International Journal of Dental Sciences
www.dentaljournal.in
E-ISSN: 2663-4708
Received: 01-06-2026; Accepted: 01-07-2026; Published: 16-07-2026
Volume 8, Issue 3, 2026, Page No. 49-52

Effect of bioceramic pulp capping materials on stem cells derived from human dental pulp: An *In vitro* Study

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DOI: https://doi.org/10.66856/ijds.2026.8.3.8086

Abstract
Aims and objectives: To evaluate and compare the cytotoxicity of bioceramic pulp capping materials i.e MTA Plus, Biodentine, and Endocem Zr on stem cells derived from human dental pulp and to assess the adhesion of the hDPSCs on these bioceramic pulp capping materials.
Methods and materials: hDPSCs were isolated from extracted maxillary premolars by using the explant method. Material disks were made using a brass mould of diameter 10 mm and thickness 2 mm. Set materials were placed in 24 well polystyrene tissue culture plates and seeded with 20,000 cells/well. After 1,3 and 7 days of incubation, cell viability was measured using MTT assay with microplate reader at 570 nm. SEM was used to visualize the attachment of hDPSCs on the surface.
Statistical analysis used: Kruskal Wallis for intragroup and Friedman's test for intergroup comparison.
Results: Biodentine showed increased cell viability after 7 days. No statistically significant difference was observed in inter and intragroup comparison. MTA Plus had higher cell attachment when compared to other materials.
Conclusion: All the 3 tested materials showed no cytotoxic effects on hDPSCs. Within the limitations of this experiment MTA Plus, Biodentine and Endocem Zr all were biocompatible with hDPSCs.

Keywords: Biodentine, Cell viability, cytotoxicity, endocem Zr, MTA Plus, SEM

Introduction
Bioceramics are the bioactive ceramic compounds that are synthesized by various chemical processes either insitu or *in vivo*.Adding to its biocompatibility, bioceramics also possess excellent antibacterial properties [1]. Other advantages is its intrinsic osteoinductive capacity, ability to form a framework as a regenerative scaffold and achieve a hermetic seal [2].The materials used for pulp capping should possess adequate biocompatibility and bioactivity to promote the activity of pulp stem cells and the pulpal healing in permanent teeth [3].
MTA, a tricalcium silicate based material, has higher success rate because it induces less pulpal inflammation and a more predictable dentin bridge is formed. MTA Plus is noncytotoxic,increased the mineralization process *in vitro* and induced the expression of osteogenic material when in contact with hDPSCs [4]. But MTA Plus has long setting time and the potential to induce discoloration of teeth.Biodentine,a tricalcium silicate based cement has a fast setting time and contains zirconium oxide as a radioopacifier.It releases calcium when in solution and acts like a source of hydroxyapatite when in contact with the synthetic tissue fluid.Endocem Zr,a calcium silicate based fast setting material contains fine size particles of pozzolan, that reacts with calcium hydroxide which is formed when the cement is hydrated and produces additional cementitious material.According to the manufacturers,the setting time for this material is within 4 minutes [5].In a clinical scenario,this feature of Endocem Zr is advantageous wherein the permanent restorative material can be placed over the pulp capping material in the same appointment [6].
Studies have shown these materials to be noncytotoxic on various cell lines such as human gingiva derived stem cells [7], human gingival fibroblasts [8] and, human periodontal stem cells [9]. Although studies have evaluated and compared the biocompatibility, the findings are limited. Pulp capping materials come in contact with the tissues and their subsequent interaction determines the regenerative process, thus more evidence on the biocompatibility of these materials especially on the adult pulp stem cells is necessary. Hence this study was conducted to evaluate and compare the cytotoxicity of MTA Plus, Biodentine, and Endocem Zr on stem cells derived from human dental pulp.Since the adhesion of cells is a preliminary requirement for proliferation and further differentiation, the study also aimed to evaluate the attachment of dental stem cells to the aforementioned materials.

Materials and methods
The study protocol was approved by the Institutional Review Board and Ethical Committee of the Institution and conducted at the Central Research Laboratory of the Institute.

Cell culture
Pulp tissue was collected from two premolars of a 15 years old healthy patient which was extracted as a part of prophylactic treatment for orthodontic reasons. Informed consent was obtained from the donor and the guardian.The isolation was carried out in a biosafety cabinet (Thermofischer scientific). The pulp tissue was minced into very small fragments with a sterile scalpel in growth medium containing Dulbecco Modified Essential medium (DMEM) supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100U/ml streptomycin.After 10 to

15 days of incubation at 37 [0]C in a humidified atmosphere of 5% CO_2 in the incubator(New Brunswick,an Eppendorf company India)the cells were transferred to a new culture flask. Cell attachment was observed under inverted microscope.(Motic microscopes,India)(Fig.1A)For cell characterizations, the cells were be incubated with fluorescein labelled antibodies (FITC) antihuman CD 24, Phycoerythrin (PE) antihuman CD 45,FITC antihuman CD44, PE antihuman CD90 and PE antihuman CD117 antibodies for 45 minutes at room temperature. After washing to remove the excess antibodies, the cells were analyzed by a FACS Caliber Flow Cytometer**.**

Pulp capping Material Preparation

MTA Plus (Prevest DenPro Limited, compounded for Avalon Biomed) powder was mixed with its gel in a Powder /Liquid ratio of 3: 1 as recommended by the manufacturers using a glass slab and spatula.300 mg of Endocem Zr (Chromadent India, for Maruchi, Korea) was mixed with 0.14 cc of physiological saline as recommended by the manufacturer using paper pad and agate spatula. Biodentine (Septodont Healthcare India Pvt.Ltd) powder was mixed as per manufacturer's instructions. Freshly mixed materials were placed in a brass mold with a diameter 10 mm and thickness 2 mm.To prevent bacterial contamination, materials were exposed to UV light for 30 minutes in the Biosafety Cabinet (Thermofisher scientific, India).

Cell viability test

Samples of each material were placed in 24 well polystyrene tissue culture plates (Tarsons, India) and cells were seeded at a concentration of 2000 cells/well in growth medium. (Fig.1B)Samples were incubated for 1, 3 and 7 days. At the end of 1, 3 and 7 days of incubation MTT solution -1mg/ml (Hi-Media laboratories Pvt. Ltd, India) was added to each well. After 3 hours of incubation MTT solution was removed and 200µl dimethyl sulfoxide was added to each well. Cell viability was measured quantitatively with a micro plate spectrophotometer (Biotek Epoch,applied biosciences)at an optical density of 590 nm.

Preparation of samples for scanning electron microscopy examination

After 3 days of incubation, samples fixed with glutaraldehyde (2.5%) for 2 hours at room temperature. Dehydration was carried out by graded series of ethyl alcohol from concentrations 70%,80%,90%,95%,100 %. Samples were placed in each concentration of ethyl alcohol for 20 minutes. Then the samples were air dried completely. Samples were coated with gold (10nm) and observed under a scanning electron microscope(Carl Zeiss,India) at 5000x magnification.

Statistical analysis

All experiments were performed in triplicates. The data are expressed as mean, standard deviation.(TABLE1)Statistical analysis was carried out using SPSS version 20.$p<0.05$ was considered significant.Non normal distribution of data was observed hence non parametric test,Kruskal Wallis was applied for intragroup comparison.Intergroup comparison was done by Friedmans test.(TABLE 2)

Results

The hDPSCs were positive for CD90, CD44, and CD117 and negative for CD24 and CD 45 antibodies.On day 3, decrease in cell viability in the biodentine group was observed when compared to MTA Plus and Endocem Zr.Whereas cell viability increased after 7 days of incubation to 119% in Biodentine group.(Fig. 2)Increase in cell viability from 24 hours to 3days and 7days was observed in MTA Plus.Endocem Zr showed an increase in cell viability on day 3.Intergroup comparisons revealed no statistically significant difference between the groups with a *p* value =0.317. Intragroup comparison was assessed by Friedman's test (TABLE 2) which shows a non-significant difference between different time intervals with a *p value* =0.233.

SEM analysis

At a magnification of 5000x, MTA Plus showed the presence of flattened cells with cytoplasmic projections spreading on the surface. Biodentine showed the presence of protruding irregular surface with the adherent cells spread over the surface but relatively lesser in density when compared to MTA Plus.Endocem Zr showed the adhesion of human dental pulp stem cells but were more rounded in appearance.[Fig 1(D,E,F)]

Discussion

Studies have shown hydraulic calcium silicate cements to be biocompatible and are able to shift the balance from inflammation to regeneration [4, 10], The release of calcium and hydroxyl ion is higher in MTA Plus than conventional MTA.It also has a prolonged release of ions for 28 days that results in better bioactivity with the formation of hydroxyapatite [11]. Biodentine has shown a significant molecular level interaction by the release of TGF β1, a key factor in the angiogenesis, progenitor cell recruitment and mineralization from human dental pulp [12]. Despite of all its advantage, a major setback for Biodentine is its poor radiopacity, which shows no discernible difference between the dentine and the material in clinical radiographs [13]. Composition of Endocem Zr includes CaO,SiO_2,Al_2O_3,ZrO_2, Fe_2O_3,Mgo [6,]. Eventhough with a lower calcium /phosphate ratio, it is capable of forming hydroxyapatite in the presence of simulated body fluids [5]. Also presence of zirconium oxide as a radioopacifier reduces the risk of discolouration of teeth.

The explant method was adopted for the isolation of dental pulp stem cells because of its relatively low cost [14], and better tolerance of the cells to isolation when compared to enzymatic digestion [15]. The cells are shown to have similar morphology and surface marker expression [16]. The culture medium used in the isolation of stem cells was DMEM. This medium was supplemented with 10% fetal bovine serum, penicillin and streptomycin. As reported by Ferrua *et al*.2015 [17] in a systematic review, DMEM is the second most commonly used medium followed by MEM and the supplements used in our study are the most common supplements cited in the studies for isolation of dental pulp stem cells. Direct contactmethod was used in our study in which the dental pulp stem cells were directly seeded on to the material disks in the culture medium. We used MTT assay in our study because it is sensitive, reliable and the cell viability can be measured quantitatively [18].

In the present study, Biodentine has shown decrease in cell viability after 3 days of incubation. These results are in accordance with the previous study by Youseef *et al*. 2019 [19]. But contradicting results were observed in another

report by Tomás-Catalá CJ et.al [1]where material eluates at different concentrations were used for the assessment of cell viability. Also it has shown to have cytotoxic effects after 3 days of incubation in a study by Bortoluzzi *et al*. 2015 [20]. After 7 days of incubation, though statistically insignificant, an increase in cell viability was observed with Biodentine in the current study. This is in accordance with the previous study by Peng *et al*.2011 [21] where the results showed that Biodentine at a concentration of 10mg/ml had greater cell viability after 7 days. But no statistically significant difference was observed after 3 and, 7 days in a study by Koseoglu et. al 2013 [22]. In the present study, Endocem Zr showed similar percentage of cell viability throughout the experimental period. These are in agreement with the results of the study by Lee *et al*. 2020 [7]. Also Kim et.al 2015 [23]found no significant difference in viability of human deciduous pulp stem cells cultured on the material for 1,7 and 14 days.

In our study, MTA Plus showed no significant increase or decrease in cell viability during the given time period. Similar results were seen with the human gingival fibroblasts for a study period of 24, 48 and 72 hours with no statistical difference [8]. Another report has shown MTA Plus to be biocompatible at 1:8 dilution after 24 hours of exposure [9].There are no previous reports assessing the viability of hDPSCs on MTA Plus for 7 days.Hence our study provides an additional information regarding the biocompatible nature of the material for a longer time period.

Under SEM, Biodentine showed the presence of dental pulp stem cells on its surface. The results are in close agreement with the previous studies but the cells were spreading and flattened on the surface of the biomaterial [24] [3],. Among the 3 biomaterials, MTA Plus showed relatively higher cell attachment with flattened cells and cytoplasmic projections when observed under SEM.Though cell adhesion has been studied in other MTA like material,there has been no study evaluating the dental pulp stem cell attachment on MTA Plus under SEM.In the present study after 3 days,Endocem Zr showed the attachment of cells on the surface but were round in appearance.This result is in consensus with the results of a study by Chung *et al.* 2015 [6], where the authors observed similar morphology of the hDPSCs with Endocem Zr. Authors attributed these results to the greater release of calcium ions increasing the alkalinity, comparatively lesser release of VEGF, angiogenin and FGF-2. On the contrary, spindle shaped cells were seen in other studies by the authors with this material after 7 days of culture [23].The greater incubation period could be a reason for the difference in results obtained in these studies.

The difference in the results could be due to the difference in methodology, duration of incubation and the method of contact of the material i.e either direct or indirect.In our study set materials were placed in direct contact with the hDPSCs whereas clinical procedure of pulp capping involves the placement of freshly mixed material on exposed pulp.Additionally various other cells and factors present in the dentin pulp complex modulate the biocompatibility of these materials in-*vivo*.

Table 1: Descriptive statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Mta Plus	6	101.0250	.02739	101.00	101.05
Biodentine	6	89.1333	32.7198	58.9	119.0
Endocem Zr	6	100.1733	.26853	100.00	100.52
Time	2	120.00	67.882	72	168

Table 2: Friedman test for intragroup comparison

	Mean Rank	N	Chi-Square	df	P value
Mta plus	2.50	6	3.000	2	0.223
Biodentine	2.00	6	3.000	2	0.223
Endocem zr	1.50	6	3.000	2	0.223

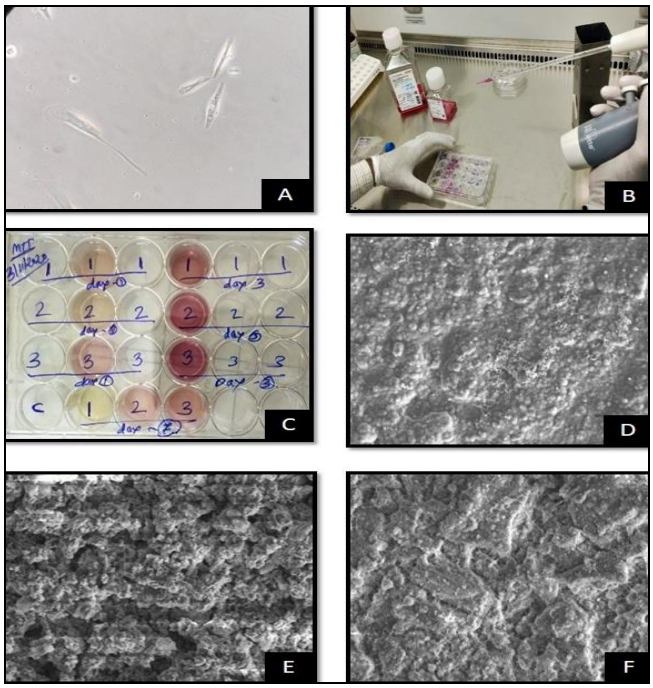

Fig 1: A. Dental pulp stem cells seen under inverted microscope. B. Materials incubated with stem cell C. Cell plates after addition of DMSO SEM Images of materials 5000X magnification MTA Plus (D)Biodentine (E) Endocem Zr(F)

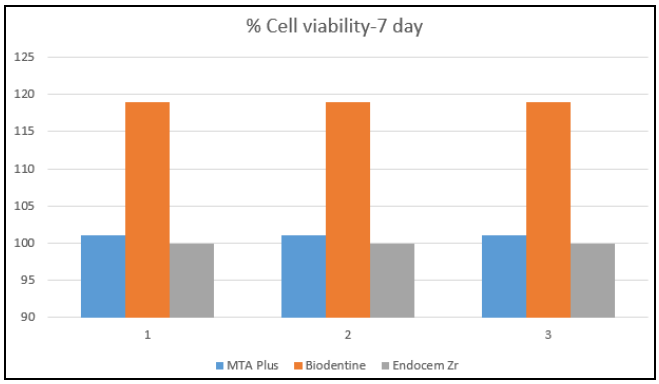

Fig 2: Bar graph representing percentage of cell viability. Biodentine showed an increase in cell viability on day 7, when compared to MTA Plus and Endocem Zr.

Conclusion

MTA Plus, Biodentine, and Endocem Zr had no cytotoxic effects on human dental pulp stem cells during the experimental period. Further studies should be done to evaluate the properties of these bioceramic pulp capping materials on pulp stem cells that are of clinical importance.

Acknowledgement
Declaration of interest: 'None'

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