Viability of Stem Cells Isolated from Human Exfoliated Deciduous Teeth in Response to Calcium-containing Pulp Capping Materials

Pornchanok Intavongsri[1], Waleerat Sukarawan[*1], and Thanaphum Osathanon[2]

[1]Department of Pediatric Dentistry, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
[2]Department of Anatomy, Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand
*Corresponding author, E-mail: wsukarawan@hotmail.com

Abstract

The effects of different calcium-containing pulp capping materials including Dycal®, ProRoot® MTA, and Biodentine™ on the viability of stem cells from human exfoliated deciduous teeth *(*SHEDs*)* were compared. The materials were prepared using a mold, followed by incubation with culture media for 24 hours. The extraction medium was filtered and subsequently stored at -20 °C. Prior to the experiments, the release of calcium ions in the extraction medium was measured. Cells were cultured using the extracted medium with serial dilutions at various concentrations*:* 100%, 50%, 25% and 10%. The MTT assay was employed to evaluate cell viability. Our study found that Dycal® released fewer calcium ions than ProRoot® MTA and Biodentine™. Following the ISO 10993 protocol for non-cytotoxic pulp capping materials, treatment with 25% of the medium extracted from ProRoot® MTA and Biodentine™, and 10% of the medium extracted from Dycal® resulted in 70% or higher cell viability. ProRoot® MTA and Biodentine™ demonstrated higher biocompatibility with SHEDs compared to Dycal®, which showed cytotoxicity. These results suggest that Biodentine™ and ProRoot® MTA have better biocompatibility than Dycal®.

Keywords: *SHEDs, Cytotoxicity, Cell Viability, Pulp Capping Material, Calcium*

1. Introduction

Pulp capping materials are used in vital pulp therapy to promote the development of a protective layer over the exposed vital pulp (Gandolfi et al., 2015), prevent bacterial penetration, minimize inflammation, and encourage dentin bridge formation. These materials need to be biocompatible and bioactive in order to facilitate dental pulp stem cell function and pulp recovery (Paula et al., 2018; Manaspon et al., 2021).

Calcium (Ca^{2+}) is an essential element in the mineralization process and serves as an active component in pulp capping materials (Song et al., 2017). Different calcium concentrations released from pulp capping materials are hypothesized to have the most influence on cellular responses, although this remains unproven. For instance, many cell types, such as bone marrow stromal stem cells (BMSCs), osteoblasts, human mesenchymal stem cells (MSCs), dental pulp stem cells (DPSCs), and stem cells from exfoliated deciduous teeth (SHEDs), have been examined for the effects of extracellular calcium alone on cell growth (Cheng et al., 2021; Maeno et al., 2005; Liu et al., 2009; An et al., 2012; Phlinyos, 2021). Previous research revealed no correlation between different Ca^{2+} concentrations and the cell proliferation of SHEDs (Phlinyos, 2021). Similarly, Ca^{2+} concentrations in cultures of DPSCs had little effect on cell proliferation (An et al., 2012).

The American Academy of Pediatric Dentistry has released clinical guidelines outlining the purpose and objective of alternate pulp therapy techniques for primary and young permanent teeth, which are often employed to treat accidentally damaged pulp without other symptoms. Calcium hydroxide, a gold standard material, has been utilized as a pulp capping material for permanent teeth (Janebodin, Horst, & Osathanon, 2010). Dycal→ is a self-setting calcium hydroxide material used in pulp-capping processes, causing secondary dentine formation when directly in contact with the pulp due to its alkaline pH (Poggio et al., 2014). Mineral trioxide aggregate (MTA) is a recently developed bioceramic material that has applications in endodontics that are similar to calcium silicate-based cements (CSCs). Calcium and silicate are the primary constituents of CSCs. Several manufacturers hold the belief that the use of CSCs can alleviate the issues associated with MTA, including challenging manipulation, extended curing periods, diminished tooth discoloration, and satisfactory radiopacity. These advancements have made it possible to utilize CSCs in essential pulp therapy procedures (such as ProRoot→ MTA), and Biodentine□, a bicomponent material, is a promising alternative to calcium hydroxide-based materials, offering advantages for direct pulp-capping and potentially enhancing long-term tooth vitality, as it contains tricalcium silicate, calcium carbonate, and zirconium oxide (Poggio et al., 2014). These pulp capping materials vary not only in the type of calcium contained, but also in their calcium concentrations and many other additives.

Numerous studies have investigated the impact of pulp capping materials, including their effects on cell viability, using dental pulp stem cells from permanent teeth. However, only a limited number of studies have employed dental pulp stem cells from primary teeth. Proliferation varies between DPSCs and SHEDs. There are also distinct reactions to the various pulp capping materials due to the modification of growth factor release, activation of signaling pathways, and regulation of transcription (Dahake et al., 2020).

This in vitro study aimed to investigate the impact of various calcium-containing pulp capping materials, such as Dycal→, ProRoot→ MTA, and Biodentine™, on the viability of SHEDs. This data will clarify the effects of the different pulp capping materials on SHEDs in order to develop pulp capping materials that can sustain the viability of cells and promote the proliferation of SHEDs.

2. Objectives

To compare the effects of different pulp capping materials on the cell viability of SHEDs.

3. Materials and Methods

The isolation procedure for SHEDs has been approved by the Human Research Ethics Committee at the Faculty of Dentistry, Chulalongkorn University (No. 038/2023). Healthy primary teeth from young patients were removed as part of the dental treatment plan, specifically for teeth with prolonged retention, and preserved in a culture medium. Dental pulp tissues were removed from the teeth and cell isolation was carried out, placing them on tissue culture plates with Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (HyClone, USA), 1% L-glutamine, 100 units/mL Penicillin, and 100 g/mL Str (Gibco, USA). The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. The media was replaced every 48 hours. Upon reaching cell confluence, cells were removed using 0.25% trypsin-EDTA (Gibco, USA). This study used cells from Passages 3–6. To characterize the cells, cultured cells were tested for properties as multipotent mesenchymal stem cells by plastic adhesion, shape, and morphology, as well as their multipotential differentiation ability, using in vitro mineralization. Surface protein expression of mesenchymal stem cell markers and hematopoietic markers was analyzed via flow cytometry. Cells were detached using a 0.25% trypsin-EDTA solution to achieve individual cell suspensions. After centrifugation, the culture media supernatant was removed. The cells were washed with sterile phosphate-buffered saline (PBS) and marked with primary antibodies linked to fluorescent dye, such as anti-

human CD44 (BD Bioscience Pharmingen, USA), PerCP-CyTM5.5-conjugated anti-human CD90 (BD Bioscience Pharmingen, USA), and PerCP-conjugated anti-CD45 (BD Bioscience Pharmingen, USA). Flow cytometry analysis was performed with a FACSCalibur flow cytometer using CellQuest software from BD Bioscience, USA.

Materials and extraction medium preparation

Dycal→ and ProRoot→ MTA were purchased from Dentsply International Inc., DE, USA. Biodentine™ was purchased from Septodont, CO, USA. All materials were prepared according to the manufacturer's specifications and inserted into a cylindrical mold (5 mm in height and 2.5 mm in radius, 1.18 cm^2 in surface area, and 0.1 cm^3 in volume). The mixes were prepared in a sterile atmosphere and stored at room temperature for 24 hours. Each material was extracted from the mold and placed into 1 mL of culture medium following ISO 10993 Part 12 guidelines and incubated for 24 hours at 37 °C in a 5% CO_2 atmosphere. Each extraction medium was filtered (0.1 m) and stored at -20 °C until use. The release of calcium ions of the extraction medium was measured before the experiments.

In vitro release of calcium ions

Calcium concentration in the extracted medium was measured by a calcium detection kit (Calcium Colorimetric Assay, Sigma-Aldrich, MO, USA). Samples of the extracted medium were collected after 6 and 24 hours. The samples were inserted in microtubes with 50 µl of DMEM, and the control was growth medium without any materials.

Cell viability assay

Subsequent media in various dilutions of these extraction media were prepared for analysis. SHEDs were seeded in 24-well plates in culture medium at a density of 12,500 cells/well for 24 hours; subsequently, the culture medium was changed with each pulp capping material's extraction medium at 100, 50, 25, and 10% concentrations. Cell growth viability changes were analyzed after 24 hours using the MTT assay. To promote formazan crystal formation, the liquid in each well was taken out and substituted with 300 µl of MTT solution (USB Corporation, USA) for 15 minutes at 37 °C in a humidified atmosphere containing 5% CO_2. The formazan was dissolved using eluting agents such as dimethyl sulfoxide and glycine buffer. The optical density (OD) of the solutions was measured at 570 nm using a microplate reader (ELx800; BIO-TEK®). Then, the cell number percentage was calculated. Cells cultured in growth media served as the control group. The concentration of extraction medium that resulted in 70% cell viability or higher was considered non-cytotoxic following the ISO 10993 protocol for non-cytotoxic pulp capping materials.

Statistical Analyses

The experiments were replicated using cells from a minimum of three different donors (n = 3) for biological replication and based on similar previous studies. All data were presented as the mean ± standard deviation (SD). The Shapiro-Wilk test of normality was used to confirm the distribution of the data. The results of the in vitro release of calcium ions and MTT assays were statistically analyzed using two-way ANOVA and Tukey's multiple comparison tests of concentration and time among experimental groups. One-way ANOVA and Tukey's post-hoc tests were used to perform multiple comparisons among experimental groups at each time point in the in vitro calcium ion release data. Statistical analyses were performed using Prism 8 software by GraphPad Software in California, USA. A p-value less than 0.05 was considered to indicate a significant difference.

4. Results and Discussion

4.1 Results

Isolated cell characterization

The presence of SHEDs was confirmed and characterized by spindle and fibroblast-like morphology, adhesion to plastic tissue culture surfaces, expression of mesenchymal stem cell surface markers, and multipotential differentiation ability. SHEDs were highly positive for CD44 (99.65%) and CD90 (99.36%). Lower expression was observed for CD45 (1.58%) (Figure 1).

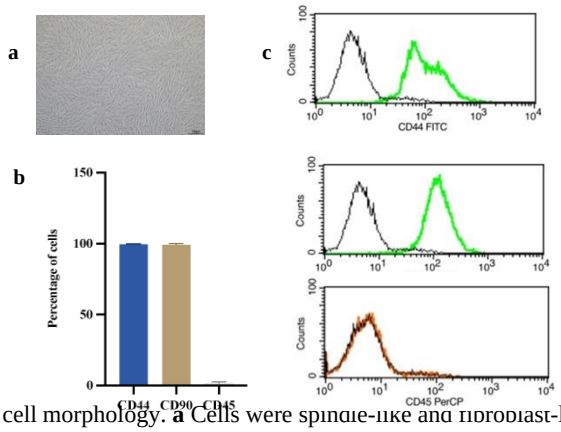

Figure 1 Characterization of cell morphology. **a** Cells were spindle-like and fibroblast-like in growth medium (original magnification: x4). **b** Surface marker expression on SHEDs was investigated by flow cytometric analysis. Isolated cells expressed mesenchymal stem cell surface markers, including CD44 and CD90, whereas cells did not express CD45. **c** Percentage of cells expressing surface markers.

Calcium release assay

All materials included calcium as a component and exhibited a time-dependent increase in the release of calcium ions, as shown in Figure 2. Biodentine™ had the highest level of calcium ion release compared to the other materials at all time points. There was no statistically significant difference in the release of calcium ions between ProRoot→ MTA and Biodentine™ at each time point. However, at 24 hours, Dycal→ exhibited significantly lower calcium ion release compared to Biodentine™, which had the highest calcium ion release among all materials.

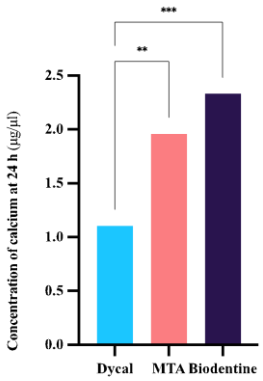

Figure 2 The release of calcium ions from Dycal→, ProRoot→ MTA, and Biodentine was quantified at 24 h. (**P < 0.01; ***P < 0.0005) analyses using one-way ANOVA.

Cell viability assay

Cell viability was assessed by an MTT test. SHEDs were cultured in different concentrations (100%, 50%, 25%, and 10%) of each pulp capping material's extraction media for 24 hours. Cells maintained in normal growth medium were used as controls. The results indicated that the cell viability in Dycal→ extraction medium treatments was not markedly increased at high concentrations. ProRoot→ MTA and Biodentine show no statistically significant differences in cell viability between each percentage of concentrations. Moreover, Biodentine extracted medium treatment resulted in a high percentage of cell viability compared to the others.

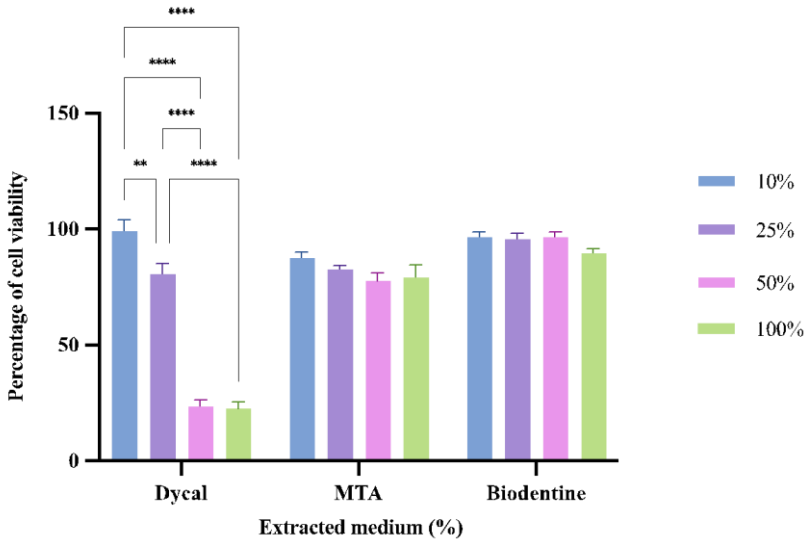

Figure 3 Cell viability after exposure to the extracted medium. The MTT assay was utilized to determine viability at 24 hours after the SHEDs were exposed to extraction medium from Dycal→, ProRoot→ MTA, and Biodentine. (**P < 0.005; ****P < 0.0001) analyses using two-way ANOVA.

4.2 Discussion

Biocompatibility is an essential consideration when choosing a material for critical pulp treatment because of its direct contact with vital tissues (Lee et al., 2014). Various quantitative and qualitative assessments are available for evaluating pulp capping materials. This study evaluated the effects of three commonly used pulp capping materials, namely Dycal→, ProRoot→ MTA, and Biodentine, which are often considered. Ca^{2+} is a key ingredient believed to stimulate or improve dental pulp healing or regeneration upon interaction with resident dental pulp stem cells (Yaemkleebbua et al., 2018). Calcium ions play a significant role in regulating essential cellular functions, such as cell proliferation, differentiation, and migration, through calcium signaling pathways.

In the present study, we observed that Biodentine released the highest amount of calcium ions. In agreement with previous findings by Manaspon et al. (2021) and other studies, at 24 hours, Biodentine ProRoot→ MTA demonstrated higher calcium ion than Dycal→. Meanwhile, there was no statistically significant difference in the release of calcium ions between ProRoot→ MTA and Biodentine. Therefore, our study focused on evaluating the cellular response to pulp capping materials at day 1.

This study demonstrated that Dycal→ is cytotoxic in vitro, whereas ProRoot→ MTA and Biodentine exhibit better biocompatibility with SHEDs. These findings concur with previous studies indicating that neither Biodentine nor ProRoot→ MTA were cytotoxic to SHEDs. These results are consistent with previous. Specifically, the findings of Manaspon et al. (2021), who reported no adverse effects of ProRoot→ MTA and Biodentine on DPSCs. However, Sequeira et al. (2018) reported that while ProRoot→ MTA did not affect apical papilla cells, 100% Biodentine extraction medium induced cell death. One possible explanation for the cytotoxic effects of Dycal→ is the elevation in pH levels, which leads to a significant increase in hydroxyl ions and subsequent cellular death.

In the cell viability analysis, our study shows that treatment with 25% extracted medium from ProRoot→ MTA and Biodentine and 10% extracted medium from Dycal→, after 24 hours, resulted in cell viabilities of 70% or higher, indicating non-cytotoxicity according to the ISO 10993 protocol. Therefore, in this study, we focused on evaluating the effects of pulp capping materials at non-cytotoxic concentrations. These findings align with previous studies, which demonstrated that treatment with 10% Dycal→, ProRoot→ MTA, and Biodentine™ resulted in cytotoxicity levels of 70% or higher and promoted cell proliferation in DPSCs (Manaspon et al., 2021).

These findings are similar to previous studies which showed that neither Biodentine™ nor ProRoot→ MTA were cytotoxic for SHEDs. Furthermore, these results conformed to previous studies on DPSCs (Kim et al., 2020). The authors reported that Biodentine™ lacked Sr, Al, and S, which are associated with the cytotoxic properties of other direct pulp capping materials (Tomás-Catalá et al., 2018). While Sequeira et al. (2018) reported that ProRoot→ MTA did not affect apical papilla cells either, 100% Biodentine™ extraction medium was found to induce cell death. In the explanation of Dycal→'s cytotoxic effects, Zohre Moradi et al. (2023) suggested that a necrotic layer contacting the pulp, caused by the high pH of Dycal→ and calcium hydroxide-based products, might be responsible.

Our present study is crucial as we aim to identify the most biocompatible pulp capping materials for primary teeth among those currently available. It highlights the significant differences in the biocompatibility effects of the three pulp capping materials. Because these findings are limited to an in vitro model system, it is essential to underscore the importance of exercising caution when using such materials in clinical practice, as they have the potential to induce irritations and cellular damage. This information holds value for the development of pulp capping materials that aim to preserve cell viability and promote the proliferation and functional differentiation of SHEDs.

5. Conclusion

Our study revealed that ProRoot→ MTA and Biodentine™ are more effective in promoting cell survival than Dycal→. Therefore, they could serve as viable alternatives for pulp capping. However, further in vivo clinical studies are necessary to confirm these findings.

6. Acknowledgements

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