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In Vitro evaluation of alginate, calcium iron silicate and extracellular matrix based scaffold for periodontal regeneration

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ABSTRACT

INTRODUCTION: Chronic periodontitis progressively disrupts periodontal tissues, eventually leading to tooth loss. In vertical bone loss defects, therapy includes surgical procedures, such as open flap debridement associated with scaffolds, grafts or membranes. The aim of the study is to fabricate a novel alginate, calcium iron silicate and ovine extracellular matrix based scaffold for periodontal regeneration

MATERIALS AND METHODS:
To 2% alginate solution 1mg/ml of CaFeSiO (calcium iron silicate) and ECM (extracellular matrix) was added to the solution. The solution is stirred to form homogeneous solution. The cross linked hydrogel was immersed in 0.15M $CaCl_2$ solution. The Ca^{+2} ion diffuses into the gel and crosslink with the alginate. The cross-linked hydrogel is washed twice and stored at 4 ^{0} C for further use. Scaffold characterisation was done by means of xray diffraction, raman spectrum and scanning electron microscope. Then hemocompatibility assay, swelling analysis, MTT compatibility test and differentiation test was done

RESULT: Dominated $2Ca_2SiO_4CaCO_3$ crystalline phase was noted owing to the highest composition of silica and calcium. Least swelling ratio was possessed by CaFeSiO ECM scaffold at 16.92%. Calcium iron silicate ECM scaffold has good agreement with red blood cells with minimal lysis of 1.2 % at the concentration of 10 mg/mL and differentiation test showed maximum proliferation of cells

CONCLUSION: The novel CaFeSiO ECM scaffold significantly enhances cell proliferation; it is highly hemocompatible, biocompatible and has enhanced mineralisation properties. Hence, incorporation of bioactive bioceramics induces the stability as well as enhances the regeneration properties to the tendon derived ECM.

KEYWORDS: Tissue engineering, Extracellular matrix, Alginate, Scaffold, Periodontal regeneration, Dental

INTRODUCTION

The multi-tissue system known as the periodontal apparatus, which holds teeth to the jawbones, is made up of the dental root cementum, the periodontal ligament, the gingiva with the dentogingival sulcus, and the alveolar bone(1). Chronic periodontitis gradually disrupts the periodontal tissues, which finally leads to tooth loss. Severe periodontitis affects 11% of persons worldwide and is harmful to the general public's health (2,3). The cementum that supports the teeth, the periodontal ligament and the alveolar bone are all impacted by the infectious disease known as periodontitis. Deep intrabony pocket patients with bony defects and furcation involvement are treated with surgical periodontal flap surgery using bone grafts and scaffolds.(4,5).

Multicomponent scaffolds are utilized in tissue engineering to construct tissues like bone, cartilage, and periodontal tissues (6).The development of the multicomponent scaffold approach has made the simultaneous regeneration of two or more tissues more feasible and effective. Multiple soft and hard tissues (periodontal ligament, gingiva, cementum, and bone) must respond in union in order for periodontal regeneration to produce a positive clinical result. For periodontal regeneration, tissue-engineered constructions must have complex 3-dimensional shapes, be of an appropriate size, and exhibit biomechanical stability over time (7,8)

Scaffolds made of extracellular matrix (ECM) are thought to be superior options due to their capacity for regeneration and ability to reduce inflammation (9). Cell survival, proliferation, and migration are encouraged by the binding of ECM proteins to cell surface integrins and other receptors. Clinical research shows that this results in effective tissue regeneration. It has also been demonstrated that cartilage ECM proteins from ovine sources can successfully direct stem cell differentiation in vitro.(10,11) Bioceramics like calcium silicate serve as a bioactive substance for bone regeneration. In addition to being osteoconductive and noncytotoxic, it possesses good bioactivity and degradability(12-16). The aim of the study is to fabricate a novel alginate, calcium iron silicate and ovine extracellular matrix based scaffold for periodontal regeneration.

MATERIALS AND METHODS

Preparation of calcium iron silicate

0.3 moles of tetraethyl orthosilicate was dissolved in 50 mL ethanol and water mixture (40mL of double distilled water added to 10mL ethanol). 0.03 mol nitric acid added to speed up the gelation process. 0.3 moles of calcium nitrate was dissolved in 50 mL of double distilled water and added into the aforementioned TEOS. 0.1 mol of iron nitrate was dissolved separately using 50mL of DD water and then added into the sources of silica and calcium. Dried at 100°C for 12 hours to remove the moisture content then sintered at 600°C for 3 hours to stabilize the crystalline phase formation.

Preparation of particulated cartilage ECM

Articular cartilage for the fabrication of CM particles was obtained from the articular joint of goat, as presented in published work [10]. Briefly, cartilage was fragmented, and further fine ECM microparticles were fabricated by pulverizing/devitalizing the cartilage ECM in pieces with a cryogenic mill (6770 Freezer-Mill, SPEX). The ECM-derived particles were Ivophilized (FreeZone-Triad, Labconco, USA), physically crosslinked and sterilized overnight using dehvdrothermal treatment at 110°C under vacuum

Fabrication of ECM/CaFeSiO incorporated Alginate hydrogel scaffold

Alginate based hydrogels were fabricated using divalent cations such as Ca^{+2}, Mg^{+2}, etc. as the ionic crosslinking agent. A stock solution of 2% alginate solution was prepared. To 2% alginate solution 1mg/ml of CaFeSiO and ECM was added to the solution. The solution is stirred to form homogeneous solution. The crosslink alginate, the hydrogel is immersed in 0.15M $CaCl_2$ solution. The Ca^{+2} ion diffuses into the gel and crosslink with the alginate. The cross-linked hydrogel is washed twice and stored at 4^{0} C for further use.

X ray Diffraction

X-ray diffraction (XRD) is a powerful technique to elucidate the crystallinity and phase of the synthesized material. The structural elucidation was examined with powder X-ray diffraction (model) with a voltage of 40 kV and a current of 30 mA using a Cu Kα X-ray radiation source (1.5406 Å) in the 2Θ range of 10°-80°

Scanning electron microscope (SEM) and Energy-dispersive spectrometry (EDS) analysis

The morphology, size and the elements present in the nanoparticles were analyzed using scanning electron microscope integrated with energy-dispersive spectrometry analyzer. The morphological characteristics of the sample was observed using scanning electron microscopy (SEM, JEOL, Tokyo, Japan) by coating with platinum via a sputter-coater at ambient temperature. The elements presented in the sample were determined by an energy-dispersive spectrometry analyzer integrated in the scanning electron microscope

SEM imaging of ECM-derived particles

ECM microparticles were observed and analyzed using scanning electron microscopy (SEM) Particles were fixed in 4% paraformaldehyde solution overnight. Microparticles were dehydrated through successive graded ethanol baths (10-100%), fixed in aluminium stubs, coated with gold and examined under a field emission scanning electron microscope (Tescan Mira FEG-SEM XMU, Libusina, Czech Republic). Images were analyzed with Image J to quantify microparticle size. Calcium silicate bioactive materials was prepared using tetra ethyl ortho silicate calcium nitrate,titanium tetra is propoxate, and zirconium nitrate. Fabricated sol dried using hot air oven and thermal treated at 600 °C for the materials stabilisation.

Hemocompatibility Assay

The solution was exposed to the three membranes, and the rates of hemolysis were assessed using a positive and negative control. The RBCs in the control sample exhibit total rupture in the positive control and complete protection in the negative control. In this experiment, 950ul of double-distilled water was combined with 50ul of blood as a positive control. As a negative control, 950ul of phosphate buffer saline (PBS) and 50ul of blood were combined. Positive controls result in full RBC rupture and haemoglobin release, which turns the fluid completely crimson. Only mechanical injury, caused by PBS in the negative control, results in limited hemolysis.

Swelling Ratio

In order to determine the hydrogel scaffolds' water content (percent) through test, 10 mg of freeze-dried scaffolds were immersed in 500µl lX of PBS at 37 °C. These scaffolds were removed from the PBS after 24 hours, weighed, and dabbed with a Kimwipe to remove any excess water from the surface. The following equation was used to determine the percentages of the swelling ratio.

$$\text{Swelling ratio (SR)} = ((W_w - W_0)/W_0) \times 100\%$$

The initial dry weight and the wet weight are W_0 and W_w respectively.

Cell Viability Assay [MTT] Analysis

10 mg of sample were prepared. The prepared samples were immersed in DMEM/F12 media formulated with 10 % FBS and 1% Penicillin-streptomycin. The media were collected after 24 hrs of immersion and treated with cells to test the compatibility. After 24hrs of culture, 10 µL/100 mL of MTT reagent (5 mg/mL stock) was added to the cultured cells and then incubate for 4 h to allow the formation of the formazan dye at 37℃. The medium is exchanged to DMSO (200 µL) and stand for 10min. The reaction product was transferred to a 96 well ELISA plate and A590 was measured with ELISA plate reader.

Alizarin Red Staining And Quantification

The cells are seeded onto the hydrogel (UV treated) and treated with differentiation media containing DMEM F12 supplemented with 10 mM β-glycerophosphate and 0.05 mM Ascorbic acid that aids osteogenic matrix formation. Alizarin red staining was performed to determine calcium deposition. Cell cultures were fixed with 3.7% formaldehyde in PBS for 30 min and then processed for Alizarin red staining. Specimens were incubated with 2% Alizarin red solution for 10 min. After that, cells were washed twice with 1X PBS. The image acquisition was carried out with a digital camera. For quantitative analysis after the image acquisition, 200 µl of DMSO was added to each well. The reaction product was transferred to a 96 well ELISA plate and the quantity of Alizarin was measured in a spectrophotometer at 405 nm.

STATISTICAL ANALYSIS

All values are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. A one-way ANOVA (analysis of variance) was used to test for significant differences, and multiple comparisons were performed using Scheffe's method. Statistical significance was set at $p < 0.05$.

RESULTS

1.X- Ray Diffraction

X-ray diffraction patterns are the tool to analyze the crystalline phase of synthesized calcium iron silicate oxide bio-ceramics. Silica is an amorphous material, even though due to the crystallization of calcium iron on the surface of silica, initiates the semi-crystalline attribution. In which it was found the crystalline phases of $CaFe_2O_{5.12}$ (JCPDS -49-1555) and $2Ca_2SiO_4CaCO_3$ (JCPDS - 04-0640). Dominated $2Ca_2SiO_4CaCO_3$ crystalline phase was noted owing to the highest composition of silica and calcium.

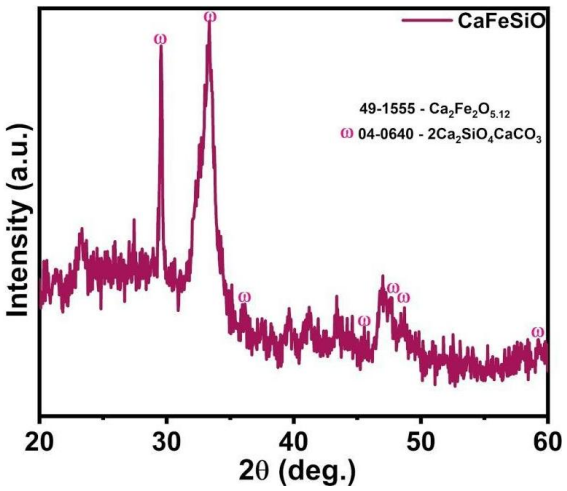

Figure 1 - X-ray diffraction Pattern of Iron infused calcium silicate ECM scaffold

2.Raman Spectra

A Raman spectrum is one of the significant tools to understand the function group properties of the fabricated materials. Synthesized Calcium silicate integration with functional group was investigated by the Raman spectra. Silica bonding with oxygen is the major bonding in this material that was evidently observed in the region around 960 cm^{-1} (Si-O-Si) that authenticates the formation of silicate based material. To further confirm this, Q^4 Si-O-Si stretching was observed in 1045 cm^{-1}.

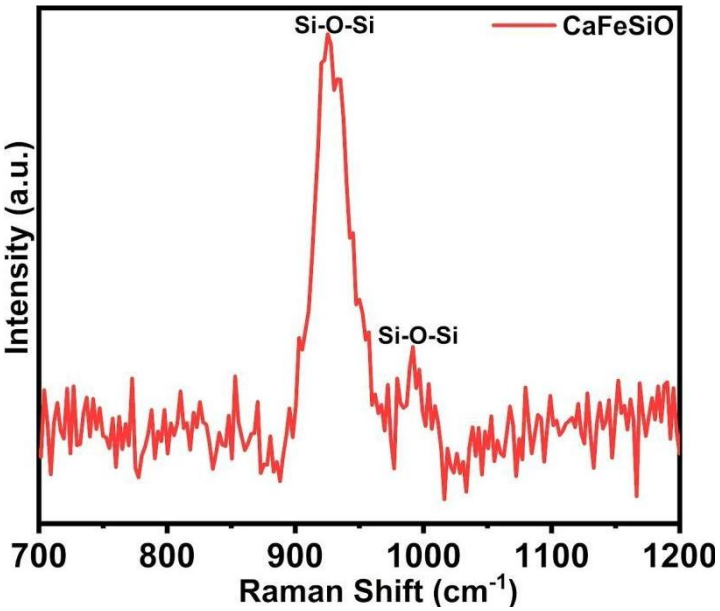

Figure 2 - Raman Spectrum of synthesized CaFeSiO ECM scaffold

3.Field Emission Scanning Electron Microscopy & Energy Dispersive Spectroscopy

FE-SEM is one of the significant tools to analyze morphology parameters. Similarly, EDX is used to study the elemental composition of the prepared materials. Calcium iron silicate is a bio-ceramic based material therefore a small spherical aggregate on the silicate gel matrix was noted through FE-SEM. Respective calcium, silicate, iron, carbon and oxygen authenticate the material formation via EDX spectra. Since silica is a base matrix, calcium may crystallize on the silica balls. Therefore a higher percentage of calcium was obtained than the silicate in the EDS spectra.

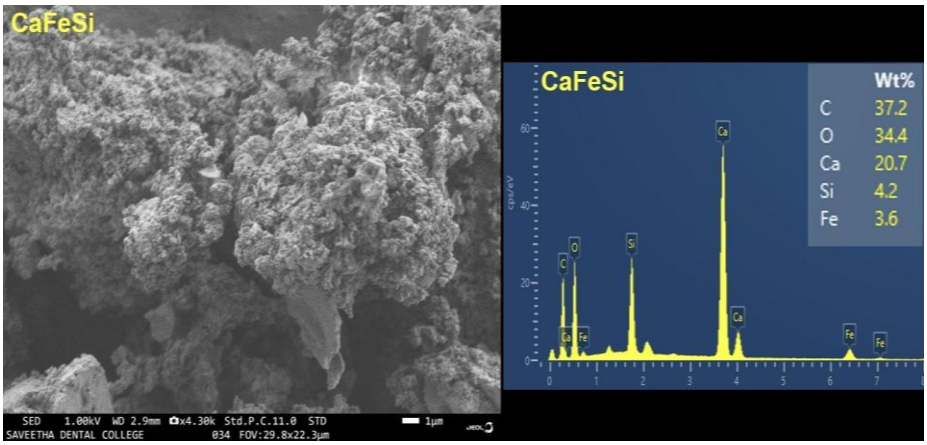

Figure 3 – Morphological and Elemental composition of the synthesized iron infused calcium silicate ECM scaffold

4.Hemocompatibility Assay

Blood compatibility is a primary tool to analyze the compatibility of synthesis calcium iron silicate ECM scaffold with red blood corpuscles. According to ASTM (American Standard for Testing Materials) upto 5 % lysis is acceptable, which indicates the materials that damages RBCs below 5% can be authorized for bio-medical applications. Hence, this calcium iron silicate ECM scaffold has good agreement with red blood cells with minimal lysis of 1.2 % at the concentration of 10 mg/mL. From this result it can be observed that CaFeSiO ECM scaffold is a potential material for regenerative application.

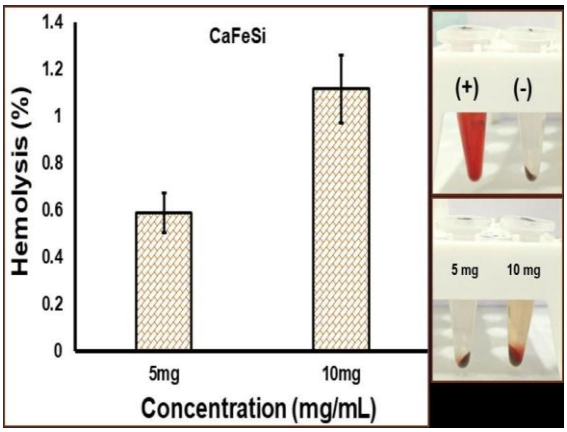

Figure 4 – Blood Compatibility assessment of synthesized CaFeSi (Histogram and micro-centrifuge tube represents the compatible RBCs)

5.Swelling Analysis

The amount of fluid absorbed or imbibed by each sample is calculated and the values are compared as shown. We see that the least swelling ratio is possessed by CaFeSiO ECM scaffold at 16.92%.

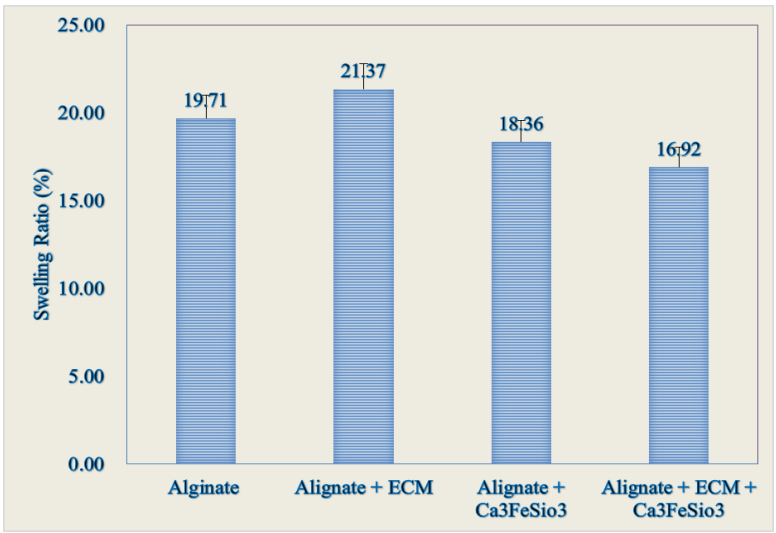

Figure 5 – Swelling test graph

6. MTT Compatibility Analysis:-

It was noticed that the highest degree of cell viability was present in the sample containing alginate and ECM

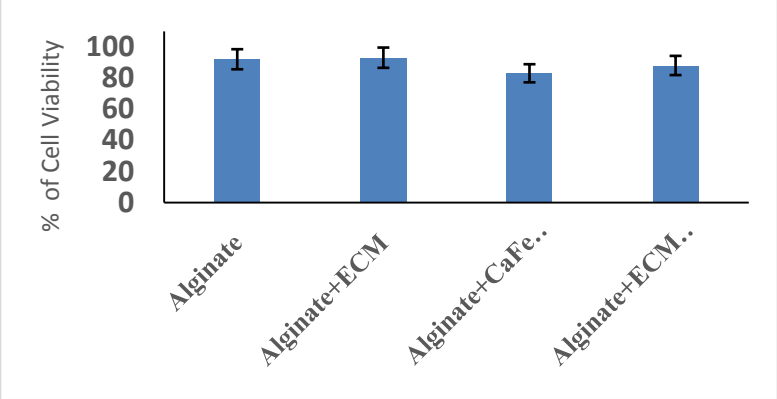

Figure 6. - The viability rates of the PDLSCs in the samples are analyzed by the MTT assay and the values are compared as shown.

7.DIFFERENTIATION ANALYSIS

The results showed the maximum proliferation of cells was for the ECM/CaFeSiO incorporated Alginate hydrogel scaffold

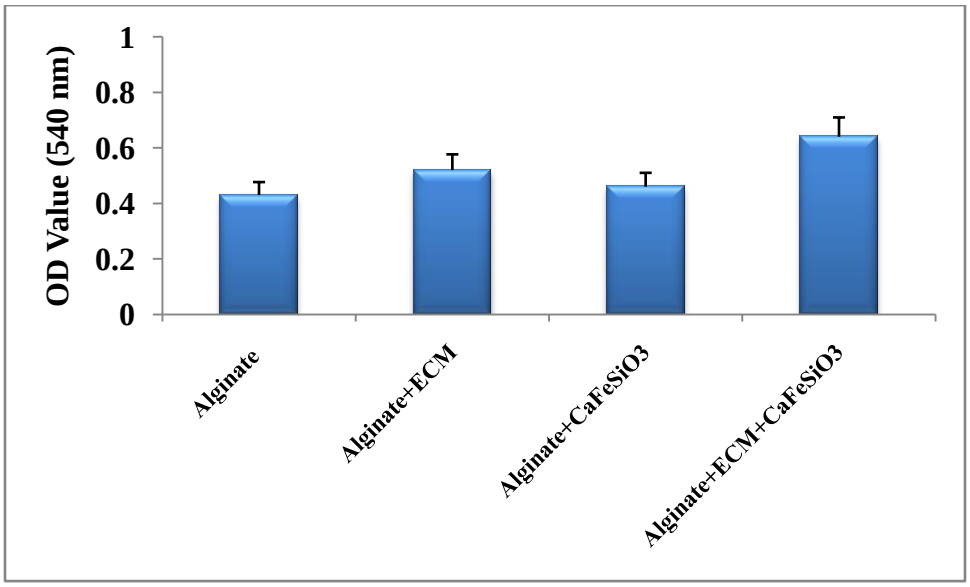

Figure 7 – Differentiation test of samples

DISCUSSION

The periodontium is a complex structure, and the management of periodontitis depends on its coordinated regeneration. The capacity to mimic the periodontal tissue composition and structure is a prerequisite for periodontal tissue engineering methods(17,18). Due to their excellent biocompatibility and bioactivity, as well as their moderate mechanical capabilities for supporting cells, biological scaffolds have gained substantial interest in the field of periodontal tissue engineering in recent years(19). Decellularized extracellular matrix scaffolds, which are biomaterials made from human or animal organs/tissues after the removal of immunogenic cellular components by decellularized technologies, are one of the different types of scaffolds that are receiving attention(20,21).

In our study, bioactive calcium silicate materials were synthesized by sol-gel method. Further we analyzed the structural, morphological and biocompatible properties of the material. $CaFe_2O_{5.12}$ and $2Ca_2SiO_4CaCO_3$ mineral phases was observed through XRD. Interconnected small spherical and rods were visibly noted on the silicate network along with relevant elemental compositions from EDS Spectra. Acceptable blood compatibility was procured from the blood compatibility assessment. The swelling ratio was around 16.92%. for the CaFeSiO ECM. This proves that the addition of bioactive calcium iron silicate decreases the swelling rate and increases the effectiveness of the scaffolds with the extracellular matrix of tendon and enhances the nutritional flow. From the differentiation analysis, we see that there is more tendon cell formation in case of theCaFeSiO ECM scaffold sample than the control. This proves that our scaffold has very high tenogenic potential and this can help in potential regeneration of periodontal tissues.

In a number of tissue engineering techniques, scaffolds and decellularized cell-derived extracellular matrix have been used to increase the bioactivity of the latter (23). In a previous study, electrospun polycaprolactone, chitosan and ECM from human periodontal Ligament Stem Cells derived scaffolds were developed and it was found to enhance cell proliferation(24,25).

Our study describes the first application of calcium iron silicate scaffolds in combination with ECM from the ovine tendon source for periodontal regeneration and illustrates its potential as a novel alternative approach to the management of periodontitis. The suitability of using the novel scaffold as a secure treatment modality for the treatment of periodontal abnormalities including intrabony defects and furcation defects must be thoroughly investigated by animal models in future.

CONCLUSION

The novel CaFeSiO ECM scaffold significantly enhances cell proliferation; it is highly hemocompatible, biocompatible and has enhanced mineralisation properties. Hence, incorporation of bioactive bioceramics induces the stability as well as enhances the regeneration properties to the tendon derived ECM. Thus, our novel scaffold is a promising candidate for regeneration of periodontal tissues.

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CONFLICT OF INTEREST

The author declares that there was no conflict of interest in the present study.

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