Biocompatibility of three new calcium silicate-based endodontic sealers on human periodontal ligament stem cells

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Abstract

Collado-González M, García-Bernal D, Oñate-Sánchez RE, Ortolani-Seltenerich PS, Lozano A, Forner L, Llena C, Rodríguez-Lozano FJ. Biocompatibility of three new calcium silicate-based endodontic sealers on human periodontal ligament stem cells. *International Endodontic Journal*, **50**, 875–884, 2017.

Aim To evaluate the biocompatibility of three calcium silicate-based endodontic sealers, Bioroot BC Sealer (Septodont, Saint-Maur-des-Fosses, France), Endoseal MTA (EndoSeal, Maruchi, Seoul, Korea) and Nano-ceramic Sealer (B&L Biotech, Fairfax, VA, USA) (NCS), on human periodontal ligament stem cells (*h*PDLSCs).

Methodology Human periodontal ligament stem cells were cultured in the presence of various endodontic sealer eluates for 24 h. Cell viability was determined using the MTT assay. Cell death and changes in phenotype induced by the set endodontic sealer eluates were evaluated through flow cytometry. Also, an *in vitro* scratch wound-healing model was used to determine their effects in cell migration. Finally, to assess cell morphology and attachment to the different sealers, *h*PDLSCs were directly seeded onto the material surfaces and analysed by scanning electron microscopy (SEM). One-way analysis of variance (ANOVA) followed by a Bonferroni post-test was performed ($P < 0.05$).

Results At 24 h, cell spreading was evident in the presence of Bioroot BC Sealer (BR) and Nano-ceramic Sealer (NCS), but not Endoseal MTA (ES). At 72 h, BR and NCS exhibited high and moderate cell proliferation, respectively, whereas ES revealed low rates of cell proliferation ($P < 0.05$). Similar results were obtained in a cell death assay. In addition, *h*PDLSCs maintained their mesenchymal phenotype in all conditions although their capacity to migrate was higher in the presence of BR. Finally, SEM studies revealed a good degree of proliferation, cell spreading and attachment, especially when using BR and NCS discs.

Conclusions BR and NCS were associated with better cytocompatibility than ES. Further *in vitro* and *in vivo* investigations are required to confirm the suitability of these calcium silicate-based endodontic sealers for clinical application.

Keywords: calcium silicate-based endodontic sealers, cytotoxicity, human periodontal ligament stem cells, mineral trioxide aggregate.

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Introduction

Calcium silicate-based endodontic sealers have been recently introduced and shown to produce appropriate biological responses (Eldeniz *et al.* 2016, Prati & Gandolfi 2015). These bioactive sealers represent a

new concept in root filling, and their interaction with periapical tissues is considered important (Costa *et al.* 2016). Due to the presence of periodontal ligament cells in periapical tissues, these cells have been used for *in vitro* studies of biocompatibility with endodontic sealers (Accardo *et al.* 2014, Diomede *et al.* 2014, Camps *et al.* 2015).

Human periodontal ligament stem cells (*h*PDLSCs) are a multipotent stem cell population that is able to differentiate into both cementoblast cells and collagen-forming cells, as *in vitro* and preclinical studies have previously demonstrated (Tour *et al.* 2012, Liu *et al.* 2015, Torii *et al.* 2015). Their phenotypic characterization has shown them to have high expression levels of the mesenchymal stem cell (MSC) markers, CD105, CD73 and CD90, but not of the hematopoietic markers CD45, CD34, CD14, CD19 and HLA class II (Rodríguez-Lozano *et al.* 2011).

The composition of endodontic sealers plays an important role in their biocompatibility. The presence of resin in other sealers containing MTA, such as MTA Fillapex (Angelus Indústria de Produtos Odontológicos S/A, Londrina, PR, Brazil), reduced cell survival rates (Braga *et al.* 2015, Mestieri *et al.* 2015, Zhou *et al.* 2015), whilst Endoseal MTA (EndoSeal, Maruchi, Seoul, Korea), a new sealer without resin, had lower cell viability than ProRoot (PMTA; Dentsply, Tulsa, OK, USA) (Lim *et al.* 2015). Although Endoseal MTA is another type of MTA-based root canal sealer, the composition and particle size of different MTAs are not the same. Recently, Bioroot BC Sealer (Septodont, Saint-Maur-des-Fosses, France) was demonstrated to show good biocompatibility with periodontal ligament cells (Camps *et al.* 2015, Eldeniz *et al.* 2016).

However, no studies have been conducted to analyse the possible cytotoxic effects induced by the new MTA-sealer, Nano-ceramic Sealer (B&L Biotech, Fairfax, VA, USA). It therefore seems appropriate to question whether its different composition could affect the cytotoxicity of MTA sealers and their bioactivity.

Cytotoxicity is defined as the capacity of a material to impact on cellular viability. Therefore, cytotoxicity tests are primary biocompatibility tests that determine the lysis of cells, the inhibition of cell growth and other effects on cells caused by test substances (Peters 2013). A number of these test methods have been combined to establish standards (ISO standards). The goal was to improve the comparability of data generated in different test laboratories as well as to save costs and test animals by avoiding duplicate testing (Schmalz *et al.* 2016).

The aim of this study was to compare and assess the cytotoxicity of three different calcium silicate-based endodontic sealers: Bioroot BC Sealer, Endoseal and Nano-ceramic Sealer in contact with human periodontal ligament stem cells. The null hypothesis was that there would be no significant cytotoxic effects between calcium silicate-containing endodontic sealers.

Materials and methods

Sealer extracts

The following calcium silicate-based sealers were used to prepare the extracts:

1. *Bioroot RCS,* powder: tricalcium silicate, zirconium oxide and excipients. Aqueous solution: calcium chloride and excipients.
2. *Endoseal MTA:* calcium silicates, calcium aluminates, calcium sulphate, radiopacifier, thickening agent.
3. *Nano-ceramic Sealer:* calcium silicates, zirconium oxide, filler and thickening agent.

The sealers were mixed according to the manufacturers' instructions. Discs of each sealer were fabricated under aseptic conditions in sterile cylindrical rubber moulds with a 5-mm diameter and a 2-mm height. Excess flash material was removed with a sterile scalpel. The materials were then sterilized using ultraviolet irradiation for 15 min and stored in an incubator at 37 °C for 48 h to achieve complete setting. To simulate the clinical situation of an endodontic sealer present in the apical region of the root and extruded over the apex, where there are fluids, the evaluation of these materials was carried out by incubation of the cultured cells with extracts or eluates of the sealers, according to the International Standard ISO 10993-5. The extraction of the different materials' eluates was performed in sterile chemically inert closed containers using aseptic techniques, in general accordance with ISO 10993-12. The vehicle of the extraction was DMEM culture medium supplemented with 10% foetal bovine serum (Gibco Invitrogen, Paisley, UK), L-glutamine (PAA Laboratories, Pasching, Austria) and penicillin/streptomycin (PAA Laboratories) (complete medium). After extraction, materials were stored in culture medium for 24 h at 37 °C in a humid atmosphere containing 5% CO_2. According to the International Organization for Standardization (ISO) standards, the ratio between the surface of the sample and the volume of the medium was

6 cm^2 mL^{-1}. The extraction media were then collected at the end of this period and passed through a 0.22-μm filter (Merck Millipore, Billerica, MA, USA). Subsequently, various dilutions (1/1, 1/2, 1/4 vol/vol) of these extraction media were prepared with DMEM fresh medium.

Isolation and culture of *h*PDLSCs

Human periodontal ligaments (*h*PDL) were obtained from impacted third molars (n : 12) from ten healthy subjects. Donors gave written informed consent according to the guidelines of the Ethics Committee of the research Institution. The *h*PDL was scraped from the middle third region of the root surface. After extraction, *h*PDL was washed with Ca^{2+}/Mg^{2+}-free Hank's balance salt solution (Gibco, Gaithersburg, MD, USA) and subjected to collagenase-A digestion (3 mg mL^{-1}) (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. *h*PDL cells were cultured as described previously (Bueno *et al.* 2013). Red blood cells and other nonadherent cells were removed and washed 2–3 times with PBS, and fresh medium was added to allow further growth. The adherent cells were grown to 80% of confluence and were defined as passage zero (P0). For subsequent passaging, cells were washed with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) (Gibco Invitrogen) and detached by incubating with 0.25% trypsin-EDTA solution (Gibco Invitrogen) for 2–5 min at 37 °C. Culture medium was added to inactivate the trypsin activity. Finally, PDLSCs were centrifuged at 300 ***g*** for 5 min and plated in 75-cm^2 flasks at a density of 5×10^3 cells per cm^2.

Direct contrast-phase microscopic observation

Human periodontal ligament stem cells were plated on 24-well plates as described above (Rodríguez-Lozano *et al.* 2017) and cultured in the presence of various endodontic sealer eluates for 24 h. Microscopic images of the cells were then acquired using an inverted contrast-phase microscope (Nikon, Tokyo, Japan).

MTT assay

The proliferation rate of *h*PDLSCs growing in the presence of the different endodontic sealer eluates was evaluated using the MTT assay (MTT Cell Growth Kit; Chemicon, Rosemont, IL, USA) after 24, 48 and 72 h of culture, using *h*PDLSCs cultured in complete medium as controls. After incubation, MTT was added to each well for the final 4 h of the experiment, which was stopped by the addition of dimethyl sulfoxide. The absorbance at 570 nm (Abs570) was measured using an automatic microplate reader (ELx800; Bio-Tek Instruments, Winooski, VT, USA) using Abs_{690} as the reference wavelength. Each condition was analysed in quintuplicate.

Flow cytometry analysis of the expression of mesenchymal stem cell surface markers

The expression of mesenchymal stem cell surface molecules was analysed on cultures of *h*PDLSCs in passages 2–4 by flow cytometry. Briefly, cells were seeded at a density of 3.0×10^4 cells per cm^2 in 48-well plates previously coated with different eluates for 72 days at 37 °C. Then, cells were detached using a 0.25% w/v trypsin-EDTA solution, rinsed with PBS and incubated in the dark at 4 °C for 30 min with fluorescence-conjugated specific monoclonal antibodies for CD73, CD90 and CD105 (Miltenyi Biotec, Bergisch Gladbach, Germany), as recommended by the International Society of Cellular Therapy (ISCT) to confirm the mesenchymal phenotype of the cells (Horwitz *et al.* 2005, Dominici *et al.* 2006). Lack of expression of the hematopoietic markers CD14, CD20, CD34 and CD45 was also analysed. Nonspecific fluorescence was measured using specific isotype monoclonal antibodies. Then, cells were acquired using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) and analysed using Kaluza analysis software (Beckman Coulter, Inc., Brea, CA, USA).

Determination of cellular death (Annexin-V/7-AAD staining)

Human periodontal ligament stem cells were cultured on the different eluates for 72 h, followed by double staining with PE-conjugated Annexin-V and 7-AAD (BD Biosciences). The percentage of live (Annexin-V−/7-AAD−), early apoptotic (Annexin+/7-AAD−) or late apoptotic and necrotic cells (Annexin-V+/7-AAD+ and Annexin-V−/7-AAD+) were analysed in a BD FACS Canto flow cytometer. Subsequently, the percentages of each population were calculated.

Cell migration assay

The effects of endodontic sealer eluates on the migration of *h*PDLSCs were examined by means of a wound-healing assay. Briefly, 5×10^4 *h*PDLSCs were

seeded into 60-mm culture plates and allowed to reach a complete confluent monolayer. Cells were incubated for 24 h in DMEM medium without FBS before a wound was inflicted by making a scratch through the confluent layer of cells using a 200-μL pipette tip. After wounding, cells were washed with PBS to remove cell debris and incubated with various dilutions of endodontic sealer eluates up to 48 h to allow cell migration back into the wound area. Images of the scratch area were taken at 0, 24 h and 48 h post-wound infliction using a phase-contrast microscope (Nikon, Tokyo, Japan). Images were analysed by ImageJ (NIH, Bethesda, MD, USA).

Scanning electronic microscopy (SEM)

Different samples of Bioroot RCS (BR), Endoseal MTA (ES) and Nano-ceramic Sealer (NCS) were shaped into 1.6-mm-thick discs of 5 mm diameter using rubber moulds. Fifteen discs of each material were prepared and subdivided into three groups. Each group contained five parallel samples. *h*PDLSCs were directly seeded onto each disc at a density of 5×10^4 cells per mL. After 3 days of culture, the samples seeded with *h*PDLSCs were removed from culture wells, rinsed with PBS and fixed with 3% glutaraldehyde, dehydrated in a graded series of ethanol concentrations through 100%, immersed in 100% hexamethyldisilazane, air-dried, mounted on aluminium stubs and sputter coated with gold/palladium. Finally, gold/palladium-coated specimens were examined by SEM. The magnification used was 100×.

Statistical analysis

Data from the MTT assay and cell migration were performed using SPSS version 15.0 statistical software (SPSS, Inc., Chicago, IL, USA). Data were tested for normal distribution by the Kolmogorov–Smirnov test, and group comparisons were analysed by one-way analysis of variance (ANOVA) followed by a Bonferroni post-test. All values are presented as the mean ± SD. A P value <0.05 was considered significant.

Results

Direct microscopic observation and proliferation rate

Initially, discs of the endodontic sealers were prepared using rubber moulds (Fig. 1a). Next, *h*PDLSCs were exposed to endodontic sealer eluates for 24, 48 and 72 h. At 24 h, microscopic observation showed that, in the presence of ES eluates, had a significantly lower number of attached cells than the control, whereas in the presence of NCS and BR, moderate and high levels of attached cells were observed, respectively (Fig. 1b). Indeed, cell proliferation in the presence of BR in concentrations of 1, 1/2 and 1/4 was significantly higher than ES and NCS at 72 h ($P < 0.05$). The presence of ES eluates significantly decreased cell proliferation compared to the control at 24, 48 and 72 h ($P < 0.001$) (Fig. 2).

Mesenchymal phenotype expression and cell viability

In order to confirm the mesenchymal phenotype of isolated *h*PDLSCs from primary *h*PDL cultures and to determine possible phenotypic changes after their culture in the different endodontic sealer eluates, flow cytometry studies were carried out. With all the endodontic sealer tested, the MSC surface molecules, CD73, CD90 and CD105, were expressed at levels greater than 95%, whereas the expression of the hematopoietic markers, CD34 and CD45, was less than 5% (Fig. 3).

Representative two-dimensional dot plots of the distribution of vital (Annexin-V- 7-AAD−), early apoptotic (Annexin-V + 7-AAD−), late apoptotic and necrotic cells (Annexin-V + 7-AAD+ and Annexin-V− 7-AAD+) in untreated *h*PDLSCs, and in *h*PDLSCs after exposure to endodontic sealer eluates are shown in Fig. 4. BR revealed more than 87% of viable cells after exposure. NCS exhibited high rates of cell viability in concentrations of 1/2 and 1/4 and ES exhibited especially low rates of cell viability at concentrations of 1 and 1/2 (<10%).

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 BR eluates for 24 h promoted a cell migration level similar to that observed using complete medium (control). As shown in Fig. 5, treatment with extracts of BR significantly 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 the more concentrated dilutions of NCS (undiluted or 1/1 and 1/2), cell migration was significantly lower than the control ($P < 0.01$).

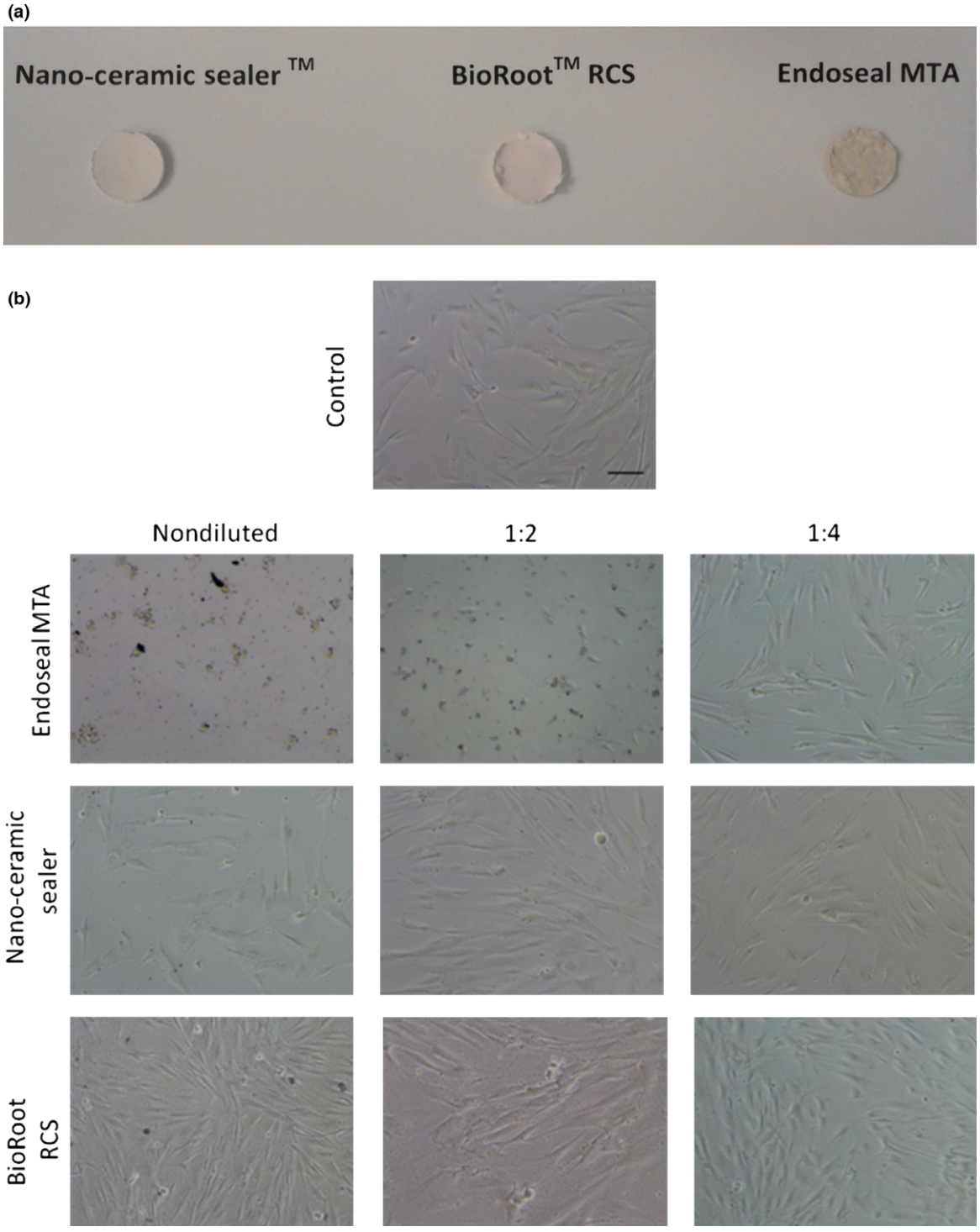

Figure 1 Behaviour of *h*PDLSCs exposed to the sealer extracts. (a) Macroscopic view of endodontic sealer discs. (b) Microphotographs showing *h*PDLSCs exposed to endodontic sealer eluates at 24 h. In the presence of ES eluates, had a lower number of attached cells than the control, whereas in the presence of NCS and BR, moderate and high levels of attached cells were observed, respectively. Phase-contrast microphotographs at 100×.

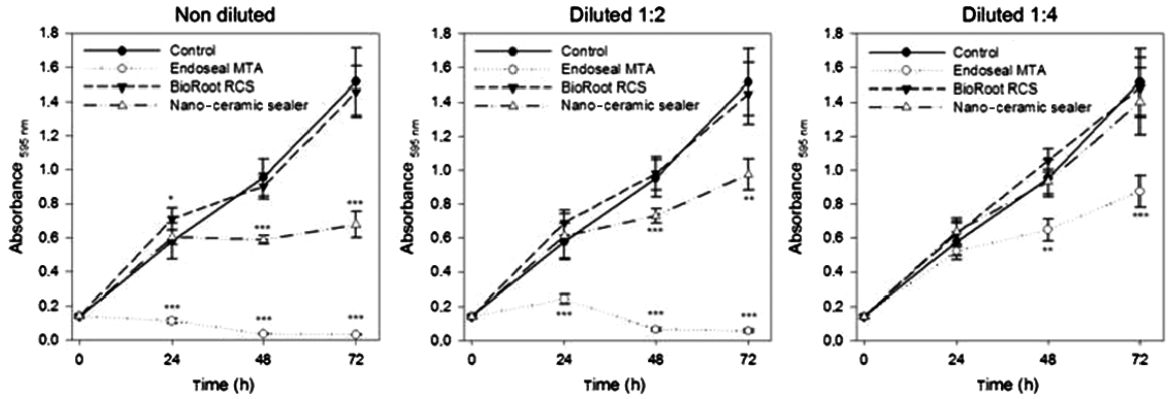

Figure 2 Cell proliferation and viability were determined using the 3-(4,5-dimethyl-thiazol)-2,5-diphenyl-tetrazolium bromide (MTT) assay; ES or NCS allowed a level of proliferation significantly lower compared to control (***$P < 0.001$). Also, level of *h*PDLSCs proliferation in the presence of undiluted BR was significantly higher to obtain using complete medium (control) at 24 h, whereas was similar than using control from 48 h. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

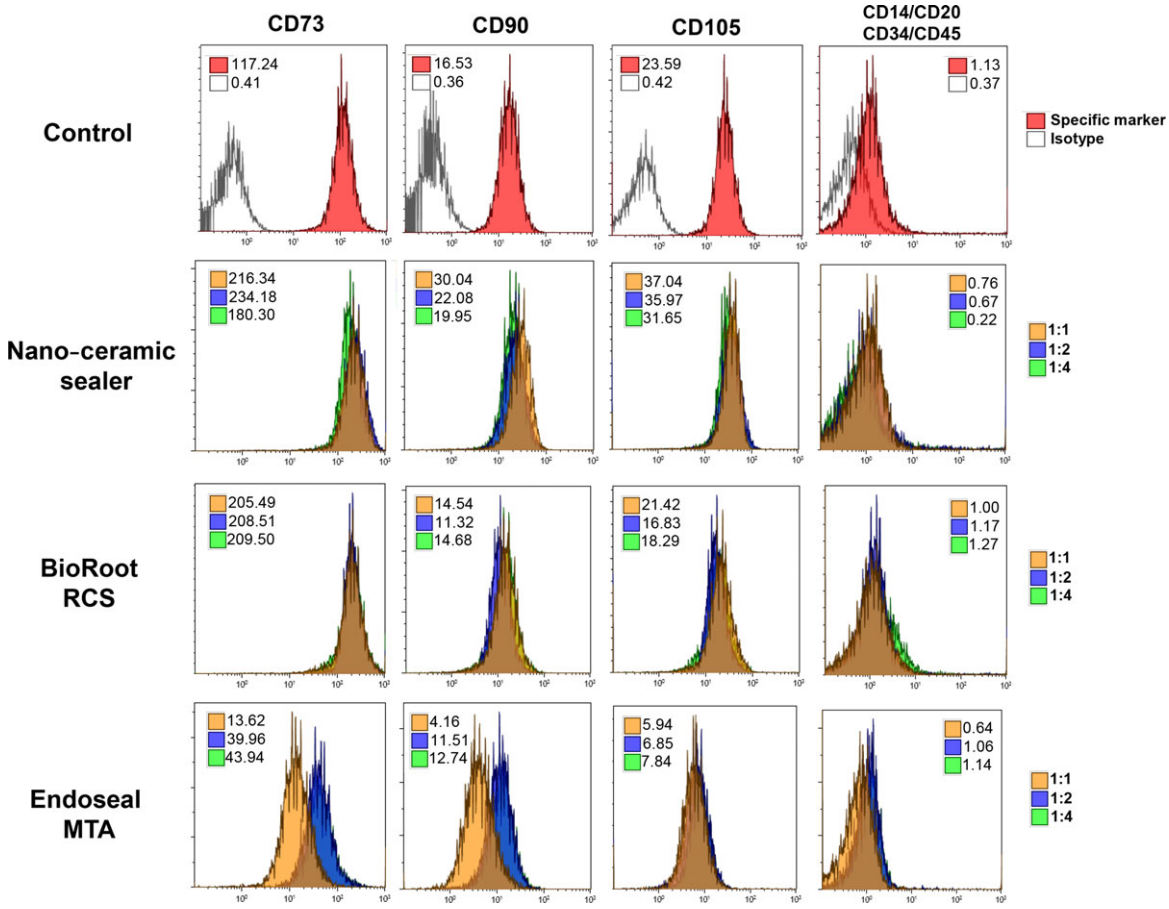

Figure 3 Mesenchymal phenotype analysis of *h*PDLSCs after culture on endodontic sealer discs by flow cytometry. After 72 h of culture, cells were detached and labelled with fluorescence-conjugated specific antibodies for the mesenchymal surface markers CD73, CD90 and CD105, and the hematopoietic markers CD14, CD20, CD34 and CD45. Insert numbers represent the mean fluorescence intensity values from viable cells. Histograms show representative flow cytometry results obtained after three independent experiments.

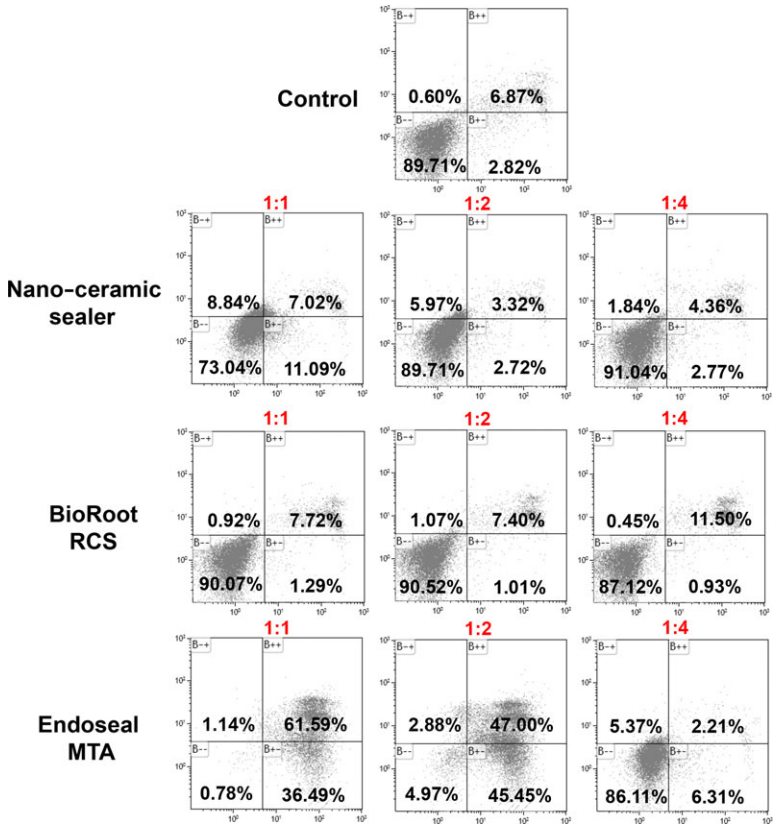

Figure 4 *h*PDLSCs were cultured on BR, NCS and ES and plastic (control) for 72 h. The *h*PDLSCs were then labelled with Annexin-V and 7-AAD and analysed by flow cytometry. Insert numbers within the different quadrants represent the percentages of live (Annexin-V−/7-AAD−), early apoptotic (Annexin-V+/7-AAD−) or late apoptotic and necrotic cells (Annexin-V+/7-AAD+ and Annexin-V−/7-AAD+). Dot plots show representative flow cytometry results obtained after three independent experiments.

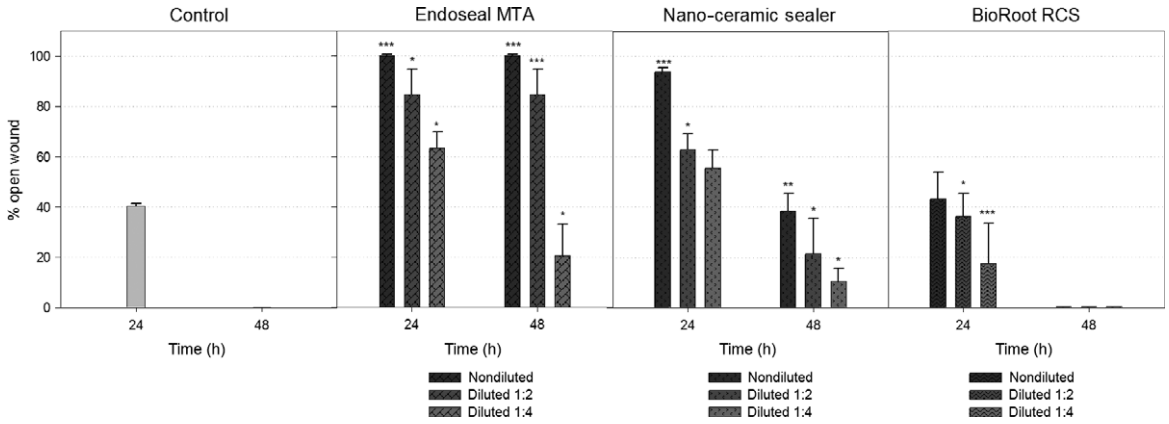

Figure 5 Cellular migration ability of *h*PDLSCs exposed to endodontic sealer extracts of BR, NCS and ES evaluated by an *in vitro* scratch wound-healing model; Confluent *h*PDLSCs were wounded and incubated with different dilutions of the endodontic sealer extracts up to 48 h. Cell migration was represented as the percentage of the open wound area for each condition and compared to control. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

However, *h*PDLSCs treated with ES did not migrate up to 24 h and only demonstrated slight migration using the 1/4 dilution after 48 h ($P < 0.005$).

*h*PDLSCs attachment on endodontic sealer discs

Surface analysis of the experimental materials was performed. The morphology and adhesion of *h*PDLSCs on the surface of BR, NCS and ES is shown in Fig. 6. The morphology of the cells seeded on BR and NCS had similar characteristics, with substantial cell spreading and the production of extracellular matrix. However, using ES discs, cell attachment was limited, with only a few round cells appearing on the surface of the material. These scanning electron microscopic findings are coherent with the MTT assay and cellular viability results.

Discussion

The results of this study showed that extracts from Bioroot BC Sealer, Endoseal MTA and Nano-ceramic Sealer had dose- and time-dependent effects on the biological responses of *h*PDLSCs. Due to the excellent properties of mineral trioxide aggregate (MTA), new endodontic materials based on MTA have emerged recently (Bin *et al.* 2012, Butt *et al.* 2014, Zhu *et al.* 2014, Lymperi *et al.* 2015, Utneja *et al.* 2015). However, the different compositions of MTA sealers are often unknown, and sealers have provided differing results for their biocompatibility *in vitro* and *in vivo* (Güven *et al.* 2013, Chang *et al.* 2014).

This study evaluated three commercially available materials, BR, ES and NCS, which were tested to evaluate their cytocompatibility on *h*PDLSCs. Human periodontal ligament stem cells were used for this study because periodontal tissues are involved in biological apical sealing (Silva *et al.* 2014). Moreover, *h*PDLSCs have important advantages such as being easily obtained from human teeth, are able to self-renew and differentiate into many cell types (Ding *et al.* 2015, Liu *et al.* 2015). Another benefit of using human mesenchymal cells is that they permit the action mechanism of new biomaterials or drugs to be tested on a patient's own cells, providing unprecedented human models to study their response (Jung *et al.* 2014, Rodríguez-Lozano *et al.* 2015). Previous reports found differences in responses between cells of various origins in the presence of endodontic sealer eluates. For example, Kim & Shin (2014) found that endoseal promoted higher proliferation on human gingival fibroblasts than on MG-63 cells, whereas Ehsani *et al.* (2012) revealed different responses between MG-63 and Sao-2 in the presence of AHPlus and 2Seal eluates. So, the *in vitro* method chosen to test the cytocompatibility of MTA sealers could significantly influence the results, whilst the difference between the susceptibility of each cell line to the type of elutes should not be ignored.

The MTT assay and the method of the double staining of cells with Annexin-V and 7-AAD were chosen to evaluate cell proliferation and cell viability, respectively. The advantages of the MTT procedure are the simplicity, accuracy, reliability and time-saving (Malkoc *et al.* 2010). Annexin-V staining precedes the loss of membrane integrity, which accompanies the latest stages of cell death, resulting from either apoptotic or

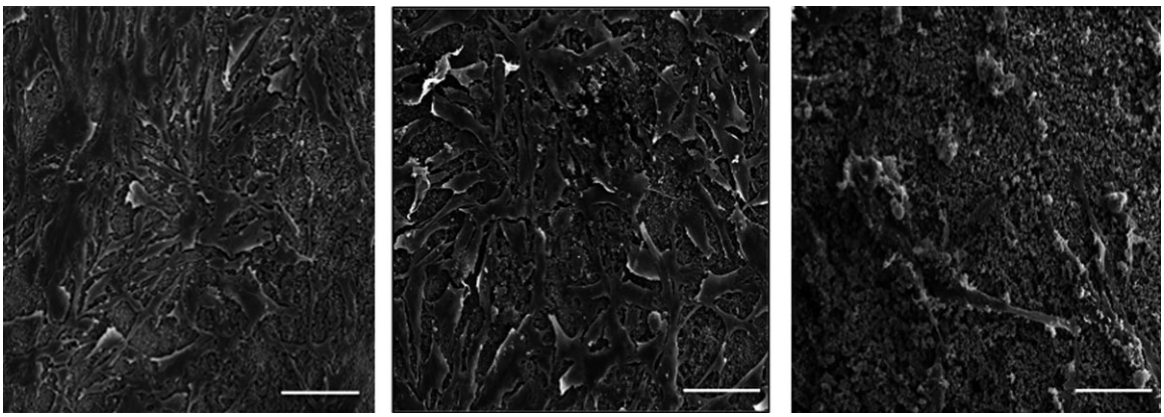

Figure 6 Scanning electron microscopic images of cells on BR, NCS and ES. The morphology of the cells seeded on BR and NCS had similar characteristics, with substantial cell spreading and the production of extracellular matrix. However, using ES discs, cell attachment was limited. Scale bar: 100×.

necrotic processes (Rodríguez-Lozano *et al.* 2015). Therefore, Annexin-V staining is typically used in combination with a vital dye such as 7-AAD to allow to identify the apoptosis in its different stages. Moreover, expression of mesenchymal stem cell markers and cell migration are two important processes to be analysed in regenerative medicine (Hayashi *et al.* 2015).

BR exhibited excellent cytocompatibility in terms of cell proliferation, cellular viability and cellular migration. These findings were consistent with results of previous studies. Dimitrova-Nakov *et al.* (2015) showed that mouse dental pulp exposed to BR continuously displayed the same morphology as control cells, and the cell sheet remained uniform. BR did not affect cell growth since the cell cultures reached confluence at 7 days after plating in the presence or absence of BR. On the other hand, Camps *et al.* (2015) evidenced that BR revealed less toxicity than pulp canal sealer in periodontal ligament stem cells and pointed to a significant increase in VEGF and FGF-2 secretion. However, it was considered necessary to make further studies of biocompatibility with human cells and those derived from periodontal tissues. ES exhibited low rates of cell proliferation and cell viability in concentrations of 1 and 1/2 (<10%). The results disagree with previous reports on ES, which suggested an acceptable degree of biocompatibility for ES (similar to MTA) (Kim & Shin 2014, Lim *et al.* 2015), although they did not use human periodontal ligament cells.

On the other hand, cell attachment on the material surface is regarded as the key determinant for assessing the performance of a new biomaterial (Rasekh *et al.* 2013). In the present study, scanning electron microscopy revealed a limited degree of cell adhesion on ES discs, whereas on BR and NCS discs, *h*PDLSCs covered the surface of the discs. In general, NCS revealed good cytocompatibility although less than BR. These results confirm that there are differences between commercially available bioceramic sealers (Güven *et al.* 2013, Candeiro *et al.* 2015), suggesting that unknown filler and thickening agents could play an important role in terms of biocompatibility. The lack of studies evaluating the cytocompatibility of nanoceramic sealers precludes direct comparison with the results of the present investigation.

Conclusions

BR and NCS exhibited better cytocompatibility than ES. Further *in vitro* and *in vivo* investigations are required to confirm the suitability of these calcium silicate-based endodontic sealers for clinical application.

Acknowledgements

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Conflict of interest

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

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