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In vitro osteogenesis on fluorcanasite glass-ceramic with three different chemical compositions

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Abstract This study aimed at investigating in vitro osteogenesis on three fluorcanasite glass-ceramic compositions with different solubilities (K3, K5, and K8). Osteoblastic cells were obtained from human alveolar bone fragments and cultured under standard osteogenic condition until subconfluence. First passage cells were cultured on K3, K5, and K8 and on Bioglass® 45S5 (45S5—control). Cell adhesion was evaluated at 24 h. For proliferation and viability, cells were cultured for 1, 4, and 10 days. Total protein content and alkaline phosphatase (ALP) activity were measured at 7, 14, and 21 days. Cultures were stained with Alizarin red at 21 days, for detection of mineralized matrix. Data were compared by ANOVA followed by Duncan's test. Cell adhesion, cell proliferation, viability, total protein content, and ALP activity were not affected by fluorcanasite glass-ceramic composition and solubility. Bone-like formation was similar on all fluorcanasite-glass ceramics and was reduced compared to 45S5. The changes in the chemical composition and consequently solubility of the fluorcanasite glass-ceramics tested here did not significantly alter the in vitro osteogenesis. Further modifications of the chemical composition of the fluorcanasite glass-ceramic would be required to improve bone response, making this biomaterial a good candidate to be employed as a bone substitute.

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Introduction

To address the problem of bone loss, caused by bone diseases, traumas and ageing, grafting techniques are widely employed. Although fresh autogenous bone remains the optimal material to restore structural and functional integrity to the bone tissue, donor site morbidity and limited sites for supplying the tissue must be taken into consideration [1–4]. When allografts are made available, the risk of disease transmission, immunogenicity, and loss of biological and mechanical properties secondary to its processing raise doubts about the success of transplantation [5]. In this context, the development of synthetic bone substitutes is much needed.

Advances in biomaterials research over the last 30 years have given new and innovative materials for the rehabilitation and reconstruction of the skeletal defects [6]. There is now a range of bone graft substitutes and ceramics and glasses have been widely used in many clinical conditions as a bone substitute and are now extensively used in dentistry. The bioactive glass Bioglass® 45S5 (45S5) has been known for many years as the bioactive material with the highest bioactivity index [7]. It has been demonstrated that 45S5 affects osteoblast activities that ultimately result in enhanced bone formation both in vitro and in vivo [8]. Despite its beneficial effects on bone healing, the use of 45S5 and other bioactive glasses for bone tissue

engineering applications has been limited due to their relatively poor mechanical properties [9]. Ideally, a glass-ceramic for bone substitute and augmentation should be osteoconductive and have good mechanical properties [10].

Fluorcanasite glass-ceramics have been shown to present a high strength and fracture toughness due to a crystalline microstructure of interpenetrating blades and may be designed to degrade at a controlled rate to provide space for new bone formation [11]. In a previous study of the in vivo bone response, the fluorcanasite glass-ceramic was unstable in the biological environment because the excessive dissolution of the glassy matrix and release of particulate debris [12]. As a result, the fluorcanasite glass-ceramic tested should not be considered as a good candidate for implant applications. Solubility of the glass-ceramics may be tailored by modifying its chemical composition [13]. Indeed, the solubility of the fluorcanasite glass-ceramics varied with alkali content being low for the low potash content glasses [14]. It has been suggested that the bone response may be improved by reducing the solubility of the fluorcanasite glass-ceramics [12]. Therefore, the present study aimed at evaluating the in vitro osteogenesis on three fluorcanasite glass-ceramic compositions with different solubilities, using osteogenic cells from human alveolar bone.

Material and methods

Glasses

Materials were prepared as described in previous studies [15, 16]. Briefly, fluorcanasite glass-ceramic materials based on the general formula $SiO_2.K_2O.CaO.CaF_2.Na_2O$ (Table 1) were produced using a melt route. Discs 12 mm in diameter and 2 mm high were produced using the lost-wax casting technique, plastic profiles were invested in a refractory material (Whip-mix gypsum bonded investment, Whip-mix Corp., Louisville, NY), which once set was heated to 700 °C to burn out the plastic profile; the investment was then cooled to 590 °C prior to casting. The glass was melted at 1,200 °C using electrical resistance melting and then centrifugally cast into the investment mould (Degussa TS3 casting machine, Hanau, Germany). After devesting and grit blasting the glass surface with 50-μm alumina the rods were heat treated for 1 h at 520 °C and 1 h at 860 °C. The fluorcanasite glass-ceramics were manufactured with three different chemical solubilities by varying the soda/potash ratio (Table 2). The samples were examined at the time of processing for any defects such as porosity. Any specimen showing signs of porosity was discarded at the time of manufacture. Discs of 45S5, based on the $SiO_2.CaO.Na_2O.P_2O_5$ composition, of the same dimensions to the fluorcanasite glass-ceramic samples were similarly produced and used as a control material. All discs were polished with silicon carbide paper (1200), cleaned in an ultrasonic bath and autoclaved before using in the cell culture experiments.

Culture of osteoblastic cells

Human alveolar bone fragments (explants) were obtained from healthy donors, using the research protocols approved by the Committee of Ethics in Research. Osteoblastic cells were obtained from these explants by enzymatic digestion using collagenase type II (Gibco – Life Technologies, Grand Island, NY, USA) as described by Mailhot and Borke [17]. These cells were cultured in α-minimum essential medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 50 μg/mL gentamicin (Gibco), 0.3 μg/mL fungizone (Gibco), 10^{-7} M dexamethasone (Sigma, St. Louis, MO, USA), 5 μg/mL ascorbic acid (Gibco), and 7 mM β-glycerophosphate (Sigma). Such culture conditions favored the development of the osteoblast phenotype [18, 19]. Subconfluent cells in primary culture were harvested after treatment with 1 mM ethylenediamine tetraacetic acid (EDTA) (Gibco) and 0.25% trypsin (Gibco); cells from the first passage were cultured in 24-well culture plates (Falcon, Franklin Lakes, NJ, USA) on glass discs at a cell density of 2×10^4 cells/disc.

Table 2 Chemical solubility of the fluorcanasite glass-ceramics K3, K5, and K8, according to Stokes et al. [14]

Composition	[K]/[K + Na]	Chemical solubility (μg/cm^2)
K3	0.2	544 ± 296
K5	0.3	2488 ± 48
K8	0.5	389 ± 128

Table 1 Composition of the fluorcanasite glass-ceramics (K3, K5, and K8) and the control Bioglass 45S5 in molar percent

Material	SiO_2	CaF_2	Na_2O	K_2O	CaO	P_2O_5
45S5	45	0	24.5	0	24.5	6
K3	60	10	12	3	15	0
K5	60	10	10	5	15	0
K8	60	10	7	8	15	0

During the culture period, cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air; the medium was changed every 3 or 4 days.

Cell adhesion

For evaluation of cell adhesion, cells were cultured for 24 h, enzymatically (1 mM EDTA, 1.3 mg/mL collagenase type II, and 0.25% trypsin—Gibco) detached and counted using a haemocytometer (Housser Scientific Company, Horsham, PA, USA). Cell adhesion was expressed as a percentage of initial number of cells.

Cell proliferation and viability

For proliferation, cells were cultured for 1, 4, and 10 days, and counted as described above. Viable and non-viable cells were detected by trypan blue and counted using a haemocytometer (Housser Scientific Company). Cell proliferation was expressed as number of cells $\times 10^4$/disc and cell viability was expressed as a percentage of the viable cells.

Total protein content

Total protein content was calculated at 7, 14, and 21 days in culture, according to the Lowry method [20]. The wells were filled with 2 mL of 0.1% sodium lauryl sulfate (Sigma). After 30 min, 1 mL of this solution from each well was mixed with 1 mL of Lowry solution (Sigma) and left for 20 min at room temperature. After this period, it was added to 0.5 mL of the solution of phenol reagent of Folin and Ciocalteau (Sigma). This stood for 30 min at room temperature and the absorbance was then measured (CE3021 – Cecil, Cambridge, UK) at 680 nm and the total protein content was calculated from a standard curve and expressed as μg/mL.

ALP activity

ALP activity was assayed as the release of thymolphthalein from thymolphthalein monophosphate using a commercial kit (Labtest Diagnostica SA, MG, Brazil). Samples of the same solutions used for calculating total protein content were assayed for measuring ALP activity according to the kit instructions. Briefly, 50 μL of thymolphthalein monophosphate was mixed with 0.5 mL of diethanolamine buffer, 0.3 mmol/mL, pH 10.1, and left for 2 min at 37 °C. After this period, it was added to 50 μL of the lysates from each well and these stood for 10 min at 37 °C and then 2 mL of a solution of $Na_2.CO_3$ 0.09 mmol/mL and NaOH 0.25 mmol/mL was added to allow color development. After 30 min, absorbance was measured at 590 nm and ALP activity was calculated from a standard curve using thymolphthalein to give a range from 0.012 to 0.40 μmol thymolphthalein/h/mL. Results were calculated and data were expressed as ALP activity normalized by the total protein content measured at 7, 14, and 21 days.

Bone-like formation

After culturing for 21 days, the cells were washed three times with PBS at 37 °C. The attached cells were fixed in 10% formalin for 2 h at room temperature and rinsed once in the same buffer. After fixation, the specimens were dehydrated through a graded series of alcohol and stained with Alizarin red S (Sigma), which stains areas rich in calcium. The samples were then examined by using a Leica fluorescence microscope (Leica) outfitted with a Leica DC 300F digital camera under epifluorescence. The acquired digital images were processed with Adobe Photoshop software, version 7.0.1. Ten ×10 microscopic fields in each sample were randomly selected and the mineralized area was measured using an image analyzer (Image Tool – University of Texas Health Science Center, San Antonio, TX, USA). Bone-like formation was expressed as a percentage area of the discs.

Statistical analysis

All experiments were done in quintuplicate. Data were compared by two-way analysis of variance (ANOVA) using Duncan's multiple range comparison test with the exception of cell attachment and bone-like nodule formation that were compared using one-way ANOVA. All results are presented as mean ± standard deviation and differences at $p \leq 0.05$ were considered statistically significant.

Results

Cell adhesion was not affected ($p = 0.49$) by the chemical composition (Fig. 1). Cell proliferation was not affected ($p = 0.75$) by the chemical composition, and was affected by the period of culture ($p = 0.0001$), in the following order: 1 day < 4 days < 10 days (Fig. 2). Cell viability was not affected ($p = 0.15$) by chemical composition, and was affected ($p = 0.005$) by period of culture in the following order 1 day = 4 days < 10 days (Fig. 3). Total protein content was not affected ($p = 0.55$) by the chemical composition, and was affected by period of culture ($p = 0.0001$), in the following order: 4 days < 7 days < 14 days < 21 days (Fig. 4). ALP activity was not affected ($p = 0.60$) by the chemical composition, and was affected by period of culture ($p = 0.0001$), in the

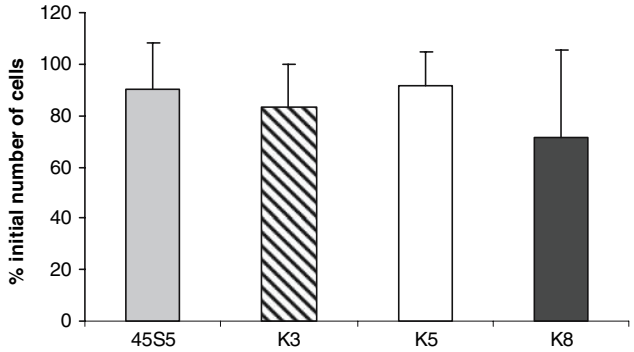

Fig. 1 Cell adhesion expressed as a percentage of initial number of cells at 24 h. Data are reported as mean ± standard deviation (n = 5)

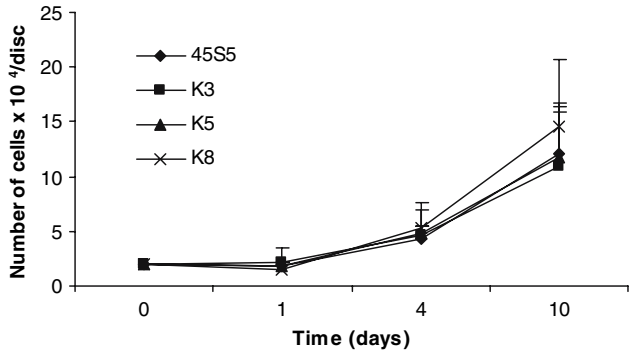

Fig. 2 Growth curve of cells at 1, 4, and 10 days. Data are reported as mean ± standard deviation (n = 5)

following order: 21 days = 14 days = 4 days < 7 days (Fig. 5). Bone-like formation (Fig. 6) was greater (p = 0.0001) on 45S5 than on all fluorcanasite glass-ceramic compositions (Fig. 7).

Discussion

In this work, the in vitro osteogenesis on fluorcanasite glass-ceramics with three different chemical solubilities

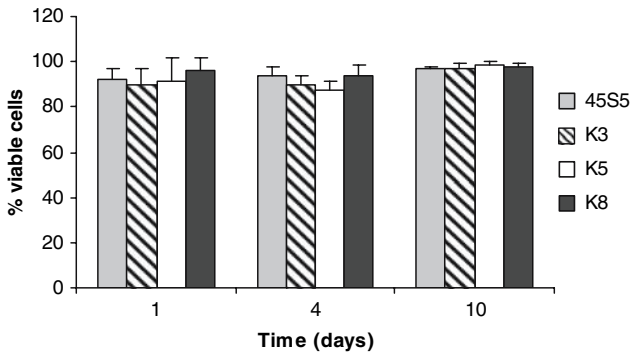

Fig. 3 Cell viability expressed as a percentage of viable cells at 1, 4, and 10 days. Data are reported as mean ± standard deviation (n = 5)

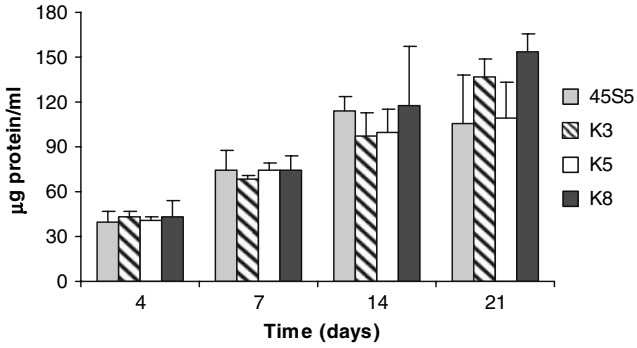

Fig. 4 Total protein content expressed as µg protein/mL at 7, 14, and 21 days. Data are reported as mean ± standard deviation (n = 5)

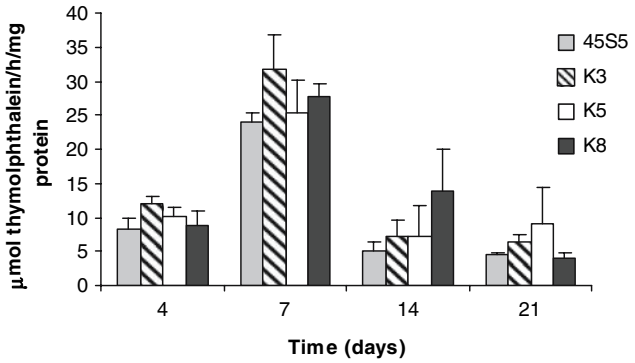

Fig. 5 Alkaline phosphatase activity expressed as µmol thymolphthalein/h/mg protein at 7, 14, and 21 days. Data are reported as mean ± standard deviation (n = 5)

was evaluated with the use of human osteoblastic cell cultures grown in experimental conditions known to favor osteoblast differentiation, namely, expression of ALP activity and production of a mineralized matrix. This method has been used in previous study of material biocompatibility testing [21]. The results showed that, irrespective of composition and solubility, all fluorcanasite glass-ceramics are biocompatible in this experimental model, considering that they allowed cell adhesion, proliferation, and differentiation. Additionally, it was observed that all fluorcanasite glass-ceramics and 45S5 presented similar cell responses with exception of bone-like nodule formation, which was greater on 45S5. Considering that all the samples were manufactured under the same conditions, surface topography was likely to be similar for the three fluorcanasite glass-ceramic compositions and the control 45S5, excluding factors other than surface chemistry that could influence osteoblastic cell response.

In order for osteoblastic progenitor cells to proceed with the wound healing cascade, proliferation, differentiation, and tissue maturation, cells need to adhere to substrata first [22]. This attachment process depends upon a series of

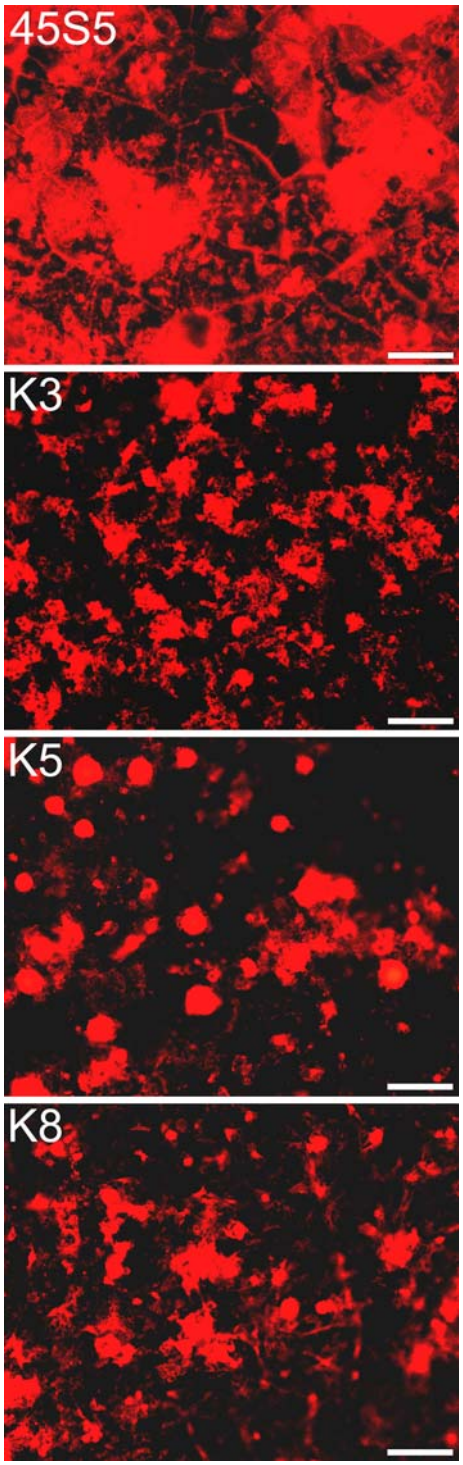

Fig. 6 Epifluorescence of alveolar bone-derived osteogenic cultures on fluorcanasite glass-ceramics and Bioglass® 45S5 (45S5) at day 21, stained with Alizarin red S for histochemical detection of calcium deposits. Typical aspects of mineralized matrix were observed on all surfaces (red areas). Bars = 100 μm

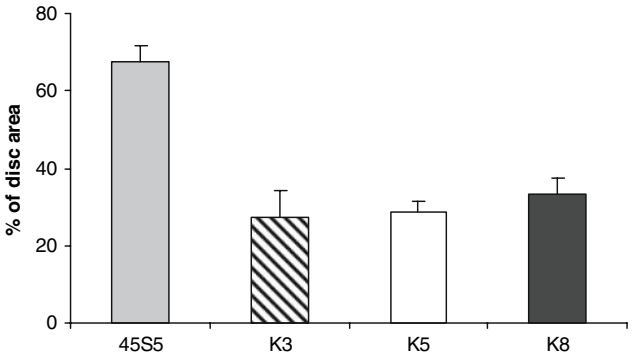

Fig. 7 Bone-like formation expressed as a percentage of the disc area at day 21. Data are reported as mean ± standard deviation (n = 5)

complex cell–matrix–substrate interactions, which involves adsorption of glycoproteins to the substrate surface, cell contact, attachment and spreading, mediated by integrin expression and binding to RGD-containing proteins [22]. Additionally, various material-related aspects have been demonstrated to affect such interactions, including surface topography, chemistry, and energy/wettability [21, 23]. This study did not show any effect of fluorcanasite glass-ceramics composition and solubility on cell adhesion. It is possible to speculate that the amount of products released from the glass surfaces during the initial periods of culture is not sufficient to produce effects on adhesion proteins that could affect cell adhesion. Besides, it is also possible that cell adhesion in culture is not seriously influenced by any characteristic of the material, since it has been shown that materials with different degrees of biocompatibility produce little or no differences in cell attachment [24].

In surface-reactive materials such as glass ceramic, the surface design changes during the culture time due to the release of inorganic compounds and adsorption of proteins and inorganic ions from the medium. Some studies show that the time-dependent variation of the surface structure acts in the regulation of the osteoblastic cell growth and synthesis [8, 25, 26]. The reduction of the chemical solubility of the fluorcanasite glass-ceramics K3 and K8 did not significantly affect cell proliferation, viability and secretory activity, which were similar to those of 45S5. In contrast with our results, it was observed that calcium phosphate glass ceramics with different surface degradation rates produced different patterns of both cell proliferation and synthesis [23]. Such different findings could be due to the methods used to modify the solubility as we changed the amount of potash whereas Dias et al. [23] incorporated K_2O or TiO_2. Additionally, osteoblastic cells from human alveolar bone were used in the present study while human bone marrow cells were employed by the other authors [23].

Both composition and solubility of fluorcanasite glass-ceramics did not affect the ALP activity, which was similar to that of 45S5, suggesting that intermediary markers of osteoblast differentiation are not sensitive to

the changes in the surface of fluorcanasite glass-ceramics. However, the production of mineralized matrix was similar among all fluorcanasite glass-ceramics and was reduced compared to 45S5. The highest amount of mineralized matrix on 45S5 could be explained by the optimal apatite formation on its surface, probably because of phosphate presence in its composition, which promotes an alkaline and favorable environment for deposition of mineralized matrix [27]. Such results do not sustain the hypothesis of a previous study by Rocha Barros et al. [12] evaluating a material similar in composition with K5, which suggested that the reduction of the solubility of the fluorcanasite glass-ceramic could improve its biological behavior. It is possible that the relatively low solubility of the K3 and K8 compositions as determined by Stokes et al. [14] is still too high to allow more in vitro osteogenesis on its surfaces. Considering that, one possible alternative to improve the biological properties of the fluorcanasite glass-ceramics could be the substitution of sodium by calcium or the incorporation of phosphate [10]. Indeed, the incorporation of a low percentage of P2O5 has been suggested to induce bioactivity in the canasite system [28].

Conclusions

The changes in the chemical composition and consequently solubility of the fluorcanasite glass-ceramics tested here did not significantly alter the in vitro osteogenesis using culture of osteoblastic cells from human alveolar bone fragments as a model. Further modifications of the chemical composition of the fluorcanasite glass-ceramic would be required to improve bone response, making this biomaterial a good candidate to be employed as a bone substitute.

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