The effect of three different pulp capping cements on mineralization of dental pulp stem cells

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This study evaluated the osteogenic differentiation of human dental pulp stem cells in response to substances released by the pulp capping agents, Biodentine (BD), mineral trioxide aggregate (MTA) and two-paste calcium hydroxide cement (CHC), along with their physicochemical characteristics. The dimensional stability test showed that of the materials studied, only BD met the standards recommended by the International Organization for Standardization (ISO) for pulp capping materials and thus can be used safely. In the chemical tests, BD was the most stable material. In the Alizarin red S test, BD formed the higher amount of mineralized nodules in the mineralizing medium and also formed mineralized nodules in a non-mineralizing medium. BD releases substances that can significantly induce formation of the human dental pulp stem cell-mineralized extracellular matrix, with physicochemical characteristics that are more conducive to pulp repair than those of MTA and CHC.

Keywords: Adult stem cells, Dental pulp capping, Pulp capping and pulpectomy agents

INTRODUCTION

Maintenance of pulp vitality in restorative procedures has become increasingly feasible due to emerging advanced treatments. These techniques use calcium silicate-based pulp capping materials, such as mineral trioxide aggregate (MTA) and Biodentine (BD), which have been highlighted compared to calcium hydroxide-based materials, such as calcium hydroxide cement (CHC).

The knowledge of physicochemical characteristics of pulp capping materials is important for choosing the most appropriate material for each clinical situation, as well as for obtaining a positive biological response from the injured dental pulp. In all pulp capping cases, the clinical objective is to maintain pulp vitality. This depends on the material used and the response of stem or progenitor cells present in the pulp tissue underneath the injured area.

Harada et al.¹ demonstrated the important role that stem cells play in vivo when dental pulp is injured, even if the injury is of low intensity and distant from the pulp tissue. This response includes differentiation and migration of stem cells to the injured dental pulp area. Therefore, determining how these cells respond to capping materials and whether they can differentiate into extracellular matrix-forming cells capable of mineralization is important. Additionally, ascertaining the fundamental physicochemical characteristics of pulp capping materials, such as dimensional stability, pH and calcium ion release, is vital, as they directly affect the pulp repair process²-⁴.

This study evaluated the osteogenic differentiation of human dental pulp stem cells in response to substances released by BD compared with those released from MTA and CHC. The fundamental physicochemical characteristics of pulp capping materials that affect cell differentiation were also analyzed.

MATERIALS AND METHODS

Ethical requirements
This study was approved by the local Human Research Ethics Committee under Protocol CAAE no. 79445117.1.0000.0018.

Experimental groups and Biomaterials preparation
This was an experimental study, conducted exclusively in the laboratory. The physicochemical and biological characteristics of the dental materials proposed for pulp capping were evaluated according to the following experimental groups:
Group 1: BD;
Group 2: MTA;
Group 3: Two-paste CHC.

Biomaterials preparation
The BD (Septodont, Saint-Maur-des-Fossés Cedex, France) was prepared by mixing five drops of liquid with the powder (pre-measured in a capsule) using an amalgamator.

The white MTA (Angelus, Londrina, PR, Brazil) was prepared at the ratio of one part MTA powder to two parts liquid.
drops of sterile distilled water.

The two-paste CHC (Hydeal, Technew, Rio de Janeiro, Brazil) was prepared with an equal ratio of base and catalyst pastes.

Physical test: Dimensional stability

The materials were placed in matrices in a humidifier and oven-dried at 37°C. The initial and final lengths of each specimen were measured with a digital caliper (Mitutoyo, Suzano, São Paulo, Brazil). Samples were stored for 30 days (Fig. 1). The volumes and percentage dimensions of the specimens were calculated, as follows: specimen volume=πhπr² and percentage volume=\[(V_{30}−V)/V\]×100, where h is thickness, π=3.14, r is the radius, V is the initial volume, and V_{30} is the final volume after 30 days.

Chemical tests: pH and calcium ion release

The tested materials were placed in 1-mm-diameter and 1-mm-long polyethylene plastic tubes, with only one side open. After weighing, each specimen was immersed into deionized water and incubated in an oven at 37°C for the experimental times of 3 and 24 h and 3, 7, 14 and 28 days. An empty polyethylene plastic tube was used as control. The pH was measured using a pH meter (Thermo Scientific, South Logan, UT, USA). The calcium ion release analysis was done by using a Vista-MPX CCD simultaneous inductively coupled plasma optical emission spectrometer (ICP-OES; Varian, Mulgrave, Australia, Fig. 2).
**Biological test:** Osteogenic differentiation — Alizarin red S assay (ARS)

1. **Cell culture**
   Aliquots of human dental pulp stem cell line (hDPSC-4) of permanent tooth were used. The clonogenic culture medium comprised alpha-modified minimum Eagle’s medium (α-MEM; Gibco Life Technologies, Grand Island, NY, USA) supplemented with 15% mesenchymal stem cell-qualified fetal bovine serum (MSC-FBS, Gibco); 100 µg/mL streptomycin (Invitrogen/Gibco, Grand Island, NY, USA), 100 U/mL penicillin (Invitrogen/Gibco); 0.1 mM ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA) and 2 mM L-glutamine (Gibco). Cells were maintained in 5% CO₂ and incubated at 37°C.

2. **Conditioned media**
   The culture medium was placed in contact with the materials during 24 h in an incubator with a humid atmosphere at 37°C. For conditioning the culture medium 0.2 g of each experimental biomaterial was placed in contact with 1 mL of clonogenic medium and then, used in a dilution of 10%⁶,⁷. The conditioned media containing all substances released by BD, as well as those released during the MTA and CHC setting were sterilized using a 0.22-µm filter⁸.

3. **Experimental groups of the biological tests**
   Descriptions of the experimental groups subjected to the ARS are presented in Table 1.

4. **Functional differentiation — ARS**
   Mineralized deposit formation was measured using the ARS (Sigma Aldrich). Cells were plated (5×10³ cells per well) in 48-well culture plates. The stem cells received osteogenic differentiation stimulus 24 h after plating. Half of the conditioned medium was removed and replaced with fresh clonogenic or mineralizing medium, depending on the experimental group (Table 1) until 14 days. For the qualitative analysis, images were taken using a digital camera of an inverted phase microscope (Digital Sight DS-U3, DS-Fi 1, Nikon, New York, NY, USA). Quantitative analysis was performed using a spectrophotometer (Synergy H1, BioTek Instruments, Winooski, VT, USA) at a 550 nm absorbance.

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**Statistical analysis**
The data were compared using analysis of variance (ANOVA), complemented with the Tukey’s test (p<0.05). The Pearson’s correlation test was used for pH and calcium release analyses.

## RESULTS

**Physical test: Dimensional stability**
The two-paste CHC lost the greatest volume at 4.58%, which differed significantly from that of MTA and BD (p<0.05), which gained 0.24% and lost 0.58% in volume, respectively, as shown in Fig. 3.

![Fig. 3](image-url) Dimensional stability assessed by means of percent volume (%) of the evaluated materials. The (*) indicates significant differences between the materials (p<0.05).

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### Table 1  Description of the experimental groups by the material and culture medium used for the ARS

| Experimental group | Biomaterial                     | Culture medium                        |
|--------------------|---------------------------------|---------------------------------------|
| Control+           | Positive control (ideal conditions for mineralization) | Fresh mineralizing medium              |
| Control−           | Negative control (ideal culturing conditions)          | Fresh clonogenic medium                |
| CHC CM             | Calcium hydroxide cement        | CHC-conditioned clonogenic medium      |
| CHC MM             | Calcium hydroxide cement        | CHC-conditioned mineralizing medium    |
| MTA CM             | Mineral trioxide aggregate      | MTA-conditioned clonogenic medium      |
| MTA MM             | Mineral trioxide aggregate      | MTA-conditioned mineralizing medium    |
| BD CM              | Biodentine                      | BD-conditioned clonogenic medium       |
| BD MM              | Biodentine                      | BD-conditioned mineralizing medium     |
Chemical tests: pH and calcium ion release

Comparing the pH results for the materials across the different experimental times (Fig. 4-A) revealed significant differences among the materials at the earliest points, i.e., after 3 and 24 h. After 3 h, BD had a pH of 6.94, differentiating it from CHC (p<0.01) and MTA (p<0.05), which had similar pH values. After 24 h, the CHC pH was 8.32, which differed from the MTA (p<0.05) and BD (p<0.01) groups, which had similar pH values. The pH values of the materials did not significantly differ over the longer experimental times of 3, 7, 14 and 28 days. Figure 4-A shows the pH curve for all time periods. Notably, the peak pH values (i.e. the maximum pH values reached by each material) occurred after 7 days. The results showed that the lowest and most neutral pH values were observed in the BD group, while the CHC and MTA groups had similar pH values that were significantly higher than those of the BD group, as shown in Fig. 4-A.

The materials differed significantly in calcium ion release at 3 h and 7 and 14 days. After 3 h, BD released 9.5 ppm of calcium. This was significantly more than CHC and MTA. Over 7 and 14 days, CHC released the most calcium. This significantly differed from MTA and from BD, which were the lowest calcium ion release values, as shown in Fig. 4-B. The calcium ion release of the evaluated groups did not significantly differ at any of the other experimental times — 24 h, 3 and 28 days. Figure 4-B shows the calcium ion release curve. Notably, the peak calcium ion release values, or the maximum calcium ion release that the materials values achieved, were found after 14 days. In the first 3 h, BD showed high calcium ion release, in contrast with CHC and MTA, which showed low calcium ion release. Throughout the experimental period, MTA released the least calcium release. Figures 5-A, -B and -C show the positive

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**Fig. 4** pH and calcium ion release curves at 3, 24 h, 3, 7, 14 and 28 days.
A: pH curves of the evaluated materials B: Calcium ion release curves of the evaluated materials. Different letters indicate significant differences between the materials (p≤0.05).

**Fig. 5** Pearson correlations between pH and calcium ion release among the tested materials.
A: CHC experimental group correlation. B: MTA experimental group correlation. C: BD experimental group correlation. Different letters indicate significant differences between the materials (p≤0.05).
Fig. 6 Representative phase photomicrographs of the Alizarin red S test results. A–H: Phase photomicrographs from all experimental groups. Mineralization nodules are stained red. A, C, E, G: Clonogenic medium; B, D, F, H: Mineralizing medium; A, B: control; C, D: CHC; E, F: MTA and G, H: BD.

Fig. 7 Graphical representation of the amount of mineralization in the Alizarin red S test after 14 days. Different letters indicate significant differences (p<0.05).

DISCUSSION

This study evaluated three pulp capping agents, comparing their physical, chemical and biological characteristics, which are important variables in pulp repair. The BD releases substances that have significant potential for inducing the formation of a human dental pulp stem cell-mineralized extracellular matrix and have physicochemical characteristics that are more conducive to pulp repair than MTA and CHC.

Dimensional stability is an important characteristic in pulp capping cements because adequate sealing is necessary. Changes in cement volume, such as shrinkage, generate losses in the material’s dimension, resulting in no marginal adaptation, thus leading to subsequent bacterial infiltration. The filling material guidelines suggested by ISO 6876 (2002) state that dimensional stability may not exceed a 1% length reduction or a 0.1% material volume expansion.

Our study evaluated the dimensional stability of the materials by analyzing the loss or gain in volume and examining the entire specimen by measuring characteristics such as diameter and thickness. This was not limited to vertical direction, which was a limitation reported in studies using other dimensional stability testing methods.

Our results showed that CHC lost more volume (4.58%) than MTA and BD, which is likely due to high solubility of the CHC in liquid media, as has been widely reported in the literature. MTA gained a volume of 0.24%, which may be related to its tendency to absorb water that has also been described in the literature. BD lost less volume (0.58%). Although no studies have evaluated the dimensional stability of BD, some have compared its marginal infiltration to that of other materials and found favorable results, suggesting that this small volume loss may not affect its marginal adaptation. BD was the only material that met ISO standard requirements, which state that the material may not lose more than 1% or expand by more than 0.1% in volume. Thus, BD is the only pulp capping agent that met these requirements.

Biological tests: Osteogenic differentiation by ARS

Figure 6 presents representative phase photomicrographs of the ARS results of all experimental groups. The amount of mineralized material of each group is graphically represented in Fig. 7. The negative control group (C CM) showed no mineralized nodules. The positive control group (C MM) showed red-stained mineralized nodules. The BD MM group formed the most mineralized nodules (p<0.05). The photomicrographs show more red-stained nodules in the BD group.
capping material that can be used safely relative to its dimensional stability.

The results of the chemical tests performed in this study show that BD had a more neutral pH (slightly basic) and a greater ability to release calcium ions, while MTA released the least amount of calcium. These findings are clinically important, because the literature shows that the bioactivity of pulp capping agents is associated with their pH and ability to release calcium ions. Studies have reported that more calcium ions and slightly basic pH values are associated with increased expression of osteopontin and bone morphogenetic protein (BMP-2) and stimulate the release of proteoglycans, metalloproteinases and growth factors of the mineralized dentin matrix, triggering a signaling cascade, by which undifferentiated pulp stem cells migrate to the injury site, proliferate and differentiate, consequently forming extracellular matrix, which mineralizes leading to pulp regeneration.

The ARS confirmed the chemical tests. The BD group showed the greatest functional differentiation, forming many mineralized nodules. Notably, greater osteogenic differentiation occurred in cultures under mineralizing culture medium, which is more similar to clinical situations, because the material comes in contact with the dentin. The same was not observed when using BD in the clonogenic medium, suggesting that BD releases more calcium ions when in contact with a mineralized medium such as dentin. This is relevant because more calcium ions being available for the pulp stem cells at a neutral pH (slightly basic) promotes differentiation and mineralization, meaning that BD can produce a more structured dentin bridge than that formed by CHC, as demonstrated in previous studies. Studies have shown that calcium silicate-based materials induce forming a homogeneous dentin bridge (non-porous/compact), and pulp responses have almost no inflammation. BD has been found to produce a thicker, more voluminous mineralized extracellular matrix than MTA, possibly due to the physicochemical characteristics of the former. A recent study demonstrated the potential of BD for regenerating the dentin-pulp complex by strongly inducing material mineralization.

Importantly, calcium ion release and pH presented strongly positive correlation. This association was less intense in the BD group, which would have fewer consequences in forming dystrophic calcifications because the pH was more neutral. High pH levels induce dystrophic calcifications in the exposed pulp area at a distance and can obliterate the pulp chamber. This degenerative change to the pulp can reduce the response capacity of dental pulps and can hinder any future endodontic treatment.

Analyzing the pH and calcium ion release curves revealed that peak pH occurs after 7 days, whereas peak calcium ion release occurs after 14 days. This information is clinically valuable, because it can guide treatment time. Fourteen days are required for the pulp capping materials to achieve the greatest calcium ion release for pulp repair.

## CONCLUSION

BD was found to be a safe pulp capping material, with satisfactory physical, chemical and biological characteristics evaluated in vitro. BD releases substances that can significantly induce formation of the human dental pulp stem cell-mineralized extracellular matrix, with physicochemical characteristics that are more conducive to pulp repair than those of MTA and CHC. Clinical studies are needed to confirm its effects when capping human pulp, to consider this material as a replacement for the current capping materials.

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