DEVELOPMENT OF A DEMINERALIZED DENTIN MATRIX HYDROGEL FOR DENTAL PULP REGENERATION

A Thesis

by

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ABSTRACT

Regenerative endodontics is one of the most exciting new developments in endodontics. Pulp regeneration can be achieved basically by using a triad of a reliable cell source capable of differentiating, a suitable scaffold, and signaling molecules that direct the cells for proliferation and cellular differentiation. Injectable scaffolds are favorable for regeneration of pulp-dentin complex as the surgery is minimally invasive, easy to place in small and irregular pulp canals, have a homogenous distribution, helps cell adhere, proliferate and differentiate into new functional tissue and reduces the risk of infection. We aim to develop a biomimetic injectable biomaterial that would facilitate pulp regeneration and have the potential for clinical application. We hypothesized that demineralized dentin matrix, which mainly consist of collagen and non- collagenous proteins, will provide a favorable microenvironment for pulp-dentin regeneration. Characteristics of demineralized dentin matrix hydrogel along with the in vitro experiments have been studied, which makes the assumptions clear that it mimics the extracellular matrix of the dentino-pulpal tissue and helps in maintaining the cellular viability along with cell proliferation and differentiation. In conclusion, we can say that the demineralized dentin matrix provides a suitable environment for the dental pulp stem cells to grow and differentiate in different lineages. More studies should be conducted for further characterization of the demineralized dentin matrix for translational use.

DEDICATION

I would like to dedicate my thesis to my parents, Rajendra Singh Ahuja & Poonam Ahuja and my brother, Arjun Ahuja who have always supported me and encouraged me in all my endeavors. I would also like to thank all my friends who stood there with me and without whom this wouldn't be possible.

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Xiaohua Liu and Dr. Chunlin Qin from the Department of Biomedical Sciences at Texas A & M University College of Dentistry and Dr. Poorya Jalali from the Department of Endodontics at Texas A & M University College of Dentistry.

All work for the thesis was completed independently by the student under the advisement of Dr. Xiaohua Liu of the Department of Biomedical Sciences at Texas A & M University College of Dentistry.

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NOMENCLATURE

α-MEM	Minimum essential medium, Alpha
ALP	Alkaline phosphatase
ATR-FTIR	Attenuated total reflection - Fourier transform Infrared
BMP	Bone morphogenetic protein
Ca^{2+}	Calcium ions
CO_2	Carbon dioxide
DDM	Demineralized dentin matrix
DI water	Deionized water
DMSO	Dimethyl sulfoxide
DPSCs	Dental pulp stem cells
EBT	Eriochrome Black T
ECM	Extracellular matrix
EDS	Energy-dispersive X-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EthD-1	Ethidium Homodimer-1
FGF	Fibroblast growth factor
FTIR	Fourier transform infrared
H & E	Hematoxylin and eosin

HCl	Hydrochloric acid
ILGF	Insulin-like growth factor
$M_{Calcium}$	Molarity of calcium
M_{EDTA}	Molarity of EDTA
$MgCl_2$	Magnesium chloride
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	Sodium hydroxide
NH_3NH_4Cl Buffer	Ammoniacal buffer
NIH	National Institute of Health
OCT	Optimal cutting temperature compound
PBS	Phosphate-buffered saline
pNPP	para-Nitrophenylphosphate
SEM	Scanning electron microscopy
SIBLINGs	Small intergrin-binding ligand N-liked glycoproteins
TGF-β1	Transforming growth factor beta-1
$V_{Calcium}$	Volume of calcium
V_{EDTA}	Volume of EDTA
VEGF	Vascular endothelial growth factor

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

1. INTRODUCTION

Regenerative medicine can be defined as ‘A field of medicine concerned with developing and using strategies aimed at repair or replacement of damaged, diseased, or metabolically deficient organs, tissues, and cells via tissue engineering; cell transplantation; and artificial organs and bioartificial organs and tissues’ (National Library of Medicine, 2004). The definition for the regenerative medicine has been redefined accordingly by various authors focusing on the interdisciplinary approach of the field which helps in the repair and replacement or even regeneration of the cells, tissues and organs. The various disciplines currently included are stem cell biology, genetics, tissue engineering, developmental biology, cellular and molecular biology, science of tissue and organ transplantation, and material science. These disciplines help in repair, replacement and regeneration for restoring the impaired function may it be cells, tissues or organs (Daar and Greenwood 2007).

Regeneration of tissues and organs is limited in humans unlike some animals, still bone and liver can be regenerated to some extent. Tissue engineering is one technique which helps regenerate the lost tissues or organs. NIH defines “tissue engineering evolved from the field of biomaterials development and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues. Basically, it is a triad of scaffolds, cells and bioactive molecules. The scaffold provides an appropriate environment for the cells to attach and to proliferate for the

regeneration of the tissues or organs. The bioactive molecules help the cells differentiate into specific lineages according to the specific tissues. An ideal scaffold should be biocompatible, biodegradable (Mikos, McIntire et al. 1998), ease of manufacturing and sufficient mechanical properties to sustain surgical procedures and mechanical loading along with providing space for cellular proliferation and differentiation (O'Brien 2011).

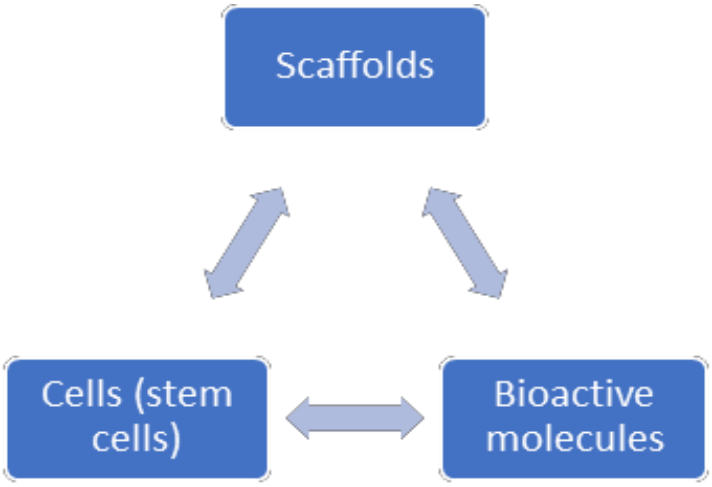

Figure 1 Tissue engineering triad.

1.1 Regenerative endodontics

The dental pulp is a non-mineralized soft connective tissue situated in the central part of the tooth. It consists of various cells like odontoblast in a fibrous matrix along with vascular, lymphatic and nervous elements. The dental pulp is a highly- specialized tissue which maintains the vitality of the tooth. But it has a limited capacity to repair and regenerate pulpal tissues and dentin. Dental carries, trauma to the tooth, attrition, severe abrasion and micro-leakage in large filling, etc can cause microbial invasion and infection of the vital pulpal tissues. Treatment options like direct and indirect pulp

capping procedures take help of the innate reparative capacity of the pulp whereas root canal treatment is advised in severely decayed or infected tooth (where pulp necrosis occurs). The pulp capping procedures uses calcium hydroxide or similar agents that help in building a dentinal bridge that helps in the innate mechanism of dental repair (Nyborg 1955, Stanley and Lundy 1972, Cox, Bergenholtz et al. 1982, Heys, Fitzgerald et al. 1990, Oguntebi, Heaven et al. 1995).

Root canal treatment is a procedure in which the necrotic tissue is removed and the cleaned and shaped canals are filled in with gutta percha or similar inert material. Although root canal treatment is the most common treatment used, it has complications like tooth fracture, re-infection, tooth discoloration and failure of treatment. Regenerative endodontics treatment seeks to reestablish the pulp-dentin complex and restore the neurovascular system in a tooth. Thus, the tooth regains its inherent immune and sensory functions, both of which are instrumental to the defense and viability of the tooth.

Regenerative endodontics is one of the most exciting new developments in endodontics. Pulp regeneration can be achieved basically by using a triad of a reliable cell source capable of differentiating, a suitable scaffold, and signaling molecules that direct the cells for proliferation and cellular differentiation into pulpal tissues.

1.2 Need for injectable scaffolds

Injectable scaffolds are favorable for regenerative techniques because of their minimal invasive nature and hence less patient discomfort. When selecting a scaffold, it

is necessary to know the shape and size of the defect, in cases where the shape of the defect is irregular the scaffold might not be able to place properly.

Injectability gives a benefit when the defect size is smaller and irregular. Dental pulp canal is a small space and can be irregular, or curved. And so, an injectable scaffold for example a hydrogel may be beneficial as it is less invasive and can be easily placed in small and irregular canals (Wintermantel, Mayer et al. 1996, Hou, De Bank et al. 2004). Sometimes, seeding of the cells might be challenging and might not be equally distributed, but an injectable material will help in a homogenous distribution of the cells and let it adhere, proliferate and differentiate into a new functional tissue. Injectable tissue also reduces the risk of infections (Hou, De Bank et al. 2004).

1.3 Demineralized dentin matrix

Extracellular matrix (ECM) of the native tissues provides a suitable environment for the tissue regeneration and remodeling. Collagen I is the basic component of the demineralized dentin matrix will act as natural polymeric scaffolding material which has the capability to support proliferation of various cell types like chondrocytes, fibroblast, bone marrow stem cells, osteoblast and many more (Mizuno and Glowacki 1996, Mizuno, Ushida et al. 1998, Makhluf, Mueller et al. 2000, Navarro, Mizuno et al. 2001). The protein analysis of the dentin matrix also identifies various protein components (Park, Cho et al. 2009, Chun, Lee et al. 2011, JÃƒgr, Eckhardt et al. 2012). Some of these proteins are known to regulate the reparative cycle of the dentin (Lee, Colombo et al. 2015). For example, TGF-β1 is known to induce dentinogenesis (Cassidy, Fahey et

al. 1997, Sloan, Perry et al.2000). Also, bone morphogenetic proteins help in upregulating the dentin synthesis and secretion (Rutherford and Gu 2000, Nakashima 2005, Casagrande, Demarco et al. 2010). Other factors such as fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), insulin like growth factor (ILGF) helps the mesenchymal stem cells to differentiate in osteogenic or odontogenic lineages, also initiating angiogenesis (Finkelman, Mohan et al. 1990, Cassidy, Fahey et al. 1997, Roberts Clark and Smith 2000, Sloan, Perry et al. 2000, Casagrande, Demarco et al. 2010, Lee, Colombo et al. 2015). The phosphorylated proteins such as the SIBLING family (DSP, DPP, OPN, BSP, DMP-1) are proteins which help in the early reparative process and signals the cells for cellular proliferation, survival, differentiation and also, mineral deposition in the dentin (Smith, Scheven et al. 2012). There are proteins and factors that also control the cell signaling and regulate the inflammatory process which is very important for the repair and regeneration (Lee, Colombo et al. 2015).

Unlike hard tissues, dental pulp is made up of diverse cell population and so the scaffold should be such selected that it reflects these specific matrix conditions and cell lineages along with the biological, functional and mechanical requirements. The bioactive molecules in the dentin tissue, if able to restore even small amounts of some of these molecules in the scaffold will help increase the bioactivity of the material and also, help better cellular differentiation. Previous studies have shown that extracellular matrix is a favorable environment for regeneration of tissues like cartilage (Wu, Ding et al. 2015), bone (Sawkins, Bowen et al. 2013), skeletal muscle (Valentin, Turner et al. 2010), cardiac tissues (Singelyn and Christman 2010), spinal cord (Tukmachev,

Forostyak et al. 2016) etc. Demineralized and /or decellularized dentin matrix has been used for the regeneration of bone as it retains certain number of bioactive molecules and shows osteo-conductive potential (Reis-Filho, Silva et al. 2012, Bakhshalian, Hooshmand et al. 2013, Li, Yang et al. 2013). It has also been favorable for cartilage regeneration (Yagihashi, Miyazawa et al. 2009).

So, it is likely that demineralized dentin matrix will be a good scaffold for the regeneration of the pulpal tissues as dentin and pulp are closely related tissues and might induce differentiation of the stem cells into the various cells of the pulp. The ECM matrix will also provide a physical support for the cell proliferation and differentiation. It is also possible for the demineralized matrix to retain the various proteins which will help in the regenerative process. However, to date, no injectable demineralized dentin matrix has been developed.

1.4 Aims and objectives

In our study, we aim to develop and characterize the demineralized dentin matrix hydrogel and we also wish to see the cell survival, proliferation and the ability to differentiate and stay on the gel system in vitro. We suggest the use of dental pulp stem cells along with the gel and demineralized dentin matrix extract will be a good environment for the development of the dental pulp and have the potential for clinical application.

Specific Aim 1- Development and Characterization of Demineralized dentin matrix hydrogel.

Specific Aim 2- *In vitro* characterization of demineralized dentin matrix treated solution and *in vitro* studies on the surface and inside the hydrogel.

1.5 Problems anticipated

Previous studies (the solubilization of bone and dentin collagen by pepsin effect of cross linkages and non-collagen components) have shown that the dentin matrix collagen is almost completely insoluble and only small amount can be extracted by neutral salts or dilute acidic solutions (Carmichael, Dodd et al. 1977, Van Strijp, Klont et al. 1992). The inter-molecular linkages of the dentin collagen make it insoluble and enhances the strength of the fibrils. Pepsin have been used in various studies to solubilize the collagen from skin and bone (Carmichael, Dodd et al. 1977). Pepsin is only 5.6% soluble with pepsin in slight acidic medium, increasing the temperature increases the solubility but increases the degradation of the collagen (Van Strijp, Klont et al. 1992). Hence no soluble form of the dentin collagen had been possible. We here aim to use the bioactive molecules present in the dentin matrix along with collagen I to form a hydrogel which will help for the mechanical strength in the narrow and irregular spacing of the root canal.

2. MATERIALS AND METHODS

2.1 Development of demineralized dentin matrix hydrogel

To test the hypothesis, we need to develop and treat the demineralized dentin matrix hydrogel so that it is biocompatible and along with it facilitate cell proliferation and differentiation. Freshly extracted bovine anterior incisors were collected and stored in -80°C for further use. The bovine incisors were de-frozen and the attached periodontal ligament and the dental pulp was cleaned using a round bur and root canal instruments with continues irrigation using DI water. After the teeth were completely cleaned and root canal was properly filed, the tooth enamel and the cementum was removed using a sharp diamond disc. The remained dentin was used for further experiments. For demineralization, the remainder dentin was cut in small pieces of around 2-3 mm in size, snap frozen with liquid nitrogen and ground with the help of a grinder. The ground particles were sieved and only size 32-500 μm was used. Demineralization was performed using 0.5N HCl at room temperature until completely demineralized. A series of wash using DI water was done to remove the residual amount of acid. The demineralized dentin matrix particles were freeze-dried and stored in -80°C for further use.

Preparation of DDM extract- The freeze-dried DDM particles were washed in PBS twice and then in α-MEM culture medium for 2 times. 10mg/ml of the dried DDM particles were incubated in the α-MEM medium for 72 hours at 37°C and 5% CO2 (Liu,

Xu et al. 2016). The DDM extract for further diluted to 1mg/ml and 0.1mg/ml for further experiments.

Demineralized dentin matrix hydrogel- The extracted demineralized dentin matrix extract was used to prepare demineralized dentin matrix hydrogel. Collagen I pre-gel solution was used to prepare the hydrogel. The pre-gel solution is determined to an appropriated concentration along with the DDM extract, so that the final concentration of the pre-gel has either 0.1mg/ml, 1mg/ml and 10mg/ml of the DDM extract. The pre-gel solution is diluted by $1/10^{th}$ by 0.1M of NaOH (Sodium hydroxide) and $1/9^{th}$ of 10X PBS at 4°C. Gelation was induced by increasing the temperature from 4°C to 37°C till a stable gel is formed (Freytes, Martin et al. 2008, Wolf, Daly et al. 2012, Sawkins, Bowen et al. 2013, Wu, Ding et al. 2015, Tukmachev, Forostyak et al. 2016).

2.2 Calcium EDTA titration

Titration by a standard solution of ethylenediaminetetraacetic acid (EDTA) is one of the classic methods for determining the calcium content of the solution. Here we take the residual solution of the demineralization acidic solution and calculate the calcium that has been leached out of the dentin matrix particles. A metallochromic indicator (Eriochrome Black T EBT) is used to determine the endpoint of the EDTA titration. When combined with the metal ions, the indicator forms a complex ion and the color of the solution changes from blue to red. EDTA is a stronger agent than the EBT which complexes the metal ions displacing it from the EBT and hence the solution

color changes again to pure blue.

We need a standardized solution freshly prepared of 0.01 M disodium EDTA with $MgCl_2$ and a standard Ca^{2+} solution for determining the exact molarity of the EDTA. Also, 12M concentrated HCl, 8.5M NH_3-NH_4Cl Buffer and Eriochrome Black T indicator is needed.

25ml of the standard calcium solution (approx. 0.01022 M of Ca^{2+}) is taken in a beaker and the pH is adjusted ~7 by sodium hydroxide. 10 ml of the buffer solution added followed by 2-3 drops of EBT indicator. Titration of the calcium solution is done by the standard EDTA solution until the color changes from wine red, through purple, to pure blue. The experiment is performed in triplicates. The average molarity was determined by considering the formula.

$$M_{calcium}V_{calcium} = M_{EDTA}V_{EDTA}.$$

The same standard EDTA solution is used for the further experiment of determining the calcium content of the residual acidic solution of demineralization. 5ml of the acidic solution is diluted in 250ml of DI water. 50 ml of the aliquoted solution is taken and neutralized to pH 7 using sodium hydroxide, 10 ml of the buffer solution is added along with 2-3 drops of EBT indicator. Titration is performed with the same EDTA solution until the color changed to pure blue (same as the reference color). The same experiment is performed in triplicates. The volume EDTA used for titration multiplied by EDTA molarity is equal to molarity of EDTA in mmoles which is equal to molarity of Ca2+ in mmoles. The calculation for the determination of the calcium concentration can be defined as

Volume of EDTA(ml)*Molarity EDTA = EDTA(mmoles) =Ca^{2+}(mmoles)

Ca^{2+} (mmoles)*40.078gram/mole = Ca^{2+}concentration in milligrams, aliquote

(Ca^{2+} concentration of aliquote) (250 ml/50ml) = Ca^{2+} concentration of solution

2.3 Histology

The freeze-dried dentin matrix particles were used for histological examination. The DDM particles were soaked in sucrose solution (buffer) and then embedded in OCT for cryo-section and the sections were taken of 10μm each. The sections were stained with H & E to look for the structure of the matrix. Images were taken under a simple microscope.

2.4 Scanning electron microscopy

Freeze dried dentin matrix was also sputter coated with gold to look under scanning electron microscopy and EDS analysis was also performed specifically to look for calcium content. For EDS freeze-dried mineralized dentin was taken as a control. The hydrogel formed along with the demineralized dentin matrix was also freeze-dried and sputter coated with gold to look for the structure of the hydrogel complex under the scanning electron microscopy.

2.5 FTIR

ATR-FTIR is an analytical technique to determine or identify the material by using the total internal reflective property of a material. We used FTIR to confirm the dentin matrix particles used are completely demineralized and resembles collagen I. It was compared with the freeze-dried mineralized bovine dentin particles.

2.6 Cell proliferation

Dental pulp stem cells were cultured till confluent and used for cell proliferation studies at P5 (passage 5). The cells were cultured in α MEM with 10% fetal bovine serum and 1% penicillin/ streptomycin. DPSCs were seeded in a 96 wells-plate, 5 X 10^3 cells per well at 37°C overnight. DDM treated culture medium was used in the concentration of 0.1mg/ml, 1mg/ml and 10mg/ml and no DDM was taken as a negative control. Each group was performed in triplicates. Cell viability was assessed by MTT assay at day 1,4 and 7, the MTT is bio-reduced by the cells to the formazan and incubated for 4 hours at 37°C and then soluble in DMSO (Liu, Xu et al. 2016). The absorbance is then observed at 570nm and 630nm. The final absorbance is the difference between the two observances. Similar studies were also performed on the hydrogel surface to see the viability of the cells on the gel system (Sawkins, Bowen et al. 2013, Wu, Ding et al. 2015)

2.7 Cell differentiation

Dental pulp stem cells were cultured until passage 4 (P4) of its confluency and tested for differentiation capability of the cells with DDM treated medium. DPSCs were seeded in a 6 well-plate, 2 X 10^5 cells per well and then incubated at 37°C with either untreated medium or DDM treated medium of the concentration of 1 mg/ml for 7, 14 and 21 days. Differentiation activity was defined by scrapping off the cells and transferred into the assay buffer followed by performing the ALP activity test by the ALP kit. After adding the pNPP, the sample supernatant is incubated at 37°C for 1 hour and read under 405nm for absorbance (Li, Yang et al. 2013, Liu, Xu et al 2016).

Differentiation can also be determined by the Alizarin red staining where the similar number of DPSCs i.e. 2 X 10^5 cells per well is seeded along with treated or untreated medium. After 14 and 21 days, the cells are fixed with 4 % paraformaldehyde and stained with alizarin red. The deep red color indicated mineralized tissue. Thus, indicating osteoblastic differentiation of the cells (Liu, Xu et al. 2016).

2.8 Turbidimetric gelation kinetics

Turbidimetric gelation kinetics were performed on the hydrogel by injecting the pre-gel solution and its absorbance was read at 490nm at 37°C every 3 mins for 1 hour with the help of an ELISA plate reader. The normal absorbance was calculated by the formula

$$(A-A_0)/(A_m-A_0)$$

where A is the actual reading at a time point, A_0 is the initial absorbance and Am is the maximum absorbance. A linear curve is graphed and the time at 50% absorbance is defined as the half gelation time ($t_{1/2}$) and the initial linear portion on the curve is the lag time for the gel to start gelation (Freytes, Martin et al. 2008, Sawkins, Bowen et al. 2013, Wu, Ding et al. 2015).

2.9 Cell viability inside the hydrogel

The cell survival rates of the DPSCs inside the gel is determined by live/dead staining (Wu, Ding et al. 2015). The dental pulp stem cells along with the pre-gel solution are cultured together and live dead staining is done for day 1, 4 and 8. The culture medium is changed every other day. 4µM of EthD-1 working solution is used along with 2µM of calcein for the Live/dead assay. The gel is incubated along with the working solution for almost 1 hour and then viewed under fluorescence microscopy for the labelled cells.

3. RESULTS

3.1 Demineralization

3.1.1 Calcium EDTA titration

According to the methods described the dentin matrix was demineralized and tested for further studies. Our aim here is to effectively demineralize the dentin in shortest possible time and able to achieve some amount of bioactivity which will further help the dental pulp stem cells to grow and have some cell lineages. The calcium titration studies show that the demineralization procedure using HCl is fast and effective. The graph in Fig 2 shows that the calcium content in the matrix is leached out almost to half in the first couple of hours and keeps on decreasing rapidly with almost negligible amounts of calcium in the matrix particles in the first 10-12 hours. Thus, standardizing the demineralization time to 10-12 hours with 0.5 M HCl.

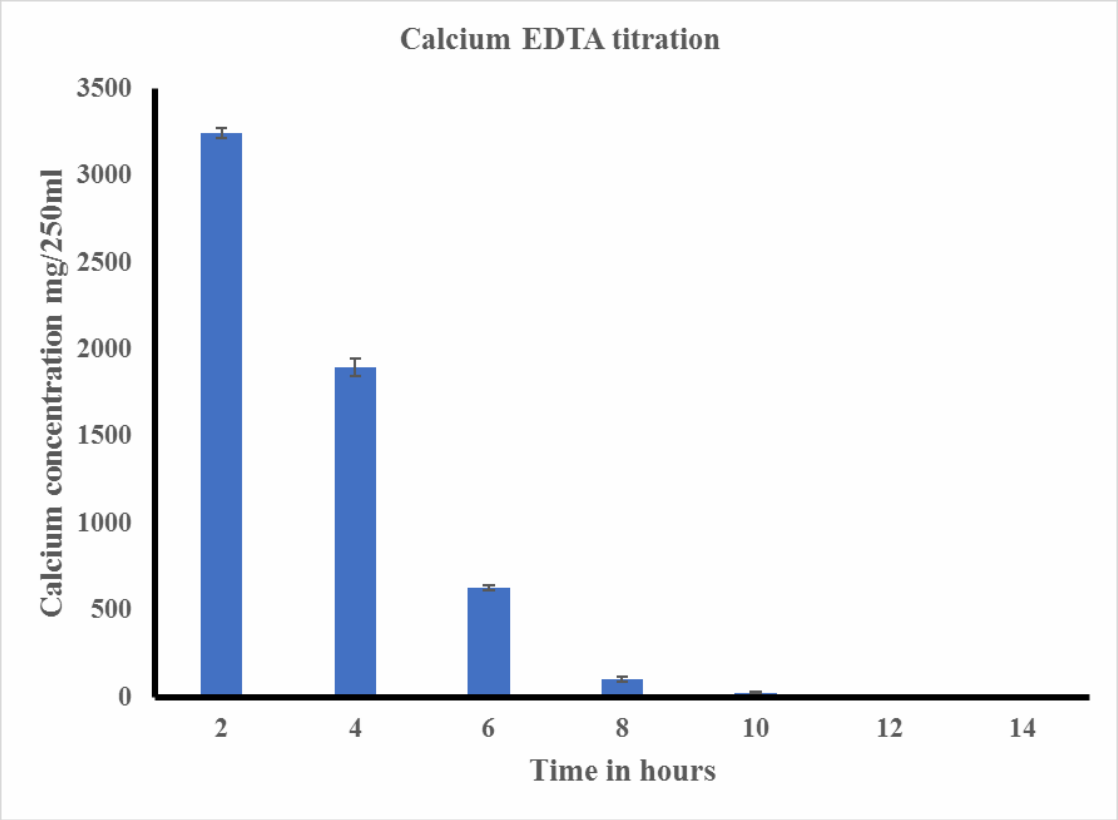

Figure 2 Calcium EDTA titration. This figure shows the decrease in the calcium concentration leached out of the dentin matrix. Negligible amounts after 10-12 hours of demineralization at room temperature with 0.5 N HCl.

3.1.2 Structural analysis of demineralized dentin matrix particles

The structural analysis of the dentin is explained by H & E staining of the dentin particle sections and viewed under a light microscope. The histological sections with H & E staining is shown in fig.3.a along with scanning electron microscopy images in fig.3.b of the demineralized dentin matrix particles showing the natural tubular structure of the dentin. Initial preparation of the dentin and demineralization results in the lack of the mineral deposition and the absence of the odontoblast (cellular structures) in the dentinal structure. In our study, we aim to mimic the matrix structure which is porous for the cells to proliferate and have a favorable environment for the regeneration to occur.

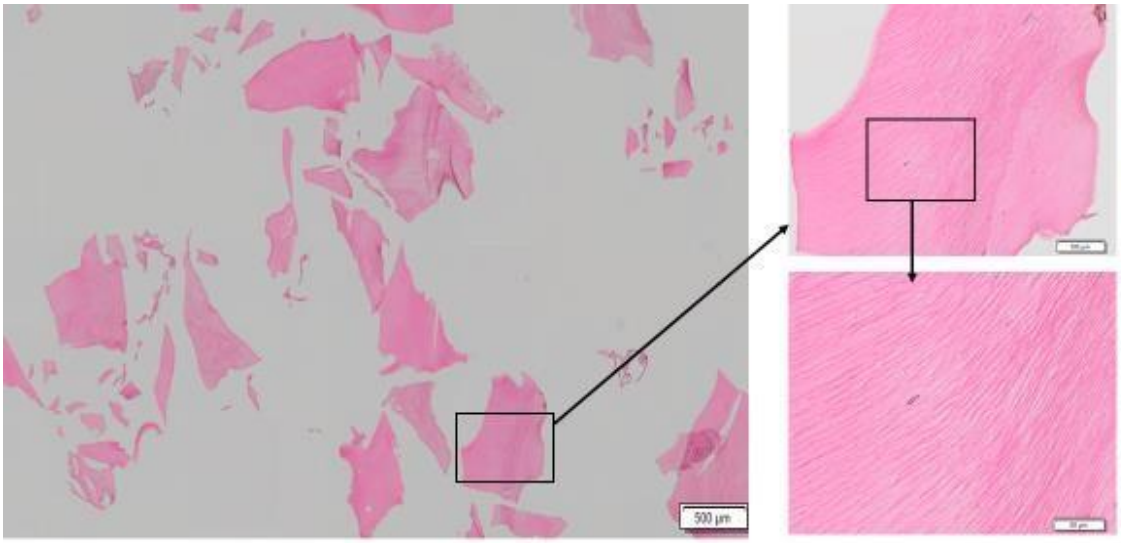

Figure 3 a. H & E staining. The sections shows normal tubular structure of dentin with absence of odontoblastic structures.

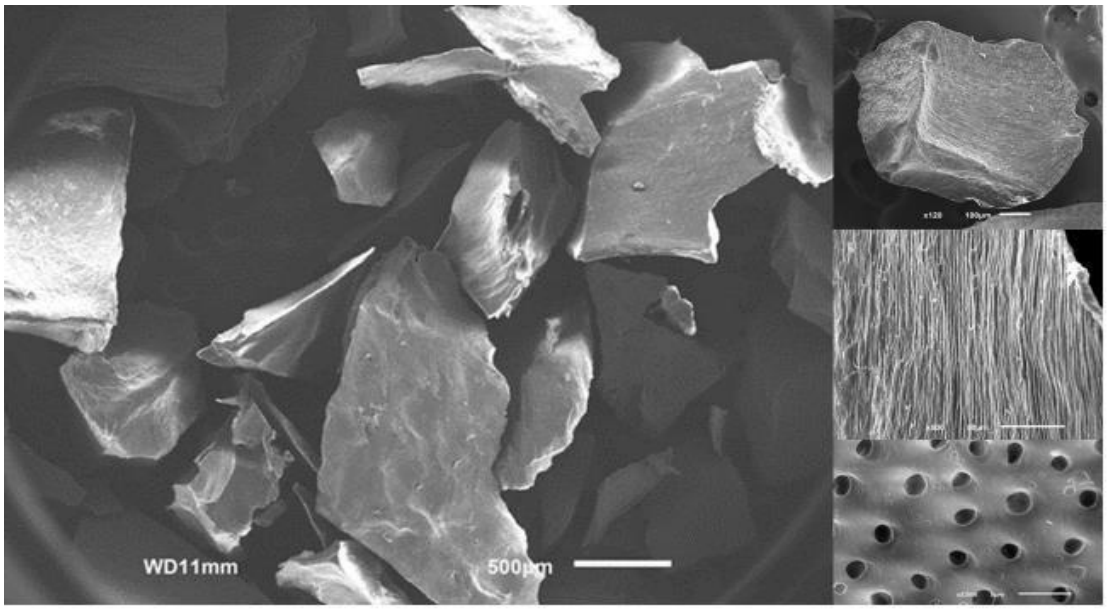

Figure 3 b. SEM imaging. The image shows the demineralized dentin matrix particles under scanning electron microscopy. The image shows the grounded particles in which the tubular structure of the dentin is defined and absence of any mineralized tissues.

3.1.3 Scanning electron microscopy with EDS analysis

The EDS analysis also compares the mineralized and demineralized matrix particles. In fig. 4 we can see the SEM image of the mineralized tissue and the tubular structure of dentin in the demineralized dentin particles. The calcium content of the mineralized and demineralized dentin is graphed and mapped using the EDS analysis which clearly shows the absence of any calcium content in the demineralized dentin matrix particles.

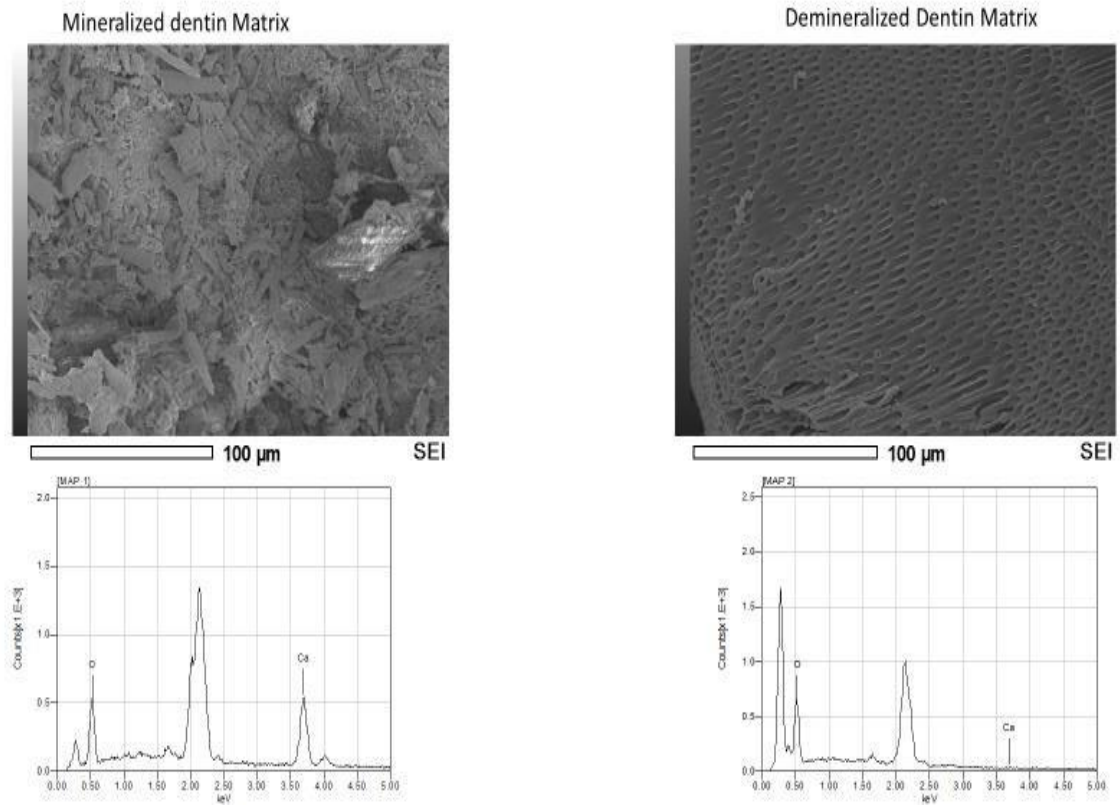

Figure 4 a. EDS analysis. The figures represents EDS analysis of mineralized and demineralized dentin matrix with Ca content. The SEM image shows the difference between the mineralized and demineralized dentin matrix where the demineralized dentin shows well organized dentinal tubules. The graph confirms the loss of calcium ions in the process of demineralization.

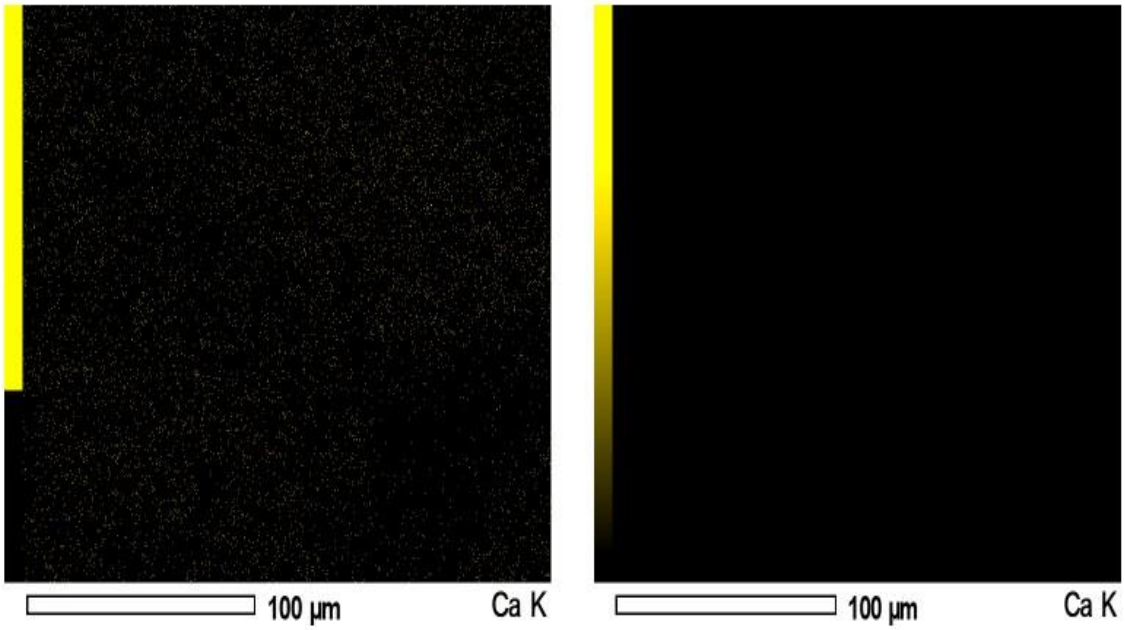

Figure 4 b. Element Mapping in EDS analysis. The picture represents EDS analysis of mineralized and demineralized dentin matrix with calcium content. This image shows the element mapping for calcium. The yellow color in the mineralized dentin shows the presence of calcium atoms, whereas no color in demineralized dentin shows complete demineralization.

3.1.4 ATR-FTIR analysis

ATR-FTIR is also one method to determine the sample. In our experiment, we use the freeze dried mineralized and demineralized dentin particles to see the structural component. The demineralized dentin matrix resembles to the collagen I with similar peaks determining that collagen I (Feng, Zhao et al. 2013) being the majority of our sample as expected

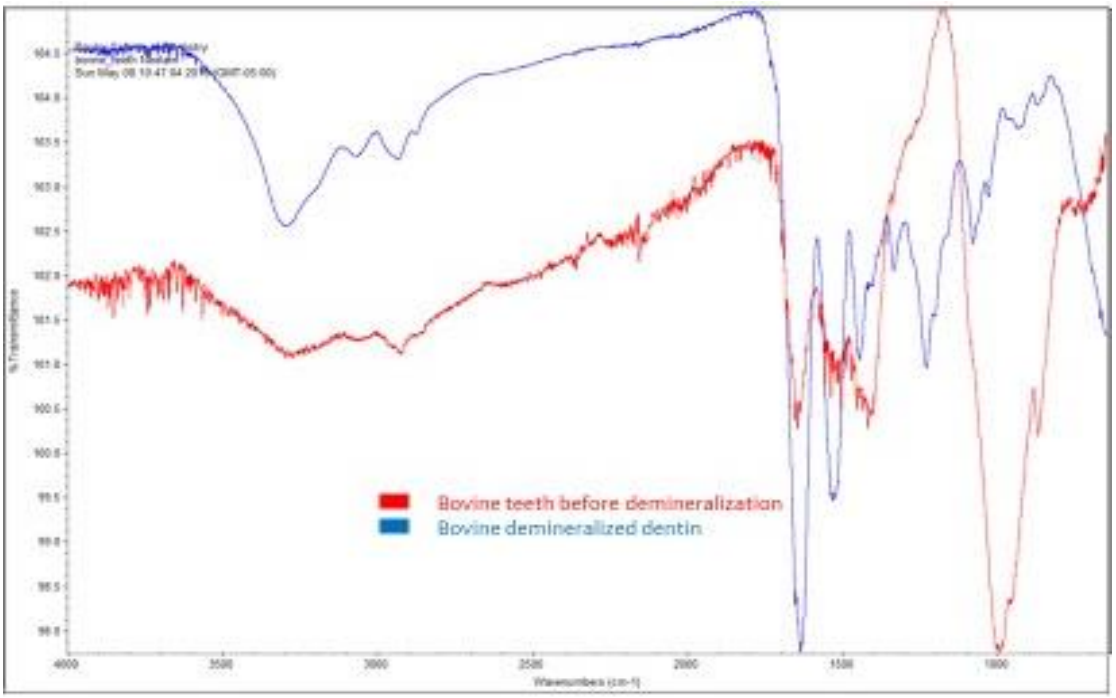

Figure 5 FTIR. This represents FTIR for bovine mineralized and demineralized dentin matrix where the demineralized dentin matrix shows similarity to the pepsin soluble collagen type I with similar peaks.

3.2 In vitro studies of demineralized dentin matrix treated solution

Biocompatibility, cytotoxicity of the material, cell viability, proliferation and differentiation capabilities are to be tested for the DDM extract as well as the hydrogel complex. To check for the cellular proliferation and vitality in the presence of the DDM extract can be characterized by cell proliferative assay such as MTT assay. The MTT is reduced by the cells into a formazan product that is purple in color which is then dissolved in a common solvent such as DMSO. The absorbance is measured at 2 wavelengths and the difference between them is the final absorbance. Fig. 6 shows the cell proliferation of the DDM extracts with the concentration of 0.1mg/ml, 1mg/ml and 10mg/ml. The control group is cells with medium which is not treated with DDM. The results suggest that the cell proliferation is similar or slightly increased with the presence of the extract from demineralized dentin suggestive of biocompatibility and ability to enhance the cellular proliferation.

We also wanted to test whether the DDM helps differentiate the stem cells. To test the differentiation capabilities of the material, ALP activity assay and Alizarin red staining was performed at 14 and 21 days (2 and 3 weeks). In Fig.7. the ALP assay shows significant activity by 14 days as compared to the control group (without DDM treatment) even when no differentiation medium was used. By the end of 21 days the differentiation activity was still maintained in the presence of DDM. Fig. 8 also shows the similar results with alizarin red staining, as the group with the demineralized dentin did show a deep red color suggestive of an increased activity and mineral deposition.

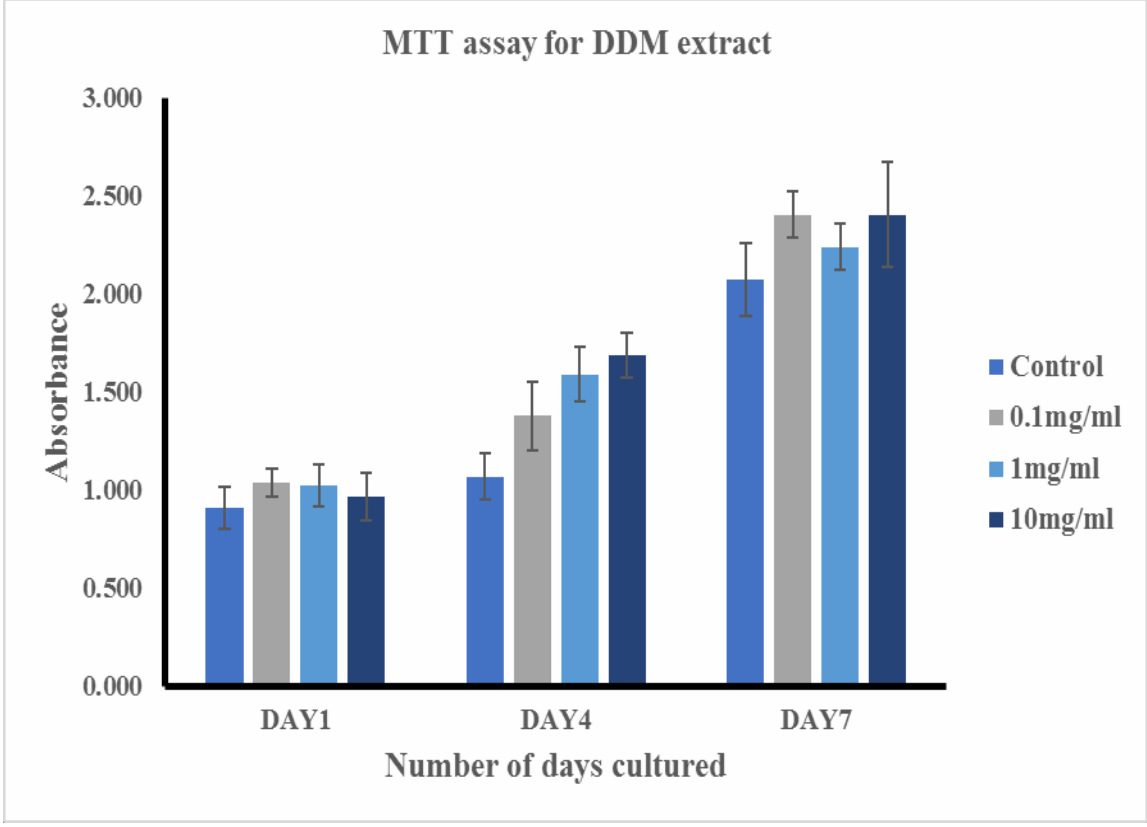

Figure 6 MTT assay. The graph shows the cell proliferation of dental pulp stem cells along with 0.1mg/ml, 1mg/ml and 10mg/ml of demineralized dentin matrix treated medium. All groups along with the no DDM treated medium shows the gradual proliferation of the cells from 1 day to 7 days of time.

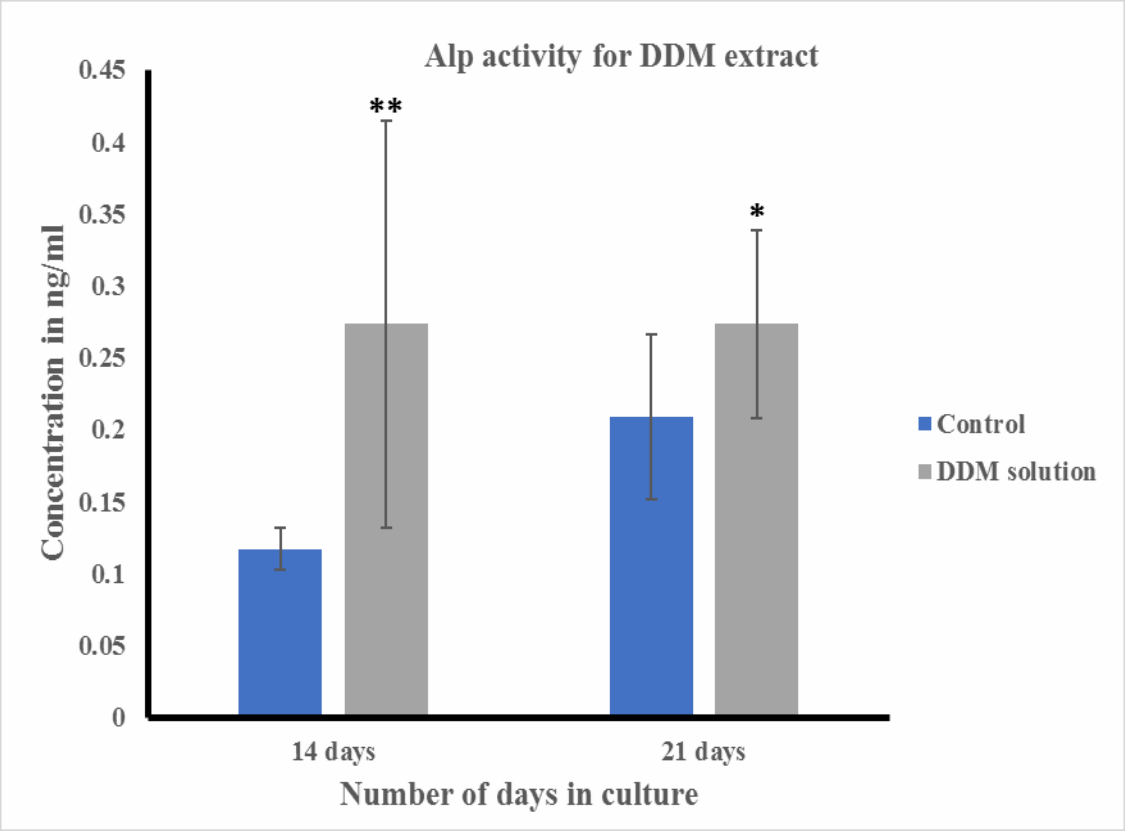

Figure 7 ALP assay. The above graph is the Alkaline phosphatase activity (ALP) showing the differentiation capability of the DPSCs in the presence of the DDM concentrate is significantly greater that the negative control (cells only) at 14 days while maintaining the differentiation of the cells over 21 days.

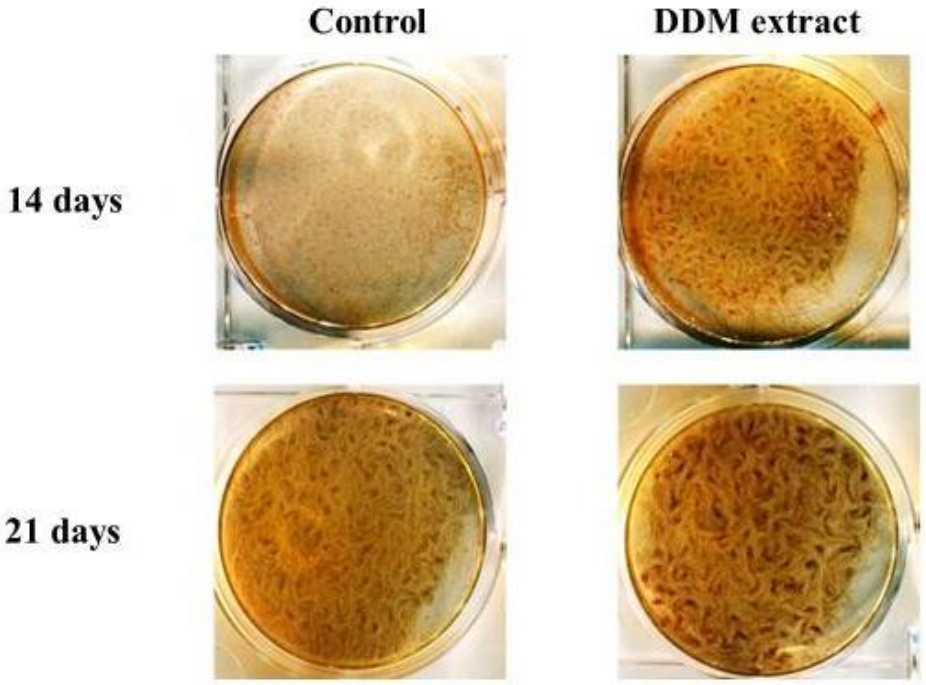

Figure 8 Alizarin red staining. The picture shows the Alizarin red staining which represents the osteoblastic differentiation of the cells with the presence of the DDM treated medium. In this figure the deep red color represents the mineralization content.

3.3 Hydrogel formation, morphology and characterization

Collagen I and demineralized dentin extract were used for making a hydrogel. The collagen I and demineralized dentin pre-gel solution was neutralized using sodium hydroxide at 4°C and gelation was induced by increasing the temperature to 37°C. The pre-gel solution was incubated at 37°C for approximately 1 hour. Fig. 9. shows the hydrogel formation. The first image represents the pre-gel solution (check flowability) and the latter is after the incubation at 37°C for 1 hour.

To characterize the properties and also to look for the biocompatibility of the hydrogel structure studies were performed. Firstly to mimic the extracellular environment for regeneration, a material which is porous in nature is highly indicative. The SEM image of the hydrogel in fig. 10 shows the collagen fibrils intertwined with each other to form the gel structure along with a high amount of porosity of almost 98-99%. The gelation kinetics were measured with the help of turbidimetric analysis under an ELISA plate reader shown in fig. 11. The resultant formula for the absorbance leads us to say that the setting time for the gel was approximately 29 minutes whereas a stable gel was obtained after 45 minutes at 37

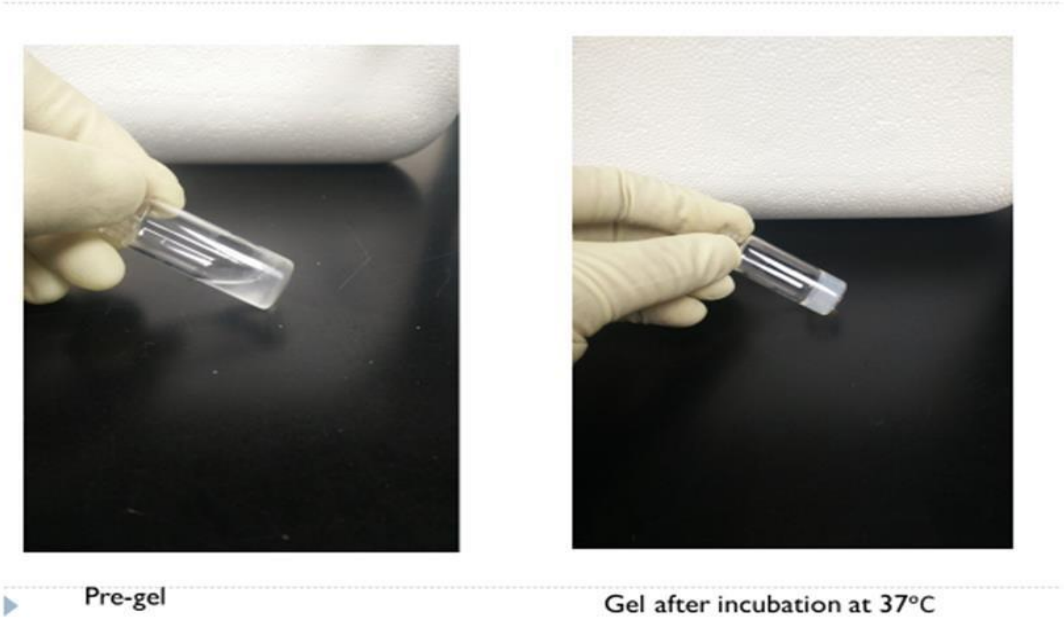

Figure 9 Gel formation. Neutralized pre-gel solution containing DDM extract and collagen I when incubated at 37°C for 1 hour forms a stable hydrogel. The above figure shows the flowability of the pre-gel solution at 4°C and formation of a hydrogel at 37°C

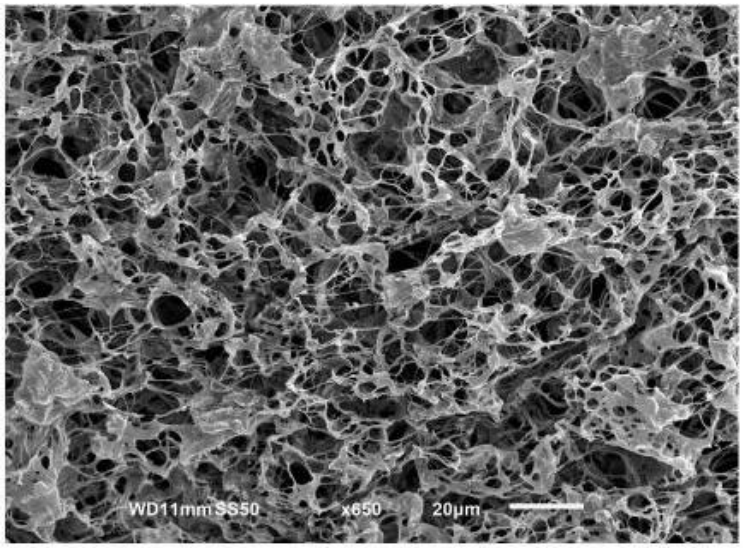

Figure 10 a. SEM image for hydrogel. Image shows SEM image with the final concentration of the gel being 3mg/ml along with the DDM extract. The SEM shows the intertwining of the collagen fibrils to the form the hydrogel structure.

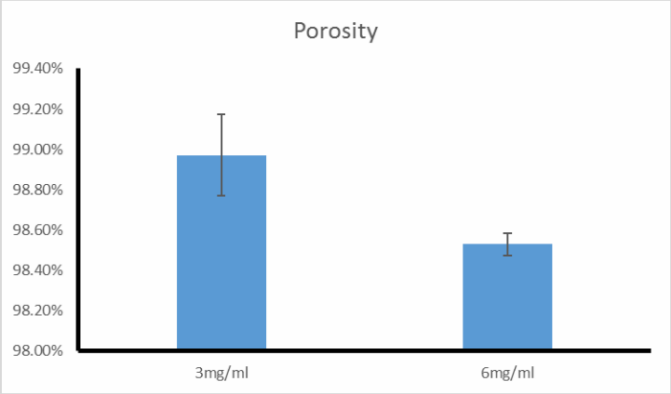

Figure 10 b. Porosity of hydrogel. Also the porosity of the 3mg/ml and 6 mg/ml is very similar being 99% and 98% approximately, respectively.

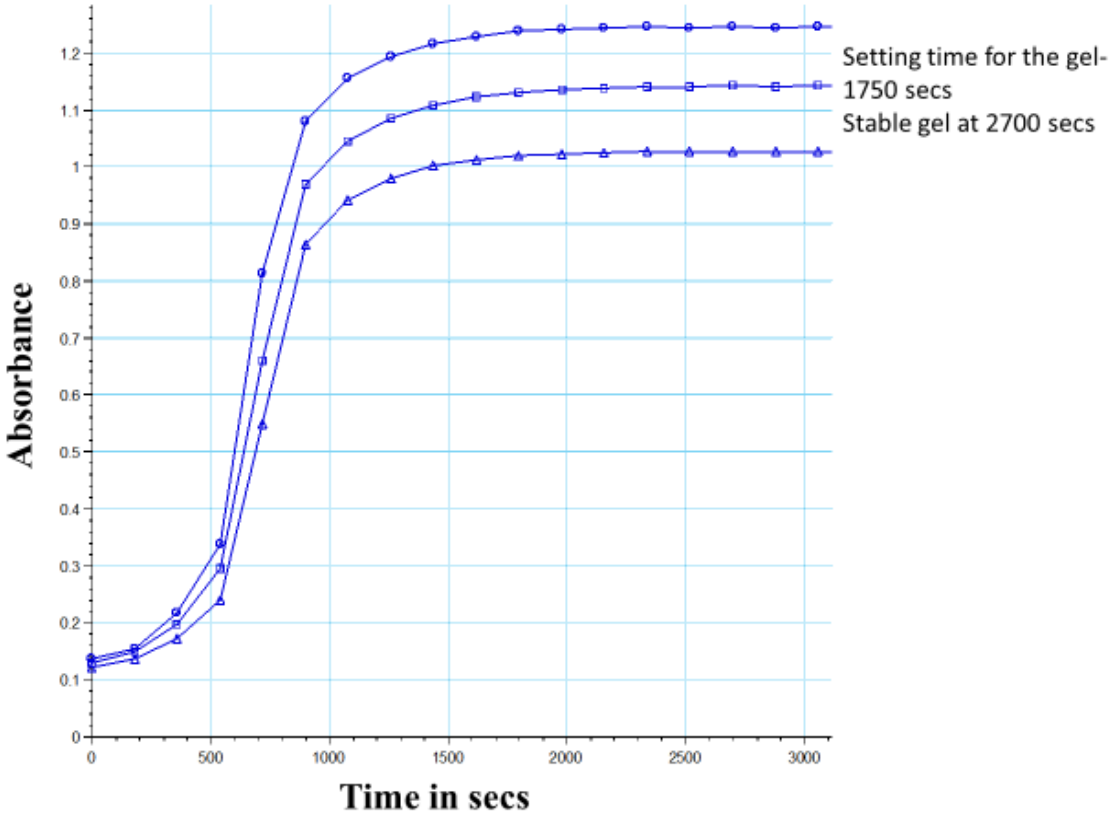

Figure 11 Turbidemetric gelation kinetics graph. The graph above shows the turbidimetric kinetics of the hydrogel. The initial linearity in the curve is the lag time needed for the gel to start gelation at 37°C. The time at 50% absorbance is the half gelation time and the linearity after reaching the peak shows the setting time for the gelation to occur which is 1750 seconds that is approximately 29 mins and a stable gel is formed at 2700 seconds that is 45 mins.

3.4 In vitro studies for demineralized dentin matrix hydrogel

To confirm that the demineralized dentin matrix is a favourable environment for the dental pulp stem cells to survive, it is necessary to perform *in vitro* studies on the hydrogel system. Ideally the hydrogel should be such that when cells are cultured on the surface of the gel, they should attach and proliferate. The cells should be viable for a specific period of time which allows them to proliferate, differentiate and form a new tissue. The viability of the cells inside the hydrogel was determined by the Live/dead staining in Fig. 12.

Collagen I was the positive control. Resultant images under the fluorescence microscopy did not show difference in the two gels. Initial 1 day gel showed unattached DPSCs while day 4 and 8 images had proliferative and more attached cells inside the gel system. There were few dead cells in the overall comparison of both groups showing that the demineralized dentin matrix gel supports the stem cells similar to the collagen I gels making it biocompatible for further use. Also MTT assay for cellular proliferation in fig.13. show the similar conclusive result of similar proliferative rate of the dental pulp stem cells on the surface of the hydrogel.

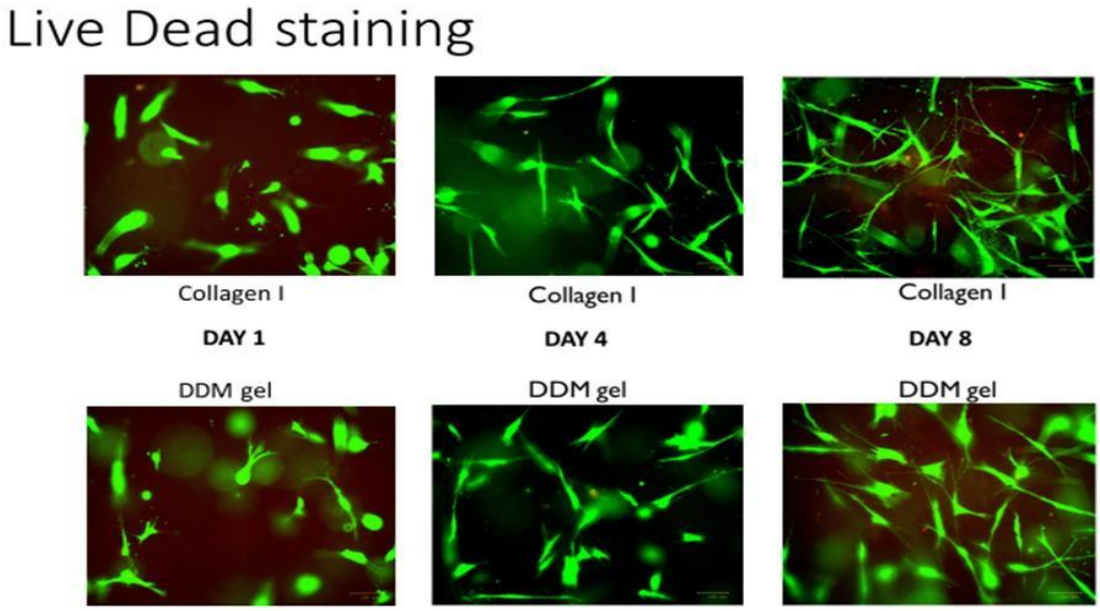

Figure 12 Live/Dead staining inside hydrogel. The image shows DPSCs inside the Collagen I hydrogel and DDM hydrogel has similar structure and viability representing that DDM hydrogel is a favorable environment for the stem cells. The images shows the initial unattached DPSCs on the surface of the hydrogel while as time passes the cells are more attached and elongated on the surface of the hydrogel.

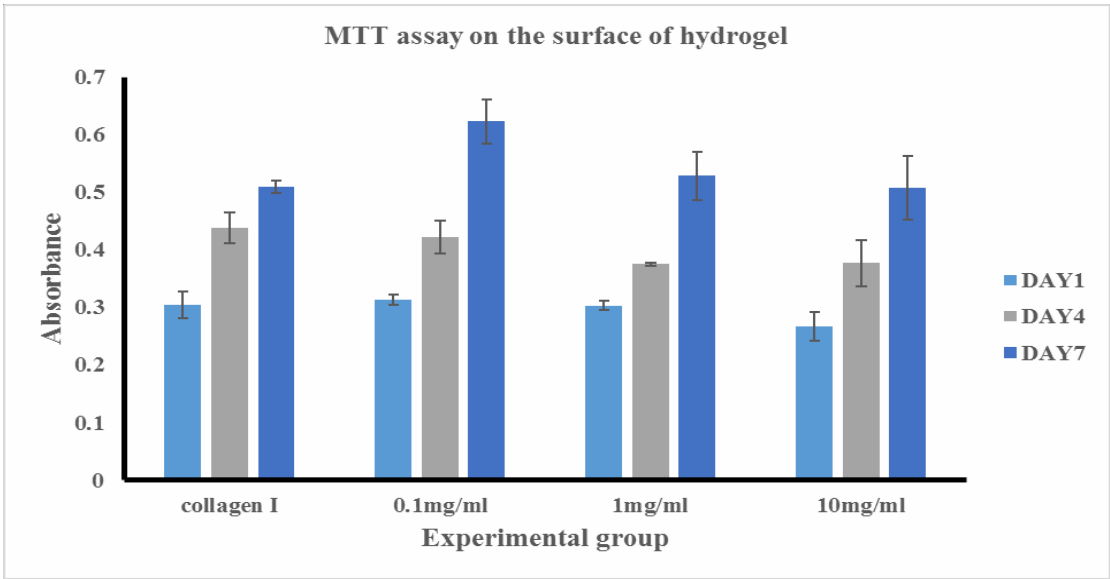

Figure 13 MTT assay on hydrogel. The graph shows the cellular proliferation on the surface of the hydrogel system with the concentration of 0.1mg/ml, 1mg/ml and 10mg/ml. Collagen I which is considered as a control for the three groups. The MTT assay shows gradual proliferation of the cells on the gel surface again suggestive of biocompatibility of the DDM hydrogel material with the DPSCs.

4. SUMMARY AND CONCLUSION

4.1 Summary

To summarize the above data, despite the problems anticipated in the solubility of the dentin to extract collagen and other bioactive factors facilitating the regeneration of dentinal and pulpal tissues, the collagen I derived by other means can be used along with the DDM extract to develop a hydrogel system mimicking the extracellular matrix of the pulp and dentin. The injectability of the material will help in ease of the technique operating for the placement of the scaffold along with the cells.

Also, with minimum discomfort and chances of infection. The DDM extract proves itself capable of helping proliferation and inducing differentiation which tells us that it will help the dental pulp stem cells to differentiate in different lineages for regeneration of natural like tissues. The characterization and morphology of the hydrogel also confirms that it will support the viability of the cells and has enough porosity for the newly formed tissue. Thus, summarizing that demineralized dentin matrix is easy to manufacture, is biocompatible and facilitated proliferation, has a capability for differentiation and hence regeneration of a tissue.

4.2 Conclusion

In conclusion, we can say that demineralized dentin matrix provides a favorable environment for the dental pulp stem cells to proliferate and differentiate in vitro. The problems that have or might occur in this material can be divided in to mainly two categories, related to the generation of the material and other would be the host response or regeneration of full length root canal.

Extracting proper concentration of collagen and bioactive molecules from the extracted teeth is a challenging task and requires meticulous preparation of the tooth dentin. Also, temperature controlled gelation should be induced for proper applications. These problems can be solved by using a protocol that helps extraction of the required material with minimum harm to it. Proper sterilization techniques and used of antimicrobial agents will help improve the host response. As expected the demineralized and decellularized material is biocompatible, help cellular proliferation and mimic the ECM like environment of the pulp. We also expect some of the bioactive molecules present in the dentin be incorporated with the collagen in the matrix to have cellular differentiation. Lastly, we expect pulp regeneration not only at the apical end but throughout the root canal space with the different cellular structures.

4.3 Future directions

To further evaluate, we plan to do studies that will show the regenerative capabilities of the demineralized dentin matrix hydrogel for pulp regeneration in vivo. For this, *in vivo* studies showing ectopic pulp regeneration in tooth roots that are subcutaneously placed in immuno-deficient mice and harvested after 3 weeks for further histological and immunohistochemistry procedures. We expect that demineralized dentin matrix as a naturally derived scaffold be biocompatible and favorable for the dental pulp stem cells to grow and differentiate *in vivo.* Our ultimate goal being our material to be used in clinical applications, it is necessary that a series of experiments *in vitro* and *in vivo* to be performed to better understand the material and its capabilities to be effective in a clinical background.

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