SHED´s Response to Various Pulpotomy Materials: Cytotoxicity and Gene Expression Analysis

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Purpose*: To examine the cytotoxicity and genetic expression of SHEDs cultured in eluates of various calcium silicate based pulpotomy materials.* ***Study design*** *: MTT assay, flow cytometry, alizarin red staining and scratch assay was used to assess the cellular viability, apoptosis, calcium matrix deposits and cell migration respectively. The gene expression of ALP, OCN and BMP -2, were measured with rtPCR. One way ANNOVA and Bonferroni post test was used for statistical analysis. Results: MTT assay analysis reported that all the test specimen had no cytotoxic effects. The highest number of live cells [%] was found in RetroMTA. The highest percentage of cell migration was observed in SHEDs cultured in EndoCem Zr. The mean absorbance for calcium matrix deposition was higher or similar in all test specimens, when compared to control groups. The expression of BMP -2 and OCN were significantly higher in cells exposed to RetroMTA and NeoMTA respectively after 24 hrs of incubation. After 72 hrs of incubation the mRNA expression of ALP was significantly higher in MTA.****Conclusions:*** *SHEDs cultured in eluates of various calcium silicate based cements exhibited cytocompatibility and maintained odontogenic like phenotype differentiation in SHEDs.*

Keywords: Cytotoxicity, SHEDs, MTA, Biodentine, EndoCem Zr, NeoMTA, RetroMTA, Gene expression

INTRODUCTION

Pediatric dentistry aims at restoring primary teeth with pulp injuries, in a sound condition till its normal exfoliation. In this regard, the vital pulp therapy, primarily focuses on the treatment of pulp injuries that are reversible in nature, preservation of the primary tooth until its exfoliation time, as well as maintenance of function and vitality.[1,2] Various approaches have been put forward to maintain vitality in deciduous teeth with deep decay, like vital pulpotomy and direct or indirect pulp capping.[3,4,5]

Few dental materials that have the potential to form a calcific barrier and retain the vitality of exposed pulp have been described as bioinductive materials in research studies involving regenerative endodontics.[6] If these bioinductive materials, can induce a positive response from the host, it is regarded as being bioactive, provided the bioactive material also elicits a biological response near the interface and encourages bond formation between the material and the tissue.[5,7]

Stem cells that have been acquired through isolation of exfoliated deciduous teeth's dental pulp tissue or dental pulp explants, have demonstrated immunosuppressive characteristics.[8,9] The cytotoxicity of Dental Pulp Stem Cells (DPSCs) isolated from permanent dentition and cultured in various pulpotomy agents has been investigated by various authors; however, a limited number of studies have employed stem cells derived from exfoliating primary teeth.[10]

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One of the most commonly used pulpotomy agent is Mineral Trioxide Aggregate [MTA], that demonstrates quite a few advances over [$Ca(OH)_2$] and Zinc Oxide Eugenol.[11,12] Although MTA had several benefits, it also has certain drawbacks – high material cost, long setting time, tough handling attributes and tooth discoloration[13]. Thus, there is a growing demand for the formulation of a new kind of MTA devoid of these issues.

Of late, Calcium Silicate-based Cements (CSCs) have garnered much attention because of their similarity with MTA and their application in conditions where MTA has been specified. One example is Biodentine which has been commercially accessible since 2009. Biodentine is seen to have an appropriate degree of biocompatibility with dental pulp stem cells,[14,15] even though its impact on SHEDs has not been examined adequately.

EndoCem Zr [Maruchi, Wonju, Korea] is regarded to be a pozzolan-based, white colored MTA material that avoids the long setting time as well as tooth discoloration associated with conventional MTA. As per Chung C et al,[16] when compared with ProRoot MTA, EndoCem Zr is associated with more cytotoxicity and lower expression pertaining to vascular endothelial growth factor and angiogenin, but its impact on SHEDs is not well known.

NeoMTA [NuSmile, USA] is a new finer tricalcium silicate powder that contains TaO as a radio-pacifier agent and a H_20-based gel for good manipulation properties.[17] However, its response to SHEDs has not been evaluated comprehensively. Retro MTA [Bio MTA, Seoul, Korea] is regarded to be a hydraulic bioceramic that does not have Portland cement in its content. Similar to ProRoot MTA[18], studies have confirmed cell viability as well as direct attachment on human pulp tissue and calcium discharge from the material, however, its impact on SHEDs need to be examined further. Researchers have studied DPSCs genetic expression related to biological effects of various CSCs.[19] However, genetic expression of SHEDs cultured in CSCs needs to studied in detail.

Hence the present experiment was conducted to examine and compare the cytotoxicity & genetic expression analysis of MTA, Biodentine, EndoCem Zr, Neo MTA & Retro MTA on SHEDs.

MATERIALS AND METHOD

The present in vitro experimental trial referred to research reporting guidelines given by Nagendrababu V *et al*, 2021.[20] The estimation of the sample size used for the present trial was based on the method of "resource equation".[21] All the tests were performed as three independent experiments with triplicates for each experimental group and result.

Preparation of test specimens

White Angelus MTA (Londrina, Brazil), Biodentine (Septodont, France), EndoCem Zr (Maruchi, Korea), NeoMTA (NuSmile, USA) and Retro MTA (Bio MTA, Seoul, Korea) set CSCs were assessed. These CSCs were tested using the International Standard Organization (ISO) 10993-521[22] method, which involved incubating cultured cells with eluates.1gm of cement and subsequent amount of liquid to maintain the powder: liquid ratio recommended by the manufacturers was mixed. The set CSCs were placed in 12 well plates [3.0 mm height and 314.0 mm^2 area] and were incubated for 15 hours at 37^0 C, 95% humidity and 5% CO_2, to confirm complete polymerization. Sterilization was maintained by exposure to UV light with power of 30 W under laminar flow for 30 minutes.

DMEM (HiMedia, India) supplemented with streptomycin was used as extraction media. These eluates were obtained after passing via 0.22 µm Merck Millipore's filter (USA). Following that, three diluted [1/4, 1/2, and 1/1 vol/vol] eluates were considered for experiment in order to observe a dose response relationship.[23]

Isolation and characterization of SHEDs

The current experiment had been accepted by the Institution's Research & Ethical Board (IREB/2019/PHD/PEDO/01). The selected parents or guardians of 6 to 9-yrs old pediatric patients visiting department of pediatric & preventive dentistry, signed an informed consent, allowing the exfoliating primary teeth (n = 8 teeth) to be included in the study. The extracted teeth were cleaned with normal saline and transported to HiMedia Laboratories (Ghatkopar, India) for isolation of stem cells. Barbed broaches were used to separate the pulp tissues from teeth. The pulp tissues were disinfected by Hank's Balanced Salt Solution (Gibco, USA). After that, collagenase-A (3 mg mL) (Sigma-Aldrich, USA) was used for enzymatic digestion at 37 °C for 60 minutes. Cells (1.5 x 10^4cells/cm^2) thus collected were placed in a complete medium in 25-cm^2 plastic culture flasks (BD Biosciences, USA) and incubated for 72 hours in the presence of 5% CO_2, at 37^0 C. Discarding of red blood cells as well as other non-adherent cells was carried out. To promote, better growth, fresh medium was introduced. Passage zero [P0] was described as the growth of adherent cells to 80% confluence. Phosphate Buffer Saline (PBS) was used to clean the cells and in order to enable subsequent passaging, cells were detached via incubation for 2-5 mins at 37^o C, with 0.25% trypsin solution. The culture medium was introduced to deactivate the trypsin action. Centrifugation of SHEDs for a period of 5 minutes at 500 g was carried out and 5 x 10^3 cells cm^{-2} were seeded in 75-cm^2 flasks. Prior to the experiments, SHEDs phenotypes were evaluated by flow cytometer, utilizing particular antibodies for CD 90 (BD Biosciences, Pharmingen), CD 105 (Abcam, Cambridge, UK), CD 73 (Santa Cruz Biotechnology Inc, USA), CD 34 (BD Biosciences, Pharmingen), CD 45 (BD Biosciences, Pharmingen), and HLA -DR (BD Biosciences, Pharmingen).[24]

Cell viability assay

The EZ count MTT cell assay kit (HiMedia, India) was used to assess the cell viability of SHEDs cultured in different eluates after 24, 48 and 72 hrs of culture. As per ISO 10993 standards,[22] SHEDs cultured in 1 mM hydrogen peroxide was considered as positive control and DMEM as negative control. MTT was introduced in all concerned wells and cultured for 240 minutes, following which the procedure was terminated by introducing dimethyl sulphoxide [25]. A microplate reader (BioTek Instrument, USA) was used to determine AB_{570} nm with a reference wavelength at AB_{630}.

Detection of apoptosis and necrosis

As recommended by the manufacturer, the BD Annexin V-FITC Apoptosis Detection Kit [BD Biosciences, USA] was used to determine the percentage distribution of live (7-AAD negative / Annexin-V negative), early apoptotic (7-AAD negative /Annexin-V positive) late apoptotic (7-AAD positive /Annexin-V positive) and necrotic cells (7-AAD positive/Annexin-V negative) by flow cytometry. [BD Biosciences, USA]. The procedural steps recommended by the manufacturer were followed for performing this experiment.

24 well plate was used to seed 2 x 10^6 mL^{-1} SHEDs and incubated for 72 hrs in various eluates.[26] SHEDs cultured in DMEM and H_20_2 (1 mmol L^{-1}) were represented as negative control and positive control specimen respectively.

Cell migration assay

Wound healing assay was used to assess the response of SHEDs cultured in various eluates for cell migration. Briefly, 5 x10^4 SHEDs were seeded onto 6 well plate and promoted to achieve an entire confluent monolayer before culturing in DMEM medium for 24 hrs. Using a 200 µl pipette tip, a 'wound' was created through the confluent cell layers, by making a scratch. To remove the cell debris, the cells were cleaned with PBS and cultured with different eluates for 72 hrs, to promote migration of cells, back into the injured area. The phase-contrast microscope (Nikon, Japan) was used to capture the photographs of the wound region at 0, 24 hrs, 48 hrs and 72 hrs post-wound day. ImageJ software (NIH, USA) was used to evaluate the acquired figures.[27]

Analysis of matrix calcium deposition

Alizarin Red Staining technique was used to evaluate the SHEDs cultured in different eluates for calcium deposition after 7, 14 and 21 days of incubation. Alizarin adheres to Ca^{+2} salts and utilized to detect Ca^{+2} containing bone forming cells. These cells were subjected to 70% ethanol fixation at 4°C, for 60 minutes. 2% Alizarin Red staining (Sigma AB, Sweden) was performed for 30 minutes. Ultrapure H_20 was used thrice to clean the cells. In order to test the degree of staining, 10% CetylPyridinium Chloride (1 mL) (Sigma AB, Sweden) was introduced to every well, followed by 20 minutes incubation.[28] An electronic microplate reader [BioTek Instrument, USA] was used to measure the absorbance of the eluted stain at 550 nm.

Gene expression analysis

In 12-well plates, 2 x10^6 cells mL^{-1} were plated. As per recommendations of manufacturer, TRIzol (Invitrogen, USA), based the RNA extraction was carried out from SHEDs, after 24 hrs of exposure to the various eluates, diluted in osteogenic (5 mmol L^{-1} b-glycerophosphate and 50 lg mL^{-1} L-ascorbic acid) and non-osteogenic medium (DMEM). The qPCR gene expression (StepOne, Applied Biosystems, USA) was used in this investigation. Primer references were as below: ALP (Hs01029144_m1), GAPDH (Hs02758991_g1) OCN (Hs01587814_g1), and BMP-2 (Hs00154192_m1), (Applied Biosystems, Life Technologies). The levels of mRNA expression was determined with ΔΔCt method (fold expression = $2^{-(\Delta\Delta Ct \pm SD)}$)[29-31] We also analyzed the same eluate in osteogenic and non-osteogenic media for the assessment of variations in mRNA expression.

Statistical Analysis

The data was evaluated with the SPSS Inc. program (Windows, version 23.0 Chicago, USA). For comparing mean measurements of test specimens, a one-way ANOVA test was used. Bonferroni post hoc test examined manifold pair-wise individual comparisons among groups. To denote the variations between means, a 5% level of significance was considered. The data was interpreted as a mean standard deviation.

RESULTS

Characterization of SHEDs

In the present study, flowcytometric analysis of SHEDs showed high expression of positive marker CD 73 (95.90 %), CD 90 (96.60 %) and moderate expression was observed for CD 105 (33.30 %). SHEDs progeny did not express negative markers CD 34 (1.60%), CD 45 (0.77%) and HLA-DR (0.60%).

Cell viability assay

The cell viability test showed that eluates of all test specimens at various dilution caused no cytotoxic effects as the absorbance was greater, compared to the negative control for all time intervals – 72 hrs, 48 hrs and 24 hrs. In the present study, at 1:1 dilution – the cell viability of MTA [48 hrs (p = 0.048)], EndoCem Zr [72 hrs (p = 0.006)], 48 hrs (p = 0.001 & 24 hrs (p = 0.006)], NeoMTA [72 hrs (p = 0.006) 48 hrs (p = 0.006) & 24 hrs (p = 0.028),] and RetroMTA [48 hrs (0.008)] was statistically higher than negative control specimens at respective time intervals.

At 1:2 dilution – the cell viability of MTA [72 hrs (p = 0.001), 48 hrs (p = 0.003) & 24hrs (p = 0.001),], Biodentine [72 hrs (p = 0.001), 48 hrs (p = 0.017) & 24 hrs (p = .001),)], EndoCem Zr [72 hrs (p = 0.001)], 48 hrs (p = 0.049) & 24 hrs (p = 0.002)], NeoMTA [72 hrs (p = 0.001), 48 hrs (p = 0.003) & 24 hrs (p = 0.003),] and RetroMTA [72 hrs (p = 0.001) ,48 hrs (p = 0.001) & 24 hrs (p = 0.001)] was statistically higher than negative control specimens at respective time intervals. When compared to negative control, the cell viability of Biodentine [72 hrs (p = 0.001), & 48 hrs (p = 0.001)], RetroMTA [72 hrs (p = 0.008)] and MTA [72 hrs (p = 0.001) & 48 hrs (p = 0.039)] was statistically higher at respective time intervals, at 1: 4 dilution (Figures 1,2,3).

Figure 1: Comparison of absorbance [mean ± SD] between different time intervals and Eluates of various test specimens at 1:1 dilution.

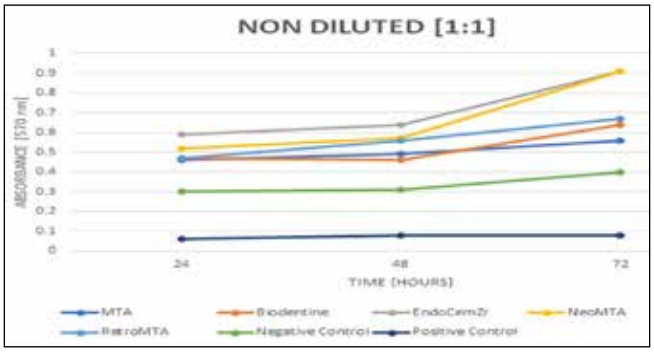


Figure 2: Comparison of absorbance [mean ± SD] between different time intervals and Eluates of various test specimens at 1:2 dilution.

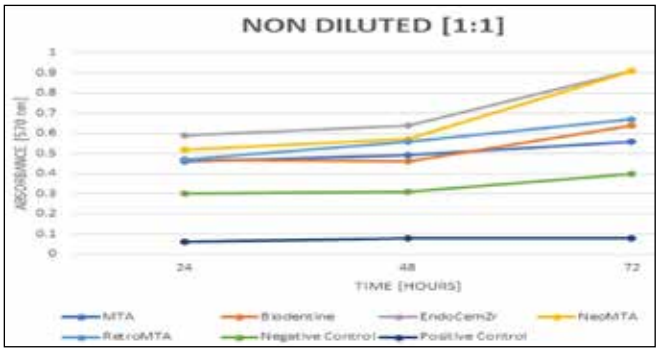
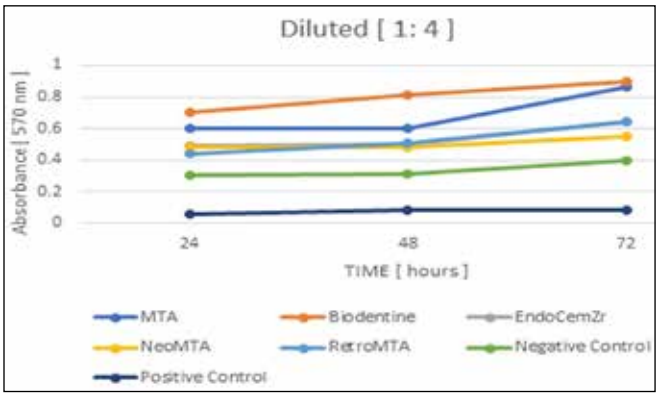


Figure 3: Comparison of absorbance [mean ± SD] between different time intervals and Eluates of various test specimens at 1:4 dilution.



Detection of apoptosis and necrosis by flow cytometry

The mean ± SD (%) difference between eluates of various CSCs, when compared to control groups (positive & negative) were statistically significant for % of live cells (p = 0.001), early (p = 0.001) and late (p = 0.046) apoptotic cells. The maximum number of live cells (%) were found in RetroMTA (96.90 ± 1.46), followed by negative control (96.48 ± 2.01), NeoMTA (95.67 ± 1.79), EndoCem Zr (94.50 ± 1.55), Biodentine (94.38 ± 0.36), MTA (92.72 ± 1.70) and positive control (26.17 ± 10.36) (Figure 4).

Cell migration assay

The images for each test specimen at different time intervals were obtained and analyzed for 'Relative Open Wound' through ImageJ software. The difference in percentage of 'Relative Open Wound' (pixel/mm^2) (mean ± SD) between eluates of all test specimens were statistically significant at all the time intervals – 72 hrs, 48 hours and 24 hrs (p = 0.001). The maximum amount of cell migration was reported in SHEDs cultured in EndoCem Zr (24.19 ± 3.89), followed by RetroMTA (31.29 ± 2.32), NeoMTA (33.53 ± 5.12), Biodentine (36.75 ± 2.98) & MTA (38.37 ± 1.21) after 72 hours of time interval. Pair wise comparison of 'Relative Open Wound' between all eluates was statistically significant than control specimen at all time intervals (Figure 5).

Figure 4: Representative 2D flow cytometry dot plot of data derived from FITC – AnV and 7-AAD stained SHEDs cultured in various CSCs.

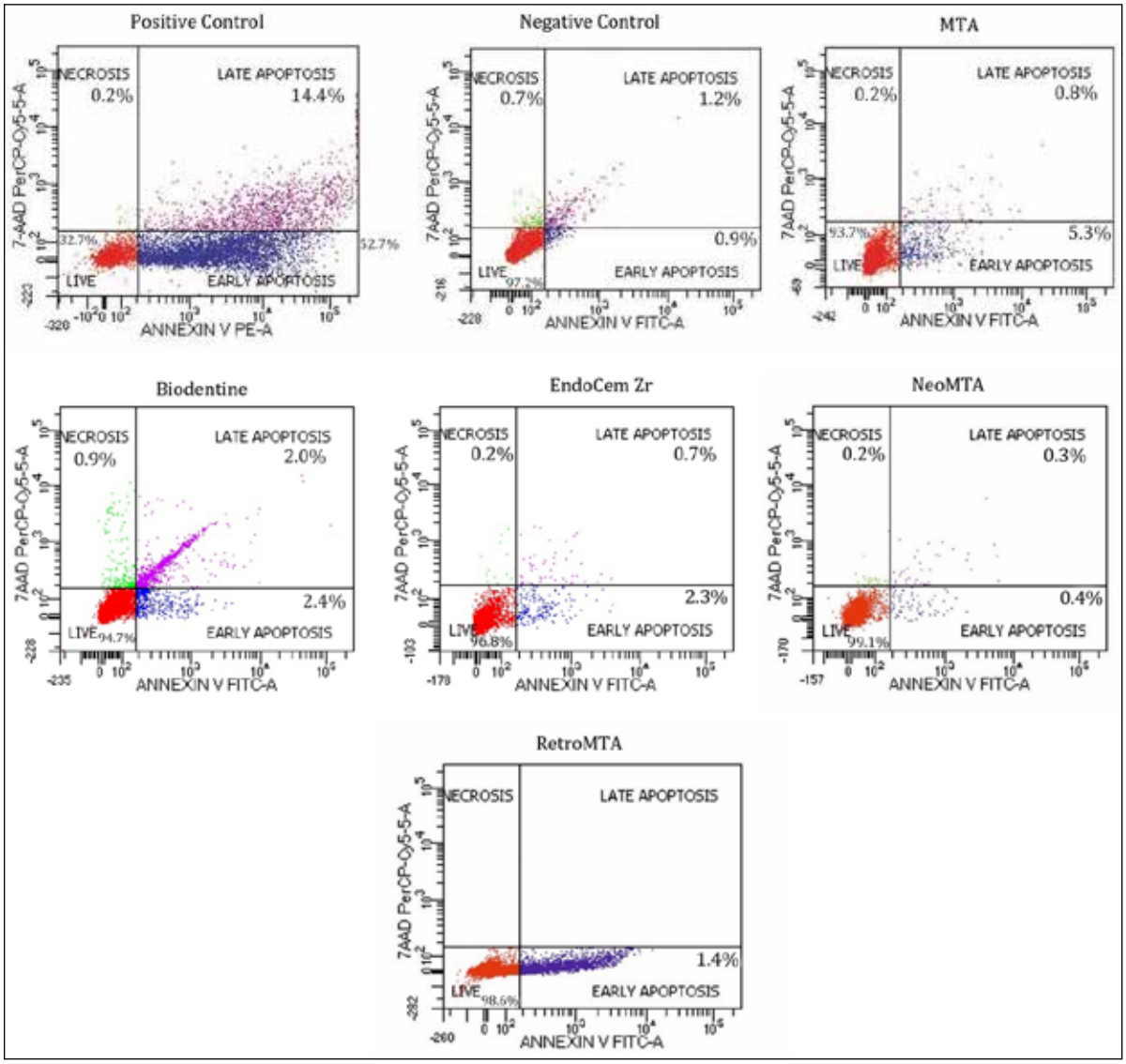
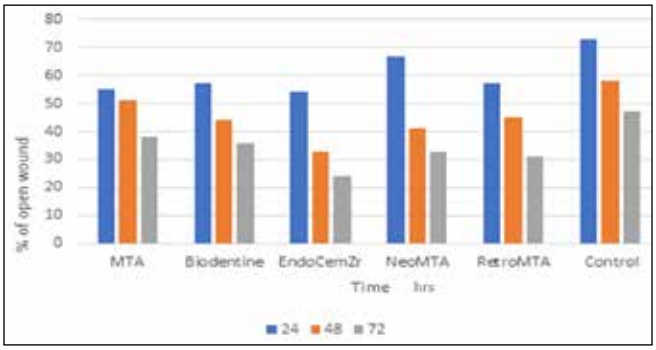


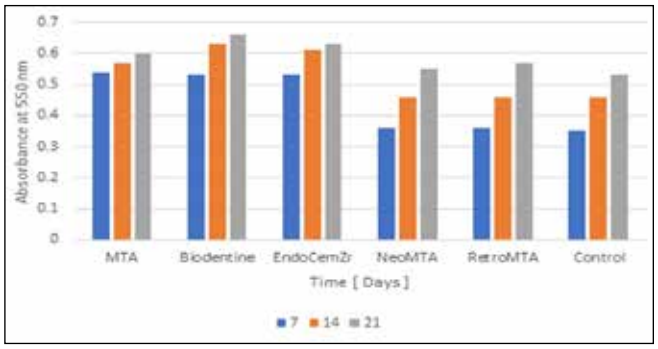
Figure 5: Comparison of percentage [mean ± SD] of 'Relative Open Wound' [pixel/mm²] of various test specimens at different time intervals.



Analysis of matrix calcium deposition

Images of Alizarin Red staining of SHEDs along with eluates of various test specimens, at different time intervals are presented (Fig VI). The difference in absorbance (mean ± SD) of calcium matrix deposition between eluates of all test specimens were statistically significant at all the time intervals – 21 days ($p = 0.012$), 14 days ($p = 0.020$) and 7 days ($p = 0.004$). Pair wise comparison of calcium deposition for SHEDs cultured in Biodentine was statistically significant when compared to control after 21 days of incubation. The mean absorbance for calcium matrix deposition was greater or similar to the control in all test specimens. The maximum amount of absorbance for calcium deposits were seen in SHEDs cultured in Biodentine (0.66 ± 0.07), followed by EndoCem Zr (0.63 ± 0.06), MTA (0.60 ± 0.06), RetroMTA (0.57 ± 0.10) and NeoMTA (0.55 ± 0.04) (Figure 6).

Figure 6: Alizarin red staining of SHEDs cultured in eluates of various test specimens at different time intervals.



mRNA expression analysis

Non osteogenic medium

The highest mRNA expression (mean ± SD) of ALP (1.93 ± 0.07), OCN (3.58 ± 0.07) and BMP-2 (7.21 ± 0.07) was reported in SHEDs cultured in RetroMTA, after 24 hrs of incubation. The expression levels of ALP, OCN and BMP-2 decreased drastically, at the end of 72 hrs incubation in eluates of all test specimens.

A statistically significant difference was found between the eluates of all the different CSCs for the mRNA expression levels [mean ± SD] of all the target genes (BMP-2, OCN and ALP) after 24 hrs of culture. However, after 72 hrs of incubation, the eluates of all the different CSCs showed a statistically significant difference for the target gene BMP-2 (Figures 8,9).

Figure 7: Comparison of absorbance [mean ± SD] for matrix calcium deposition of eluates of various test specimens at different time intervals.

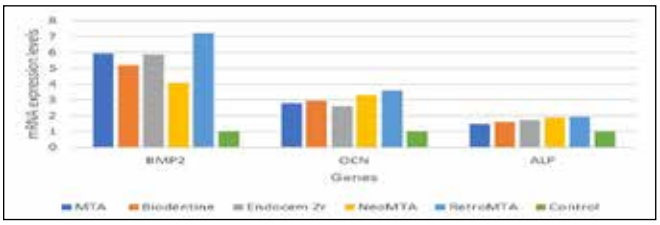


Figure 8: Comparison of mRNA expression levels [mean ± SD] of target genes in SHEDs exposed to eluates of various test specimens in non- osteogenic media after 24 hours.

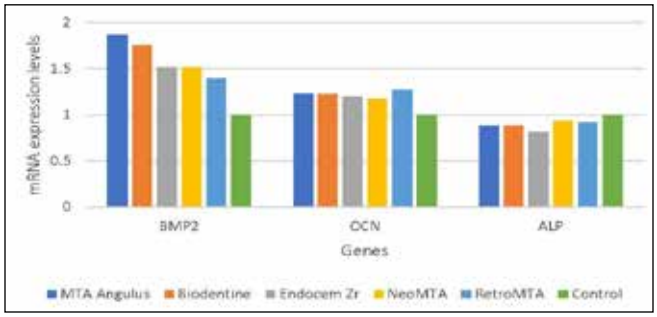
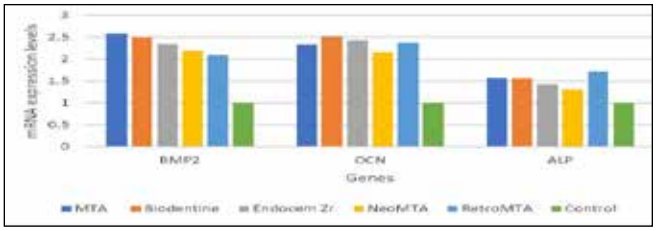


Figure 9: Comparison of mRNA expression levels [mean ± SD] of target genes in SHEDs exposed to eluates of various test specimens in non- osteogenic media after 72 hours.

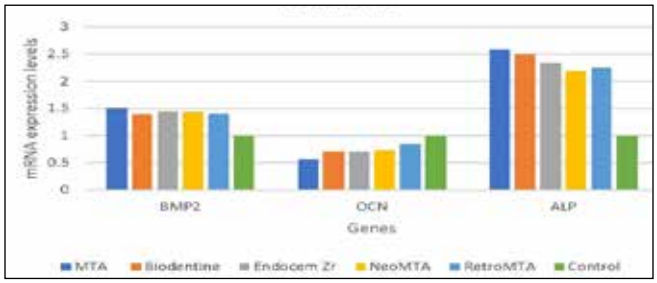


Osteogenic medium

The highest level of mRNA expression of OCN and BMP-2 were reported in SHEDs cultured in Biodentine (2.51 ± 0.07), MTA (2.58 ± 0.07) after 24 hrs of incubation. SHEDs cultured in MTA (2.58 ± 0.07) reported highest mRNA expression levels of ALP after 72 hrs of incubation.

After 24 hours of culture, a statistically significant difference was discovered between the eluates of all the different CSCs for the mRNA expression levels [mean SD] of all the target genes (BMP-2, OCN, and ALP). However, after 72 hrs of culture, a statistically significant difference in ALP and BMP-2 expression levels was discovered between the eluates of all the different CSCs (Figure 10).

Figure 10: Comparison of mRNA expression levels [mean ± SD] of target genes in SHEDs exposed to eluates of various test specimens in osteogenic media after 24 hours.



DISCUSSION

Considering that vital pulp treatment comprises direct contact between tissue and treating medicament, biocompatibility characteristics of medicaments must be considered carefully. The present study used various *in vitro* cytotoxicity tests to obtain information concerning the safe application of these bioinductive/biomaterials for vital pulp treatment for primary teeth. Unlike the previous studies[32] which used animal cell lines, the current study utilized SHEDs.

MSCs population is expected to be positive for CD105, CD 73, and CD 90 as per the phenotype requirements of ISCT. Furthermore, such cells should not express CD 14, CD 45, CD 34, CD19, HLA class II, CD 79a or CD11b.[33] The present study used CD 45, HLA-DR, and CD 45 as negative markers, while CD105, CD 73, and CD 90 were used as positive markers. Phenotypical analysis of SHED in present study, showed high positivity for positive markers CD 73 [96.69 %], CD 90 [97.70 %] and low positivity was observed for CD 105 [34.33 %] which is commonly expressed by endothelial progenitors. These results were in consistent with other studies[34-36]

Several techniques facilitate the assessment of cytotoxicity and cell proliferation. Erythrocin B staining and Trypan Blue exclusion techniques are straightforward and cost-effective techniques for determining cell viability [or death]. Nevertheless, these techniques lack the required sensitivity and are infeasible for high-speed assessment. Tetrazolium compounds are employed to produce a quantifiable colorimetric assay to evaluate human cell proliferation and survival. Hence, MTT assay has been used extensively to the extent that it is now the typical technique for assessing cell viability. Therefore, the present study used the same technique for determining cell viability. Cells exposed to drugs for up to 72 hours are assessed to determine drug's maximum potency and efficacy.[37] Therefore, the MTT assay was evaluated at 24, 48, and 72-hour intervals.

The EZcount™ MTT Cell Assay kit identifies the extracellular decrease caused by metabolically vital cells in H_2O soluble MTT, a yellow colored dye to form insoluble crystals of formazan. Lactate dehydrogenase is a mitochondrial enzyme that facilitates this reaction. These formazan crystals express purple color, whose intensity is directly related to the number of live cells, that can be counted spectrophotometrically at 570 nm.[38]

The results of cell viability test of the present study were in accordance with study conducted by Dahake *et al* (2020)[39] who reported that SHEDs cultured in Biodentine showed more cell viability and proliferation than MTA. Jung J *et al* (2020)[40] revealed SHEDs cultured in Biodentine had a cell viability of about 0% in 100% extracts and extreme cytotoxicity in 50% extracts. Yun J *et al* (2019)[41] reported that the cell viability and proliferation of SHEDs cultured in EndoCem Zr to be more than RetroMTA. This contradicted the results of the present study wherein the cell viability and proliferation of SHEDs cultured in RetroMTA was more than EndoCem Zr.

A study conducted by Tsai *et al* (2018)[42] revealed that SHED's viability is hindered by post-set MTA in the first three days of culture. Hasweh *et al* (2018)[43] revealed that at concentrations of 2.0, 0.2, and 0.02 mg/ml of Biodentine™, the cell viability ranged from 81% to 114% compared to the control, while a sudden increase was observed from 5th to 6th day. However, at the concentration of 20 mg/ml, there was a decrease in cell viability on 2nd day (85%), 4th day (18%), and a slight increase was observed (31%) at 6th day of the treatment.

The viabilities of SHED treated Biodentine and MTA were less than the positive control after 72, 120, and 168 hrs of treatment (p 0.05), according to Araujo LB et al (2018)[44]. The viability of SHEDs cultured in MTA was higher than that of the negative control and Biodentine group after 7 days of treatment (p 0.05). Athanasiadou *et al* (2018)[45], reported a dilution dependent stimulation of cell growth after 72 hrs in SHEDs cultured in Biodentine eluates; however, this reached statistical significance (p 0.01) only for dilutions from 1:16 to 1:64. On evaluating cell proliferation at 72 hrs to 120 hrs, there was a statistically significant reduction in cell proliferation on the fifth day, which was statistically significant (p 0.01) for all dilutions. Collado G *et al* (2017)[10] revealed that as compared to control specimen, cell viability in the presence of Biodentine eluates was significantly greater, as was cell viability using MTA after 48 hrs of culture (p 0.01).

In the present study, SHEDs reported highest cell viability and proliferation when cultured in NeoMTA followed by RetroMTA, EndoCem Zr, Biodentine and MTA at 1:1, 1:2,1:1,1:4 and 1:4 dilutions respectively after time interval of 72 hours of treatment. Hence the subsequent experiment involving the test specimens were performed with these dilutions only. Nonetheless, cell viability and proliferation are just one component of biocompatibility and cannot be used to assess whether a substance is biocompatible or not. As a consequence, CSC cell apoptosis, migration, and calcium deposition were studied to learn more about cytocompatibility.

Apoptosis, also known as type I programmed cell death, is a tightly controlled mechanism that is essential for tissue homeostasis. Intra- or extra-cellular stimuli can initiate apoptosis through the activation of internal or external pathways. The present study used reagents–Annexin V and 7-AAD for detecting apoptosis and necrosis, respectively. Annexin V-FITC kit provides fluorescent identification of Annexin V attached to apoptotic cells and along with flow cytometry, is used for quantitative assessment.[46-47]

Apoptosis was identified with the help of dual staining with 7-AAD and Annexin-V in this experiment. Annexin-V stains start appearing before degradation in membrane integrity [which occurs during the final phases of cell death] caused due to necrosis or apoptosis. Hence, Annexin-V staining is traditionally employed along with a dye [like 7-AAD] to pinpoint apoptosis and its stages.

The results of Apoptosis of the present experiment were consistent with studies conducted by Tsai CL *et al* (2018),[42] who reported that after 48 hours of incubation, SHEDs in direct contact with MTA induced significant early apoptosis. Collado G *et al* (2017)[10] revealed that the eluates of Biodentine and MTA Angelus were accompanied with > 87% of live cells after 72 hrs of incubation. This result was in line with the present study, wherein more than 92 % of viable cells were reported in SHEDs cultured in MTA and Biodentine after 72 hrs.

For tissue homeostasis, cell migration and the healing of wounded tissues is mandatory. Hence, the bioinductive material must promote the movement of cells to restore or preserve dental pulp. Therefore, the present study assessed the cell migration of SHEDs cultured in various eluates. They were assessed using wound healing assay, or scratch assay at 24-hr, 48-hr, and 72-hr intervals. It is also referred to as a 'scratch assay' since a cell monolayer is scratched, and a microscope is used for capturing images .[48]

This method offers several advantages. Firstly, it is a cost-effective and straightforward method that facilitates real-time assessment. This method also facilitates a potent directional movement, thereby simplifying data quantification. There is a challenge concerning the assay concerning scratch size and depth. Manual scratching may cause 'ragged' edge contours, which are not simple to analyse. Additionally, damaging the intended area may also cause inadvertent damage to neighbouring cells, thereby leading to imprecise wound sizes.[48]

Studies on cell migration conducted by Hasweh *et al* (2018)[43] reported that migration of cells treated with Biodentine was determined based on the pattern of wound closure. Cells treated with the following concentrations of Biodentine (2 mg/ml, 0.2 mg/ml, and 0.02 mg/ml) showed variable migration mobility. However, Biodentine concentrations at 0.2 mg/ml and 0.02 mg/ml showed the best migration mobility and the best wound closure after 24 hrs in, comparison with the blank control. Araujo *et al* (2018)[44] reported that as compared to calcium hydroxide and positive control media, more SHEDs migrated to Biodentine and MTA. Collado *G et al* (2017)[10] reported that Biodentine and MTA demonstrated cell migration at 48 hrs, particularly at 1:4 dilution. The above findings were consistent with the cell migration results of the present study.

Alizarin Red staining is based on the anthraquinone dye. The technique requires functionality and colorimetric assay, and is used extensively to assess the calcium deposits in cell cultures. 1 mL of 10% cetylpyridinium chloride was used, in the present study to quantify the level of staining.[49]

The results of calcium deposition of the present experiment, were in accordance with study conducted by Dahake *et al* (2020)[39] who reported that SHEDs cultured in Biodentine had more mean absorbance for calcium deposition than MTA as against negative and positive control, at the end of 14 days of incubation. Athanasiadou *et al* (2018)[45] reported that SHEDs cultured in Biodentine exhibited a dilution-dependent increment of in vitro biomineralization potential, after 14 days of treatment. Collado G *et al* (2017)[10] reported that the SHEDs cultured in Biodentine demonstrated greater degree of Alizarin Red staining when compared to the control sample after 7 days of incubation. Biodentine also showed a noteworthy rise in calcification as against control and MTA, after 21 days of culture.

In the present experiment, all the test specimens demonstrated a rise in mRNA expression for BMP-2 after 24 hrs incubation in non-odontogenic as well as odontogenic medium. However, post incubation of 72 hours, distinct reduction with regards to BMP-2 expression in non-odontogenic as well as osteogenic medium could be seen in all test specimens, which could suggest negative feedback related to the early rise noted on day 1. Previous research has shown that expressing BMP-2 along with its receptor in hDPCs would speed up the development of these cells into odontoblasts.[50-51] OCN expression levels decreased for all test specimen after 72 hrs incubation as compared to 24 hrs incubation in non-osteogenic medium as well as osteogenic medium. Low ALP activity could be seen on the first day of exposure. However, post incubation for 72 hrs, the ALP expression increased for SHEDs that were exposed to eluates, including the control sample in osteogenic environment. Greater ALP gene expression can be linked to the hydroxyapatite nucleation procedure, in which the osteogenic medium induces the ALP enzyme with a basic pH, and a rise in Ca^{2+} amount encouraged by all CSCs.

The results of genetic expression analysis of the present study, were in collaboration with studies conducted by Rodrigues EM 2016, who reported that the expression of OCN and BMP-2 mRNA was significantly greater in SHEDs cultured in MTA than MTA P after 24 hrs. After 72 hrs, the mRNA expression of ALP was suggestively greater in MTA P than MTA.[52]

CONCLUSION

MTA, Biodentine, EndoCem Zr, NeoMTA and RetroMTA reported cell viability and encouraged cell proliferation, calcium deposition, cell migration, and odontogenic like phenotype differentiation in SHEDs. Hence these cements are not cytotoxic.

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