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Original Research Article

Self-Setting Calcium Sulfate-Phosphate Bioactive Cement As "Barrier Graft" In Periodontal Defect Management: Preclinical Evaluation

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Alveolar bone loss associated with periodontitis is a common dental problem. Surgical debridement combined with the prevention of epithelial overgrowth by placing a 'barrier membrane' allows for bone regrowth. In cases of severe alveolar bone loss, grafting with a bioactive material is preferred alongside the barrier membrane. Various synthetic bone graft materials are currently in use. A calcium sulfate cement composition enriched with phosphate ions, developed for periodontal repair and designated by the acronym "CASPA," has been designed. This self-setting, moldable, and biocompatible cement showed promising responses in previous *in vitro* studies. This report presents the preclinical evaluation of CASPA cement. Initially, the response of human periodontal ligament cells to this formulation was assessed through a direct contact cytotoxicity test and MTT assay in comparison with conventional calcium sulfate (gypsum-based) cement. Cell viability, adhesion, and morphology were also tested. The osteogenic induction potential of the cements was investigated through staining (Alizarin red and Von Kossa) and osteogenic marker expression. The periodontal defect healing potential of CASPA cement was evaluated in a rat maxillary alveolar bone defect model in comparison with conventional gypsum material. The newly developed bioactive calcium sulfate cement (CASPA) exhibited improved biological properties compared to conventional calcium sulfate (gypsum) cement. Biocompatibility was excellent both *in vitro* and *in vivo* in rats. CASPA showed a slower resorption rate that was on par with the rate of bone formation, ensuring site stability and complete defect healing. In clinical use, the conformal filling of the cement obviates the need for a barrier membrane. CASPA acts as a 'barrier-graft' and leads to better bone regeneration than conventional calcium sulfate.

Introduction

Alveolar bone loss associated with periodontitis remains a leading cause for adult tooth loss. Untreated microbial infections lead to inflammatory responses resulting in elevated osteoclastic activity leading to the resorption of the alveolar bone supporting the teeth [ýý1]. The treatment strategy is already established. First approach is the cleaning of the defect and controlling infection. This will allow the multipotent endogenous stem cells in periodontal ligament to differentiate and initiate the innate regenerative responses [2]. To facilitate this, a barrier membrane will be provided over the defect area to prevent epithelial overgrowth and to promote connective tissue attachment between the tooth root cementum and the alveolar bone. This is known as Guided Tissue Regeneration (GTR) of the periodontal defect [3]. A resorbable membrane is preferred so that the intervention for removal could be avoided. However, it should remain in place with sufficient mechanical strength till the grafted defect is remodeled with new bone. This is too stringent requirement to achieve practically.

In case of severe alveolar bone loss manifesting as two-wall and/or three-wall defects, grafting with a bioactive material is preferred under the membrane [4]. Bone grafts require certain desirable properties such as adequate mechanical properties, interconnected pores with optimal pore size to promote cellular and vascular infiltration, and tunable resorption rates at par with regenerating bone tissues. Conventional autografts, allografts and xenografts had issues of donor site morbidity, availability, and unwanted immune responses. Therefore, synthetic bioceramic grafts were introduced for periodontal regenerative treatment strategies. Though the untreated bioceramics lacked biological cues, they were welcomed because of off-the-shelf-availability, adequate mechanical properties, lack of immunogenicity and surface osteogenic property [5]. Alloplastic (or synthetic) graft materials are mainly fabricated from calcium phosphates (hydroxyapatite and tricalcium phosphate) and bioglass (calcium phospho silicate) materials in ceramic fine granule form. Calcium sulfates, though not optimally a bioceramic, has

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barrier functions and bone regenerative abilities as adjunct to commonly used bioceramics [6]. They can be designed as new generation alloplasts which are mouldable and self-setting cement formulations, which are biocompatible and osteoconductive. These are based on calcium phosphates, calcium sulfates and their combination. These cement-based grafts give the convenience of conformal filling in the desired contours, and some products in this class have been introduced in the market [6,7].

In comparison with the ceramic counterparts, the calcium phosphate cement is reported to show the special property of 'osteo-transduction' in which the boundaries of the filled cement in contact with the defective bone resorbs in the same pace as the growth of the new bone. The cement graft gets replaced by bone in a progressive way and get. The mouldability of the cement enables the conformal filling into the periodontal defect. Once filled upto the ridge height, it will ward off the epithelial tissue and maintain the integrity till the osteotransduction is complete. This way, the self-setting mouldable cements serve the role of "barrier-graft" [8]. Interestingly, a composition of a calcium sulfate cement enriched with phosphate ions and designed as a bone cement also showed the 'osteotransduction' property [9]. This composition with the acronym as "CASPA" (CAlcium Sulfate-Phosphate bioActive) has been suggested for periodontal repair, in an in vitro study. On testing, in the presence of in vitro cultured hPDL (human Periodontal Ligament) cells, this cement was seen to promote mineral deposition without additional supplements, and the response was superior to sintered hydroxyapatite ceramics [10].

The present study represents the results of the pre-clinical evaluation of CASPA cement. In this study, the response of hPDL cells to this formulation was assessed through cytotoxicity evaluation (Direct contact test) and metabolic activity (MTT assay), in comparison with conventional calcium sulfate (gypsum based) cement. Cell viability, cell adhesion and morphology on the cement samples were also evaluated. Osteogenic induction potential of the cements was investigated through Alizarin red staining, Von Kossa staining and osteogenic marker expression. Thereafter, the periodontal defect healing potential of the CASPA cement was evaluated in vivo, in a rat maxillary alveolar bone defect model in comparison to conventional gypsum material.

Materials and Methods

Sample preparation

The powder part of CASPA cement was made through multi-step chemical synthesis process described elsewhere [9]. Initially, gypsum powder was prepared through wet reaction between $Ca(NO_3)_2 \cdot 4H_2O$ and concentrated H_2SO_4 (97–99%). The precipitation was done in isopropyl alcohol (99.5%) medium, following a proprietary drowning-out process in order to obtain uniform low-dimensional (5-10 micron sized) crystals. The precipitate was washed with isopropyl alcohol (99.5%) and heated at 125°C to obtain calcium sulfate hemihydrate powder. disodium hydrogen orthophosphate dihydrate powder was added in a quantity 2.5 wt. % and mixed in a ball mill to make the Bioactive Calcium Sulfate (CASPA) cement. The resultant cement powder was sterilized at 100°C in a hot air oven for 24 hours. The cement powder was mixed with sterile deionized distilled water in the prescribed powder liquid ratio (0.6ml/gm) to form the cement. Commercially available high pure gypsum was heated at 125°C to obtain the hemihydrate form which served as the control material (CAS). Same liquid medium in the same wetting ratio was used to make the control cement.

The setting time of the cements were determined using a custom made Vicat type apparatus, mentioned in an earlier work [8]. It consists of a steel needle of 1 mm diameter moving vertically under a constant load of 100 g force. A stainless-steel trough of 5 mm depth is kept below to hold the paste and a dial gauge was used to record the movement of the needle to an accuracy of 1/100 of an mm. The cement powder was mixed with deionized distilled water at a powder liquid ratio of 1 g/600µl for 1 min, and loaded into the sample holder. The needle was allowed to penetrate into it and the penetration depth readings was taken at time intervals of 30 s. An initial steep decrease in penetration depth is taken as the setting time.

For cell culture experiment, cement discs were prepared by placing the mixed mass in circular silicon molds of 4 mm inner diameter and 1mm height, in a sterile environment. Once set, the cement discs were carefully unmolded, and further sterilized at 100°C in a hot air oven for 24 hours.

Periodontal ligament cell isolation and characterization

The isolation and culture of human periodontal ligament cells (hPDLCs) were carried out after obtaining necessary approvals from the Institutional Ethics Committee, Sree Chitra Tirunal Institute of Medical Science and Technology, Thiruvananthapuram. Anonymous, discarded, extracted teeth were collected from Govt. Dental College, Thiruvananthapuram, for the cell isolation.

Isolation of periodontal ligament cells

The collected teeth were transferred in a tissue collection medium composed of sterile Phosphate Buffered Saline (PBS) containing antibacterial and antifungal agents. The teeth samples were transferred to a class II Biosafety cabinet and the periodontal ligament tissue adhering to the tooth root surface were gently scrapped off and collected in a sterile tissue culture petri dish. The tissue fragments were cultured using Minimum Essential Medium á modification (áMEM) with 10% Fetal Bovine Serum (FBS), and 100 IU Penicillin/ Streptomycin (Gibco). The explants were monitored daily for cell outgrowths under inverted phase contrast microscope. Once the hPDL cells from the explant reached sufficient quantity, the cells were sub cultured and transferred to cell culture flasks to obtain sufficient number of cells for experiments.

Characterization of periodontal ligament cells

The hPDLCs in passage 2 were seeded onto 1cm^2 glass coverslips at a density of 5×10^3 cells/cm^2 and cultured for 48h. The cells were fixed using 4% Paraformaldehyde (PFA) solution at room temperature for 1h, rinsed with PBS, permeabilized using 0.1% triton X100 for 1 min, and nonspecific antibody blocking was carried out using 10% Bovine Serum Albumin (BSA) for 30 minutes. Thereafter, the cells were incubated overnight at 4 ºC with the corresponding primary antibodies to characteristic marker proteins - Vimentin, Cementum Membrane Protein (CEMP), Scleraxis, Dentin Matrix Protein 1 (DMP1), CD 90 (Thy 1), CD 105 (Endoglin), CD 73 (ecto-5'-nucleotidase) and Stro 1. After incubation, the unbound primary antibodies were removed by rinsing and the cells were incubated with corresponding secondary antibodies (AlexaFluor 488 and AlexaFluor 546, Abcam) for 1 h in dark, at room temperature. The unbound secondary antibody was rinsed off using PBS and the cell nuclei were stained using Hoechst 33258 (0.5ig/ml in PBS) for 1 min. The stained cells were viewed using inverted fluorescence microscope (Leica DMI 6000, Germany) and Confocal Laser Scanning microscope (CLSM) (Olympus) and images were taken.

Cell – material interactions

The interactions of hPDLCs to the cements are to be studied to understand the cytocompatibility for the use as alveolar bone graft substitutes. For the purpose, CASPA and CAS cements were subjected to cytotoxicity test (in direct contact mode) and metabolic activity assessment (through MTT assay) using the isolated characterized hPDL cells, *in vitro*. Cell viability and morphology on the materials were analysed using staining techniques in optical microscope. In addition, the cell adhesion on the material surface was observed in scanning electron microscopy.

Direct contact test

The cells were seeded on 24 well cell culture plates (Nunc, Thermofischer) at a density of 3×10^4 cells per well and cultured for 24 h. The set cement discs of 4 mm diameter were carefully placed over the cell monolayer and incubated for 24 h. The cell response was analyzed under an inverted phase contrast microscope (Nikon TS100, Japan) and the cytotoxicity was graded from 0 to 4 (0 no cytotoxicity, 1 – slight cytotoxicity, 2 mild cytotoxicity, 3 moderate cytotoxicity, and 4 severe cytotoxicity) based on the morphology, cell lysis, cell detachment and vacuolization of the cells around the material.

MTT assay

The metabolic activity of hPDL cells in presence of the materials were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. The cells were seeded into 24 well cell culture plate at a density of $3´10^4$ cells per well and cultured for 24 h, after which, the CAS and CASPA discs (4 mm diameter) were carefully placed on the cell layer and cultured for 24 h. The hPDLCs cultured without the test materials were taken as the negative control (cell control) and the cells treated with 0.13% phenol was taken as the positive control (toxic control). The discs were removed from the cell monolayer, the culture medium was removed, and the cells were incubated with 200il of freshly prepared 1mg/ml MTT solution for 2-4 h in the dark. The formazan crystals formed by the metabolically active cells were dissolved in 200 il isopropanol and absorbance was measured as optical density values in a microplate reader (Biotek, USA) at 570 nm. The percentage metabolic activity of the cells was calculated from the optical density (OD) values obtained, using the equation,

$$\text{Cell activity (\%)} = [OD_{test}/OD_{control}] \times 100$$

Cell morphology and adhesion on the material surface

The cell morphology, adhesion and spreading on the cement samples was confirmed using SEM to visualize the lamellipodia and even minute filopodial extensions. The cells cultured on CAS and CASPA discs for 24 h were fixed with 2.5 % glutaraldehyde for 1 h, washed five times with PB solution [0.2M disodium hydrogen phosphate and sodium dihydrogen phosphate in deionised water], and serial dehydration was done with ascending concentrations of ethanol (30%, 50%, 70%, 90% and 100%) and isoamyl acetate. The samples were dried in a critical point dryer and sputter coated with gold, before SEM imaging (FEI Quanta).

Osteogenic induction potential of the cements

To understand the osteogenicity of the materials, the hPDL cells were cultured *in vitro* in presence of the materials and mineralization of the cells were observed. The cells in passage 3 were seeded into 6 well cell culture plates at a density 10^4 cells/cm^2, the respective cement discs were placed carefully over the cell monolayer, and cultured for 7, 14 and 21 days using regular cell culture medium (áMEM). The hPDL cells cultured alone (without the test samples) in regular cell culture medium, was taken as negative control. The mineralization of hPDL cells in the presence of the cements was evaluated histologically using Alizarin red and Von Kossa staining techniques. The expression of specific osteogenic markers by hPDL cells in the presence of the cements was carried out by ICC/IF staining.

Alizarin red staining

The calcium deposits by hPDL cells in the presence of the cement discs was evaluated using Alizarin red dye that can chelate calcium deposits so that the mineralized nodules appear red. The biomaterial discs were carefully removed from the cell monolayer at 7, 14 and 21 days, washed with sterile PBS and fixed using 4% PFA. The cell monolayer was rinsed with PBS and incubated with freshly prepared 4% alizarin red solution of pH 4.2, for 30 min at room temperature. The solution was discarded, samples were rinsed with deionized water, viewed under microscope (Nikon) and imaged to visualize the calcium deposits stained red.

Von Kossa staining

The phosphate deposition by hPDLCs in the presence of CAS and CASPA was evaluated by Von Kossa staining using 2% silver nitrate solution. The cells in control and test groups at 7, 14, and 21 days were fixed with 4% PFA for 1 h. The fixed cells were rinsed with PBS, incubated with freshly prepared 2% silver nitrate solution for 30 min at room temperature, exposed to UV light for 30 min, and rinsed in deionized water. The brown-black deposits for phosphates were visualized under brightfield microscope (Nikon TS100, Japan) and images were taken.

Osteogenic marker expression

The osteogenic marker expression by the hPDL cells on CAS and CASPA cements were carried out by ICC/IF staining. The cells were seeded onto sterile 1 cm^2 glass cover slips coated with a thin layer of the respective cement. The cells were fixed at day 7 with 4% PFA, rinsed with PBS, permeabilized and non-specific antibodies were blocked using 10% BSA. The cells were the incubated overnight at 4ºC, with primary antibodies to Alkaline Phosphatase, Osteonectin, DMP 1 and Collagen Type I. The unbound primary antibodies were rinsed off and the cells were incubated with the corresponding secondary antibodies (AlexaFluor 488 and AlexaFluor 545), for 1 h in dark. The cell nuclei were counterstained using Hoechst 33258. The cells were viewed under Fluorescence microscope using suitable filters and images were taken.

Pre-clinical study of the cement

Preclinical usage tests using suitable animal models need to be carried out for clinical translation. The in vivo pre-clinical study was conducted after obtaining the necessary Institutional Animal Ethics Committee clearance (IAEC), and all the procedures were carried out ethically. The CASPA cement was evaluated as alveolar bone graft substitute in a rat maxillary alveolar bone defect model with CAS as control.

Animal selection, defect creation and alveolar bone graft placement

Sprague Dawley rats of either sex, weighing above 350 g were selected for the studies. Total 3 defect sites were dedicated to each test group and sham control. Since bilateral defects were created, a total of six animals were used for the study. Each defect site was taken as a single unit and the time duration was fixed at 1month /4 weeks.

Briefly, the selected animals were weighed and anaesthetized using Ketamine 75 mg/kg body weight and xylazine 10 mg/kg body weight, taken in the same syringe and administered subcutaneously on either thigh. Once anaesthetized, the animals were placed on their backs securely on a special surgical platform and the jaws were kept open using elastic bands. The mucosa adjacent to the mesio-buccal root of the first molar was reflected along with the periosteum. Approximately 4 mm of alveolar bone surrounding the tooth root was removed carefully using tungsten carbide burs of 0.8 mm diameter at a speed of 2300 rpm, under ambient cooling using cold saline irrigation. The respective cements were mixed using a sterile glass slab and spatula the defects were packed with the corresponding cement to conformally fill the defects, so that they were in close contact with the tooth root and alveolar bone, till the gingival attachment level. The gingival tissues were repositioned and sutured with 6-0 Vicryl (polyglactin 910; Ethicon). The bone defects without the cements were considered as the sham control.

Histopathology evaluations

At the end 1 month, the animals were euthanized using CO_2 inhalation. The craniofacial area containing the maxillary alveolar bone was harvested and fixed in 10% neutral buffered formalin by completely immersing the tissue specimen for 48 h. After fixation, gross dissection of the maxillary alveolar bone was carried out and the specimens were embedded in clear Poly Methyl Methacrylate (PMMA). The PMMA embedded tissue specimens were sectioned using a hard tissue microtome (Struers Accutome 100) to get PMMA embedded sections of 100 µm thickness. The specimens were viewed under microscope and the selected sections were stained using Van Gieson (for bone) and Stevenel's blue for osteoblasts cells. The stained cells were viewed and images were taken.

Results

Biomaterial preparation and characterization

The pictures of the morphological observation of the conventional gypsum powder (CAS) and the newly synthesized gypsum (CASPA) using SEM are given in figure 1. CAS particles displays notable diversity in both size and morphology, whereas CASPA powder shows fine sized particles in a narrow range in sub-micron scale. These powders are used to make the cement after the thermal conversion to hemihydrate of calcium sulfate.

The calcium sulfate hemihydrate powder was obtained by heating the calcium sulfate dihydrate (prepared from the wet synthesis technique) at 130°C for 18 h. Bioactive calcium sulfate was obtained by thoroughly

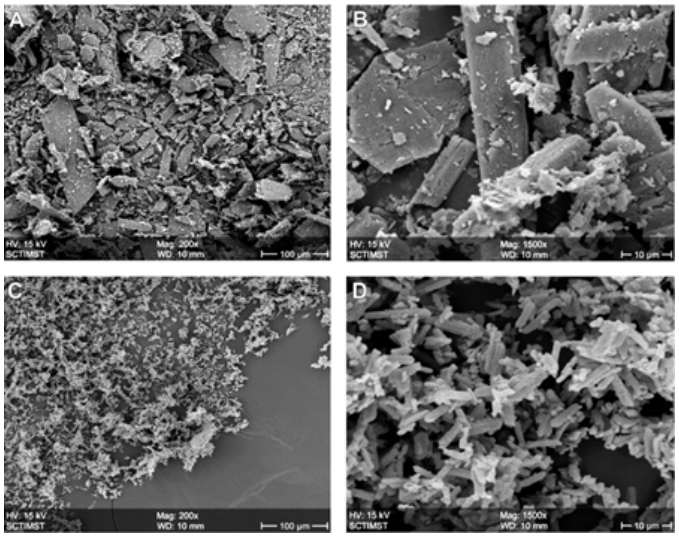

Figure 1: Morphological comparison of the CAS (pictures A and B) and CASPA (pictures C and D). A and C are in 200X and B and D are in 1500X magnification. CAS particles, the conventional material, have a notable diversity in both size and morphology. CASPA powder obtained through the proprietary drowning out method, shows a uniform particle morphology in the size range 5-10 microns in length

mixing 2.5% by weight, disodium hydrogen orthophosphate in powder form and a uniform cement powder was obtained. The cements in powder form, when mixed with deionized distilled water, set to a fast-setting moldable mass. The CAS exhibited a working time of 2 minutes and a setting time of 6 ± 1 minute. The CASPA cement exhibited a working time of 3 minutes and a setting time of 8 ± 2 minutes.

Periodontal ligament cell isolation and characterization

The cell outgrowths from the periodontal ligament explants were observed between 7 and 21 days. The cells were spindle in shape with fibroblast morphology. The cells were plastic adherent and attained confluency within 48 to 72 h after cell outgrowths were noted.

The hPDLCs showed positive expression of the type III intermediate filament protein vimentin and the mesenchymal stem cell (MSC) markers CD 73, CD 90, CD 105 and stro 1. In addition, the hPDLCs showed positive expression for dentin matrix protein (DMP1), the ligament specific protein Scleraxis, and cementum membrane protein (CEMP) confirming their tissue of origin (figure 2).

Cell – Material Interactions

Cytotoxicity and cytocompatibility evaluations

The phase contrast images of hPDL cells cultured in direct contact with the bioactive cement discs shows no evidence of cell death (Cytotoxicity grade 0). The cells in the test group were adherent and maintained their spindle morphology throughout the experiment, similar to the control cells (figure 3A). The hPDL cell metabolic activity assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction

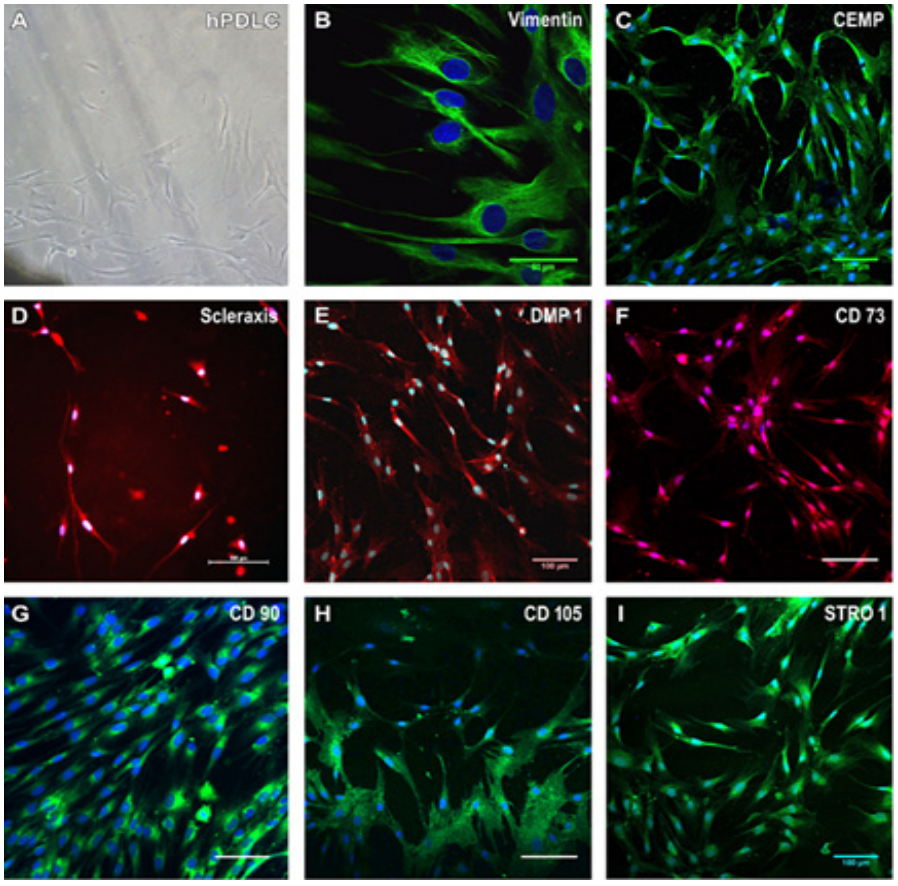

Figure 2: hPDLC outgrowth from tissue explant (A) and expression of specific markers by hPDL cells. B – Vimentin, C – Cementum Membrane Protein (CEMP), D – Scleraxis, E – Dentin Matrix Protein (DMP 1), F – CD 73, G – CD 90, H – CD 105, I – SRO 1

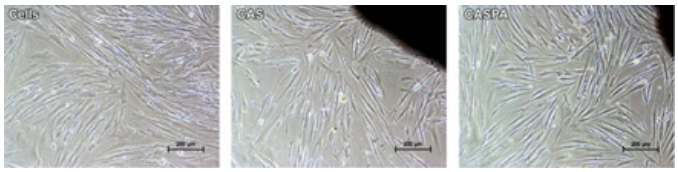

Figure 3A: hPDL cells in the presence of the cements showing no evidence of cytotoxicity

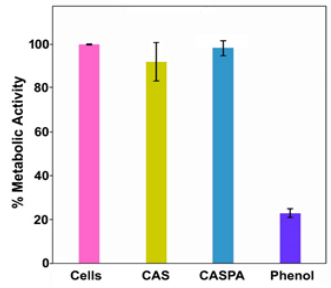

Figure 3B: Metaboloic activity of the samples through MTT Assay

assay showed a greater than 80% metabolic activity of the hPDL cells in the presence of the test materials CAS and CASPA (figure 3B).

Cell adhesion and morphology on cements

The hPDLCs seeded onto the CAS and CASPA discs, when viewed and imaged in SEM, confirmed the morphology of the cells, and evidence of cell spreading by means of filopodial and lamellipodial extensions were seen (figure 4).

Osteogenic induction potential of the cements

The osteogenic potential of the bioactive cements was studied using the primary human periodontal ligament cells. The cells showed evidence of mineralization and expressed osteogenic markers in the presence of the cement, even without an induction medium, confirming the osteoinduction properties of the cements. The detailed results are as follows:

Alizarin red staining

The hPDL cells cultured in the presence of CAS and CASPA discs showed evidence of calcium deposits at 7, 14 and 21 days (figure 5). hPDL cells in the presence of both CAS and CASPA exhibited mineralization as evidenced by the alizarin red stained calcium nodules, compared to the untreated control. This shows the mineralization potential of the cells in the presence of the biomaterials. This also gives an indication of the osteogenic induction potential of the cements.

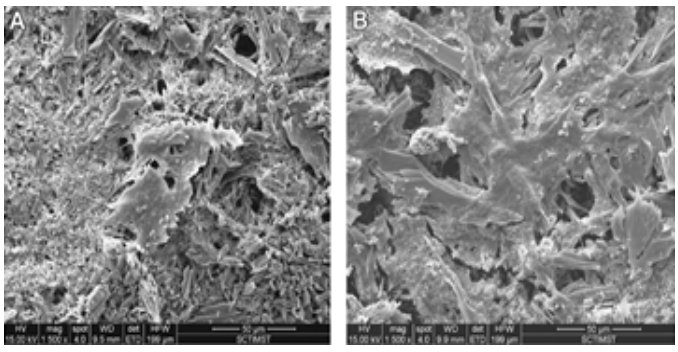

Figure 4: hPDL cells morphology and adhesion on the cement discs : Picture A - CAS; Picture B - CASPA

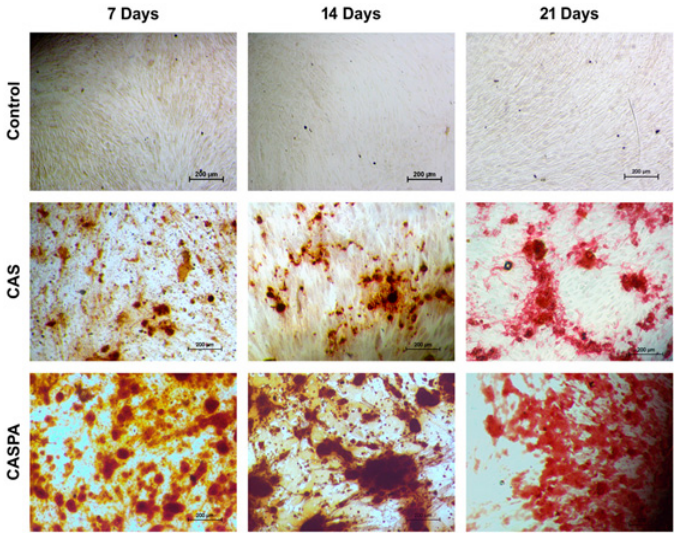

Figure 5: Alizarin red staining showing red calcium deposits by hPDL cells in the presence of CAS and CASPA cements

Von Kossa staining

The Von Kossa staining showed the presence of mineralized deposits by hPDL cells cultured in the presence of CAS and CASPA cements. Furthermore, the cells cultured in the presence of CASPA showed evidence of early mineralization at 14 days (figure 6). This is in accordance with the alizarin red values, which also showed evidence of early mineralization.

Osteogenic marker expression

The ICC/IF evaluation of hPDL cells for osteogenic markers in the presence of the CAS and CASPA cements showed positive expression of alkaline phosphatase, an enzyme responsible for the osteogenic differentiation of the cells. The ICC/IF also revealed the positive expression of osteonectin, the non-collagenous protein that promotes osteoblast differentiation and extracellular matrix assembly. Collagen matrix deposition was confirmed by the positive expression of Collagen I. The positive expression of the dentin matrix protein (DMP1), the extracellular matrix protein that regulates phosphate metabolism and bone formation also confirms the differentiation potential of the hPDL cells in the presence of the bioactive cements (figure 7).

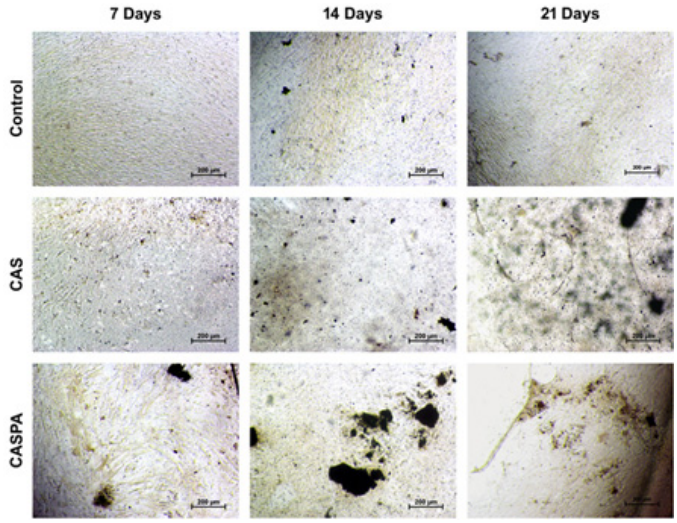

Figure 6: Von Kossa staining showing phosphate deposits by hPDL cells in the presence of CAS and CASPA cements

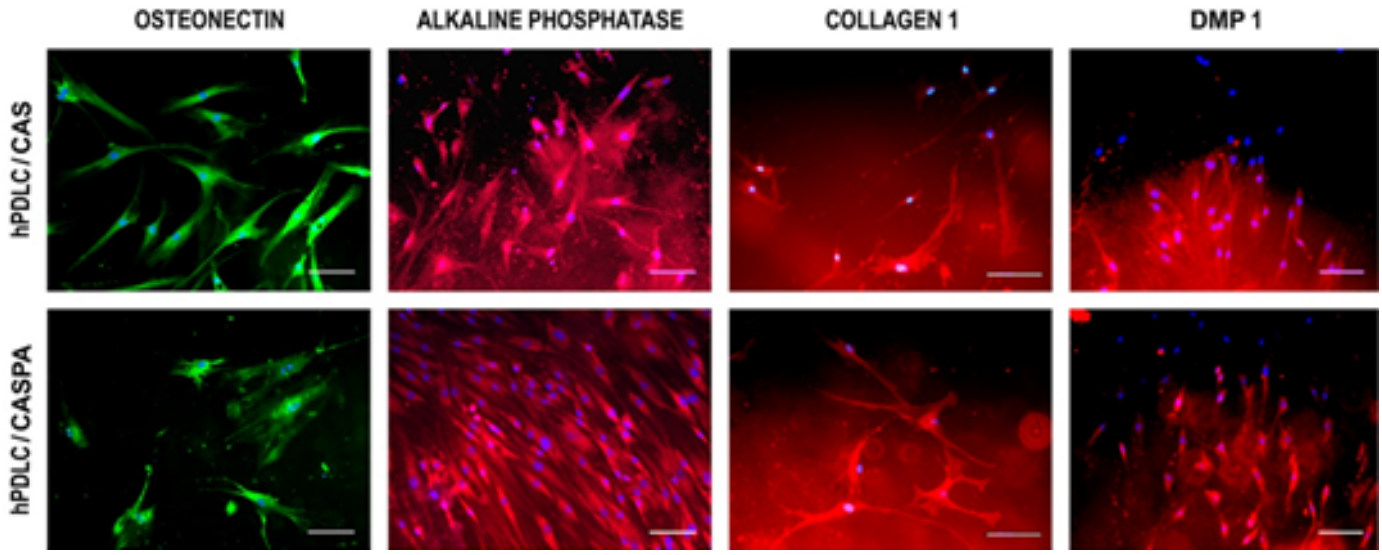

Figure 7: Expression of osteogenic markers by hPDLCs in the presence of CAS and CASPA cements

Histopathology evaluations of *in vivo* implantation of cements

The *in vivo* preclinical usage test in rat maxillary alveolar bone defect model was designed and carried out with calcium sulfate (CAS) and bioactive calcium sulfate (CASPA) as the alveolar bone graft substitutes. In this model, the period of 1 month is sufficient to show the bone regeneration at the defect site.

The histopathology evaluation of CAS and CASPA cements at 1 month duration is represented in **figure 8**. Evidence of new bone formation could be seen in the presence of CAS and CASPA cements, when compared to the SHAM control. In the CASPA group, the presence of remnant cement particles was noted in defect site, with new bone formation at the cement – bone interface. Resorption of the cement was noted, which was found to be at par with the new bone formation. The periodontal ligament space was maintained in width, without any evidence of external root resorption or ankylosis. In CAS cement group, the new bone formed was trabecular without any traces of the cement particles, suggesting an early and complete resorption of the cement. Here also, the periodontal ligament space was maintained without evident signs of ankylosis.

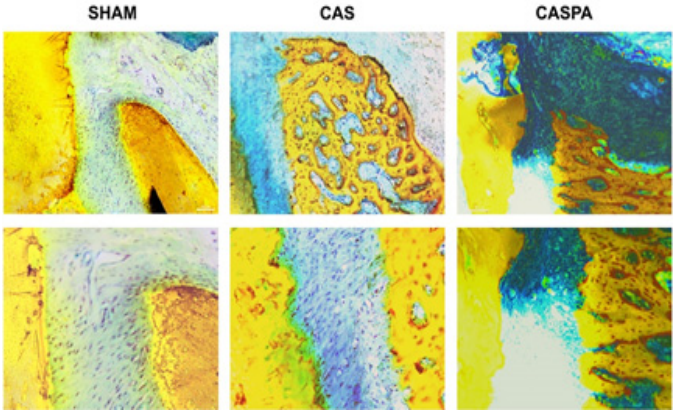

Figure 8: Histopathology evaluation of cements showing new bone formation with aligned periodontal ligament attachment. The periodontal ligament space is maintained with no evidence of ankylosis

Discussion

Regeneration of diseased alveolar bone in the presence of bioceramic biomaterials involves complex cellular and molecular interactions between the resident progenitor cells and the biomaterials [11]. The aim of this study was to develop and evaluate *in vitro* and *in vivo*, the cellular and tissue level responses to a resorbable bioactive cement for the regeneration of periodontal bone lost in periodontitis. The advantage of resorbing and osteoconductive cements is that they can act as a "barrier-graft", which provides a physical barrier that can prevent epithelial migration into the bony defect and serves as a graft [8 -10]. Sterile Calcium sulfate powder mixed with sterile liquid placed as a barrier over the bone graft in the GTR/GBR procedure exhibited similar healing compared to routinely used collagen barrier membrane [12]. Calcium sulfate cement emerged as a cost-effective bone graft substitute and bone filler with dental applications in treatment procedures for periodontal defects, as fillers around dental implants and for sinus augmentation [12,13]. However, calcium sulfate cements had certain disadvantages including inadequate mechanical strength, rapid resorption (faster than the formation of new bone at the defect), and local accumulation of sulfate ions and pH changes that can sometimes affect bone healing [14,15]. The cement tested in this study, CASPA was developed to overcome these limitations by modifying the particle morphology and infusing phosphate ions into them. Incorporation of phosphate ions is proven to improve the mechanical and biological properties of calcium sulfate cements [9,10].

The SEM observations of CASPA cement showed the particle size and distribution. The microstructure, i.e., the size and distribution of the particulates of the cement, influence it's *in vivo* performance related to resorption and bioactivity. Smaller particle sizes and larger porosities (gaps in between the particles) ensure better bioactivity and resorption [11].

The working time of a cement is the time elapsed from the start of mixing of the cement, until the setting mass reaches a semi-hard stage. This is the time available for the manipulation of the cement without dimensional changes, and indicates initiation of the setting reaction. Final setting time represents the time elapsed from the start of mixing till the cement becomes a rigid mass that cannot be manipulated. The final setting time indicates the completion of the hydration reaction of the cement. Both the cements exhibited adequate working and setting times optimal for placing the cements in surgical sites wherein they conform to the defect size as bone grafts. Once set to a solid mass, the cements can act as barriers preventing the epithelial migration [10].

Human periodontal ligament cells (hPDL cells) constitute the most suitable cell system to study the cytocompatibility and specific regenerative responses to the cements in vitro. A healthy population of hPDL cells were obtained through the standardised isolation and culture protocol. The cell phenotype was established by the presence of hPDL cell specific markers such as Cementum Membrane Protein (CEMP), and Scleraxis (figure 2) [16,17]. The fibroblast nature of the cells was confirmed by the positive expression of Vimentin (figure 2) [18]. Dentin matrix protein 1(DMP 1) is a non-collagenous protein of SIBLING (small integrin-binding ligand, N-linked glycoproteins) family, that is present in dental pulp and periodontal ligament. DMP 1 can enhance cell adhesion through RGD domains and induce differentiation of periodontal ligament cells. DMP 1 plays an important role in the differentiation of osteoblasts and biomineralization with its strong affinity to calcium and function as a nucleation protein facilitating hydroxyapatite nucleation and crystal formation by functioning as a nucleation protein in the ECM (figure 2) [19]. The presence of a sub population of stem cells within the heterogenous periodontal ligament cells was confirmed by the presence of the Mesenchymal stem cell markers such as CD 73, CD 105, CD 90 and STRO 1 (figure 2)[20].

The set cements exhibited adequate cytocompatibility and no evident cytotoxicity in the presence of hPDL cells (figure 3A) which is supported by MTT Assay (figure 3B). The cells retained their surface morphology and adapted closely to the cement surface, as evidenced by SEM images (figure 4). When cultured in the presence of the cements, the hPDL cells showed increased mineralization evidenced by Alizarin Red and Von Kossa staining (figure 5,6). The positive expression of the osteogenic markers – osteonectin, alkaline phosphatase, collagen 1 and DMP 1 confirms the osteogenic ability of the cements (figure 7) [23, 19].

The barrier-graft function of CASPA can be validated only through *in vivo* animal experiments. The surgically created intra-bony periodontal defect filled with both bioactive calcium sulfate (CASPA) and conventional calcium sulfate (CAS) cements showed enhanced bone formation. However, the CAS cement group showed premature resorption with larger trabeculae and no evident cement remnants visible in histopathology images (figure 8). This is in accordance to the literature reports of rapid resorption of calcium sulfate cements [9]. The CASPA group showed remnants of cement particles indicative of slower resorption of the cement, in tune with new bone formation and mineralization, with denser new bone formations in relation to the cements. Both the cements successfully prevented the epithelial overgrowth as evidenced by the adequate periodontal ligament space between the cementum and the alveolar bone, without any evidence of epithelial cells, thereby confirming the potential "barrier-graft" function [8]. Inflammatory cells were also absent reiterating the *in vivo* biocompatibility of the cements.

Conclusions

The newly developed bioactive calcium sulfate cement CASPA exhibited improved biological properties when compared to conventional calcium sulfate (gypsum) cement. Both cements showed excellent biocompatibility *in vitro* and *in vivo*. Both could be successfully used as barrier grafts for GTR/GBR treatment procedures for periodontal and alveolar bone regeneration. The resorption rate of CASPA was slower, at par with bone formation, an important requirement for bone graft biomaterials. This study advocates the use of CASPA, the bioactive calcium sulfate, for periodontal because it acts as a "barrier-graft" and lead to better bone regeneration than conventional calcium sulfate. However, carefully planned case studies and clinical trials are needed to further validate the biomaterial before routine clinical utilization.

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