
Assessment of the osteoblastic cell response to a zinc glass reinforced hydroxyapatite composite (Zn-GRHA)

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Abstract: Hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ and tricalcium phosphate (TCP) bioceramics have been used as graft materials. However, optimal biological performance has not been established yet and zinc, being a biosafe, biocompatible element, could favour for specific osteoblastic cell response. Therefore, this paper investigates the preliminary results and potential impact of zinc glass reinforced hydroxyapatite (Zn-GRHA) on a human osteoblastic cell system. The biological behaviour of Zn-GRHA samples was assessed by confocal laser scanning microscopy, while material characterisation was performed by SEM-EDX and XRD analysis. Established cultures reported an increased proliferation and a confluent cell layer in some areas of the material surface at day two. Cells were spread all over the material surface and established multiple cell-to-cell interactions relying on prominent cytoplasmic processes. At day six, confluent cell layers were verified on the Zn-GRHA material's surface, reporting an improved biological response, compared to control (hydroxyapatite).

Keywords: zinc glass-reinforced hydroxyapatite; Zn-GRHA; cell proliferation.

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1 Introduction

Zinc oxide (ZnO) is a bio-safe, biocompatible material for biomedical applications (Wang, 2004). Zinc has been considered to be an essential mineral for animals and humans since mid-20th century. It is known to be an essential trace element for ossification, fetal growth and development (Spadaro et al., 1970; Prasad, 1998; Salih et al., 2007; Uckan et al., 2001). Zinc deficiency affects cell-mediated immunity and leads to activation of monocytes-macrophages; in addition, this element may play an important role as an antioxidant (Prasad, 1998, 2000; Prasad et al., 2004). Hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ and tricalcium phosphate (TCP) bioceramics have been used as graft materials *in vivo* and they interact directly with bone (Walsh et al., 2008; Hing et al., 2007; Okuda et al., 2007; Weinand et al., 2006; Caria et al., 2007; Sopyan et al., 2007; Rush, 2005; Silva et al., 2005; Fujita et al., 2003). However, synthetic HA does not contain all the ions found in natural bone nor does it exhibits all the characteristics of living bone. In order to modify the HA, incorporation of bivalent cation Zn^{2+} could be favourable for bone repair. Zinc containing HA enhanced the osteogenesis and osteoconduction on a critical size defect in the rat calvaria (Clasans-Maia et al., 2008) and stimulatory effect of zinc-releasing calcium phosphate implant on bone formation in rabbit femora has been reported (Kawamura et al., 2002). It has also been shown that zinc doped HA improves osteoblast cell adhesion compared to undoped HA (Webster et al., 2002). Finally, zinc can also have useful antibacterial effects and it follows that zinc can be used to boost the integration of bone grafts in injury sites as well as prevent the infection risks that can arise from these procedures (Osinaga et al., 2003; Bright et al., 2002).

Literature reports on different types of synthesis methods for the production of Zn-HA materials (Jaroch and Clupper, 2007; Fujii et al., 2006; Otsuka et al., 2000). The authors previously reported a liquid phase sintering route using $\text{ZnO-P}_2\text{O}_5\text{-CaO-Na}_2\text{O-CaF}_2$ glass in an HA matrix. Earlier, the authors have studied $\text{CaO-P}_2\text{O}_5\text{-Na}_2\text{O-CaO-CaF}_2$ host glass reinforced HA without ZnO and registered/patented it as Bonelike[®]. Bonelike[®] is an osteoconductive synthetic graft material for the enhancement of ossification in biomedical applications and has been extensively reported (Santos et al., 1995, 1999; Knowles et al., 1996; Hussain et al., 2007; Gutierrez et al., 2007, 2008).

Therefore, this paper investigates the preliminary results and potential impact of zinc doped glass reinforced hydroxyapatite (Zn-GRHA) in osteoblastic function.

2 Materials and methods

2.1 Preparation of ZnO glass

A ZnO based glass with a chemical composition of 10ZnO-60P₂O₅-15CaO-5Na₂O-10CaF₂ (%mol) has been prepared by melting the following mixtures of analytical grade ZnO, P₂O₅, CaHPO₄, Na₂CO₃ and CaF₂ raw materials (Sigma Aldrich 99.99%) in a Pt crucible for about an hour in an electrical furnace at 1450°C. By employing the conventional method and then finally using standard crushing and sieving techniques, a powder with less than 75 micron size was obtained.

2.2 Preparation of HA

HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ has been prepared with the following chemical reaction: $10\text{Ca}(\text{OH})_2 + 6\text{H}_3\text{PO}_4 \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 18\text{H}_2\text{O}$.

Briefly, appropriate quantities of analytical grade calcium hydroxide, $\text{Ca}(\text{OH})_2$ and ortho phosphoric acid, H_3PO_4 have been mixed independently with deionised water. Then $\text{Ca}(\text{OH})_2$ solution was transferred into H_3PO_4 solution for about four hours with constant mixing (100 rpm) maintaining the pH 10.50, at the end. After 24 hours, the material was filtered to remove the excess water and then dried at 60°C for approximately 48 hours. Finally, the dried product was crushed and sieved for less than 75 micron size particles. HA disks were prepared by uni-axial pressing technique.

2.3 Preparation of Zn-GRHA composite

The Zn-GRHA composite was obtained by mixing 2.5% of glass with HA in isopropanol. The obtained composite was dried at 60°C and then sintered at 1300°C for one hour and finally using standard crushing and sieving techniques, the authors prepared few discs for cell culture studies by uni-axial pressing technique. Prior to cell seeding, HA and Zn-GRHA discs were sterilised in a steam autoclave at 120°C for 30 minutes.

2.4 Materials characterisation

X-ray diffraction (XRD) was performed on powder samples of glass, Zn-GRHA and HA by using Siemens D 5000 diffractometer with Cu-K_α radiation ($\lambda = 1.5418\text{\AA}$). The scans were made in the range of $25\text{--}40^\circ$ (2θ) with a step size of 0.02° and a count time of 2 sec/step. A scanning electron microscopy (JEOL JSM 630IF) equipped with an energy dispersive analyzer (SEM/EDS technique) was used to evaluate the microstructure of these samples.

2.5 Cell culture studies

MG63 cells, human osteosarcoma-derived osteoblastic cells, were cultured in α -minimal essential medium supplemented with 10% foetal bovine serum, ascorbic acid ($50\text{ }\mu\text{g.ml}^{-1}$), penicillin-streptomycin (100 IU.ml^{-1} and 10 mg.ml^{-1} , respectively) and fungisone ($2.5\text{ }\mu\text{g.ml}^{-1}$), at 37°C , in a humidified atmosphere of 5% CO_2 in air, in 50 cm^2 flasks. The medium was changed twice a week until sub-confluence. For materials' seeding, adherent cells were enzymatically released (0.05% trypsin–0.25% EDTA) and resuspended in complete medium. Cells were cultured at a density of $10^4\text{ cells.cm}^{-2}$, for six days on the surface of HA and Zn-GRHA discs, that had been previously immersed in complete culture medium for one hour. Seeded material samples were evaluated throughout the incubation time, at days two and six, for cell morphology and cell growth, by confocal laser scanning microscopy (CLSM).

At days two and six, the colonised HA and Zn-GRHA discs were fixed in 3.7% methanol-free formaldehyde and permeabilised with 0.1% Triton[®]. Cell cytoskeleton filamentous actin (F-actin) was visualised by treating the fixed cells with Alexa Fluor[®] 480-conjugated phalloidin dye (1:100 in PBS, 20 minutes), after initial incubation with bovine serum albumin (10mg.ml^{-1} in PBS one hour) – in order to block all non-specific

sites. Cultures were counterstained with propidium iodide (10 mg.ml^{-1}) for cell nuclei labelling, for ten minutes. Labelled cultures were mounted in Vectashield[®] and examined with a Leica SP2 AOBS (Leica Microsystems) microscopy.

3 Results and discussions

3.1 Physical and morphological analysis of ZnO glass, Zn-GRHA and HA materials

Based on the glass composition and glass amount added to HA, the final concentration of zinc in the Zn-GRHA material prepared was 0.4%. The range of zinc concentration for an optimal biological response is supposed to be very narrow as previously reported [24]. Figure 1 shows the EDX analysis of zinc glass material. Despite the EDX detection of zinc in the prepared glass, when observing the microstructure of the Zn-GRHA material, the zinc element was not detected. This was due to the fact that the Zn-GRHA material contains only 2.5% (m/m) of ZnO glass.

Figure 1 EDX analysis of $10\text{ZnO}-60\text{P}_2\text{O}_5-15\text{CaO}-5\text{Na}_2\text{O}-10\text{CaF}_2$ glass (see online version for colours)

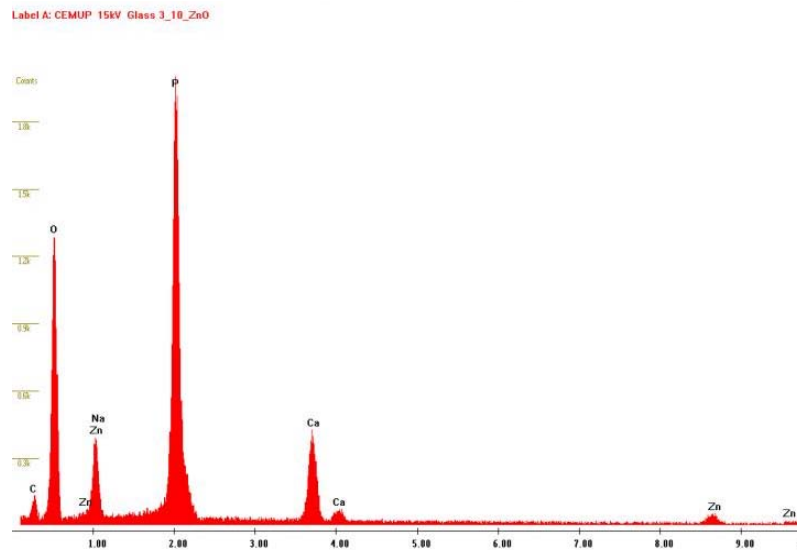
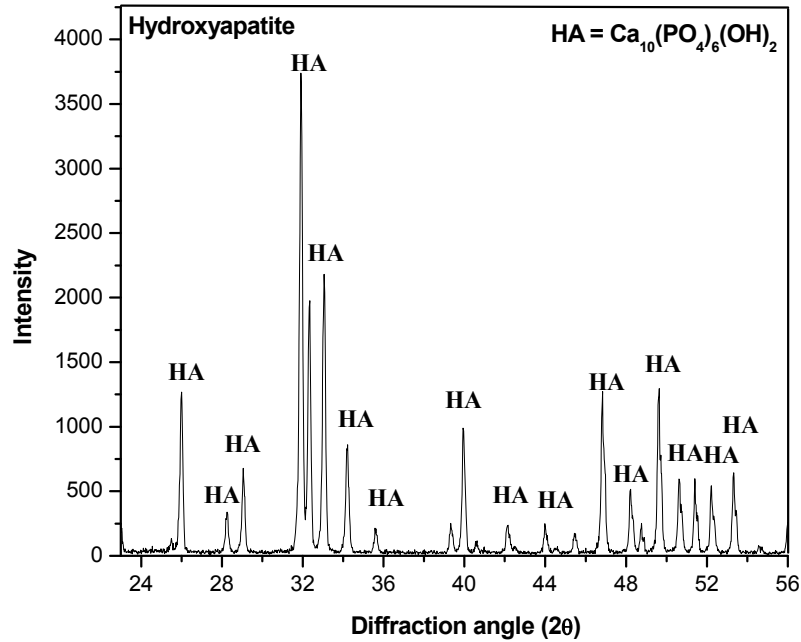
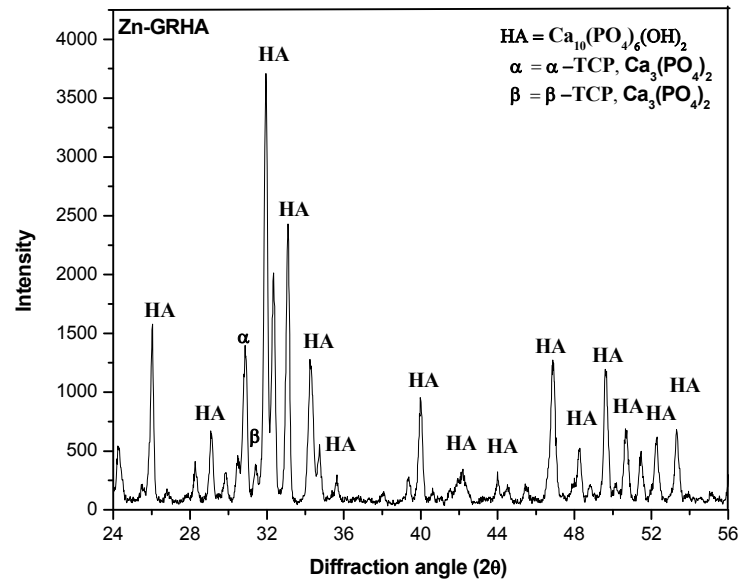


Figure 2 shows the XRD of hydroxyapatite powder produced, which shows that the material is pure phase HA. The XRD of Zn-GRHA composite is shown in Figure 3. From this diffractogram, the following three phases can be identified: HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, β - and α -TCP, $\text{Ca}_3(\text{PO}_4)_2$. No zinc containing phases were detected. It means that the zinc ions should be incorporated in the lattice structure of HA or TCP phases. Further, Rietveld analysis of the XRD spectrum obtained should be done to confirm this incorporation by quantifying the lattice parameters and the distortion index.

Figure 2 XRD of HA material**Figure 3** XRD of zinc glass reinforced hydroxyapatite (Zn-GRHA) material

The presence of TCP phases in the composition of the Zn-GRHA were expected, since previous studies of these authors have already demonstrated that the liquid sintering process that occurs due to the presence of a glass in the sintering process of HA leads to the reaction of HA with a glass and therefore part of it is converted into TCP phases.

SEM observation of the surface of Zn-GRHA and HA materials have shown that both materials are almost fully dense materials.

3.2 Colonisation of Zn-GRHA and HA with MG63 cells

Biological behaviour of Zn-GRHA and HA samples was assessed by CLSM, as shown in Figure 4. CLSM images of seeded Zn-GRHA samples at day two [Figure 4(a)] showed that the material surface was colonised with MG63 cells, reporting already areas of high cell density. Cells proliferated actively with culture time and at day six the material surface was completely covered by dense multiple cell layers [Figure 4(c)]. Comparatively, the number of cells that attached to HA samples was significantly lower, both at days two and six [Figure 4(b) and (d)]. On both materials, cells displayed a normal morphology, with cytoplasmic flattening and spreading, prominent nucleus, numerous filipodia and extensive cell-to-cell contact, as evident at a higher magnification, as shown in Figure 5. These observations regarding the enhanced osteoblast adhesion and proliferation on Zn-GRHA samples are in line with previous *in vitro* studies performed on various Zn-containing substrates, namely Zn(2%)-doped HA (Fujita et al., 2003), Zn-organoapatite coating (Storrie and Stupp, 2005), Zn-releasing calcium phosphate ceramics (Ikeuchi et al., 2003) and Zn-phosphate-based glasses (Salih et al., 2007).

Figure 4 CLSM images of Zn-GRHA and HA samples cultured with MG63 cells for two and six days (see online version for colours)

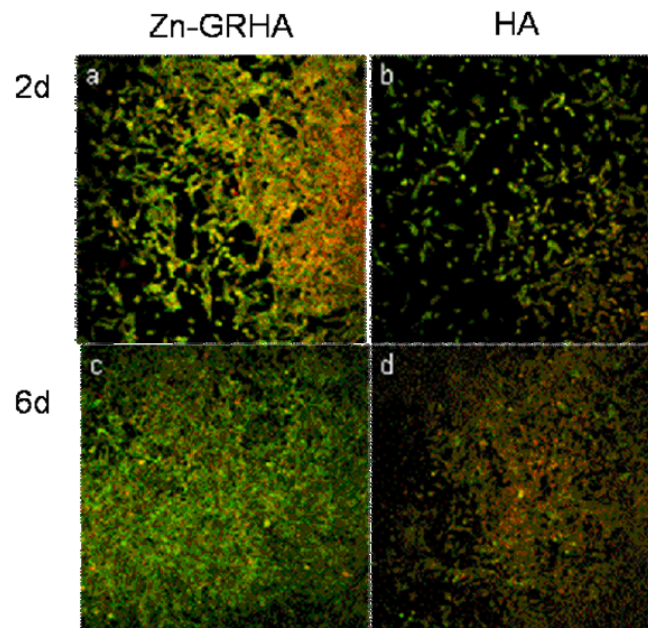
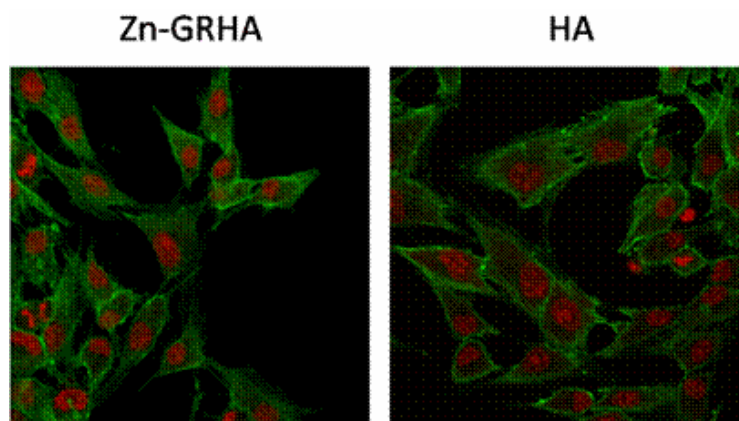


Figure 5 High magnification CLSM images of Zn-GRHA and HA samples cultured with MG63 cells for two days (see online version for colours)



Zinc is an essential trace element, usually a cofactor in metalloenzymes and proteins. Alkaline phosphatase, which is involved in the mineralisation of the collagenous bone matrix, is a Zn-metalloenzyme (Yoon et al., 1989). Zinc appears to play a significant role in bone metabolism having stimulatory effects on bone formation *in vitro* and *in vivo* (Hall et al., 1999; Kawamura et al., 2003), as well as an inhibitory effect on osteoclastic bone resorption *in vitro* (Moonga and Dempster, 1995). Also zinc has been reported to have an antibacterial effect when added to glass-ionomer-based cements (Osinaga et al., 2003) and ceramic coatings (Bright et al., 2002) – a useful property in decreasing bone infection risk associated with the repair of skeletal defects.

4 Conclusions

This paper reports preliminary results of the potential impact of zinc-glass reinforced hydroxyapatite (Zn-GRHA) on osteoblastic cell behaviour. Regarding biological evaluation by CLSM, an increased cell number was verified in the Zn-GRHA materials, compared to pure phase HA. Accordingly, at six days of culture, multiple cell layers were visualised on the surface of this material. Hence, the partial addition of zinc may provide the Zn-GRHA system with appropriate biodegradation as well as the enhancement of bone formation. Therefore, further *in vitro* and *in vivo* studies are underway to assess the osteoblastic response by using Zn-GRHA material.

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