ZrO\textsubscript{2} phase was interconnected without local isolation. Moreover, the FHA phase was also continuous, whereas some parts were relatively large (~3–5 \(\mu\)m in size), which was susceptible to act as a fracture origin. By contrast, when pure HA powder was mixed with ZrO\textsubscript{2} at the same ratio (50:50) and sintered under the same conditions as the FHA–ZrO\textsubscript{2}, the resultant structure was highly porous with a porosity of >10%, as shown in Figure 2(B). Hereafter, this HA–ZrO\textsubscript{2} composite was not considered for further mechanical tests or cellular assessments, because of its high porosity.

Figure 3 shows the XRD pattern of the FHA–ZrO\textsubscript{2} composite after pressureless sintering at 1400°C for 3 h [Fig. 3(A)], in comparison with that of the HA–ZrO\textsubscript{2} composite sintered under the same conditions [Fig. 3(B)]. In the FHA–ZrO\textsubscript{2} composite, only the FHA and ZrO\textsubscript{2} phases were observed. However, in the HA–ZrO\textsubscript{2} composite, considerable amounts of \(\beta\)-TCP and calcium zirconate (CaZrO\textsubscript{3}) phases appeared, whereas only small HA peaks remained. These TCP and CaZrO\textsubscript{3} phases were attributed to the thermal reaction between HA and ZrO\textsubscript{2} as follows:\textsuperscript{3}

\[
\text{Ca}_{10} (\text{PO}_4)_6 (\text{OH})_2 [\text{HA}] (s) + \text{tetra-ZrO}_2 (s) = 3\text{Ca}_3 (\text{PO}_4)_2 [\beta\text{-TCP}] (s) + \text{CaZrO}_3/\text{cubic-ZrO}_2 (s) + \text{H}_2\text{O} (g) \tag{3}
\]
In contrast to the HA–ZrO$_2$ composite, the FHA–ZrO$_2$ composite maintained its initial phases (FHA and tetra-ZrO$_2$) because of the high thermal stability of FHA with respect to ZrO$_2$. The prevention of the degradation reaction facilitated by fluorine substitution had a crucial role in producing an apatite–ZrO$_2$ dense composite (Fig. 2) by pressureless sintering alone.

Mechanical properties

Figure 4(A–D) shows the mechanical properties of the FHA–ZrO$_2$ composite in comparison with those of pure HA and FHA. The flexural strength of the FHA–ZrO$_2$ was much higher (310 MPa) than that of both pure HA and FHA (80–90 MPa), as shown in Figure 4(A). The fracture toughness of the FHA–ZrO$_2$ was as high as 3.4 MPa$m^{1/2}$, a noticeably higher value as compared with those of pure HA and FHA (0.8–0.9 MPa$m^{1/2}$), as represented in Figure 4(B). The strength and fracture toughness of the FHA–ZrO$_2$ composite were about 4 times higher than those of pure HA and FHA, illustrating clearly the strengthening and toughening effects of ZrO$_2$ on the pure apatite. The Vickers hardness of the FHA–ZrO$_2$ composite was also highly improved (10.2 GPa) as compared with that of pure HA and FHA (5–6 GPa) [Fig. 4(C)]. The elastic modulus of the composite also increased (160 GPa) [Fig. 4(D)].

Figure 3. XRD patterns of the (A) FHA–ZrO$_2$ and (B) HA–ZrO$_2$ 50:50 composites after pressureless sintering at 1400°C for 3 h in air. Legends are (○) FHA or HA, (□) ZrO$_2$, (❖) TCP, and (■) CaZrO$_3$. The HA–ZrO$_2$ composite showed considerable degradation to TCP and CaZrO$_3$ phases, in contrast to FHA–ZrO$_2$ composite, in which FHA and ZrO$_2$ phases were maintained.

Figure 4. Mechanical properties of the FHA–ZrO$_2$ 50:50 composite in comparison with those of pure FHA and HA: (A) flexural strength $S$, (B) fracture toughness $T$, (C) hardness $H$, and (D) elastic modulus, $E$. The flexural strength was measured using a biaxial testing configuration, the fracture toughness and hardness by the Vickers indentation method, and the elastic modulus by the ultrasonic pulse method. All specimens were densified nearly completely (residual pores <2%), and the mechanical tests were performed on the samples polished to 1 μm. Data represent mean ± 1 SD for $n = 5$ (strength and elastic modulus) and for $n = 10$ (fracture toughness and hardness).
but the increment was not as high as the improvements observed in the other properties. The hardness and elastic modulus of the composite were about 2 and 1.5 times, respectively, higher than those of pure HA and FHA. There was little difference between HA and FHA in terms of any of the mechanical properties.

Figure 5 shows the SEM morphology of the fractured surface (A) and indentation crack formation (C) on the FHA–ZrO$_2$ composite, in comparison with those (B and D, respectively) of pure HA. The fractured surface of the FHA–ZrO$_2$ composite was highly rough and complex, consisting of both intergranular and transgranular fracture modes [Fig. 5(A)]. In contrast, the pure HA was fractured in quite a flat manner, being completely transgranular [Fig. 5(B)]. The superior strength of the composite with respect to the pure apatites was well explained by these different fracture modes. When a Vickers indentation was brought to bear on the FHA–ZrO$_2$ composite at 9.8 N, only slight crack initiation was observed [Fig. 5(C)]; however, on the pure HA, the cracks propagated extensively under the same load [Fig. 5(D)], showing the superiority of the FHA–ZrO$_2$ composite in terms of its crack resistance.

Cell proliferation

The biological properties of the FHA–ZrO$_2$ composite were assessed by measuring the in vitro cellular responses, using osteoblast-like MG63 cells. Figure 6 shows the electron micrographs of MG63 cells grown on the FHA–ZrO$_2$ composite, as well as those on the HA and FHA pure phases, after culturing for 3 days.
The cells spread and grew favorably on the composite sample, in a number of cytoplasmic extensions [Fig. 6(A)]. The growth morphology on the composite was quite similar to that on pure FHA and HA [Fig. 6(B) and (C), respectively].

The cell proliferation was quantified by directly measuring the cell numbers using a hemocytometer, as well as by monitoring the [3H]-thymidine incorporation into the DNA of the replicating cells. Figure 7(A) shows the cell numbers counted after culturing for up to 7 days. On all of the samples, the MG63 cells proliferated actively throughout the culture period. No statistically significant (considered at \( p < 0.05 \)) difference was observed between the samples.

Figure 7(B) shows the incorporation of [3H]-thymidine into the DNA of the replicating cells for the last 4 h of each culture period. The levels were the highest at days 3–5, and decreased slightly or changed little thereafter. These results mirrored the total cell population data shown in Figure 7(A), that is, rapid initial growth followed by a further slow increase in cell number. A significant difference (considered at \( p < 0.05 \)) was only observed on the FHA–ZrO2 sample at day 3 with respect to the FHA.

**Figure 6.** SEM morphologies of the MG63 cells grown on (A) FHA–ZrO2 50:50 composite, (B) FHA, and (C) HA after culturing for 3 days.

**Figure 7.** MG63 cell proliferation levels on each sample after culturing for up to 7 days. (A) Cell number counted using a hemocytometer, and (B) [3H]-thymidine incorporation into the DNA of replicating cells (mean ± 1 SD, \( n = 3 \)). Statistically significant difference (\( p < 0.05 \)) was observed, in (B): on FHA–ZrO2 compared with FHA (\( F \)) at day 3.
Cell differentiation

The MG63 osteoblastic functionality on the FHA–ZrO₂ composite was assessed in terms of ALP, OC, and collagen production by the cells, as shown in Figure 8(A–C). In all of the samples, the ALP expression levels increased at day 10 compared with those at day 5 [Fig. 8(A)]. Among the samples, the FHA–ZrO₂ composite exhibited a significantly lower (considered at \( p < 0.05 \)) ALP level at day 10 compared with both the HA and FHA. In comparing FHA with HA, the ALP level on FHA was significantly lower (considered at \( p < 0.05 \)) than that on HA at day 10, although there was no significant difference at day 5.

Figure 8(B) shows the OC production by the cells after culturing for up to 10 days. The cells on all of the samples produced significantly higher (considered at \( p < 0.05 \)) OC levels at day 10 compared with day 5. The OC level on the FHA–ZrO₂ composite was quite similar to that on the pure HA for all periods; however, in comparison to the pure FHA, the level on the FHA–ZrO₂ composite was significantly lower (considered at \( p < 0.05 \)) only at day 5. The OC production on FHA appeared to be higher than that on HA, but the difference was not significant.

Figure 8(C) shows the collagen synthesis by the cells after culturing for up to 10 days. Unlike in the case of the ALP and OC production, the collagen synthesis did not vary much between the different culture periods for any of the samples. The collagen synthesis on the FHA–ZrO₂ composite at day 10 was significantly lower (considered at \( p < 0.05 \)) than that on FHA, but little difference was observed when compared with that on HA for both periods. The collagen synthesized by the cells was higher on FHA than on HA at day 10, but the difference was not significant.

DISCUSSION AND CONCLUSION

This study was undertaken to investigate the feasibility of the FHA and ZrO₂ dense composite for use in hard tissue applications, in terms of its mechanical and biological properties. The apatite composites with ceramic oxides (zirconia and alumina), intended to utilize in load-bearing compartments, have had a crucial drawback in their fabrication, because of the severe thermal reaction between the apatite and oxides at increased temperatures resulting in phase degradation and poor densification.³−⁷ This problem was more substantial under normal sintering conditions in the absence of external pressure or water vapor, under which conditions the product came to possess high porosity and poor mechanical properties, negating the role of the oxides as reinforcing materials. These series of degradation phenomena mainly originate from the intrinsic thermal instability of the HA structure at increased temperatures, that is, the dehydration of the hydroxyl groups and subsequent phase decomposi-
tion. In the presence of ceramic oxides or metals, the decomposition of HA is accelerated, with Ca$^{2+}$ ions being diffused out of the HA structure and forming other Ca byproducts. The TCP and CaZrO$_3$ phases produced in this HA–ZrO$_2$ composite resulted from such a series of thermal reactions. The decomposition process itself is highly deleterious to the production of dense ceramic bodies. Furthermore, the production of CaZrO$_3$/cubic-ZrO$_2$ poses a more serious problem in stabilized-ZrO$_2$ ceramics, in the sense that it results in the attenuation of the typical strengthening and toughening mechanism, that is, the phase transformation of tetragonal ZrO$_2$ (stabilized with yttria in this study) to the monoclinic phase. As such, until now, these factors have limited research into the apatite–ZrO$_2$ dense composites, casting doubt on the feasibility of using them in load-bearing applications.

In this respect, the introduction of fluorine within the HA lattice and the formation of FHA solid solutions were highly effective in suppressing the thermal degradation reaction and maintaining the initial tetragonal ZrO$_2$ and apatite phases. This was attributable to the high thermal stability of the FHA structure, because the F substitution for OH enhances the crystal symmetry and, thus, the structural stability. The F ion is just the right size to fit between three Ca atoms, and it lies on the (001) mirror planes so as to increase the crystal symmetry, whereas the OH ion in the apatite lattice is slightly displaced from the mirror plane. The high structural and thermal stability of FHA with respect to ZrO$_2$ facilitated the production of an almost completely dense composite (>98% in theoretical density) under normal sintering conditions. The efficacy of FHA can be extended through the fabrication of dense apatite composites containing other reinforcements, such as alumina and metals. In the same manner, the fluorine-substituted apatite had an effective role as a buffer coating layer in preventing the reaction and maintaining the phase stability of calcium phosphate coatings on zirconia substrate systems. Along with the processing aspects, the fluoride ion has specific biological functions, in the prevention of dental caries and the enhancement of crystallization and mineralization of apatite crystals in bone formation and, thus, the treatment of osteoporosis.

The FHA–ZrO$_2$ 50:50 composite produced in this study had considerably higher mechanical properties than the pure apatites (HA and FHA). This improvement was more noticeable in terms of both the strength and fracture toughness (4 times higher), whereas the elastic modulus did not increase much (1.4 times higher), reflecting the effectiveness of ZrO$_2$ as a reinforcing material, because of its high strength (>1000 MPa) and toughness (~7–10 MPam$^{1/2}$) whereas possessing a relatively low elastic modulus (~210 GPa: much lower than that of Al$_2$O$_3$ and similar to that of metal alloys). Practically, considering the mechanical properties of cortical bone (S = 50–150 MPa, T = 2–12 MPam$^{1/2}$, and E = 7–30 GPa), the values obtained on FHA–ZrO$_2$ 50:50 (S = 310 MPa, T = 3.4 MPam$^{1/2}$, and E = 160 GPa) make it a promising material for use as a hard tissue implant. However, because these values represent the indices in static conditions, the performance of the composite under dynamic load in the presence of body fluid needs to be clarified and remains an area for further study.

Along with the mechanical integrity, the biological properties of the composite should be addressed. Practically, there has been no in-depth study on the biological properties, either in vitro or in vivo, of the apatite–ceramic oxide dense composites, mainly because of the difficulty of producing dense composites. Herein, because the FHA–ZrO$_2$ composite was completely dense and its surface was polished to 1 μm, the physical variations (porosity and roughness) could be minimized for the cellular assessments. At the first estimation, the bioactivity of apatite is expected to decrease to a certain degree with the addition of ZrO$_2$, because the ZrO$_2$ ceramic is nearly bioinert, whereas the apatite is well known for its bioactivity. In other words, the biocompatibility of the FHA–ZrO$_2$ composite might be the result of a trade-off between the mechanical properties of ZrO$_2$ and the bioactivity of apatite. In addition, the effect of fluoride on the biological properties needs to be considered, even though little difference was observed in the mechanical properties between HA and FHA. With this in mind, this study was conducted as a first step toward the biological evaluation of the FHA–ZrO$_2$ 50:50 composite using osteoblast-like MG63 cells in vitro. Despite being a cell line, MG63 is derived from human osteoblasts, and has been well characterized, providing a useful and reproducible tool to investigate the effects of biomaterials and to understand the mechanisms of cellular response to materials. This has certain advantages over primary cells with regard to experimental reproducibility and repeatability.

The cell proliferation rate and viability were observed by counting live cells directly and assessing the incorporation of $^3$H-thymidine into the DNA of replicating cells. Whereas counting the cells provided information on the total cell population, measuring the incorporation of $^3$H-thymidine provided a parameter that reflected the ability of the cells to synthesize DNA at a specific culture period, that is, the metabolic activity and cell viability. Despite the slight difference between these two concepts, the trends were quite similar with regard to the test samples and culture periods. For all of the samples, the proliferation rate was highest at days 3–5 and decreased slightly thereafter. The decrease in proliferation rate was related to the cell confluence on the substrate. Although there was a slight variation among the sam-
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The FHA composite with 50% ZrO₂ had lower osteoblastic phenotype expressions than the pure FHA and HA, although the cell viability on the composite was comparable to that on the pure ceramics. A significant difference (considered at $p < 0.05$) was observed in ALP at day 10 (compared with both HA and FHA), OC at day 5 (compared with FHA), and collagen production at day 10 (compared with FHA). However, it is notable that the FHA–ZrO₂ composite exhibited all the phenotypes expressed by the MG63 cells, and moreover, the expression levels showed quite similar trends to those on the HA and FHA pure ceramics with respect to the culture period. Regarding the in vitro cellular responses to the pure ZrO₂ ceramic, most authors reported that it is favorable to cell growth and viability, without having any harmful effect on the cell responses, whereas others reported up-regulations in the differentiation of osteoblastic and osteogenic cells compared with that on a control polymer or titanium metal. Moreover, its in vivo phenomena never showed any adverse reactions, and sometimes good bone-bonding ability to host tissues was reported. Based on these reports, it can at least be concluded that the ZrO₂ ceramic should not adversely affect the cellular responses by itself. However, with respect to bioactive HA, ZrO₂ would be expected to have a somewhat negative effect, because HA ceramics are known to enhance all aspects of the bone cell responses, from the proliferation to the expression of many phenotypes and mineralization, that is, the whole bone-forming process.

The biologically inert ZrO₂ phase, added at a ratio of 50 vol % in relation to the FHA, did not up-regulate the osteoblastic cellular functionality as compared with that on the pure apatites, but the composite exhibited the traits of osteoblastic responses in quite a similar manner as the pure phases did. In the case of the fluorine-substituted HA and ZrO₂ composite, which retained a dense body by a simple pressureless sintering, an appropriate compromise needs to be reached between the bioactive apatite and the inert ZrO₂ in order to obtain a composition that does not reduce the bioactivity significantly, but retains sufficient mechanical integrity. Although further tests are needed to ascertain whether this material can perform similarly with regard to osteogenic cells in vitro and host tissues in vivo, at least from these findings, it can be concluded that the osteoblast-like MG63 cells on the FHA–ZrO₂ composite had good viability and maintained their cellular functional activity. Therefore, in terms of their biocompatibility, the mechanical performance, and biological properties, the FHA–ZrO₂ composites should find widespread use as load-bearing hard tissue implants.

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References
