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Experimental Calcium Silicate-Based Cement with and without Zirconium Oxide Modulates Fibroblasts Viability

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The aim of this study was to verify whether the use of zirconium oxide as a radiopacifier of an experimental calcium silicate-based cement (WPCZO) leads to cytotoxicity. Fibroblasts were treated with different concentrations (10 mg/mL, 1 mg/mL, and 0.1 mg/mL) of the cements diluted in Dulbecco's modified Eagle's medium (DMEM) for periods of 12, 24, and 48 h. Groups tested were white Portland cement (WPC), white Portland cement with zirconium oxide (WPCZO), and white mineral trioxide aggregate Angelus (MTA). Control group cells were not treated. The cytotoxicity was evaluated through mitochondrial-activity (MTT) and cell-density (crystal violet) assays. All cements showed low cytotoxicity. In general, at the concentration of 10 mg/mL there was an increase in viability of those groups treated with WPC and WPCZO when compared to the control group ($p<0.05$). A similar profile for the absorbance values was noted among the groups: 10 mg/mL presented an increase in viability compared to the control group. On the other hand, smaller concentrations presented a similar or lower viability compared to the control group, in general. A new dental material composed of calcium silicate-based cement with 20% zirconium oxide as the radiopacifier showed low cytotoxicity as a promising material to be exploited for root-end filling.

Keywords: cell viability, fibroblasts, zirconium oxide, MTA

Introduction

A cement to be used for endodontic purposes, both in conventional treatment, such as in surgical treatment, should be well tolerated by the periapical tissue, have dimensional stability, be easily manipulated, provide radiopacity, and effectively seal root canals (1). One of the materials used in endodontics is mineral trioxide aggregate (MTA), which was developed at Loma Linda University and has shown excellent biological properties, satisfactory physical and chemical properties, and discrete antimicrobial activity (2).

It is well known that the main component of MTA is calcium silicate-based cement. Several studies (3-5) have shown similar results for tissue response and were compared using MTA and experimental calcium silicate-based cements. However, it has been hypothesized that the arsenic present in experimental calcium silicate-based cement could be released and become harmful to the tissues. One study showed the release of arsenic was low and similar to that of MTA (4), while another study verified that neither experimental calcium silicate-based cement nor MTA presented genotoxicity (5).

One shortcoming of calcium silicate-based cement is its poor radiopacity, which precludes its radiographic visualization (6). Therefore, this material requires the addition of a radiopacifying agent.

The use of bismuth oxide (BO), which has higher porosity when associated with an experimental calcium silicate-based cement compared to calcium silicate-based cement alone (7), is commonly used as a radiopacifier, although it interferes with cement hydration (8). In addition, BO is a yellow powder, and when separated from the cement, it may turn a grayish color that can promote tooth darkening if used in coronal-pulp capping (9).

The association of other radiopacifying agents has also been studied (10), and the majority of those studies resulted in radiopacity beyond the minimum recommended by American National Standards Institute/American Dental Association (ANSI/ADA) 2000 (11), including zirconium oxide. Zirconium oxide is a biocompatible and nontoxic white powder (12). When associated with calcium silicate-based cement, it acts as an inert material and does not react with the byproducts of hydration (13).

This study verified whether the use of zirconium oxide as a radiopacifier of an experimental calcium silicate-based cement (WPCZO) would lead to cellular cytotoxicity.

Material and Methods

Tested Materials and Extract Preparation

The tested materials were white calcium silicate-based

cement (WPC; Votoran, Cubatão, SP, Brazil), white calcium silicate-based cement with zirconium oxide (WPCZO; Votoran, Cubatão, SP, Brazil and Merck & Co., Inc., Whitehouse Station, NJ, USA, respectively), and white MTA angelus (MTA; Angelus, Londrina, PR, Brazil).

The association of calcium silicate-based cement and zirconium oxide was performed using a ratio of 80% white calcium silicate-based cement and 20% radiopacifying agent by weight (8). The radiopacifying agents were subjected to analysis of particle size, and when required, the calcium silicate-based cements were sieved until the final particles measured approximately 0.062 mm (Bronzinox, SP, Brazil) in order to yield a more uniform particle size before mixing (8). The materials were mixed at a ratio of 1 g of powder per 0.3 mL of liquid (distilled water). The mixture obtained was added to the culture medium, Dulbecco's modified Eagle's Medium with 10% fetal bovine serum (DMEM 10% FBS), at an initial ratio of 10 mg/mL. Each solution was placed in an incubator overnight. After this period, the remaining extracts were obtained from the stock solution (10 mg/mL) by serial dilution to the following concentrations: 0.1 mg/mL and 1 mg/mL.

Cytotoxicity Test

Cell Culture

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was performed as described by Mosmann (14). Briefly, NIH-3T3 fibroblasts (American Type Culture Collection - ATCC - mouse embryonic cell lineage) were cultivated in DMEM 10% FBS, with 1% penicillin/streptomycin. Cells were plated at a density of 1.6 x 10^3 per well in 96-well plates (TPP®, Trasadingen, Switzerland) and then incubated at 37 °C and 5% CO_2. After 12 h, adherent cells were treated with different concentrations of extracts prepared with the cement diluted in DMEM 10% FBS, according to International Organization for Standardization (ISO) standard 10993-5 (15). Untreated cells (control group) were maintained in DMEM 10% FBS.

MTT Assay

Cells were plated and treated as described above for 12, 24, and 48 h. After each period, the medium, per well, was removed and new medium (no serum) was added, followed by the addition of 20 µL of MTT (0.5 mg/mL in PBS - phosphate-buffered saline), and incubated for 4 h at 37 °C. Formazan precipitate was solubilized with 100 µL/well of isopropyl alcohol. Assays were performed in triplicate. The plates were read in a spectrophotometer (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) at 540 nm.

Crystal Violet Assay

Cells were plated and treated as described above for 12, 24, and 48 h. After each period, the medium was removed and the cells were washed with PBS and placed in absolute ethanol and glacial acetic acid (3:1, vol/vol) for 10 min at room temperature. Next, cells were stained with crystal violet 0.1% (wt/vol) for 10 min at room temperature. Excess dye was washed with distilled water. The dye was extracted with 10% acetic acid. Assays were performed in triplicate. The plates were read in a spectrophotometer (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany) at 550 nm.

Statistical Analysis

Data are presented as mean ± standard deviation of at least 3 independent experiments. All statistical analyses were performed with the Prism GraphPad 5.0 (Prism Software, GraphPad, San Diego, CA, USA) on a personal computer. Parametric analysis was performed by variance analysis followed by ANOVA and Tukey tests. Statistical significance was considered if $p<0.05$.

Results

MTT Assay

In general, with the concentration of 10 mg/mL there was an increase in viability of those groups treated with WPC and WPCZO when compared to the control group. A similar profile for the absorbance values was noted among the groups. After 12 h of treatment, every group treated with 10 mg/mL presented an increase in viability compared to the control group (Fig. 1). Furthermore, the other 2 concentrations of WPCZO increased the proliferation of fibroblasts after 12 h of treatment. It was noted that MTA 10 mg/mL didn't cause an increase in proliferation after 24 h, contrary to the 2 experimental groups of the same concentration (Fig. 1).

As in the previous period, the WPCZO also presented increasing viability at the 1 mg/mL concentration ($p< 0.05$). After 48 h, only groups treated with WPC and WPCZO at 10 mg/mL presented an increase in viability compared to the control group (Fig. 1). On the other hand, both groups with smaller concentrations presented a decrease in viability ($p<0.05$).

Crystal Violet Stain

After 12 h, almost every group presented an increase in viability, except WPC at a concentration of 0.1 mg/mL (Fig. 2). In the 24-hour period, it was observed that every group at 10 mg/mL concentration (Fig. 2) presented greater viability compared to the control group ($p<0.05$). After 48 h, only the WPC group at 10 mg/mL presented a statistically significant ($p<0.05$) increase in viability.

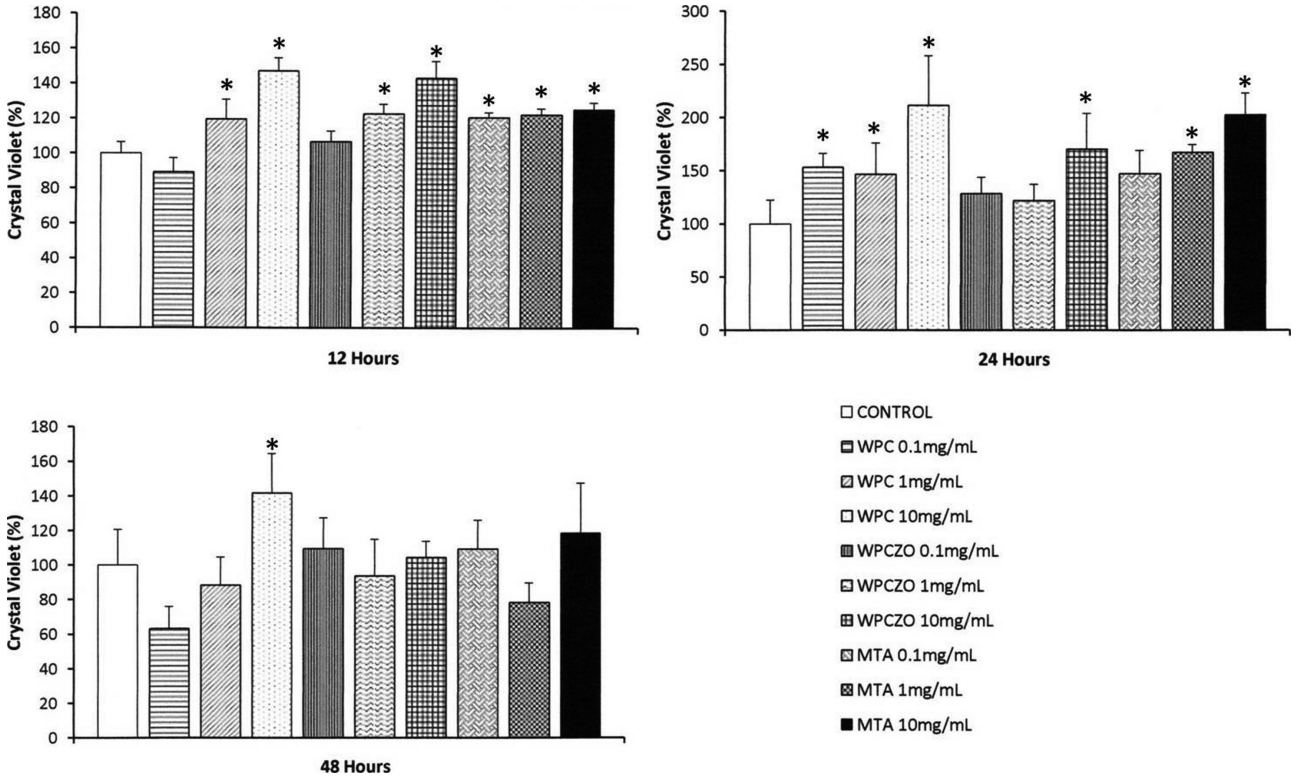

Figure 1. Viability of cells (%) revealed by MTT when in contact for 12, 24, and 48 h with different concentrations (10 mg/mL, 1 mg/mL, and 0.1 mg/mL) of the materials: white Portland cement (WPC), white Portland cement with zirconium oxide (WPCZO), and white MTA angelus (MTA), as well as a control group (CONTROL). Significant differences from the control group are represented by an asterisk (*; p≤0.05).

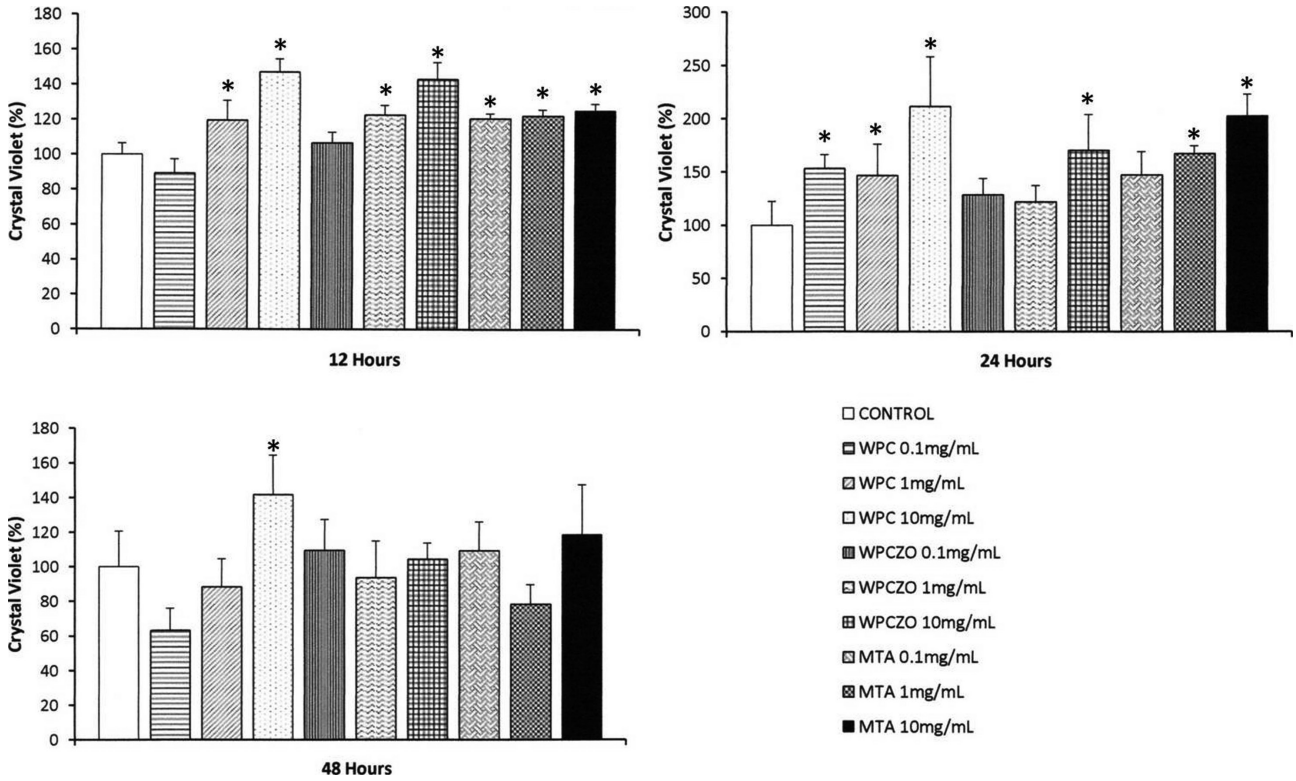

Figure 2. Viability of cells (%) revealed by crystal violet when in contact for 12, 24, and 48 h with different concentrations (10 mg/mL, 1 mg/mL and 0.1 mg/mL) of the materials: white Portland cement (WPC), white Portland cement with zirconium oxide (WPCZO), and white MTA Angelus (MTA), as well as a control group (CONTROL). Significant differences from the control group are represented by an asterisk (*; p≤0.05).

Discussion

Biological compatibility is one of the most important properties of endodontic materials. The final result of endodontic treatment depends on the tissue response to these materials (16). This study evaluated the effect of the addition of zirconium oxide on the cytotoxicity of experimental calcium silicate-based cement. In this study, cell viability was determined by MTT assay, based on the capacity of mitochondrial enzymes in living cells to convert salt MTT (yellow) to formazan crystals (dark blue), and by using crystal violet, which is a test based on the ability of DNA from viable cells to capture the pigment (gentian violet).

The *in vitro* tests employing cell culture are advantageous due to their simplicity, quickness, low costs (17), reproduction in reduced time, and the increased control they offer over the experimental conditions (pH maintenance, CO_2 concentration, and levels of signaling molecules) (18). Furthermore, *in vitro* models can reduce or eliminate the use of animals and the associated ethical implications (18).

On the other hand, *in vitro* viability tests (MTT, crystal violet, and neutral red) have some limitations, including the impossibility of keeping the cells alive after the procedure and the inability to quantify and detect the type of cell death (17). Actually, these tests can give a specific idea of the viability, cellular proliferation, and metabolism compared to a control group. This information (viability, proliferation, and metabolism) is important to show what the behavior of a cell is after an experimental protocol. We used 10% FBS for all groups because this condition can maintain cellular proliferation/viability and allow comparison between control and experimental groups, especially with the MTT assay (14).

Studies examining biocompatibility, genotoxicity, and arsenic release showed similar results between MTA and WPC (4,5,7,10), corroborating the findings of this study on cultured fibroblasts.

Zirconium oxide has been studied by many authors, including our research group (19), and the results are promising because of its ability to maintain physical, chemical, and biological properties (20). In this context, the present study generated results that support its application, revealing the cytotoxicity of WPCZO as similar to the control group.

The present results showed the high concentration of material (10 mg/mL) in all groups caused an increase in viability, particularly after 12 and 24 h. By comparison, another study (20) showed MTA and WPC caused an increase in viability after 12 and 24 h. The same study also showed that, after 48 and 72 h, the WPC groups treated with different concentrations of bismuth oxide presented higher viability than the MTA and untreated WPC groups. Our results were similar for some groups with the highest concentration, although viability decreased at lower concentrations.

The 24-h MTT results were consistent with the literature, showing MTA did not inhibit cell viability and showing no difference from the control group (21).

Other studies showed WPC has no cytotoxic behavior and exhibits cell viability similar to that of the control group (5,22). Our results revealed that, even in some concentrations (1 and 10 mg/mL), WPC did not reduce cell proliferation when compared to the control group, in accordance with previous reports (5,22). Other authors have also shown lower *in vitro* cytotoxicity of MTA and WPC for various cell types, such as mesenchymal stem cells, osteosarcoma cell lines, osteoblasts, and gingival fibroblasts (21-24).

Zirconium oxide, as other radiopacifiers, has shown no genotoxicity when evaluated on murine osteoblasts, probably indicating this element may not present a cytotoxic behavior (13). Compared to that study, the WPCZO showed no toxic effect either; in fact, at some concentrations (10 and 1 mg/mL) and a period of 24 h, it caused an increase in viability. WPC treatment resulted in an increase in cellular viability, and we speculate this could be due to some specific alteration in cell-signaling pathways, as some authors have already reported such alterations in those mechanisms as a result of increased cell viability (21-25).

Some molecular mechanisms of the action of cements and endodontic materials have been studied (22-25), but few questions have been addressed so far among them, the up- or down-regulation of genes related to mineralization (such as osteonectin and bone sialoprotein), flash regulatory molecules (iNOS, COX-2) (23), bone morphogenetic proteins 2 and 4 (BMP-2 and BMP-4), fibroblast growth factor 4 (FGF4) (25), and matrix metalloproteinases (24).

Regardless of the mechanism involved, this study could demonstrate that the addition of zirconium oxide does not cause changes in the biological properties of WPC and that it could be a potential radiopacifying agent for WPC.

Zirconium oxide may be an alternative radiopacifying material for root-end filling, and the white coloration of the material offers favorable esthetics if used as a pulp-capping material.

Resumo

O objetivo deste estudo foi verificar se o uso do óxido de zircônia como radiopacificador de um cimento experimental à base de silicato de cálcio (WPCZO) levou a citotoxicidade. Os fibroblastos foram tratados com diferentes concentrações (10 mg/mL, 1 mg/mL e 0,1 mg/mL) dos cimento diluídos em meio Eagle modificado por Dulbecco (DMEM) durante períodos de 12, 24, e 48 horas. Os grupos testados foram: cimento

Portland (WPC), cimento Portland branco com óxido de zircônio (WPCZO) e MTA Angelus branco (MTA). No grupo controle as células não foram tratadas. A citotoxicidade foi avaliada por meio da mitocondrial-atividade (MTT) e ensaio de densidade celular (cristal violeta). Todos os cimentos apresentaram baixa citotoxicidade. Em geral, na concentração de 10 mg/mL, houve um aumento na viabilidade desses grupos tratados com WPC e WPCZO quando comparado com o grupo controle ($p < 0,05$). Um perfil semelhante para os valores de absorvância foi observado entre os grupos: 10 mg/mL apresentaram um aumento da viabilidade em relação ao grupo controle. Por outro lado, as concentrações menores apresentaram uma viabilidade semelhante ou inferior em comparação com o grupo controle, em geral. Um novo material odontológico composto de cimento à base de silicato de cálcio com 20% de óxido de zircônio, como o radiopacificador, apresentou baixa citotoxicidade e pode ser explorado como um material promissor para retrobturações.

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