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# GuttaFlow Bioseal promotes spontaneous differentiation of human periodontal ligament stem cells into cementoblast-like cells

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## ABSTRACT

**Objectives.** To evaluate *in vitro* the cementogenic potential and the biological effects of GuttaFlow Bioseal, GuttaFlow 2, MTA Fillapex and AH Plus on human periodontal ligament stem cells (hPDLSCs).

**Methods.** Cell viability, cell migration and cell morphology assays were performed using eluates of each material. To evaluate cell attachment, hPDLSCs were directly seeded onto the material surfaces and analyzed by scanning electron microscopy (SEM). The effects of endodontic sealers on cementum protein 1 (CEMP1), cementum-derived attachment protein (CAP), bone sialoprotein (BSP), ameloblastin (AMBN), amelogenin (AMELX) and alkaline phosphatase (ALP) gene expression on hPDLSCs were investigated by qPCR and immunofluorescence (IF). Statistical analysis was performed with analysis of variance and Bonferroni or Tukey post-test ( $\alpha < 0.05$ ).

**Results.** More than 90% of viable cells were obtained using extracts of GuttaFlow Bioseal and GuttaFlow2 after 72 h of culture. By contrast, AH Plus and MTA Fillapex induced significantly lower levels of cell viability. GuttaFlow2 and GuttaFlow Bioseal promoted wound closure in a concentration-dependent manner, comparable to that observed with control extracts ( $*p < 0.05$ ). However, with AH Plus and MTA Fillapex, cell migration was significantly lower than in the control ( $***p < 0.0001$ ). SEM analysis pointed to an organized stress fiber assembly and high degree of cell adhesion on GuttaFlow Bioseal disks but low rates on GuttaFlow2, MTA Fillapex and AH Plus. When hPDLSCs were cultured with GuttaFlow Bioseal-conditioned media, qPCR assays and IF showed a higher level of AMELX, AMBN, CEMP1 and CAP expression than the control ( $*p < 0.05$ ), whereas no such expression was observed in the other sealers.

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*Significance.* Our results showed that GuttaFlow sealers were more cytocompatible than AH Plus and MTA Fillapex, while GuttaFlow Bioseal favored cementoblast differentiation of hPDLSCs in the absence of any growth factors.

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## 1. Introduction

Bioactive-based materials have recently been introduced as root repair cements and root canal sealers [1–3]. These bioactive materials represent a new concept in root filling and have the ability to release calcium ions and calcium hydroxide, and to form an interfacial layer between the cement and dentinal wall. They can also lead to the formation of apatite crystals over the surface of the material in a synthetic tissue fluid environment such as phosphate buffer saline [2]. These materials often interact with periapical tissues and may allow, or even stimulate, the deposition of cementum, producing a biological seal and inducing the healing process [4].

Importantly, periapical tissues contain a variety of mesenchymal stem cells (MSCs), which have shown potent anti-inflammatory and immunomodulatory properties and the ability to differentiate into specialized cell lineages including osteoblasts, cementoblasts, adipocytes, and chondrocytes using different inductive culture media [5,6]. These cells have a demonstrated ability to form alveolar bone, cementum, gingiva, periodontal ligaments, peripheral nerves, and blood vessels *in vivo* [7]. Due to the presence of periodontal ligament stem cells in the periapical tissues, these cells have previously been used for a variety of *in vitro* studies into the bioactivity of different endodontic sealers [8,9].

Previous reports point to new cementum formation adjacent to dental materials when placed in contact with periodontal tissues. These materials include mineral trioxide aggregate (MTA) [1,10,11], calcium silicate-based cements [12], and hydroxyapatite [13]. However, there is no specific information on the cementogenic potential of calcium silicate-based sealers on mesenchymal progenitor cells during the repair of periodontal ligament and surrounding tissue, and the molecular mechanisms underlying the cementogenesis induced by various dental materials are unclear [11].

Calcium silicate-based sealers are new root canal sealers that have demonstrated good biocompatibility, anti-microbial activity and apatite forming ability [14–16]. MTA Fillapex, (Angelus Industria de Produtos Odontologicos S/A, Londrina, PR, Brazil), is a two-paste material containing tricalcium silicate particles (MTA), silicon dioxide and bismuth oxide [14]. Although it shows good physicochemical properties, MTA Fillapex appears to be even more cytotoxic than the epoxy resin-based sealer AH Plus [17]. On the other hand, GuttaFlow Bioseal, (Coltene/Whaledent AG, Altstatten, Switzerland), an evolution of its predecessor GuttaFlow2, is a polydimethylsiloxane with gutta-percha powder combined with calcium silicate particles that was launched in late 2015. While previous reports have demonstrated the good biological and physicochemical properties of GuttaFlow Bioseal [18–20], to

date, no studies have been conducted to analyze its cementogenic potential.

This study was designed to assess and compare the biological effects and cementogenic potential of different endodontic sealers in contact with human periodontal ligament stem cells (hPDLSCs): MTA-based endodontic sealer (MTA Fillapex) and the two novel silicone-based sealers GuttaFlow2 and GuttaFlow Bioseal. A commonly used root canal sealer, AH Plus (Maillefer Dentsply, Ballaigues, Switzerland), was used as the reference material.

## 2. Materials and methods

### 2.1. Endodontic sealer extracts

The materials tested were GuttaFlow Bioseal (Coltene/Whaledent AG, Altstatten, Switzerland), GuttaFlow2 (Coltene/Whaledent AG, Altstatten, Switzerland), MTA Fillapex (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil) and AH Plus (Dentsply DeTrey GmbH, Konstanz, Germany).

The materials were mixed according to the manufacturers' instructions. Discs of each endodontic sealer were shaped under aseptic conditions in sterile cylindrical rubber molds 5-mm in diameter and a 2-mm high, sterilized using ultraviolet irradiation for 15 min and stored in an incubator at 37 °C for 48 h to achieve complete setting. The eluates of the different materials were extracted in sterile conditions, using DMEM culture medium as extraction vehicle. The extraction procedure was as follows: the materials were stored in the culture medium for 24 h at 37 °C in a humid atmosphere containing 5% CO<sub>2</sub>. The ratio of material surface area to medium volume was set at approximately 1.5 cm<sup>2</sup>/mL in accordance with the guidelines of the International Organization for Standardization 10993–5. The extraction medium was filtered with sterile filters of 0.22 μm diameter pores, and several dilutions (undiluted, 1/2, 1/4) were prepared.

### 2.2. Isolation, culture and characterization of hPDLSCs

hPDLSCs were isolated and characterized as described previously [16,21]. Human periodontal ligaments (hPDL) were obtained from impacted third molars from ten healthy subjects. Donors gave written informed consent according to the guidelines of the Ethics Committee of our Institution. The hPDL was scraped from the middle third region of the root surface. After extraction, hPDL was washed with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hank's balance salt solution (Gibco, Gaithersburg, MD, USA), and subjected to collagenase-A digestion (3 mg/mL) (Sigma–Aldrich, St. Louis, MO) for 1 h at 37 °C. Then, were cells seeded in 75-cm<sup>2</sup> culture flasks (Corning, New York, USA)

containing alpha minimum essential ( $\alpha$ -MEM) medium supplemented with 100 U/mL penicillin and streptomycin; and 10% foetal bovine serum (FBS) in an incubator at 37 °C and 5% CO<sub>2</sub>. This study was carried out using cells from passage 4 onward. To analyze cell surface antigen expression, 10<sup>5</sup> cells per well were seeded on 6-well plates, and 24 h were allowed for adhesion. After 72 h, cells were harvested and identified following the guidelines of the International Society of Cellular Therapy (ISCT) to confirm their stem cell mesenchymal phenotype [22]. Surface antigens of hPDLSCs were revealed by flow cytometry using specific fluorescence-conjugated antibodies. The antibodies used were CD73-APC (clone AD2), CD90-FITC (clone DG3), CD105-PE (clone 43A4E1), CD14-PerCP (clone TÜK-4), CD20-PerCP (clone LT20.B4), CD34-PerCP (clone AC136) and CD45-PerCP (clone 5B1) (Human MSC Phenotyping Cocktail, Miltenyi Biotec, Bergisch Gladbach, Germany). The antibodies were diluted following the manufacturer's specifications (10  $\mu$ L of the Human MSC Phenotyping Cocktail per 10<sup>6</sup> cells). Flow cytometry analysis was performed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA). Also, to evaluate the *in vitro* trilineage mesenchymal differentiation, hPDLSCs were differentiated toward the adipogenic, osteogenic and chondrogenic mesodermal lineages. Adipogenic differentiation was accomplished by culturing cells in StemMACS AdipoDiff media (Miltenyi. Biotec GmbH, Bergisch Gladbach, Germany) for 21 days. After, hPDLSCs were fixed with 4% paraformaldehyde (PFA) in PBS and stained with Oil Red O solution (Sigma-Aldrich, St. Louis, MO, USA) to detect accumulation of intracellular lipid vacuoles. To differentiate hPDLSCs to the osteogenic lineage, cells were cultured in StemMACS OsteoDiff media (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) for 21 days. After, cells were fixed with 4% PFA in PBS and stained with Alizarin Red (Sigma-Aldrich, St. Louis, MO, USA) to detect calcium deposits. Finally, chondrogenic differentiation was stimulated by culturing hPDLSCs in StemMACS CondroDiff media (Miltenyi. Biotec GmbH, Bergisch Gladbach, Germany) for 21 days. After, cells were fixed with 4% PFA in PBS to visualize mucopolysaccharides and acidic mucins by Alcian Blue staining (Sigma-Aldrich, St. Louis, MO, USA).

### 2.3. Cell viability

hPDLSCs were cultured in the presence of the different eluates for 72 h, followed by double staining with FITC-conjugated Annexin-V and 7-AAD (Immunostep, Salamanca, Spain). Percentages of live (Annexin-V<sup>-</sup>/7-AAD<sup>-</sup>), early apoptotic (Annexin-V<sup>+</sup>/7-AAD<sup>-</sup>) or late apoptotic and necrotic (Annexin-V<sup>+</sup>/7-AAD<sup>+</sup> and Annexin-V<sup>-</sup>/7-AAD<sup>+</sup>) cells were determined by flow cytometry. Subsequently, the percentages of each population were calculated.

### 2.4. Cell migration

The capability of cells to migrate in the presence of the distinct endodontic sealer extracts was evaluated by means of wound-healing assays. Cells were seeded in 60-mm diameter culture plates at a density of 5  $\times$  10<sup>4</sup> hPDLSCs per well in complete medium with FBS and subsequently incubated for 24 h to ensure a confluent monolayer of cells. To perform the

wound-healing assay, a scratch was made in each well using a sterile pipette tip. Then, the cell monolayers were washed with PBS to remove cell debris, and incubated after the addition of eluates of each material at various dilutions. To quantify and compare the cell migration rates, images of the scratched area were captured at times 0, 24 and 48 h using a phase-contrast microscope. Migration assays were carried out five times. Two images were taken per well at each indicated time. Image J software (NIH, Bethesda, Maryland, USA) was used to analyze the area covered and not covered by cells.

### 2.5. Cell morphology in presence of eluates

To investigate morphological changes, hPDLSCs were seeded directly on glass coverslips at a low density and cultured in culture medium containing the eluates of the different materials. Then, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.25% Triton-X-100 (Sigma-Aldrich, St. Louis, MO, USA). To stain the F-actin cytoskeleton and nuclei, cells were incubated with CruzFluor594-conjugated phalloidin (Santa Cruz Biotechnology, Dallas, TX, USA) and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich), respectively. Confocal microscopy (Zeiss, Oberkochen, Germany) was used for visualization of the staining.

### 2.6. Scanning electronic microscopy (SEM)

Different samples of AH Plus, MTA Fillapex, GuttaFlow2 and GuttaFlow Bioseal were shaped into 1.6-mm thick disks of 5 mm diameter using rubber molds. Fifteen disks of each material were prepared and subdivided into 3 groups, each containing 5 parallel samples. The hPDLSCs were directly seeded onto each disk at a density of 5  $\times$  10<sup>4</sup> cells/mL. After 72 h of culture, the samples seeded with hPDLSCs were removed from the culture wells, and fixed with 3% glutaraldehyde for 4 h. Then, the samples were dehydrated in a graded series of ethanol concentrations up to 100%, immersed in 100% hexamethyldisilazane, air dried, mounted on aluminum stubs, and sputter coated with gold/palladium (Bio-RADPolaron e5400 SEM Sputter Coating System, Kennett Square, PA, USA). Finally, gold/palladium-coated specimens were examined by SEM, using a magnification of 100 $\times$ .

### 2.7. qPCR analysis

The qPCR study was performed to analyze changes in the expression of cementoblastic/osteoblastic-related genes after exposure to endodontic sealers for 7 days. Importantly, too, a negative control (without extracts) and a positive control for osteogenic differentiation using Human OsteoDiff Medium; Miltenyi Biotec) were carried out. Total RNA was isolated using a RNeasy Mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 1  $\mu$ g of total RNA by using an iScript<sup>TM</sup> Reverse Transcription Supermix for RT-qPCR (Biorad). Real-time PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems) using SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> (Takara, Clonteh). A 20  $\mu$ L reaction was set up?? with the following PCR conditions: (cDNA synthesis) 5 denaturation steps/cycles

at 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and finally 95 °C for 1 min, 60 °C–95 °C for 10 min. Primer sequence for human genes encoding cementum protein 1 (CEMP1), cementum-derived attachment protein (CAP), alkaline phosphatase (ALP), bone sialoprotein (BSP), amelogenin (AMELX), ameloblastin (AMBN) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were as follows (forward/reverse): CEMP1 (5'-GGGCACATCAAGCACTGACAG-3'/5'-CCCTTAGGAAGTGGCTGTCCAG-3'); CAP (5'-TTTTTCTGGTCGCGTGGACT-3'/5'-TCACCAGCAACTCCAACAGG-3'); ALP (5'-TCAGAGCTCAACACCAACG-3'/5'-TTGTACGTCTTGGAGAGGGC-3'); BSP (5'-TGCCTTGAGCCTGCTTCCT-3'/5'-CTGAGCAAAATTAAGCAGTCTTCA-3'); AMELX (5'-CACCTGCAGCCTCATCACC-3'/5'-GTGTTGGATTGGAGTCATGG-3'); AMBN (5'-AGCCATGTTCCAGGATTTG-3'/5'-TGCACCTCCTTCTTCGTTCT-3'); -GAPDH (5'-TCAGCAATGCCTCCTGCAC-3'/5'-TCTGGGTGGCAGTGATGG-3').

All experiments were performed in triplicate and expression levels of the abovementioned molecules were obtained using delta-delta ct method, normalizing for GAPDH.

## 2.8. Immunocytofluorescence analysis

Protein expression of CEMP1 and CAP were evaluated after 7 days of treatment with endodontic sealers. Also, a negative control (without extracts) and a positive control for osteogenic differentiation using Human OsteoDiff Medium; Miltenyi Biotec) were carried out. Cultured cells were plated at  $1 \times 10^5$  cells/well in fourwell chamber slides. Then, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.25% Triton-X-100 (Sigma–Aldrich, St. Louis,

MO, USA). Cultures were incubated with the following primary antibodies at 4 °C overnight: rabbit polyclonal anti-CAP (1:100; LifeSpan Biosciences, Seattle, WA) and rabbit polyclonal anti-CEMP1 (1:100; Abcam, Cambridge, United Kingdom). After washing with PBS, the samples were incubated with the following secondary antibodies for 30 min at RT in the dark: Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (both diluted 1:1,000; Thermo Fisher Scientific). The samples were washed with PBS thrice for 5 min each and mounted with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Images were obtained by using a confocal laser-scanning microscope (LSM-510; Carl Zeiss, Oberkochen, Germany).

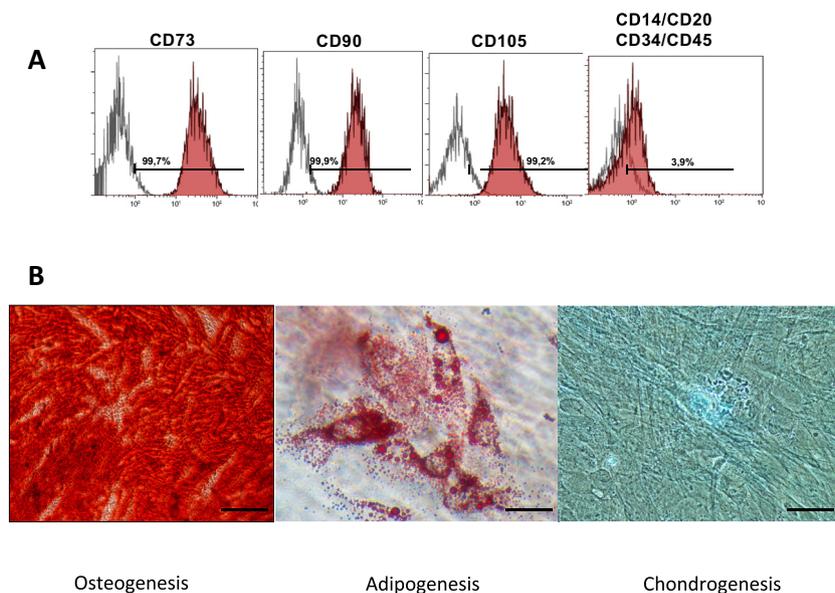
## 2.9. Statistical analysis

Data from the cell migration and PCR assays were analyzed using SPSS version 22.0 statistical software (SPSS, Inc., Chicago, IL, USA). Each experiment was performed with five replicates and carried out at least twice. Statistical differences between the control and cell migration in the presence of different material extracts were analyzed. Also, statistical differences between the migration rates of different extracts were analyzed. In both cases, analyses were assessed by ANOVA and Tukey's test ( $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ). A  $p$  value  $< 0.05$  was considered significant.

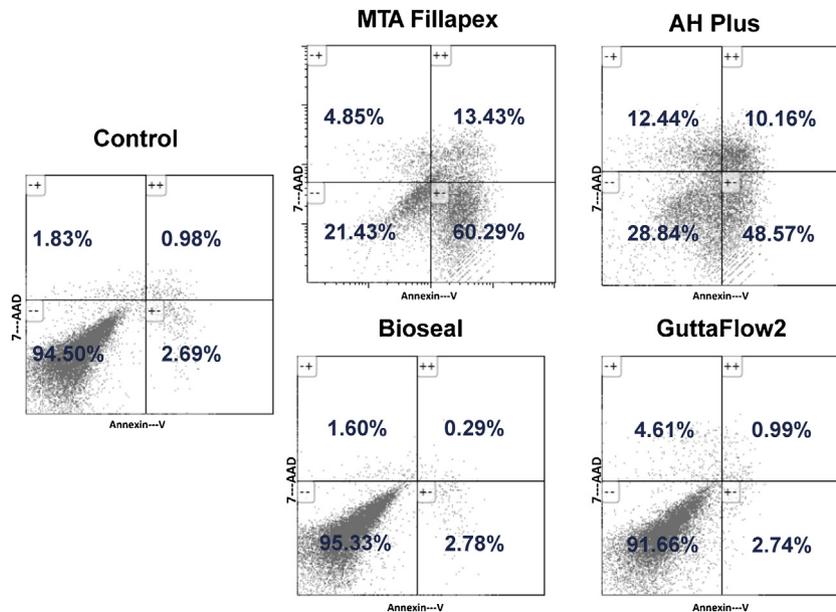
## 3. Results

### 3.1. Isolation and characterization of hPDLSCs

After isolation and culture of hPDLSCs, cell surface marker expression was analyzed by flow cytometry. As reported pre-



**Fig. 1 – Mesenchymal phenotype analysis of hPDLSCs by flow cytometry (Fig. 1A). Histograms display fluorescence intensity as measurement parameter on the x-axis and the cell count on the y-axis. The number in the FACS graph corresponds to the average of MFI (mean fluorescence intensity) for the marker analyzed. (B) Multilineage differentiation potential toward the adipogenic, chondrogenic and osteogenic lineages is shown.**



**Fig. 2** – hPDLSCs were cultured with endodontic sealer extracts and plastic (control) for 72 h, labeled with Annexin-V and 7-AAD, and analysed using flow cytometry. Numbers within the different quadrants represent the percentages of live (Annexin-V<sup>-</sup>/7-AAD<sup>-</sup>), early apoptotic (Annexin-V<sup>+</sup>/7-AAD<sup>-</sup>) or late apoptotic and necrotic (Annexin-V<sup>+</sup>/7-AAD<sup>+</sup> and Annexin-V<sup>-</sup>/7-AAD<sup>+</sup>) cells. Dot-plots display representative flow cytometry results from three independent experiments.

viously, more than 95% of viable hPDLSCs showed a positive expression of the mesenchymal markers, CD73, CD90 and CD105, and a lack of expression of the hematopoietic markers CD14, CD20, CD34 and CD45 (Fig. 1A). hPDLSCs also showed multipotent properties including adipogenic, chondrogenic and osteogenic differentiation potentials (Fig. 1B).

### 3.2. Determination of endodontic sealer-induced cytotoxicity

Representative 2-dimensional dot plots of the distribution of live (Annexin-V<sup>-</sup>/7-AAD<sup>-</sup>), early apoptotic (Annexin-V<sup>+</sup>/7-AAD<sup>-</sup>), or late apoptotic and necrotic (Annexin-V<sup>+</sup>/7-AAD<sup>+</sup> and Annexin-V<sup>-</sup>/7-AAD<sup>+</sup>) cells in untreated hPDLSCs, or exposed to different eluates of the endodontic sealers, are shown in Fig. 2. Similar to the cells cultured in the absence of eluates (Control), more than 90% of viable cells were obtained using undiluted extracts of GuttaFlow Bioseal and GuttaFlow2 after 72 h of culture. By contrast, AH Plus and MTA Fillapex induced a significant decrease of cell viability compared to control cells (28.84% and 21.43% of living cells, respectively).

### 3.3. Cell migration

Cell monolayers were wounded by a scraper and allowed to heal in the presence or absence of endodontic sealer extracts. Treatment with the different dilutions of Guttaflow2 and GuttaFlow Bioseal eluates for 24 h promoted a cell migration level similar to that observed using complete medium (control). As shown in Fig. 3, treatment with extracts of GuttaFlow2 and GuttaFlow Bioseal promoted wound closure in a concentration-dependent manner, comparable to that observed in control extracts ( $p < 0.05$ ). However, after 48 h of

incubation with AH Plus and MTA Fillapex, cell migration was significantly lower than in the control ( $**p < 0.0001$ ).

### 3.4. Cell morphology

Cell adhesion and morphology were analyzed by staining hPDLSCs with phalloidin (red fluorescence) and DAPI (blue fluorescence) to visualize the actin cytoskeleton and cell nuclei, respectively. In the control group, hPDLSCs showed a gradual increase in growth over time, an extended morphology and a high content of F-actin, reaching confluence after 72 h of culture (Fig. 4A). Importantly, the cells treated with the undiluted extracts of GuttaFlow Bioseal and GuttaFlow 2 exhibited a similar organized and stretched stress fiber assembly. However, the cells treated with extracts of MTA-Fillapex and AH Plus showed a reduction in cell numbers and a very low stretched stress fiber assembly.

### 3.5. Cell attachment to the materials

The scanning electron micrographs of the cells in direct contact with the materials are shown in Fig. 4B. After 72 h of culture, hPDLSCs showed a suitable degree of attachment and merged with each other to reach subconfluence in GuttaFlow Bioseal and a moderate level of the same in GuttaFlow2. In contrast, when using AH Plus and MTA Fillapex, cell attachment was limited, with abundant round cells appearing on the surface of the material (scale bar: 50  $\mu$ m).

### 3.6. qPCR analysis

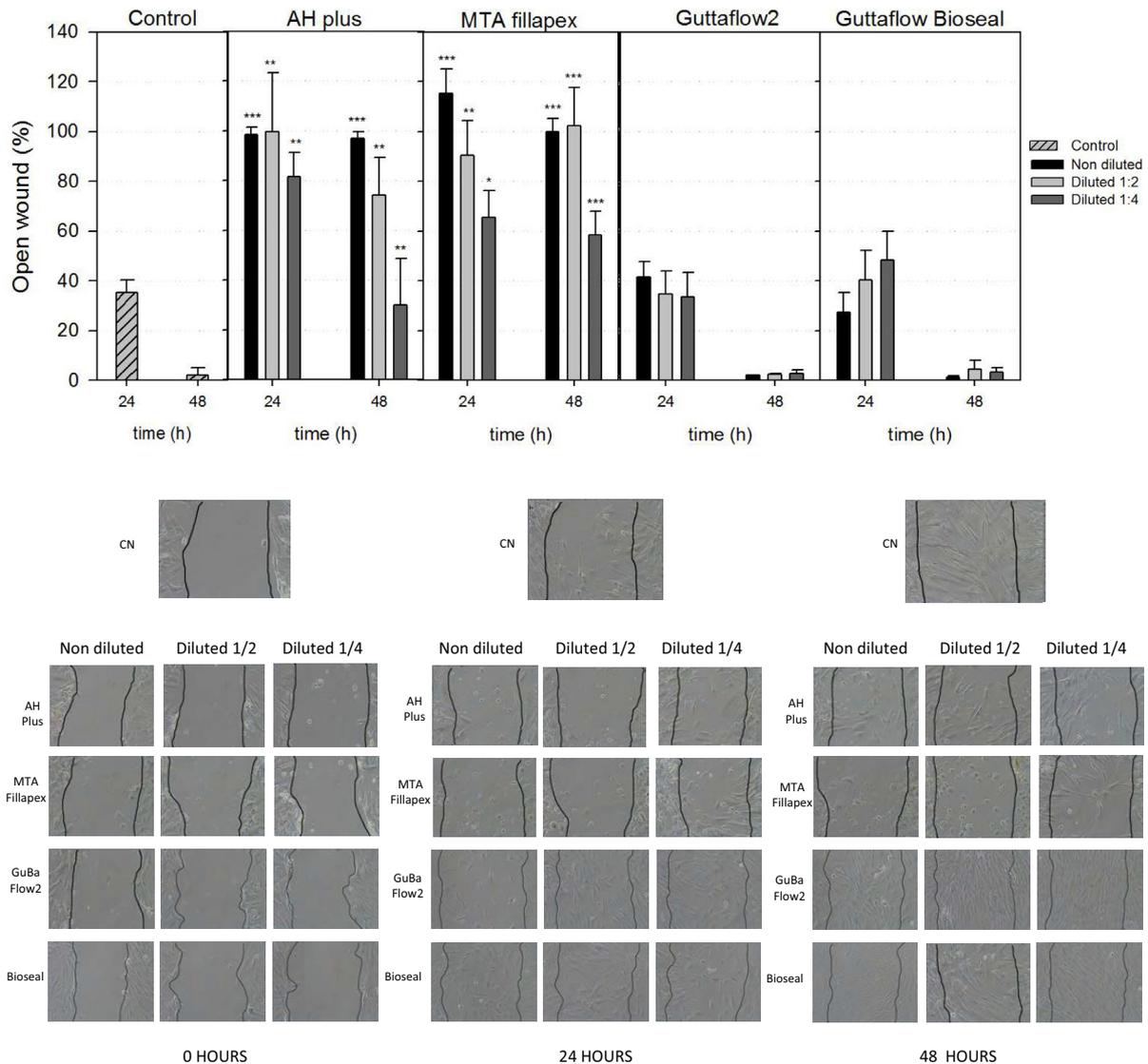
After 7 days of culture, cementogenic genes (CEMP-1, CAP) and ALP expression in cells grown in the presence of the different materials were analyzed using quantitative real time

PCR (qRT-PCR) (Fig. 5). Since the cells died in the presence of MTA Fillapex and AH Plus (see previous experiments) gene expression in these extracts was not analyzed. GAPDH was used as a housekeeping gene to normalize the qPCR results. The expression of ALP was seen to be significantly upregulated when hPDLSCs were exposed to Osteodiff medium (positive control) ( $*p < 0.05$ ). However there was no significant difference in ALP expression between the untreated hPDLSCs (control) and cells exposed to GuttaFlow Bioseal ( $p = 0.536$ ;  $p = 0.485$ , respectively). In a CAP pairwise comparison with the control, the results showed that CAP expression was significantly upregulated after exposure to GuttaFlow Bioseal and Osteodiff (both  $**p < 0.01$ ), although there was no significant difference between untreated hPDLSCs and cells exposed to GuttaFlow2 ( $p = 0.559$ ). Moreover, CEMP1 expression was significantly upregulated when hPDLSCs were exposed to GuttaFlow Bioseal ( $*p < 0.05$ ), whereas there was no significant

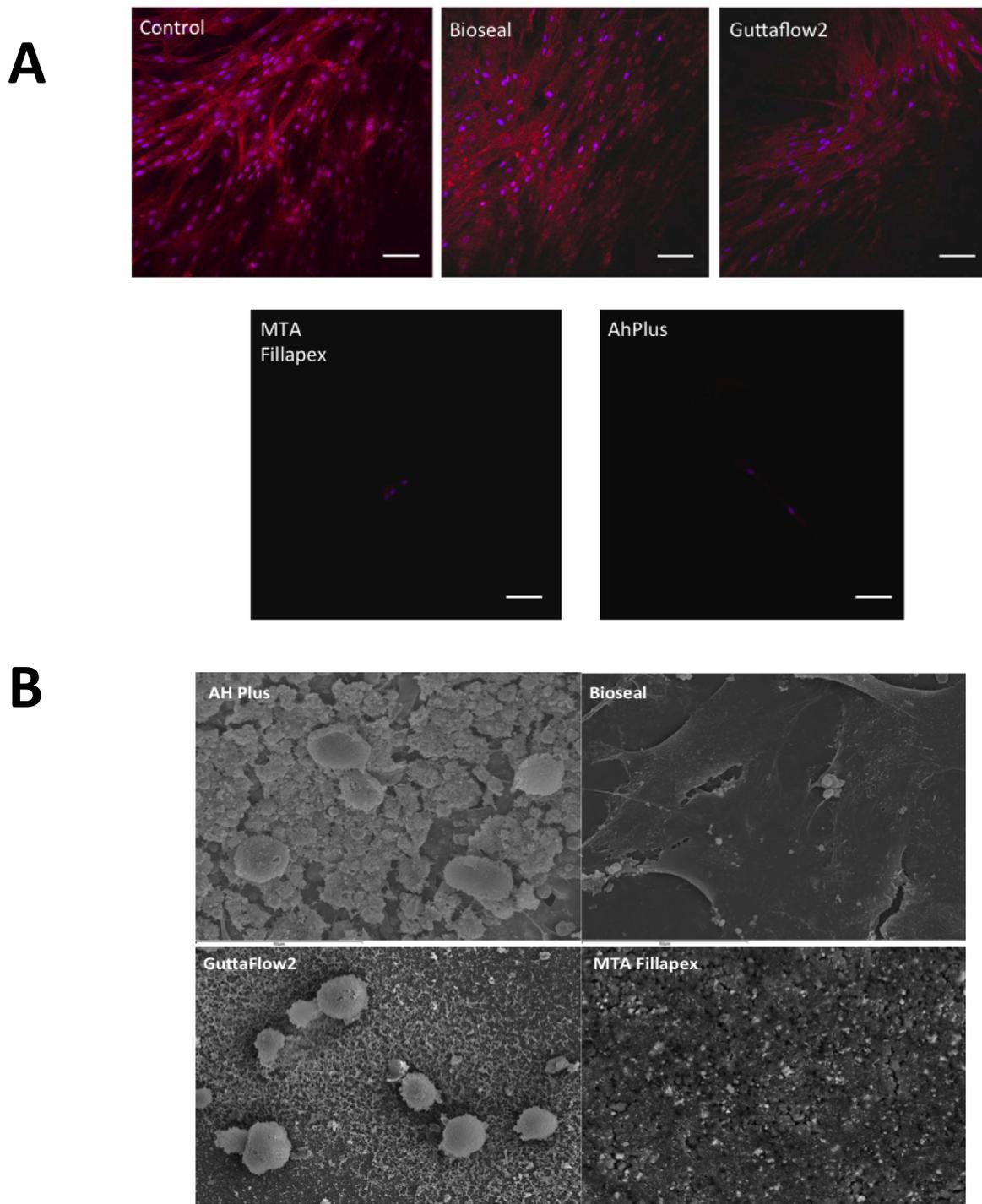
difference between cells exposed to Control and Osteodiff or GuttaFlow2 ( $p = 0.081$ ,  $p = 0.09$ , respectively). Finally, AMELX, AMBN, BSP expression was significantly upregulated when hPDLSCs were exposed to GuttaFlow Bioseal ( $*p < 0.05$ ), whereas there was no significant difference between cells exposed to Control (Fig. 6).

### 3.7. Immunofluorescence

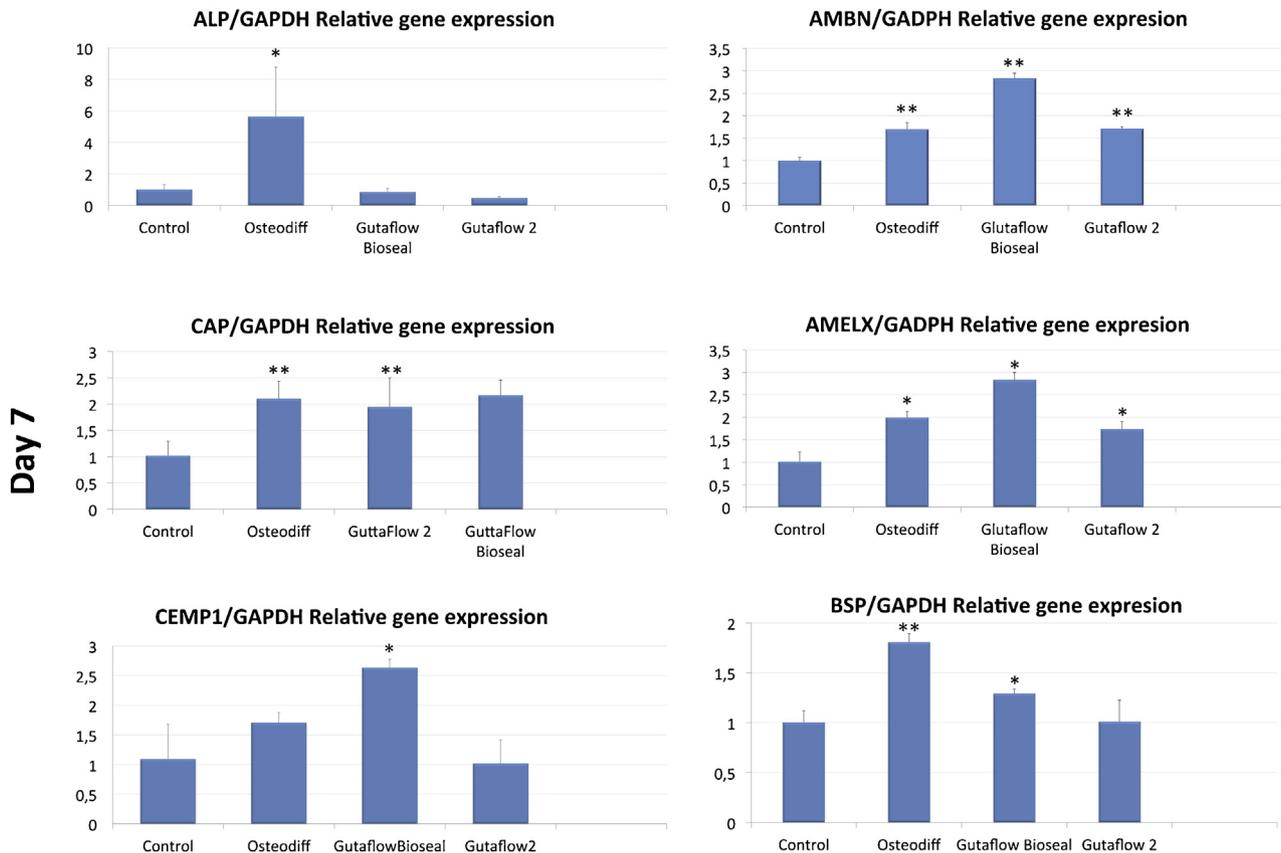
Cementogenic differentiation of hPDLSCs was measured by immunofluorescence staining (Fig. 4). Immunofluorescence assay revealed that the expression of CEMP1 (green fluorescence) and CAP (red fluorescence) in GuttaFlow Bioseal-treated hPDLSCs was stronger than in the cells of GuttaFlow2 and Osteodiff. In contrast, there was not expression of CEMP1 and CAP in AH Plus, MTA Fillapex and control groups. This result



**Fig. 3 – Migration of hPDLSCs exposed to extracts of AH Plus, MTA-Fillapex, GuttaFlow2 and GuttaFlow Bioseal evaluated by in vitro scratch wound healing assay. Confluent hPDLSCs were wounded and incubated with different dilutions of the material extracts for up to 48 h. Cell migration is represented as the percentage of the open wound area for each condition compared with the control. ( $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ ) analysed by one-way ANOVA.**



**Fig. 4** – Representative immunofluorescence micrographs revealing the cytoskeletal organisation of hDPSCs exposed to control and endodontic sealers. Cytoskeletal F actin filaments were stained with phalloidin (red), and nuclei were stained with (DAPI) (blue). Scale bar = 150  $\mu\text{m}$  (A). The morphology of cells on the surface of the material was analyzed by scanning electron microscopy. Scanning electron microscopic morphology images of hPDLSCs attached to AH Plus, GuttaFlow Bioseal, GuttaFlow2 and MTA Fillapex surfaces were obtained at 72 hours. Scale bar: 50  $\mu\text{m}$  (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 5 – Expression of cementum and osteogenic genes in PDLSCs in the presence of several endodontic cements and osteodiff (positive control). (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ) analysed by one- way ANOVA.**

confirms the spontaneous cementoblastic differentiation of GuttaFlow Bioseal-treated hPDLSCs observed by qPCR.

#### 4. Discussion

Because the ultimate goal of therapy of inflamed periapical tissues of endodontic origin is the regeneration of a healthy periodontal ligament (PDL) with surrounding sound alveolar bone [23], the biocompatibility and the cementogenic potential of new endodontic sealers need to be tested. The present study revealed that GuttaFlow Bioseal displays better cytocompatibility and cementogenic potential than GuttaFlow2, MTA Fillapex, and AH Plus ( $p < 0.05$ ).

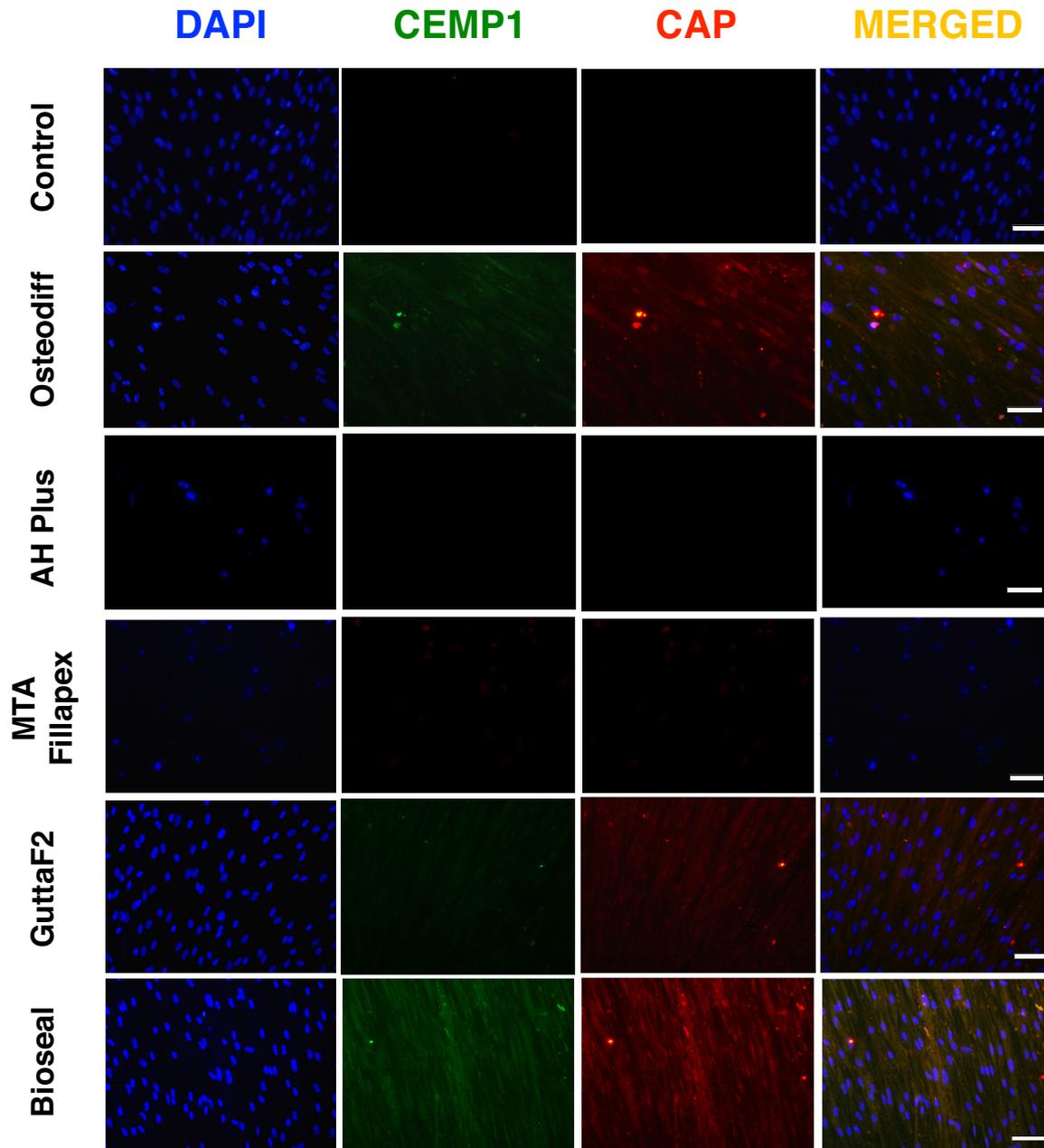
As mentioned above, cementogenesis plays a very important role in periodontal/periapical regeneration. Cell-based therapy using PDLSCs has shown promising results [24,25]. Although primary cell cultures and transformed strains are used for *in vitro* studies, primary PDLSCs are needed for cell-based therapy in periapical regeneration [26,27]. For these reasons, hPDLSCs were used in the present study.

The biocompatibility of new materials is the first step necessary before cells can differentiate and produce an extracellular mineralized matrix on a substrate. For this purpose and to evaluate the possible cytotoxicity of the different endodontic sealers, we investigated the viability of hPDLSCs cultured in the presence of the different extracts by measuring the binding of Annexin-V and 7-AAD, two-color flow

cytometry analyses usually used to determine the stage of cell apoptosis. The results revealed that GuttaFlow Bioseal and GuttaFlow2 did not induce apoptosis, thus preserving cell viability. However, AH Plus and MTA Fillapex displayed a potent cytotoxic effect and caused membrane permeability-related apoptosis and necrosis. In agreement with our results, Saygili et al. [20] found that GuttaFlow sealers were less cytotoxic than MTA Fillapex and AH-Plus.

The capacity of cells to migrate and home to a site of injury is an important factor in cell therapy and the ability of materials to promote this effect is an important indicator for evaluating the bioactivity of new biomaterials [28]. Cell migration assays pointed to better biological effects of GuttaFlow Bioseal and GuttaFlow2 at all the dilutions tested than MTA Fillapex and AH Plus. These findings were consistent with our viability results. Notably, in a previous study, we observed lower cell migration with AH Plus and MTA Fillapex than that obtained using a tricalcium silicate sealer [16].

Cell migration requires cytoskeletal reorganization and focal adhesion molecule expression, so the optimum state of cells is a critical aspect that contributes to cell migration and finally to periapical tissues repair [29]. In the present study, immunofluorescence staining revealed that cells treated with GuttaFlow sealer extracts exhibited better morphological characteristics and cytoskeletal organization patterns than cells that were treated with AH Plus and MTA Fillapex. Such differences in cell morphological characteristics



**Fig. 6 – Guttaflow Bioseal enhanced CEMP1/CAP protein expression of hPDLSCs in vitro. CEMP1 and CAP immunofluorescence staining of hPDLSCs in presence of Control (without material), Osteodiff (positive control), AH Plus, MTA Fillapex, GuttaFlow2 and GuttaFlow Bioseal. Blue fluorescence (DAPI) represents nuclei; Green fluorescence represents CEMP1, whereas red green fluorescence represents CAP. Scale Bar: 100 $\times$ .**

may be attributed to the properties of each material. In fact, SEM results showed a large number of flattened and spreading cells covering the surface of GuttaFlow Bioseal, whereas using AH Plus and MTA Fillapex, cell attachment was limited, with only a few round cells or some cellular detritus appearing on the surface of the materials. Other authors have previously reported a similar cell degree of attachment with MTA Fillapex, AH Plus, GuttaFlow Bioseal and GuttaFlow 2 [8,18,30].

Regarding cementogenic gene expression, CEMP-1 and CAP are most strongly associated with cementogenesis, whereas ALP plays a role in bone matrix mineralization and has

been used as an early marker gene during osteogenesis and dentinogenesis [31–34]. CEMP-1 has shown itself to be a cementoblast phenotype marker and a regulator of hPDLSC commitment into cementoblast-like cells, being reduced when hPDLSCs differentiate into osteoblasts [7]. CAP detection was associated with newly formed mineral tissues [27]. Also, recent studies have suggested that the expression of enamel-associated molecules might play a role during cementogenesis [31,35,36]. In our study, the osteogenic differentiation medium induced osteogenic gene expression (ALP) on hPDLSC, and a downregulation of the cementogenic gene CEMP1. This effect

suggests that the osteogenic stimulus induces hPDLSCs to commit toward osteoblasts while suppressing cementogenic pathways. On the other hand, we found that GuttaFlow Bioseal promoted CEMP1, CAP, AMELX and AMBN overexpression, and a reduced ALP expression on hPDLSCs. Extensive data have shown the high osteo/cementoinductive potential of MTA, based on its ability to induce bone formation in ectopic locations [12,37,38]. In fact, Bartols et al. [23] demonstrated the regeneration of the periodontal ligament induced by MTA in humans. However, there are no studies with tricalcium silicate sealers that attempt to ascertain their cementogenic potential.

To our knowledge this is the first study on GuttaFlow Bioseal and its cementogenic potential. Although the results presented suggest that GuttaFlow Bioseal may be considered a promising endodontic sealer, more *in vitro* and *in vivo* studies are needed to verify whether its *in vitro* cementogenic potential on hPDLSCs corresponds to an increase and/or acceleration of cementum repair in the periapical region.

## 5. Conclusion

Taken together, our results show that GuttaFlow sealers were associated with a higher biocompatibility than AH Plus and MTA Fillapex. Of particular note was that GuttaFlow Bioseal favored the cementoblast differentiation of hPDLSCs in the absence of any growth factors.

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