Original Article

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Dentistry Section

Evaluation of Cell Adhesion and Proliferation of Human Dental Pulp Stem Cells to Different Root End Filling Materials: An In-vitro Study

SHRESHTHA MUKHERJEE[1], GEETA HIREMATH[2], ANIL BAPU BARGALE[3], PRIYA HORATTI[4]

ABSTRACT

Introduction: Recent advancements in dental materials have led to the development of various restorative cements. The amalgamation of various biomimetic cements becomes necessary to improve the physico-chemical properties of the existing cements. Mineral Trioxide Aggregate (MTA) Plus and Chitosan are two such materials which are combined and chosen to check their effects on dental stem cell adhesion and proliferation.

Aim: To compare and evaluate the cell adhesion and proliferation of human dental pulp stem cells on MTA Plus, MTA Plus-Chitosan Conjugate, and Chitosan by directly culturing the cells on the materials.

Materials and Methods: This in-vitro study was conducted at the Central Research Laboratory in Department of Conservative Dentistry and Endodontics, S.D.M College of Dental Sciences, Dharwad, Karnataka, India over a period of 1.5 years from the month of June 2020 to December 2021. The samples were divided into four groups: Group A-MTA Plus mixed with the proprietary gel (9 specimens), Group B-MTA Plus mixed with 2% Chitosan gel (9 specimens), Group C-2% Chitosan Gel (9 specimens), and Group D-Control Group (Growth Medium). Non carious extracted permanent human premolars for orthodontic purposes were collected and sectioned for the extraction of pulp tissue. The tissue was placed in a basic medium Dulbecco modified essential medium-High Glucose (DMEM-HG). Dental Pulp Stem Cells were subjected to differentiation using differentiation media (Hi-Media) containing Dexamethasone. Cell proliferation and viability were checked using trypan blue, and cell adhesion was checked using crystal violet under an inverted light microscope. To evaluate if there were any changes in the proliferation of viable cells at 24 hours, 48 hours, and 72 hours among different materials, the Wilcoxon's Signed-rank Test was used. Statistical significance was set at $p \leq 0.05$.

Results: The cells were able to adhere to the biomaterial and showed a spindle-shaped morphology, which was observed under the inverted light microscope. ALP activity was measured using a photometer after cell differentiation. MTA Plus-Chitosan conjugate showed a 94% increase in cell proliferation after 48 hours (n=86/91). MTA Plus showed a 91% increase in cell proliferation after 48 hours (n=109/120).

Conclusion: Chitosan can be used as a vehicle with MTA since their conjugate showed greater proliferation activity after stem cell culture.

Keywords: Cell proliferation, Chitosan, Mesenchymal stem cell, Odontoblasts

INTRODUCTION

Caries is an irreversible microbial disease that leads to demineralisation of the inorganic content and dissolution of the organic component of the tooth. Untimely management often leads to the progression of caries and subsequently leads to pulpal damage. During this process, the natural repair process is stimulated, leading to the formation of new odontoblasts that produce new dentin. This observation suggests the possibility that dental pulp contains mesenchymal cells that provide a source of cells that facilitate repair [1].

This observation led to various studies on the mesenchymal cells that can help in the regeneration of damaged cells. The isolation of stem cells from extracted teeth was first reported by Gronthos, leading to studies on the effect of various materials on dental pulp stem cells [2]. Their attachment and spreading on material surfaces are important aspect that play a crucial role in dental pulp repair. Cell adhesion to bioactive materials is important in stimulating signals that regulate cell differentiation, migration, and survival [3]. Higher expression of fibroblast growth factor and transforming growth factor β was observed in Dental Pulp Stem Cells (DPSCs) when cell proliferation increased [4].

Dental restorative materials can affect the surrounding structures of teeth they come in contact with (enamel, dentin, cementum, bone) either directly or indirectly. Ions are released from the material into the pulp via open dentinal tubules, stimulating tissue response either during or after the setting process. Therefore, restorative materials should be assessed not only for their chemical, physical, and mechanical characteristics but also for their biocompatibility [5]. Any assessment of biocompatibility requires an evaluation of cytotoxicity, i.e., the effect of a material on cell viability [4].

Over the years, various materials have been tried and tested for repair purposes. The most commonly and frequently used material in recent years is Mineral Trioxide Aggregate (MTA). MTA is a calcium silicate-based material that consists of tricalcium silicate, dicalcium silicate, tricalcium aluminate, bismuth oxide, calcium sulfate, and gypsum. It has been widely used in conservative and endodontic treatment, including root end filling, direct pulp capping, and regenerative endodontic procedures. Moreover, the versatile properties of MTA, including biocompatibility, antibacterial, and anti-inflammatory properties, make it a promising candidate for tissue engineering applications [6,7]. Unfortunately, it suffers from several deficiencies, such as poor workability and prolonged setting time [8].

To overcome these deficiencies, newer materials have been introduced into the market. MTA Plus (Prevest Denpro) is a material similar to MTA with a finer particle size and better handling properties that have been released into the market. Another material widely used in the medical field for various applications is Chitosan.

Chitosan is a natural biodegradable polymer produced by the deacetylation of chitin. It is derived from naturally occurring sources, such as the exoskeletons of insects, crustaceans such as crabs, shrimps, prawns, lobsters, and the cell walls of some fungi such as aspergillus and Mucor [9]. Chitosan is an ideal material for biomedical applications because of its distinctive biological properties, including good biodegradability, biocompatibility, osteoconductivity, and antimicrobial properties. It is also a candidate material for hard tissue repair and regeneration [10].

Chitosan, as a vehicle, has been widely discussed in research papers due to its property to coat and protect the molecules of dental materials from degradation. It serves as a semi-synthetic extracellular matrix to provide an amenable environment for cellular adherence and remodelling [11].

Studies must be performed, especially on human cells, to ensure that these new materials are biocompatible when in close contact with living tissues. The compounds in them may either interfere with the healing process or repair the damaged tissue. The incorporation of various particles into each other can not only improve their physiochemical properties but also their biocompatibility, pH, sealing ability, and calcium-releasing ability [8].

Thus, the present study develops a novel approach of using chitosan as a vehicle to mix MTA Plus. The aim of the study was to compare and evaluate the cell adhesion and proliferation of human dental pulp stem cells on MTA Plus, MTA Plus-Chitosan Conjugate, and Chitosan by directly culturing the cells on the materials.

MATERIALS AND METHODS

This in-vitro study was conducted at the Central Research Lab in Department of Conservative Dentistry and Endodontics, S.D.M College of Dental Sciences, Dharwad, Karnataka, India, over a period of 1.5 years. The cultivation of stem cells was started in June 2020 and completed in December 2021. The study protocol was approved by the Institutional Review Board and Ethical Committee of the Institution (IRB NO- 2019/P/CONS/71).

Inclusion and Exclusion criteria: Inclusion criteria included non carious extracted vital permanent human teeth. Exclusion criteria were dental caries involving the dental pulp, fractured teeth, sclerosed teeth, calcified pulp chambers, and non vital teeth.

Sample size calculation [12]:

$$n=\frac{2s_p^2[z_{1-\alpha/2}+s_{1-\beta}]^2}{\mu_d^2}$$

$$S_p=\frac{s_1^2+s_2^2}{2}$$

where,

s_1^2 : Standard Deviation in First Group

s_2^2 : Standard Deviation in Second Group

μ_d^2 : Mean Difference between the samples

α : Significance Level

$1-\beta$: Power

Study Procedure

Sample preparation: The MTA Plus powder was mixed with its gel as recommended by the manufacturers. For conjugates, each material was mixed with 2% chitosan gel. The materials were grouped as follows:

Group A: MTA Plus mixed with the proprietary gel (9 specimens)

Group B: MTA Plus mixed with 2% Chitosan gel (9 specimens)

Group C: 2% Chitosan Gel (9 specimens)

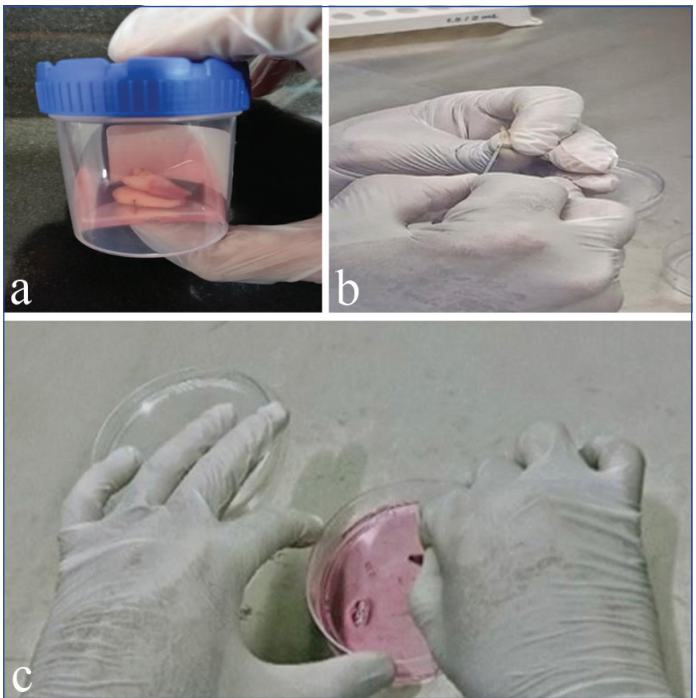
Group D: Control Group (Growth Medium)

All biomaterials were condensed into specimen molds (2±0.1 mm diameter×2±0.1 mm). Each group had 9 specimens as the study was done in triplicates in three different experiments at three different time intervals, i.e., 24, 48, and 72 hours [13,14]. They were then incubated in a CO_2 incubator at 37°C with a 100% humidified atmosphere for 24 hours to ensure complete setting.

1. **Extraction of dental pulp:** A total of 27 non carious extracted permanent human premolars for orthodontic purposes were collected from the Department of Oral Surgery [Table/Fig-1a]. The teeth were then disinfected with a 3% sodium hypochlorite solution for two minutes and rinsed with Phosphate Buffer Saline (PBS) thrice. The teeth were decoronated at the cementoenamel junction, and the pulp was exposed. The sectioned teeth were placed into the Transport Media (TM) containing basic medium Dulbecco Modified Essential Medium-High Glucose (DMEM-HG) supplemented with 20% Fetal Bovine Serum (FBS) and 1% Antibiotic Antimycotic (ABAM) (Hi-Media- A002A-20 mL) and transferred to the Central Research Laboratory for subsequent processing and culture [8].

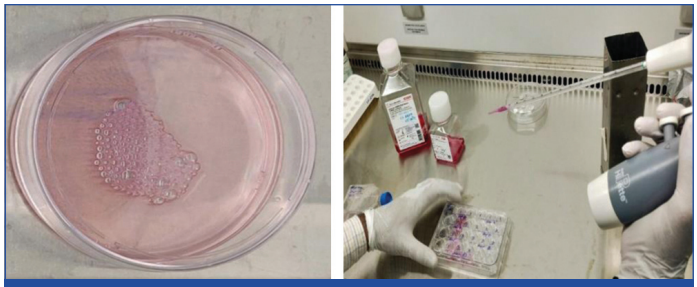
   A 100 mm petri plate (Hi-Media) was set up for the processing of each tooth in a biohazard laminar flow hood (Thermofisher) under aseptic conditions. Sectioned teeth were decanted in a petri plate. Teeth were held with sterile forceps, and pulp tissue was gently extirpated using an endodontic H-file #30 (MANI, Inc, USA) [Table/Fig-1b]. The pulp tissue was placed in PBS containing a 1% antibiotic-antimycotic solution (Hi-Media) in a petri plate for 10 to 20 minutes and then washed with PBS (Hi-Media) for 10 minutes each.

2. **Preparation of dental pulp stem cells:** The pulp tissue was then transferred into a petri plate containing DMEM-HG with 20% FBS and minced into 1-2 mm^3 using a surgical blade [Table/Fig-1c]. The minced fragments were plated in a T-25 flask containing DMEM-HG supplemented with 20% FBS, 1% antibiotic and antimycotic, 1 mM sodium pyruvate, and 2 mM L-glutamine (Hi-Media) [Table/Fig-2]. Explants were cultured at 37°C in a humidified incubator with 5% CO_2.



[Table/Fig-1]: a) Extracted human permanent premolars. b) Decoronated at CEJ for the removal of pulp tissue. c) Pulp tissue minced into fragments with the culture media.

Colonies were observed daily under an inverted microscope (Motic) for any contamination or cell growth via migration from explants. When cells reached 70 to 80% confluency, they were either used for an assay or cryopreserved for later use.



[Table/Fig-2]: Cell seeding into 24-well tissue culture plates.

Dental pulp stem cells were subjected to differentiation using differentiation media (Hi-Media) containing Dexamethasone (DMEM-HG+10% FBS+2% ABAM, Dexa-40 ng/mL). The cells were allowed to differentiate for 10-15 days, and the morphology was monitored daily.

3. **Verification of dental pulp stem cells to be odontoblasts:** Alkaline Phosphatase (ALP) activity was carried out for newly-differentiated odontoblast-like cells using the ALP assay kit. This test confirmed that the differentiated cells were odontoblasts, and the differentiated cells were used to perform the experiment [8].

Estimation of Alkaline Phosphatase (ALP) [15-17]: The ALP activity was carried out for newly-differentiated odontoblast-like cells using the ALP assay kit.

Alkaline Phosphatase (ALP) Assay protocol:

1. Cells were washed with Phosphate Buffered Saline (PBS) and then homogenised in 200 µL buffer.
2. The cellular debris was separated by centrifugation at 5000 rpm for five minutes.
3. The reaction mixture was prepared in Eppendorf tubes.
4. The colour intensity was measured at 510 nm using a multiwell spectrophotometer.
5. The ALP activity was measured by comparing it with a standard solution.
6. The results are expressed in IU/L.

Observation: Absorbance (A) readings were noted as shown in [Table/Fig-3]. Four test tubes were marked as follows: Blank (B), Standard (S), Test (T), and Control (C). The enzyme activity was expressed in terms of IU/L, where 7.1 is for the conversion of KA units to IU/L:

$$\text{Phenol liberated by 0.1 mL of lysate} = \frac{\text{AT-AC}}{\text{AS-AB}} \times \frac{0.01 \text{ mg}}{0.1 \text{ mL}} \times 100$$

$$\text{Phenol liberated by 0.1 mL of lysate} = \frac{\text{AT-AC}}{\text{AS-AB}} \times 10 \times 7.1$$

AS-Absorbance of Standard sample

AB-Absorbance of Blank

AC-Absorbance of Control

AT-Absorbance of Test

4. **Cell proliferation and viability assay with trypan blue [18]:** Cells that reached 60-65% confluency were briefly trypsinised and resuspended in complete media. Equal volumes of cell suspension and 0.4% trypan blue (Gibco) were mixed, and 10 µL of the prepared sample was loaded using micropipettes in the chamber of a hemocytometer. Viable and nonviable cells were counted manually. Counting was performed in duplicate as shown in [Table/Fig-4].

Calculations for cell proliferation [19]:

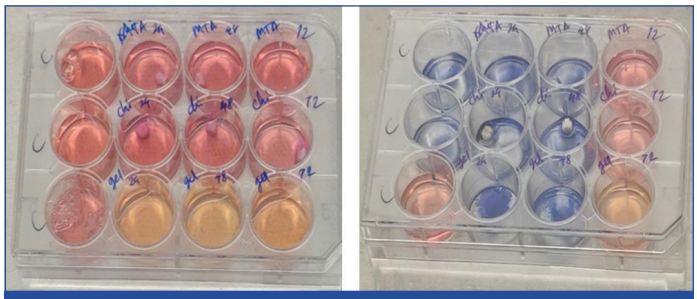
Percentage of viable cells=Number of viable cells/Total number of cells×100

Average of cells/square=Number of viable cells/Number of squares

Dilution factor=Final volume/Volume of cells

Reagent (mL)	Blank	Standard	Test	Control
Buffer	1.1	1.1	1.0	1.0
Substrate	–	–	1.0	1.0
Serum	–	–	0.1	–
Standard (Phenol 1 mg/100 mL)	–	1.0	–	–
Water	1.0	–	–	–
Incubated at 37°C for 15 minutes				
Sodium hydroxide	0.5	0.5	0.5	0.5
Serum	–	–	–	0.1
Sodium bicarbonate	1.2	1.2	1.2	1.2
Mixed thoroughly				
4-Amino antipyrine	1.0	1.0	1.0	1.0
Potassium ferricyanide	1.0	1.0	1.0	1.0

[Table/Fig-3]: Absorbance (A) readings for the Alkaline Phosphatase (ALP) Assay.



[Table/Fig-4]: Cells with biomaterials before and after adding tryphan blue.

Concentration of viable cells=average of cells per square×Dilution factor×10^4

5. **Crystal violet assay for cell adhesion [20]:** The crystal violet assay was conducted to check cell adhesion. The materials were coated on the 12-well plates. Differentiated odontoblastic cells from the third and fourth generations were seeded into biomaterial cell culture plates at a density of 10^4 cells and incubated in a 5% CO_2 humidified atmosphere at 37°C for 24, 48, and 72 hours. The images were captured on a Motic camera at 20X magnification. Simultaneously, in another plate, cells were stained with 0.2% crystal violet, and the viable cells were counted manually using a hemocytometer. This assay is useful for obtaining quantitative information about the relative density of cells adhering to multi-well cluster dishes as crystal violet will stain the Deoxyribonucleic Acid (DNA).

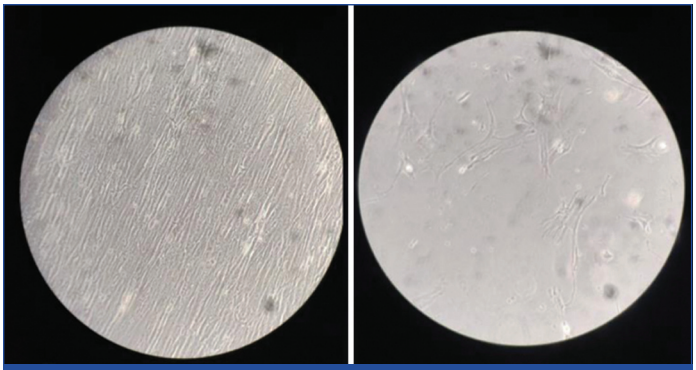
RESULTS

The Human Dental Pulp Stem Cells (hDPSCs) were differentiated into odontoblasts using differentiation medium. ALP activity was measured using a photometer after cell differentiation. Increased ALP activity indicates that cells have differentiated into odontoblasts since they are highly expressed in osteo/odontogenic differentiated cells. [Table/Fig-5] shows the numerical data in the graph for ALP activity.

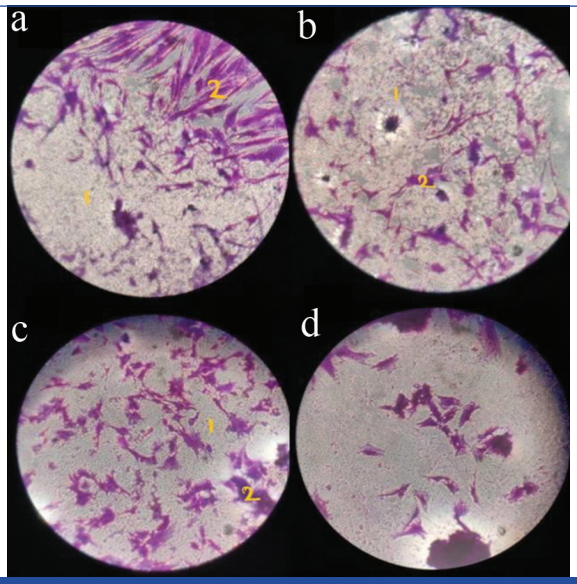
DPSC	Mean±SD	Open Database Connectivity (ODBC)	Mean±SD
51.63636	66.7±14.55	96.81818	139.8±37.27
67.77273		161.3636	
80.68182		129.0909	

[Table/Fig-5]: Statistical analysis of ALP activity (IU/L).

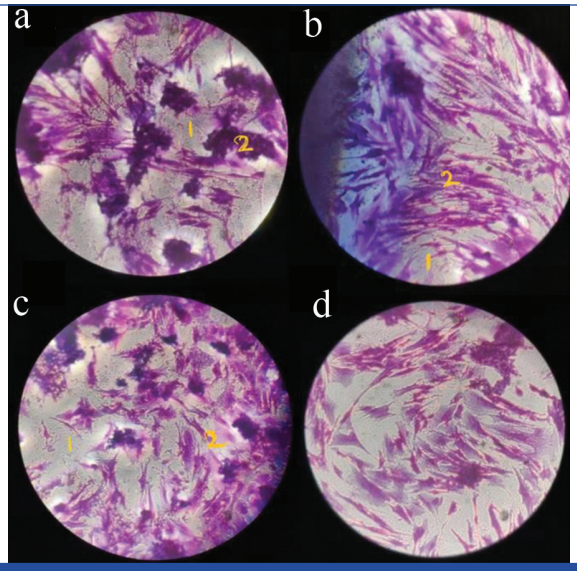
Cell adhesion: Initial light microscopy investigation of odontoblasts showed a stellate shaped homogeneous fibroblast-like appearance and long cytoplasmic processes as shown in [Table/Fig-6]. The cells formed colonies indicating that they were adherent to each other. The cells were stained with crystal violet to check for cell adhesion as shown in [Table/Fig-7-9a-d] at 24 hours, 48 hours, and 72 hours for MTA Plus, Chitosan, MTA plus Chitosan Conjugate, and the control group, respectively.



[Table/Fig-6]: Dental pulp stem cells (a) and odontoblasts (b) under inverted light microscope.



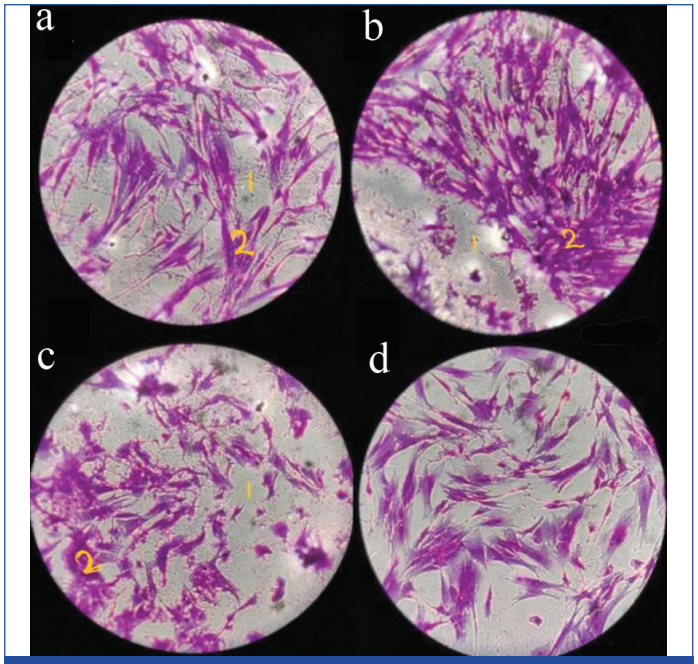
[Table/Fig-7]: Light microscopic images (20X) of cell adhesion to biomaterials using crystal violet at 24 hours: a) MTA Plus; b) MTA Plus- Chitosan; c) Chitosan gel; and d) Control medium
1. Biomaterials.
2. Odontoblast-like cells.



[Table/Fig-8]: Light microscopic images (20X) of cell adhesion to biomaterials using crystal violet at 48 hours: a) MTA Plus; b) MTA Plus- Chitosan; c) Chitosan gel; and d) Control medium
1. Biomaterials
2. Odontoblast like cells.

This shows the affinity of cells towards the material surfaces indicating their anchorage to the biomaterials. The cells showed no signs of cell death or infection.

Cell proliferation: Cell viability was calculated as the number of viable cells in lacs/mL at different time intervals as shown in [Table/Fig-10-13] for Group A, B, C, and D, respectively.



[Table/Fig-9]: Light microscopic images (20X) of cell adhesion to biomaterials using crystal violet at 72 hours: a) MTA Plus; b) MTA Plus- Chitosan; c) Chitosan gel; and d) Control medium
1. Biomaterials
2. Odontoblast like cells.

STATISTICAL ANALYSIS

In the present study, there were three paired groups, hence the Friedman test was appropriate to use within the groups. In order to evaluate if there were any changes in the proliferation of viable cells at 24 hours, 48 hours, and 72 hours among different materials, the Wilcoxon's Signed-rank Test was used. Statistical significance was set at $p \leq 0.05$.

Cell death: The number of dead cells was counted manually for all three groups. For Group-A, cell death was reported to be 20%, 13%, and 0.09% after 24, 48, and 72 hours, respectively. For Group-B, cell death was reported to be 15%, 10%, and 0.05% after 24, 48, and 72 hours, respectively. For Group-C, cell death was reported to be 0%, 0%, and 10% after 24, 48, and 72 hours. The cells showed very less signs of apoptosis around the biomaterials compared to their proliferation rate, indicating their cytocompatibility with them.

DISCUSSION

In response to a biomimetic material at the dentin-pulp interface, DPSCs form new odontoblasts which differentiate at the site of injury to synthesise an atubular reparative dentin. Thus, a cascade of events is induced by various growth factors that involve migration, proliferation, and differentiation of these dental pulp stem cells [21].

The present study used two materials: MTA Plus and Chitosan. Chitosan was used as a vehicle with the hypothesis of surface modification of the calcium silicate cement (MTA Plus) [8]. The basic idea behind this concept was to extract the biomimetic properties of MTA Plus and Chitosan. The scaffold formed by the chitosan will enmesh the calcium hydroxyl ions released by the MTA and thus prolong the release of these ions into the dentin matrix [22]. Moreover, a hydrogel form of chitosan is used since it is easier to mix the MTA powder with it.

In present study, the hDPSCs after differentiation revealed a spindle-shaped morphology with a characteristic nucleus situated at the basal third of the body, similar to another study [23]. This differentiation was confirmed using the ALP assay. ALP is also one of the markers of the differentiation of DPSCs and plays an important role in the formation of calcified tissue and extracellular matrix [24]. The results of this assay showed increased ALP activity after seven days as shown in [Table/Fig-5]. This confirmed that the differentiated cells were odontoblasts.

Cell adherence was evaluated by conducting a wash assay using crystal violet. Adhesion assays measure the contacts between a cell and extracellular adhesion proteins [25]. During the assay, dead detached cells are washed away. The remaining attached live cells are stained with crystal violet. The amount of crystal violet staining in the assay is directly proportional to the cell biomass that is attached to the plate. Crystal violet staining is a quick and versatile assay for screening cell viability under diverse stimulation conditions [26]. In present study, cells have shown a spreading type of morphology around the biomaterials, indicating that they are well adhered to each other as well as the material. The morphology seen under the light microscope showed a spindle shape with cytoplasmic processes extending onto the material surfaces [Table/Fig-7-9]. There was a minimal amount of cell death around the materials, thus indicating that all the materials allow cell proliferation. Similar studies were performed by Zhu L et al., and Abou ElReash A et al., where the hDPSCs showed a similar type of cell morphology after differentiation [27,28].

Cell proliferation was evaluated by the dye exclusion test using Trypan blue. It is one of the gold standard methods of cell counting since it is versatile, cost-effective, and accurate when used for small cell populations [29]. Trypan blue is used to count dead cells; the dye is excluded from cells with intact membranes, so only dead cells are stained blue. The present study showed an increase in cell proliferation after 24, 48, and 72 hours among MTA Plus, MTA Plus-Chitosan conjugate, Chitosan Gel, and Control groups [Table/Fig-10-13]. Statistics displayed a statistically significant increase in cell proliferation from 24 hours to 72 hours (p=0.042) and 48 hours to 72 hours (p=0.042) but no statistically significant difference from 24 hours to 48 hours (p=0.08) in MTA Plus (Group-A). The increase in viable cells in the MTA Plus group was continuous without any decline as shown in [Table/Fig-10]. After 72 hours, the MTA Plus group showed a higher rate of cell proliferation when compared to other groups. A study by Tamaki showed that hDPSC proliferation on calcium silicate cements increased after day 6 [30]. A similar study showed an increase in cell proliferation with MTA Plus after day 3 [31].

Similarly, MTA Plus-Chitosan conjugate and Chitosan Gel groups displayed a statistically significant increase in cell proliferation from 24 hours to 72 hours (p=0.042) and 48 hours to 72 hours (p=0.039). Both groups showed a higher proliferation rate at 48 hours.

This can be explained based on the fact that chitosan decreased inflammatory mediators and increased cell proliferation of pulpal cells, as shown in a study conducted by Renard E et al., [32]. Percot A et al., stated that chitosan is mainly composed of amino acids such as aspartic and glutamic acids, lysine, and histidine, which are responsible for the biocompatibility of chitosan [33]. Also, external mechanical forces can be transmitted across the cell surface and through the cytoskeleton via adhesion molecules like integrin, that can influence cell proliferation on its surface [34]. MTA Plus, being a calcium silicate cement, induces various growth signalling pathways like Extracellular signal-regulated Kinases (ERKs), Jun N-terminal Kinase (JNK) etc., which are responsible for cell proliferation [35]. When mixed with chitosan, the latter acts as a high-strength scaffold that supports hDPSCs' attachment and proliferation. In a study conducted by Moreau JL and Xu HH after day 1, the stem cells were able to adhere and spread on the chitosan scaffold, thus forming cell-cell junctions. After 14 days, their stem cells had greatly proliferated [36].

There is no statistically significant difference in the number of proliferating cells for Group B and C after 48 hours compared to Group A and D. This can be explained based on studies conducted by Mao JS et al., and Hamilton V et al., who showed

MTA plus (Group-A)	Viable cells					Dead cells	Total	Viable cells	Average viable cells/square	No. of viable cells/mL
	1st square	2nd square	3rd square	4th square	5th square					
24 h	8	8	6	6	0	7	35	28	5.6	112000
48 h	12	16	24	5	11	11	79	68	13.6	272000
72 h	23	27	26	18	15	11	120	109	21.8	436000

[Table/Fig-10]: Number of viable cells in lacs for Group-A.
*The number of viable cells for MTA Plus showed an increase in cell viability at 48 hours. Significant increase of cell proliferation from 2,72,000 cells/mL to 4,36,000 cells/mL was noted at 72 hours

MTA plus+-Chitosan (Group-B)	Viable cells					Dead cells	Total	Viable cells	Average viable cells/square	No. of viable cells/mL
	1st square	2nd square	3rd square	4th square	5th square					
24 h	5	11	5	4	9	6	40	34	6.8	136000
48 h	13	19	27	10	9	9	87	78	15.6	312000
72 h	17	22	19	14	14	5	91	86	17.2	344000

[Table/Fig-11]: Number of viable cells in lacs for Group-B.
*Significant increase of cell proliferation from 1,36,000 cells/mL to 3,12,000 cells/mL was noted at 48 hours. However, there was no significant increase of cells from 48 to 72 hours

Chitosan gel (Group-C)	Viable Cells					Dead Cells	Total	Viable cells	Average viable cells/square	No. of viable cells/mL
	1st square	2nd square	3rd square	4th square	5th square					
24 h	8	5	11	8	5	0	37	37	7.4	148000
48 h	12	13	5	7	4	0	41	41	8.2	164000
72 h	13	23	15	17	9	9	86	77	15.4	308000

[Table/Fig-12]: Number of viable cells in lacs for Group-C.
*There was no significant increase of cell proliferation at 24 and 48 hours for Group-3. However, there was significant increase of cells at 72 hours

Control (Group-D)	Viable cells					Dead cells	Total	Viable cells	Average viable cells/square	No. of viable cells/mL
	1st square	2nd square	3rd square	4th square	5th square					
24 h	6	7	5	4	10	4	36	32	6.4	128000
48 h	9	14	9	8	5	6	51	45	9	180000
72 h	16	13	19	25	18	10	101	91	18.2	364000

[Table/Fig-13]: Number of viable cells in lacs for Group-D.
*There was significant increase in cell proliferation from 1,80,000 to 3,64,000 cells/mL after 48 h

that chitosan did not show significant fibroblast cell growth on its surface. Chitosan has a high surface energy, so even though it shows high cell adhesion, the cells began to degrade the surface of chitosan, which inhibits further cell migration and proliferation on its surface [34,37].

This study concluded that the basic function of chitosan is to act as a vehicle onto which the cultured cells can adhere and proliferate when they come in contact with a biomimetic material like MTA Plus. The scaffold will support the growth factors induced by the MTA Plus. The hydrogel form used in this case will preserve the viability of incorporated cells. Also, the interconnected porous structure will favor nutrient and oxygen diffusion required for cell proliferation [38]. However, cell proliferation will decrease eventually over a period of time.

Within the limitations of the study, it was noted that the novel, non toxic, biodegradable natural polymer, Chitosan, increased cell adhesion and proliferation when used with MTA Plus. Chitosan, when conjugated with MTA Plus, also increased the apatite forming ability of the calcium silicate cement. Further studies need to be conducted to evaluate other properties of Chitosan alone as well as in conjunction with other novel materials.

Limitation(s)

No confirmatory studies were done for the differentiation of odontoblasts (gene profiling), adhesion (flow cytometry, Scanning Electron Microscopy (SEM) studies), and proliferation of cells (Optical Density). Only cell adhesion and proliferation were evaluated in the present study, so further studies should be done to evaluate the other properties of these biomimetic pulp capping materials on pulp stem cells that are of clinical importance.

CONCLUSION(S)

It can be concluded that MTA Plus, MTA Plus-chitosan conjugate, and Chitosan gel showed cell adhesion at 24, 48, and 72 hours. MTA Plus-chitosan conjugate (Group B) showed an increase in cell proliferation after 24, 48 hours, and 72 hours. MTA Plus (Group A) showed an increase in cell proliferation from 24 to 72 hours. Chitosan gel (Group C) showed a significant increase in cell proliferation after 48 hours. All three groups, MTA Plus, MTA Plus-chitosan conjugate, and chitosan gel, showed a significant amount of cell proliferation, indicating that they were less toxic to cells. Thus, present research provides valuable insights into the biocompatibility and potential regenerative effects of various root end filling materials in dental procedures. Particularly, Chitosan can be used as a vehicle with MTA since their conjugate showed greater proliferation activity after stem cell culture.

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REFERENCES

[1] Volponi AA, Pang Y, Sharpe PT. Stem cell-based biological tooth repair and regeneration. Trends in Cell Biology. 2010;20(12):715-22.
[2] Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human Dental Pulp Stem Cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences. 2000;97(25):13625-30.
[3] Khalili AA, Ahmad MR. A review of cell adhesion studies for biomedical and biological applications. Int J Mol Sci. 2015;16(8):18149-84.
[4] Ersahan S, Oktay EA, Sabuncuoglu FA, Karaoglanoglu S, Aydın N, Suloglu AK. Evaluation of the cytotoxicity of contemporary glass-ionomer cements on mouse fibroblasts and human dental pulp cells. European Archives of Paediatric Dentistry. 2020;21(3):321-28.
[5] An S. The emerging role of extracellular Ca2+ in osteo/odontogenic differentiation and the involvement of intracellular Ca 2+ signaling: From osteoblastic cells to dental pulp cells and odontoblasts. J Cell Physiol. 2019;234(3):2169-93.
[6] Al-Hezaimi K, Salameh Z, Al-Fouzan K, Al Rejaie M, Tay FR. Histomorphometric and micro-computed tomography analysis of pulpal response to three different pulp capping materials. J Endod. 2011;37(4):507-12.
[7] Chacko DV, Kurikose DS. Human pulpal response to mineral trioxide aggregate (MTA): A histologic study. Journal of Clinical Pediatric Dentistry. 2006;30(3):203-09.
[8] Tu MG, Ho CC, Hsu TT, Huang TH, Lin MJ, Shie MY. Mineral trioxide aggregate with mussel-inspired surface nanolayers for stimulating odontogenic differentiation of dental pulp cells. J Endod. 2018;44(6):963-70.
[9] Moussaoui Y, Mnasri N, Elaloui E, Salem RB, Lagerge S, De Menorval LC. Preparation of chitosan gel. In: EPJ Web of Conferences. EDP Sciences; 2012;29;00034.
[10] Ahmadi F, Oveisi Z, Samani SM, Amoozgar Z. Chitosan based hydrogels: Characteristics and pharmaceutical applications. Research in Pharmaceutical Sciences. 2015;10(1):01-16.
[11] Sandford PA, Zikakis JP, Brine CJ. Advances in chitin and chitosan. Elsevier Applied Science; 1992.
[12] Lemeshow S, Hosmer DW, Klar J, Lwanga SK. World Health Organization. Adequacy of sample size in health studies. Chichester: Wiley; 1990.
[13] Vaux DL, Fidler F, Cumming G. Replicates and repeats- what is the difference and is it significant? A brief discussion of statistics and experimental design. EMBO reports. 2012;13(4):291-96.
[14] Robasky K, Lewis NE, Church GM. The role of replicates for error mitigation in next-generation sequencing. Nat Rev Genet. 2014;15(1):56-62.
[15] Belfield A, Goldberg DM. Revised assay for serum phenyl phosphatase activity using 4-amino-antipyrine. Enzyme. 1971;12(5):561-73.
[16] Kind PR, King E. Estimation of plasma phosphatase by determination of hydrolysed phenol with amino-antipyrine. J Clin Pathol. 1954;7(4):322.
[17] Hansen PW. A simplification of Kind and King's method for determination of serum phosphatase. Scand J Clin Lab Invest. 1966;18(3):353-56.
[18] Piccinini F, Tesei A, Arienti C, Bevilacqua A. Cell counting and viability assessment of 2D and 3D cell cultures: Expected reliability of the trypan blue assay. Biol Proced Online. 2017;19:01-02.
[19] Spector DL, Goldman RD, Leinwand LA. Cells: A laboratory manual. Cold Spring Harbor Laboratory Press; 1998.
[20] Feoktistova M, Geserick P, Leverkus M. Crystal violet assay for determining viability of cultured cells. Cold Spring Harb Protoc. 2016;2016(4):343-46.
[21] Ricucci D, Loghin S, Lin LM, Spångberg LS, Tay FR. Is hard tissue formation in the dental pulp after the death of the primary odontoblasts a regenerative or a reparative process? J Dent. 2014;42(9):1156-70.
[22] Hashmi A, Zhang X, Kishen A. Impact of Dentin Substrate Modification with Chitosan-Hydroxyapatite Precursor Nanocomplexes on Sealer Penetration and Tensile Strength. J Endod. 2019;45(7):935-42. Doi: 10.1016/j.joen.2019.03.021. Epub 2019 May 9. PMID: 31078326.
[23] Hao J, Narayanan K, Ramachandran A, He G, Almushayt A, Evans C, et al. Odontoblast cells immortalized by telomerase produce mineralized dentin-like tissue both in vitro and in vivo. Journal of Biological Chemistry 2002;277(22):19976-81.
[24] Liu Q, Cen L, Yin S, Chen L, Liu G, Chang J, et al. A comparative study of proliferation and osteogenic differentiation of adipose-derived stem cells on akermanite and β-TCP ceramics. Biomaterials. 2008;29(36):4792-99.
[25] Chiba K, Kawakami K, Tohyama K. Simultaneous evaluation of cell viability by neutral red, MTT and crystal violet staining assays of the same cells. Toxicology In vitro. 1998;12(3):251-58.
[26] Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K. A combined assay of cell vability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull. 1996;19(11):1518-20.
[27] Zhu L, Yang J, Zhang J, Peng B. A comparative study of BioAggregate and ProRoot MTA on adhesion, migration, and attachment of human dental pulp cells. J Endod. 2014;40(8):1118-23.
[28] Abou ElReash A, Hamama H, Grawish M, Saeed M, Zaen El-Din AM, Shahin MA, et al. A laboratory study to test the responses of human dental pulp stem cells to extracts from three dental pulp capping biomaterials. Int Endod J. 2021;54(7):1118-28. Doi: 10.1111/iej.13495. Epub 2021 Mar 7.
[29] Strober W. Trypan blue exclusion test of cell viability. Current protocols in Immunology 1997;21(1):111:A3.B.1-A3.B.3.
[30] Hayashi Y, Kawaki H, Hori M, Shintani K, Hasegawa T, Tanaka M, et al. Evaluation of the mechanical properties and biocompatibility of gypsum-containing calcium silicate cements. Dent Mater J. 2021;40(4):863-69.
[31] Rodrigues EM, Gomes-Cornélio AL, Soares-Costa A, Salles LP, Velayutham M, Rossa-Junior C, et al. An assessment of the overexpression of BMP-2 in transfected human osteoblast cells stimulated by mineral trioxide aggregate and Biodentine. Int Endod J. 2017;50:e9-e18.
[32] Renard E, Amiaud J, Delbos L, Charrier C, Montembault A, Ducret M, et al. Dental pulp inflammatory/immune response to a chitosan-enriched fibrin hydrogel in the pulpotomised rat incisor. Eur Cells Mater. 2020;40:74-87.
[33] Percot A, Viton C, Domard A. Characterization of shrimp shell deproteinization. Biomacromolecules. 2003;4(5):1380-85.
[34] Mao JS, Cui YL, Wang XH, Sun Y, Yin YJ, Zhao HM, et al. A preliminary study on chitosan and gelatin polyelectrolyte complex cytocompatibility by cell cycle and apoptosis analysis. Biomaterials. 2004;25(18):3973-81.
[35] Sanz JL, Rodríguez-Lozano FJ, Lopez-Gines C, Monleon D, Llena C, Forner L. Dental stem cell signaling pathway activation in response to hydraulic calcium silicate-based endodontic cements: A systematic review of in vitro studies. Dent Mater. 2021;37(4):e256-68.
[36] Moreau JL, Xu HH. Mesenchymal stem cell proliferation and differentiation on an injectable calcium phosphate-chitosan composite scaffold. Biomaterials. 2009;30(14):2675-82.

[37] Hamilton V, Yuan Y, Rigney DA, Puckett AD, Ong JL, Yang Y, et al. Characterization of chitosan films and effects on fibroblast cell attachment and proliferation. Journal of Materials Science: Materials in Medicine. 2006;17(12):1373-81.

[38] Busilacchi A, Gigante A, Mattioli-Belmonte M, Manzotti S, Muzzarelli RA. Chitosan stabilizes platelet growth factors and modulates stem cell differentiation toward tissue regeneration. Carbohydrate Polymers. 2013;98(1):665-76.

PARTICULARS OF CONTRIBUTORS:
1. Postgraduate Student, Department of Conservative Dentistry and Endodontics, S.D.M College of Dental Sciences, Dharwad, Karnataka, India.
2. Associate Professor, Department of Conservative Dentistry and Endodontics, S.D.M College of Dental Sciences, Dharwad, Karnataka, India.
3. Assistant Professor, Department of Biochemistry, S.D.M College of Medical Sciences and Hospital, Dharwad, Karnataka, India.
4. Professor, Department of Conservative Dentistry and Endodontics, S.D.M College of Dental Sciences, Dharwad, Karnataka, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Shreshtha Mukherjee,
Flat-202A, Shubh Nirman CHS, Plot-1, Sec-3, New Panvel,
Navi Mumbai-410206, Maharashtra, India.
E-mail: shreshtha_pnik22@yahoo.com

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