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ORIGINAL ARTICLE

Cytotoxicity of Reparative Endodontic Cements on Human Periodontal Ligament Stem Cells

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ABSTRACT

Objective: To compare the cytotoxicity of commercial reparative endodontic cements on human periodontal ligament stem cells (hPDLSCs). **Material and Methods:** The culture of hPDLSCs was established. Cell density was set at 2×10^4 cells/well in 96-well plates. Extracts of Biodentine, Bio-C Repair, Cimmo HD, MTA Repair HP and White MTA were prepared. Then, the extracts were diluted (pure, 1:4 and 1:16) and inserted into cell-seeded wells for 24, 48, and 72 h to assess cell viability through MTT assay. hPDLSCs incubated with culture medium alone served as a negative control group. Data were analyzed by Two-Way ANOVA and Tukey's test (α=0.05). **Results:** At 24 h, pure extract of MTA Repair HP and Biodentine 1:16 presented higher cell viability compared to control. Lower cell viability was found for pure extract of Cimmo HD, MTA Repair HP 1:4 and 1:16, and White MTA 1:16. At 48 h, pure extract of Bio-C Repair and MTA Repair HP presented higher cell viability compared to control. At 72 h, only the pure extract of MTA Repair HP led to higher cell proliferation compared to control. **Conclusion:** Biodentine, Bio-C Repair and MTA Repair HP were able to induce hPDLSCs proliferation. Cimmo HD and White MTA were found to be mostly cytotoxic in hPDLSCs.

Keywords: Endodontics; Dental Cements; Periodontal Ligament; Cytotoxicity Tests, Immunologic.

Introduction

Calcium silicate-based cements (CBSC) have received a lot of interest from endodontists due to their biocompatibility and bioactivity. As a result, these materials have been indicated in root-end filling, pulp capping, pulpotomy, apexogenesis, apexification and in cases of root perforation, retro filling, direct pulp protection, restoration of deciduous tooth and cavities with enamel without dental support (dentine substitute) and non-conventional endodontic treatment without gutta-percha [1-13].

Mineral trioxide aggregate (MTA) is CBSC extensively used in endodontics [1,2]. However, MTA presents some drawbacks such as long setting time, inducement of tooth discoloration and difficult handling [3,4]. To overcome it, several new bioactive endodontic cements have been introduced to the market. Among these materials are Biodentine (Septodont, Saint-Maur-des-Fossés, France), Bio-C Repair (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, and Brazil), Cimmo HD (PBS Cimmo Soluções em Saúde, Pouso Alegre, MG, Brazil) and MTA Repair (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil).

Biodentine is CSBC presented in powder and liquid system [5-7]. Biodentine presents good sealing ability, favorable biological and physical properties, increased biocompatibility and a wide range of clinical applications [8]. Bio-C Repair is a ready-for-use CSBC [9-11]. According to the manufacturer, besides presenting the same indications of conventional MTA, Bio-C Repair is easy to be applied and does not contribute to discoloration, acts as a barrier against microorganisms, and stimulates tissue healing.

MTA Repair HP is based on the chemical formulation of conventional MTA but with improved physical properties related [12]. It is composed of a powder and a mixing liquid with a plasticizer agent. The bismuth oxide radiopacifier in the conventional MTA was replaced for calcium tungstate in the MTA Repair [12,13]. Cimmo HD is a biological cement developed with the same base as MTA or Biodentine, but with natural elements as additives to increase the cement resistance and without radiopacifier [14].

The biological properties of new endodontic materials should be assessed to minimize adverse effects [15]. *In vitro* cytotoxicity tests are used to detect toxic effects caused by material or its extract in cell culture [12]. Therefore, this study was conducted to assess the *in vitro* toxicity of Biodentine, Bio-C Repair, Cimmo HD, MTA Repair HP and White MTA. The null hypothesis tested was that the calcium silicate-based cements would present a similar effect on the cell viability.

Material and Methods

Primary Culture of hPDLSCs

The experimental protocol was approved by the Ethics Committee of the School of Dentistry of the University of São Paulo (Protocol# 3.895.056). The hPDLSCs were obtained from the cell biobank of the School of Dentistry of the University of São Paulo and cultured in Minimum Essential Medium α (α-MEM) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 100 µg/mL penicillin, 100 µg/mL streptomycin, and 0.5 mg/mL amphotericin B at standard culture conditions (37 °C, 100% humidity, 5% CO2 and 95% air) [16]. hPDLSCs cells from passages four to eight were used for MTT Assays. hPDLSCs cells were seeded at $2x10^4$ cells per well.

Characterization of hPDLSCs

The characterization of hPDLSCs was performed by immunostaining and evaluated by flow cytometry. The cells were seeded at 5 x 10^5 concentration and incubated in 5% FBS/PBS 1x at 4 °C in the dark for 1 hour with OCT4-FITC, SOX2-FITC, STRO-1-FITC (Abcam, Cambridge, UK), CD90-FITC (eBioscience, San Diego, CA, USA), CD34-FITC (Biolegend, San Diego, CA, USA), CD31-PE, CD-44-PE (eBioscience, San Diego, CA, USA) and CD146-PE (Biolegend, San Diego, CA, USA) antibodies for 30 min at 4 °C. Unstained control was used to set gates. A total of 10-50,000 events were recorded and data analyzed in FlowJo software (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) [16].

Specimen and Extract Preparation

All materials (Table 1) were manipulated according to the manufacturers' instructions and were inserted into a round metal appliance designed for the production of discs measuring 5 mm wide and 3 mm high. Materials were allowed to set for 24 h in a humid atmosphere and aseptic conditions. After setting, each specimen was immersed into 1 mL of α-MEM with 10 % FBS and incubated for 72 h. The specimens were then discarded and the pure extracts were filtered by 0.22-µm pore size membranes (Millipore; Billerica, MA, USA) [17,18].

Table 1. Tested materials.

Materials	Manufacturer	Composition	Proportion
Biodentine	Septodont, France.	Powder: Tricalcium silicate, zirconium oxide, calcium oxide, calcium carbonate, brown pigment, red pigment and brown iron oxide Liquid: dehydrated calcium chloride and purified water	Five drops of the liquid for one capsule
Bio-C Repair	Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil	Calcium silicate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide, dispersing agent	Ready to use
Cimmo HD	Cimmo Soluções em Saúde, Pouso Alegre, MG, Brazil	calcium oxide, calcium carbonate, magnesium oxide, dicalcium silicate, aluminum oxide, sodium oxide, potassium oxide and pozzolan	1 blister for and 1 drop of the liquid.
MTA Repair	Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil	Powder: Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide and calcium tungstate Liquid: Water and plasticizer	1 package of MTA Repair HP for 2 drops of the liquid.
White MTA®	Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil	Powder: Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, bismuth oxide Liquid: Distilled water	1 sachet of White MTA for 1 drop of distilled water.

Cell Stimulation with Materials Extracts

The pure extracts (1) were diluted (1:4 and 1:16) in α-MEM supplemented with 10% FBS. hPDLSCs were counted and seeded at 2 × 10^4 cells/well in 96-well plates in α-MEM supplemented with 10% FBS in triplicate. After 24 h, the cells were incubated with 100 µL of the extracts dilutions or medium only (negative control).

Cell Viability

Cell viability was assessed through 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay. The hPDLSCs were stimulated with the extracts for 24, 48, and 72 h. The medium was renewed

after 48 h. The cell supernatant was replaced by 20 µL of a 5 mg/mL solution of MTT in phosphate-buffered saline, followed by 180 µL of α-MEM with 10% FBS. Cells were incubated for 4 h and MTT solution was replaced by 100 µL of dimethyl sulfoxide (Synth, Diadema, SP, Brazil). Optical density was determined using a plate reader (Synergy HT, Biotek, Instruments, Inc. Winooski, VT, USA) at the wavelength of 570 nm.

Statistical Analysis

Normal data distribution was verified through the Shapiro-Wilk normality test and data were analyzed by two-way analysis of variance (Two-Way ANOVA) followed by Tukey's test (α= 0.05). Data are presented as mean ± standard deviation. All statistical analyses were performed using GraphPad Prism 7.00 (GraphPad Software, Inc., CA, US).

Results

The characterization of the hPDLSCs is shown in Figure 1. The results show high expression of mesenchymal stem cell surface markers CD-44 (100%), CD-90 (95.7%), and CD-146 (93.4%) and low expression of CD-31 (6.69%), CD-34 (7.99%), OCT-4 (19.2%) and SOX-2 (13.4%). STRO-1 was 48.2%.

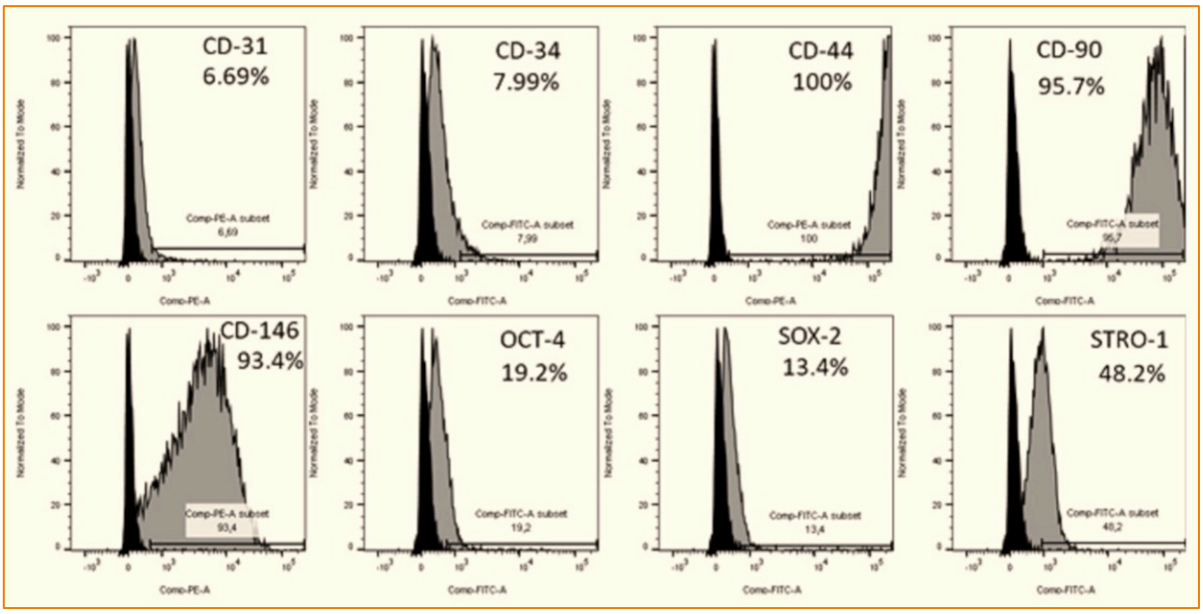


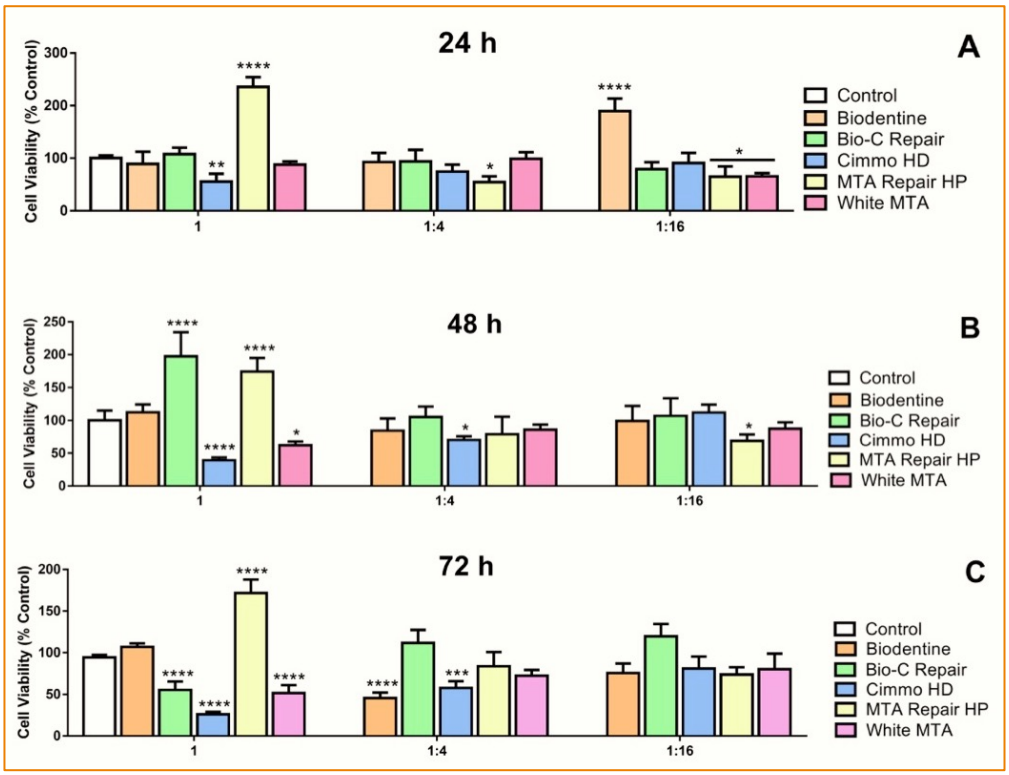
Figure 1. Characteristics of the hPDLSCs detected by flow cytometry. hPDLSCs stained with surface markers are shown as brown curves, and control hPDLSCs with no staining are shown as black curves.

Figure 2 show the cell viability according to MTT assay in SCAPs after 24 (A), 48 (B) and 72 (C) hours of exposure to different dilutions (1, 1:4 and 1:16) of the extracts of Biodentine, Bio-C Repair, Cimmo HD, MTA Repair HP and White MTA.

At 24 h, pure extract of MTA Repair HP when pure and Biodentine 1:16 presented higher cell viability ($p<0.0001$). Compared to control, lower cell viability was found for pure extract of Cimmo HD ($p<0.01$), MTA Repair HP® 1:4 and 1:16 ($p<0.05$), and White MTA 1:16 ($p<0.05$).

In the 48 h period, pure extract of Bio-C Repair and MTA Repair HP presented higher cell viability than control ($p<0.0001$). On the other hand, lower cell viability was observed for pure extract of Cimmo HD ($p<0.0001$) and White MTA ($p<0.05$), Cimmo HD 1:4 ($p<0.05$) and MTA Repair HP® 1:16 ($p<0.05$).

At 72 h of stimulation, only the pure extract of MTA Repair HP led to higher cell proliferation compared to control ($p<0.001$). The cell viability of pure extract of Bio-C Repair, Cimmo HD and White MTA were lower than control group ($p<0.0001$). For the 1:4 dilution, Biodentine and Cimmo HD had lower cell proliferation than control ($p<0.0001$).



Statistically significant differences are indicated by (*) when $p<0.05$, (**) when $p<0.01$, (***) when $p<0.001$ and (****) when $p < 0.0001$ compared to the control group. Two-Way ANOVA with Tukey test ($\alpha<0.05$).

Figure 2. Cell viability according to MTT assay in SCAPs after 24 (A), 48 (B) and 72 (C) hours of exposure to pure (1), 1:4 and 1:18 dilutions of the extracts of Biodentine, Bio-C Repair, Cimmo HD, MTA Repair and White MTA. hPDLSCs incubated in culture medium alone served as the negative control. The results show mean and standard deviation of the experiments performed in triplicate.

Discussion

An ideal calcium silicate-based material must present low cytotoxicity. In this study, we evaluated the toxicity of Biodentine, Bio-C Repair, Cimmo HD, MTA Repair HD and White MTA on stem cells of the human periodontal ligament. The null hypothesis was rejected as the cements presented different toxicity profiles in the hPDLSCs. Overall, the results showed that Biodentine, Bio-C Repair HP and MTA Repair induced cell proliferation, whereas Cimmo HD and White MTA were mostly cytotoxic.

The endodontic treatment consists of the disinfection and filling the root canal with synthetic, inert or bioactive materials capable of inducing the formation of mineralized tissue and thus, contributing to tissue healing [19-21]. Interestingly, during endodontic procedures, despite the possibility of placing the bioactive cements in contact with stem cells, an influx of undifferentiated stem cells from the apical papilla and periradicular tissues into the root canal system may occur [22,23].

In this study, the cytotoxicity of the cements were evaluated in periodontal ligament stem cells, which the phenotypic characterization is in agreement with previous studies [24-26]. Several published studies have pointed to the use of these cells to assess the toxicity of new endodontic materials *in vitro* [11,17,27-31]. These

cells reside around teeth, have a higher proliferation rate and possess the ability of multipotent differentiation such as osteogenesis, adipogenesis and chondrogenesis [27]. Furthermore, by working with cements extracts in different dilutions, a possible dose-response of the cements on the hPDLSCs could be inferred, as the cements release soluble components that may be diluted by surrounding tissue fluids *in vivo* [17,32,33].

Biodentine is a calcium silicate-based cement, synthesized to be used in direct contact with the periodontal ligament [27]. Previous studies have shown that Biodentine enhanced the proliferative activity of hPDLSCs [27,30]. This was also observed in our study in the first 24 h. Higher concentrations (20 mg/mL) of Biodentine™ were found to be more cytotoxic on PDLSCs. Lower concentration, however, increased the proliferation of hPDLSCs [27]. Thus, this corroborates our study, in which a dose-response regarding cell proliferation was observed.

In a study in which hPDLSCs were exposed to several dilutions of Bio-C Repair, similar cell viability was found for this cement compared to the control in 24 h [11]. This was also observed in this study. Interestingly, pure extract of Bio-C Repair in 48 h and Bio-C Repair at 1:4 and 1:16 dilutions presented higher cell viability compared to the control. Thus, the differences found might be attributed to the evaluated experimental periods. Furthermore, physicochemical analyses, including pH and ion release, are required to better explain our results.

In this *in vitro* cytotoxicity study, the cell viability was significantly increased for MTA Repair HP compared to control up to 72 h. Corroborating our findings, studies have shown increased proliferation of human dental pulp stem cells (hDPSCs) [12] and hPDLSCs [34] in the presence of MTA Repair HP [12,28]. In hDPSCs, however, the cell proliferation was lower than Biodentine [12]. Controversially, in a report [35], this material was slightly cytotoxic and did not lead to the proliferation of hPDLSCs.

In this study, White MTA and Cimmo HD presented lower cell viability in all experimental periods. As observed in our study, the literature shows that White MTA was not able to induce cell proliferation in L929 fibroblast cell lines [15] and periodontal ligament fibroblasts [17]. To the best of our knowledge to date, there is no published report on the effect of Cimmo HD on stem cells. White MTA and Cimmo HD were found to present good performance *in vivo* [14,36,37]. Notably, the results of this *in vitro* study must be interpreted with caution given that variations in experimental procedures and conditions may produce conflicting results and the concentration of toxic substances may decrease by tissue fluids under clinical situations [31].

Besides supporting the literature on Biodentine and White MTA, to the best of our knowledge, this is the first study that has evaluated the biological properties Bio-C Repair, Cimmo HD and MTA Repair on human periodontal ligament stem cells. Thus, it helps to illuminate the properties of these materials. However, as limited evidence is currently available regarding to the outcomes of most of the calcium silicate-based assessed in this study, a broader *in vitro* experimental approach to clarify the biological and physicochemical properties of these materials, as well as the short and long-term assessment of their performance *in vivo* is necessary to better understand the behavior of these materials.

Conclusion

Biodentine, Bio-C Repair and MTA Repair HP were able to induce hPDLCs proliferation whereas Cimmo HD and White MTA were found to be mostly cytotoxic up to 72 h.

Authors' Contributions

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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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