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Chemical composition and bioactivity potential of the new Endosequence BC Sealer formulation HiFlow.

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Abstract

Aim To evaluate in a laboratory setting the effects of Endosequence BC Sealer HiFlow (Brasseler USA, Savannah, GA, USA), a novel calcium silicate-based sealer developed for use in warm canal filling techniques, on human periodontal ligament stem cells (hPDLSCs).

Methodology Eluates of EndoSequence BC Sealer HiFlow (BCHiF) (Brasseler USA), EndoSequence BC Sealer (BCS) (Brasseler USA) and AH Plus (AHP) (Dentsply DeTrey GmbH, Konstanz, Germany), were placed in contact with hPDLSCs. The characterisation of the chemical elements of the root canal sealers was assessed using Scanning Electron Microscopy and Energy Dispersive X-ray analysis (SEM-EDX). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was used to determine the ion release of the sealers. MTT assay and wound healing techniques were used to determine cell viability and migration, respectively. Cell morphology and cell attachment were assessed using a direct contact technique of hPDLSCs onto the surface of the sealers and analysed by SEM. The bioactivity potential was carried out with the Alizarin Red and qPCR testing methods. The statistical differences were evaluated using one-way ANOVA and Tukey´s test ($p<0.05$).

Results ICP-MS and EDX revealed significantly more zirconium in BCHiF than BCS ($p<0.05$), whereas BCS had slightly higher levels of Ca^{2+} than BCHiF ($p<0.05$). The cell viability assay revealed no relevant differences between BCS and BCHiF when compared with the control group ($p>0.05$). Both BCS and BCHiF had similar rates of cell migration to the control group at 24 and 48 hours. Cell morphology and adhesion capacity were also similar for BCS and BCHiF groups, while the AHP group was associated with reduced adhesion capacity. The Alizarin Red assay revealed a significant difference between the BCS and the control group ($p<0.001$), as well as for the BCHiF group ($p<0.001$). Finally, BCS and BCHiF promoted overexpression of osteo/cementogenic genes.

Conclusions In general, EndoSequence BC Sealer HiFlow possesses suitable biological properties to be safely used as a root canal filling material and promote increased expression of oste/cementogenic genes by hPDLSCs.

Introduction

Hydraulic cements based on calcium silicate composition are now well established clinical options when approaching pulp or periapical reparation/regeneration due to their excellent antimicrobial properties, sealing capacity, biocompatibility, and bioactivity (Fagogeni *et al.* 2019, Giacomino *et al.* 2019). The biocompatibility and biomineralization potential of calcium silicate-based materials are hallmarks of these materials and make them suitable for uses such as direct pulp capping (Tomás-Catalá *et al.* 2017), perforation repair, root-end filling, and apical plugs for teeth with open apices (Donnermeyer *et al.* 2019).

Calcium silicate-based materials display a reparative/regenerative ability needed to overcome pulpal damage when facing pulp exposure after a carious lesion, for example, in primary teeth as well as in mature permanent teeth (Linu *et al.* 2017). These materials used during vital pulp treatments induce proliferation of dental pulp stem cells and the formation of a reparative dentine bridge, leading to pulpal healing (Liu *et al*. 2015, Al-Saudi *et al.* 2019). Apical plugs with those cements are associated with the formation of a natural hard tissue barrier of cementum and potential to provide a biological seal of the apical root canal (Palma *et al.* 2017). As a consequence of the outstanding biological and clinical properties of hydraulic materials, new endodontic sealers based on their composition have been introduced over recent years.

The outcome of root canal treatment in part depends upon the ability of the root canal filling to prevent subsequent bacterial ingress and therefore protect the periapical tissues from disease (Santos *et al.* 2014). To achieve complete filling, root canal sealers are used in combination with a core material, usually gutta-percha, which can be used in cold lateral compaction or in warm techniques (Schilder 2006). Warm canal filling techniques require the use of root canal sealers that are expected to be heated during the process. However, not all sealers are suitable for warm techniques. Previous studies reported that sealers such as MTA Fillapex (Angelus, Londrina, PR, Brazil) and Apexit Plus (Ivoclar Vivadent AG, Schaan, Liechtenstein) are suitable with warm gutta-percha filling techniques, whereas others such as AH Plus suffer from property alterations when heated which may compromise their clinical performance (Camilleri 2015). In addition, the exposure of hydraulic sealers to high temperature causes a reduction in their physical properties such as setting time and flowability (Qu e*t al.* 2016).

Additionally, warm techniques also increase the risk of material extrusion through the periapical foramen, mainly the sealer (Peng *et al.* 2007). Furthermore, some authors suggest using ultrasonic vibration to improve the equitable distribution of the calcium silicate-based sealer along the root canal (Kim *et al.* 2018), also increasing as well as the risk of extrusion. Both clinical procedures raise the probability of direct contact and interfacial interaction between sealers and periodontal ligament cells; therefore the study of the biological properties of these materials becomes an important issue (Kaur *et al.* 2015).

New root canal hydraulic sealers continuously develop in order to combine proper sealing and bioactive properties. Endosequence BC Sealer (BCS) (Brasseler USA, Savannah, GA, USA) is a well known premixed ready-to-use injectable calcium-silicate based material developed for root canal filling and sealing (Hess *et al.* 2011), with suitable physicochemical properties that harden in the presence of wet locations such as dentinal tubules (Candeiro *et al.* 2012). A new formulation of Endosequence BC Sealer has been

modified into Endosequence BC Sealer HiFlow (BCHiF)(Brasseler USA, Savannah, GA, USA) to obtain a suitable calcium-silicate based sealer to use in warm canal filling techniques. This new sealer, according to the manufacturer, shows a lower viscosity when heated and is more radiopaque than its predecessor. However, there is no information on its biological properties.

This study aimed to evaluate the biological effects of BCHiF compared with its predecessor BCS and an epoxy-resin based root canal sealer AH-Plus (AHP) in a laboratory setting. The null hypothesis was that there is no difference between the materials in their bioactivity potential and cytotoxicity on human periodontal ligament stem cells.

Material and Methods

Sealer Eluates

The hydraulic sealers tested in this laboratory investigation were EndoSequence BC Sealer HiFlow (BCHiF) (Brasseler USA), EndoSequence BC Sealer (BCS) (Brasseler USA) and AH Plus (AHP) (Dentsply DeTrey GmbH, Konstanz, Germany). Their composition, as supplied by their respective manufacturers, are presented in Table 1.

All three sealers were mixed under aseptic conditions and following the manufacturers´ indications. Each sealer was placed in preformed moulds of 2-mm height and 5-mm diameter, sterilized by using ultraviolet radiation for 15 minutes and stored for 48 hours in an incubator at 37°C, 5% CO_2 and 95% humidity to achieve complete setting (n=30), immersed in Hank's balanced salt solution (HBSS) (Koutroulis *et al*. 2019). After this time, specimen disks were stored within the culture medium (DMEM) for 24 h at 37°C, 5% CO_2 and humid atmosphere. This procedure was carried out following the International Organization for Standardization (ISO) guideline 10993-12. The ratio of the specimen surface area was 1.5 cm2/mL (ISO 10993-5). Prior to using these extracts in the MTT assay, migration and Alizarin Red experiments, the extracts were filtered through a 0.22µ pore size mesh and prepared undiluted, diluted 1:/2 and diluted 1:/4.

Isolation and culture of hPDLSCs

The study was approved by the Ethical Committee of the University of Murcia (ID:2199/2018). Wisdom molars (n=10) were extracted and transported to the laboratory in Minimum Essential Medium Alpha (α-MEM; Gibco, Invitrogen, Carlsbad, CA, USA) solution containing 1% antibiotics (Sigma Aldrich, St. Louis, MO, USA) and fungizone maintained at 4ºC. Next, after washing three times with PBS, the periodontal tissues were scraped from the middle and the apical part of the root surface and were cut into small fragments with surgical blades. The fragment tissues were digested with an enzymatic solution (Collagenase type I (Gibco)) during 1 hour at 37ºC. Then, periodontal cells were seeded in Minimum Essential Medium Alpha (α-MEM; Gibco) with 10% foetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigma). Culture medium was replaced every three days. Cells at passages 2-4 were used for subsequent experiments (Rodríguez-Lozano *et al.* 2017). The expression of cell surface markers was detected using FACS (Calibur Flow Cytometer, BD Biosciences, San José, CA, USA). Flow cytometry was used to analyze the immunophenotype of cells at passage 3. Briefly, hPDLSCs (2 x10^5) were trypsinized, washed with PBS, and then incubated for 15 min at 4°C with monoclonal antibodies

conjugated with fluorescent dyes. The following antibody cocktails were used: MSC-positive cocktail (CD90, CD105 and CD73) and MSC-negative cocktail (CD34, CD14, CD20 and CD45) (Miltenyi Biotec, Bergisch Gladbach, Germany). Results were evaluated using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Scanning Electronic Microscopy and Energy-dispersive X-ray Analysis

Samples of BCS, BCHiF and AHP were shaped into 5-mm in diameter and 2-mm high using sterile rubber moulds and were immersed in HBSS in a ratio of six cm^2/mL and stored at 37°C for 24 hours. Then, disks were coated with carbon in a CC7650 SEM Carbon Coater unit (Quorum Technologies Ltd, Laughton, UK) and each sample was examined using a scanning electron microscope (SEM) (Jeol 6100 EDAX, Peabody, MA, USA) connected to a secondary electron detector for energy dispersive X-ray analysis (EDX; Oxford INCA 350 EDX, Abingdon, UK) by using computer-controlled software (INCA energy version 18, Inca Oxford Instruments, Abingdon, UK).

Assessment of Inductively Coupled Plasma Mass Spectrometry of Sealer Extracts

Three disks of 5-mm in diameter and 2-mm high samples from each material were stored in 5 mL Milli-Q water. The presence of calcium, iron, zirconium, silicon and tungsten was determined using inductively coupled plasma-mass spectrometry (ICP-MS- Agilent 7900, Stockport, UK).

Cell Viability Assay

Cell viability in contact with the tested materials was assessed using a proven reliable test, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. As stated above, eluates from 3 discs of each material were collected after 24h immersion in the culture medium. Shortly after, 1×10^4 hPDLSCs were added to 96-well plates with 180 µL of DMEM and stored for 24 hours. Then, the cells were placed in contact with the diluted extracts (undiluted, 1:2 and 1:4) and incubated for 24, 48 and 72 hours at 37 ºC in a 5% CO_2 conditions. At the indicated period times, 1 mg/mL of MTT substance was applied and incubated for 4h. Then, 0.2 mL of dimethyl sulfoxide (DMSO) was added to each well. This reaction is needed to solubilize the formazan crystals obtained as a result of MTT reduction by the cells that are still alive after contact with the materials. The cover was removed, and the light absorption in each well was evaluated by spectrophotometer (Synergy H1, BioTek, Winooski, VT, USA) at 570 nm (Abs570).

Cell migration assay

To assess the effect of different sealers extracts on hPDLSCs migration, scratch migration assay was performed. $2x10^5$ hPDLSCs /well were seeded onto six-well plates (n=3) and proliferated to achieve confluency. A scratch was made with a 200 µL-pipette tip, and each well was washed three times to remove cell debris using PBS. The wound closure was observed in the absence (control group) or presence of the different sealers´ eluates (1:1; 1:2 and 1:4). The migration analysis of the scratched area was observed at 24, 48 and 72 h. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to measure the percentage of wound area closed/open after 24, 48 or 72 h relative to the total wound area measured at 0 h in the same well. Migration distances were analyzed separately during periods 0–24 h (migration during first 24 h period), 24–48 h (during second 24 h period) and 48-72 h (during third 24 h

period). A “relative wound closure” area (RWC) was calculated (RWC [%] = wound closure area [pixel] X 100 [%]/x [pixel] in order to avoid any scratch width variation.

Cell morphology and cell adhesion analysis

Fifteen discs of 2-mm height and 5-mm diameter of the three sealers were obtained and subdivided into three groups (n=5). A total of $5x10^4$ hPDLSCs were directly added to each disk´s surface and cultured for 72 hours. Then, cells were fixed with 4% glutaraldehyde in PBS for four hours and dehydrated, air-dried, and sputter-coated with gold/palladium. Finally, cell morphology was evaluated using 100X and 300X magnifications by SEM.

RT-qPCR gene expression analysis

To evaluate the expression of cementoblastic/osteoblastic-related genes (ALP, CEMP-1, and CAP), $2x10^4$ hPDLSCs /well were seeded onto twelve-well plates (n=3) and stimulated with undiluted extracts of endodontic sealers during seven days. For this purpose, six discs were immersed in culture medium for 24h. Medium without extracts served as negative control and an optimized differentiation medium to generate cementoblast/osteoblasts-like cells, OsteoDiff media (Miltenyi Biotec, Bergisch Gladbach, Germany), as a positive control. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 1 µg of RNA by using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad) following the manufacturer’s instructions. Changes in gene expression were calculated by the 2-ΔΔCT method. Primer sequence for human genes encoding cementum protein 1 (CEMP1), cementum-derived attachment protein (CAP), alkaline phosphatase (ALP), Runt-related transcription factor 2 (RUNX2) 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´- TCAGAAGCTCAACACCAACG-3´/5´-TTGTACGTCTTGGAGAGGGC- 3´); RUNX2 (5`- TCCACACCATTAGGGACCATC-3`/5`-TGCTAATGCTTCGTGTTTCCA-3`); GAPDH (5´-TCAGCAATGCCTCCTGCAC-3´/5´-TCTGGGTGGCAGTGATGG- 3´).

Alizarin Red Staining

Mineralization capacity of endodontic sealers was evaluated using Alizarin red staining. $2x10^4$ hPDLSCs /well were seeded onto twelve-well plates (n=3) and proliferated until achieving confluency. Then, cells were stimulated with undiluted extracts of BCS, BCHiF and AHP during 21 days. Medium without extracts served as negative control and OsteoDiff media (Miltenyi Biotec) as a positive control. At the end of the experimental period, the cells were washed with PBS and fixed for 1h using 70% ethanol. They were then incubated with 2% Alizarin Red solution (Sigma AB, Malmö, Sweden) at room temperature in the dark for 30 min. Finally, the absorbance value at 550 nm was measured using the microplate reader.

Statistical analysis

Data were presented as the mean ± standard deviation (SD). All analyses were carried out using Graph-Pad Prism (version 8.1.0, GraphPad Software, San Diego, CA, USA). Normal data with equal variance was

analysed using one-way analysis of variance (ANOVA) and Tukey´s test. Significance was defined when $p < 0.05$. All assays were performed at least three times.

Results

Characterisation of hPDLSCs immunophenotype

hPDLSCs were isolated, cultured and passaged successfully. FACS analysis revealed high expression (>95%) of MSCs specific surface markers (CD73, CD90 and CD105), and low expression (<5%) of CD34, CD45, CD14, and CD20 (Figure 1).

Scanning Electronic Microscopy and Energy-dispersive X-ray Analysis

SEM-EDX analysis provided the qualitative semi-quantitative elemental composition of the surface of each material, represented in Figure 2. BCS and BCHiF had the same elemental composition. C, O and Si were similar % in both sealers, as for the amount of Ca^{+2} and Zr a variation was found. BCHiF contained higher % of Zr than BCS. On the other hand, the % of Ca^{+2} in BC Sealer was significantly higher when compared with the amount of Zr ($p<0.05$). As for AHP, the main difference in terms of composition remains in the presence of W, as reported in our previous studies (Collado-González *et al.* 2017).

Assessment of Inductively Coupled Plasma Mass Spectrometry of Sealer Extracts

Results of the multi-elemental analysis are shown in Table 2 where, as shown with the SEM-EDX technique, a significantly higher concentration of Zr was found in BCHiF when compared with BCS and AHP as well ($p<0.05$). Both BC Sealers contained significantly higher concentration rates of Ca^{+2} than the resin-based sealer ($p<0.05$), as expected.

MTT assay

The viability of hPDLSCs cultured with medium combined with different concentrations of the extracts of each sealer was detected by the enzymatic reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Fig.3). The undiluted extracts of BCHiF and BCS increased cell viability rates significantly over the control group levels at 24 hours ($p<0.01$) whereas the AHP group at this time-point decreased cell viability rates ($p<0.001$). At 48 and 72 hours, neither of the two BC sealers groups suffered any significant variation of viability rates when compared with the control group. Once more, at these time-points, the AHP group decreased cell proliferation rates ($p<0.001$). With dilution ½, BCHiF and BCS groups increased cell viability in the first 24hour period-time ($p<0.05$). However, neither at the 48 hours nor 72 hours period times were the differences significant between the BC Sealers groups and the control group in terms of cell viability rates. AHP group was associated with a significant decrease in cell viability rates at all time-periods studied in this dilution. With dilution ¼, no significant differences were found with the control group for both of the BC Sealers groups except for the AHP group, with the results for this dilution being the same as for the previous dilutions. These results evidence that BCHiF and BCS had no cytotoxic effect on hPDLSCs.

Cell migration assay

At all period-times and all dilutions studied, cell migration rates in the BCS group were similar to that of the control group and no detectable differences were found (Fig.4). In the BCHiF group, only at 24 hours in the

non-diluted group, significant differences were found ($p<0.01$) meanwhile, no significant differences were revealed at 48 nor 72 hours in any dilution when compared with the control group wound closure. On the contrary, at all period-times and all dilutions, the AHP group exhibited significant differences ($p<0.001$), being unable to heal the wound when compared with the control group (without extracts). These results indicate that both BC Sealers had similar migration values to those of the control.

Cell morphology and cell attachment analysis

As shown in Figure 5, the morphology of hPLSCs and their attachment to the surface of the materials, after 72 hours of culture, were analyzed by scanning electron microscopy. The results exhibited a high degree of cells bonded and spreading through the surface of both BCHiF and BCS. The morphology of these cells in contact with these materials suggested an active adhesion interaction with the surface since multiple prolongations and a flattened morphology was observed. No cells attached to the surface of AHP were found.

qPCR analysis

At day 7, ALP, CEMP-1 and RUX2 expression were significantly higher in BCS and BCHiF groups when compared to osteodiff and control group ($p<0.001$). In addition to that, expression of CAP was higher in the Osteodiff group (positive control) when compared to the groups BCS, BCHiF and negative control groups ($p<0.05$; $p<0.01$;$p<0.001$,respectively)(Fig. 6). GAPDH was used to normalize the results. Because AHP provoked cell death (see previous experiments), qPCR analysis in this group was not analyzed.

Alizarin Red Staining

The mineralization capacity of tested materials was detected by Alizarin Red staining. As shown in Fig. 7, BCHiF, BCS and Osteodiff groups, produced significantly more calcium deposits than the control only after 21 days of culture ($p<0.001$). The greatest mineralization capacity was seen with the BCS group compared with BCHiF and Osteodiff groups ($p<0.001$ respectively). On the other hand, no calcium deposits were detected in the AHP group, with significantly lower rates than that of the control group ($p<0.01$).

Discussion

As the use of calcium silicate-based sealers continues to increase, new bioceramic formulations attempt to achieve the ideal physicochemical, mechanical and biological properties. Several studies have been published on the physicochemical and mechanical properties of hydraulic cements when used in combination with warm gutta-percha techniques (Camilleri 2015, Boyadzhieva *et al.* 2017). A new hydraulic sealer formulation has been developed recently named Biosequence BC Sealer HiFlow (BCHiF), and the manufacturer asserts that it can be used with warm gutta-percha techniques without risking its efficacy.

Since the biological properties of this new sealer have not been tested yet, in this laboratory study, the cytocompatibility and bioactivity potential of BCS, and the new calcium silicate sealer formulation, BCHiF were analysed. AHP was chosen as reference material as it is one of the most commonly used and investigated root filling cements (Santos *et al.* 2019). In general, excellent cytocompatibility was observed with BCHiF, as well as with BCS. The results revealed that AHP had lower cytocompatibility when

compared to the control group and with the other tested materials. These results are consistent with previous studies (Candeiro *et al.* 2015, Graunaite *et al.* 2018, Benetti *et al.* 2019).

As stated in the latest review of the ISO 7405:2018, prior to conducting cytotoxicity studies as part of the biocompatibility tests, material characterization is required before biological testing is performed. In this study, following other published studies (Jimenez-Sanchez *et al.* 2019) scanning electron microscopy with energy-dispersive X-ray analysis was carried out to evaluate the surface of all three sealers, and inductively coupled plasma mass spectrometry (Bulska & Wagner 2016) was conducted to monitor the ion release of the sealer extracts. In this study BCS and BCHiF had the same elemental composition. C, O and Si showed similar % in both sealers,but a variation was found in the amount of Ca^{+2} and Zr. A higher concentration of Zr was found in BCHiF when compared with BCS and AHP as well. BCS releases higher concentration rates of Ca^{+2} than the resin-based sealer AHP, in accordance with previous reports (Candeiro *et al.* 2012). Due to the results presented in this study, the chemical evaluation of BCHiF can be compared with that of its predecessor. Further studies should be made regarding the concentration of Zr in BCHiF and its influence on the biological healing process.

The biological reaction of cells in contact with these materials can be evaluated by a cell migration assay (Yarrow *et al.* 2004). In the present study, cell migration rates with BCS were similar to those with the control group, meanwhile with AHP, hPDLSCs were unable to migrate in order to close the wound ($p<0,001$). These same migration results are shown in a study conducted by Seo *et al*. (2019) with human dental pulp stem cells. hPDLSCs exposed to BCHiF extracts revealed no significant differences with those in contact with BCS nor the control group.

Cell adhesion to biomaterials is essential in cell communication and interactions and is of main importance in the process of cell differentiation (Khalili & Ahmad 2015). Cell morphology, when attached to the surface of a biomaterial, can be a predictable sign of cell function and differentiation (Bacakova *et al.* 2004). As a consequence, as described by other authors (Zhang *et al.* 2013), hPDLSCs were seeded onto the surfaces of the three sealers in order to observe cell morphology and cell adhesion to these materials using a scanning electron microscope (Chen *et al.* 2016). The results revealed adequate attachment of hPDLSCs to both BCS and BCHiF. As described in previous reports, no cells were observed attached to the surface of AHP discs (Collado-González *et al.* 2017, Rodriguez-Lozano *et al.* 2017).

Bioactivity is also defined as the cellular effects induced by the release of biologically active substances and ions from the biomaterial allowing the biomineralization. In the widest meaning, bioactivity is a desire property of calcium silicate-based sealers due to have a biological effect or be biologically active and form a bond between the tissue and the material (Vallittu *et al.* 2018).. In this study, bioactivity assays as qPCR and Alizarin red assays were assessed to evaluate the bioactivity potential of these hydraulic materials. It has recently been demonstrated that GuttaFlow Bioseal had increased expression of CEMP-1, CAP and ALP (Rodriguez-Lozano *et al.* 2019). It may be speculated that the biological properties and bioactivity of the materials are influenced by their composition (D'Anto *et al.* 2010, Gandolfi *et al.* 2015). In fact, Giacomino *et al.* (2019) reported the osteogenic effect of the BCS by an increase in ALP and DMP-1–expressing cells, significant gene expression up-regulation of osteogenic genes and mineralization

potential. Unsurprisingly, in the study, both BCHiF and BCS promoted greater osteo/cementogenic genes expression than the control group. Interestingly, BC sealer groups exhibited greater mineralization capacity than the Osteodiff group (positive control), with more visible calcium deposits. On the contrary, there was no mineralization in the cells treated with AHP as this material is associated with extensive cell death. These results may be related to the significantly higher calcium release observed for both calcium silicate-based sealers (Zordan-Bronzel *et al.* 2019). Although this seems to be counterintuitive, it is possible that the high alkalinity of the calcium silicate-based sealer media can up-regulate alkaline phosphatase activity and enhance mineralization (Wu *et al.* 2018). Moreover, the main component of both calcium silicate-based sealers is calcium silicates, whose mineralization capacity has previously been reported (Zordan-Bronzel *et al.* 2019), which is in line with the present results. Based on this mineralization boost provided by the exposure of hPDLSCs to BC sealer it can speculated that clinically this can induce hard tissue deposition by periodontal ligament cells in the areas of contact with the sealer, reduce the size of the root canal portal of exit and improve the biological seal.

Conclusions

In general, EndoSequence BC Sealer HiFlow is a biocompatible root canal filling material when put in contact with hPDLSCs. BCHiF had similar results to its predecessor BCS in terms of cytocompatibility, cell migration, cell adhesion and bioactivity potential.

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Conflict of Interest statement

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Figure Legends

Figure 1 Flow cytometric characterization. FACS analysis showed high expression (>95%) of MSCs specific surface markers (CD73, CD90 and CD105), and low expression (<5%) of CD34, CD45, CD14, and CD20.

Figure 2 EDX analysis. Evaluation of the chemical composition (spectra) and the element distribution (elemental mapping) of BCHiF (*Column A*), BCS (*Column B*) and AHP (*Column C*) conducted with energy-dispersive X-ray spectroscopy. BCHiF contains higher % of Zr than BC Sealer. The % of Ca^{+2} in BCS is higher when compared with the amount of Zr. As for AHP, the main difference in terms of composition remains in the presence of W.

Figure 3 MTT assay. Determination of cell viability was carried out by an MTT assay. After the contact of hPDLSCs with the three sealers extracts in all dilutions at 24, 48 and 72 hours, the absorbance results are shown in these graphics. Absorbance values were significantly different from to the control group (*p< 0.05; ** p< 0.01; *** p< 0.001, respectively, by one-way ANOVA and Tukey's post-hoc test.). Values with BCHiF group and BCS group were similar to those of the control group. AHP group showed significant differences with all BC sealer groups and the control group, showing the lowest cell viability rates, as expected.

Figure 4 Scratch migration assay. The closure of the space created in the wound healing technique after the contact of the eluates of all three sealers with the hPDLSCs after 24, 48 and 72 hours is represented in this figure by a composition of the photographs analyzed by the Image J program and representation by a bar graphic after the statistical analysis. Cell migration rates were expressed as the open wound area percentage for each condition compared with the control (*p<0.05; **p<0.01; ***p<0.001, respectively). One-way ANOVA and Tukey's post-hoc test

Figure 5 SEM analysis. Photomicrographs showed hPDLSCs cultured on the discs´ surface of BCS(A), BCHiF (B) and AHP(C) for a 72 h period time. Photomicrographs show hPDLSCs fully adhered to the surface´s disk of BCS and BCHiF, with a polyhedral shape extended, and displaying dendritic extensions. No cells were attached to the AHP surface disk. Scale bars: 100X and 300X.

Figure 6 RT-qPCR gene expression analysis. Gene expression profiles of hPDLSCs treated with the test materials showing expression of ALP, CEMP, RUNX2 and CAP genes. Values indicated with a * represent significant differences between the groups. (*p<0.05; **p<0.01; ***p<0.001, respectively). One-way ANOVA and Tukey's post-hoc test

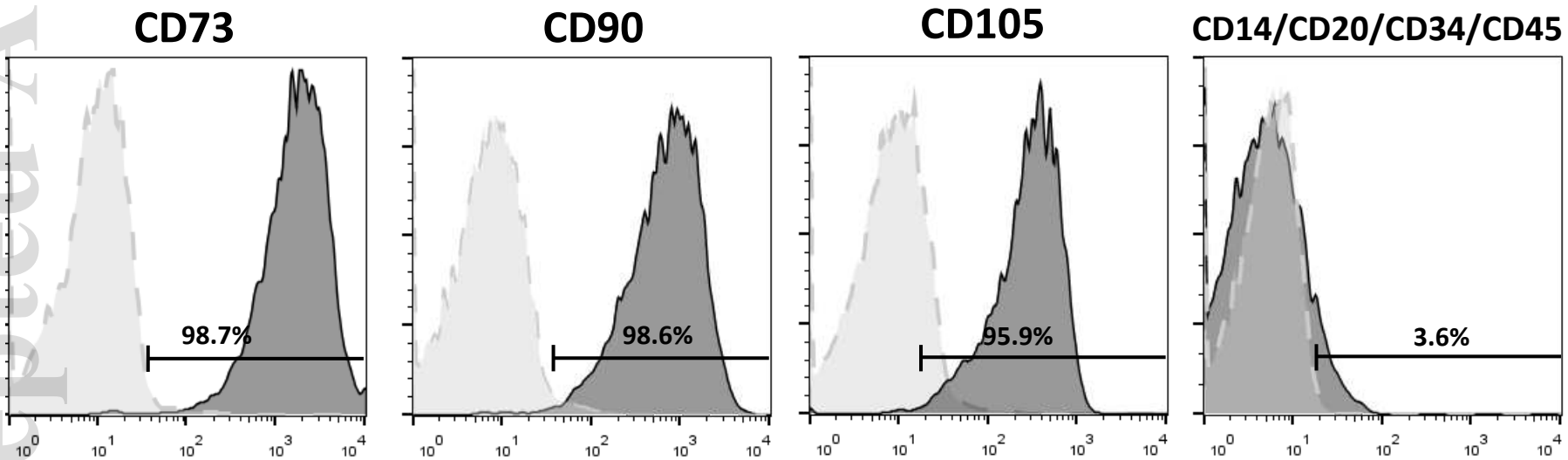
Figure 7 Mineralization assay. Alizarin Red staining to evaluate the bioactivity potential of BCS, BCHiF and AHP. Values indicated with a * represent significant differences between the groups. (*p<0.05; **p<0.01; ***p<0.001, respectively). One-way ANOVA and Tukey's post-hoc test

Table 1 Tested materials

Materials	Manufacturer	Composition	Lot Number
Endosequence BC Sealer	Brasseler USA Savannah, GA, USA	Zirconium oxide, calcium silicates, calcium phosphate monobasic, calcium hydroxide, filler and thickening agents.	(10)18002SP
Endosequence BC Sealer Hi Flow	Brasseler USA Savannah, GA, USA	Zirconium Oxide, Tricalcium Silicate, Dicalcium Silicate, Calcium Hydroxide and fillers	(10)1802SPWF
AH Plus	Dentsply DeTrey, Konstanz, Germany	Epoxy paste: diepoxy, calcium tungstate, zirconium oxide, aerosol, and dye Amine paste: 1-adamantane amine, N'dibenzyl-5 oxanonandiamine-1,9, TCD-diamine, calcium tungstate, zirconium oxide, aerosol, and silicone oil	1705000999

Table 2 Assessment of ICP-MS of endodontic sealer eluates.

	28 Si [He]	44 Ca [He]	56 Fe [He]	91 Zr [He]	182 W[He]
Sample Name	**Conc. [ppm]**	**Conc. [ppm]**	**Conc. [ppm]**	**Conc. [ppm]**	**Conc.[ppm]**
AH Plus	2,08 ±0.02^{B}	1,79 ±0.00^{AB}	<0.000	1,24 ±0.00^{AB}	4267±0.00^{AB}
Endosequence BC Sealer	8,09 ±0.00^{BC}	67,22 ±0.02^{B}	<0.000	1,55 ±0.02^{BC}	120,87 ±0.00^{BC}
Endosequence BC Sealer Hiflow	1,91±0.01^{C}	63,53±0.00^{A}	<0.000	3,85±0.00^{AC}	50,65 ±0.00^{AC}
Uppercase A indicates significant difference (p< 0.05) between AH Plus and Hiflow					
Uppercase B indicates significant difference (p< 0.05) between AH Plus and Endosequence BC Sealer					
Uppercase C indicates significant difference (p< 0.05) between Hiflow and Endosequence BC Sealer					



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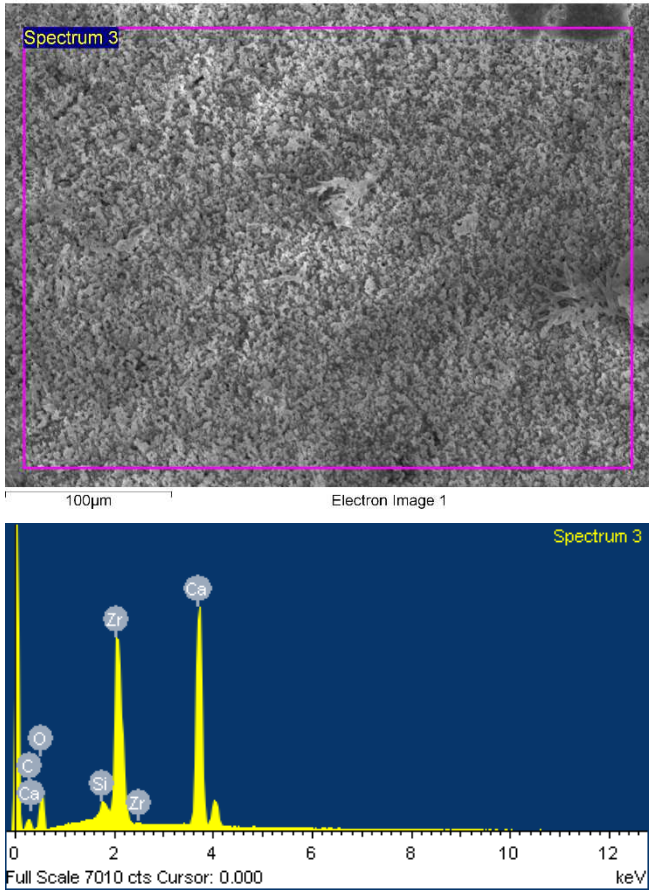
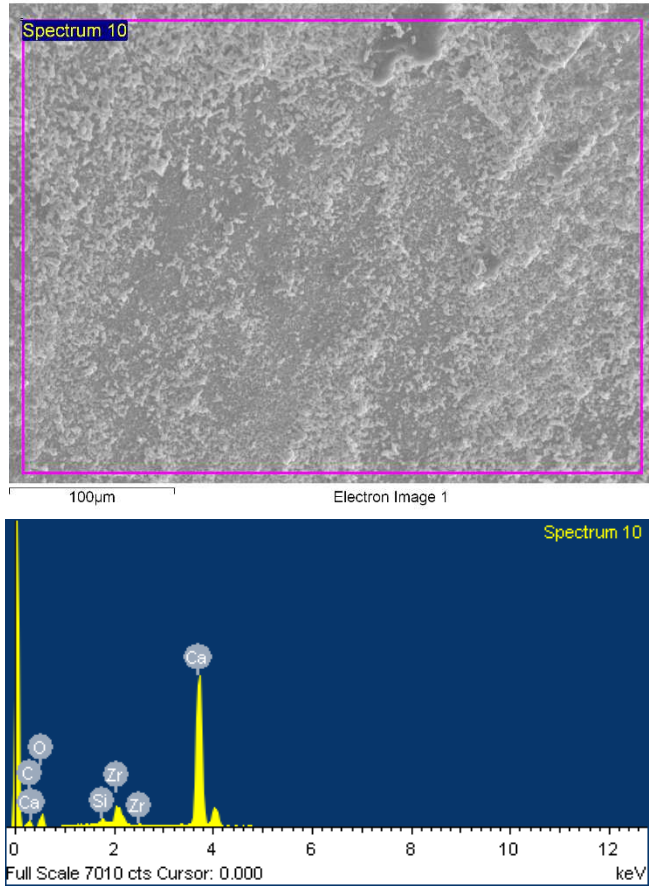


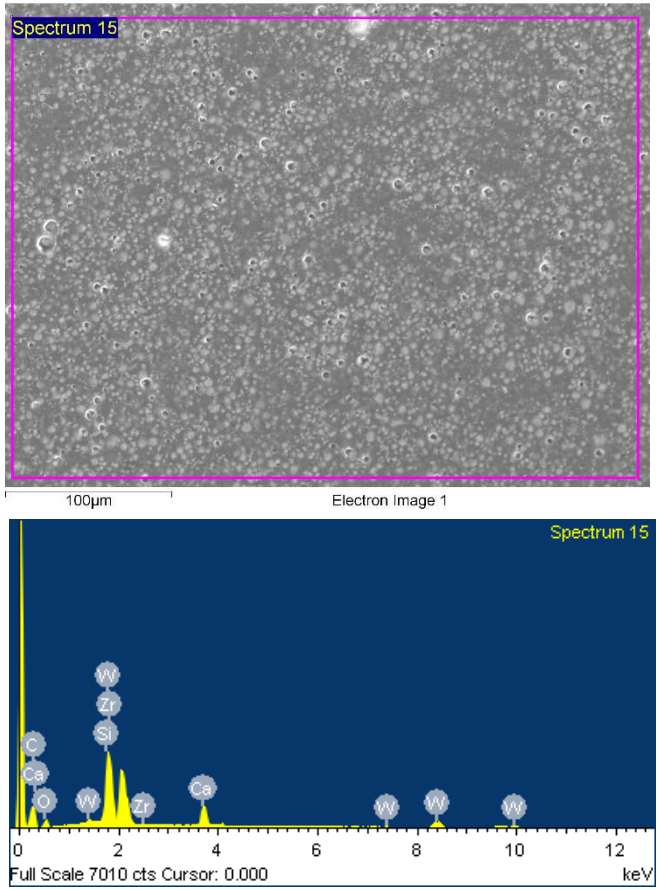
Element	Weight%	Atomic%
O K	33.89	65.05
Si K	0.52	0.56
Ca K	28.66	21.96
Zr L	36.93	12.43
Totals	100.00	

B

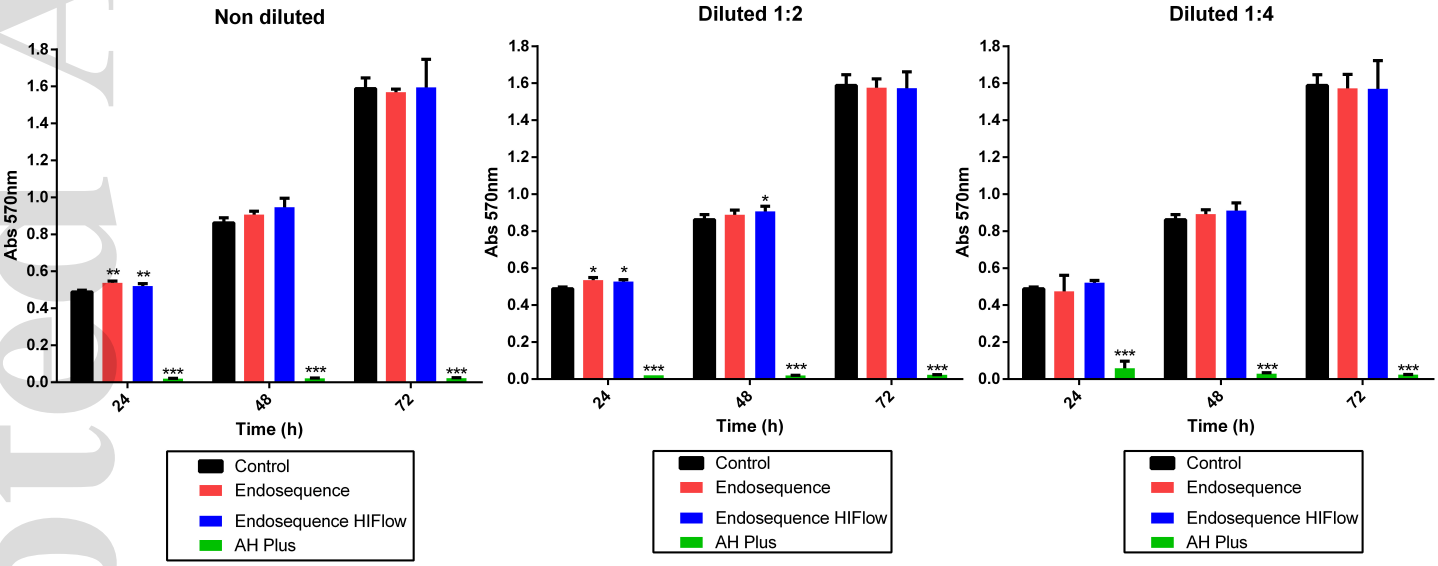


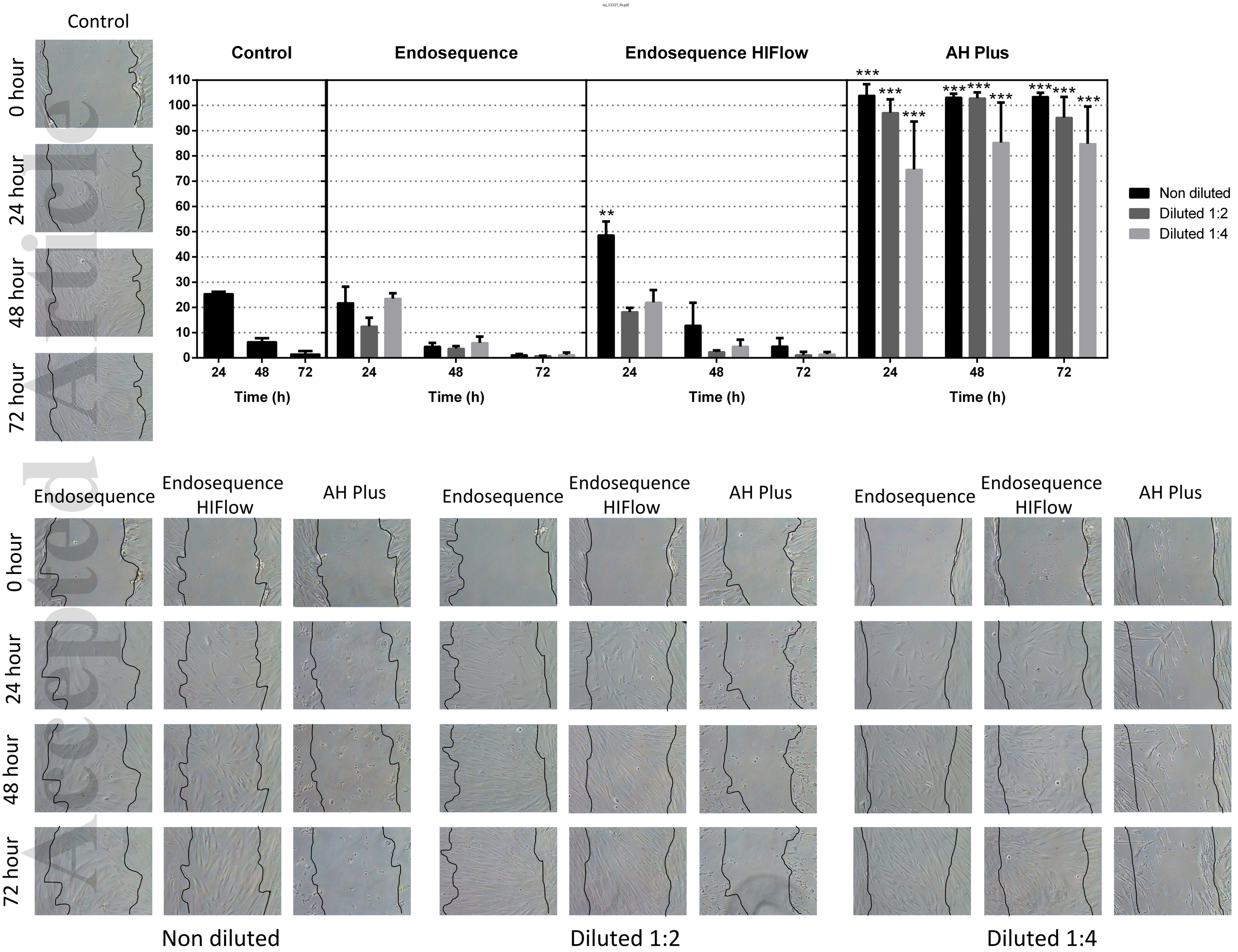
Element	Weight%	Atomic%
O K	37.56	62.34
Si K	0.87	0.83
Ca K	50.91	33.73
Zr L	10.66	3.10
Totals	100.00	

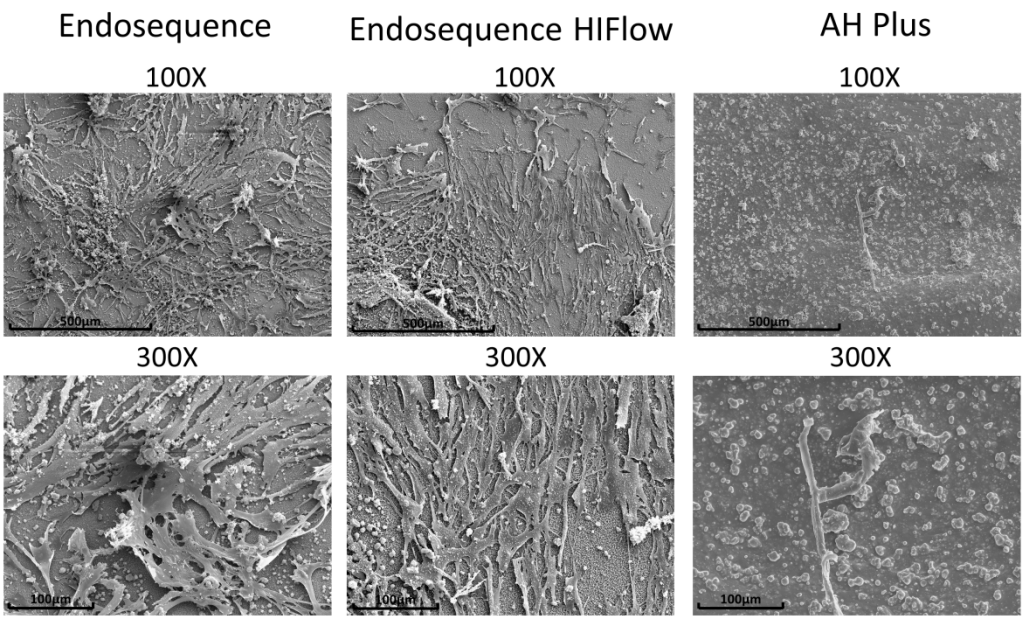
C



Element	Weight%	Atomic%
C K	53.23	82.01
O K	9.22	10.67
Si K	1.08	0.71
Ca K	3.35	1.55
Zr L	16.95	3.44
W M	16.17	1.63
Totals	100.00	







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