The Effect of Fibrin on the Differentiation of Dental Pulp Stem Cells

ABSTRACT

An emerging area in dentistry, regenerative endodontic therapy (RET) has been shown to be effective in treating immature permanent teeth. Normally accompanied by induced periapical bleeding, the procedure of root elongation has demonstrated a thickening of the canal walls, apical closure, and root lengthening, indicating its success as a regenerative process. Such regeneration in the tooth lies within the ability to restore function using the stem cells resident in the dental pulp (DPSCs). It is therefore critical to establish a model for this process, so that it can be developed for application to teeth in the general population. The challenge, however, is to determine which procedures will induce DPSC differentiation along an odontogenic lineage. As collagen is the natural matrix for the DPSC and fibrinogen is the major component of blood, an enhancement of DPSC differentiation may be achieved by inducing a clot. As a result, we developed a platform for probing this model, first using gelatin (far less expensive than collagen) to explore the influence of fibrin. DPSCs were plated on a scaffold with 9:5 gelatin/fibrinogen, where mTG and thrombin were used to crosslink the gelatin and fibrinogen, and incubated for approximately one month in osteogenic media without dexamethasone. RTqPCR demonstrated a significantly greater upregulation of osteocalcin in the DPSCs on such scaffolds, OCN being a critical component and definitive indicator of osteo/odontogenic differentiation. Consistent with this up-regulation, scanning electron microscopy indicated extensive bio-mineralization on the fibrin scaffolds. The deposition of DPSCs on collagen substrates is currently being evaluated.

LITERATURE REVIEW

Pluripotent stem cells have many potential uses in regenerative medicine. Of particular interest are dental pulp stem cells (DPSCs) because of their ability to differentiate into neurons, cardiomyocytes, chondrocytes, osteoblasts, liver cells and pancreatic β cells. Their relatively low immunogenicity makes them ideal for applications in regenerative medicine. Today, some of the diseases that can be cured with DPSCs include Type 1 diabetes, neurological diseases, immunodeficiency diseases, and diseases of bone and cartilage. This broad range serves to prove their versatility in differentiation, which is a large benefit to using DPSCs. One possible issue in the therapeutic applications is the progressive shortening of the telomeres that reduces the suitability of transplantation. However, DPSCs do allow for possible future work in whole tooth regeneration. Along with treatment of disease, DPSCs are now being used in orthopedic surgeries as well. While still primarily experimental, neuronal disorders can be treated with DPSC because of their ability to differentiate into functionally active neurons. Different factors, including growth factors, transcriptional factors, and extracellular matrix proteins, can induce differentiation into cell types including odontoblasts, osteoblasts, chondrocytes, cardiomyocytes, neuron cells, adipocytes, corneal epithelial cells, melanoma cells, and insulin secreting Beta cells [1].

However, one area in which dental pulp stem cells have proved relatively helpful is that of root elongation in regenerative endodontic therapy (RET). The repair and restoration of teeth can be widely researched and tested with human subjects because of its low risk and generally healthy subjects. Therefore, the endodontic regenerative medicine is a major field of study in the broader field of regenerative medicine, as the stem cells from teeth can also be used in deriving other regenerative therapies.

The success of root elongation in treating immature permanent teeth can be seen by the resulting thickening of the canal walls, apical closure, and root lengthening. The cause of this success is the access to DPSCs within those teeth during the process. [8, 11] To extend this process to general teeth, we must first model it. Dental root genesis is important in the study of regenerative medicine because of the interaction with surrounding tissue and nerve bundles. Genetic moderation of osteodentinogenic capacity determines root formation, so the stem cells used would have to have the correct differentiability. However, DPSCs have already successfully been used to form a bio-root in the molar of a pig. [9] According to an experiment in revascularization of an immature tooth, fibrin may be a potentially ideal scaffold for the process because it enhances cell proliferation and differentiation and can act as a matrix for tissue ingrowth. [10]

This study's focus was on the ability of the extracellular matrix to affect the proliferation and differentiation of DPSCs. Fibrin scaffolds have been found to be highly suitable for dental pulp regeneration because they promote a pulp-like tissue formation. Just some of the benefits of fibrin include its superior cytocompatibility and physiological degradation kinetics and nontoxic degradation products. Its formation of an extracellular matrix makes it ideal for culturing DPSC *in vitro*. One consideration that must be taken when using fibrin as a scaffold is its absence of antibacterial activity; it should be combined with an antibacterial substance to prevent microorganisms from hindering the regeneration of dental pulp. For this purpose, PenStrep was included in our initial cell solution; an addition of an antibacterial substance to the matrix itself was deemed unnecessary because of the *in vitro* nature of our experiment. A natural insoluble protein, fibrin is produced from the polymerization of fibrinogen by thrombin enzymes in blood clot formation. The resulting fibrous network, originally intended to begin hemostasis in the

wound healing process, can be applied to creating a scaffold for cell culturing. Another benefit of fibrin usage is its relatively facile structural property manipulation by controlling concentration and ionic strength. This allows for optimization to mimic the viscoelastic properties of connective tissue ECM, fast nutrient diffusion, and homogenous cell encapsulation [2]. The impact of fibrin scaffolds on dental pulp regeneration led us to study their potential impact on dental pulp stem cells. If endodontic conditions can be nearly recreated, this opens up numerous possibilities for stem cell differentiation.

An injectable hydrogel of fibrin with polyethylene glycol (PEG) side chains has been used as a scaffold for dental pulp stem cells and resulted in high cell viability and observable influence in odontogenic differentiation in a study designed specifically for applications in regenerative endodontics [3]. Fibrin glue (fibrin and thrombin solution) has also been shown to increase proliferation of DPSCs [5]. Other studies have used PEG modification of fibrin to decelerate the otherwise rapid degradation of fibrin in extracellular matrix. The resulting hybrid hydrogel has some benefits, such as photo-cross-linking and tunability; however, increasing PEG-DA (polyethylene glycol diacrylate) cross-linking results in a decrease in cell viability and adhesion [3-4].

Gelatin, on the other hand, can be combined with fibrin in a hydrogel to influence morphology, decelerate degradation, and optimize cellular activities such as proliferation and differentiation [6]. Although the exact nature of the effect of gel stiffness on DPSC differentiation remains unclear, one point of agreement is that the modification of gel stiffness can influence the mode of differentiation [2]. Gelatin would likewise make the hydrogel a more suitable bioink, allowing for potential applications in the bioprinting of complex tissue with locally induced differentiation. The pore size in such a hydrogel is close to that of a human

dentinal tubule. Cell viability in a gelatin-fibrin hydrogel is also significantly higher; according to one study, over 90% [7]. As a result, while the many benefits of PEG modification of fibrin to overcome the rapid degradation were recognized, gelatin was implemented in the hydrogels to maximize biocompatibility and observe the effects of a gelatin-fibrin scaffold on the proliferation and differentiation of DPSCs.

METHODS

Dental Pulp Stem Cell Culturing

Due to their applications in odontogenic regenerative medicine, potentially augmented upon interaction with fibrin, dental pulp stem cells (DPSCs) were the cell type employed during the investigation. In preparation for plating, 13 y.o. DPSCs were cultured for 3-4 days, prior to transfer to the hydrogels. 15 mL of MEM Alpha, a nutrient-filled medium, was added to a 75 cm^3 flask, after being warmed to 37°C in a *** hot water bath. Once the medium was introduced (the serological pipette never touching the inside of the flask), 0.5 mL of DPSCs, stored in liquid nitrogen, were transferred to the flask, freed by dimethyl sulfoxide (DMSO) – washed away with PBS shortly after. The 75 cm^3 flask, kept horizontal, was then transferred to a 37°C 5% CO_2 biology incubator (***). In order to allow for proliferation, maintain proper pH, and remove any remaining DMSO, the MEM Alpha medium was changed every other day, with the DPSCs being washed three times with PBS beforehand, until the cells reached approximately 80% confluence, quantified using a hemocytometer and observed under an *** optical microscope.

Preparation of Hydrogels

In order to see the impact of fibrinogen on the differentiation and the proliferation of the DPSCs, two sets of hydrogels were created: gelatin hydrogels and gelatin + fibrinogen (GELFIB) hydrogels. The general composition ratio of the latter gels was 9 gelatin (15%) : 5 fibrinogen (12 mg/mL) : 1 (mTG (10%) + thrombin (1.5%)), the concentrations expressed as weight by volume; for the gels solely containing gelatin, thrombin was not included and PBS was added in place of fibrinogen. As a result, in order to fill each well with 2 mL solution, 1.2 mL of gelatin, 0.67 mL of fibrinogen, and 0.13 mL was to be added to each well. Thus, in order to create 30 hydrogels in total, each set of gels required 18 mL of gelatin, 10.05 mL of fibrinogen (in the case of GELFIB – PBS for gelatin), and 1.95 mL of mTG (with 3µL of thrombin being added to GELFIB).

In order to prepare the necessary 36 mL of gelatin solution, 1.5 mg of gelatin was first massed using a Mettler Toledo balance, to which 10 mL of PBS was added into a 15 mL test tube; this was repeated four times in order to obtain 40 mL of gelatin solution. Each tube was then secured on an incubating rotating waver at 60 °C and removed only when the gelatin was completely dissolved in solution, a process lasting approximately 1 hour. Removing each tube from the waver one at a time, 10 mL of gelatin was transferred to a 20 mL syringe attached and filtered through a 0.22 µm filter into a separate test tube. This filtration process was repeated four times until two test tubes, labeled by hydrogel group, each contained approximately 20 mL of gelatin; these were placed in the 37°C water bath until use.

In order to obtain the 12 mg/mL solution of fibrinogen, 10.05 mL of PBS was transferred to 120.06 mg of fibrinogen, stored in -40°C. Once the solution was prepared, it was transferred to -20°C, where it was stored until being heated to 37°C and filtered through a 20 mL syringe and 0.22 µm filter. 10.05 mL of this fibrinogen solution was transferred to the GELFIB 50 mL test tube, while 10.05 mL of PBS was placed in the gelatin-only counterpart.

10% microbial transglutaminase (mTG), an enzymatic cross linker for gelatin, was prepared by transferring 0.5 mg of mTG, initially stored at 4°C, to 5 mL PBS. In order to avoid light exposure and thus degradation, the mTG was placed in a test tube covered with aluminum foil. Once the PBS was added, following dissolution of mTG, the solution was filtered using a 5 mL syringe and 0.22 µm filter; the mTG solution was then incubated at 37°C 5% CO_2. Once warm, 1.95 mL of mTG was transferred, in the absence of light, to the 50 mL gelatin-only hydrogel test tube. 1.95 mL of mTG was then transferred to the GELFIB test tube, along with 3µL thrombin, stored in -80°C.

After mixing each hydrogel solution, 2 mL of each was added to its respective wells in a total of 6 12-well plate, with there being 15 hydrogels for each the gelatin-only and gelatin-fibrinogen samples: 3 RT-qPCR (x2 – for Days 12 and 31), 1 SEM (x2– for Days 12 and 31), 1 EVOS (x2– for Days 12 and 31), 3 Rheology, 2 NoDPSCs. Gels were washed and 2 mL MEM Alpha was added to each well 5 times, after which, all plates were placed into the incubator at 37°C 5% CO_2.

Plating DPSCs onto Hydrogels

Prior to plating the DPSCs onto the 20 cell-containing hydrogels, an augmented MEM Alpha medium mixture was created. To the MEM Alpha being used, 50 mL FBS (fetal bovine serum), 5 mL PenStrep, 5 mL β-glycerol phosphate, and 1 mL L-ascorbic acid.

The DPSCs were removed from the incubator and, after being checked under the microscope, the medium above was aspirated. In order for the DPSCs to fully detach from the bottom of the flask, 3 mL of trypsin was added to the flask, which was then placed in the incubator for 3 minutes. After doing so, 5 mL of the augmented MEM Alpha solution was added

and mixed in the flask, after which the entire solution was transferred to a 15 mL test tube which was centrifuged at a speed of ½ g for 8-10 minutes. The resulting surfactant was aspirated, and 7 mL of the MEM Alpha was added to the test tube.

Viewing 10µL of this cell solution under the *** optimal microscope indicated a cell concentration of 38.5 x 10^4 cells/mL. Desiring 8000 cells per each well and thus 12 x 10^4 cells for each of 2 test tubes created (containing 30 mL), it was determined that 0.312 mL of the cell solution was needed for every 29.7 mL of MEM alpha. As a result, 0.312 mL of the cell solution was added to each of two test tubes containing 29.7 mL MEM Alpha; both test tubes were placed in the centrifuge for 8-10 minutes at ½ g. The well-plates containing the hydrogels were then removed from the incubator, and 2 mL of the cell solution was added to each well intended to contain cells (all but the Rheology and NoDPSCs wells); the medium was, however, switched for those gels. The gels were replaced to the incubator and the MEM Alpha (10% FBS, 1% PenStrep, etc.) was changed, by aspirating and adding 2 mL to each well, every other day until days 12 and 31, the day depending on which the gel's respective well plate.

EVOS Fluorescence Microscopy

In order to be able to view the DPSCs under the EVOS fluorescent microscope on days 13 and 31 after DPSC plating, the cells had to be stained, a process involving 3.7% formaldehyde, 0.4% triton, and AF488 and DAPI stains (staining occurred on the days of EVOS observation). Prior to doing so, the two hydrogels intended for EVOS observation on that particular day were washed three times using PBS. As 3.7% of formaldehyde was needed, the stock solution had to be diluted by 10 times. 2 mL of 37% formaldehyde was added to 18 mL PBS, from which 2 mL of the 3.7% formaldehyde solution was transferred to the gelatin-only

and GELFIB hydrogels. Fifteen minutes after adding formaldehyde, intended to kill and maintain the cells at their current state, the gels were once again washed thrice with PBS. In order to obtain a 0.4% solution of triton, a solution intended to enhance the permeability of the DPSC membranes, 0.5 mL of the stock 4% triton solution was added to 4.5 PBS. 2 mL of 0.4% was then added to the EVOS wells and after 7.5 minutes, the solution was aspirated.

After aspirating the PBS-triton solution and washing the gels, being diluted by 200x, by adding 10µL of dye into 2mL of PBS, 1 mL of AF488, an actin fiber dye, was added to each of the wells. To prevent the interaction of light with the dye, this process occurred in the dark, and the well plate was covered with aluminum for a period of 20 minutes. Once again avoiding exposure to light, 2 µL of DAPI, a nucleus-staining agent, was added to 2 mL of PBS to dilute the stain by 1000x. After washing the gels three times with PBS, 1 mL of DAPI was added to each well. After 3.5 minutes, the gels were once again washed with PBS and 1.5 mL PBS was added to the gels, the well plates being covered prior to observation by EVOS (20x).

Scanning Electron Microscopy

As with EVOS, the DPSCs were viewed under the scanning electron microscope (SEM) on days 12 and 31 following DPSC plating on the hydrogels. As preparation for SEM, which occurred on each of the established days, the cells designated as such had to be dehydrated. In order to do so, 2 mL of distilled water was added to the two wells, the plate was shaken, and the water was aspirated, a process repeated four times. After doing so, the SEM gelatin and gelatin + fibrinogen gels, remaining in the well plates, were placed in a vacuum and SEM was performed, following which was analyzed using Energy-Dispersive X-ray analysis.

Reverse Transcription - Quantitative Polymerase Chain Reaction

Examining the effect of fibrin, the product of fibrinogen and thrombin, on the differentiation and proliferation of DPSCs was most directly measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR), in which, after transcription of RNA to DNA and following amplification through PCR, the DNA encoding for the ALP, OCN, DSPP, COL1A1 proteins was measured through the determination of the linear phase of the fluorescence intensity graph resulting from this mRNA amplification. However, in order to do so, RNA first needed to be extracted from the cells, which even before so, were collected from the gels. In order to do so, after washing the three RT-qPCR gels three times with PBS, a cell scraper was used to scrape off the cell film from each of the RTPCR gels, the film being placed in a cuvette. To aid in lysing of the cells, necessary prior to RNA isolation, 600 µL of QiAzol Lysis Reagent was added to the cell films, which were placed in -80°C, until RNA isolation using and RNeasy Plus Universal Mini Kit. Once RNA was collected, RT-qPCR was performed, obtaining, on days 12 and 31, quantitative data regarding the impact of fibrin on DPSC differentiation.

Following initial competition, the investigation was repeated for a second trial, during which data was once again collected on days 12 and 31.

RESULTS

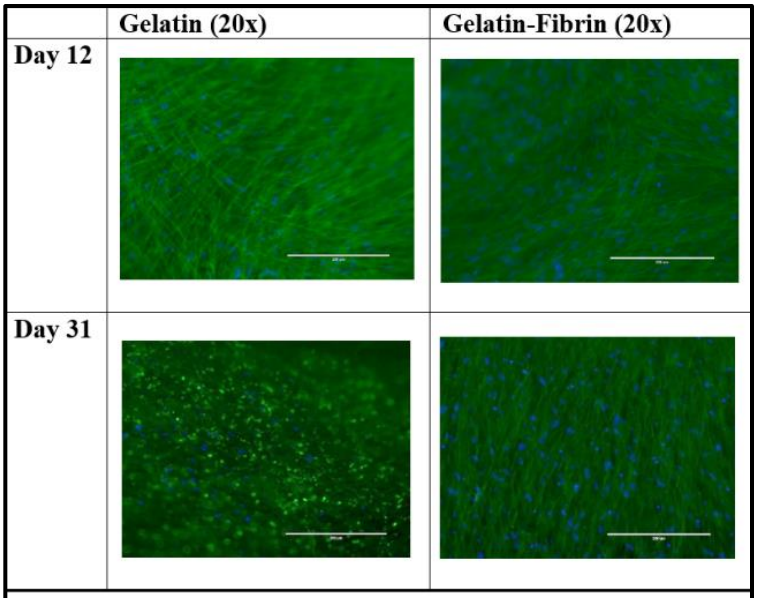

Figure 1. EVOS Fluorescence Microscopy. Images display DPSCs exposed to gelatin hydrogels w/ and w/o fibrin on days 12 and 31 (20x magnification).

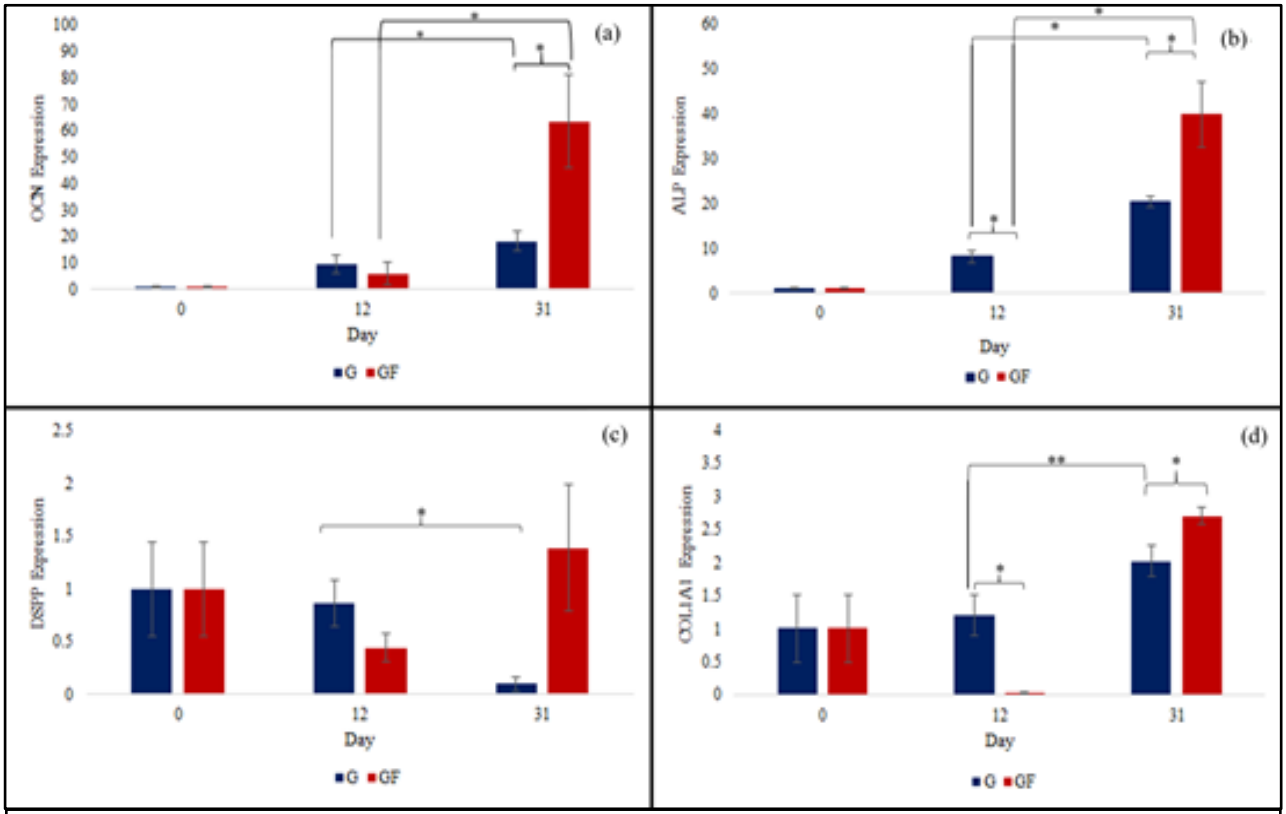

Figure 2. Reverse Transcription - Quantitative Polymerase Chain Reaction (RT-qPCR) demonstrating non-standardized mRNA expression of osteocalcin (OCN) – (a); alkaline phosphatase (ALP) – (b); dentin sialophosphoprotein (DSPP) – (c); alpha-1 type I collagen (COL1A1) – (d) genes in cultures exposed to gelatin hydrogels with and without fibrin.

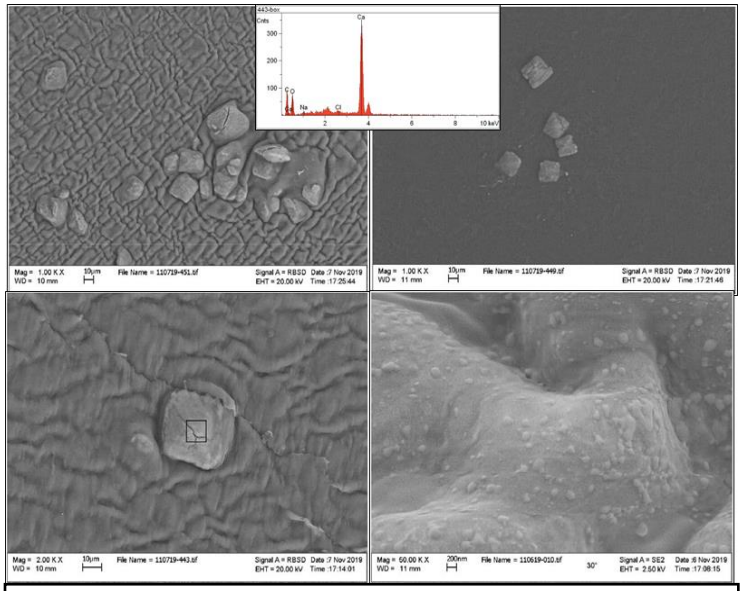

Figure 3. Scanning Electron Microscopy (SEM) image representation and EDX analysis of DPSCs differentiation and biomineralization on gelatin hydrogels – DAY 12

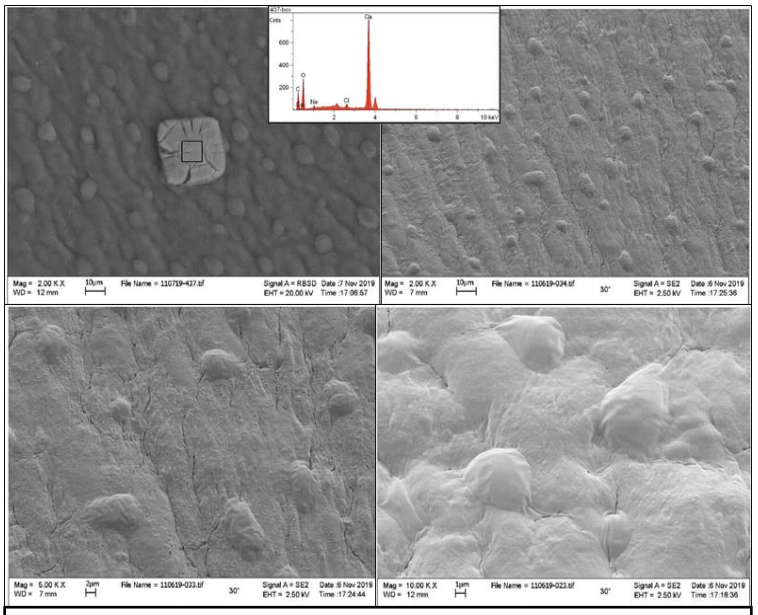

Figure 4. Scanning Electron Microscopy (SEM) image representation and EDX analysis of DPSCs differentiation and biomineralization on gelatin-fibrin hydrogels – DAY 12

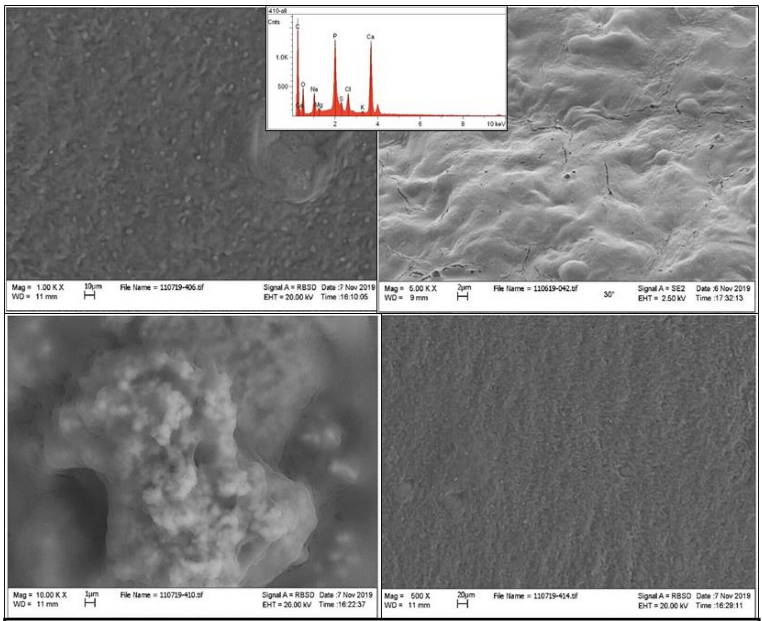

Figure 5. Scanning Electron Microscopy (SEM) image representation and EDX analysis of DPSCs differentiation and biomineralization on gelatin hydrogels – DAY 31

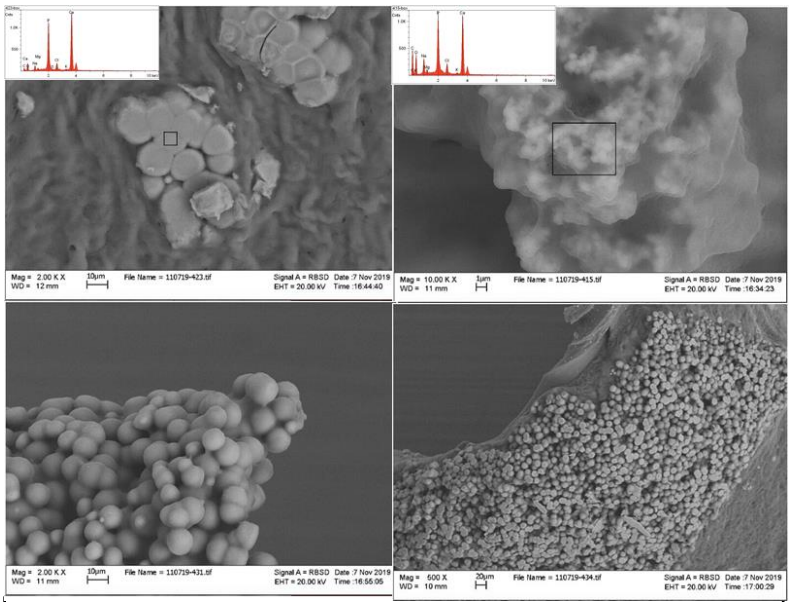

Figure 6. Scanning Electron Microscopy (SEM) image representation and EDX analysis of DPSCs differentiation and biomineralization on gelatin-fibrin hydrogels – DAY 31.

DISCUSSION and CONCLUSION

Containing nerves, blood vessels, and connective tissue, the dental pulp is critical in ensuring appropriate functionality of the tooth. As a result, dental pulp stem cells have a promising future in the of regenerative endodontic therapy (RET), currently aimed at treating immature permanent teeth with necrotic pulp/apical periodontitis. Although such a condition has been traditionally treated with apexification procedures, root elongation accompanied by induced periapical bleeding has demonstrated a thickening of the canal walls, apical closure, and root-lengthening thus resulting in its recognition as the appropriate clinical practice. However, in order to improve the regeneration of the pulp-dentin complex and extend this therapy to afflicted mature teeth, the specific function of blood in initiating DSPC differentiation along an odontogenic lineage must be understood. This was evaluated in this investigation as a means of providing insight which may facilitate the creation of a versatile grow-factor-rich scaffold which may be applied to mature teeth in the general population.

As collagen is the natural matrix for the DPSC and fibrinogen is the major component of blood, an enhancement of DPSC differentiation may be achieved by inducing a clot. As a result, we developed a platform for probing this model, first using gelatin (far less expensive than collagen) to explore the influence of fibrin. In order to assess this, a cross linked 9:5 gelatin-fibrin scaffold containing β-Glycerophosphate was constructed to discern the presence of a correlation between fibrin, a major plasma protein, and the outcome of dental pulp stem cell (DPSC) differentiation. Developed using 9% w/v gelatin and 4 mg/mL bovine fibrinogen, cross-linked by 10% mTG (microbial transglutaminase) and 5 units/ml thrombin, respectively, the hydrogels were cultured in osteogenic media. in the absence of dexamethasone. In vitro, dexamethasone is often added to induce osteogenic differentiation; however, since DEX is a

dangerous steroid in vivo we therefore proposed this study in part to determine if there are alternate triggers for differentiation.

Following the second and fourth weeks in culture in both trials of the study, RT-qPCR was performed to quantitatively analyze mRNA expression for genes indicative of DPSC osteo/odontogenic differentiation: osteocalcin (OCN), alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP), and alpha-1 type I collagen (COL1A1). EVOS Fluorescence and Scanning Electron Microscopies were employed in viewing differentiation among stained DPSCs and consequent biomineralization, respectively.

The genetic moderation of osteo/odontogenic capacity determining root formation in the widely accepted practice of root elongation, this study provided insight into the significance of fibrin in the blood as an inducer of DPSC differentiation along an osteo/odontogenic lineage, establishing the proposed model as plausible and necessary of further investigation.

As evident in *Figure 1*, a significant contrast in the quantity of nuclei at a particular magnification was seen between those cells exposed to hydrogels with and without fibrin, the sample with indicating a greater change in nuclei. This visual depiction of the health and growth of the DPSCs is further supported by SEM and EDX, through which it was evident that gelatin alone does not trigger differentiation. The cells visibly produce face-centered cubic $CaCO_3$ mineral deposits in the presence of solely gelatin, which are detrimental since they form blockages and do not reinforce the tooth. On the contrary, addition of fibrin had a dramatic effect positive impact, with large amounts of Ca_3PO_4 deposits observed in thick layers on the sample, Ca_3PO_4 being the natural component of bone and dentin. It is likewise interesting to note biomineralization which was present on gelatin not involving DPSC, indicating gelatin's

similarity to collagen, thus supporting gelatin as a model for a collagen scaffold to be developed and improved upon.

Consistent with the SEM and EDX results, OCN demonstrated significant upregulation when DPSCs were exposed to fibrin, a conjecture not only supported by the inexistence of overlap between standard error bars, but also through a result of $p<0.05$ in a t-test. OCN being the primary indicator of osteo/odontogentic differentiation, fibrin has been shown to in fact be a critical component of the process of root elongation, demonstrating its necessity as a basis for the future development of hydrogel scaffolds. This is likewise supported by the statistically significant upregulation of ALP and COL1A1, and the change in DSPP levels. As indicated in the figures, the genes associated with osteogenic and odontogenic differentiation follow the expected sequence for upregulation which results in eventual biomineralization, significantly greater in the DPSCs on the gelatin-fibrin scaffolds, thus indicating the attractiveness of these fibrin-gelatin scaffolds as a clinical alternative in root canal therapy.

In addition to scanning electron microscopy indicating greater long-term bio-mineralization on the part of the DPSCs exposed to fibrinogen, RTqPCR demonstrated a significantly greater up-regulation of the genes critical in osteo/odontogenic differentiation. Due to the success of this initial model, deposition of DPSCs on the natural matrix of collagen substrates will be evaluated to further solidify the composition of an ideal matrixial scaffold which may enhance DPSC cell proliferation and differentiation along a lineage allowing for functional root elongation and thus tooth regeneration in the general population, for immature and mature teeth alike. Furthermore cross-linking ratios for the hydrogel matrix may be examined and Raman spectroscopy may be conducted to further discriminate between the hard tissues of bone, dentin, and enamel produced.

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