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Calcium silicate-based cements affect the cell viability and the release of *TGF-β1* from apical papilla cells

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This study investigated the cytotoxicity and release of Transforming Growth Factor Beta 1 (*TGF-β1*) from cultured human apical papilla cells (APCs) after application of four bioactive materials. Culture of APCs was established and used for cytotoxic and quantitative assays. Extracts of Biodentine, Bio-C Repair, MTA Repair and White MTA were prepared and diluted (1, 1:4 and 1:16) and used for MTT assays up to 72 h. Total TGF-β1 was quantified by ELISA. Data were analyzed by ANOVA and Tukey's test ($\alpha = 0.05$). For Biodentine, at 24 h and 48 h, cell viability was lower than control ($p < 0.05$). At 72 h, only undiluted extract of Biodentine were cytotoxic ($p < 0.05$). At 24 h, a cytotoxic effect was found for undiluted and 1:4 dilution of Bio-C Repair ($p < 0.05$). At 48 h, however, Bio-C Repair at 1:4 and 1:8 dilution showed higher cell viability ($p < 0.05$). At 24 and 48 h, the cell viability for undiluted MTA Repair were higher than control ($p < 0.05$). For White MTA, at 24 and 48 h, all dilutions were cytotoxic ($p < 0.05$). All cements led to reduced release of total *TGF-β1* from the APCs ($p < 0.05$). In conclusion, cell viability varied depending on the material and dilution. Only Bio-C repair and MTA repair led to higher cell viability of APCs. All materials induced a decrease in the release of total TGF-β1 from the APCs.

Key Words: Endodontics, dental cements, cytotoxicity tests, transforming growth factor beta

Introduction

Since the introduction of mineral trioxide aggregate (MTA), calcium silicate-based cements (CSBCs) have been seen as materials of choice in several endodontic applications (1). Despite MTA presents superior laboratory and clinical performance compared to previous endodontic materials (e.g. calcium hydroxide), it has poor handling properties (1). Thus, new CSBCs formulations, with improvements in manipulation and insertion, are constantly launched in the market (2).

Biodentine (Septodont, France) is a calcium silicate-based cement used in several clinical applications (3). This material presents good physical and biologic properties (3). Compared to its precursor (White MTA - Angelus, Brazil), MTA Repair HP (Angelus, Brazil) shows excellent biological and mechanical properties (4,5). Bio-C Repair (Angelus, Brazil) is a ready-for-use calcium silicate-based cement that acts as barrier against microorganisms, stimulates tissue healing, besides not causing discoloration (4).

In regenerative endodontic procedures, there is an influx of undifferentiated stem cells into the root canal system (6) probably originated from the apical papilla and periradicular tissues (7). CSBCs present the ability to promote regeneration of pulpal and periradicular tissues (8). The interaction between bioactive materials and cells may influence stem cell survival, promote their proliferation and differentiation and lead to tissue repair (2,8).

Cells from the human apical papilla (APCs) represents a group of mesenchymal stem cells residing in the root apex of immature permanent teeth. These cells present the ability to regenerate dentin-like tissues (9). Interestingly, APCs were found to express Transforming Growth Factor Beta 1 (*TGF-β1*) (10) which plays a central role in regulating a broad range of cellular responses including cell growth and differentiation (11).

To date, there is not much published literature on the effects of CSBCs on APCs (12-15). To the best of our knowledge, the effects of Bio-C Repair, MTA Repair and White MTA on the cell viability and release of *TGF-β1* by APCs have not been examined. Therefore, the purpose of our study was to investigate the cytotoxicity and release *TGF-β1* from APCs after application of Biodentine, Bio-C Repair, MTA Repair and White MTA. The null hypotheses tested were: (i) there would be no difference in cell viability between the CSBCs and the negative control group (ii) the CSBCs would not interfere in the TGF-β1 release from the APCs.

Material and Methods

Culture of Apical Papilla Cells

The Ethics Committee of the School of Dentistry of the University of São Paulo (Protocol #3.821.657) approved this study. The APCs were obtained from the cell biobank of the School of Dentistry of the University of São Paulo. APCs were cultured in Minimum Essential Medium α (α-MEM) (Invitrogen) with 10% fetal bovine serum (FBS) (Gibco) and antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin, 0.5 mg/mL amphotericin B – Invitrogen) at standard culture conditions (37°C, 100% humidity, 5% CO2 and 95% air) (16). APCs from passages four to eight were used for MTT and ELISA assays. Cell density was set at 2 $\times 10^4$ cells/well in 96-well plates.

Preparation of conditioned medium

Biodentine, Bio-C Repair, MTA Repair and White MTA were manipulated according to the manufacturers' recommendations (Table 1) and inserted into a round metal appliance to produce specimens of 5 x 3 mm. All the cements were manipulated under aseptic conditions according to ISO 10993-12:2012 (E) recommendations (17). Materials were allowed to set for 24 h. After setting, each specimen was immersed into 1 mL of α-MEM with 1% FBS and incubated up to 72 h. The specimens were then discarded and the 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, Londrina, PR, Brazil	Calcium silicate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide, dispersing agent	Ready to use
MTA Repair®	Angelus, 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, Londrina, PR, Brazil	Powder: Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, bismuth oxide Liquid: Distilled water	1 sachet of MTA-Angelus® for 1 drop of distilled water.

Cell stimulation with materials extracts

The extracts were diluted (1, 1:4 and 1:16) in α-MEM with 1% FBS. APCs were counted and seeded. After 24 h, the cells were incubated with 100 µL of the extracts dilutions (experimental groups) or medium only (negative control group). Following the ISO 10993-12:2012 (E) recommendations, the cytotoxicity tests were performed in triplicate and at different time intervals (17).

MTT assay

The APCs were stimulated with the extracts of the CSBCs for 24, 48 and 72 h. The cell supernatant was replaced by 20 µL of 5 mg/mL of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) in PBS and then, 180µL of α-MEM was added. Cells were incubated for 4 h

and the solution was replaced by 100 µL of dimethyl sulfoxide (Synth, Diadema, SP, Brazil). Optical density was determined at 570nm.

Quantification of *TGF-β1*

The APCs were stimulated with the extracts of the CSBCs (1, 1:4 and 1:8 dilution) for 24 h. *TGF-β1* was quantified in the supernatants by ELISA (DY240-05, R & D Systems). Acidification was performed by adding 20 µL of 1 N HCl for 100 µL of the culture supernatants. The samples were incubated at room temperature for 10 min. Then, the solution was neutralized with 20 µL of 1N NaOH / 0.5 M HEPES and pH range was checked (7.2 - 7.6). The concentrations read based on the standard curve were multiplied by the dilution factor (1.4 x).

Statistical analysis

Shapiro-Wilk normality test and Levene's test was used to assess normal data distribution and homogeneity of variances, respectively. Analysis of variance (ANOVA) and Tukey's test ($\alpha = 0.05$) was used for data analysis. Data are presented as mean ± standard deviation. Statistics were performed using GraphPad Prism 7.00 (GraphPad Software, Inc., CA, US).

Results

MTT assay

According to the ISO 10993-5:1999 (E) recommendations, biomaterials that promote reduction in cell viability by more than 30% are considered cytotoxic (19). At 24 h (Figure 1A), the cell viability for undiluted MTA Repair at 24 were higher than control ($p < 0.05$). With the exception of Bio-C Repair 1:16, all material presented a cytotoxic effect on the APCs ($p < 0.05$). In the 48 h period (Figure 2B), undiluted MTA Repair and Biodentine at 1:4 dilution presented higher cell viability compared to control ($p < 0.05$) and with the exception of Biodentine 1:16, the other material and dilutions presented lower cell viability compared to control ($p < 0.05$), which was found to be cytotoxic. At 72 h (Figure 1C), only Bio-C Repair 1:4 led to higher cell viability compared to control ($p < 0.05$). A cytotoxic effect, however, was found for all undiluted CSBCs and Biodentine at 1:4 dilution ($p < 0.05$).

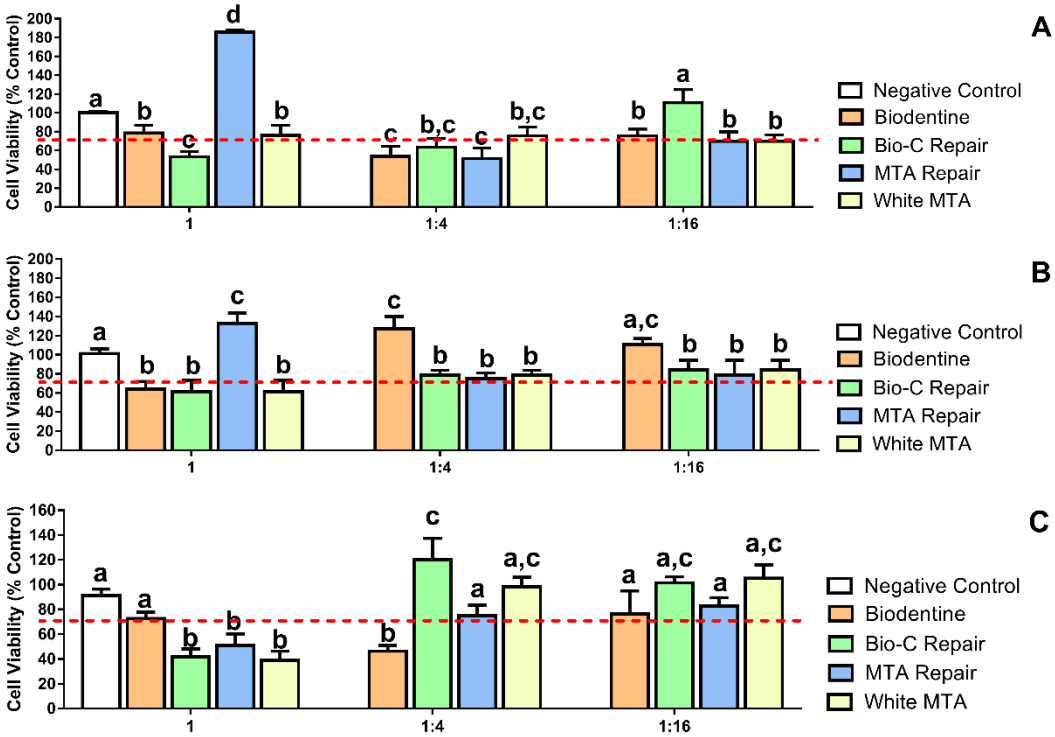

Figure 1. Cell viability rate (% of negative control) according to MTT assay in APCs after 24 (A), 48 (B) and 72 (C) hours of exposure to different dilutions (1, 1:4, and 1:16) of the extracts of Biodentine, Bio-C Repair, MTA Repair and White MTA. APCs incubated in culture medium alone served as the negative control. The results show mean and standard deviation of the experiments performed in triplicate. Different letters represent significant differences between groups in each dilution extract. Two-Way Analysis of variance followed by Tukey test ($\alpha = 0.05$). The horizontal dashed line indicate 70% cell viability

Total *TGF-β1*

All dilutions of the CSBCs presented lower *TGF-β1* values compared to control ($p < 0.05$) (Figure 2). For Biodentine and white MTA, the lower concentration of *TGF-β1* was found with 1:4 and 1:16 dilutions ($p < 0.05$). Bio-C Repair showed values of a *TGF-β1* in dose-response manner with the high dilution (1:16) presenting high quantification of *TGF-β1* ($p < 0.05$). The MTA Repair at 1:16 dilution presented higher values of *TGF-β1* compared to undiluted and 1:4 dilution. The last, was lower than the undiluted extract ($p < 0.05$).

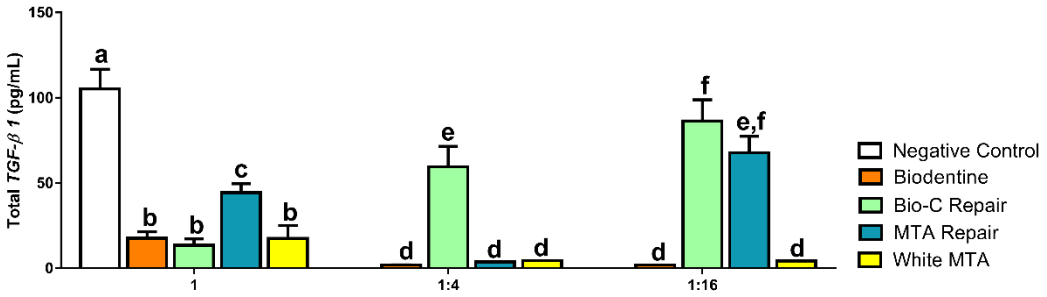

Figure 2. Total TGF-β1 according to ELISA assay in APCs after 24 hours of exposure to 1, 1:4 and 1:16 dilution of Biodentine, Bio-C Repair, MTA Repair and White MTA. APCs incubated in culture medium alone served as the negative control group. The results show mean and standard deviation of the experiments performed in triplicate. Different letters represent significant differences between groups. Two-Way Analysis of variance followed by Tukey test ($\alpha = 0.05$).

Discussion

In this study, the null hypotheses were rejected. The results showed significant differences in cell viability of the APCs were found between the CSBCs and the negative control group. In addition, the *TGF-β1* release from the APCs was significantly influenced by the application of the CSBCs. Both cytotoxic and quantitative assays varied depending on the material and dilution.

Different dilutions were evaluated to infer a possible dilution that occurs *in vivo* to better understand the cytotoxicity of the tested CSBCs (18). TGF-β1 may promote or inhibit cell activity depending on the concentration available (20). The effect of *TGF-β1* on mesenchymal stem cells *in vitro* depends on the specific culture conditions involved (20). Thus, for this purpose, an analysis of different dilutions of CSBCs is also important.

In this study, Biodentine promoted a significant increase in metabolic activity/cell viability of the APCs at 48 h. This is consistent with previous reports (12-14). A study with human dental pulp stem cells (hDPSCs) showed that Biodentine enhances its proliferation (21). Controversially, Biodentine also showed significant cytotoxicity effect (22) against hDPSCs. This was also observed in our study when APCs were exposed to Biodentine.

Undiluted Bio-C repair led to higher cytotoxicity in the APCs. However, at 48 h, the cell viability for 1:4 and 1:8 dilutions was higher than negative control. In a study with Human Periodontal Ligament Stem Cells (hPDLCs) (2), eluates of Bio-C Repair presented higher cell viability than other CSBCs. However, it was not superior to control. In addition, in L929 fibroblasts (4) exposed to Bio-C Repair, greater cell viability at most of the periods was observed. To the best of our knowledge, literature lacks on studies evaluating the cytotoxicity effect of this material on APCs.

MTA Repair showed higher cell viability in the undiluted extract compared to control up to 48 h. The other concentrations were or cytotoxic or equal to control. Corroborating these findings, in studies in which *h*DPSCs (5) and L929 fibroblasts (4) were incubated with extracts of MTA Repair, significant increase in cell viability compared to the control were observed.

Even though MTA is not relatively new compared to the other materials in this study, reports regarding its effect on APCs are scarce (15). In this study, undiluted White MTA showed lower cell viability compared to control in the first 48 h. At 72 h, only the undiluted extract presented lower cell viability. This is supported by a study (15) in which no significant differences were found when APCs were cultured in the presence of White MTA in the first 72 h. In other study conducted with human periodontal ligament fibroblasts (18) no significant differences were found after incubation with White MTA for up to 72 h. As also observed in this study, the dilution affects the cytotoxicity of the material (18).

TGF-β1 is a multifunctional cytokine that plays an important role in several biological processes, being involved in the repair/regeneration and inflammatory processes (23). For the assays, we decided use 1% FBS for preparation of conditioned medium and stimulation of APCs as FBS contains high level of *TGF-β1* (23). This would also allow a better understanding in the condition *in vivo*. Moreover, in low-serum medium, APCs maintained levels of cell viability and cellular morphology unchanged (24).

It is known that APCs express *TGF-β1* (10). However, reports of the effects of CSBCs on *TGF-β1* release from these cells are scarce (25). The results of this study showed that the CSBCs led to lower concentration of total *TGF-β1* in the cell supernatant. Interestingly, *TGF-β1* was found to inhibit the proliferation, differentiation, and mineralization events in APCs (25). The effects of Bio-C Repair, MTA Repair and White MTA on the release of *TGF-β1* by APCs are largely unknown. Thus, to the best of our knowledge, this is the first study evaluating the influence of these materials on the release of *TGF-β1* by APCs.

In this study, the differences in cytotoxicity between the CSBCs might be due to their chemical composition as well as their solubility. Thus, an assessment of the components released from the cements on the extracts could help us to better understand the results for cytotoxicity and total *TGF-β1*. As limited evidence is currently available regarding the outcomes of most of the CSBCs assessed in this study, a broader *in vitro* experimental approach is necessary to clarify the biological and physiochemical properties of these materials.

Besides the results reported herein help to illuminate the properties of calcium silicate-based materials on apical papilla cells, they must be interpreted with caution as variations in experimental procedures may produce conflicting results. Moreover, the application of the body of knowledge obtained from *in vitro* studies into clinical situations has been a great challenge as *in vitro* studies present limitations, which were previously reported in literature (26). Thus, results from long-term clinical observations are also necessary to better understand the behavior of these materials.

In general, the CSBCs investigated in this study affected the cell viability and release of *TGF-β1* from human APCs. Biodentine, Bio-C repair and MTA repair led to higher metabolic activity/cell viability of APCs. All materials induced a decrease in the release of total *TGF-β1* from the APCs. These interactions could help in the development of regenerative strategies aimed at root growth and development in immature teeth for endodontic treatment (25).

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Resumo

Este estudo investigou a citotoxicidade e liberação do Fator de Crescimento Transformador Beta 1 (*TGF-β1*) em células da papila apical humana (APCs) cultivadas após a aplicação de quatro materiais bioativos. A cultura de APCs foi estabelecida e usada para ensaios citotóxicos e quantitativos. Extratos de Biodentine, Bio-C Repair, MTA Repair e White MTA foram preparados e diluídos (1, 1: 4 e 1:16) e usados para ensaios de MTT por até 72 h. O *TGF-β1* total foi quantificado por ELISA. Os dados foram analisados por ANOVA e teste de Tukey (α = 0,05). Para o Biodentine, em 24 h e 48 h, efeito citotóxico foi observado ($p < 0,05$). Em 72 h, apenas o extrato não diluído de Biodentine teve efeito citotóxico ($p < 0,05$). Em 24 h, valores mais baixos de viabilidade celular foram encontrados para o extrato não diluído e diluidi 1:4 de Bio-C Repair ($p < 0,05$). Em 48 h, no entanto, Bio-C Repair na diluição 1:4 e 1:8 mostrou maior viabilidade celular ($p < 0,05$). A viabilidade celular para MTA Repair não diluído em 24 e 48 h foi maior que o controle ($p < 0,05$). Para White MTA, às 24 e 48 h, a viabilidade celular em todas as diluições foram citotóxicas ($p < 0,05$). Todos os cimentos levaram à redução da liberação de *TGF-β1* total das APCs ($p < 0,05$). Em conclusão, a viabilidade celular variou dependendo do material e da diluição. Biodentine, Bio-C Repair e MTA Repair levaram a uma maior viabilidade celular de APCs. Todos os materiais induziram uma diminuição na liberação de *TGF-β1* total das APCs.

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