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# *In vitro* Evaluation of Osteoblasts Exposed to Different Bioceramic Root Canal Sealers

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## Abstract

**Background:** This study aimed to evaluate the cytotoxicity and cell viability of two calcium silicate-based sealers.

**Material and Methods:** Test specimens were made in a silicone matrix and divided into 4 groups (n=3) according to the sealer evaluated: control; Bio-C Sealer; EndoSequence BC and AH Plus. The sealers were weighed, sterilized with ethylene oxide, and cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and 1% antibiotic-antimycotic for 24 h (37±1°C). MTT and alkaline phosphatase (ALP) assays were performed in osteoblast-like SAOS-2 cells (Alizarin red staining). Data were analyzed by ANOVA and Tukey's test (p<0,05).

**Results:** The greater cell vitality was observed in the control group at 24 h and in AH Plus at 48 h. At 72 h, there was no significant difference in MTT assay results between the groups (p>0.05). At 7 days, the control group had the best ALP assay result. At 10 days, the best results were found for the control group, Bio-C Sealer, and AH Plus, with a significant difference from EndoSequence BC (p<0.05).

**Conclusion:** AH Plus sealer had higher levels of cell viability and bioactivity similar to the calcium silicate-based sealers, EndoSequence BC and Bio-C Sealer at 24 and 48 h.

**Keywords:** Alkaline Phosphatase; Cell Survival; Endodontics; Osteoblasts; Root Canal Filling Materials

## Introduction

The endodontic sealers have the function of filling in the irregularities between the dentinal walls and the gutta-percha and the areas of complexity of the root canal [1,2]. Due to the intimate contact with the periapical tissues, the biocompatibility and the tissue response to these materials can influence the final result of the root canal treatment [3,4], being able to help or stimulate the repair of injured tissues [4]. The epoxy resin–based sealer AH Plus (Dentsply DeTrey GmbH, Konstanz, Germany) is the gold standard sealer regarding physical properties (excellent radiopacity, reduced polymerization shrinkage, reduced solubility, adhesion to dentin, and adequate sealing ability). However, it does not have bioactive properties [5] or osteogenic potential [6].

Therefore, in the last years premixed calcium phosphate silicate–based sealer had been constantly developed [7,8].

These materials are known to be non-toxic, with antimicrobial properties, stable and maintain adhesion to dentin walls [9-12]. However, biocompatibility and bioactivity have been the focus of investigations.

EndoSequence BC (Brasseler, Savannah, USA) is a premixed calcium phosphate silicate-based composed of calcium silicates, zirconium oxide, calcium phosphate monobasic, calcium hydroxide, filler, and thickening agents [13]. It has a high rate of calcium ion release, and adequate flow capacity, with an alkaline pH [14].

Another exemple of premixed calcium phosphate silicate–based sealer is the Bio-C Sealer (Angelus, Paraná, Brazil). It is available in a single syringe and consists of calcium silicate, calcium aluminate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide, and dispersing agents [15].

To date, there are no studies that have addressed the cell viability of EndoSequence BC and Bio-C Sealer using immortalized human osteoblast-like SAOS-2 cells. Therefore, the purpose of the present study was to determine the cell viability (by MTT assay) and osteogenic process (by ALP assay) of EndoSequence BC and Bio-C Sealer, using AH Plus as the reference material for comparison. The null hypothesis was that both calcium phosphate silicate–based sealers would show cell viability and osteogenesis similar or inferior to those of AH Plus.

## Material and Methods

For the sample calculation, the G * Power v3.1 for Mac (Heinrich Heine, Universität Düsseldorf, Dusseldorf, Bundesland, Germany) was used and the Wilcoxon-Mann Whitney test from the T test family was selected. Data from a previous study evaluating root canal preparation that used mandibular incisors [11] were used and the effect size in the present study was established (=1.20). An alpha type error of 0.05, a beta power of 0.80 and an N2/N1 ratio of 1 were also stipulated. A total of 3 specimens per group were indicated as the optimal size needed to notice significant differences.

Human osteoblast-like SAOS-2 cells (HTB-85TM) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). To establish and maintain the cultures, the cell culture medium Dulbecco-modified Eagle medium (DMEM) (Gibco, Grand Island, NY) was supplemented with 100 mg/mL penicillin G, 50 mg/mL streptomycin (Gibco), and 10% fetal bovine serum (Gibco). Osteoblast-like SAOS-2 of the second to third passage were used for both assays.

The groups were divided according to the endodontic sealer tested (n=3): Bio-C Sealer (Angelus Indústria de Produtos Odontológicos S/A), EndoSequence BC (Brasseler), AH Plus (Dentsply) (positive control) and untreated SAOS-2 cells (medium only) (negative control).

The sealers were prepared according to the manufacturer instructions and poured into silicone moulds of 3-mm deep, 5-mm width and 14-mm height (PELCO® 21 Cavity EM Embedding Mold; Ted Pella Inc., Redding, Canada). Given the difficulty in completing the setting reaction of the calcium phosphate silicate–based sealers, moistened cotton pellets were placed next to the incubator to increase the ambient humidity and accelerate the setting time of the sealers, thus allowing the study to be performed. Two pieces of wet cloth were placed between the molds and glass plates, and the glass plates were secured by a clamp. The entire assembly was placed in a ziplock container with adequate water to cover the molds and stored in a water bath (37C) for 24 hours. The clamp was then removed, and the assembly remained in the water-filled container for 24 hours.

Once the setting reaction was complete, each test specimen was weighed, sterilized with ethylene oxide, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum and 1% antibiotic-antimycotic for 24 h in an incubator at 37°C, thus obtaining a conditioned medium.

After this period, plated cells (density of 110 cells/mm2) were supplemented with the conditioned medium in the proportion of 0.2 g/mL (ISO 10993) and transferred to a centrifuge tube containing 5 mL of McCoy's 5A medium (Sigma, St. Louis, MO, USA) and centrifuged at 336g for 3 min.

The supernatant was discarded, and the cells were cultured in 25-cm2 culture flasks (Sarstedt, Hildesheim, Germany) containing McCoy's 5A medium (Sigma) supplemented with 15% fetal bovine serum (Nutricell Nutrientes Celulares, Campinas, SP, Brazil), 100 IU/mL of penicillin (Sigma), and 50 µg/mL of streptomycin (Sigma). The culture medium was changed every 2 days, and culture progression was analyzed by phase microscopy using an inverted microscope (Nikon Eclipse TS100, Tokyo, Japan). During the entire culture period, the cells were stored at 37°C in a humidified atmosphere containing 5% CO2 and 95% air.

### Cell Proliferation Assay

To assess cell proliferation, the Trypan blue exclusion test (Sigma, Steinheim, Germany) was performed at 24, 48, and 72 h after the cell cultures were plated under the conditions described above.

After exposure, cells were enzymatically removed from the plates and the cell precipitate resulting from centrifugation was suspended in 1 mL of medium. After

removing 10 µL of the cell suspension, 10 µL of Trypan blue was added (Sigma, Steinheim, Germany) and 1 µL of this solution was placed in a hemocytometer (Neubauer-Fisher Scientific, Pittsburgh, PA, USA) and taken to an inverted phase-contrast microscope (Nikon Eclipse TS100) for cell observation and counting.

The total number of cells in each well at the different assessment time points was obtained using the following mathematical equation:

$$\text{Total number of cells} = \frac{\text{Number of cells counted X Initial Vol. X Dilution X } 10^4}{\text{Number of squares used for counting}}$$

### Cell Viability Assay

Cell cultures were tested for cell viability using the MTT assay. This assay evaluated the ability of metabolically active cells to reduce MTT by converting yellow tetrazolium salts (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals and, therefore, the ability of viable cells to cleave the tetrazolium ring of MTT by the action of dehydrogenase enzymes present in active mitochondria, thus forming formazan crystals.

At 24, 48 and 72 h after plating on the different surfaces of the materials, 10 µL of the MTT solution (5 mg/mL-Sigma, USA) diluted in serum-free DMEM culture medium was added to the treated cultures, which were incubated for 3h at 37°C. Subsequently, 100 µL of 10% dimethyl sulfoxide (DMSO) solution was added.

After crystal solubilization, quantification was performed at 590 nm using an ELX800 microplate reader (Epoch; BioTek Instruments, Inc., Vermont, USA), and optical density was measured.

### Alkaline phosphatase (ALP) activity

At 7 and 14 days, in situ ALP activity was evaluated by Fast Red staining [9,10]. The culture medium was removed, and the wells were rinsed with Hank's solution (Sigma, East Sussex, UK) warmed to 37°C, followed by addition of 1 mL/well of 120 mM Tris buffer (Sigma, Wisconsin, USA), pH 8.4, containing 1.8 mM Fast Red TR (Sigma, Missouri, USA), 0.9 mM naphthol AS-MX phosphate (Sigma, Missouri, USA), and 1:9 dimethyl formamide (Dinâmica, São Paulo, SP, Brazil). Plates were maintained for 30 min in a humidified atmosphere of 5% CO2 and 95% air.

The proportion of Fast Red-positive areas was determined based on macroscopic images of cultures, digitally obtained with a high-resolution camera (Canon EOS Digital Rebel, 6.3 megapixels, with EF 100 mm f/2.8 macro lens) and using Image Tool software (University of Texas Health Science Center, San Antonio, TX, USA), converting them to binary images. Data were presented as the percentage of Fast Red-positive areas.

### Mineralization

At 7 and 14 days after culture, the medium was removed, and the cultures were washed with buffered saline solution and fixed in 70% alcohol for 1h at 4°C. Cultures were stained with 2% Alizarin red (Sigma, Missouri, USA), pH 4.2, for 10 min at room temperature and then washed abundantly with deionized water obtained from a deionizer (Merck, Darmstadt, Germany) to remove excess dye. Plates were left half open to dry. Quantification of mineralization was performed as described by Gregory, et al. [16], 280 µL of 10% acetic acid was added to each well, and the plates were shaken for 30 min at room temperature.

Subsequently, the contents of each plate were transferred to a polypropylene tube heated to 85°C for 10 min and then kept on ice for 5 min. The tubes were centrifuged at 20,000g for 15 min and 100 µL of the supernatant was transferred to a 96-well plate, along with 40 µL of 10% ammonium hydroxide to neutralize the acid. Absorbance was measured at 405 nm using an Epoch spectrophotometer (BioTek Instruments, Inc.). Mineralized matrix formation was expressed as absorbance.

### Statistical Analysis

To verify the normal distribution of the data, the Shapiro-Wilk test was used. One-way analysis of variance (ANOVA) and Tukey's test were used to analyze the data. The level of significance was set at a = 5% (GraphPad Prism, San Diego, United States).

## Results

Table 1 shows the mean and standard deviation of cell viability and proliferation. At 24 hours, the control showed greater cell vitality compared to EndoSequence BC ($p<0.05$) while the AHPlus showed greater cell proliferation compared to the other groups ($p<0.05$). At 48 hours, AHPlus showed greater cell proliferation compared to the calcium phosphate silicate–based sealers ($p>0.05$) and greater viability compared to Endossequence BC ($p<0.05$). At 72 hours, the control showed greater cell proliferation compared to the other groups ($p>0.05$), while no difference was observed between groups in the cell vitality analysis ($p>0.05$).

| Period | Control | AH Plus | EndoSequence BC | Bio C Sealer |
|---|---|---|---|---|
| 24 h | 0.51 x $10^4$ (±0.03x$10^4$)$^{abA}$ | 0.57 x$10^4$ | 0.44 x$10^4$ | 0.48 x$10^4$ |
| | | (±0.06 x$10^4$)$^{aA}$ | (±0.02 x$10^4$)$^{bA}$ | (±0.03 x$10^4$) $^{abA}$ |
| 48 h | 0.63 x$10^4$ | 0.73 x$10^4$ | 0.53 x$10^4$ | 0.61 x$10^4$ |
| | (±0.04 x$10^4$)$^{abB}$ | (±0.02 x$10^4$) $^{aB}$ | (±0.03 x$10^4$)$^{bB}$ | (±0.06 x$10^4$)$^{bB}$ |
| 72 h | 0.84 | 0.73 x$10^4$ | 0.66 x$10^4$ | 0.69 x$10^4$ |
| | (±0.03 x$10^4$)$^{aC}$ | (±0.03 x$10^4$)$^{aB}$ | (±0.04 x$10^4$)$^{bC}$ | (±0.03 x$10^4$)$^{bB}$ |

Lowercase letters indicate statistical difference between groups for the same period. Upper letters indicate statistical differences between periods for the same group (p<0,05).

**Table 1:** Means and standard deviations of cellular proliferation of Bio-C Sealer, EndoSequence BC and AH Plus sealers.

Table 2 shows the mean and standard deviation of mineralization tests. Regarding the ALP test (Fast Red staining), at 7 days, the control showed a significant difference compared to EndoSequence BC (p<0.05) and at 10 days, the Bio-C Sealer and AH Plus groups showed a significant difference compared to EndoSequence BC (p<0.05).

| Period | Control | AH Plus | EndoSequence BC | Bio C Sealer |
|---|---|---|---|---|
| 24 h | 0.76 x $10^4$ (±0.02x$10^4$)$^{aA}$ | 0.58 x$10^4$ | 0.50 x$10^4$ | 0.51 x$10^4$ |
| | | (±0.03 x$10^4$)$^{abA}$ | (±0.11 x$10^4$)$^{bA}$ | (±0.15 x$10^4$) $^{abA}$ |
| 48 h | 0.79 x$10^4$ | 1.02 x$10^4$ | 0.53 x$10^4$ | 0.82 x$10^4$ |
| | (±0.07 x$10^4$)$^{abA}$ | (±0.10 x$10^4$) $^{aB}$ | (±0.01 x$10^4$)$^{bA}$ | (±0.16 x$10^4$)$^{abA}$ |
| 72 h | 0.83 | 0.64 x$10^4$ | 0.59 x$10^4$ | 0.74 x$10^4$ |
| | (±0.11 x$10^4$)$^{aA}$ | (±0.04 x$10^4$)$^{aA}$ | (±0.14 x$10^4$)$^{aA}$ | (±0.08 x$10^4$)$^{aA}$ |

Lowercase letters indicate statistical difference between groups for the same period. Upper letters indicate statistical differences between periods for the same group (p<0,05).

**Table 2:** Means and standard deviations of the MTT test for Bio-C Sealer, En doSequence BC and AH Plus sealers.

The Fast Red and Alizarin Red data are presented in table 3. After 7 days, the best result in the Alkaline Phosphatase test (Fast Red) was for the control with a significant difference in relation to EndoSequence BC (p<0.05). After 10 days, the best results were for the control, Bio-C Sealer and AH Plus with a significant difference compared to EndoSequence BC (p<0.05) Table 3.

| Period | Control | AH Plus | EndoSequence BC | Bio C Sealer |
|---|---|---|---|---|
| 7 days | 0.31 x $10^4$ (±0.07x$10^4$)$^{aA}$ | 0.18 x$10^4$ | 0.14 x$10^4$ | 0.23 x$10^4$ |
| | | (±0.01 x$10^4$)$^{abA}$ | (±0.03 x$10^4$)$^{bA}$ | (±0.07 x$10^4$) $^{abA}$ |
| 10 days | 0.39 x$10^4$ | 0.35 x$10^4$ | 0.24 x$10^4$ | 0.38 x$10^4$ |
| | (±0.01 x$10^4$)$^{aB}$ | (±0.02 x$10^4$) $^{aB}$ | (±0.03 x$10^4$)$^{bB}$ | (±0.02 x$10^4$)$^{aB}$ |

Lowercase letters indicate statistical difference between groups for the same period. Upper letters indicate statistical differences between periods for the same group (p<0,05).

**Table 3:** Means and standard deviations of the Fast red test for Bio-C Sealer, EndoSequence BC and AH Plus sealers.

Regarding the Alizarin red assay, Bio-C Sealer and EndoSequence BC groups showed the highest Alizarin red staining compared to AH Plus and control at 7 days. At 14 days, there was no significant difference between groups (p>0.05) Table 4.

| Period | Control | AH Plus | EndoSequence BC | Bio C Sealer |
|---|---|---|---|---|
| 7 days | 0.14 x $10^4$ (±0.04x$10^4$)$^{aA}$ | 0.12 x$10^4$ | 0.34 x$10^4$ | 0.41 x$10^4$ |
| | | (±0.03 x$10^4$)$^{aA}$ | (±0.02 x$10^4$)$^{bA}$ | (±0.04 x$10^4$) $^{bA}$ |
| 14 days | 0.07 x$10^4$ | 0.08 x$10^4$ | 0.08 x$10^4$ | 0.07 x$10^4$ |
| | (±0.01 x$10^4$)$^{aB}$ | (±0.01 x$10^4$) $^{aA}$ | (±0.01 x$10^4$)$^{aB}$ | (±0.01 x$10^4$)$^{aB}$ |

Lowercase letters indicate statistical difference between groups for the same period. Upper letters indicate statistical differences between periods for the same group (p<0,05).

**Table 4:** Means and standard deviations of the Alizarin red assay for Bio-C Sealer, EndoSequence BC and AH Plus sealers.

## Discussion

This study aimed to evaluate the cytotoxicity and osteogenic capacity of endodontic cements EndoSequence BC and Bio-C Sealer. Based on the results, the null hypothesis was accepted, as AHPlus demonstrated greater cell viability and bioactivity similar to calcium phosphate silicate–based sealers.

Biocompatibility and bioactivity are important properties for endodontic sealers, as they may contact periapical tissues and affect repair. In this context, a biocompatible sealer, in addition to promoting tissue repair, should preferably stimulate the reorganization of damaged structures [16].

Therefore, in vitro cytotoxicity tests are important to better understand the biological risks associated with these materials [17,18]. Several methods are available to assess cell viability or proliferation after direct or indirect exposure to materials, including tetrazolium salt-based assays that are widely used alone or in combination with other assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is the most commonly used tetrazolium compound, and the MTT reduction assay is considered the gold standard for cytotoxicity testing, in addition to having good agreement with other cell viability assays [19].

In the present study, AH Plus showed greater cell viability in the first 24 h than the other sealers, while the results of the MTT assay favored the untreated control group. This result can be explained by the release of calcium ions by calcium phosphate silicate-based may increase its intracellular concentration [20], which had implications in most aspects of cell physiology and has been associated with cell death regulation [21-23].

At 48 h, AH Plus showed higher cell viability than the control group, whereas EndoSequence BC showed the lowest cell viability at the same time point. This result differs from previous studies Rodriguez Lozano FJ [24] demonstrated that the tested calcium phosphate silicate–based sealers were superior to AHPlus in terms of cell viability. However, the determination of cell viability depends on the physical and biochemical properties of cells. Cell viability depends on the type of material, culture medium, and incubation time to which the cells are exposed. [24].

Both tested studies were performed on different cell types (human gingival fibroblast and human periodontal ligament stem cell), which may have contributed to the different results. Thus, works that evaluated cell viability in osteoblast precursor cells and pulp stem cells showed similar results to the present study [13].

At 72 h, increased cell viability was observed for EndoSequence BC, which is consistent with the study by Lopez-Garcia, et al. [25], who achieved significance for this result. In the present study, however, there were no significant differences between the tested sealers at 72 h. This may indicate initial differences in the use of these materials for cell viability, but similar performance in the long term, which was also demonstrated in the studies by De-Deus, et al. [26] and Nair, et al. [27] when comparing calcium phosphate silicate–based sealers with other materials.

Another aspect evaluated in this study was the bioactivity, which corresponds to the ability of a material to bind to tissue [28]. In this study, the Alizarin red assay were evaluated to evaluate the bioactivity potential of these hydraulic materials, which is influenced by its composition [29].

At 7 days, the bioceramic cements showed greater mineralization than AH Plus and the control, which is in agreement with previous studies [24,30]. This result may be related to the release of calcium by calcium phosphate silicate–based sealers [31], increasing the pH of the medium and possibly regulating alkaline phosphatase activity and increasing mineralization [32]. However, at 14 days the results were similar for all groups, but slightly higher for AH Plus and EndoSequence BC. The low solubility of AHPlus may have resulted in less exposure of cells to the sealer, contributing to the reduction of cytotoxicity, as previously reported [33,34].

Added to this, alkaline pH can contribute to osteogenic potential, biocompatibility and antibacterial activity, therefore, endodontic sealers can contribute to hard tissue formation by activating ALP, which requires an alkaline pH [12,34]. However, it should be noted that high pH during sealer setting can damage adjacent tissues, thereby affecting cell viability with reactions similar to chemical burns [35]. In this regard, previous studies have shown that EndoSequence BC and Bio-C Sealer have a more alkaline pH than AH Plus [35,36]. This factor may be associated with the superior results of AH Plus in the present study, since the setting time of calcium phosphate silicate–based sealers is longer, maintaining the alkaline pH for a longer time.

This study has limitations related to its laboratory nature and the fact that it was restricted to only one dilution. However, in the absence of in vivo studies, in vitro laboratory study is important. Given the above, caution and common sense are needed when extrapolating the results to the clinic.

## Conclusion

Based on the methodology and results presented, it is possible to conclude that the AH Plus sealer showed higher levels of cell viability than the calcium phosphate silicate–based sealers, EndoSequence BC and Bio-C Sealer, for 24 and 48h and bioactivity similar to that of the calcium phosphate silicate–based sealers in immortalized human odontoblast.

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