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Biocompatibility and osteogenic assessment of experimental fluoride-doped calcium-phosphate cements on human dental pulp stem cells

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ABSTRACT

Objectives: This study investigated the impact of some specific experimental calcium phosphate cements doped with different fluoride salts (FDCPCs) concentrations on the basal functions of human Dental Pulp Stem Cells (hDPSCs). Furthermore, this study also examined the migration, as well as the mineralisation through osteogenic differentiation.

Methods: Experimental FDCPCs were formulated using different concentrations of calcium/sodium fluoride salts [(5 wt%: VS5F), (10 wt%: VS10F), (20 wt%: VS20F)]. A fluoride-free calcium phosphate (VS0F) was used as a control. The hDPSCs were assessed to evaluate their self-renewal and migration activity in the presence of eluates of the different FDCPCs. A viability assay in osteogenic conditions was carried out, along with the differentiation potential through Alkaline Phosphatase Activity (ALP), and Alizarin Red Staining (ARS). Moreover, the gene expression of specific markers (RUNX2, ALP, COL1α1, OCN, OPN, DSPP, MEPE, and DMP-1) was also evaluated.

Results: All the tested FDCPD had no influence on cell migrations, but they caused a decrease in cell viability in osteogenic conditions when not diluted. Conversely, the eluants of VS20F showed a positive effect on stem cell differentiation. This result was corroborated through ALP activity, ARS assay. Moreover, upregulation of specific gene markers such as RUNX2, DMP-1, and DSPP was observed in hDPSCs, especially when treated with VS20F.

Significance: The experimental FDCPC tested in this study exhibits a dose-dependent capacity to promote mineralisation in osteogenic environment. The FDCPC-VS20F seems to be the most promising experimental material suitable for developing of pulp-capping materials with osteogenic and bioactive properties.

1. Introduction

The development of innovative ion-releasing inorganic (e.g. Bioactive glasses, modified calcium phosphate) and their incorporation fillers for resin-based dental materials has gained the attention of several dental material manufacturers and researchers [1–3]. Indeed, this seems to be the next frontier in the development of innovative dental materials to improve the longevity of dental restorations and protect the remaining dental hard structures (e.g. enamel and dentine) from recurrent caries lesions [4,5]. However, it is important to state that in dentistry, in particular in preventive (PD) and minimally invasive dentistry (MID), materials are acknowledged as "bioactive" [6] when these can release and recharge specific ions, such as calcium (Ca), phosphate (PO) and fluoride (F); a constant liberation of such ions must induce remineralisation through apatite-like deposition and confer some antimicrobial activity [7]. Modern "bioactive" materials should not only replace the tissues destroyed by caries, but they need to be able to remineralise via apatite formation the remaining caries-affected dentine preserved during MID procedures (e.g. selective caries removal), as well as optimising the integrity and sealing of the bonding interface. Furthermore, their clinical use should also favour a reduction of biofilm formation, as well as prevent the infiltration of microorganisms within

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the margins of restorations, and formation of secondary caries lesions [8,9]. It is important to consider that when performing selective caries removal in deep cavities, the main objective is to avoid the exposure of the pulp; this procedure may allow clinicians to maintain the pulp tissue integrity and preserve its functional and biological activities. Unfortunately, it is not so uncommon that during such caries excavation procedures in deep cavities, it may be necessary to perform indirect or direct pulp capping therapies. In the case of direct pulp capping, it is important to state that there are several clinical controversies if performing an endodontic treatment or traying to save the tooth by placing on the exposed pulp a protective, highly biocompatible material able to induce the formation of dentinal tissue (i.e. reparative dentine bridge). Conversely, when facing a case of indirect pulp capping, it is a common procedure to apply pulp-protection materials on the dentine able to provide an optimal seal and minimise bacteria leakage, so preserving the vitality of the pulp [10,11].

It is well known that an ideal material for pulp capping, along with excellent biocompatibility and bioactivity properties, it ought to have also low solubility, dimensional stability, antibacterial properties, radiopacity, and adequate compression resistance [11,12]. However, the most common material used for such a dental treatment is calcium hydroxide, which has good biocompatibility, and excellent antimicrobial properties, but very high solubility. Conversely, mineral trioxide aggregate (MTA) cements have very low solubility, excellent biocompatibility and excellent antimicrobial properties. [13,14] Moreover, the use of such materials may stimulate a response from the pulp tissue and initiates the reparative process, which is characterised by the migration of stem cells to the lesion with subsequent odontogenic differentiation and synthesis of a mineralised barrier [15].

Calcium phosphate cements (CPCs) have been often employed as a biocompatible/bioactive scaffold for bone regeneration since 1920 [16]. Moreover, some specific CPCs have been advocated as promising pulp-capping materials for direct and indirect application, which can encourage dentine regeneration. In this regard, it is also important to highlight that the association between CPCs and calcium silicates has led to the generation of several endodontic biomaterials for bone, tooth revascularisation, and experimental pulp capping procedures due to synergistic effects in terms of high release of calcium and phosphates ions, as well as mild alkaline pH [17,18].

Recently, some experimental calcium phosphates have been used to generate resin-based cements with a high ability to remineralise caries-affected dentine [19]. Furthermore, experimental fluoride-doped calcium phosphates (FDCP) have been proven to possess an excellent ability to convert into biocompatible fluoride-containing apatite-like crystals when immersed in simulated body fluid [3]. It was also demonstrated that the incorporation of these latter FDCP in resin-based materials could improve their biocompatibility and confer specific antimicrobial properties [2].

The remaining question about such newly developed FDCP is, can they help to preserve the vitality of the pulp? It is important to anticipate that several biomaterials containing fluoride have been previously tested on various oral cell lines. The most common outcome is that cell cytotoxicity is fluoride dose-dependent; the higher the fluoride releases the greater the cytotoxic effect on cells [20–23].

Thus, this study aimed at investigating the impact of some specific experimental cements based on calcium phosphate doped with different concentrations of fluoride salts on the basal functions of human Dental Pulp Stem Cells (hDPSCs) . Furthermore, it was also elucidated the influence of such innovative cements on migration and wound healing of the hDPSCs, as well as the activation of mineralisation processes. This latter objective was accomplished by examining the alkaline phosphatase activity (ALP), alizarin red staining (ARS), as well as the gene expression of specific markers (RUNX2, ALP, COL1α1, OCN, OPN, DSPP, MEPE and DMP-1). This study hypothesised that the experimental FDCPCs tested in this study would have suitable biocompatibility and osteogenic properties to be considered for the creation of innovative biomaterials for pulp capping procedures.

2. Materials and methods

2.1. Preparation of fluoride-doped calcium-phosphate cements – specimens extracts

Several FDCPCs were generated using different concentrations of fluoride salts, as previously described in the literature [2,3]. In brief, calcium hydroxide (10 wt%), calcium and sodium fluoride salts (1:1) at different concentration (5 wt%, 10 wt%, or 20 wt%) were incorporated into a 1:1 molar ratio of beta-calcium-phosphate (β-TCP: $Ca_3(PO4)_2$) and monocalcium phosphate monohydrate (MCPM: $Ca_3(H_2PO4)_2$). All these components were mixed in distilled water for 3 min and left undisturbed at room temperature (22 °C) for 12 h to achieve a complete setting reaction. The resultant FDCPCs (VS5F; VS10F; VS20F) and a fluoride-free (VS0) calcium phosphate cement, which was used as the control material. All the tested cements were milled and sieved as previously described in the literature (average particle size 20–50 μm) and used in the form of powder during the entire study. The morphological (TEM/SEM-EDX) and chemical characteristics (FTIR; EDX; NMR) of the cements used in this study were also reported in the literature [3].

Subsequently, the experimental materials were used according to ISO 10993–12 [24], so they were dispersed in a Dental Pulp Stem Cell Growth Medium (DPSC-GM) (Lonza, PT-4516 containing human Dental Pulp stem cell Growth Supplement, PT-3927) at the concentration of 200 mg/mL. After incubation (37 °C for 24 h at 5 % CO_2), the eluate was recovered and sterilised using a 0.2-μm filter (Merck Millipore, Tullagreen, Carrigtwohill, Co. Cork, Ireland). Serial dilutions of powder extracts were prepared (1:1-non-diluted, 1:5, 1:10, 1:50, 1:100) in DPSCs medium. The same preparation was conducted using an osteogenic medium (DPSC-GM containing 10^{-8} M dexamethasone, and 8 mM *β*-glycerophosphate) and growth medium without serum.

2.2. Human dental pulp stem cells culture

hDPSCs were purchased from Lonza (PT-5025, Swiss, Basel, Switzerland), cultured and preserved following the manufacturer's instructions in DPSC-GM, or induced to osteogenesis using the osteogenic medium described above, in an incubator at the temperature of 37 °C and 5 % CO_2. In this study, hDPSCs from passages 4–7 were used.

2.3. Evaluation of clonogenic activity

To evaluate the clonogenic activity of hDPSCs in the presence of eluates of the experimental FDCPCs (VS0; VS5F; VS10F; VS20F), a colony-forming unit fibroblast (CFU-F) assay was performed [25]. Briefly, 2×10^2 cells/well were cultured into 6 well, in growth medium at 37 °C and 5 % of CO_2. The extracts at 1:50 dilutions were added after 24 h and cells were fixed 7 days after treatment, stained with crystal violet for 10 min at room temperature, and then washed with sterile water until clear. The number of colonies in each well was determined by manual counting, using an optical microscope (LEICA DMi6000). Colonies with $n \geq 30$ cells were considered. Untreated cells were used as control. Data were presented as fold-changes compared to the control.

2.4. Wound healing assay

The effect of the FDCPCs on hDPSCs migration was assessed through a wound healing test [26]. The cells were cultured into 6-well plates until confluency, with the growth medium replaced with serum-free medium for 2 h. A wound was generated manually with a 1000 μL pipette tip, and cells were treated with the extracts at 1:50 dilutions in a serum-free medium. The cell cultures were photographed with an optical microscope (LEICA DMi6000) at the zero-time point (T0) and after 72 h (T72) to quantify the wound closure area using ImageJ software

(National Institutes of Health, Bethesda, MD, USA). Ten images were analysed for each specimen. Cells without any extracts of FDCPCs served as controls. Results were shown as the percentage of wound closure calculated as (area T0-area T72)/area T0×100.

2.5. Viability assay in osteogenic medium

The cytotoxicity of the FDCPC extracts on hDPSCs was evaluated with MTT [3-(4,5- dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide] assay, according to manufacturer's instructions (Sigma-Aldrich, M5655). The cells were placed into a 96-well at an initial density of 5×10^3 cells per well, and subsequent adhesion of the cells, appropriate eluates in the osteogenic medium were added at different dilutions (1:1, 1:5, 1:10, 1:50, 1:100) and incubated for 72 h. The MTT solution (5 μg/mL) was added and after 4 h at 37 °C and 5 % CO_2, 100 μL acidic isopropanol (0.04 N HCl in absolute isopropanol) was added. Absorbance was measured at 570 nm using a microplate reader (Tecan, Grödic, Austria). Cells cultured with hDPSCs osteogenic medium were used as control [27].

2.6. ALP - Alkaline phosphatase activity

hDPSCs were cultured in a 48-well plate at a density of 2.5×10^4 cells/well. The cells were left undisturbed to adhere, and subsequently treated with 1:50 dilution of the eluates in an osteogenic medium for 14 days. At different time points from the treatment (3, 7, 10, 14 days), ALP activity was determined using an Alkaline Phosphatase Activity Assay Kit (Elabscience, E-BC-K091) according to the manufacturer's instructions. The outcomes were calculated and then expressed as enzymatic units/mg protein.

2.7. Mineralized deposits through alizarin red staining

The cells (hDPSCs) were cultured in a 48-well plate at a density of 2.5×10^4 cells/well. After reaching the confluence, 1:50 dilution of the eluates in the osteogenic medium was added for 14 days. The accumulated calcium deposition was analysed using an alizarin red stain solution as described in a previous study [28]. Photographs were taken using an optical microscope equipped with a digital camera at 10X magnification (LEICA DMi6000). To quantify the alizarin red, 10 % acetic acid was added to the specimens for 30 min under continuous shaking, and then specimens were evaluated at 405 nm.

2.8. Gene expression of osteogenic and odontogenic markers (q-PCR)

The RNA was extracted from cell cultures using the TriZOL^R Reagent (Invitrogen) as per the manufacturer's instructions. Reverse transcription was conducted using 1.0 μg total RNA and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4387406). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659) and primers as listed in Table 1. The amplification was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, 4376357) with the following cycling conditions: cDNA denaturation and polymerase activation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The data analysis was conducted in comparison with the GAPDH control gene following the comparative $2^{-\Delta Ct}$ method. The normalised expression was calculated as fold change mRNA level versus control.

2.9. Statistical analysis

The results were presented as mean (± SEM) of at least three independent experiments, as indicated in the legends of figures shown in this article. Statistical analysis of the data was performed using a One-way analysis of variance (One-way ANOVA) followed by a Tukey's test for multiple comparisons using GraphPad Prism 9.0 software (GraphPad Software, Inc., La Jolla, CA, USA). A *p-value* lower than 0.05 was considered significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3. Results

3.1. hDPSCs clonogenic capacity and migration evaluation

The results on the clonogenic capacity of the tested FDCPCs are depicted in Figs. 1A and 1B. In particular, it was observed that the 1:50 eluate of VS20F reduced 1.5-fold the self-renewal ability of the cells as compared to control (0.67 %; $p = 0.0169$), 1.76-fold as compared to VS0 (1.18 %; $p = 0.0003$) and 1.68-fold as compared to VS5F (0.92 %; $p = 0.004$). Conversely, no statistically significant differences were observed between VS10F and VS20F.

Conversely, the results of the migration ability of hDPSCs using the wound healing assay after 72 h are presented in Figs. 1C and 1D. In this case, it was possible to observe that none of the tested materials (1:50 diluted extracts) had a significant effect on the hDPSCs migration.

3.2. Cell viability in osteogenic condition

The results of the tested FDCPCs on hDPSCs viability in the osteogenic medium at different dilutions (1:1, 1:5, 1:10, 1:50, 1:100) are depicted in Fig. 2. The results showed that the undiluted eluates caused a significant decrease in the cell viability in VS0 (percentage of cell viability: 35.9 %) and VS5F (percentage of cell viability: 45.8 %). VS10F and VS20F showed a significant reduction of the cell viability (22.83 % and 24.09 %, respectively), as compared to the control group ($p < 0.0001$). Notably, no significant differences were observed between the FDCPC containing fluoride. Furthermore, at 1:5 dilutions VS20F (percentage of cell viability 27.6 %) had a reduction in cell viability of 3.6-fold compared to the control ($p < 0.0001$) and approximately 3-fold compared to the fluoride-free VS0 cement (percentage of cell viability: 80.4 %; $p = 0.0014$) and VS5F (percentage of cell viability: 83.2 %; $p = 0.0008$). Conversely, VS10F showed a reduction of viability 2-fold to that of untreated cells (percentage of cell viability: 54.9 %; $p = 0.0044$). At 1:10 dilution, VS20F (percentage of cell viability: 32.9 %) had a significant reduction in cell viability by 3-fold

Table 1
Primer sequences for qPCR analysis.

Primer	Forward sequence	Reverse sequence
Runx2	F - ATGTGTGTTTGTTTCAGCAGCA	R - TCCCTAAAGTCACTCGGTATGTGTA
OCN	F - TGAGAGCCCTCACACTCCTC	R - ACCTTTGCTGGACTCTGCAC
OPN	F - CAGTTGTCCCCACAGTAGACAC	R - GTGATGTCCTCGTCTGTAGCATC
ALP	F - TCAGAAGCTCAACACCAACG	R - TTGTACGTCTTGGAGAGGGC
Col1α1	F - CCCGGGTTTCAGAGACAACTTC	R - TCCACATGCTTTATTCCAGCAATC
MEPE	F - GGTTATACAGATCTTCAAGAGAGAG	R - GTTGGTACTTTCAGCTGCATCACT
DSPP	F - AGA AGGACCTGGCCAAAAAT	R - TCT CCT CGG CTA CTG CTG TT
DMP−1	F - TGGGGATTATCCTGTGCTCT	R - TACTTCTGGGGTCACTGTCG
GAPDH	F - TCAGCAATGCCTCCTGCAC	R - TCTGGGTGGCAGTGATGGC

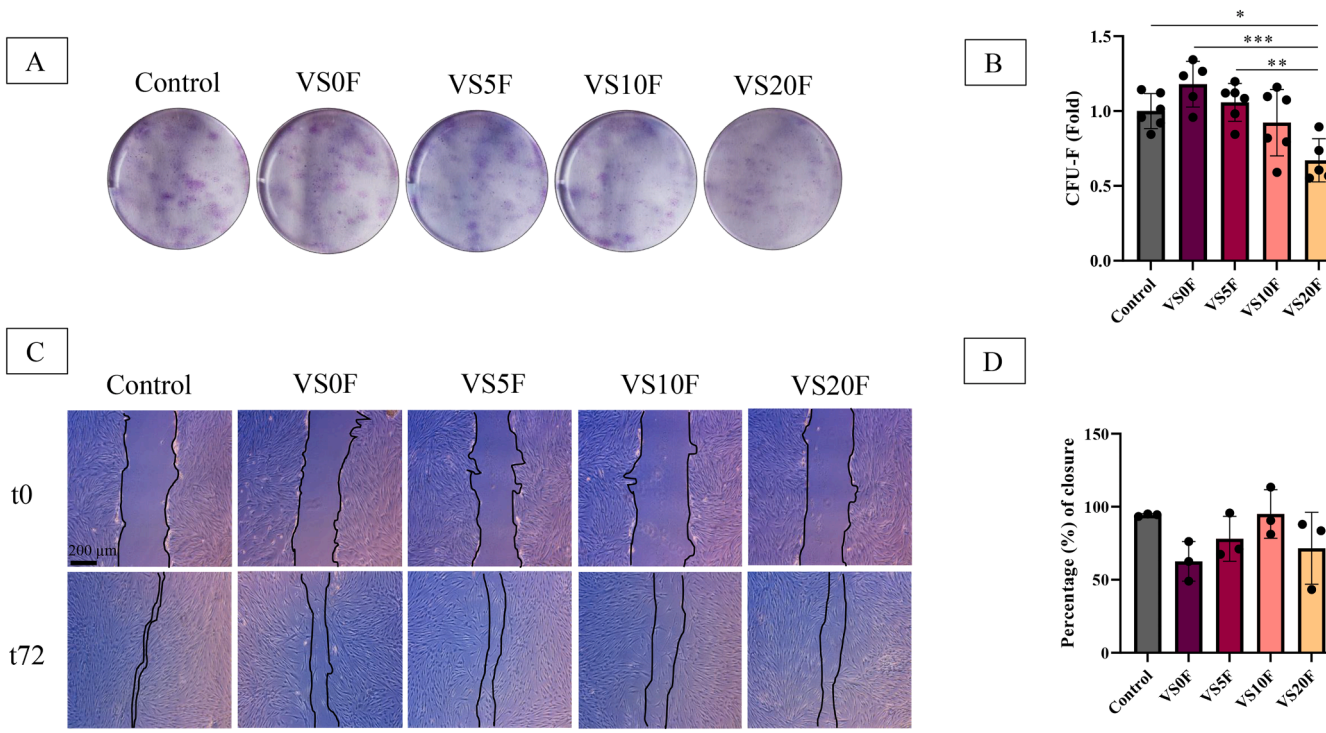

Fig. 1. : Evaluation of the ability to form colonies assessed with crystal violet (n = 5 or 6) indicated a reduced capacity for colony formation in cells treated with the VS20F (A). Evaluation of migration ability after 72 h of treatment assessed with wound healing assay (n = 3) revealed no significant difference between the tested cements and the control (B). scale bar 200 µm. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$.

compared to the control group ($p < 0.0001$), and 2-fold compared to VS (percentage of cell viability: 72.1 %; $p = 0.0074$) and VS5F (percentage of cell viability:71.7 %; $p = 0.008$). In contrast, VS10F caused a decrease in viability of 1.6-fold compared to the untreated cells (percentage of cell viability: 60.9 %; $p = 0.0075$). Furthermore, all fluoride-containing FDCPC did not affect cell viability at 1:50 and 1:100 dilutions.

3.3. Osteogenic differentiation

The results of the ability of the tested FDCPCs to influence osteogenesis (ALP) are shown in Figs. 3A and 3B. It was detected an increase of ALP activity already at day 7 in cells treated with VS20F, compared to control (ALP values: control: 0.025; VS20F: 0.74; p-value < 0.0001). Conversely, for all the other tested FDCPC containing fluoride, the peak of ALP activity was reached at day 10, but at a lower extent than VS20F (Figs. 3A and 3C). In particular, ALP activity in VS0 increased 2.4-fold, VS5F 1.7-fold, and VS10F 3-fold compared to untreated cells (ALP values: control: 0.12; VS0: 0.29; VS5F: 0.20; VS10F: 0.37; p-value < 0.0001). VS20F showed only a slight increase compared to the control (ALP value 0.14; p-value = 0.0046). Furthermore, a reduction in ALP expression was observed after 14 days in all the treatments as compared to the control group (p-value < 0.0001), with the ALP activity remaining higher in VS20F compared to the other FDCPC containing fluoride (p-value < 0.001) (Figs. 3A and 3D). These findings were supported by the capacity of hDPSCs to form mineralisation nodules (Figs. 3E and 3F). Indeed, there was an increase of 8.6-fold in VS20F compared to the control group ($p = 0.0007$) and 8-fold compared to VS0 ($p = 0.0004$), VS5F ($p = 0.0008$) and VS10F ($p = 0.0009$).

3.4. Gene expression of osteogenic and odontogenic markers

To support the differentiation outcomes, the gene expression analysis on osteogenic and odontogenic markers was evaluated after 14 days of differentiation in the presences of the eluants of the FDCPCs diluted at 1:50. As shown in Fig. 4, the mRNA levels of RUNX2 resulted 1.6-fold significantly higher in VS20F compared to the control group ($p = 0.030$). VS10F showed a similar trend to that of VS20F. The mRNA levels of OCN in VS20F were 2-fold greater compared to untreated cells ($p = 0.0105$) and to VS10F ($p = 0.0053$), 4.4-fold more than the VS0 ($p = 0.0003$) and 3.4-fold more compared to VS5F ($p = 0.0006$). Furthermore, considering the odontogenic markers, only the mRNA level in MEPE resulted approximately 8-fold higher in VS20F compared to the control ($p = 0.0004$), VS0 ($p = 0.0006$) and VS5F ($p = 0.0007$), while it was 2-fold greater compared to VS10F ($p = 0.0223$). The other osteogenic and odontogenic differentiation markers analysed, such as COL1α1, OPN, DSPP and DMP-1 did not show any significant difference between the tested FDCPCs and the control group.

4. Discussion

It has been advocated that the incorporation of fluoride in biomaterials at concentrations relatively low may enhance the overall biocompatibility of such materials, as well as favour cellular metabolic stability [29]. Indeed, after evaluating the cytocompatibility of fluoride-containing dental varnish on Human Gingival Fibroblasts (HGFs), it was demonstrated that the cell viability decreased at increased concentrations of fluoride [20]. Moreover, a further previous study reported changes in the sensitivity of three human oral cell types (HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) in the presence of sodium fluoride (NaF); the cell viability was NaF dose-dependent [21]. On the other hand, the cytotoxic of glass ionomer cements (GICs) on human dental pulp stem cells (hDPSCs) seems to be correlated to the release of fluoride, rather than aluminium and strontium ions released in the eluates [22]. More recently, the cytotoxicity of silver diamine fluoride on hDPSCs was also recently assessed; such a treatment used at high concentrations reduces the viability of hDPSCs [23].

The cytotoxicity of experimental FDCPCs tested in the current study, which was previously evaluated through MTT assay [3], exerted

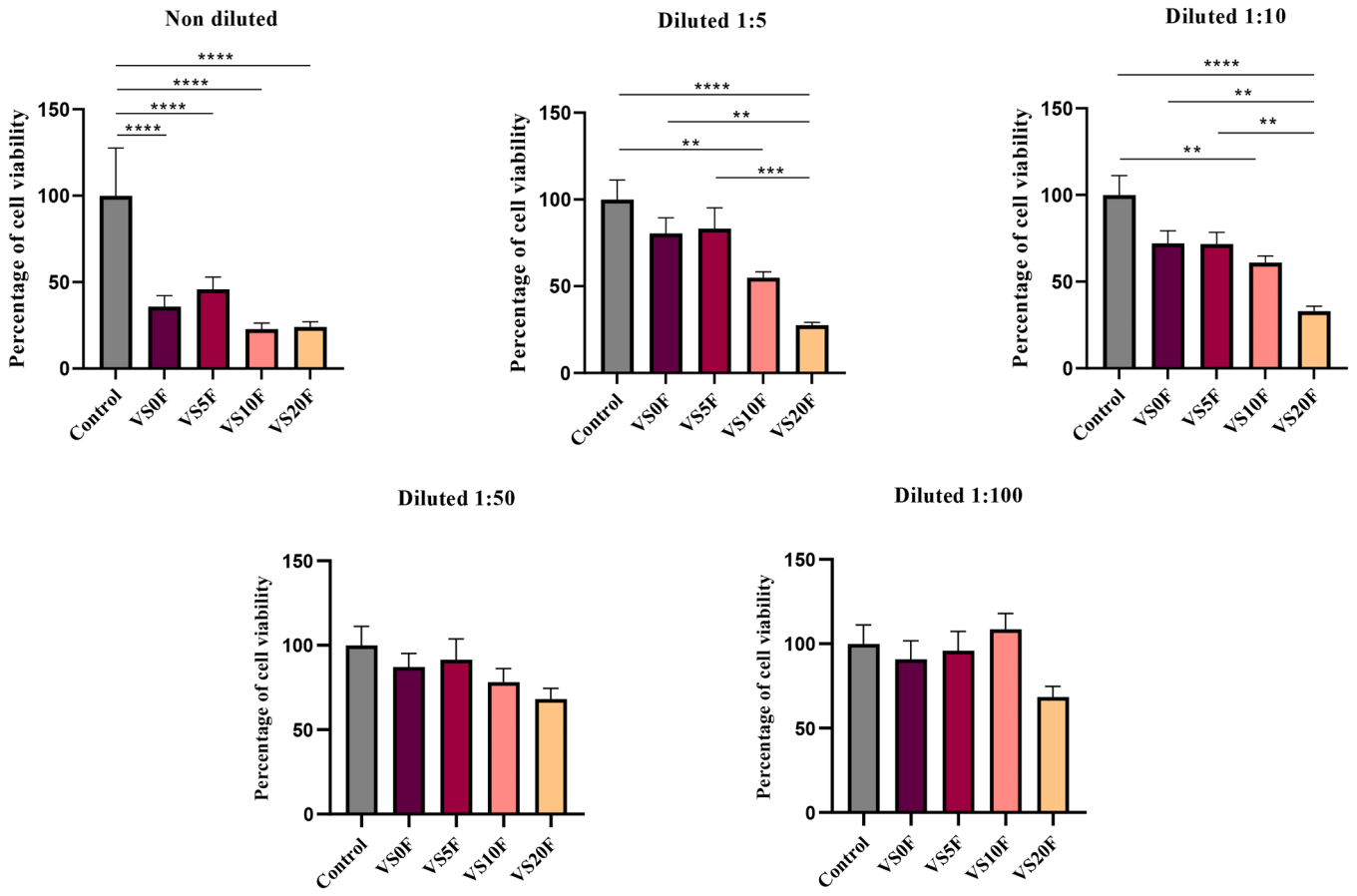

Fig. 2. : Cell viability in osteogenic medium of powder extracts (VS0; VS5F; VS10F; VS20F) at different dilutions (1:1 (non-diluted), 1:5, 1:10, 1:50, 1:100) (n = 3) evaluated with MTT assay after 72 h. The undiluted eluates at all fluoride percentages caused a significant decrease in cell viability compared to the control group. In addition, at 1:5 and 1:10 dilutions VS20F decreased the cell viability compared to the control, VS0, VS5F, while VS10F reduced the cell viability only compared to the untreated cells. * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$.

important toxicity on human dental pulp stem cells (hDPSCs) at higher concentrations of fluoride; VS20F presented the greatest release of fluoride ions when immersed in SBF [3]. The same authors also reported an increase in cell vitality for all the FDCPCs when the eluates were diluted at 1:50 and 1:100 compared to the control; this was the main reason why in the current study cell assays were performed using 1:50 dilutions of the eluates. However, to better understand the suitability of such FDCPCs as potential innovative biomaterials for direct and indirect pulp capping application, we considered it necessary to assess the influence they could have on the basal functions of hDPSCs (e.g. cytotoxicity in osteogenic conditions) and cell migration.

It was advocated through in vitro and in vivo studies that biocements able to release a considerable amount of calcium ions can induce osteogenesis and odontogenesis by supporting the attachment, proliferation, and differentiation of cells, as well as improving deposition of mineralised matrix [30,31]. Thus, in this study it was also elucidated the influence of the tested FDCPCs to activate the mineralisation processes in hDPSCs. For this latter aim, the formation of mineralisation nodules was assessed through the quantifying of the Alkaline Phosphatase (ALP) activity and analysis of the expression patterns of specific osteogenic and odontogenic markers, such as RUNX2, COL1α1, OCN, OPN, DSPP, MEPE, and DMP-1 [32].

Previous studies have defined the importance of such markers evaluated in this study [32,33]. For instance, ALP is considered an early indicator for bone and tooth differentiation, whereas osteocalcin (OCN) is a marker of the late stages of osteoblast (bone-forming cell) differentiation, and its production signifies the onset of matrix deposition. Dentine sialophosphoprotein (DSPP) is the initial translation product of the DSPP gene that is then cleaved into dentine phosphoprotein and dentine sialoprotein. The runt-related transcription factor (RUNX2) is an osteoblast-specific transcription factor that is essential for the differentiation of mesenchymal stem cells into mature osteoblasts/odontoblasts (tooth-forming cells) and for maintaining their differentiated function during bone and tooth formation and remodelling. The DMP-1 is involved in the regulation of dentine collagen, while the matrix extracellular phosphoglycoprotein (MEPE) expression by dental pulp stem cells (DPSC) is a marker for early odontogenic differentiation; when odontoblasts are mature, MEPE becomes down-regulated. On the other hand, osteopontin (OPN) plays an important role in osteoblast differentiation and bone matrix mineralisation. Moreover, OPN promotes the differentiation of osteoblasts and the deposition of hydroxyapatite crystals within the bone extracellular matrix. Finally, COL1α1 (Collagen type I alpha 1 chain) represents the most abundant collagen type found in the bone extracellular matrix. It is an early marker of osteoblast differentiation and an important component in cell differentiation of osteoblasts and odontoblasts [32,33].

Our characterisation efforts yielded several distinguished findings. For instance, it was observed that VS20F presented a decreased self-renewal capability, possibly due to its ability to release a high amount of fluoride ions compared to the other tested FDCPCs [3]. A further explanation would be that VS20F reduced cells' self-renewal ability because of possible induction of the differentiative stimulus. Indeed, previous studies demonstrated a significant decrease in mean CFU-F found in B6 and C3H mice treated with 50 ppm of fluoride [34]. It has been suggested that the hDPSCs migration occurs during pulp capping [35] and that pulp capping materials solubilise dentine, leading to the

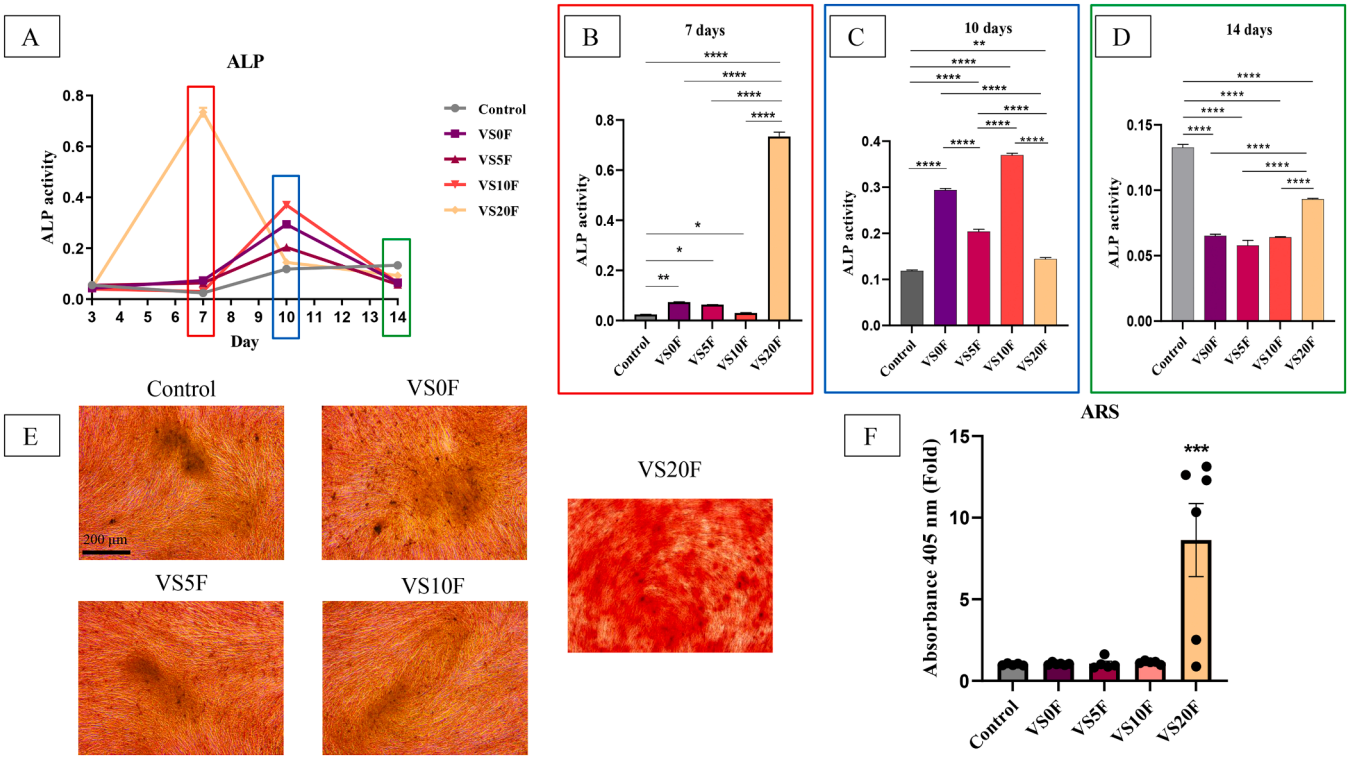

Fig. 3. : Alkaline phosphatase activity shown as enzymatic activity (n = 3) (A) and alizarin red representative staining and quantization (n = 6) (B) after 14 days of treatments. Cells treated with VS10F fluoride exhibited an upregulation in ALP activity and the ability of hDPSCs to form mineralization. scale bar = 200 µm. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$.

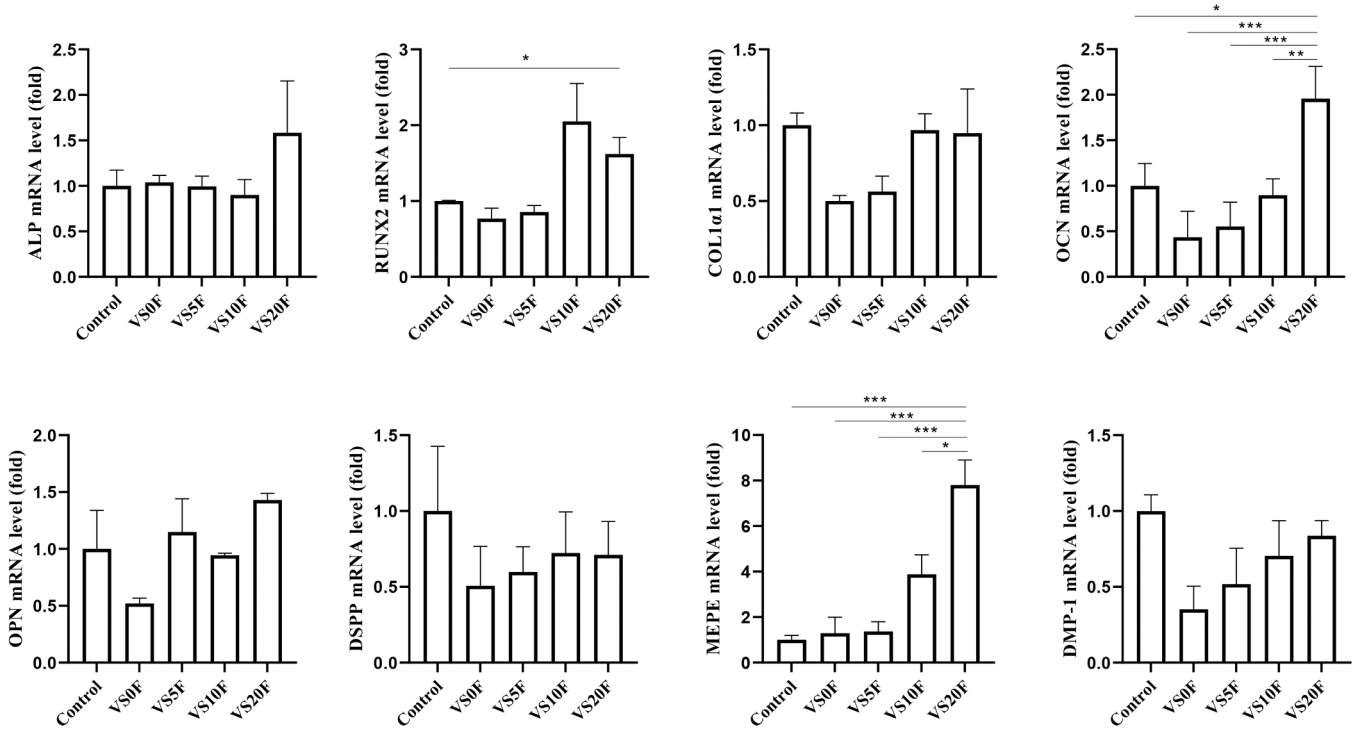

Fig. 4. qPCR for osteogenic and odontogenic marker genes (n = 3 per group). * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$.

release of different growth factors that encourage biological events [36], such as cell migration, improving pulp healing. The current study demonstrated that none of the tested FDCPC triggered any significant effect on cell migration, suggesting that fluoride-doped calcium phosphates do not affect the cytoskeletal function of hDPSCs. Similarly, various studies demonstrated that cell migration rates for calcium silicate cements such as MTA (Mineral Trioxide Aggregate: a mixture of a refined Portland cement and bismuth oxide and trace amounts of SiO_2,

CaO, MgO, K_2SO_4, and Na_2SO_4), Repair HP (endodontic bioceramic reparative cement with high plasticity composed of mineral oxides in the form of fine hydrophilic particles) and TheraCal (a light-curing, resin-modified calcium silicate used as pulp capping materials and as a protective liner) were similar to the control group, while only Biodentine (a calcium silicate-based cement formed by highly purified tricalcium silicate, zirconium oxide, calcium oxide, calcium carbonate and iron oxides pigment) showed the highest capability on hDPSCs migration [37,38].

Conversely, a recent study on the biocompatibility of ammonia-free silver fluoride products on hDPSCs demonstrated an improved cell migration when potassium iodate was combined with silver fluoride [39]. Such a different outcome compared to our results may be related to the combination of fluoride to other compounds, which may have caused different cell responses.

The eluates of the tested FDCPCs used undiluted reduced significantly the viability of hDPSCs in osteogenic medium. When the eluates were diluted at 1:5 and 1:10, VS20F presented lower cell viability compared to the control, VS0, and VS5F; only a slight reduction in viability was observed in VS10F compared to untreated cells. The dilutions offered us the possibility to discern the different impacts of the diverse fluoride content in the tested FDCPC on cell viability. Previous investigations demonstrated that fluoride-free VS0 was unable to convert completely into apatite-like crystals, while VS5F formed the smallest apatite crystals and exhibited the lowest release of fluoride ions, with no significant variation observed over time when compared to the other FDCPCs. Moreover, VS10F showed a significant release of fluoride ions, with the cumulative values increasing notably over time; these remained lower compared to those of VS20F [3]. These findings confirm the idea that higher fluoride percentages impair cell viability and supported that lower fluoride release impairs cell growth [40]. The current outcomes are per existing literature suggesting that the proliferation of human osteoblast-like (HO) cells increases by approximately 10 % when in contact with fluoridated hydroxyapatite (FHA), while higher concentrations of fluoride can impede cell growth [41]. Moreover, none of the FDCPCs influenced the cell viability at 1:50 and 1:100 dilutions, probably attributable to the formation of fluoride-containing apatite, which appears to occur particularly when fluoride is present at specific low concentrations [42].

As previously mentioned, in this study it was also assessed the impact of the experimental FDCFCs on hDPSCs osteogenesis. In this regard, cells treated with 1:50 dilution extracts exhibited elevated ALP activity only material on day 7, especially in the presence of VS20F. Previously, when investigating the ALP activity in other commercial pulp capping materials, it was observed that the enzyme expression in hDPSCs increased with TheraCal LC treatment for 7 days, which then decreased in the following days. Conversely, Biodentine was the only material to show a gradual increase in ALP activity with the highest values on days 10 and 14 days [43]. Our results showed that the ALP activity in hDPSCs treated with VS20F increased on day 7 and declined over time. This trend could be explained as the ALP activity represents an early marker for osteogenic differentiation and that possibly it can be reduced over time, upon mineralisation process occurs [44]. We believe that ALP activity began earlier (day 7) in cells treated with VS20F and by day 14, it decreased compared to the control group in which the enzyme activity starts to be observable. The results obtained during the ALP assay performed in the current study were corroborated by the improved cell ability to form mineralisation nodules when treated with VS20F, in comparison to the control and the other FDCPC tested in this study. This indicates that fluoride release, in combination with calcium and phosphate ions in VS0, VS5F and VS10F may not be “synergically” appropriate to exert a significant influence on cell-induced mineralisation. Indeed, regarding the outcomes observed in VS20F, we hypothesise that the significant formation of calcium precipitates may be referred to as the higher presence of fluoride ions released into the media, along with the presence of phosphate and calcium ions, which likely facilitated the mineralisation process. As already reported in the literature, the efficacy of fluoride for remineralisation depends both on dose and in particular on the ability of the treatment to use fluoride efficiently [45]. In literature it has been also reported that hDPSCs treated with ProRoot MTA (root repair material consisting of fine, hydrophilic particles that set in the presence of moisture) and TheraCal increased mineralisation compared to Biodentine, Dycal (calcium hydroxide radiopaque material used for cavity lining and pulp capping.), and control. [43,46] Further previous studies demonstrated that ProRoot MTA and Biodentine improved mineral nodule formation in several cell lines, such as human mandibular-derived mesenchymal stem cells and stem cells isolated from apical papilla [47,48]. Furthermore, gene expression analysis of odontoblast and osteoblast markers showed that RUNX, OCN, and MEPE resulted upregulated in VS20F as compared to the control, VS0F, VS5F, and VS10F.

A similar expression pattern of hDPSCs treated with Biodentine and ProRoot MTA was observed. Specifically, RUNX2 and OCN mRNA levels were upregulated in the Biodentine treatments, while only RUNX2 was overexpressed in the cells stimulated with ProRoot® MTA [45].

Furthermore, in a previous study, OCN upregulation was detected in the elevated extracellular calcium ion condition [49]. Similarly to our results, Asgary et al. [50] demonstrated that the ALP, DMP1, and DSPP mRNA expression levels were not upregulated in hDPSCs treated with MTA, and calcium-enriched cements as compared to a control cell group in the osteogenic medium.

Therefore, the hypothesis that the FDCPC tested in this study would have potential biocompatibility and osteogenic properties to be considered as a suitable biomaterial for pulp capping procedures must be only partially accepted.

5. Conclusions

The experimental fluoride-doped calcium phosphate cements exhibit a dose-dependent capacity to promote osteogenesis. Thus, given the outcomes obtained in this study, VS20F may be the most promising experimental FDCPCs to develop pulp-capping materials with osteogenic and bioactive properties. However, it is important to consider that high release of fluoride can also endanger the viability of pulp cells, hence it would be necessary to perform further biological, animal, and in vivo studies to understand the mechanism of action and signalling pathways activated by this new biomaterial, as well as to ascertain its real suitability as a pulp capping material in a real clinical scenario.

Declaration of Competing Interest

The author has no financial conflicts to disclose or personal relationships that could have appeared to influence the work reported in this paper.

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