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Original Article

Response of stem cells derived from human exfoliated deciduous teeth to Bio-C Repair and Mineral Trioxide Aggregate Repair HP: Cytotoxicity and gene expression assessment

Viral Maru[1], Manisha Madkaikar[2], Ashita Gada[2], Vivek Pakhmode[3], Dimple Padawe[4], Salil Bapat[5]

Departments of [1]Pediatric Dentistry and [4]Pediatric and Preventive Dentistry, Government Dental College and Hospital, [2]Director, ICMR - National Institute of Immunohematology, [3]D.M.E.R, Joint Director, [5]Department Public Health Dentistry, SMBT Dental College, Hospital and Research Center, Mumbai, Maharashtra, India

ABSTRACT

Background: The aim of this study was to investigate and compare the cytotoxicity and gene expression of Bio-C Repair, Mineral Trioxide Aggregate (MTA) HP Repair, and Biodentine on stem cells derived from exfoliated deciduous teeth.

Materials and Methods: In this *in vitro* study MTT assay was used to assess the cellular viability at three different dilutions. The gene expression of Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), osteocalcin [OCN], and dentin matrix protein-1 (DMP-1) was measured with real-time polymerase chain reaction after 7 days, 14 days, and 21 days of incubation. One-way analysis of variance and Bonferroni posttest were used for statistical analysis (p=0.05).

Results: After 72 h of incubation at dilution 1:4, stem cells derived from human exfoliated deciduous teeth (SHEDs) cultivated in Biodentine, followed by Bio-C Repair and MTA Repair HP reported with highest cellular viability. The highest mRNA expression of Runx2, ALP, OCN, and DMP-1 was reported in SHEDs cultured in Biodentine (after 21 days of incubation).

Conclusion: Bio-C Repair and MTA HP Repair are biocompatible and capable of odontogenic differentiation similar to Biodentine when cultured in stem cells derived from exfoliated primary teeth.

Key Words: Bio-C Repair, cytotoxicity, gene expression, Mineral Trioxide Aggregate HP Repair, stem cells derived from human exfoliated deciduous teeth

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Address for correspondence:
Dr. Viral Maru,
Department of Pediatric Dentistry, Government Dental College and Hospital, Mumbai, Maharashtra, India.
E-mail: viralmaru@yahoo.co.in

INTRODUCTION

Direct pulp capping and pulpotomy are frequently performed as vital pulp therapies for carious primary teeth. Mineral trioxide aggregate (MTA) has been reported as the most suitable material for the same, while other experimental cement are being assessed.[1] Although MTA has several benefits, it also has certain drawbacks – high material cost, long setting time, tough handling properties, and tooth discoloration.[2] To compensate for the MTA's shortcomings, Biodentine was promoted as an alternative. In cases of pulp capping, this material can be used similarly to MTA, to encourage the development of a calcified bridge.[3]

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A bioceramic repair medication-Bio-C Repair was invented to make the surgical operation even easier. This bioceramic repair agent has exhibited bioactive outcomes similar to MTA.[4] To encourage pulp repair and stem cell activity, these protective materials must be biocompatible and bioactive.[5] Bioactive pulp-capping cement should possess adequate biocompatibility and bioactivity to promote dental pulp stem cell activity and pulp healing in primary teeth.[6] Stem cells derived from human exfoliated deciduous teeth (SHEDs) have the capacity to induce bone formation, generate dentin, and differentiate into other nondental mesenchymal cell derivatives *in vitro*. In contrast to DPSCs, SHEDs exhibit higher proliferation rate, increased population doublings, osteoinductive capacity *in vivo,* and an ability to form sphere-like clusters.[7] However, various researches have looked at the cytotoxicity of pulp-capping materials with stem cells obtained from permanent tooth pulp tissue; however, none of the studies have employed SHED. As a result, the goal of this research was to assess and compare the responses of SHEDs to MTA Repair HP, Bio-C Repair, and Biodentine. The null hypothesis stated that no significant differences in cytotoxicity and gene expression would exist between these pulp-capping agents.

MATERIALS AND METHODS

The present *in vitro* experimental investigation referred to the research reporting guidelines provided by Nagendrababu *et al*., 2021.[8] The "resource equation" was used to calculate the sample size for the current trial.[9] The present research involved three different trials wherein all the tests were performed in triplicate for each experimental group.

Preparation of test specimens

MTA Repair HP (Angelus, Brazil), Biodentine (Septodont, France), and Bio-C Repair (Angelus, Brazil) set cement were evaluated. The International Standard Organization (ISO) 10993-5218 guidelines were used to test these calcium silicate-based cement (CSC), which involved incubating cultured cells with eluates.[10] One gram of the powder was mixed with an equivalent amount of liquid of each cement to maintain the powder: liquid ratio advised by the manufacturers. To ensure complete polymerization, the set CSC were placed in 12 well plates (3.0 mm height and 314.0 mm^2 area) and cultivated for 15 h at 37°C, 95% humidity, and 5% CO_2. Sterilization was maintained for 30 min by exposing the sample to a UV light at 30 W in a laminar flow. The extraction medium was DMEM (HiMedia, India) supplemented with streptomycin. These eluates were collected after passing through a Merck Millipore 0.22 m filter (USA). Following that, three diluted (1/4, 1/2, and 1/1 vol/vol) eluates were tested in order to examine a dose–response correlation.[11]

Isolation and characterization of stem cells derived from human exfoliated deciduous teeth

The current experiment, as well as the technique for isolating and collecting SHEDs, had been approved by the Ethical Board of the institution. The parents/guardians of children aged 6–12 years who visited the Department of Pediatric and Preventive Dentistry with exfoliating primary teeth (n = 5) signed a written consent allowing them to be included in the research. These specimens were transferred to India's National Institute of Immunohematology at Parel, Mumbai, after rinsing in normal saline. The pulp tissues of the teeth were extirpated using barbed broaches. Hank's Balanced Salt Solution disinfected the pulp tissues (Gibco, USA). Enzymatic digestion was promoted at 37°C for 60 min using 3 mg mL Collagenase-A (Sigma-Aldrich, USA). Cells (2.0 × 104 cells/cm^2) were collected and cultured for 72 h at 370 C in the presence of 5% CO2 in 25-cm^2 plastic culture flasks (BD Biosciences, USA). Nonadherent cells, such as red blood cells, were eliminated. To encourage greater growth, a new medium was used after 3–5 days**.** Passage zero (P0) was defined as the point at which adherent cells reached 80% confluence. Incubation in a 0.25% trypsin solution for 2–5 min at 37°C was used to detach cells after they had been cleaned in phosphate-buffered saline. The trypsin action was deactivated by the introduction of the culture medium. Centrifuged at 500 g for 5 min, the SHEDs were seeded into 75-cm^2 flasks.[12]

SHED phenotypes were determined using a flow cytometer and specific antibodies for HLA-DR, CD105, CD73, CD45, CD34, and CD90 (BD Biosciences, Pharmingen) prior to the experiments.

Cell viability assay

The viability of SHEDs cultivated in various eluates was examined through MTT cell assay kit after 24 h, 48 h, and 72 h of incubation (EZ count, HiMedia, India). SHEDs cultured in DMEM and 1 mM hydrogen peroxide were used as positive and

negative control specimens, according to ISO 10993 standards.[10] MTT was added to all of the wells in question and cultured for 240 min before the procedure was stopped with the addition of dimethyl sulfoxide. A microplate reader (BioTek Instrument, USA) was used to determine $AB_{570\ nm}$ with a reference wavelength at AB_{630}.[13] The cement dilution was chosen for gene expression analysis based on the results of the cell viability test.

Gene expression assessment

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to examine the expression levels of osteogenic differentiation markers. 2×10^6 cells mL^{-1} were plated in 6-well plates. As per the manufacturer's recommendations, the ribonucleic acid was isolated from SHEDs using the RNeasy Mini Kit (QIAGEN, USA) after 24 h of exposure to the various eluates, diluted in osteogenic (5 mmol L^{-1} b-glycerophosphate and 50 lg mL^{-1} L-ascorbic acid) medium. The qPCR gene expression technique was used in the present trial (StepOne, USA). qRT-PCR was performed to identify genes associated with bone formation. Runt-related transcription factor 2 (Runx2), osteocalcin (OCN), dentin matrix protein-1 (DMP-1), alkaline phosphatase (ALP), and glyceraldehyde 3-phosphate Dehydrogenase (control gene) were used. After 7, 14, and 21 days of incubation, the levels of mRNA expression were measured using the ΔΔCt method (fold expression = 2 – [ΔΔCt ± SD]).[14]

Statistical analysis

The data were analyzed using the Statistical Package for the Social Sciences software. The mean measurements of test specimens were analyzed by one-way analysis of variance test. The Bonferroni *post hoc* test was used to look at multiple pair-wise individual comparisons between groups. A 5% level of significance was used to denote the differences between the means. A mean standard deviation was calculated from the data.

RESULTS

Isolation and characterization of stem cells derived from human exfoliated deciduous teeth

The cultured SHEDs observed under light microscopy showed more round-shaped morphology after 12 days following the third passage [Figure 1]. In the current trial, flow cytometric examination of SHEDs indicated moderate expression of CD105 (35.60%) and high expression of CD90 (97.50%), and CD73 (93.60%). Negative markers CD45 (0.57%), CD34 (1.60%), and HLA-DR (0.50%) were not expressed in the progeny of SHEDs.

Cell viability assay

Because the absorbance was higher for all time intervals, when compared to the negative control, MTT assay demonstrated that all test specimens did not induce cytotoxicity. When comparing various test specimens with the negative control at all time intervals – 24 h, 48 h, and 72 h – a statistically significant difference was discovered [Figures 2-4].

After 72 h of incubation at dilution 1:4, SHEDs cultivated in Biodentine (0.86 ± 0.01), Bio-C Repair (0.85 ± 0.01), MTA Repair HP (0.84 ± 0.01), negative control (0.47 ± 0.01), and positive control (0.08 ± 0.01) showed the maximum cell viability in descending order. As a result, the test specimens were used in the subsequent experiment with a 1:4 dilution only.

Gene expression analysis [Figure 5]

After 7 days of incubation [Figure 6], SHEDs cultured in Bio-C Repair showed statistically significant expression for Runx2 (3.5 ± 0.01) and OCN (2.5 ± 0.01). Whereas SHEDs cultured in Biodentine showed statistically significant expression for ALP (1.6 ± 0.01) and DMP-1 (2.4 ± 0.01). After 14 days of incubation [Figure 7], SHEDs cultured in Bio-C Repair showed statistically

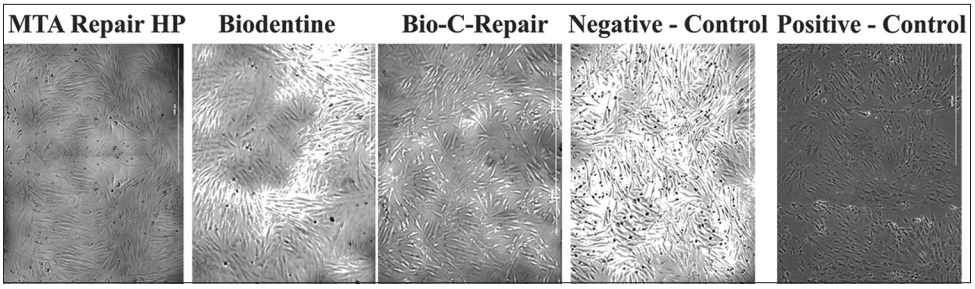

Figure 1: Morphology of SHEDs cultured in various eluates after 72 h of incubation. SHEEDs: Stem cells derived from human exfoliated deciduous teeth.

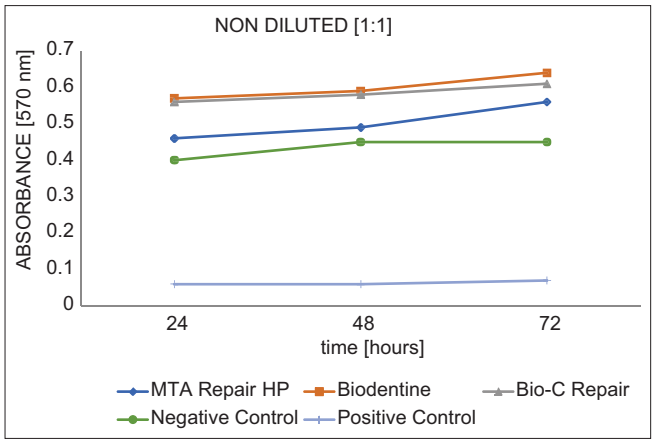

Figure 2: Comparison of absorbance (mean ± SD) between different time intervals and eluates of various test specimens at 1:1 dilution. SD: Standard deviation

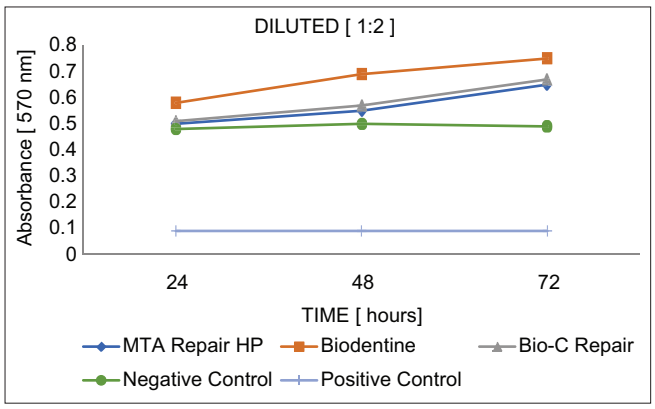

Figure 3: Comparison of absorbance (mean ± SD) between different time intervals and eluates of various test specimens at 1:2 dilution. SD: Standard deviation.

significant expression for Runx2 (3.9 ± 0.01), DMP-1 (2.8 ± 0.01), and OCN (2.7 ± 0.01). Whereas SHEDs cultured in Biodentine showed statistically significant expression for ALP (1.8 ± 0.01). After 21 days of incubation [Figure 8], SHEDs cultured in Biodentine showed statistical significance in expression for Runx2 (4.9 ± 0.01), DMP-1 (3.3 ± 0.01), ALP (2.0 ± 0.01), and OCN (3.5 ± 0.01).

For all target genes, significant variations in factor materials ($P < 0.001$) and time interval ($P < 0.001$) were observed. The interaction between the two variables was found to be statistically significant ($P < 0.001$).

DISCUSSION

Considering that vital pulp treatment comprises direct contact between tissue and treating medicament, biocompatibility characteristics of medicaments must

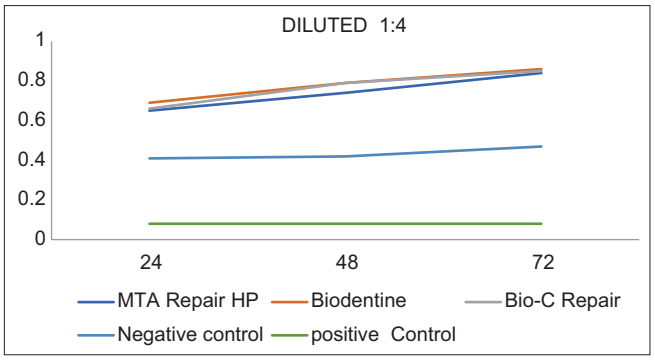

Figure 4: Comparison of absorbance (mean ± SD) between different time intervals and eluates of various test specimens at 1:4 dilution. SD: Standard deviation.

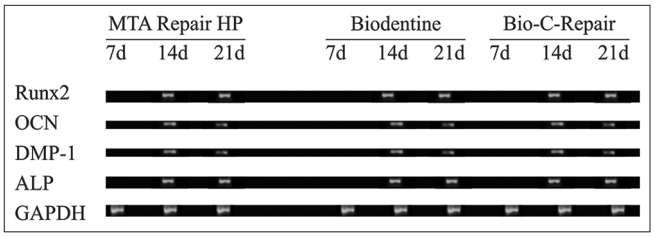

Figure 5: RT-PCR analysis of various eluates with different markers after 7, 14, and 21 days of incubation. RT-PCR: Real-time polymerase chain reaction.

be considered carefully. Several researches have been carried out to look into the possibility of compounds causing toxicity in cells, animals, and humans. For the purpose of replacing animal models, new cell culture methods have been developed.[15,16] Using extracts has the advantage of being sterile, making it easier to test their response on cells, and they replicate a clinical state where the compounds can dissolve and disperse into the periapical tissue.[17,18]

SHEDs were used in the present trial because of their ability to self-renew, simplicity of isolation and preservation, multi-lineage pluripotency, and clinical importance.[18,19] MSC population is expected to be positive for CD105, CD73, and CD90 as per the phenotype requirements of ISCT. Furthermore, such cells should not express CD14, CD45, CD34, CD19, HLA class II, CD79a, or CD11b. 17 The present study used CD45, HLA-DR, and CD45 as negative markers, while CD105, CD73, and CD90 were used as positive markers. The results of phenotypical analysis of the present study were in consistent with other studies.[20-23] The MTT assay, which follows ISO 10993 guidelines, is the gold standard for evaluating the potential cytotoxic effects of pulpotomy materials.[10] Tetrazolium compounds are employed to produce a quantifiable colorimetric assay to evaluate human cell proliferation and survival. Hence,

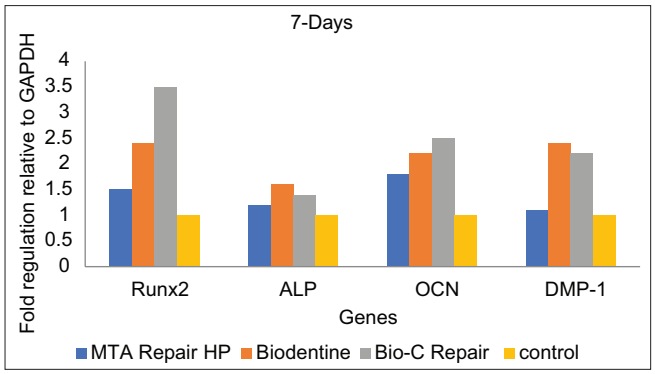

Figure 6: Comparison of mRNA expression levels (mean ± SD) of target genes in SHEDs exposed to eluates of various test specimens in osteogenic media after 7 days of incubation. SD: Standard deviation, SHEEDs: Stem cells derived from human exfoliated deciduous teeth.

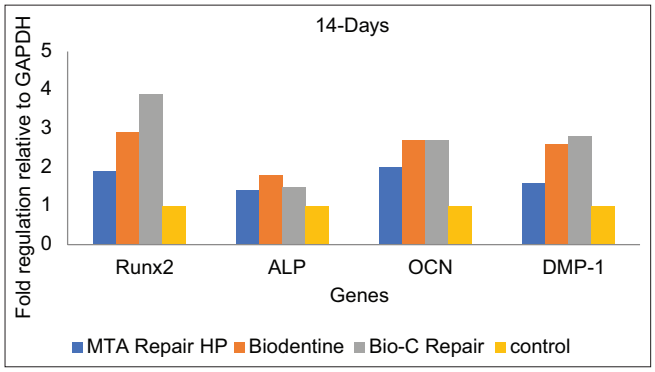

Figure 7: Comparison of mRNA expression levels (mean ± SD) of target genes in SHEDs exposed to eluates of various test specimens in osteogenic media after 14 days of incubation. SD: Standard deviation, SHEEDs: Stem cells derived from human exfoliated deciduous teeth.

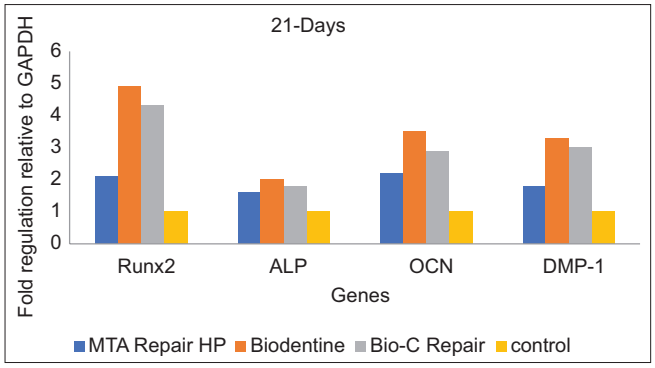

Figure 8: Comparison of mRNA expression levels (mean ± SD) of target genes in SHEDs exposed to eluates of various test specimens in osteogenic media after 21 days of incubation. SD: Standard deviation, SHEEDs: Stem cells derived from human exfoliated deciduous teeth.

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 are exposed to a medication for up to 72 h to evaluate its optimum potency and efficacy. The MTT assay was therefore conducted at 24 h, 48 h, and 72 h of incubation.[24]

Cell viability was highest for Biodentine in this trial, followed by Bio-C repair and MTA Repair HP, which was consistent with the findings of Klein-Junior *et al.*,[25] who found that bioceramic material had superior cell viability to MTA when exposed to NIH 3T3 fibroblasts. This could be because Biodentine and Bio-C Repair released much more calcium (Ca) than MTA, as recommended by the manufacturer. The formation of calcium hydroxide is critical for dentin bridge development and cementoblast differentiation but also for its antibacterial action. Other Bio-C Repair components, such as iron oxide, may have altered SHED cell viability, based on the chemical composition and solubility of each constituent of cement. When compared to calcium silicate materials containing bismuth oxide (MTA), those containing zirconium oxide (Bio-C Repair) cause less inflammation.[26]

The cell viability of Bio-C Repair was statistically significant after 72 h of incubation when compared to control in the present trial. López-García *et al.*,[27] on the other hand, reported that after 3 days of incubation, Bio-C Repair had comparable cell proliferation to the control with no substantial change. According to Benetti *et al.*,[4] when exposed to living cells, MTA Repair HP and Bio-C Repair were found to be biocompatible and had the potential for biomineralization. Cell viability experiments on L929 fibroblasts found that both materials were equally viable in the presence of each other. The variance in outcomes can be described by researches utilizing different forms of cells, each of which responds differently to the substance.[28]

Figures 6-8 show the expression of chosen osteogenic differentiation-related gene markers in the current investigation over three exposure durations. Mesenchymal stem cells must adopt the osteoblast/odontoblast phenotype in order to undergo cell proliferation, specialization, and skeletogenesis, and Runx2 is the transcription factor responsible for this.[29] For the 1st and 3rd weeks, the expression level of Runx2 was in the following order: Biodentine > Bio-C Repair > MTA Repair HP. Whereas, for the 2nd week, the expression level of Runx2 was in the following order: Bio-C Repair > Biodentine > MTA Repair HP.

OCN is an osteoblast-related gene that attaches to the Runx2-promoting site to express osteoblast-specific genes. Osteogenesis's final phase is marked by the release of this Vitamin K-dependent noncollagenous protein.[30] After 7 days of incubation, the expression of OCN was in the following order: Bio-C Repair > Biodentine > MTA Repair HP. The expression of OCN was reported in the following order after 14 days of incubation: Bio-C Repair = Biodentine > MTA Repair HP. Whereas, after 21 days of incubation, there was a substantial increase in expression of OCN for Biodentine followed by Bio-C Repair and MTA.

Dentin Morphogenic Protein 1 (DMP1) is a noncollagenous protein that causes stem cells to differentiate into odontoblast-like cells.[31] After 7 days and 21 days of incubation, expression of DMP1 was in the order: Biodentine > Bio-C Repair > MTA Repair HP. Whereas, after 14 days of incubation, expression of DMP1 was in the order: Bio-C Repair > Biodentine > MTA Repair HP.

ALP, which promotes the breakdown of phosphate monoesters at basic pH values, appears to influence preosseous cellular metabolism and the production of an extracellular environment that supports bone formation.[32] The concentrations of ALP mRNA substantially increased with time interval ($P < 0.001$ for all pairwise assessments). The concentration of ALP was also material dependent ($P < 0.001$), in the following descending order: Biodentine > Bio-C Repair > MTA at all time intervals.

These results were in accordance to a study conducted by Lozano *et al*.[33] who reported that the expression of Runx2 was higher for Bio-C Repair when compared to control after 1- and 2-week incubation. The same study reported that the expression of ALP for Bio-C Repair was less than the control after 1 and 2 weeks of incubation,[33] which contradicted the outcomes of the present trial. The lack of relevant trials assessing the cytotoxicity and gene expression of MTA HP Repair and Bio-C Repair on SHEDs is a limitation of our study.

MTA HP Repair and Bio-C Repair promote cell viability and enhance odontogenic mineralization potential on SHEDs similar to Biodentine. Hence, they could be an alternative therapeutic agent in pulp capping and pulpotomy. It can positively influence the healing of pulp followed by odontogenesis resulting in reparative dentin formation. Further, animal and clinical studies have to be explored to introduce Bio-C Repair and MTA HP Repair into clinical practice.

CONCLUSION

The response of SHEDs to Bio-C Repair and MTA HP Repair was not cytotoxic within the restrictions of this two-dimensional cell culture trial. Like Biodentine, cell proliferation and odontoblastic differentiation on SHEDs was possible with MTA HP Repair and Bio-C Repair.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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