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Carboxymethyl cellulose hydroxyapatite hybrid hydrogel used to evaluate the osteogenic and odontogenic differentiation potential of dental pulp and periodontal ligament stem cells: An in vitro comparative study

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***Abstract*---**In order to maintain optimal function, the aim of pulp treatment is to maintain the tooth structure intact. Retaining the vitality of teeth damaged due to caries or trauma is also one of the purpose of pulp treatment. In particular, the preservation of pulp vitality is essential for continuous root development and apical closure in the case of immature permanent teeth. In recent years, regenerative endodontic treatment procedures have been suggested in order to replace the damaged pulp tissue with viable tissue. The idea of regeneration of the dentin-pulp complex was introduced into endodontics to restore the vitality of young permanent teeth that have been non-vitalized due to trauma/dental caries. The concept of success in regenerative endodontic procedures is relied on three basic elements that are; stem cells, growth factors, and scaffolds. Each of these play their own important role in the regeneration of pulp-dentin complex. This invitro comparative study had intended to deal with the new experimental custom prepared semi-synthetic hybrid scaffold (CMC-HA), its acceptance for the cells (DPSCs and PDLSCs) seeded on it and osteogenic and odontogenic differentiation of both the dental pulp and periodontal ligament stem cells. The aim of this invitro

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comparative study was to evaluate the ability of 'osteogenic' and 'odontogenic' differentiation-potential of dental pulp stem cells and periodontal ligament stem cells using the new experimental custom prepared carboxymethyl cellulose hydroxyapatite hybrid hydrogel. Though many scaffold materials have been used in regenerative medicine, each of them have there own advantages and drawbacks to overcome. Thus, the objective of this invitro comparative study was to evaluate the ability of the newly introduced experimental semi-synthetic hybrid hydrogel (carboxymethyl cellulose hydroxyapatite hybrid hydrogel) on cell viability and also investigated the differentiation-potential of dental pulp and periodontal ligament stem cells into osteogenic and odontogenic forms. Thus, within the limitations of this study, the obtained results from this invitro comparative study revealed that the scaffold used (carboxymethyl cellulose hydroxy apatite hybrid hydrogel), has better cell viability for dental and periodontal ligament stem cells. The osteogenic and odontogenic differentiation potential of dental pulp and periodontal ligament stem cells on the 14th day and 21st day revealed a gradual increase in the expression of markers for both the cell lines. However, with regards to the comparative evaluation of dental pulp stem cells and periodontal ligament stem cells, a marginal increase in the expression of markers was observed in periodontal ligament stem cells.

***Keywords*---**hDPSCs (human Dental pulp stem cells), hPDLSCs (human Periodontal ligament stem cells), CMC-HA (Carboxymethyl cellulose hydroxyapatite hybrid hydrogel), ALP (Alkaline phosphatase), DMP-1 Dentin matrix protein-1, RUNX-2 (Runt-related transcription factor-2)

Introduction

Dental caries, is one of the greatest challenges to the integrity of the developing tooth that can result in irreversible pulpal damage, necrosis of the pulpal tissues and associated arrested development of the tooth root. Thus, eventually abnormal root development will impact the long-term prognosis for tooth retention. Maintaining the vitality of teeth damaged due to dental caries or trauma is also one of the objective of pulp therapy. The purpose of pulp treatment is to maintain the tooth structure intact so as to preserve its optimal function. In young immature permanent teeth, maintaining the pulp vitality is essential for continuous tooth root development and the closure of root apex.

Dental caries and traumatic injuries are common problems seen in young children with immature permanent teeth that can often lead to pulpal necrosis and failure of root formation. An immature permanent tooth is young/newly erupted permanent tooth with incomplete root apex formation. However, it takes three more years for the root development to complete after the eruption of the tooth into the oral cavity. These developing roots, shape and form is determined

by a two layered cellular structure called Hertweg's Epithelial Root Sheath (HERS) .[21]

When the pulp in these young immature permanent teeth is infected, 'Apexification' (that includes the debridement of infected pulp tissue and placement of a Biomimetic materials) has been routinely advocated. However, while Apexification can induce apical closure it cannot maintain the continuous growth of root end and pulp vitality. Hence, to overcome the shortcomings and to replace the damaged pulp tissue with a viable tissue Regenerative Endodontic Procedure has been suggested as part of treatment plan.

'Regenerative endodontics', has been defined as biological procedure designed to replace damaged, diseased or missing dental structures, including dentin and root as well as cells of the pulp-dentin complex with living viable tissues, preferably of the same origin, that restore the normal physiological functions of the pulp-dentin complex.[36] Regenerative Endodontics comprises of building blocks that include adult stem cells, growth factors, organ tissue culture, tissue engineering materials, etc.
Stem cells, are defined as a distinct subpopulation of undifferentiated cell with self-renewal and differentiation potential. There are also one of the key elements of the tissue engineering triad, the other two are; growth factors/signals and scaffold or extracellular matrix.

There are different types of human dental stem cells that can be extracted from the oral cavity which include;[1] Dental pulp stem cells(DPSCs),Stem cells from exfoliated deciduous teeth(SHED), Stem cells from apical papilla(SCAP), Periodontal ligament stem cells(PDLSCs), Tooth germ progenitor cells(TGPCs).[2]
'Growth factors', are one of the three key elements for regeneration considered nothing less important than other two. (stem cells and scaffolds)[29] These Biomolecules Combined with scaffolds and biomolecules (BM) that are delivered from demineralized dentin lattice or delivered exogenously have been found to have a significant function in revascularization by forming ideal microenvironment. These Bioactive cues that recruit the proper cells are critical in pulp regeneration (transforming growth factors [TGFs] β1, β3 for odontoblast differentiation and stimulation of dentin matrix).[28] Growth factors such as platelet-derived growth factor (PDGF), TGF, BMPs, vascular endothelial growth factor (VEGF), fibroblast growth factor and insulin-like growth factor (IGF)etc have a ability to coordinate and modulate the events of repair and regeneration.[32]
'Scaffolds', play a significant part in tissue engineering by contributing a brief, three-dimensional spatial structure and extracellular matrix (ECM) component to keep up the regenerative condition and stem cell function.[33,35]

Scaffolds are categorized into Biological (Natural) scaffolds and Artificial (Synthetic) scaffolds. Biological or natural scaffolds include; platelet rich plasma, platelet rich fibrin, collagen, chitosan, demineralized or native dentin matrix, silk, etc. Artificial (synthetic) scaffolds include; polymer of Polylactic Acid, Poly-L-Lactic Acid, Polyglycolic Acid, Poly-Epsiloncaprolactone, Glass ceramics, Bioactive glasses, etc.

Over the year's scaffolds have played an important role for creating a 'micro-environment' for cell proliferations and angiogenesis at the defect site that had to be regenerated. In recent years hydrogels have been instituted into the tissue regeneration procedures which can be composed of natural, synthetic, or semi-synthetic.

'Hydrogels' are a three-dimensional organisation made up of hydrophilic polymers that are either crosslinked by covalent bonds or held together by intramolecular and intermolecular physical attractions.[40] Recently, an custom prepared experimental semi-synthetic natural polymer acquired from the carboxymethylation of common cellulose, named carboxymethyl cellulose (CMC) was made with hydroxyapatite(HA) crystals to produce new custom prepared hybrid hydrogel for tissue engineering.[1]

Aim

Investigated and evaluated the ability of odontogenic differentiation-potential of dental pulpal and periodontal ligament stem cells, when a new custom prepared experimental carboxymethyl cellulose hydroxyapatite hybrid hydrogel was used. Investigated and evaluated the ability of osteogenic differentiation-potential of dental pulpal and periodontal ligament stem cells when experimental custom prepared experimental carboxymethyl cellulose hydroxyapatite hybrid hydrogel was used.

Objective

Though many scaffold materials have been introduced into regenerative medicine each of them have there own advantages and drawbacks. The objective of this invitro comparative study was to evaluate the ability of the new custom prepared experimental semi-synthetic hybrid hydrogel (carboxymethyl cellulose hydroxyapatite hybrid hydrogel) when used as a scaffold, on the cell and differentiation potential of dental pulp and periodontal ligament stem cells into osteogenic and odontogenic forms.

Materials

1. Scaffold. (custom prepared experimental carboxymethyl cellulose hydroxyapatite hybrid hydrogel)
2. Human dental pulp stem cells (hDPSCs)(Himedia labs, Mumbai, india) and Human periodontal stem cells (hPDLSCs)(ANSA research labs, bangalore) culturing (using modified eagles medium suspended with 10% fetal bovine serum and 1% penicillin/streptomycin, 1% trypsin.
3. phycoerythrin-(PE)-labeled mouse anti human HLA-DR, CD-34, CD-45, Allophycocyanin(APC) labelled CD-73, fluorescein isothiocyanate-(FITC)-labeled mouse anti-human CD-90 and CD-166.
4. Cell culture medium: Alpha-MEM High Glucose - (#AL111, Himedia)
5. Adjustable multi-channel pipettes and a pipettor (Benchtop, USA)
6. Fetal Bovine Serum (#RM10432, Himedia)
7. MTT Reagent (5 mg/ml) (# 4060 Himedia)
8. Dimethyl Sulfoxide(DMSO) (#PHR1309, Sigma)
9. Phytohemagglutinin(PHA-L) (Cat No.11249738001, Sigma)

10. Dulbecco's phosphate-buffered saline(D-PBS) (#TL1006, Himedia)
11. 96-well plate for culturing the cells (From Corning,USA)
12. T25 flask (# 12556009, Biolite - Thermo)
13. Adjustable multichannel pipettes and a pipettor (Benchtop, USA)
14. Stem Pro Osteogenesis Differentiation Kit(#A10072-01, Gibco life technologies, USA)
15. Human Alkaline Phosphatase/ALPL-PE antibody (FAB1448P-025, R & D Biosystems)
16. Human DMP-1 (Cat No.4129-DM-050, R and D Biosystems)
17. DSPP-FITC (LFMb-21)antibody (SC-73632-FITC, Santa Cruz biotechnology, USA)
18. RUNX-2-FITC (SC-390351-FITC, Santa Cruz biotechnology, USA)
19. 6-well plate for culturing the cells (From Corning,USA)
20. 50 ml centrifuge tubes (# 546043 TORSON)
21. 1.5 ml centrifuge tubes (TORSON)
22. 10 ml serological pipettes (TORSON)
23. 10 to 1000 ul tips (TORSON)
24. MTT Assay.
25. Flow cytometry (BD Biosciences, USA model: BD FACS Calibur)

Methodology

This study was carried out by obtaining human dental pulp stem cells from the stem cell bank(Himedia labs, Mumbai, india) and cultured to the required rate for the experiment. Human periodontal ligament stem cells(ANSA research labs, bangalore) were primary cell lines harvested from the periodontal tissue and cultured. The cells were carefully collected into 96 well cell culture plate in Alpha-MEM High Glucose (AL111, Himedia), supplemented with Fetal Bovine Serum (RM10432, Himedia), Antibiotic antimicotic solution 0.5% of 100 X liquid(Himedia) then incubated at 37°C supplemented with 2% oxygen in a humidified atmosphere of 5% CO_2 and cutured for 7days to obtain the populace for conducting the experiments.

Flowcytometry

At 7th day, cultured cells were obtained from the both the cell lines and checked for their staminal profile using flow cytometry. The cells were collected into T-25 cm^2 flask and were harvested using 1x concentration Phosphate buffer saline(PBS). They were then stained with the following:
phycoerythrin-(PE)-labeled mouse anti human HLA-DR, CD-34, CD-45, Allophycocyanin(APC) labelled CD-73, fluorescein isothiocyanate-(FITC)-labeled mouse anti-human CD-90 and CD-166.
Mouse IgG1 a isotype Control group for both the cell lines. The data was evaluated using Human Stem Cells analysis kit (Cat No.562245, BD Biosciences, USA using the software BD CellQuest Pro Ver.6.0.).

MTT Assay

The prepared experimental custom prepared hybrid hydrogel Scaffold(CMC-HA) was rinsed and washed three times with 70% absolute ethanol to ensure sterility

and then washed 3-4times with double distilled water to remove excess ethanol content from scaffolds. Finally washed again with 1x Dulbecco Phosphate Buffer Solution for 3 times.
The experimental scaffolds were then exposed to UltraViolet(UV) light for 30-40 minutes in the Biosafety Laminar Hood and ensure the sterility.

After the incubation period of 24hrs, plates were taken out from incubator, the cell images were captured and recorded using Inverted Biological Microscope at 10x Magnification. The suspended media was washed out and MTT reagent of final concentration of 0.5mg/mL of total volume per each well of 12well Plate was added. The plates were wrapped with aluminum foil to avoid exposure to light and then were returned to the incubator and incubated for 3 hours.

MTT reagent was washed then 500 µl of solubilisation solution Dimethyl Sulfoxide (DMSO) was added to the wells. Gentle stirring was done using gyratory shaker so that it enhances dissolution. Occasionally, pipetting up and down might be required to completely dissolve the MTT formazan crystals especially it is done in dense cultures. Absorbance rate was observed using spectrophotometer at 570nm and 630nm that are used as reference wavelengths.

Osteogenic Differentiation

Sterilization of the Scaffold:
The experimental hybrid hydrogel scaffold (CMC-HA) was rinsed and washed 3 times with 70% absolute ethanol to ensure sterility and then was washed 4-times with double distilled water to remove the excess ethanol content from scaffolds. Then further washed with 1× DPBS for 3 times. The scaffolds were then exposed to UV light for 30-40 minutes in the Biosafety Laminar Hood to ensure the sterility.

Cell Seeding on experimental Scaffolds/Biomaterials:
From the Fully Confluent T-25 Cm^2 flask the hDPSCs and hPDLSCs were collected by trypsinization and the viability of cells was counted using hemocytometer. The sterilized scaffolds were carefully placed in each well of 6well Plate and was Seeded with 1000µl of cell suspension containing 1x10^5 cells in each well above the experimental scaffolds along with standard control and untreated Control wells and the plates were incubated for 24hrs at 37°C in a 5% CO_2 , 2%O_2 atmosphere separately for getting 70-80% confluence.

Osteogenic differentiation assay by flow cytometry:
Seeding of cells onto the experimental scaffold was done, osteogenic differentiation media which contains (90ml of Stempro Osteocyte differentiation basal media, 9ml of Stempro Osteogenesis supplement, 1% antibiotic-antimycotic solution, 10mM/L β-glycerophosphate, 0.2mM/L ascorbate-2-phosphate and 100ηM/L dexamethasone), was added on. After an incubation period of 24hrs, the plates were taken out from the incubator and the media replaced with pre-warmed complete osteogenesis differentiation media after which incubation was continued. (Stem cells were allowed to expand as they were differentiating under osteogenic conditions. Cultures were refed with the medium for every 3days). After the specific incubation period of cultivation (14 and 21 days) the medium

was removed from all the wells and a PBS wash performed. A 200 µl of trypsin-EDTA solution was then added to the wells which were seeded with stem cells and incubated at 37°C for 3-4 minutes. Then 2ml culture medium was added to the cultures and the cells have been harvested directly into 5ml storage tubes. These tubes were centrifuged for five minutes at 300 xg at 25°C. The supernatant was carefully decant from the 5ml storage tubes and the remaining precipitate in the tubes washed with phosphate buffer saline (PBS). The cells were stained with 20µl of osteogenic and odontogenic markers, conjugated with FITC and PE and were incubated for 30minutes in dark at room temperature. The samples were finally analysed using flow cytometry.

Figure 1: carboxymethyl cellulose hydroxyapatite hybrid hydrogel

Results

Flow Cytometry (FACS) Analysis of Human Dental Pulp Stem Cells (DPSCs) And Human periodontal ligament stem cells

The mesenchymal stem cells used in the experiment were characterized for HLA-DR, CD-34, CD-45, Allophycocyanin(APC) labelled CD-73, fluorescein isothiocyanate-(FITC)-labeled mouse anti-human CD-90 and CD-166 using flowcytometry analysis. The data obtained was that the both cell lines expressed positive for CD73, CD90, CD166 and negative for CD34, CD45, and HLA-DR.

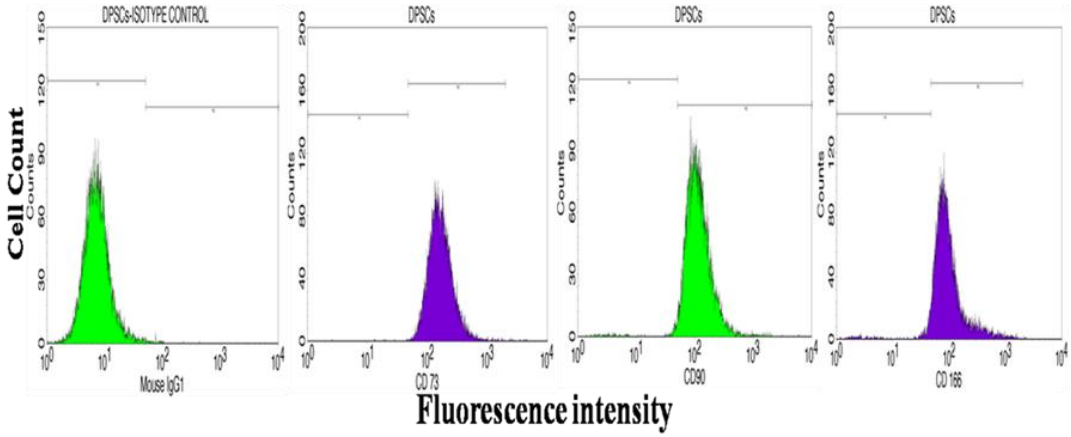


Fig 2: Positive Marker Panel of hDPSCs: (CD-73, CD-90 and CD-166)

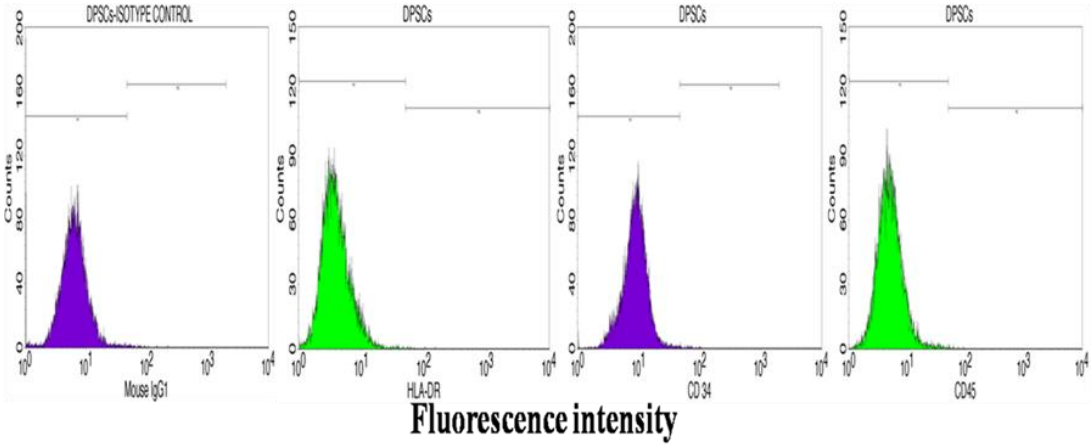


Fig 3: Negative Marker Panel hDPSCs: (HLA-DR, CD-34 and CD-45)

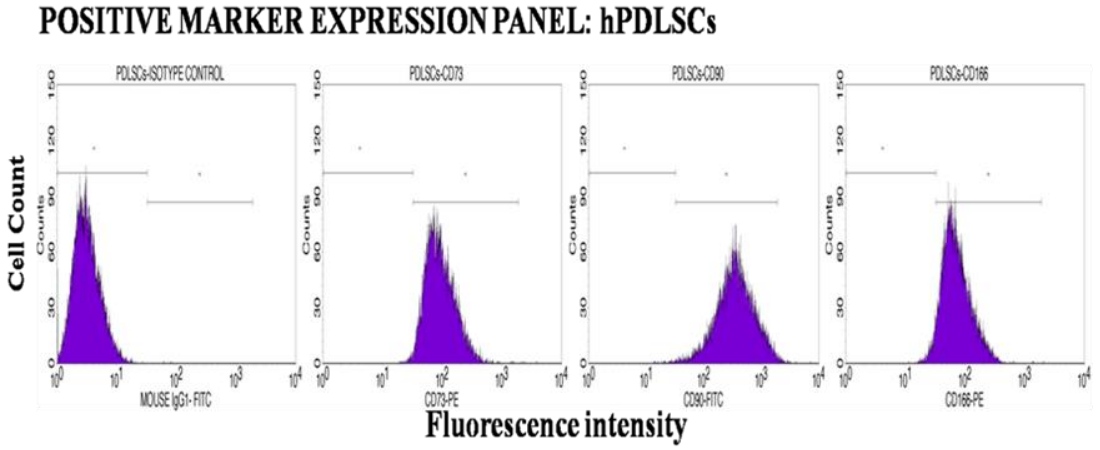


Fig 4: Positive Marker Panel of hPDLSCs: (CD-73, CD-90 and CD-166)

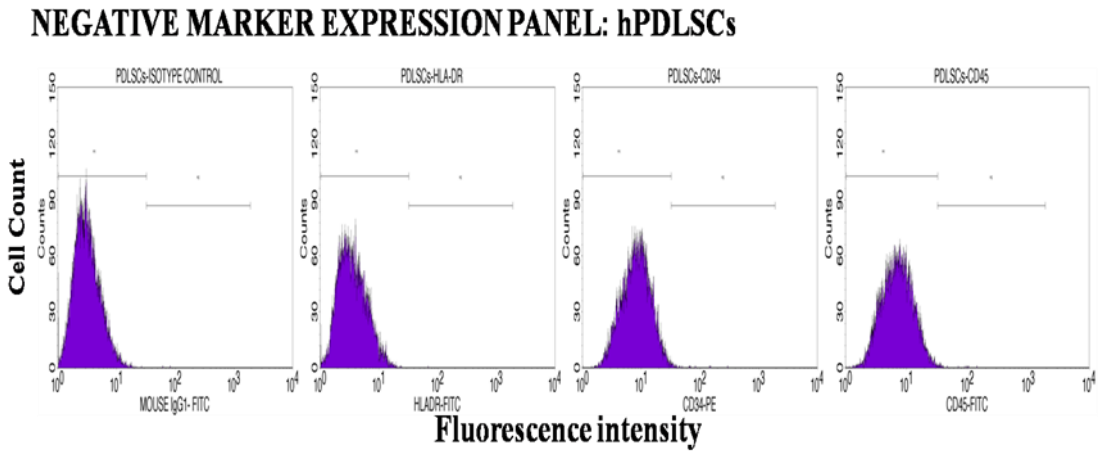


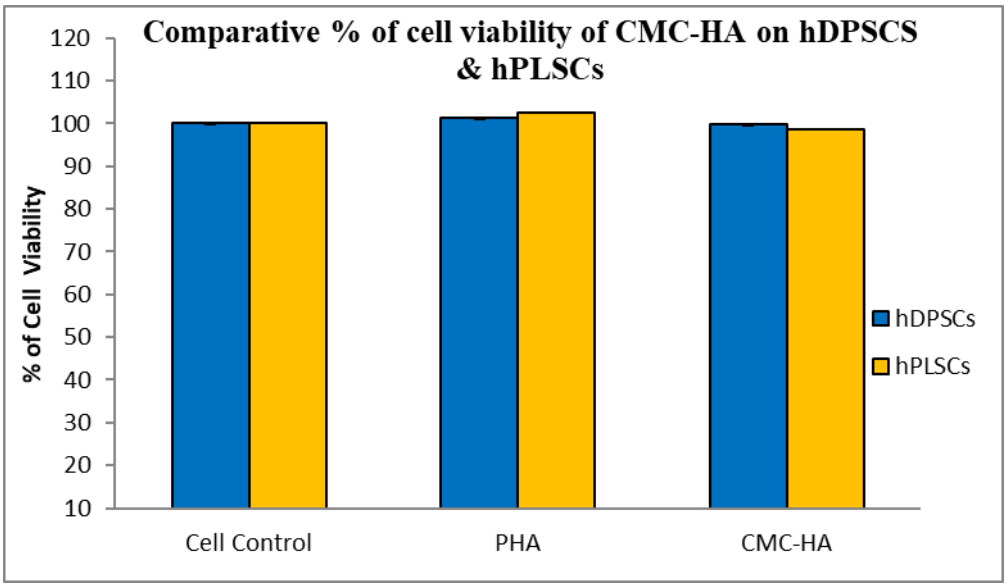
Fig 5: Negative Marker Panel hPDLSCs: (HLA-DR, CD-34 and CD-45)

MTT Assay:

The results obtained suggested that the experimental hybrid hydrogel Scaffolds/Biomaterial, CMC-HA do not showing any Cytotoxic potential properties against both the cells after the treatment 24hrs of incubation at 37^{o}C temperature and the custom prepared experimental hybrid hydrogel having the Good cell viability without affecting the cell growth properties against Human Dental Pulp Stem Cells and human periodontal ligament stem cells in invitro. The absorbance readings with calculations enclosed using spectrophotometer at 530nm and 630nm wavelengths.

Formula used to calculate the obtained optical density values of the cell groups:

$$\%\ cell\ vibility = Mean\ optical\ density\ \frac{sample - blank}{control - blank} \times 100$$



Graph 1 : Comparative % Cell Viability of CMC-HA treated Scaffold on hDPSCs and hPLSCs after incubations of 24hrs.

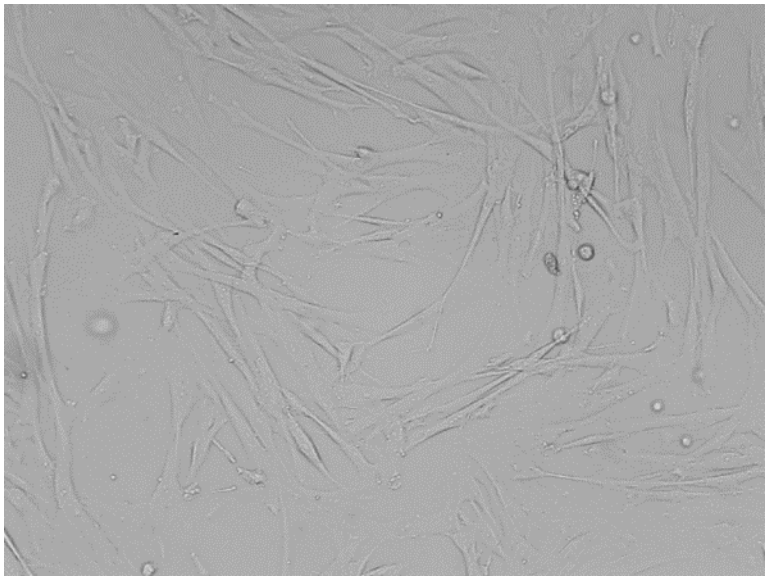


Fig 6: human dental pulp stem cells microscopic observations under 10x inverted biological microscope after 24hr incubation on CMC-HA.

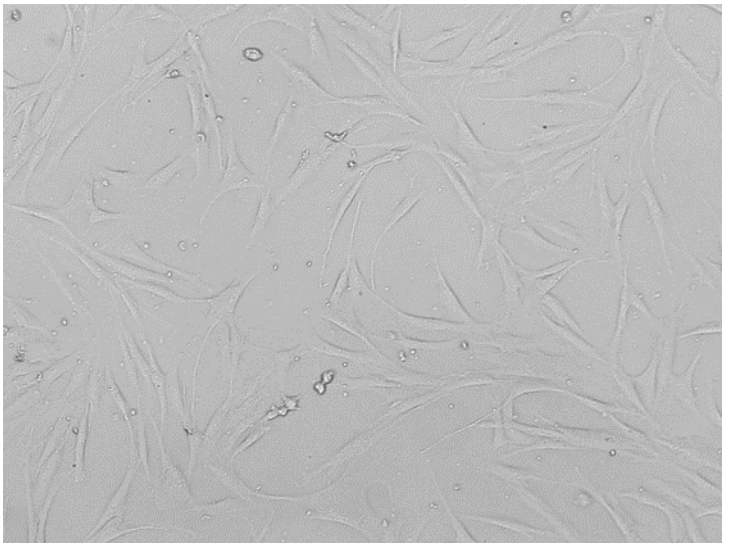
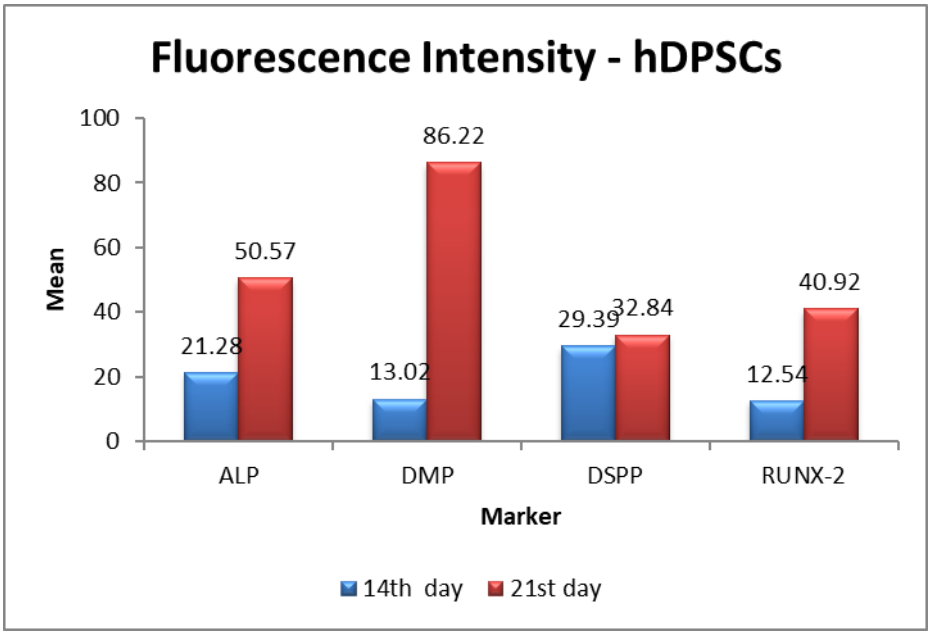
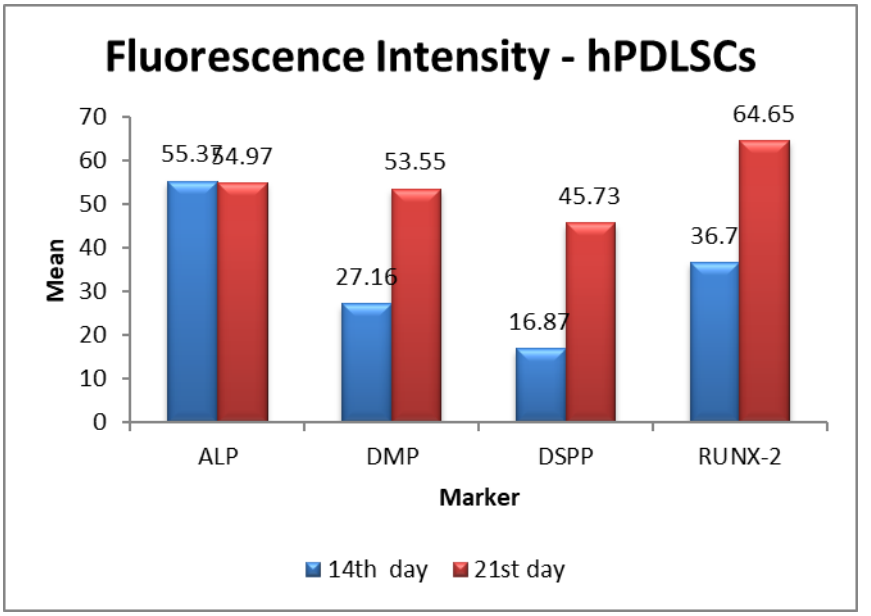


Fig 7: human periodontal ligament stem cells microscopic observations under 10x inverted biological microscope after 24hr incubation on CMC-HA.

Osteogenic and odontogenic flowcytometry analysis



Graph 2: Overlaid bar graph depicting the comparative expression of mean values of Osteogenic differentiation markers such as ALP and RUNX-2 for 14days,21days and Odontogenic differentiation markers such as DMP-1 and DSPP for 14 days,21 days seeded on CMC-HA. The study is performed on hDPSCs.



Graph 3: Overlaid bar graph depicting the comparative expression of mean values of Osteogenic differentiation markers such as ALP and RUNX-2 for 14,21 days and Odontogenic differentiation markers such as DMP-1 and DSPP for 14 and 21 days seeded on CMC-HA. The study is performed on hPDLSCs.

Osteogenic and odontogenic differentiation ability of the cells lines was evaluated on 14 and 21 days. The results obtained exhibit that both the cell lines had a gradual increase in osteogenic and odontogenic markers expression as shown in (Graphs 2and3). When compared both the cell lines on their differentiation ability results after 14day culture exhibited upregulation of Alkaline phosphatase (ALP), Dentin Matrix Protein(DMP-1) and Runt-related transcription factor-2(RUNX-2) by the periodontal ligament stem cells.

However, when compared with dental pulp stem cells, there was upregulation in Dentin Sialophospho Protein (DSPP) for periodontal ligament stem cells. At the end of 21 days, culturing of cells on osteogenic medium, the dental pulp stem cells revealed an increase in DMP-1 compared to the periodontal ligament stem cells which have shown an increase in expression of ALP, DSPP and RUNX-2. For osteogenic and odontogenic differentiation-potential of dental pulp and periodontal ligament stem cells on intervals of 14 and 21 days ANOVO and Turkey B Post Hoc Test were done to assess the differences. Both the cell lines were significant, exhibiting *p* value 0.001.

Discussion

The most desirable result of endodontic therapy is to substitute a diseased or non-vital pulp with healthy pulp tissue that would revitalise the teeth through regenerative endodontics.[28] The idea of Regenerative endodontic treatment has begun with recovering the vitality of dental pulp as a potential treatment alternative. It centres around substituting injured and diseased pulp with a

functional pulp tissue, by the utilization of biologically based procedures thereby re-establishing normal function of the pulp-dentin structure.[36]

'Regenerative endodontics', can be defined as a biological procedure designed to replace damaged, diseased or missing dental structures including dentin, root and cells of the pulp-dentin complex with living viable tissues, preferably of the same origin, that help to restore the normal physiological functions of the pulp-dentin complex.[36] The success of regenerative endodontics is evaluated when the following three goals are fulfilled. They include:
Primary: Elimination of symptoms and evidences of bony healing.
Secondary: Elimination root wall thickness and/or increased root length.
Tertiary: Positive response to vitality testing.[40]

In regenerative endodontics, there are some important ingredients that aid in restoring back the compromised pulp-dentin complex. They include:

- Stem cells
- Growth factors
- Organ-tissue cultures and
- Tissue engineering materials

However, it is the stem cells that have the unique ability to continuously divide, yield progeny cells and differentiate either into a variety of cell types or tissues.[41] Stem cells are defined as distinct subpopulation of undifferentiated cells with self-renewal and differentiation potential.[21] They can be classified into: Totipotent, Pluripotent and Multipotent cells.

Totipotent stem cells, have cell plasticity of every cell that can form into new individual. Pluripotent stem cell, can form any (more than 200) cell types.[41] True pluripotent stem cells can be found in developing embryo. However, harvesting of these cells requires destruction of embryo, hence legal and ethical concerns with such practice. Stomatic cells can be transformed into pluripotent immature stem cells called induced pluripotent stem cells(iPSC) a detailed noteworthy finding by. However, adult Mesenchymal Stem Cells are more confined in their ability to differentiate, only forming tissues of mesenchymal origin and in this way they are classified as multipotent cells.[21]

It has been proved that under certain physiological or experimental conditions that the adult mesenchymal stem cells show unique ability to renew themselves over long periods. which is achieved by cell division. Experiments have revealed that 'specialized cells' can be developed on induced mesenchymal stem cells.[24] Various populaces of adult stem cells have been recognized in tissue compartments in the oral region. Five forms of MSCs have been isolated from dental tissues and they have exhibited strong properties of proliferative and multilineage differentiation. The Isolated mesenchymal stem cells from oral tissues are; Dental Pulp Stem Cells (DPSCs), Stem Cells From Human Exfoliated Deciduous Teeth(SHEDs), Periodontal Ligament Stem Cells (PDLSCs), Dental Follicle Progenitor Stem Cells (DFPCs), Stem Cells From Apical Papilla (SCAPs), salivary gland stem cells (SGSCs), oral epithelial stem cells (OESCs), gingival-derived mesenchymal stem cells (GMSCs) and periosteal derived stem cells (PSCs).[21,1]

'Growth factors', one of the three key ingredients for regeneration is nothing less important than the former two (stem cells and scaffolds).[29]These are proteins that bind to cell receptors and cause proliferation and/or differentiation of cells. Many of the growth factors in different cell types are very flexible promoting cell division while others are more cell specific.

Scaffolds play an important role in providing a temporary and three-dimensional spatial structure and extracellular matrix(ECM) portion to preserve the regenerative environment and stem cell function.[33,35] They encourage cell organisation, vascularization, help the proliferation and differentiation of stem cells, contribute to improved and faster development of tissues, contain nutrients to promote cell survival, growth, can contain antibiotics to prevent bacterial growth in canal systems exhibit certain mechanical and biological functions.[44] Scaffolds are of two types (based on their orgin) (a)natural and (b)artificial (synthetic).

Though certain draw backs present in each of the above discussed scaffolds which lead to development of the Hydrogel scaffolds, these have been employed recently as an emerging and promising medium in regenerative medicine. A variety of natural and synthetic polymers have been used to fabricate hydrogels eg; Collagen, hyaluronic acid, chondroitin sulfate, fibrin, fibronectin, alginate, agarose, chitosan, silk, etc. To improve the properties of regularly hydrogels such as the ability to adhere cells on to the surface, non release of toxic substitutes on degradation, pore size and porosity extent etc.[58] Thus, to overcome the drawbacks of regularly used hydrogels the custom prepared experimental hybrid hydrogel was developed. In this invitro comparative study custom prepared experimental carboxymethyl cellulose hydroxyapatite hybrid hydrogel (CMC-HA) was used.(fig:1)

Though each scaffold have their own pros and cons, there is limited comparative scientific research regarding the efficiency of differentiation-potential of dental pulp and periodontal ligament stem cells, especially with the use of the newly customised hybrid hydrogel scaffold. Thus, the aim of this study was to investigate the osteogenic and odontogenic-differentiation ability of dental pulp and periodontal ligament stem cells when new custom prepared experimental semisynthetic natural polymer as a scaffold (carboxymethyl cellulose hydroxyapatite hybrid hydrogel) was used.

This study also interpreted whether both the cell lines had any marked difference (dental pulp and periodontal ligament stem cells) that is, their potential to differentiate into osteogenic/odontogenic forms when cultured on a custom prepared experimental semi-synthetic hybrid hydrogel scaffold.(carboxymethyl cellulose hydroxyapatite hybrid hydrogel CMC-HA)

Though many scaffold materials have been used in regenerative medicine, each of them has their own advantages and drawbacks to overcome. The objective of this comparative study was to evaluate the ability of the newly introduced custom prepared experimental semi-synthetic hybrid hydrogel (carboxymethyl cellulose hydroxyapatite hybrid hydrogel) on the cell viability and also investigated the

differentiation-potential of dental pulp and periodontal ligament stem cells into osteogenic and odontogenic forms.

In this study, the cells were obtained from both the cell lines and checked for their staminal profile using flow cytometry. The flow Cytometric analysis of the human dental Pulp Stem Cells determining surface markers include, HLA-DR, CD-34 and CD-45. All these markers expressed in the negative expression region. HLA-DR confirmed they are disease free. However, the surface markers, CD-73, CD-90 and CD-166 markers expressed in positive expression region and confirmed that they are truly human dental Pulp Stem Cells.(fig:2,3) Similarly, flow Cytometric analysis of the Periodontal Ligament Stem Cells surface markers such as CD-73, CD-90 and CD-166 surface markers expressed in 'Positive' expression region.(fig:4,5)

On MTT assay, the experimental hybrid hydrogel (CMC-HA) had good cell viabilty without affecting the cell growth properties against human dental pulp Stem cells and human periodontal ligament stem cells invitro. However, polysaccharide-based hydrogels designed for bone tissue engineering, need stronger mechanical proprieties to regenerate hard tissues. Research has demonstrated that the combination of hydroxyapatite with natural or synthetic hydrogels improves bioactivity, osteoinductivity and osteoconductivity.[52]

As previous investigations report that the experimental CMC-HA hybrid hydrogel exhibited the presence of HA crystal homogenously distributed inside and, on the hydrogel, surface thereby improving inherent mechanical and adhesive proprieties.[53] In this invitro comparative study another important parameter evaluated was the observation of the differentiation-potential of human dental pulp and human periodontal ligament stem cells into osteogenic and odontogenic forms. The observations were done at time interval periods of 14 and 21days. At the end of 7days, culturing on experimental CMC-HA hybrid scaffold by supplementing with osteogenic medium so as to induce the differentiation of cells and were evaluated using osteogenic and odontogenic markers.

The results obtained after 14day culture were analysed by flow cytometry. Hence the upregulation of Alkaline phosphatase (ALP), Dentin Matrix Protein-1 (DMP-1) and Runt-related transcription factor-2(RUNX-2) by human periodontal ligament stem cells was observed when compared with human dental pulp stem cells were there was an upregulation in Dentin Sialophospho Protein (DSPP). At the end of 21 days, culturing of cells on osteogenic medium the dental pulp stem cells revealed an increase in DMP-1 compared to human periodontal ligament stem cells which have shown an increase in expression of ALP, DSPP, RUNX-2.

On overview of the outcomings after both 14days and 21 days it was observed that both the cell lines exhibited a gradual increase in the osteogenic and odontogenic markers. In this invitro comparative study the results obtained revealed the ability of the custom prepared experimental hybrid hydrogel scaffold (CMC-HA) to enhance the growth of both the cell lines. As per the previous investigations the CMC-HA hydrogel exhibited good cell viability properties. Additionally, hydroxyapatite crystals osteoconductive and osteoinductive properties were also enhanced. When both the cell lines were cultured and

evaluated at different intervals of 14 and 21 days. The results obtained revealed a marginal upregulation of ALP, DMP-1, RUNX-2 by human periodontal ligament stem cells and DSPP high expression by dental pulp stem cells. where the untreated control group did not express the values as experimental groups did.

In this invitro study, the human dental pulp stem cells had exhibited better upregulation of all the osteogenic and odontogenic markers. Markers such as dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1) are the key elements for the odontoblastic differentiation. A major non-collagenous dentin specific protein DSPP is expressed and secreted by odontoblasts.[54] Another extra cellular dentin matrix protein is also involved in human dental pulp stem cells to differentiate into odontoblasts. The results in this study have expressed specific upregulation of DSPP and DMP-1 markers by dental pulp stem cells. This shows the capacity of the human dental pulp stem cells to differentiate into odontoblast cells. However, expression of osteogenic markers such as ALP, RUNX-2 also play an important role in extra cellular matrix formation and calcified tissue.[55]

Within the limitations of this study, it was observed that human periodontal ligament stem cells exhibited better upregulation of ALP, RUNX-2, DSPP, DMP-1 markers on both the interval periods(14 and 21days) of observation. When cultured on α-minimum essential medium (*α*-MEM), human periodontal ligament stem cells had greater proliferation rates and stronger osteogenic potential than cells cultured in Dulbecco's minimum essential medium (DMEM).[56] The probable reasons to the results maybe because of the amino acids, vitamins and nucleotides present in the alpha MEM medium play an important role for hPDLSCs differentiation. Additionally, hypoxic conditions(2% O_2) can also promote the osteogenic potential of hPDLSCs via, activation of p38 and ERK1/2 signalling pathway.[57]

The Osteogenic and odontogenic differentiation-potential of both cell lines was promising with the positive expression of markers. However, the human periodontal ligament stem cells had exhibited marginal higher values with respective markers.

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