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Tesis Doctoral

Análisis de la citocompatibilidad, la bioactividad y la activación de vías de señalización celular inducida por nuevos biosilicatos hidráulicos de uso en Endodoncia Regenerativa

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A Manuela, Pepe y Francisco;
siempre conmigo.

D. Leopoldo Forner Navarro, Dª María Carmen Llena Puy, Catedrático y Catedrática, respectivamente, del Departamento de Estomatología, de la Facultad de Medicina i Odontología de la *Universitat de València (Estudi General)*, y D. Francisco Javier Rodríguez Lozano, Profesor Titular del Departamento de Dermatología, Estomatología, Radiología y Medicina Física, de la Facultad de Medicina de la Universidad de Murcia,

hacen constar que,

la tesis doctoral titulada "Análisis de la citocompatibilidad, la bioactividad y la activación de vías de señalización celular inducida por nuevos biosilicatos hidráulicos de uso en Endodoncia Regenerativa", presentada por el doctorando D. José Luis Sanz Aleixandre ha sido realizada bajo nuestra dirección y reúne las condiciones necesarias para su presentación y defensa.

Lo que firmamos a los efectos oportunos,

Prof. Dr. Forner Navarro Profa. Dra. Llena Puy Prof. Dr. Rodríguez Lozano

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Publicaciones presentadas

A continuación, se presentan las referencias bibliográficas de las 5 publicaciones que comprenden el compendio de artículos presentado en el presente proyecto de tesis doctoral:

Sanz, J. L., Guerrero-Gironés, J., Pecci-Lloret, M. P., Pecci-Lloret, M. R., & Melo, M. (2021). Biological interactions between calcium silicate-based endodontic biomaterials and periodontal ligament stem cells: A systematic review of in vitro studies. *International endodontic journal*, 54(11), 2025–2043. https://doi.org/10.1111/iej.13600

Sanz, J. L., López-García, S., Lozano, A., Pecci-Lloret, M. P., Llena, C., Guerrero-Gironés, J., Rodríguez-Lozano, F. J., & Forner, L. (2021). Microstructural composition, ion release, and bioactive potential of new premixed calcium silicate-based endodontic sealers indicated for warm vertical compaction technique. *Clinical oral investigations*, 25(3), 1451–1462. https://doi.org/10.1007/s00784-020-03453-8

Sanz, J. L., Soler-Doria, A., López-García, S., García-Bernal, D., Rodríguez-Lozano, F. J., Lozano, A., Llena, C., Forner, L., Guerrero-Gironés, J., & Melo, M. (2021). Comparative Biological Properties and Mineralization Potential of 3 Endodontic Materials for Vital Pulp Therapy: Theracal PT, Theracal LC, and Biodentine on Human Dental Pulp Stem Cells. *Journal of endodontics*, 47(12), 1896–1906. https://doi.org/10.1016/j.joen.2021.08.001

Sanz, J. L., Rodríguez-Lozano, F. J., Lopez-Gines, C., Monleon, D., Llena, C., & Forner, L. (2021). Dental stem cell signaling pathway activation in response to hydraulic calcium silicate-based endodontic cements: A systematic review of in vitro studies. *Dental materials*, 37(4), e256–e268. https://doi.org/10.1016/j.dental.2021.01.025

Sanz, J. L., López-García, S., Rodríguez-Lozano, F. J., Melo, M., Lozano, A., Llena, C., & Forner, L. (2022). Cytocompatibility and bioactive potential of AH Plus Bioceramic Sealer: An in vitro study. *International endodontic journal*, 55(10), 1066-1080 https://doi.org/10.1111/iej.13805

Publicaciones complementarias

A continuación, se presentan las referencias bibliográficas de otras publicaciones relacionadas producidas durante el desarrollo del presente proyecto de tesis doctoral:

Sanz, J. L., Forner, L., Almudéver, A., Guerrero-Gironés, J., & Llena, C. (2020). Viability and Stimulation of Human Stem Cells from the Apical Papilla (hSCAPs) Induced by Silicate-Based Materials for Their Potential Use in Regenerative Endodontics: A Systematic Review. *Materials*, 13(4), 974. https://doi.org/10.3390/ma13040974

Sanz, J. L., Forner, L., Llena, C., Guerrero-Gironés, J., Melo, M., Rengo, S., Spagnuolo, G., & Rodríguez-Lozano, F. J. (2020). Cytocompatibility and Bioactive Properties of Hydraulic Calcium Silicate-Based Cements (HCSCs) on Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs): A Systematic Review of In Vitro Studies. *Journal of clinical medicine*, 9(12), 3872. https://doi.org/10.3390/jcm9123872

Ghilotti, J., **Sanz, J. L.**, López-García, S., Guerrero-Gironés, J., Pecci-Lloret, M. P., Lozano, A., Llena, C., Rodríguez-Lozano, F. J., Forner, L., & Spagnuolo, G. (2020). Comparative Surface Morphology, Chemical Composition, and Cytocompatibility of Bio-C Repair, Biodentine, and ProRoot MTA on hDPCs. *Materials*, 13(9), 2189. https://doi.org/10.3390/ma13092189

Rodríguez-Lozano, F. J., López-García, S., García-Bernal, D., **Sanz, J. L.**, Lozano, A., Pecci-Lloret, M. P., Melo, M., López-Ginés, C., & Forner, L. (2021). Cytocompatibility and bioactive properties of the new dual-curing resin-modified calcium silicate-based material for vital pulp therapy. *Clinical oral investigations*, 25(8), 5009–5024. https://doi.org/10.1007/s00784-021-03811-0

Muedra, P., Forner, L., Lozano, A., **Sanz, J. L.**, Rodríguez-Lozano, F. J., Guerrero-Gironés, J., Riccitiello, F., Spagnuolo, G., & Llena, C. (2021). Could the Calcium Silicate-Based Sealer Presentation Form Influence Dentinal Sealing? An In Vitro Confocal Laser Study on Tubular Penetration. *Materials*, 14(3), 659. https://doi.org/10.3390/ma14030659

García-Bernal, D., López-García, S., **Sanz, J. L.**, Guerrero-Gironés, J., García-Navarro, E. M., Moraleda, J. M., Forner, L., & Rodríguez-Lozano, F. J. (2021). Melatonin Treatment Alters Biological and Immunomodulatory Properties of Human Dental Pulp Mesenchymal Stem Cells via Augmented Transforming Growth Factor Beta Secretion. *Journal of endodontics*, 47(3), 424–435. https://doi.org/10.1016/j.joen.2020.12.008

Rodríguez-Lozano, F. J., Lozano, A., López-García, S., García-Bernal, D., **Sanz, J. L.**, Guerrero-Gironés, J., Llena, C., Forner, L., & Melo, M. (2022). Biomineralization potential and biological properties of a new tantalum oxide (Ta2O5)-containing calcium silicate cement. *Clinical oral investigations*, 26(2), 1427–1441. https://doi.org/10.1007/s00784-021-04117-x

Guerrero-Gironés, J., Forner, L., **Sanz, J. L.***, Rodríguez-Lozano, F. J., Ghilotti, J., Llena, C., Lozano, A., & Melo, M. (2022). Scientific production on silicate-based endodontic materials: evolution and current state: a bibliometric analysis. *Clinical oral investigations*. https://doi.org/10.1007/s00784-022-04605-8

Ortiz-Blanco, B.; **Sanz, J.L.***; Llena, C.; Lozano, A.; Forner, L. (2022). Dentin Sealing of Calcium Silicate-Based Sealers in Root Canal Retreatment: A Confocal Laser Microscopy Study. *Journal of Functional Biomaterials*, 13, 114. https://doi.org/10.3390/jfb13030114

Lozano-Guillén, A.; López-García, S.; Rodríguez-Lozano, F.J.; **Sanz, J.L.***; Lozano, A.; Llena, C.; Forner, L. (2022) Comparative cytocompatibility of the new calcium silicate-based cement NeoPutty versus NeoMTA Plus and MTA on human dental pulp cells: an in vitro study. *Clinical Oral Investigations*. https://doi.org/10.1007/s00784-022-04682-9

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Índice

RESUMEN GRÁFICO

Análisis de la citocompatibilidad, la bioactividad y la activación de vías de señalización celular inducida por nuevos biosilicatos hidráulicos de uso en Endodoncia Regenerativa

Objetivo general:

Evaluar la citocompatibilidad y las propiedades bioactivas de materiales basados en silicato de calcio con respecto a las células madre pulpares y periodontales y explorar los mecanismos detrás de su interacción.

Fase 1: Revisión sistemática:

Metodología: Síntesis cualitativa de estudios in vitro sobre la citocompatibilidad y propiedades bioactivas de los cementos y selladores basados en silicato de calcio con respecto a las células madre del ligamento periodontal humanas.
Resultados: Los cementos y selladores basados en silicato de calcio presentan una adecuada citocompatibilidad y favorecen la diferenciación osteo/cementogénica y el potencial de mineralización de las células madre del ligamento periodontal humanas.

Fase 2: Estudio experimental sobre hPDLSCs:

Metodología: Evaluación de la citocompatibilidad y propiedades bioactivas del Bio-C Sealer ION+ en comparación con el EndoSequence BC Sealer HiFlow y el AH Plus con respecto a las células madre del ligamento periodontal humanas.
Resultados: El Bio-C Sealer ION+ y el Endosequence BC Sealer HiFlow muestran una alta liberación de iones de calcio, una adecuada citocompatibilidad y propiedades bioactivas, ambas superiores a las del AH Plus, con respecto a las células madre del ligamento periodontal humanas.

Fase 3: Estudio experimental sobre hDPSCs:

Metodología: Evaluación de la citocompatibilidad y propiedades bioactivas del Theracal PT en comparación con el Theracal LC y el Biodentine con respecto a las células madre de la pulpa dental humanas.
Resultados: El Theracal PT muestra una citocompatibilidad aumentada en comparación con el Theracal LC y propiedades bioactivas comparables a las del Biodentine con respecto a las células madre de la pulpa dental humanas.

Fase 4: Estudio experimental sobre hPDLSCs:

Metodología: Evaluar la citocompatibilidad y las propiedades bioactivas del AH Plus bioceramic sealer en comparación con el EndoSequence BC Sealer HiFlow y el AH Plus con respecto a las células madre del ligamento periodontal humanas.
Resultados: El AH Plus Bioceramic Sealer y el Endosequence BC Sealer muestran una adecuada citocompatibilidad y propiedades bioactivas, ambas superiores a las del sellador AH Plus, con respecto a las células madre del ligamento periodontal humanas.

Fase 5: Revisión sistemática:

Metodología: Síntesis cualitativa de la evidencia in vitro disponible sobre la activación de vías de señalización celular durante la interacción entre los cementos basados en silicato de calcio y las células madre dentales.
Resultados: Los resultados de los ensayos sobre la expresión de marcadores relacionados con la actividad celular y la formación de nódulos mineralizados sugieren un papel activo de las vías de señalización MAPK, NF-κB, Wnt/β-catenina, BMP/Smad y CAMKII como posibles mediadores en la interacción biológica entre las células madre dentales y los cementos de silicato de calcio.

GRAPHICAL ABSTRACT

Analysis of the cytocompatibility, bioactivity and activation of cell signaling pathways induced by new hydraulic biosilicate materials for their use in Regenerative Endodontics.

General aim:
To evaluate the cytocompatibility and bioactive properties of calcium silicate-based materials on dental pulp stem cells and periodontal stem cells and to explore the mechanisms behind their interaction.

Fase 1: Systematic review:
Methodology: Qualitative synthesis of *in vitro* studies on the cytocompatibility and bioactive properties of hydraulic calcium silicate-based cements and sealers on human periodontal ligament stem cells.
Results: Calcium silicate-based cements and sealers exhibit adequate cytocompatibility and promote the osteo/cementogenic differentiation and mineralization potential of human periodontal ligament stem cells.

Fase 2: Experimental study on hPDLSCs:
Methodology: Evaluation of the cytocompatibility and bioactive properties of Bio-C Sealer ION+ compared to EndoSequence BC Sealer HiFlow and AH Plus with on human periodontal ligament stem cells.
Results: Bio-C Sealer ION+ and Endosequence BC Sealer HiFlow show high calcium ion release, adequate cytocompatibility and bioactive properties, both superior to AH Plus, on human periodontal ligament stem cells..

Fase 3: Experimental study on hDPSCs:
Methodology: Evaluation of the cytocompatibility and bioactive properties of Theracal PT compared to Theracal LC and Biodentine on human dental pulp stem cells..
Results: Theracal PT shows increased cytocompatibility compared to Theracal LC and bioactive properties comparable to those of Biodentine on human dental pulp stem cells.

Fase 4: Experimental study on hPDLSCs:
Methodology: To evaluate the cytocompatibility and bioactive properties of AH Plus bioceramic sealer compared to EndoSequence BC Sealer HiFlow and AH Plus on human periodontal ligament stem cells.
Results: AH Plus Bioceramic Sealer and Endosequence BC Sealer show adequate cytocompatibility and bioactive properties, both superior to those of AH Plus sealer, on human periodontal ligament stem cells.

Fase 5: Systematic review:
Methodology: Qualitative synthesis of the available *in vitro* evidence on the activation of cell signaling pathways during the interaction between calcium silicate-based cements and dental stem cells.
Results: The results of the assays from the included studies on the expression of markers related to cellular activity and mineralized nodule formation suggest an active role of MAPK, NF-κB, Wnt/β-catenin, BMP/Smad and CAMKII signaling pathways as possible mediators in the biological interaction between dental stem cells and calcium silicate cements.

RESUMEN / SUMMARY

Introduction

Dental stem cells are a subgroup of postnatal stem cells which display a mesenchymal stem cell-like phenotype. According to their origin, they can be classified into dental pulp stem cells, periodontal ligament stem cells, stem cells from the apical papilla, stem cells from human exfoliated deciduous teeth and dental follicle stem cells. Due to their multilineage differentiation potential and their implication in the reparative processes from the dentin-pulp complex and periodontal structures, they have aroused a substantial interest within the field of Regenerative Dentistry and Medicine for their potential application in stem cell-based treatments and tissue engineering.

The biomaterials to be applied in stem cell-based treatments and tissue engineering approaches need to exhibit certain biological properties, namely, biocompatibility and bioactivity. Biocompatibility refers to the absence of negative response from a living tissue in contact with a (bio)material. In other words, a material will be categorized as biocompatible if it is able to perform the desired function in accordance with the treatment for which it has been applied, without hindering the correct functioning and physiological conditions of the surrounding living tissues. The same term, applied at a cellular level, is referred to as cytocompatibility.

In the field of study of dental materials, cytocompatibility is often assessed by means of the analysis of the influence of a specific material on cell viability, proliferation/migration, adhesion and/or morphology. A material that interferes negatively with any of these parameters will be considered cytotoxic.

Bioactivity goes one step further and implies a positive response from a living tissue induced or stimulated by a (bio)material in contact with it. Applied to endodontic materials, bioactivity translates into the induction of the formation of a mineral attachment between the bioactive material and the dentin substrate by

means of an ionic exchange with the tissue fluids from the surrounding microenvironment. This phenomenon is known as biomineralization. On the other hand, within the study of material-stem cell interaction, bioactivity acquires an alternative meaning: the influence of the material on cell plasticity; that is, the ability of the material to induce differentiation of a stem cell to a specific lineage.

In the field of study of dental materials, both definitions of bioactivity are often assessed. Biomineralization potential is usually analyzed by quantifying the production of mineralized/calcified nodules by DSCs in contact with the study material and/or by analyzing the release of ions. Complementarily, for the analysis of the influence of a material on cellular plasticity it is common to measure the expression of genetic or protein markers of cell differentiation, so that an overexpression of a given marker is indicative of the positive influence of the study material on cell differentiation specific for that marker.

Among endodontic biomaterials, hydraulic calcium silicate-based materials appear as promising candidates for biologically based endodontic procedures and tissue engineering approaches. As a subgroup of bioceramics, these materials have exhibited both an adequate cytocompatibility and bioactive properties on cellular studies and animal models. High success rates have also been observed in clinical studies when used as pulp cappers, root-end filling materials, root repair materials and root canal sealers. During the treatments, calcium silicate-based materials will be placed in contact with surrounding tissues and their cellular component. Therefore, these materials should express adequate biological properties to not interfere or even to enhance the reparative processes responsible for the successful outcome of the treatments.

Variations in the composition of calcium silicate-based materials could lead to differences in their clinical behavior, as has been previously described. This fact, added to the constant introduction of new material compositions into the market calls for research on their biological properties prior to their clinical application in daily endodontic practice.

Aim

The primary aim of the present PhD thesis project was to evaluate the cytocompatibility and bioactive properties of calcium silicate-based cements and sealers on dental pulp and periodontal stem cells and to explore the mechanisms behind their interaction.

Within this framework, a total of 5 studies are presented. Their specific aims are as follows:

Study 1: To perform a qualitative synthesis of the available *in vitro* evidence on the cytocompatibility and bioactive properties of calcium silicate-based cements and sealers on human periodontal ligament stem cells.

Study 2: To evaluate the cytocompatibility and bioactive properties of a new calcium silicate-based sealer (Bio-C Sealer ION+) in comparison with another calcium silicate-based sealer (EndoSequence BC Sealer HiFlow) and an epoxy resin-based sealer (AH Plus) on human periodontal ligament stem cells.

Study 3: To evaluate the cytocompatibility and bioactive properties of a new dual-curing resin-modified calcium silicate cement (Theracal PT) in comparison with a light-curing resin-modified calcium silicate cement (Theracal LC) and a hydraulic calcium silicate cement (Biodentine) on human dental pulp stem cells.

Study 4: To evaluate the cytocompatibility and bioactive properties of a new calcium silicate-based sealer (AH Plus bioceramic sealer) in comparison with another calcium silicate-based sealer (EndoSequence BC Sealer HiFlow) and an epoxy resin-based cement (AH Plus) on human periodontal ligament stem cells.

Study 5: To perform a qualitative synthesis of the available *in vitro* evidence on the activation of cell signaling pathways during the interaction between calcium silicate-based cements and dental stem cells.

Materials and Methods

Study 1: An advanced search was performed in 5 databases (Medline, Scopus, Web of Science, Embase, and SciELO). *In vitro* studies which assessed the cytocompatibility and/or bioactivity of calcium silicate-based cements and or sealers on human periodontal ligament stem cells were eligible for inclusion. For the data extraction, variables were subdivided into methodological and outcome variables. Methodological variables included data on the cell variant, material/s studied and their concentration, tests performed and their duration. Outcome variables included the significant results found for each test, the time in which they were recorded (duration) and their level of statistical significance as a p-value. Quality assessment was performed using a modified CONSORT checklist for the assessment of *in vitro* studies on dental materials. A qualitative synthesis of the eligible studies was performed.

Study 2: The cytocompatibility and bioactivity of Bio-C Sealer ION+, EndoSequence BC Sealer HiFlow and AH Plus were assessed on human periodontal ligament stem cells. Tests for cytocompatibility included: MTT assay (cell viability), wound healing assay (cell migration/proliferation), and scanning electron microscopy (cell attachment and morphology). Tests for bioactivity included: RT-qPCR (cell osteo/odonto/cementogenic marker expression), Alizarin Red S staining (cell mineralization potential), and ion release (material biomineralization potential).

Study 3: The cytocompatibility and bioactivity of Theracal PT, Theracal LC and Biodentine were assessed on human dental pulp stem cells. Tests for cytocompatibility included: MTT assay (cell viability), Annexin V-FITC / 7-AAD (cell apoptosis), reactive oxygen species release (cell viability), wound healing assay (cell migration/proliferation), and scanning electron microscopy (cell attachment and morphology). Tests for bioactivity included: RT-qPCR (cell osteo/odonto/cementogenic marker expression), and Alizarin Red S staining (cell mineralization potential).

Study 4: The cytocompatibility and bioactivity of AH Plus Bioceramic Sealer, Endosequence BC Sealer and AH Plus were assessed on human periodontal ligament stem cells. Tests for cytocompatibility included: MTT assay (cell viability), wound healing assay (cell migration/proliferation), and scanning electron microscopy (cell attachment and morphology). Tests for bioactivity included: RT-qPCR (cell osteo/odonto/cementogenic marker expression), and Alizarin Red S staining (cell mineralization potential).

Study 5: An advanced search was performed in 5 databases (Medline, Scopus, Web of Science, Embase, and SciELO). *In vitro* studies which assessed the implication of cell signaling pathways in the interaction between calcium silicate-based cements and dental stem cells were eligible for inclusion. For the data extraction, variables were subdivided into methodological and outcome variables. Methodological variables included data on the subtype of dental stem cells used, materials used and their concentration, signaling pathways studied, activity assays performed and their duration, and specific markers and/or inhibitors of the pathways used for such assays. Outcome variables included the significant results found, the time at which they were recorded (duration) and their level of statistical significance as a p-value. Quality assessment was performed using a modified CONSORT checklist for the assessment of *in vitro* studies on dental materials. A qualitative synthesis of the eligible studies was performed.

Results

Study 1: Overall, the different calcium silicate-based cements and sealers assessed by the included studies exhibited at least similar results to a negative control in cytocompatibility assays. Furthermore, all calcium silicate-based cements tested for their influence in hPDLSCs plasticity resulted in an overexpression of osteogenic, cementogenic and/or odontogenic markers, suggesting their positive influence on their differentiation.

Studies 2 and 4: The tested calcium silicate-based sealers showed positive results in the cytocompatibility assays. In addition, it was observed that the higher dilutions of the materials produced better results. All calcium silicate-based

sealers exhibited an overexpression of osteo/cement/odontogenic markers and the formation of calcified nodules. The epoxy resin-based sealer exhibited negative results both in terms of cytocompatibility and bioactivity in both studies.

Study 3: The tested dual curing resin-modified calcium silicate cement and the calcium silicate-based cement showed positive results in the cytocompatibility assays. Both materials also exhibited an overexpression of osteo/cement/odontogenic markers and the formation of calcified nodules. The light curing resin-modified calcium silicate cement exhibited negative results both in terms of cytocompatibility and bioactivity.

Study 5: Overall, the expression of cell differentiation-related markers and mineralization assays evidenced the involvement of MAPK (and its subfamilies ERK, JNK and P38), NF-κB, Wnt/β-catenin, BMP/Smad and CAMKII pathways in the biological response of DSCs to the studied CSCs.

Conclusions

The results of the present doctoral thesis project have led to four main contributions:

- The cytocompatibility and bioactive properties of hydraulic calcium silicate-based materials on human periodontal ligament stem cells have been confirmed by the qualitative synthesis of the available evidence in this regard.
- The favorable biological properties of two new formulations of calcium silicate-based sealers on human periodontal ligament stem cells have been preliminarily elucidated.
- The favorable biological properties of a new dual curing resin-modified calcium silicate cement formulation on human dental pulp stem cells have been preliminarily elucidated.
- The involvement of several cell signaling pathways in the interaction between calcium silicate-based cements and dental stem cells has been described by the qualitative synthesis of the available evidence in this regard.

ABREVIATURAS

Biología celular

MSCs	*Mesenchymal stem cells* (células madre mesenquimales)
DSCs	*Dental stem cells* (células madre dentales)
DPSCs	*Dental pulp stem cells* (células madre de la pulpa dental)
PDLSCs	*Periodontal ligament stem cells* (células madre del ligamento periodontal)
SCAPs	*Stem cells from the apical papilla* (células madre de la papila apical)
DFSCs	*Dental follicle stem cells* (células madre del folículo dental)
SHEDs	*Stem cells from human exfoliated deciduous teeth* (células madre de dientes deciduos)
ALP	*Alkaline phosphatase* (fosfatasa alcalina)
BSP	*Bone sialoprotein* (sialoproteína de hueso)
CAP	*Cementum attachment protein* (proteína de adherencia al cemento)
CEMP-1	*Cementum protein 1* (proteína recombinante del cemento)
Runx2	Runt-related transcription factor 2 (factor de transcripción 2 relacionado con Runt)

Materiales dentales

CSCs	*Calcium silicate-based cements* (cementos basados en silicato de calcio)
CSSs	*Calcium silicate-based sealers* (selladores basados en silicato de calcio)
MTA	*Mineral trioxide aggregate* (agregado de trióxido mineral)

Ensayos biológicos

MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (Bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio)
SEM	*Scanning electron microscopy* (microscopía electrónica de barrido)
RT-qPCR	*Reverse transcriptase quantitative polymerase chain reaction* (reacción en cadena de la polimerasa cuantitativa con transcriptasa inversa)
ARS	*Alizarin red s staining* (tinción con rojo de alizarina s)
ROS	*Reactive oxygen species* (especies reactivas de oxígeno)
SCRM	*Single-cell Raman spectroscopy* (espectroscopia Raman unicelular)

Ensayos fisicoquímicos

EDX	*Energy dispersive x-ray spectroscopy* (espectroscopía de energía dispersiva de rayos X)
XRD	*X ray diffraction* (difracción de rayos X)
ATR-FTIR	*Attenuated total reflection – Fourier-transform infrared spectroscopy* (Espectroscopia de reflexión total atenuada-infrarroja transformada de Fourier)

Enfoques terapéuticos

OR	Odontología regenerativa
RET	*Regenerative endodontic treatment* (tratamiento de endodoncia regenerativa)
VPT	*Vital pulp treatment* (tratamiento pulpar vital)

Otros

ESE	*European Society of Endodontology* (Sociedad Europea de Endodoncia)
AAE	*American Association of Endodontists* (Asociación Americana de Endodoncistas)
PRISMA	*Preferred reporting items for systematic reviews and meta-analyses* (criterios para la presentación de datos de revisiones sistemáticas y metaanálisis)

RESUMEN GLOBAL

A continuación, se presenta un resumen global de la temática, de los principales resultados y conclusiones. El resumen se encuentra estructurado en 7 apartados: 1. Introducción; 2. Justificación; 3. Objetivos; 4. Metodología; 5. Resultados; 6. Discusión; 7. Conclusiones.

1. Introducción

La Odontología Regenerativa (OR) es un campo emergente que integra tres ramas principales: la biología celular, los biomateriales dentales y la ingeniería tisular. Su estudio y práctica involucran las ciencias básicas y clínicas que tienen como objetivo restaurar y mantener la vitalidad de los tejidos dentales y perirradiculares [1]. Dentro de la Endodoncia, el desarrollo de la OR ha supuesto un avance en su práctica clínica, en la que destaca la aparición de los procedimientos de Endodoncia Regenerativa [2, 3].

1.1. La biología celular en la OR

Dentro de la rama de la OR referente a la biología celular, se engloban todos los procesos fisiológicos de defensa que poseen los tejidos dentales y perirradiculares frente a estímulos nocivos. El estudio de los mecanismos celulares y moleculares implicados en dichos procesos podría dar lugar al desarrollo de nuevos *biologically-based regenerative procedures* (tratamientos regenerativos basados en la biología) que aprovechen sus potenciales reparadores y/o regeneradores intrínsecos [4].

La dentición temporal y permanente se encuentra expuesta a numerosos factores potencialmente nocivos. Entre ellos, la caries y los traumatismos dentales suponen un riesgo frecuente para la integridad de los tejidos que conforman el diente y la vitalidad de éste [5, 6]. Frente a ellos, el complejo dentino-pulpar cuenta con una serie de mecanismos de defensa, recogidos en el concepto de la “dentinogénesis terciaria” [7].

La dentinogénesis terciaria comprende el proceso de producción de dentina en la interfase dentino-pulpar en respuesta a un estímulo nocivo. Se conoce como dentina reactiva a aquella secretada por odontoblastos post-mitóticos supervivientes a un estímulo leve o moderado. Por otra parte, ante un estímulo grave que resulte en la necrosis local del estrato odontoblástico cercano a la lesión, será una nueva generación de células similares a los odontoblastos o

neo-odontoblastos la que secrete una matriz de dentina terciaria, conocida como dentina reparativa [8].

En este último caso, se requiere de un proceso previo de división celular, quimiotaxis, migración, adhesión y citodiferenciación de las conocidas como células madre de la pulpa dental o *DPSCs* (*dental pulp stem cells*) hacia una estirpe neo-odontoblástica, para la adquisición de la capacidad de síntesis y secreción de matriz dentinaria terciaria [9].

Las *DPSCs* son un subtipo de célula madre dental o *DSC* (*dental stem cell)*. En conjunto, las DSCs, como subgrupo de células madre postnatales, han demostrado ser positivas para los marcadores específicos de las células madre mesenquimales o *MSCs* (*mesenchymal stem cells*): Stro-1, CD146, CD106 y CD44; indicando que poseen un potencial de diferenciación multilinaje. Específicamente, se ha confirmado su potencial de diferenciación osteogénico, odontogénico, condrogénico, adipogénico y miogénico [10]. Además, se ha descrito que poseen propiedades inmunomoduladoras [11]. Por ello, han despertado el interés dentro del campo de la Odontología y Medicina Regenerativas para su potencial aplicación en los *stem cell-based therapies* (tratamientos basados en células madre) [12].

Dentro del subgrupo de las células madre derivadas de los tejidos dentales, se han categorizado 5 subtipos de células (Figura 1.1): las previamente mencionadas *DPSCs*, las células madre del ligamento periodontal o *PDLSCs* (*periodontal ligament stem cells*), las células madre de la papila apical o *SCAPs* (*stem cells from the apical papilla*), las células madre de los dientes deciduos exfoliados o *SHEDs* (*stem cells from human exfoliated dediduous teeth*) y las células madre del folículo dental *DFSCs* (*dental follicle stem cells*) [13].

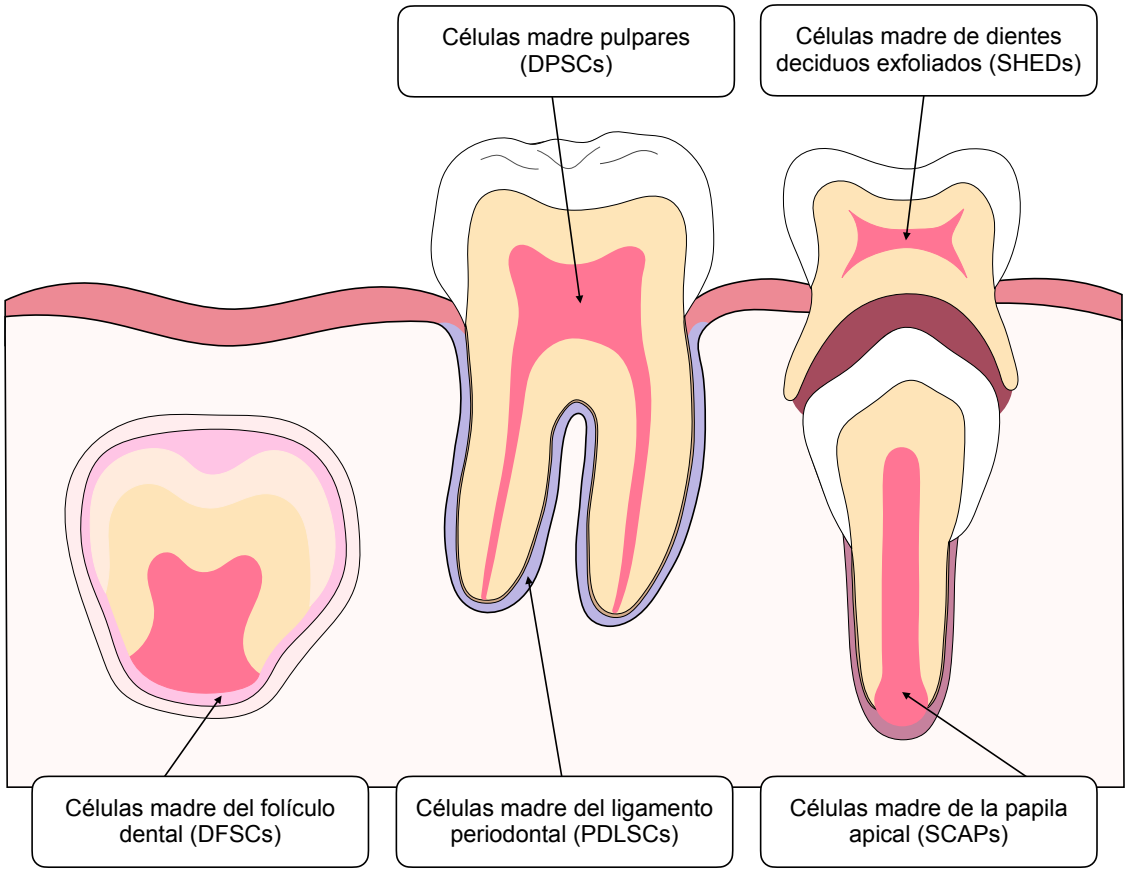

Figura 1.1. Ilustración esquemática de la clasificación de las células madre de origen dental o *dental stem cells* (*DSCs*) por su origen. Imagen original basada en la clasificación descrita por Huang et al. (2009) [13].

Las *DPSCs* fueron las primeras células madre orofaciales identificadas y aisladas a partir del tejido pulpar de dientes permanentes de pacientes adultos [14], mostrando una alta capacidad de proliferación y de diferenciación a odontoblastos; capaces a su vez de producir un tejido con una estructura similar a la dentina rodeado de un tejido intersticial similar al pulpar [15]. Más tarde, estas mismas células fueron aisladas a partir de gérmenes dentarios de terceros molares en formación, confirmándose que, en etapas de desarrollo tempranas, poseen ventajas funcionales frente a las aisladas a partir de dientes adultos [16].

Posteriormente, se caracterizaron y aislaron células madre con fenotipo mesenquimal a partir del tejido pulpar de los dientes temporales, conocidas actualmente como *SHEDs*. Estas células mostraron una mayor capacidad clonogénica, proliferativa y osteogénica que las *DPSCs* [17]. Además, en cuanto

a la diferenciación a un linaje específico, las *SHEDs* y las *DPSCs* muestran capacidades discriminadas, reflejando que existen variaciones genéticas entre las distintas fuentes del mismo subgrupo de células [18].

El folículo dental representa otra fuente para el aislamiento de un subtipo de *DSCs*: las *DFSCs*, las cuales están involucradas en el proceso de formación y desarrollo del periodonto [19]. Estas células, al igual que las *SHEDs*, poseen una gran accesibilidad, ya que pueden ser aisladas a partir de los gérmenes dentarios de los terceros molares, que habitualmente son extraídos por motivos ortodóncicos [20]. Además, han demostrado un potencial de diferenciación osteogénico [20] y cementogénico [21] en ensayos *in vivo*. Por ello, han sido propuestas como candidatas potenciales para la regeneración de los tejidos periodontales en procedimientos de ingeniería tisular [22].

El ligamento periodontal supone una fuente de otro subtipo de *DSCs*: las *PDLSCs* [23]. Varios estudios *in vitro* han descrito su potencial de diferenciación osteogénico, adipogénico y condrogénico [24–26]. En líneas generales, la evidencia disponible destaca la importancia de las *PDLSCs* en el proceso del mantenimiento de la homeostasis del periodonto y, por tanto, se consideran una fuente celular prometedora para los tratamientos reparativos que implican la reparación dichos tejidos [27, 28].

Por otro lado, los dientes en formación presentan un último subtipo de *DSCs* en el interior de la papila apical: las *SCAPs* [29]. Dichas células contribuyen a las funciones principales de la papila apical, a saber, la formación de la raíz dentaria y la pulpa radicular; entre otros [30]. Las *SCAPs* muestran una proliferación mayor que las *DPSCs* y las *PDLSCs* [31, 32], pero menor que las DFSCs [33]. Además, las *SCAPs* presentan un mayor potencial de mineralización y una mayor capacidad de regeneración de dentina en comparación con las *DPSCs* [34].

En resumen, se ha observado que las *DSCs* de distinto origen o fuente exhiben características biológicas distintas, lo que condiciona sus potenciales aplicaciones dentro del campo de la OR. Por ello, entre la bibliografía disponible,

se observa que su estudio no se realiza en conjunto, como subgrupo de *MSCs*, sino que se analizan las propiedades y posibles aplicaciones de cada subtipo celular de forma independiente [35].

1.2. Los biomateriales dentales en la OR

La segunda rama de la OR hace referencia al estudio de las propiedades de los materiales que serán puestos en contacto con los tejidos vivos, y de la respuesta de estos hacia ellos. Entre las propiedades biológicas imprescindibles en un material de aplicación en procedimientos terapéuticos basados en el paradigma de la OR destacan la biocompatibilidad y la bioactividad [36, 37].

La biocompatibilidad hace referencia a la ausencia de respuesta negativa por parte de un tejido vivo puesto en contacto con un (bio)material. En otras palabras, un material será categorizado como biocompatible si es capaz de desempeñar la función deseada de acuerdo con el tratamiento para el cual se ha aplicado, sin perjuicio del correcto funcionamiento y de las condiciones fisiológicas de los tejidos vivos circundantes. El mismo concepto, a nivel celular, se conoce como citocompatibilidad [38, 39]. Habitualmente, esta última propiedad es evaluada *in vitro* mediante el análisis de la influencia del material de estudio sobre la viabilidad, proliferación/migración, adhesión y/o morfología celular (Figura 1.2ª). Aquel material que interfiera en alguno de estos parámetros de forma negativa será considerado como citotóxico [40].

La bioactividad supone un paso más allá, e implica una respuesta positiva por parte de un tejido vivo inducida o estimulada por un (bio)material en contacto con éste. Este término ha sido utilizado ampliamente entre la bibliografía sobre los materiales dentales, diluyéndose su significado original. Técnicamente, dentro de la ciencia de los biomateriales, la bioactividad refleja la capacidad de un material de formar hidroxiapatita en su superficie [41].

Aplicado a los materiales de uso endodóntico, la bioactividad se traduce en la inducción de la formación de una unión mineral entre el material bioactivo y el sustrato dentinario, mediante un intercambio iónico con los fluidos tisulares del

microambiente circundante. A este fenómeno se le conoce como biomineralización [41].

Por otra parte, dentro del estudio de la interacción material-célula madre, la bioactividad adquiere un significado alternativo: la influencia del material en la plasticidad celular; es decir, la capacidad del material de inducir la diferenciación de una célula madre a un linaje específico. En referencia a la interacción entre los materiales de uso endodóntico y las *DSCs*, estos serán considerados bioactivos si inducen la diferenciación de uno o más subtipos de *DSCs* a un linaje osteogénico, odontogénico, cementogénico y/o angiogénico; entre otros [42].

Por lo tanto, para la evaluación *in vitro* de la bioactividad de un material de uso endodóntico, han de tenerse en consideración ambas definiciones. Entre los estudios sobre la interacción material-*DSC*, el potencial de biomineralización se analiza, habitualmente, mediante la cuantificación de la producción de nódulos mineralizados/calcificados por parte de las *DSCs* en contacto con el material de estudio y/o mediante el análisis de la liberación de iones.

Por otra parte, para el análisis de la influencia sobre la plasticidad celular es común la medición de la expresión de marcadores genéticos o proteicos de diferenciación celular; de tal manera que una sobreexpresión de un determinado marcador es indicativa de la influencia positiva del material de estudio en la diferenciación celular específica para dicho marcador [43]. Por ejemplo, una sobreexpresión de la fosfatasa alcalina o *ALP* (*alkaline phosphatase*) [44], y/o de la osteonectina u *ON* (*osteonectin*) [45] son marcadores indicativos de osteodiferenciación (Figura 1.2B).

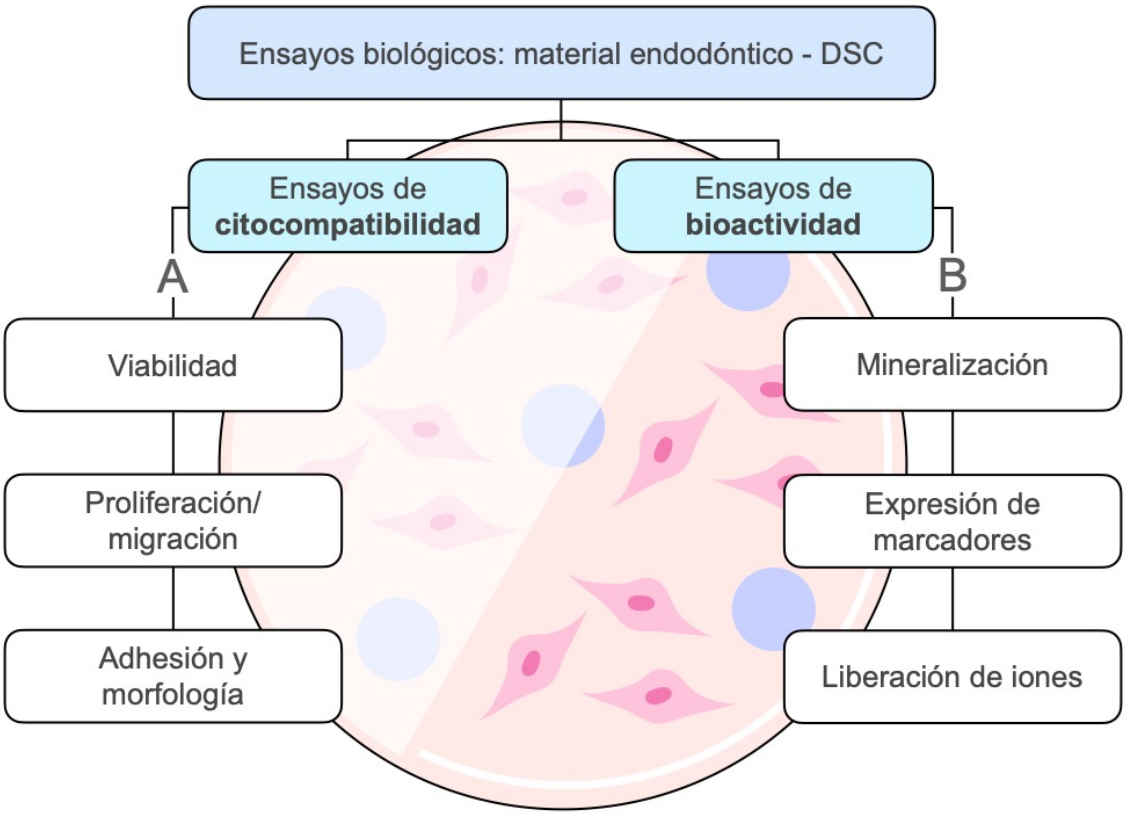

Figura 1.2. Ilustración esquemática simplificada de los principales ensayos *in vitro* para el estudio de la citocompatibilidad (A) y bioactividad (B) de los materiales endodónticos con las DSCs. Imagen original.

Son numerosos los materiales que han sido propuestos para su aplicación en los procedimientos regenerativos con base biológica, los cuales pueden subdividirse en inorgánicos y orgánicos. Entre los inorgánicos encontramos materiales como los vidrios bioactivos [46], los fosfatos de calcio [47] y los silicatos de calcio [48]; entre otros. Y entre los orgánicos destacan materiales como el chitosán o quitosano [48], o la fibroína de seda [49]; entre otros. Dentro del campo de la Endodoncia, cabe destacar a los materiales basados en silicato de calcio, debido al reciente incremento en su popularidad y producción científica al respecto [50].

Los cementos de silicato de calcio o *calcium silicate-based cements* (*CSCs*) [51] y los selladores de silicato de calcio o *calcium silicate-based sealers* (*CSSs*) [52] son un subtipo de materiales biocerámicos. Estos materiales son categorizados como hidráulicos, es decir, se hidratan y fraguan en contacto con el agua e

interaccionan con los fluidos del microambiente circundante. Son de especial interés en la terapéutica endodóntica por sus propiedades biológicas favorables: son altamente citocompatibles, son capaces de formar hidroxiapatita en su superficie y producen hidróxido de calcio como subproducto de su fraguado [53]. Gracias a esta última propiedad, poseen las ventajas propias del hidróxido de calcio, a saber, propiedades antimicrobianas por su alto pH y la liberación de iones calcio e hidroxilo. Clínicamente, dicha liberación iónica se traduce en la inducción del fenómeno de biomineralización entre su superficie y el sustrato dentinario [54].

Los materiales basados en silicato de calcio de los que disponemos actualmente surgieron tras la introducción de otro material hidráulico en el campo de la Endodoncia, el agregado de trióxido mineral o MTA (*mineral trioxide aggregate*). El MTA es un material basado en el cemento de Portland, compuesto esencialmente por silicato tricálcico, silicato dicálcico y aluminato tricálcico. Mostró una adecuada biocompatibilidad y bioactividad además de elevadas tasas de éxito clínico como material reparador [55, 56]. Dicho material es considerado como el precursor de los conocidos comúnmente como “nuevos materiales biocerámicos”, un término amplio de uso popular que hace referencia a los compuestos basados en silicato de calcio de introducción posterior al MTA.

Actualmente, los materiales hidráulicos de uso endodóntico disponibles comercialmente pueden clasificarse según su composición esencial (Figura 1.3). En general, presentan cuatro componentes principales: un cemento, que generalmente está basado en el cemento de Portland o en silicatos de calcio; un radioopacificador, que permitirá la identificación del material en una imagen radiográfica; un vehículo, que puede ser acuoso en el caso de los productos presentados en formato polvo-líquido o no-acuoso en los productos “pre-mezclados”; y una serie de aditivos enfocados en la mejora de las propiedades fisicoquímicas, mecánicas y/o biológicas de los compuestos [57].

El formato polvo-líquido puede requerir un mezclado manual o el uso de una vibradora para su mezclado. En este último caso, el producto se presentará en forma de cápsula. El formato pre-mezclado se presenta en forma de jeringa,

permitiendo su aplicación directa en el conducto. Los productos en formato polvo-líquido comenzarán su reacción de fraguado una vez mezclados, mientras que los “pre-mezclados” lo harán una vez puestos en contacto con los fluidos tisulares [58].

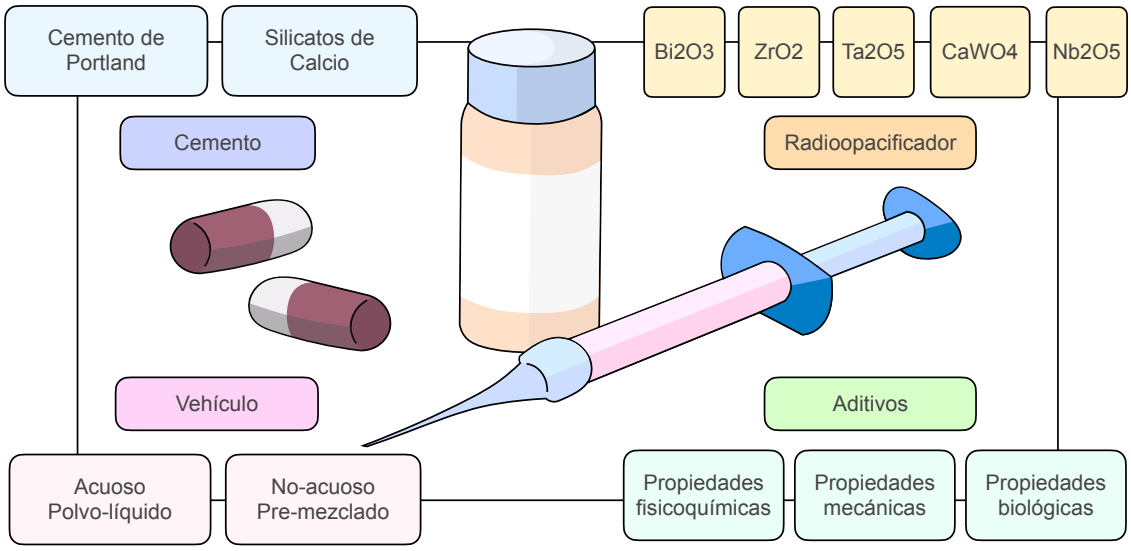

Figura 1.3. Esquema de la composición general de los materiales hidráulicos de uso endodóntico. Imagen original basada en la clasificación de los materiales hidráulicos según su composición química de Camilleri (2020) [57].

La variación en la composición de los materiales hidráulicos podría dar lugar a diferencias en su comportamiento clínico. Por ejemplo, se ha sugerido que las diferencias en el agente radioOpacificante pueden influir en las propiedades biológicas de dichos materiales, entre otras [59]. Entre los radioOpacificantes más utilizados encontramos, clásicamente, al óxido de bismuto (Bi_2O_3), el cual ha ido sustituyéndose en las nuevas composiciones de materiales hidráulicos por otros agentes, debido a su asociación con altas tasas de discoloración tanto del material que lo contiene como de los tejidos circundantes [60]. Más tarde, el dióxido de circonio (ZrO_2) surgió como alternativa, mostrando tasas de discoloración inferiores [61]. Actualmente, están apareciendo nuevos materiales al mercado con nuevos radioOpacificadores, como el wolframato de calcio ($CaWO_4$) o el óxido de tantalio (Ta_2O_5) [62, 63]; y otros se encuentran en fase experimental, como el óxido de niobio (Nb_2O_5) [64, 65].

Por otro lado, la incorporación de aditivos en la composición de los materiales hidráulicos puede suponer un cambio en su manejo, presentación y en sus propiedades fisicoquímicas, mecánicas y/o biológicas [66]. Un ejemplo a destacar son los materiales basados en silicatos de calcio modificados con resina. La incorporación de resina supone una serie de cambios, como la necesidad de una polimerización fotoactivable o dual del componente resinoso, la potencial mejora en su manejo y propiedades mecánicas, y el posible incremento en la citotoxicidad por la presencia de monómeros resinosos [67].

Debido a la posible influencia de los cambios y de la incorporación de aditivos en la composición los materiales hidráulicos de uso endodóntico en sus propiedades y a la constante introducción de nuevos materiales al mercado, se ha observado un incremento exponencial en la bibliografía disponible sobre estos materiales en los últimos años [68].

1.3. La ingeniería tisular en la OR

El tercer ámbito de estudio de la OR corresponde a la ingeniería tisular. A grandes rasgos, esta disciplina integra el campo de la biología y la ingeniería con el objetivo de regenerar -y no únicamente reparar- tejidos dañados o perdidos [69]. En este contexto, resulta imprescindible diferenciar la reparación de la regeneración, términos que habitualmente son sujetos a controversia. La regeneración tisular implica el restablecimiento de la estructura histológica y de la función original del tejido, mientras que la reparación hace referencia a la sustitución del tejido perdido por otro, habitualmente fibroso o cicatricial, que puede dar lugar a la pérdida de la función biológica del tejido afectado [70].

La aplicación de la ingeniería tisular se basa en la combinación de tres pilares fundamentales: una fuente adecuada de células madre, factores de crecimiento y un andamiaje para controlar el desarrollo del tejido objetivo (Figura 1.4).

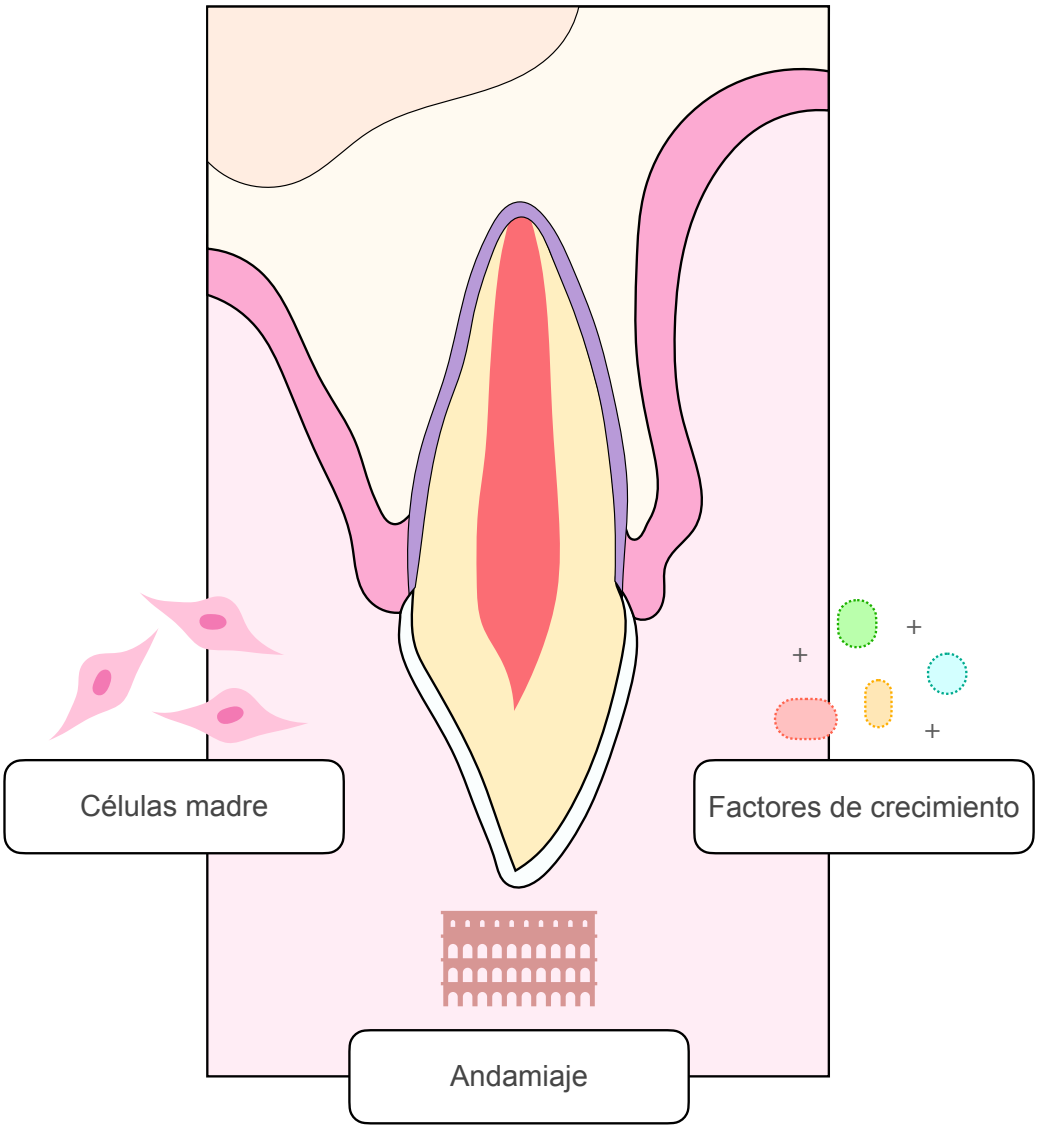

Figura 1.4. Ilustración esquemática de los tres pilares fundamentales de la ingeniería tisular dental. Imagen original basada y adaptada de Hargreaves *et al*. (2013) [69].

El primer elemento o pilar consiste en una fuente de células capaces de diferenciarse en el componente tisular deseado. El segundo elemento se centra en los factores de crecimiento u otros mediadores inductores. Las células madre, como se ha descrito previamente, tienen la capacidad de diferenciarse en una serie de fenotipos celulares. Esta diferenciación depende de su linaje, pero también de la exposición a estímulos ambientales como factores de crecimiento, la matriz extracelular, hipoxia u otras condiciones [71, 72]. De esta manera, el entorno es un factor crítico en la regulación de la diferenciación celular.

El tercer y último pilar implica el soporte de los demás elementos mediante un andamiaje. Esta estructura ha de ser capaz de proporcionar una liberación local de factores de crecimiento y/o inducir la cascada de señalización que permita la diferenciación celular [73].

Dentro del campo de la Endodoncia, se aplican numerosos conceptos de la ingeniería tisular en los procedimientos destinados a la reparación o regeneración del tejido dentino-pulpar y perirradicular. Existen dos enfoques principales: el *cell-free approach* (enfoque libre de células) y el *cell-based approach* (enfoque basado en células). El primero recoge todos aquellos procedimientos que implican el *cell homing* (reclutamiento celular) de *DSCs* residentes en el tejido pulpar o periapical, mientras que el segundo involucra el trasplante de células madre al diente afectado [74, 75].

Actualmente, el enfoque basado en células se encuentra en fase experimental, mostrando resultados prometedores en estudios con modelos animales [76, 77]. Recientemente, dos estudios clínicos han mostrado resultados positivos tras el trasplante de *DPSCs* [78] y *SHEDs* [79] en dientes con pulpitis irreversible y necrosis pulpar; respectivamente. En ambos casos, se ha descrito un restablecimiento de la respuesta al estímulo mediante tests eléctricos pulpares. La implementación de los enfoques basados en células en la clínica diaria en Endodoncia dependerá mayormente de la simplificación del procedimiento y la reducción de su coste [80].

Por otro lado, en los tratamientos endodónticos actuales, entre los que destacan el tratamiento de conductos, el tratamiento pulpar vital, la apicoformación, el tratamiento endodóntico regenerativo o “revascularización”, la reparación de perforaciones o defectos radiculares y la microcirugía endodóntica, se encuentran presentes en gran medida los conceptos del enfoque libre de células. Esto es debido a que, fundamentalmente, la reparación o regeneración tisular y/o la resolución de lesiones existentes será mediada por el componente celular intrínseco del complejo dentino-pulpar y de los tejidos perirradiculares [81, 82].

1.4. La Endodoncia Regenerativa

Definida por la Asociación Americana de Endodoncia o *American Association of Endodontists* (AAE) como los "procedimientos de base biológica diseñados para sustituir fisiológicamente las estructuras dentales dañadas, incluida la dentina y las estructuras radiculares, así como las células del complejo dentino-pulpar," [83] la Endodoncia Regenerativa ha despertado el interés en el mundo académico y clínico en los últimos años. Este creciente interés ha resultado en un aumento sustancial de la bibliografía en relación con los tratamientos recogidos dentro de este enfoque [84].

Entre ellos, destaca el propio tratamiento de endodóntico regenerativo o "revascularización", descrito habitualmente en la literatura como *regenerative endodontic treatment* (*RET*) [85]. Este tratamiento surgió como alternativa a la apicoformación para el tratamiento del diente permanente inmaduro no vital, cuyo objetivo es la creación de una barrera apical, bien mediante la inducción de la formación de una barrera mineralizada mediante el uso de hidróxido de calcio o bien mediante un "tapón" de un material basado en silicato de calcio o cemento de Portland [86]. A diferencia de la apicoformación, el tratamiento endodóntico regenerativo persigue la promoción del desarrollo radicular y el refuerzo de las paredes dentinarias radiculares mediante el depósito de tejido mineral [69].

Existen numerosos protocolos para este tipo de tratamientos, los cuales comparten una serie de componentes principales: la desinfección del sistema de conductos radiculares, la inducción de sangrado desde el tejido periapical al interior de los conductos, la creación de un andamiaje y la aplicación de una barrera y restauración coronales [87]. El sangrado resulta en la formación de un coágulo sanguíneo en el interior de los conductos, que actuará como vehículo y soporte de un gran componente celular con potencial de diferenciación y biomineralización. Por ello, este tratamiento podría categorizarse como un procedimiento basado en el enfoque libre de células o de reclutamiento celular [74].

La neoformación tisular observada tras este tratamiento se ha atribuido a las *DSCs* presentes entre el componente celular del coágulo sanguíneo, entre las cuales se encuentran las *SCAPs* y las *PDLSCs* [88]. Éstas, presentes en un ambiente con una carga microbiana reducida tras la desinfección, rodeadas de una fuente potencial de factores de crecimiento en la matriz dentinaria y escudadas bajo una barrera coronal con propiedades biológicas favorables, deberían ser capaces de diferenciarse y mediar la producción de tejido mineralizado; atendiendo a los conceptos de la ingeniería tisular [89] (Figura 1.5).

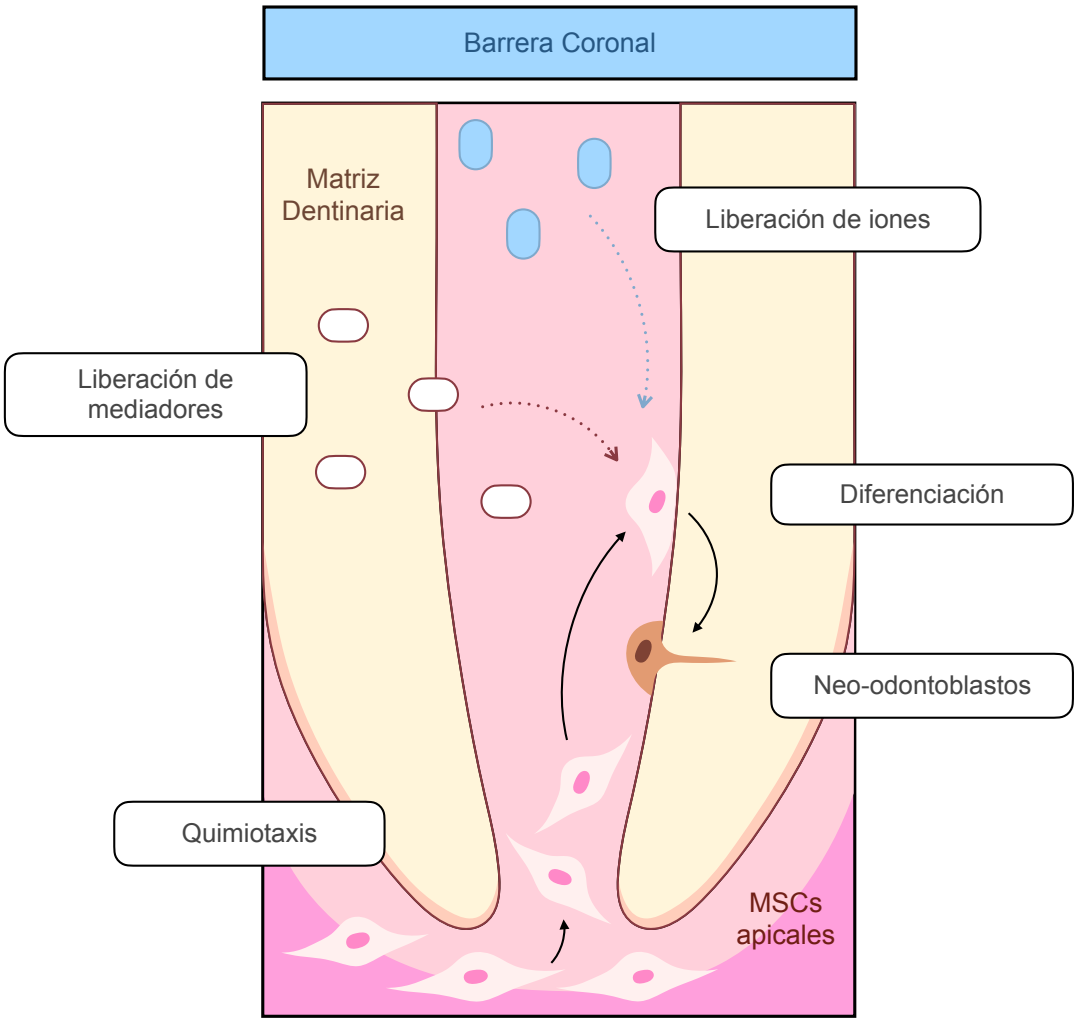

Figura 1.5. Ilustración esquemática simplificada de la biología celular detrás de la biomineralización observada tras el tratamiento de endodoncia regenerativa. La liberación de mediadores en la matriz dentinaria y la liberación de iones por parte de una barrera coronal bioactiva actúan como factores para la quimiotaxis y diferenciación de las *MSCs* apicales a células secretoras como los neo-odontoblastos. Imagen original basada y adaptada de Smith *et al*. (2016) [89].

Actualmente, siguiendo las recomendaciones de la *AAE* y la Sociedad Europea de Endodoncia o *European Society of Endodontology* (*ESE*), los materiales de elección como barreras coronales en el tratamiento endodóntico regenerativo son los *CSCs*, debido a su elevada citocompatibilidad y bioactividad [90, 91].

Paralelamente, el tratamiento pulpar vital o *vital pulp treatment* (*VPT*) sigue una premisa similar al tratamiento endodóntico regenerativo, aplicada a la terapéutica del diente vital. Dicho tratamiento tiene como objetivo mantener la vitalidad pulpar tras la inflamación histológica reversible del tejido pulpar ante un estímulo nocivo, y su éxito se basa en el potencial reparador intrínseco del componente celular del complejo dentino-pulpar [92]. En este caso, las *DPSCs* serán las principales células mediadoras de la reparación producida tras el tratamiento pulpar vital en dientes permanentes [9].

De nuevo, existen varios protocolos para el tratamiento pulpar vital, los cuales comparten los siguientes aspectos: limpieza químico-mecánica de la lesión, eliminando el tejido dentinario infectado y -si se produce una exposición pulpar- la porción de tejido pulpar diagnosticado con afectación irreversible, aplicación de un protector pulpar con propiedades biológicas favorables y restauración coronal [3].

Los diferentes procedimientos del tratamiento pulpar vital pueden clasificarse atendiendo al grado de conservación tisular: La protección del tejido dentinario viable sin exposición pulpar se conoce como recubrimiento indirecto, la protección de una exposición pulpar puntual tras la limpieza y desinfección de la lesión se conoce como recubrimiento directo, y la eliminación del tejido pulpar diagnosticado con afectación irreversible y la protección de aquel con afectación reversible o en estado de salud se conoce como pulpotomía. La pulpotomía, a su vez, puede sub-clasificarse como parcial o cameral si se elimina parte del tejido pulpar cameral o en su integridad; respectivamente [93].

Tanto la *AAE* como la *ESE*, en sus respectivas declaraciones de posición, coinciden en que la evidencia apunta a los *CSCs* como los protectores pulpares de elección [94, 95]. Las propiedades bioactivas de este subgrupo de materiales,

junto con el potencial de diferenciación y reparación de las *DPSCs*, resultan en la formación de una interfase mineralizada entre el tejido y el protector pulpar [96] (Figura 1.6). Este proceso se encuentra mediado por una cascada de eventos moleculares, recogidos dentro del proceso de la dentinogénesis reparativa [89]. Además, el desarrollo de un ambiente bioactivo gracias a los *CSCs* puede proveer una liberación local de mediadores y activar cascadas de señalización celular para regular la diferenciación y la reparación tisular [69].

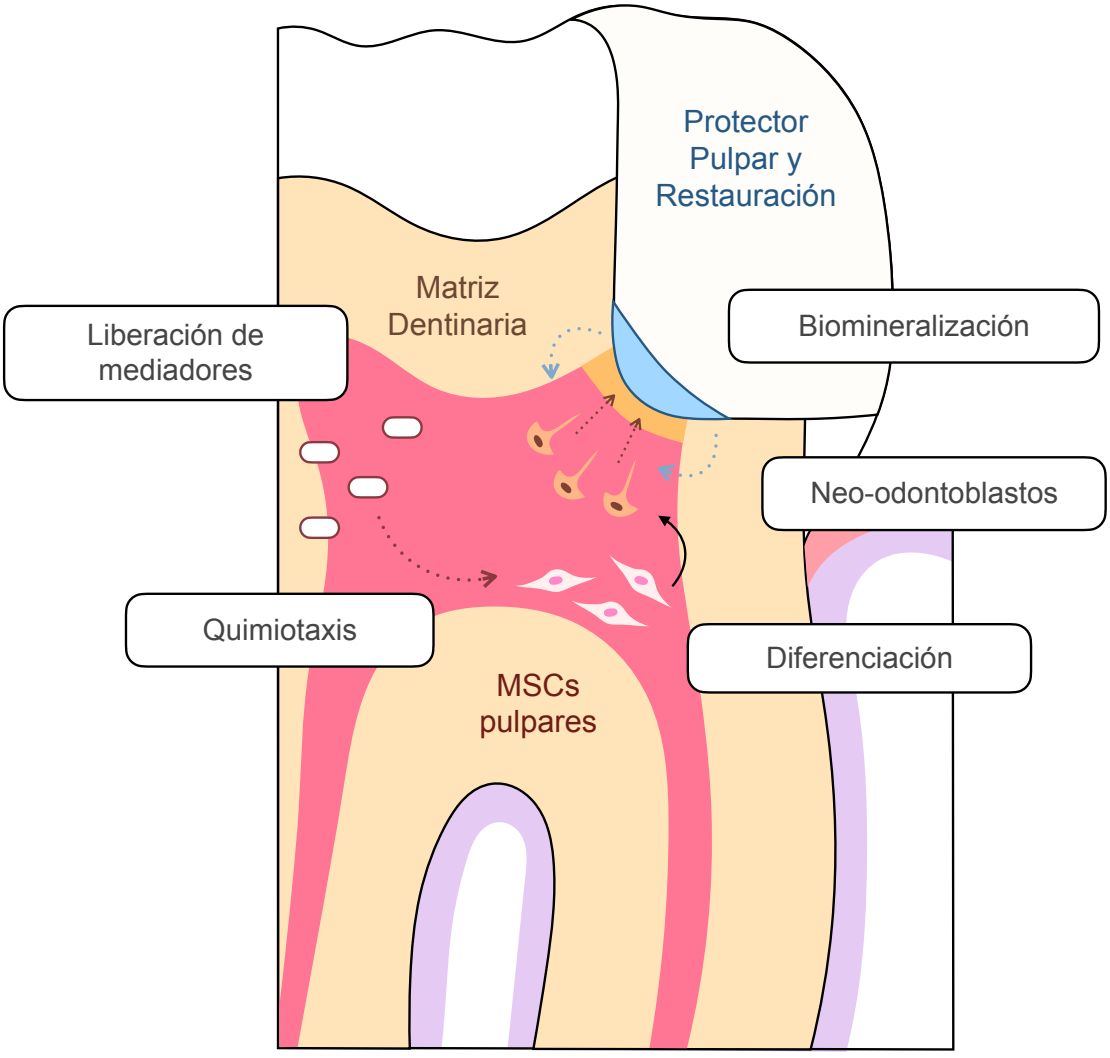

Figura 1.6. Ilustración esquemática simplificada de la biología celular detrás de la biomineralización observada tras el tratamiento pulpar vital. La liberación de mediadores en la matriz dentinaria y la liberación de iones por parte de un protector pulpar bioactivo actúan como factores para la quimiotaxis y diferenciación de las *MSCs* pulpares a células secretoras como los neo-odontoblastos. Imagen original basada y adaptada de Bjørndal *et al*. (2019) [96].

El mecanismo de diferenciación de las DSCs y la actividad secretora de los odontoblastos durante el proceso de la dentinogénesis reparativa comprende la activación de una serie de vías de señalización celular [89]. Entre ellas, las vías de la familia MAPK (y sus subfamilias p38, ERK y JNK), TFG-Smad, NF- κB, Wnt-Catenina y P13K/AKT/mTOR parecen actuar como mediadoras en este proceso [97–101]. Estas vías funcionan como reguladoras de la actividad de las *DSCs*, mediante la activación secuencial de complejos proteicos intermedios que culminan con la translocación de factores de transcripción al núcleo celular y la consiguiente activación de genes específicos para la proliferación celular, la diferenciación, la actividad secretora y/o la respuesta inflamatoria [9]. Estudios recientes han explorado la influencia de los *CSCs* en dichas vías de señalización [102]. No obstante, los procesos moleculares exactos implicados en la relación entre los *CSCs* y las *DSCs* aún están por dilucidar.

Por otra parte, los conceptos de la ingeniería tisular y los procedimientos regenerativos de base biológica también pueden aplicarse a otros tratamientos endodónticos, como el tratamiento de conductos, la microcirugía endodóntica y la reparación de perforaciones y defectos radiculares. El tratamiento de conductos tiene como objetivo principal la desinfección químico-mecánica del sistema de conductos y el posterior sellado tridimensional de éste con materiales biocompatibles [103]. Con ello, se persigue evitar la aparición de patología periapical o favorecer la resolución de aquella ya existente. La resolución de la patología periapical, de nuevo, será mediada por el componente celular local [104], entre el cual se encuentran las *PDLSCs*.

Durante el tratamiento de conductos, el material de relleno, a saber, la gutapercha y los selladores endodónticos, pueden extruirse de forma variable por el foramen apical o por los conductos secundarios. Por tanto, dichos materiales deben tener propiedades biológicas favorables para permitir o incluso favorecer la respuesta reparadora celular [105]. El mismo concepto puede extrapolarse a la microcirugía endodóntica y a la reparación de perforaciones o defectos radiculares ya que, en ambos casos, el material de sellado se encontrará en contacto directo con el tejido periodontal [106–108].

Los materiales basados en silicatos de calcio aparecen como candidatos para actuar como materiales selladores (*CSSs*) o reparadores (*CSCs*) en el tratamiento de conductos, y en la microcirugía endodóntica y la reparación de perforaciones o defectos radiculares; respectivamente. A grandes rasgos, la evidencia disponible respalda su uso como materiales extra-radiculares, mostrando resultados favorables en ensayos celulares, modelos animales y estudios clínicos [109].

En conjunto, cabe destacar la importancia del estudio de la relación entre los materiales basados en silicato de calcio y las células madre de origen dental dentro del campo de la Endodoncia Regenerativa, ya que dicha interacción puede estar presente en todos los tratamientos endodónticos previamente descritos y será crucial para su éxito.

2. Justificación

El desarrollo del campo de la Endodoncia Regenerativa ha supuesto una gran evolución y mejora de los enfoques terapéuticos endodónticos y de los materiales dentales destinados para tal fin. Consecuentemente, disponemos en la actualidad de una gran variedad de materiales de uso endodóntico. Esto, añadido a la constante introducción de nuevos materiales al mercado resulta en una falta de evidencia científica que respalde su uso clínico.

El uso de las nuevas composiciones de materiales basados en silicato de calcio en Endodoncia es un reflejo de dicha cuestión. Como subgrupo de biomateriales, los cementos y selladores basados en silicato de calcio han mostrado unas propiedades biológicas favorables tanto en ensayos *in vitro* como en modelos animales y estudios clínicos. No obstante, la evidencia sugiere que los cambios en la composición esencial de dichos materiales o la incorporación de aditivos pueden producir cambios en sus propiedades y comportamiento clínico. Por ello, existe la necesidad del análisis independiente y específico de cada nueva formulación de material.

Los ensayos *in vitro* suponen la vía preliminar para el análisis de los materiales dentales de manera previa a su avance a modelos animales o estudios clínicos. En referencia al estudio de las propiedades biológicas de los materiales dentales, los ensayos celulares son de utilidad para predecir la respuesta de estos hacia el componente celular de los tejidos con los que entrarán en contacto en su aplicación clínica.

En los procedimientos de Endodoncia Regenerativa, las células madre dentales son de especial interés, ya que intervienen en el proceso de reparación y/o regeneración observada tras dichos tratamientos. No obstante, la presencia de varios subtipos de células madre dentales de distinto origen y localización, y las diferencias en sus propiedades y potencial de diferenciación justifican su estudio individual; atendiendo a la relevancia clínica con el material a estudiar. De tal forma que el estudio de los protectores pulpares sería relevante junto con el de

las células madre de origen pulpar y, también, el estudio de las barreras coronales, cementos reparadores y selladores endodónticos lo sería con el de las células madre de origen periodontal.

De cara al futuro, el estudio en profundidad y caracterización de los mecanismos que hay detrás de la interacción entre los materiales basados en silicato de calcio y las células madre dentales puede suponer un avance crucial para el desarrollo de nuevas composiciones de materiales destinadas a favorecer dichos mecanismos.

Teniendo en cuenta los argumentos previamente descritos, se presenta en este proyecto de tesis doctoral, en primer lugar, una revisión sistemática sobre las propiedades biológicas de los materiales basados en silicato de calcio con respecto a las células madre periodontales, como visión actualizada sobre la evidencia disponible en este campo.

Seguidamente, se describen tres estudios experimentales en los que se analizan las propiedades biológicas de tres nuevos materiales basados en silicato de calcio con respecto a dos subtipos de células madre dentales: dos selladores basados en silicato de calcio con respecto a células madre periodontales y un cemento de silicato de calcio modificado con resina con respecto a células madre de origen pulpar. Dichos estudios analizan, de forma preliminar, las propiedades biológicas de los materiales descritos, con el objetivo de suplir la falta de evidencia científica que respalde o desaconseje su uso clínico.

En quinto lugar, se finaliza con una revisión sistemática sobre la activación de vías de señalización celular por parte de los cementos basados en silicato de calcio; con el objetivo de dilucidar de forma preliminar la evidencia disponible sobre los mecanismos detrás de la interacción entre este subgrupo de materiales y las células madre dentales, y abrir una nueva línea de investigación al respecto.

3. Objetivos

3.1. Objetivo general

Evaluar la citocompatibilidad y las propiedades bioactivas de materiales basados en silicato de calcio con respecto a las células madre pulpares y periodontales y explorar los mecanismos detrás de su interacción.

3.2. Objetivos específicos

3.2.1. Realizar una síntesis cualitativa de la evidencia *in vitro* disponible sobre la citocompatibilidad y propiedades bioactivas de los cementos y selladores basados en silicato de calcio con respecto a las células madre del ligamento periodontal humanas.

3.2.2. Evaluar la citocompatibilidad y propiedades bioactivas de un nuevo sellador basado en silicato de calcio (Bio-C Sealer ION+) en comparación con otro sellador basado en silicato de calcio (EndoSequence BC Sealer HiFlow) y un sellador basado en resina epoxi (AH Plus) con respecto a las células madre del ligamento periodontal humanas.

3.2.3. Evaluar la citocompatibilidad y propiedades bioactivas de un nuevo cemento de silicato de calcio modificado con resina de polimerización dual (Theracal PT) en comparación con un cemento de silicato de calcio modificado con resina de fotopolimerizable (Theracal LC) y un cemento de silicato de calcio hidráulico (Biodentine), con respecto a las células madre de la pulpa dental humanas.

3.2.4. Evaluar la citocompatibilidad y las propiedades bioactivas de un nuevo sellador basado en silicato de calcio (AH Plus bioceramic sealer) en comparación con otro sellador basado en silicato de calcio (EndoSequence BC Sealer HiFlow) y un cemento basado en resina epoxi (AH Plus) con respecto a las células madre del ligamento periodontal humanas.

3.2.5. Realizar una síntesis cualitativa de la evidencia *in vitro* disponible sobre la activación de vías de señalización celular durante la interacción entre los cementos basados en silicato de calcio y las células madre dentales.

4. Metodología

A continuación, se presenta un resumen de la metodología de los estudios recogidos en el presente proyecto de tesis doctoral, dividido por fases correspondientes a cada estudio y a cada objetivo planteado.

Para las Fases 1 y 5, correspondientes a las revisiones sistemáticas, se presenta un resumen de las principales secciones metodológicas recogidas dentro del protocolo PRISMA (*Preferred Reporting Items for Systematic reviews and Meta-Analyses*) [110, 111] para la presentación de datos de revisiones sistemáticas y metaanálisis: los criterios de inclusión reflejados en la pregunta PICOS [112], las bases de datos y estrategia de búsqueda, la selección de estudios, la extracción de datos y el análisis de calidad. Véase la sección "*Materials and Methods*" de los anexos 1 y 5 para una descripción de la metodología de las revisiones.

Para las Fases 2, 3 y 4, correspondientes a los estudios experimentales, se presenta un resumen de la metodología empleada: materiales y líneas celulares utilizadas, ensayos de citocompatibilidad y de bioactividad realizados. Véase la sección "*Materials and Methods*" de los anexos 2, 3 y 4 para una descripción de la metodología de los estudios.

Los tres estudios experimentales comparten las siguientes características:

- Los materiales de estudio fueron preparados siguiendo las instrucciones de sus respectivos fabricantes.
- Se prepararon discos de los materiales fraguados o polimerizados con medidas estandarizadas y diluciones de los materiales fraguados o polimerizados en proporción 1:1, 1:2 y 1:4 para los ensayos biológicos;
- Los grupos de estudio consistieron en la línea celular elegida cultivada en un medio acondicionado con los discos o las diluciones de los materiales;
- El grupo control negativo consistió en la línea celular elegida cultivada en un medio sin acondicionar;
- El grupo control positivo consistió en la línea celular elegida cultivada en un medio de osteodiferenciación.

4.1. Fase 1 (objetivo 3.2.1): Revisión sistemática sobre la citocompatibilidad y propiedades bioactivas de los cementos y selladores basados en silicato de calcio con respecto a las células madre del ligamento periodontal humanas.

4.1.1. Protocolo o "*reporting guidelines*":

La presentación de datos de la presente revisión se realizó de acuerdo al protocolo PRISMA 2020 [110].

4.1.2. Pregunta PICOS:

Tabla 4.1.1. Distribución de la pregunta PICOS

Población / *population* (P)	Células madre del ligamento periodontal humanas
Intervención / *intervention* (I)	Medios de cultivo tratados con selladores o cementos a base de silicato de calcio
Comparación / *control* (C)	Medios de cultivo no tratados
Resultado / *outcome* (O)	Viabilidad, proliferación, migración, diferenciación y mineralización de las *PDLSCs*
Diseño del estudio / *study type* (S)	*In vitro*

4.1.3. Bases de datos consultadas:

Se realizó una búsqueda avanzada en 5 bases de datos: Medline, Scopus, Embase, Web of Science y SciELO.

Tabla 4.1.2. Estrategia de búsqueda avanzada

#1 (silicate) OR (bioceramic)
#2 (periodontal ligament stem cells) OR (PDLSCs)
#3 (((((cytocompatibility) OR (biocompatibility)) OR (bioactivity)) OR (differentiation)) OR (expression)) OR (mineralization)
#1 AND #2 AND #3

4.1.4. Selección de estudios:

El proceso de selección de estudios se realizó mediante el *software* de gestión de referencias bibliográficas Mendeley v1.19.8 (Elsevier, AMS, Países Bajos).

4.1.5. Extracción de datos:

- Variables metodológicas: datos sobre la variante celular, material/es estudiados/s y su concentración, ensayos realizados y su duración.
- Variables de resultados: los resultados significativos encontrados para cada prueba, el tiempo en el que se registraron (duración) y su nivel de significación estadística como valor de p.

4.1.6. Análisis de calidad:

Para el análisis del riesgo de sesgo de cada uno de los estudios incluidos se utilizó la guía *"Modified CONSORT checklist of items for reporting in vitro studies of dental materials"* [113].

4.2. Fase 2 (objetivo 3.2.2): Estudio *in vitro* sobre la citocompatibilidad y las propiedades bioactivas de tres selladores endodónticos: Bio-C Sealer ION+, EndoSequence BC Sealer HiFlow y AH Plus, con respecto a las células madre del ligamento periodontal humanas.

Figura 4.2.1. Características de las muestras

Tabla 4.2.1. Ensayos de citocompatibilidad

Viabilidad	MTT (bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio)
Migración	Ensayo de curación de herida
Morfología y adhesión	Microscopía electrónica de barrido (*scanning electron microscopy;* SEM)

Tabla 4.2.2. Ensayos de bioactividad

Liberación de iones	Espectrometría de masas con plasma acoplado inductivamente (*Inductively-coupled plasma mass spectrometry*; *ICP-MS*)
Expresión de marcadores	*RT-qPCR* (*reverse transcriptase quantitative polymerase chian reaction*)
Mineralización	Tinción con Rojo de Alizarina S (*Alizarin red S staining*; *ARS*)

4.3. Fase 3 (objetivo 3.2.3): Estudio *in vitro* sobre la citocompatibilidad y propiedades bioactivas de tres materiales indicados para el tratamiento pulpar vital: Theracal PT, Theracal LC y Biodentine, con respecto a las células madre de la pulpa dental humanas.

Materiales de estudio

Theracal PT
(Bisco Inc., Schaumburg, IL, EE.UU.)

Theracal LC
(Bisco Inc., Schaumburg, IL, EE.UU.)

Biodentine
(Septodont, Saint-Maur-des-Fossés, Francia)

Línea celular:
Células madre de la pulpa dental humanas (hDPSCs).

Figura 4.3.1. Características de las muestras

Tabla 4.3.1. Ensayos de citocompatibilidad

Viabilidad	MTT; tinción con Anexina V-FITC / 7-AAD; liberación de especies reactivas de oxígeno (*ROS*)
Migración	Ensayo de curación de herida
Morfología y adhesión	*SEM*; inmunofluorescencia

Tabla 4.3.2. Ensayos de bioactividad

Expresión de marcadores	*RT-qPCR*
Mineralización	*ARS*

4.4. Fase 4 (objetivo 3.2.4): Estudio *in vitro* sobre la citocompatibilidad y propiedades bioactivas de tres selladores endodónticos: AH Plus Bioceramic Sealer, EndoSequence BC Sealer y AH Plus, con respecto a las células madre del ligamento periodontal humanas.

<u>Materiales de estudio</u>

AH Plus Bioceramic Sealer (Maruchi, Taejanggongdan-gil, Gangwon-do, Corea)	Endosequence BC Sealer (Brasseler, Savannah, GA, EE.UU.)	AH Plus (Dentsply, Konstanz, Alemania)

<u>Línea celular</u>**:**

Células madre del ligamento periodontal humanas (hPDLSCs).

Figura 4.4.1. Características de las muestras

Tabla 4.4.1. Ensayos de citocompatibilidad

Viabilidad	*MTT*
Migración	Ensayo de curación de herida
Morfología y adhesión	*SEM*

Tabla 4.4.2. Ensayos de bioactividad

Expresión de marcadores	*RT-qPCR*
Mineralización	*ARS*

4.5. Fase 5 (objetivo 3.2.5): Revisión sistemática sobre sobre la activación de vías de señalización celular durante la interacción entre los cementos basados en silicato de calcio y las células madre dentales.

4.5.1. Protocolo o "*reporting guidelines*":

La presentación de datos de la presente revisión se realizó de acuerdo al protocolo PRISMA 2009 [111].

4.5.2. Pregunta PICOS:

Tabla 4.5.1. Distribución de la pregunta PICOS

Población / *population* (P)	Células madre dentales humanas
Intervención / *intervention* (I)	Medio de cultivo acondicionado con cementos basados en silicato de calcio
Comparación / *control* (C)	Medio de cultivo no acondicionado
Resultado / *outcome* (O)	Activación de la/s vía/s de señalización
Diseño del estudio / *study type* (S)	*In vitro*

4.5.3. Bases de datos consultadas:

Se realizó una búsqueda avanzada en 5 bases de datos: Medline, Scopus, Embase, Web of Science y SciELO

Tabla 4.5.2. Estrategia de búsqueda avanzada

#1 (silicate) OR (bioceramic)
#2 ((molecular) OR signaling) OR pathway
#3 (mesenchymal stem cells) OR (dental stem cells)
#1 AND #2 AND #3

4.5.4. Selección de estudios:

El proceso de selección de estudios se realizó mediante el *software* de gestión de referencias bibliográficas Mendeley 1.19.8 (Elsevier, AMS, Países Bajos).

4.5.5. Extracción de datos:

- Variables metodológicas: subtipo de células madre dentales utilizadas, materiales utilizados y su concentración, vías de señalización estudiadas, análisis de actividad realizados y su duración, y marcadores y/o inhibidores específicos de las vías utilizados para dichos análisis.
- Variables de resultados: los resultados significativos encontrados, el momento en que se registraron (duración) y su nivel de significación estadística como valor de p. También se registraron variables específicas para cada tipo de ensayo.

4.5.6. Análisis de calidad:

Para el análisis del riesgo de sesgo de cada uno de los estudios incluidos se utilizó la guía *"Modified CONSORT checklist of items for reporting in vitro studies of dental materials"* [113].

5. Resultados

A continuación, se presenta un resumen de los resultados de los estudios recogidos en el presente proyecto de tesis doctoral, dividido por fases correspondientes a cada estudio y a cada objetivo planteado.

Véase la sección "*Results*" de los anexos 1 y 5 para una descripción detallada de los resultados de las revisiones sistemáticas.

Véase la sección "*Results*" de los anexos 2, 3 y 4 para una descripción detallada de los resultados de los estudios experimentales.

5.1. Artículo 1 (Fase 1; Objetivo 3.2.1)

5.1.1. Datos generales

Tabla 5.1.1. Datos bibliométricos

Revista	*International Endodontic Journal*
Fecha de publicación	02/08/2021
Factor de impacto (*JCR* 2021)	5.165
Área	*Dentistry, oral surgery & medicine – SCIE*
Posición	8/92 (D1)

JCR: Journal Citation Reports; SCIE: Science Citation Index Expanded

Título: "*Biological interactions between calcium silicate-based endodontic biomaterials and periodontal ligament stem cells: A systematic review of in vitro studies*"

5.1.2. Metodología de los estudios incluidos

Tras la búsqueda avanzada en 5 bases de datos y el proceso de selección de estudios, se incluyeron un total de 20 estudios. Entre ellos, se evaluaron un total de 16 *CSSs* y 9 *CSCs*.

Como indicadores de citocompatibilidad, los estudios realizaron una serie de ensayos de viabilidad, migración/proliferación, morfología y/o adhesión celular. Los ensayos de viabilidad celular más utilizados fueron el ensayo *MTT*, *cell counting kit 8* y el *live/dead viability kit*. Paralelamente, los estudios que evaluaron la citotoxicidad de los materiales lo hicieron mediante un ensayo de apoptosis con Anexina V / 7-AAD. Los ensayos de migración/proliferación celular más utilizados fueron el ensayo de curación de herida y el ensayo de migración de Transwell. La morfología y adhesión se evaluó mediante SEM.

Como indicadores de bioactividad, los estudios realizaron ensayos de plasticidad celular y biomineralización. El ensayo de plasticidad celular más utilizado fue la cuantificación de marcadores osteo/odonto/cementogénicos mediante *RT-*

qPCR. Para el análisis de la biomineralización, los estudios incluidos realizaron un ensayo de tinción con *ARS*.

5.1.3. Resultados de los estudios incluidos

Del total de 16 *CSSs* evaluados, 14 mostraron resultados positivos significativos en los ensayos de viabilidad, migración, diferenciación y/o mineralización de las *hPDLSCs* en comparación con un control negativo.

Entre los *CSSs* considerados como citocompatibles, los siguientes mostraron además una formación de nódulos calcificados por parte de las *hPDLSCs* significativamente mayor en comparación con un control negativo: BioRoot RCS, Endosequence BC Sealer, Endosequence BC Sealer HiFlow, Totalfill BC Sealer, Bio-C Sealer, Bio-C Sealer ION+, C Root y Ceraseal.

De manera similar, las *hPDLSCs* exhibieron una sobreexpresión de uno o más marcadores cementogénicos, osteogénicos y/o odontogénicos con los siguientes *CSSs*: BioRoot, Endosequence BC Sealer, Endosequence BC Sealer HiFlow, GuttaFlow Bioseal, Bio-C Sealer ION+ y Ceraseal.

Entre los 9 *CSCs* evaluados, el Biodentine fue el único cemento que mostró resultados positivos significativos en los ensayos de viabilidad y migración celular en comparación con un grupo de control negativo. El resto de CSCs estudiados exhibieron resultados similares a un grupo de control negativo en los ensayos de citocompatibilidad.

Además, las *hPDLSCs* cultivadas con dichos materiales mostraron la sobreexpresión de uno o más marcadores cementogénicos, osteogénicos y/o odontogénicos. Además, las células tratadas con Bio-C Repair TotalFill BC RRM Putty mostraron resultados positivos significativos en los ensayos con ARS.

Véase "*Table 1*" (Anexo 1) para los datos referentes a la composición y fabricantes de los materiales mencionados previamente.

5.2. Artículo 2 (Fase 2; Objetivo 3.2.2)

5.2.1. Datos generales

Tabla 5.2.1. Datos bibliométricos

Revista	*Clinical Oral Investigations*
Fecha de publicación	10/07/2020
Factor de impacto (*JCR* 2020)	3.573
Área	*Dentistry, oral surgery & medicine – SCIE*
Posición	21/92 (Q1)

Título: "*Microstructural composition, ion release, and bioactive potential of new premixed calcium silicate-based endodontic sealers indicated for warm vertical compaction technique*"

5.2.2. Citocompatibilidad de los materiales estudiados

Los *CSSs* estudiados no mostraron diferencias estadísticamente significativas con el control negativo en el ensayo *MTT*. Además, las *hPDLSCs* cultivadas con las diluciones de las muestras de los *CSSs* en las proporciones 1:2 y 1:4 mostraron una proliferación/migración similar a la de las células del grupo control negativo tras 48 y 72 horas de cultivo. En contraste, las muestras de AH Plus mostraron una viabilidad y proliferación/migración celular significativamente inferiores con respecto al grupo control negativo ($p<0,001$). El análisis mediante *SEM* coincidió con estos resultados, mostrando una adhesión celular abundante con intenso crecimiento y elongación en el caso de las muestras de los *CSSs* y una baja cantidad de células junto con residuos celulares en el caso del AH Plus.

5.2.3. Bioactividad de los materiales estudiados

Para el ensayo de expresión de marcadores con RT-qPCR, se excluyó al AH Plus, debido a sus resultados negativos en los ensayos previos.

Las muestras de Endosequence BC Sealer HiFlow mostraron una sobreexpresión de los siguientes marcadores: proteína de adherencia al periodonto o CAP ($p<0,01$), proteína recombinante del cemento o *CEMP1*, *ALP* y el factor de transcripción 2 relacionado con Runt o Runx2 ($p<0,001$) en comparación con el control negativo, mientras que las muestras de Bio-C Sealer ION+ mostraron una sobreexpresión de *CEMP1* ($p<0,01$), *CAP* y Runx2 ($p<0,001$). Al mismo tiempo, las *hPDLSCs* cultivadas con Endosequence BC Sealer HiFlow mostraron una expresión mayor de *ALP* ($p<0,01$), *CEMP1* y *Runx2* ($p<0,001$) que aquellas cultivadas con Bio-C Sealer ION+ o en el medio de osteodiferenciación (control positivo). Sin embargo, la expresión de CAP fue significativamente más elevada en las células cultivadas con Bio-C Sealer ION+ o en Osteodiff ($p<0,001$).

Véase el apartado "*Discussion*" del Anexo 2 para la descripción de los marcadores previamente mencionados.

En cuanto al ensayo de biomineralización, ambas muestras de *CSSs* mostraron una mineralización significativamente superior en comparación con el control negativo y positivo ($p<0,01$). Además, dicha mineralización fue significativamente más elevada en las muestras de Bio-C Sealer ION+ que las del Endosequence BC Sealer HiFlow ($p<0,05$). Contrariamente, no se observaron diferencias entre las muestras del AH Plus y el control negativo.

Por último, ambos *CSSs* mostraron una liberación de iones de calcio significativamente superior al AH Plus ($p<0.05$), que fue a su vez más elevada en el caso del Bio-C Sealer ION+ que en el Endosequence BC Sealer HiFlow ($p<0,05$).

Artículo 3 (Fase 3; Objetivo 3.2.3)

5.3.1. Datos generales

Tabla 5.3.1. Datos bibliométricos

Revista	*Journal of Endodontics*
Fecha de publicación	21/08/2021
Factor de impacto (*JCR* 2021)	4.422
Área	*Dentistry, oral surgery & medicine – SCIE*
Posición	13/92 (Q1)

Título: "*Comparative Biological Properties and Mineralization Potential of 3 Endodontic Materials for Vital Pulp Therapy: Theracal PT, Theracal LC, and Biodentine on Human Dental Pulp Stem Cells*"

5.3.2. Citocompatibilidad de los materiales estudiados

Las *hDPSCs* cultivadas con las diluciones del Theracal LC mostraron una viabilidad y migración significativamente inferior a la del grupo control negativo ($p<0,05$). Las muestras de Theracal LC mostraron además el mayor porcentaje de células no viables en el ensayo con Anexina V-FITC / 7-AAD y de liberación de *ROS*.

Por otro lado, las *hDPSCs* cultivadas con las diluciones de Theracal PT y Biodentine en las proporciones 1:2 y 1:4 mostraron una migración similar a la del grupo control negativo. Lo mismo se observó en las muestras de Biodentine en los ensayos de viabilidad celular. Sin embargo, únicamente la dilución del Theracal PT en la proporción 1:4 mostró una viabilidad similar al control negativo tras 48h de cultivo. Además, las células cultivadas con Biodentine mostraron un menor porcentaje de apoptosis celular y de liberación de *ROS* que las células cultivadas con Theracal PT; ambos menores que las muestras de Theracal LC.

Los ensayos de imagen, tanto con *SEM* como con inmunofluorescencia, mostraron una morfología celular orientada funcionalmente y una dispersión celular superior en las células cultivadas con Biodentine y Theracal PT. Las muestras de Theracal LC exhibieron un pobre componente celular con baja dispersión y morfología aberrante.

5.3.3. Bioactividad de los materiales estudiados

Para el ensayo de expresión de marcadores con *RT-qPCR*, se excluyó al Theracal LC, debido a sus resultados negativos en los ensayos previos.

Las muestras de Theracal PT mostraron una sobreexpresión de los siguientes marcadores osteo-odontogénicos: *ON* y Runx2 ($p<0,001$), en comparación con el control negativo, mientras que las muestras de Biodentine mostraron una sobreexpresión del marcador odontogénico DSPP ($p<0,05$).

En el ensayo de biomineralización, las *hDPSCs* cultivadas con Biodentine y Theracal PT exhibieron una producción de nódulos calcificados significativamente más alta que el grupo control negativo y positivo ($p<0,001$). Sin embargo, las muestras de Theracal LC produjeron una mineralización significativamente inferior en comparación con el resto de los grupos ($p<0,001$).

Artículo 4 (Fase 4; Objetivo 3.2.4)

5.4.1. Datos generales

Tabla 5.4.1. Datos bibliométricos

Revista	*International Endodontic Journal*
Fecha de publicación	29/07/2022
Factor de impacto (*JCR* 2021)	5.165
Área	*Dentistry, oral surgery & medicine – SCIE*
Posición	8/92 (D1)

Título: "*Cytocompatibility and bioactive potential of AH Plus Bioceramic Sealer: an in vitro study*"

5.4.2. Citocompatibilidad de los materiales estudiados

Ambos CSSs mostraron una viabilidad y migración celular similar a la del grupo control, mientras que el AH Plus mostró resultados negativos significativos en ambos parámetros ($p<0,001$). Estos resultados fueron complementados por el ensayo mediante SEM, que reveló un crecimiento, dispersión y morfología adecuadas por parte de las *hPDLSCs* en la superficie de las muestras de Endosequence BC Sealer y AH Plus Bioceramic Sealer. Sin embargo, cabe destacar que un mayor número de células adheridas fue visible en las muestras de Endosequence BC Sealer. Por otra parte, la superficie de los discos de AH Plus mostró una baja cantidad de células y residuos celulares, indicativo de muerte celular.

5.4.3. Bioactividad de los materiales estudiados

Para el ensayo de la expresión de marcadores con *RT-qPCR*, se excluyó al AH Plus Bioceramic Sealer, debido a sus resultados negativos en los ensayos previos.

El análisis de la expresión de marcadores con *RT-qPCR* produjo una gran variedad de resultados. En líneas generales, las muestras de ambos *CSSs* exhibieron una sobreexpresión de, al menos, un marcador de diferenciación osteo/odonto/cementogénica.

Los resultados del ensayo de biomineralización fueron coincidentes: las *hPDLSCs* cultivadas con ambos *CSSs* mostraron una formación de nódulos calcificados superior a los grupos de control negativo y positivo ($p<0,001$). Paralelamente, las *hPDLSCs* tratadas con Ah Plus mostraron una mineralización significativamente menor en comparación con los grupos de control negativo y positivo ($p<0,05$ y $p<0,001$; respectivamente).

Artículo 5 (Fase 5; Objetivo 3.2.5)

5.5.1. Datos generales

Tabla 5.5.1. Datos bibliométricos

Revista	*Dental Materials*
Fecha de publicación	08/02/2021
Factor de impacto (*JCR* 2021)	5.687
Área	*Dentistry, oral surgery & medicine – SCIE*
Posición	7/92 (D1)

Título: "*Dental stem cell signaling pathway activation in response to hydraulic calcium silicate-based endodontic cements: A systematic review of in vitro studies*"

5.5.2. Metodología de los estudios incluidos

Tras la búsqueda avanzada en 5 bases de datos y el proceso de selección de estudios, se incluyeron un total de 13 estudios. Las *DSCs* más estudiadas fueron, en orden decreciente: *DPSCs* (8 estudios), *PDLSCs* y *SCAPs* (2 estudios) y las *DPSCs* inflamatorias (1 estudio). Los *CSCs* más estudiados fueron el ProRoot MTA (Dentsply Tulsa Dental; Tulsa, OK, EE. UU.) y el Biodentine (Septodont, Saint Maur-des-Fosses, Francia). La mayoría de los estudios analizaron la activación de la vía de señalización MAPK y sus subfamilias (ERK, JNK y P38), seguido de las vías NF-κB, Wnt/β-catenina, BMP-Smad y CaMKIII.

Para la evaluación de la implicación de las vías de señalización en la interacción *CSC-DSC*, se utilizaron dos métodos principales: la medición de la expresión de marcadores específicos para la vía de señalización estudiada, o la combinación de la utilización de un inhibidor específico para la vía de señalización estudiada y la medición de la actividad de diferenciación o mineralización celular.

5.5.3. Resultados de los estudios incluidos

La heterogeneidad metodológica entre los estudios incluidos resultó en la producción de una gran variedad de resultados. En líneas generales, la expresión de marcadores relacionados con la diferenciación celular y los ensayos de mineralización evidenciaron la implicación de las vías MAPK (y sus subfamilias ERK, JNK y P38), NF-κB, Wnt/β-catenina, BMP/Smad y CAMKII en la respuesta biológica de las *DSCs* a los *CSCs* estudiados.

6. Discusión

A continuación, se presenta una discusión global sobre la metodología, resultados y limitaciones de los estudios del presente proyecto de tesis doctoral; además de una reflexión sobre las perspectivas futuras y las líneas de investigación derivadas de dicho proyecto.

A lo largo del texto, se han incluido nuevas referencias bibliográficas además de las presentadas originalmente en los artículos, con el objetivo de proporcionar una visión actualizada de la situación en este campo de investigación.

6.1. Sobre la metodología

6.1.1. Revisiones sistemáticas (Anexos 1 y 5)

Actualmente, el análisis de las propiedades biológicas de los materiales basados en silicato de calcio en estudios celulares de laboratorio comprende la realización de una batería de ensayos de citocompatibilidad y/o bioactividad.

Para ello, la mayoría de los estudios utilizan muestras, tanto discos con medidas estandarizadas como diluciones de los materiales, obtenidas a partir de los materiales de estudio tras su fraguado en condiciones controladas [114, 115]. Clínicamente, los *CSCs* y los *CSSs* se aplican tras su mezcla o bien se aplican directamente “pre-mezclados”, por lo que la totalidad o parte del proceso de fraguado se llevará a cabo en contacto con los tejidos dentarios. Por ello, las células pulpares y periapicales pueden exponerse a dicho proceso en el *VPT* o en el tratamiento de conductos y el *RET*, respectivamente.

Consecuentemente, el uso de muestras de material incubado en condiciones controladas (fraguado) puede ser útil para predecir la respuesta celular clínica a largo plazo. Sin embargo, el uso de muestras de material recién mezclado (no fraguado) podría ser de utilidad para predecir la respuesta celular clínica inicial hacia dicho proceso de fraguado [116].

De manera previa a la realización de los ensayos biológicos, varios de los estudios más recientes perfilaron además la composición química superficial de los materiales estudiados mediante Espectroscopía de Energía Dispersiva de Rayos X (EDX). No obstante, la EDX únicamente muestra la distribución de elementos en la superficie de la muestra. Para identificar los picos de hidróxido de calcio y otras fases cristalinas en los cementos hidráulicos tras su fraguado, son necesarias otras técnicas complementarias como el análisis de difracción de rayos X (XRD) o la espectroscopia de reflexión total atenuada-infrarroja transformada de Fourier (ATR-FTIR) [117, 118].

Estudios previos han descrito que pequeñas variaciones químicas, la inclusión de un bajo porcentaje de aditivos, o incluso diferencias en el agente radioopacificante pueden modificar sustancialmente las propiedades físicas, químicas y biológicas y el comportamiento de los biomateriales basados en silicato de calcio [115]. Sin embargo, los estudios incluidos en las revisiones que analizaron la composición química superficial de los materiales no evaluaron la influencia de las diferencias en dicha composición en las propiedades biológicas mostradas por los materiales de estudio.

En cuanto a las líneas celulares, los estudios incluidos en ambas revisiones sistemáticas analizaron distintos subtipos de *DSCs* previamente caracterizadas, con el objetivo de confirmar su fenotipo mesenquimal. El estudio individual de los subtipos de *DSCs* se encuentra justificado por la literatura, en la que se muestran diferencias sustanciales en sus propiedades [119, 120].

No obstante, en una revisión reciente se destaca, además, la heterogeneidad en cuanto a la proliferación, expresión superficial proteica y potencial de diferenciación entre las células de un mismo subtipo; específicamente las *DPSCs* [121]. Entre los numerosos factores que condicionan sus propiedades destacan la edad del paciente [122, 123], la afectación periodontal del diente de origen [124] y los métodos de extracción y cultivo de las células [125, 126]. De extrapolarse al resto de subtipos de *DSCs*, esta aportación podría potencialmente justificar las diferencias observadas entre estudios sobre un mismo material y subtipo celular.

Por último, cabe destacar la heterogeneidad en la presentación de datos entre los estudios incluidos en las revisiones sistemáticas. Generalmente, los estudios presentaron datos sobre la preparación de las muestras, los ensayos realizados y el análisis estadístico. Sin embargo, mediante el uso de la guía *CONSORT* modificada para la evaluación de estudios sobre materiales dentales [113], se observaron grandes carencias en cuanto a los datos presentados. Dichas carencias disminuyen la reproducibilidad y comparabilidad de los estudios.

6.1.2. Estudios experimentales (Anexos 2-4)

En el primer estudio experimental, se comparó al Bio-C Sealer ION+ con el Endosequence BC Sealer HiFlow y con el AH Plus como material sellador endodóntico de referencia. La selección de los *CSSs* de estudio se basó en su aplicación clínica común: la obturación radicular mediante técnicas calientes. Según las instrucciones de sus respectivos fabricantes, los tres materiales de estudio se encuentran indicados para la técnica de la onda continua de calor o condensación vertical.

En el segundo estudio experimental, se comparó al Theracal PT con su predecesor, el Theracal LC. Ambos son cementos de silicato modificados con resina, diferenciándose en su polimerización: El Theracal LC es fotopolimerizable, mientras que el Theracal PT es de polimerización dual. Además, se realizó una comparación con el Biodentine como material de referencia basado en silicato de calcio. La selección del Biodentine se basó en estudios previos similares [67, 127], sus elevadas tasas de éxito en el VPT [128] y su uso extendido entre la práctica clínica en Endodoncia [129].

En el tercer estudio experimental, se comparó al AH Plus Bioceramic Sealer con el Endosequence BC Sealer y el AH Plus. Se estableció el Endosequence BC Sealer como *CSS* de referencia, basado en la metodología de estudios previos similares [130, 131]. Al igual que en el primer estudio experimental, se utilizó el AH Plus como base de comparación debido a su extensa evidencia científica y su popularidad en el campo de la Endodoncia [132]. La selección de materiales se encuentra justificada por un estudio reciente, en el que se utilizan los mismos selladores de referencia como comparadores para una nueva formulación de *CSS* [133].

Para los ensayos biológicos del primer y tercer estudio experimental, se utilizaron hPDLSCs previamente caracterizadas como líneas celulares, con el objetivo de extrapolar su respuesta *in vitro* hacia los materiales estudiados a su comportamiento en el tratamiento de conductos. Estas células han demostrado

una alta supervivencia a largo plazo, capacidad de autorrenovación y una habilidad para formar tejido mineralizado similar al hueso/cemento [134, 135].

En el tratamiento de conductos, las *hPDLCs* pueden estar expuestas directamente a la extrusión de *CSSs*. El uso habitual de los *CSSs* en el tratamiento de conductos se basa en la técnica de cono único [136] o bien, en caso de estar indicado para técnicas calientes, la onda continua de calor o condensación vertical [137]. La primera es una técnica de obturación basada en el sellador, mientras que la segunda basa su obturación en la gutapercha calentada [138]. En ambos casos, se ha descrito la posibilidad de la extrusión de sellador, tanto por la mayor cantidad de sellador en la técnica de cono único como por el menor control apical en la técnica de onda continua de calor. Por lo tanto, estos materiales deben garantizar al menos la supervivencia de las células periapicales [139].

Para los ensayos biológicos del segundo estudio experimental, se utilizaron *hDPSCs* como líneas celulares, con el objetivo de extrapolar su respuesta *in vitro* hacia los materiales estudiados a su comportamiento en el *VPT*. Dichas células juegan un papel crucial en el proceso de la dentinogénesis reparativa, y se encontrarán en contacto con los protectores pulpares en el recubrimiento pulpar directo y en la pulpotomía [9]. Por lo tanto, resulta imprescindible que los materiales de aplicación en el *VPT* posean unas propiedades biológicas adecuadas frente a ellas.

La selección de los ensayos biológicos y su duración se encuentra justificada por varias revisiones sistemáticas sobre la interacción entre las *DSCs* y los materiales basados en silicato de calcio [42, 140, 141], donde se describe que la metodología seleccionada se encuentra entre la más utilizada entre los estudios en este campo.

Por último, la selección de marcadores de diferenciación para los ensayos con *RT-qPCR* se basa, de nuevo, en la metodología de estudios previos [42, 140]. Para los ensayos con *hDPSCs*, resulta relevante el análisis de la expresión de marcadores osteogénicos y odontogénicos, como indicadores de diferenciación

y neoformación tisular aplicable al *VPT*. Para los ensayos con *hPDLSCs*, conviene añadir un tercer grupo de marcadores, a saber, los marcadores cementogénicos; debido a su ubicación específica en el periodonto y a su participación en la regeneración periodontal [130, 142].

6.2. Sobre los resultados

6.2.1. Revisiones sistemáticas (Anexos 1 y 5)

En líneas generales, los *CSCs* y *CSSs* estudiados mostraron al menos resultados similares a un control negativo en los ensayos de citocompatibilidad. Además, todos los *CSCs* evaluados para la plasticidad de las *hPDLSCs* dieron lugar a una sobreexpresión marcadores osteogénicos, cementogénicos y/o odontogénicos, lo que sugiere su influencia positiva en su diferenciación. Estos resultados concuerdan con varias revisiones sistemáticas publicadas recientemente sobre la interacción con los *CSCs* con otros subtipos de *DSCs*; mostrando resultados positivos en cuanto a la citocompatibilidad y propiedades bioactivas junto con *hDPSCs* [42, 43, 143], *hSCAPs* [140] y *SHEDs* [141, 144].

Por otro lado, se ha descrito la implicación de diversas vías de señalización en el proceso de diferenciación osteo/odontogénica de las *DSCs* [145, 146]. No obstante, la interacción de los *CSCs* con dichas vías aún está por elucidar. En la segunda revisión sistemática presentada (Anexo 5), se describe, preliminarmente, la implicación de varias vías de señalización en la interacción *DSC-CSC*. Según el conocimiento del autor, no se han realizado revisiones similares que permitan el contraste de sus resultados. Sin embargo, posteriormente a la realización de la revisión sistemática, se han llevado a cabo nuevos estudios que amplían los datos presentados.

Un estudio reciente describe la implicación de la vía de señalización *ATK* en la regulación de la adhesión y proliferación de *iDPSCs* mediada por dos *CSCs*: Biodentine y MTA [147]. Paralelamente, en otro estudio se observa que la diferenciación osteo/odontogénica de células pulpares humanas mediada por MTA implica a la vía al complejo enzimático *AMPK* [148]. Por último, cabe

destacar que la implicación de las vías de señalización en la interacción material-célula también se está estudiando sobre otros subgrupos de materiales, como los vidrios bioactivos [149].

6.2.2. Estudios experimentales (Anexos 2-4)

En los estudios experimentales primero y tercero, ambos *CSSs* estudiados mostraron resultados positivos en los ensayos de citocompatibilidad, coincidiendo con estudios previos sobre otros materiales basado en silicato de calcio [131, 150]. Además, se observó que las mayores diluciones de los materiales produjeron mejores resultados. Clínicamente, es esperable que los tejidos periapicales entren en contacto con concentraciones considerablemente menores de los selladores endodónticos y, por tanto, puedan mostrar incluso mayor citocompatibilidad [151].

La sobreexpresión de marcadores osteo/cemento/odontogénicos y la formación de nódulos calcificados exhibida por los *CSSs* estudiados refleja sus propiedades bioactivas, tanto su influencia sobre la plasticidad celular como su potencial biomineralizante. Dicha bioactividad ha sido previamente descrita en estudios previos sobre otros *CSSs* [150].

La liberación de iones mostrada por los *CSSs* estudiados en el primer estudio experimental también ha sido observada en otros *CSSs* [131, 152]. La disolución de los materiales a base de silicato de calcio y la liberación de sus componentes catiónicos principales, a saber, el Ca^{2+}, en contacto con los fluidos tisulares, puede dar lugar a un intercambio iónico que se traduce en la formación de una capa mineral superficial en el proceso de la biomineralización. Las diferencias en la liberación de iones mostrada entre los *CSSs* estudiados podrían influir en la capacidad de sellado o, al menos, en el grosor y la composición de la unión mineral formada con sustrato dentinario [153].

Por último, cabe destacar los resultados del sellador basado en resina epoxi AH Plus. Dicho material fue excluido de los ensayos de expresión de marcadores de diferenciación debido a sus resultados negativos en los ensayos de

citocompatibilidad con respecto al resto de grupos. Los resultados obtenidos coinciden con estudios previos, en los que se describe su citotoxicidad con respecto las *hPDLSCs* [154, 155].

En el segundo estudio experimental, la adecuada citocompatibilidad exhibida por las mayores diluciones de los materiales analizados se encuentra en concordancia con un estudio previo realizado por nuestro grupo de investigación sobre las propiedades biológicas del Theracal PT frente al *MTA*. En dicho estudio, tanto el Theracal PT como el *MTA* mostraron una ausencia de citotoxicidad en su mayor dilución (1:4), mientras que se observó una alta citotoxicidad en los grupos tratados con Theracal LC [66].

En trabajos previos, se ha sugerido que la citotoxicidad exhibida por el Theracal LC puede estar asociada a la presencia de monómeros resinosos residuales sin polimerizar, lo que puede dar lugar a su lixiviación [156, 157]. Por otro lado, se ha descrito que el Theracal LC, una vez polimerizado, libera aditivos como la canforoquinona. Dicho aditivo, que actúa como fotoiniciador, se ha asociado con un aumento en la producción de *ROS* por parte de fibroblastos pulpares humanos [156, 158, 159]. Complementariamente, varios estudios sobre modelos animales han descrito una respuesta inflamatoria incrementada tras la pulpotomía [160] o implantación subcutánea [161] del Theracal LC frente a materiales sin componente resinoso.

Complementariamente, tanto las hDPSCs cultivadas con Theracal PT como con Biodentine mostraron una sobreexpresión de uno o más marcadores osteo/odontogénicos, además de mostrar una producción aumentada de nódulos calcificados. Ambos resultados son indicadores de su potencial bioactivo y concuerdan con estudios previos [66].

Recientemente, se ha descrito que el Theracal PT, Theracal LC y el Biodentine son capaces de producir precipitados minerales en suero bovino fetal y de liberar iones de calcio, considerando a los tres materiales como bioactivos desde un punto de vista fisicoquímico [162]. En el presente estudio, se evaluó la bioactividad desde un enfoque celular, con el objetivo de predecir el

comportamiento clínico de los materiales analizados. Las diferencias entre los resultados de ambos estudios dan lugar a la siguiente reflexión: la capacidad de la liberación de iones y la formación de precipitados minerales puede ser independiente de la respuesta celular hacia los materiales bioactivos o, al menos, hacia los cementos de silicato de calcio modificados con resina.

6.3. Limitaciones

Teniendo en cuenta la naturaleza *in vitro* de los ensayos realizados en los estudios incluidos en ambas revisiones sistemáticas, la generalización y extrapolación de los resultados al ámbito clínico debe realizarse con cautela, ya que no se considera la influencia de posibles factores externos, como pueden ser las variaciones de pH, niveles de oxígeno o la respuesta inmune del paciente [163–166]. Por otro lado, la heterogeneidad en cuanto a la metodología utilizada por los estudios incluidos imposibilitó la realización de una síntesis cuantitativa o metaanálisis de los datos.

Actualmente, no existe más evidencia sobre las propiedades biológicas del Bio-C Sealer ION+ ni sobre el AH Plus Bioceramic Sealer, lo que imposibilita el contraste de los resultados de sus respectivos estudios con otros trabajos. Las limitaciones inherentes a la naturaleza *in vitro* de los estudios, previamente expuestas, también son aplicables a los estudios experimentales presentados.

6.4. Perspectivas futuras

Futuros estudios *in vitro* sobre las propiedades biológicas de los *CSCs* y *CSSs* junto con *DSCs* podrían incorporar nuevos análisis para completar la caracterización de los materiales previo a su uso clínico.

El uso de muestras de materiales tanto recién mezcladas como fraguadas puede ser de utilidad para analizar tanto la respuesta celular inicial como la respuesta celular a largo plazo; ya que las diferencias fisicoquímicas entre ambos tipos de muestras, como el pH [115], pueden dar lugar a diferencias en la respuesta celular.

La evaluación de las propiedades biológicas de los *CSCs* y *CSSs* desde la perspectiva de las diferencias en su composición esencial, agente radioopacificante, vehículo y/o la presencia de aditivos podría ser una línea de investigación relevante para el futuro desarrollo de nuevas formulaciones de materiales con una proporción y composición que optimice sus propiedades biológicas. Un estudio reciente ilustra dicha propuesta mediante el análisis de las propiedades biológicas de varios materiales basados en silicato de calcio con diferentes agentes radioopacificantes [59].

De hecho, en el estudio de las propiedades biológicas de otros subgrupos de materiales de aplicación en OR, como los vidrios bioactivos, se muestra una mayor tendencia al análisis de la influencia específica de la modificación en su composición, en el tamaño de partícula o la inclusión de aditivos [149, 167]. Especialmente, es habitual observar dichos análisis en los estudios sobre combinaciones de materiales y compuestos para su uso como andamiajes en procedimientos de OR [168, 169]. Esta tendencia podría aplicarse en el estudio de materiales noveles basados en silicato de calcio de uso endodóntico en un futuro.

Por otra parte, la liberación iónica de las nuevas formulaciones comerciales de *CSCs* y *CSSs* también merece ser estudiada en profundidad para elucidar los mecanismos subyacentes a sus propiedades biológicas. Clásicamente, la bioactividad como propiedad fisicoquímica del principal precursor de los materiales basados en silicato de calcio, el *MTA*, fue descrita mediante ensayos en fluido tisular sintético o simulado, compuesto por una solución salina de tampón fosfato neutro y dentina del canal radicular. Mediante este tipo de ensayo, se confirmó su capacidad de liberación de iones de calcio y la interacción de estos con el fosfato del fluido tisular para formar un mineral similar a la hidroxiapatita [170, 171]. Más tarde, se confirmó este mismo fenómeno con el Biodentine, que actúa como el material basado en silicato de calcio de referencia en gran parte de los estudios en este campo [68, 153].

No obstante, la relación entre la liberación iónica de los materiales basados en silicato de calcio y la respuesta celular todavía no se ha dilucidado.

Recientemente, se ha sugerido el papel de la liberación de calcio en la alteración de la dinámica intracelular de este elemento, que a su vez puede influir en la diferenciación celular y en su potencial de mineralización [172]. Por ello, el estudio de la respuesta de las *DSCs* a los materiales basados en silicato de calcio desde la perspectiva de la liberación iónica puede considerarse como una línea de investigación relevante.

En cuanto al uso de líneas celulares, la heterogeneidad mostrada tanto entre como intra-subtipos destaca la necesidad de la adecuada caracterización y definición de las células utilizadas. Se ha propuesto la utilización de métodos de caracterización como la citometría de flujo multiparamétrica, que permite la co-localización de varios marcadores moleculares en la superficie de cada célula [173]. Otro método propuesto es la espectroscopia Raman unicelular o *SCRM* (*Single-cell Raman Spectroscopy*). Esta técnica, según un estudio reciente, permite discriminar entre *DPSCs* con diferente capacidad proliferativa y de diferenciación a nivel *ex vivo* [174].

Las diferencias intra-subtipo de las *DSCs* previamente descritas justifican además le necesidad de proporcionar datos sobre el historial médico y odontológico de las personas donantes y de estandarizar los protocolos de aislamiento y cultivo de las líneas celulares de estudio, con el objetivo de reproducir el comportamiento celular en condiciones clínicas.

Recientemente, se ha descrito en mayor profundidad el papel de los fibroblastos pulpares en la regeneración del complejo dentino-pulpar y en el reclutamiento y diferenciación de las *DPSCs*, entre otros [175]. Estudios previos sobre las propiedades biológicas de los materiales basados en silicato de calcio han realizado ensayos con fibroblastos pulpares [176] y del ligamento periodontal [177], mostrando resultados positivos. En futuros estudios, podría explorarse la diferencia en la respuesta de los fibroblastos y las *DSCs* al tratamiento con este tipo de materiales.

Por otro lado, con el objetivo de solventar la falta de estandarización en la presentación de los datos en los estudios *in vitro* sobre materiales endodónticos,

se ha desarrollado recientemente una herramienta específica denominada *PRILE* o "*Preferred Reporting Items for Laboratory studies in Endodontology*" [178]. Dicha guía actúa como un protocolo para la presentación de datos en dichos tipos de estudios. Concretamente, el estudio presentado en el Anexo 4 sigue esta guía para la presentación de sus datos. Su utilización en futuros estudios en este campo puede resultar en un aumento en su reproducibilidad y comparabilidad.

Una vez descrita la respuesta positiva por parte de las *hPDLSCs* al cultivo junto con *CSCs* y *CSSs in vitro*, resulta imprescindible avanzar hacia modelos animales y ensayos clínicos para confirmar la adecuación de dichos materiales a la terapéutica endodóntica clínica diaria. Los materiales basados en silicato de calcio de referencia cuentan con soporte científico tanto en modelos animales [179, 180] como en ensayos clínicos [181, 182]. Cabe esperar que las nuevas formulaciones de *CSCs* y *CSSs* cuenten con este nivel de evidencia con el paso del tiempo.

Paralelamente, el estudio de los mecanismos moleculares detrás de la interacción entre las *DSCs* y los materiales basados en silicato de calcio se encuentra en un estado preliminar, como se evidencia en el Anexo 5. Recientemente, se ha explorado la función de los micro-ARN, un subgrupo de moléculas de ARN no codificantes, en la diferenciación de las *DSCs* y en la activación de las vías de señalización celular [183].

También se han observado avances en el estudio proteómico de las *DSCs*, habiéndose descrito el perfil de expresión molecular de varios subtipos celulares [120, 184]. Por último, el estudio de los exosomas secretados por las *DSCs* y su función ha mostrado un incremento en los últimos años [185, 186], resaltando la importancia de éstos en los procesos de reparación tisular y su potencial aplicación en los procedimientos de OR.

En conjunto, estas aportaciones podrían aplicarse en el estudio de la interacción entre los materiales basados en silicato de calcio y las células madre dentales y actuar como nuevas líneas de investigación.

6.5. Líneas de investigación actuales

Actualmente, tras haber dilucidado de forma preliminar varias de las vías de señalización implicadas en la interacción CSC-DSC, desde nuestro grupo de investigación estamos llevando a cabo una serie de ensayos dentro del campo de la proteómica, metabolómica y epigenética con varias formulaciones de CSCs y hDPSCs.

Los ensayos comprenden dos fases paralelas:

- Determinación de la influencia de los CSCs en el componente proteico y los metabolitos producidos por las hDPSCs.
- Determinación de la influencia de los CSCs en la expresión de microARNs por parte de las hDPSCs.

A corto plazo, esperamos contribuir al conocimiento sobre los mecanismos moleculares y genéticos que hay detrás de la interacción entre los materiales basados en silicato de calcio y las células madre dentales.

A medio y largo plazo, esperamos que la evidencia adquirida permita la transferencia de resultados y la colaboración con empresas para el desarrollo de nuevas formulaciones de materiales basados en silicato de calcio enfocadas a estimular los mecanismos previamente descritos y, en consecuencia, mejorar la predictibilidad y los resultados de los tratamientos endodónticos de base biológica.

7. Conclusiones

A continuación, se presentan las conclusiones del presente proyecto de tesis doctoral, respondiendo a los objetivos específicos previamente planteados.

Los cementos y selladores basados en silicato de calcio, como grupo de materiales endodónticos, presentan una adecuada citocompatibilidad y favorecen la diferenciación osteo/cementogénica y el potencial de mineralización de las células madre del ligamento periodontal humanas, evidenciado por los resultados de los estudios *in vitro* incluidos en la presente revisión sistemática.

Los selladores basados en silicato de calcio Bio-C Sealer ION+ y Endosequence BC Sealer HiFlow muestran una alta liberación de iones de calcio, una adecuada citocompatibilidad y propiedades bioactivas, ambas superiores a las del AH Plus, con respecto a las células madre del ligamento periodontal humanas.

El cemento basado en silicato de calcio modificado con resina de polimerización dual Theracal PT muestra una citocompatibilidad aumentada en comparación con el Theracal LC y propiedades bioactivas comparables a las del Biodentine con respecto a las células madre de la pulpa dental humanas.

Los selladores basados en silicato de calcio AH Plus Bioceramic Sealer y Endosequence BC Sealer muestran una adecuada citocompatibilidad y propiedades bioactivas, ambas superiores a las del sellador AH Plus, con respecto a las células madre del ligamento periodontal humanas.

Los cementos de silicato de calcio muestran una respuesta biológica favorable por parte de las células madre dentales. Los resultados de los ensayos sobre la expresión de marcadores relacionados con la actividad celular y la formación de nódulos mineralizados sugieren un papel activo de las vías de señalización MAPK, NF-κB, Wnt/β-catenina, BMP/Smad y CAMKII como posibles mediadores en la interacción biológica entre las células madre dentales y los cementos de silicato de calcio.

En conjunto, los resultados del presente proyecto de tesis doctoral han dado lugar a cuatro principales aportaciones:

- Se han confirmado la citocompatibilidad y las propiedades bioactivas de los materiales basados en silicato de calcio hidráulicos con respecto a las células madre del ligamento periodontal, mediante la síntesis cualitativa de la evidencia disponible al respecto.

- Se han elucidado preliminarmente las propiedades biológicas favorables de dos nuevas formulaciones de selladores basados en silicato de calcio con respecto a las células madre del ligamento periodontal.

- Se han elucidado preliminarmente las propiedades biológicas favorables de una nueva formulación de cemento basado en silicato de calcio modificado con resina de polimerización dual con respecto a las células madre de la pulpa dental.

- Se ha descrito la implicación de varias vías de señalización celulares en la interacción entre los cementos basados en silicato de calcio y las células madre dentales, mediante la síntesis cualitativa de la evidencia disponible al respecto.

CONCLUSIONS

The conclusions of this PhD thesis project are presented below, in response to the specific objectives previously stated:

Calcium silicate-based cements and sealers, as a group of endodontic materials, present adequate cytocompatibility and favor the osteo/cementogenic differentiation and mineralization potential of human periodontal ligament stem cells, evidenced by the results of the in vitro studies included in the present systematic review.

The calcium silicate-based sealers Bio-C Sealer ION+ and Endosequence BC Sealer HiFlow show a high calcium ion release, adequate cytocompatibility and bioactive properties, both superior to those of AH Plus sealer, on human periodontal ligament stem cells.

The dual curing resin modified calcium silicate-based cement Theracal PT shows an increased cytocompatibility compared to Theracal LC and bioactive properties comparable to Biodentine on human dental pulp stem cells.

The calcium silicate-based sealers AH Plus Bioceramic Sealer and Endosequence BC Sealer show an adequate cytocompatibility and bioactive properties, both superior to those of AH Plus Sealer, on human periodontal ligament stem cells.

Calcium silicate cements exhibit a favorable biological response from dental stem cells. Assay results on the expression of markers related to cellular activity and mineralized nodule formation suggest an active role of MAPK, NF-κB, Wnt/β-catenin, BMP/Smad and CAMKII signaling pathways as possible mediators in the biological interaction between dental stem cells and calcium silicate cements.

Overall, the results of the present PhD thesis project have led to four main contributions:

- The cytocompatibility and bioactive properties of hydraulic calcium silicate-based materials on periodontal ligament stem cells have been confirmed by the qualitative synthesis of the available evidence in this regard.

- The favorable biological properties of two new formulations of calcium silicate-based sealers on human periodontal ligament stem cells have been preliminarily elucidated.

- The favorable biological properties of a new dual-curing resin-modified calcium silicate-based cement formulation on human dental pulp stem cells have been preliminarily elucidated.

- The involvement of several cell signaling pathways in the interaction between calcium silicate-based cements and dental stem cells has been described by the qualitative synthesis of the available evidence in this regard.

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ANEXOS

A continuación, se presentan las publicaciones de los 5 estudios que comprenden el presente proyecto de tesis doctoral:

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REVIEW

Biological interactions between calcium silicate-based endodontic biomaterials and periodontal ligament stem cells: A systematic review of *in vitro* studies

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Abstract

Background: Most recently, the biological interactions, that is cytocompatibility, cell differentiation and mineralization potential, between calcium silicate-based biomaterials and periodontal ligament stem cells (PDLSCs) have been studied at an *in vitro* level, in order to predict their clinical behaviour during endodontic procedures involving direct contact with periodontal tissues, namely root canal treatment, endodontic surgery and regenerative endodontic treatment.

Objective: The aim of the present systematic review was to present a qualitative synthesis of available *in vitro* studies assessing the biological interaction of PDLSCs and calcium silicate-based biomaterials.

Methodology: The present review followed PRISMA 2020 guidelines. An advanced database search was performed in Medline, Scopus, Embase, Web of Science and SciELO on 1 July 2020 and last updated on 22 April 2021. Studies assessing the biological interactions of PDLSCs with calcium silicate-based sealers (CSSs) and/or cements (CSCs) at an *in vitro* level were considered for inclusion. The evaluation of the 'biological interaction' was defined as any assay or test on the cytotoxicity, cytocompatibility, cell plasticity or differentiation potential, and bioactive properties of PDLSCs cultured in CSC or CSS-conditioned media. Quality (risk of bias) was assessed using a modified CONSORT checklist for *in vitro* studies of dental materials.

Results: A total of 20 studies were included for the qualitative synthesis. CSCs and CSSs, as a group of endodontic materials, exhibit adequate cytocompatibility and favour the osteo/cementogenic differentiation and mineralization potential of PDLSCs, as evidenced from the *in vitro* studies included in the present systematic review.

Discussion: The influence of the compositional differences, inclusion of additives, sample preparation, and varying conditions and manipulations on the biological properties of calcium silicate-based materials remain a subject for future research.

Conclusions: Within the limitations of the *in vitro* nature of the included studies, this work supports the potential use of calcium silicate-based endodontic materials in stem cell therapy and biologically based regenerative endodontic procedures.

Registration: OSF Registries; https://doi.org/10.17605/OSF.IO/SQ9UY.

KEYWORDS

bioactive materials, biocompatibility, calcium silicate, endodontics, stem cells, systematic reviews and evidence-based medicine

INTRODUCTION

Biologically based regenerative procedures rely on the intrinsic reparative mechanisms of reversibly affected and/or healthy tissues and cellular populations, along with the use of biomaterials which enhance such mechanisms or at least provide a biocompatible medium for them to take place (Hargreaves et al., 2013; Prati & Gandolfi, 2015). In the field of Dentistry, the complex anatomy, composition and interaction of the various tissues which constitute the dentine–pulp complex and periodontium hinder the study and definition as to how tissue repair occurs within the tooth and its supporting structures at a molecular level (Bosshardt et al., 2015; Smith et al., 2016).

Nevertheless, following tissue-engineering concepts, the repair process can be simplified into a triad of variables: the cellular component, the use of biomaterial scaffolds/vehicles/matrix and molecular signalling regulation (Sanz et al., 2015). The cellular component involved in the neoformation of dental and surrounding tissues upon damage, that is by trauma or infection, is largely represented by various populations of dental stem cells (DSCs) with a mesenchymal phenotype (Rodríguez-Lozano et al., 2011). The availability of a series of DSC subsets has been confirmed by their isolation and characterization and classified according to their residing tissue (Huang et al., 2009).

The periodontium and, specifically, the periodontal ligament hosts a population of multipotent postnatal stem cells, categorized as periodontal ligament stem cells (PDLSCs; Seo et al., 2004). Their mesenchymal stem cell (MSC)-like characteristics have been demonstrated (Bartold & Gronthos, 2017; Dominici et al., 2006) by the *in vitro* cell surface expression of MSC-specific markers (CD10, CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146, CD166) and the absence of hematopoietic markers (CD14, CD34, CD45, CD79a), endothelial markers (CD31) and helper immune antigens (HLA-DR, CD40, CD54, CD80, CD86; Huang et al., 2009; Lindroos et al., 2008; Park et al., 2011; Trubiani et al., 2019). Furthermore, their osteogenic, adipogenic and chondrogenic differentiation potential has been reported in various *in vitro* studies (Gay et al., 2007; Xu et al., 2009; Zhang et al., 2012). Collectively, evidence highlights the importance of PDLSCs in the repair process regarding the periodontium and is therefore considered as a promising cell source for the reparative procedures which involve such tissues (Bright et al., 2015; Maeda et al., 2013).

Endodontic procedures, particularly root canal treatment (RCT), regenerative endodontic treatment (RET) and endodontic surgery (ES), involve the placement of various biomaterial compositions in direct contact with periradicular tissues and, consequently, their cellular populations (Dawood et al., 2017; Prati & Gandolfi, 2015). During RCT, the biomaterials used to create a root filling within the root canal system after its chemical-mechanical disinfection and shaping, that is endodontic sealers, may extrude to a variable extent from the apical foramen and/or secondary canals into the surrounding tissues (Aminoshariae & Kulild, 2020). RET, on the other hand, aims to promote root development in immature permanent teeth with nonvital pulps through the formation of an intracanal blood clot from the periapical tissue and placement of a biomaterial as a coronal barrier (Tong et al., 2017). The repair exhibited after RET has been associated with tissue neoformation resulting from the differentiation of multipotent stem cells found within the blood clot, namely stem cells from the apical papilla (SCAPs) and PDLSCs (Kim et al., 2018). Lastly, ES procedures such as apicoectomies and the repair of root perforations or resorption defects require the placement of biomaterials to seal the existing radicular cavities superficially. Such biomaterials will therefore be in contact with the surrounding supporting tissues (Liu et al., 2021).

Various biomaterial compositions have been investigated for such therapeutic applications, requiring specific biological properties in order to be considered as suitable for clinical use. Properties such as cytocompatibility or lack of cytotoxicity are essential to ensure cell survival and proliferation, and the provision of a biocompatible medium for repair (Fonseca et al., 2019). However, biomaterials should also ideally exhibit a bioactive nature, thereby promoting the formation of a hydroxyapatite-like superficial layer to favour the development of a mineral attachment to the surrounding mineralized tissue substrates (Gandolfi et al., 2010; Vallittu et al., 2018). As a group of biomaterials, calcium silicate-based cements (CSCs) and sealers (CSSs) have demonstrated both excellent biocompatibility and bioactive properties (Kim et al., 2015; Prati & Gandolfi, 2015; Watson et al., 2014), appearing as promising candidates for endodontic treatment and biologically based regenerative procedures (Chen et al., 2009).

The use of CSCs and CSSs in endodontic procedures has been widely investigated (Parirokh et al., 2018). Both in RET and ES, CSCs have exhibited adequate sealing ability, marginal adaptation, and hard tissue conductivity in laboratory studies and high success rates in clinical studies

(Torabinejad et al., 2018). Regarding RCT, CSSs have been reported to have similar bond strength values to the well-established resin-based sealer AH Plus (Dentsply DeTrey GmbH, Konstanz, Germany) and adequate flowability, in conformity with ISO 6876:2012 recommendations (Silva Almeida et al., 2017).

Most recently, the relationship between these biomaterials and DSCs of different origin has been studied at an *in vitro* level, in order to predict their clinical behaviour in procedures involving direct contact with biological tissues (Pedano et al., 2020; Sanz et al., 2020a; 2020b). Considering the potential application of CSCs and CSSs in RCT, RET and ES, where they will be placed in contact with periodontal tissues and their cellular component, the relationship between such biomaterials and PDLSCs should be determined beforehand.

Accordingly, the aim of the present systematic review was to present a qualitative synthesis of available *in vitro* studies assessing the biological interaction of PDLSCs and calcium silicate-based biomaterials.

MATERIALS AND METHODS

The present work was carried out following the PRISMA 2020 (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) Statement (Page et al., 2021a). The protocol of this systematic review was previously registered in Open Science Framework (OSF) Registries (https://doi.org/10.17605/OSF.IO/SQ9UY).

Eligibility criteria

Studies assessing the biological interactions of PDLSCs with calcium silicate-based sealers and/or cements at an *in vitro* level were considered for inclusion. The evaluation of the 'biological interaction' was defined as any assay or test on the cytotoxicity (i.e. cell apoptosis), cytocompatibility (i.e. cell viability, migration, proliferation), cell plasticity or differentiation potential (i.e. osteo/cemento/odontogenic marker expression) and bioactive properties (i.e. calcified nodule formation) of PDLSCs cultured in calcium silicate-based cement or calcium silicate-based sealer-conditioned media. Studies evaluating the aforementioned variables between two or more CSCs/CSSs and/or between one or more CSCs/CSSs and a control group were eligible.

The criteria for eligibility were established in accordance to the PICOS model (Page et al., 2021b), as follows: population (P): periodontal ligament stem cells; intervention (I): culture media treated with calcium silicate-based sealers or cements; comparison/control (C): untreated culture media; outcome (O): PDLSC viability, proliferation, migration, differentiation and mineralization; study design (S): *in vitro*.

Search strategy and terminology

The search strategy, study screening and selection, methodology and outcome data extraction, and inner methodological risk of bias assessment were carried out by two independent investigators (JL.S and M.M). In the event of doubt, a third researcher was called for deliberation (J.G-G).

Individual advanced search strings were formulated and performed in five electronic databases: Medline, Scopus, Embase, Web of Science and SciELO on 1 July 2020 and last updated on 22 April 2021, without year or language restrictions. The search strategy included the following terms: 'silicate', 'bioceramic', 'periodontal ligament stem cells', 'PDLSCs', 'cytocompatibility', 'biocompatibility', 'bioactivity', 'differentiation', 'expression', and 'mineralization'; combined with the Boolean operators 'OR' and 'AND'. The search term selection was based on previous works within this framework and their most cited descriptors. Complementarily, the references from the included studies were manually screened after the selection process to check for additional potentially eligible studies. The advanced search strings and the findings for both the independent and combined search fields are presented in Table S1.

Study screening and selection process

The search results were exported from each database into a reference manager software (Mendeley; Elsevier, AMS), and duplicate records were manually discarded. The resulting record titles and abstracts were reviewed using the previously mentioned inclusion criteria. Studies which fulfilled the criteria were then further assessed for eligibility for qualitative synthesis by full-text screening.

Data extraction

The data extraction process was subdivided into three separate categories: study characteristics, methodology and outcomes/results. Authors and years of publication were recorded as study characteristics. Methodological variables included, regarding PDLSCs: cell variant and passage; regarding CSCs/CSSs: tested material/s and its/their concentration; and regarding the assessment of their biological interaction: assays performed and their duration,

and the nature of the control groups used as a reference. Outcome variables included significant results found for each test, the time in which they were recorded (duration), and their significance level as a *P*-value.

Quality assessment

The 'Modified CONSORT checklist of items for reporting *in vitro* studies of dental materials' (Faggion, 2012) was used for the evaluation of inner methodological quality assessment (risk of bias) of the included studies resulting from the selection process. For each of the 14 parameters considered in the quality assessment tool, studies were individually assessed for fulfilment/nonfulfilment, and the percentage of complied items was subsequently calculated (number of complied items/total number of items x 100).

RESULTS

Search results and study selection

The results from the electronic database search and the study screening and selection process are shown in Figure 1. The individual database searches identified a total of 128 records: (Medline: 29, Scopus: 27, Embase: 18, Web of Science: 54, SciELO: 0). By means of the 'check for duplicates' tool from the reference manager software, duplicate records were manually discarded ($n = 43$). From the resulting 85 records, 67 were excluded after assessing the fulfilment of the inclusion criteria by examining the titles and abstracts. Two additional studies which met the inclusion criteria were found during the manual search of the references of the included studies. All 20 of the resulting studies were considered as eligible for qualitative synthesis after full-text evaluation (Collado-González et al., 2017a,b; Rodríguez-Lozano et al., 2017; Wang et al., 2018; Luo et al., 2018; Collado-González et al., 2019; Rodríguez-Lozano et al., 2019a,b; Lee et al., 2019; López-García et al., 2019, 2020; Abuarqoub et al., 2020; Oh et al., 2020; Olcay et al., 2020; Rodríguez-Lozano et al., 2020a,b; Zheng et al., 2020; Gaudin et al., 2020; Sanz et al., 2020c; Jing et al., 2020).

Study methodology

The list of calcium silicate-based biomaterials tested by the studies included in the present review is presented in Table 1, along with their compositions and their manufacturer or developer. A total of 16 CSSs and 9 CSCs were assessed amongst the selected studies.

The data extracted from the selected studies on the methodology used to evaluate the biological interaction between PDLSCs and CSCs/CSSs, that is cell viability, proliferation/migration, osteo/odonto/cementogenic marker

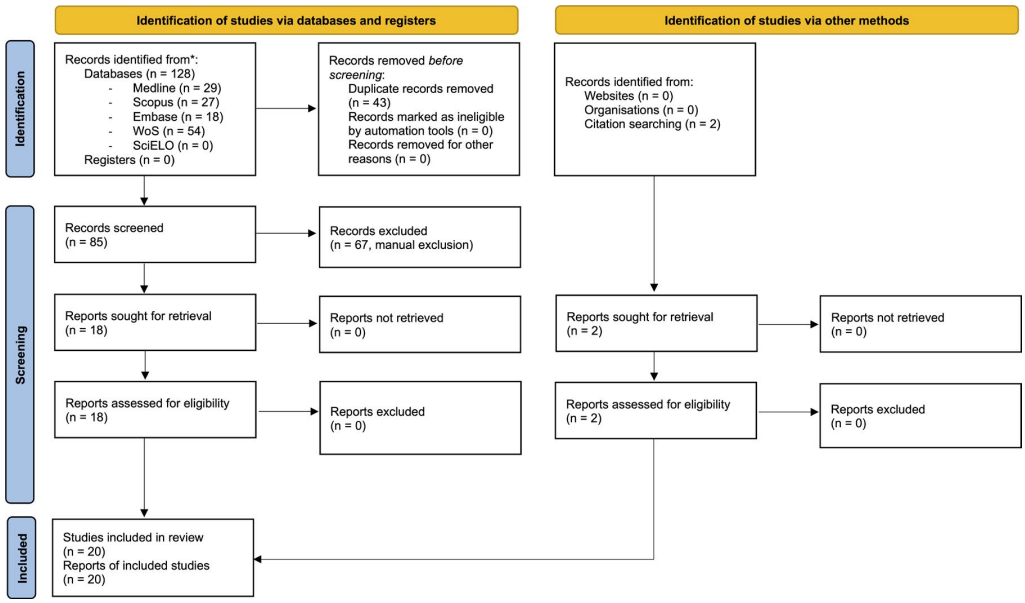

FIGURE 1 Systematic flow chart representing the study selection process. Based on the PRISMA 2020 flow diagram [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 List of CSCs/CSSs studied

Material	Type	Abbreviation	Composition	Manufacturer/ Developer	Times studied
Bio-C Repair	HCSC	BioC R	Powder: calcium silicate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide and dispersing agent (Rodríguez-Lozano et al., 2020a)	Angelus, Londrina, PR, Brazil	1
Bio-C Sealer	HCSS	BioC S	Calcium silicates, calcium aluminate, calcium oxide, zirconium oxide, iron oxide, silicon dioxide and dispersing agent[a]	Angelus, Londrina, PR, Brazil	1
Bio-C Sealer ION+	HCSS	BioC I	Calcium silicate, magnesium silicate, polyethylene glycol, zirconium oxide, silicon dioxide nanoparticles, potassium sulphate, calcium sulphate hemihydrate (Sanz et al., 2020c)	Angelus, Londrina, PR, Brazil	1
Biodentine	HCSC	BD	Powder: tricalcium silicate, dicalcium silicate, calcium carbonate, calcium oxide, iron oxide, zirconium oxide. Liquid: calcium chloride, hydrosoluble polymer[a]	Septodont, Saint-Maur-des-Fosses, France	3
Bioroot RCS	HCSS	BR	Powder: tricalcium silicate, zirconium oxide and excipients. Aqueous solution: calcium chloride and excipients (Gaudin et al., 2020)	Septodont, Saint-Maur-des-Fosses, France	3
C-Root	HCSS	CR	Powder: calcium silicate compound, strontium silicate, calcium dihydrogen phosphate, potassium dihydrogen phosphate, magnesium oxide, zirconium dioxide. Liquid: polyethylene glycol, water-free liquid (Jing et al. 2020)	Beijing C-root Dental Medical Devices Co. Ltd, Beijing, China	1
Ceraseal	HCSS	CS	Calcium silicates, zirconium oxide, thickening agent (López-García et al., 2020)	Meta Biomed Co., Cheongju, Chungcheong, South Korea	2
Endocem MTA	HCSC	EC MTA	Powder: calcium oxide, silicate oxide, aluminium oxide, other metallic oxides, bismuth oxide (Rodríguez-Lozano et al., 2019a)	Maruchi, Wonju, Gangwon, South Korea	1
Endoseal MTA	HCSS	ES MTA	Calcium silicates, calcium aluminates, calcium sulphate, radiopacifier, thickening agent (López-García et al., 2020)	Maruchi, Wonju, Gangwon, South Korea	3
Endoseal TCS	HCSS	ES TCS	Tricalcium silicate, phyllosilicate mineral, zirconium oxide, dimethyl sulphoxide (Oh et al., 2020)	Maruchi, Wonju, Gangwon, South Korea	1
Endosequence BC Sealer	HCSS	BCS	Zirconium oxide, calcium silicates, calcium phosphate monobasic, calcium hydroxide, filler and thickening agents (Rodríguez-Lozano et al., 2020a,b)	Brasseler USA Savannah, GA, USA	2
Endosequence BC Sealer HiFlow	HCSS	BCHiF	Zirconium oxide, tricalcium silicate, dicalcium silicate, calcium hydroxide, fillers (Sanz et al., 2020b)	Brasseler USA Savannah, GA, USA	2
GuttaFlow Bioseal	CSS	GFB	Gutta-percha powder, polydimethylsiloxane, platinum catalyst, zirconium dioxide, silver (preservative), colouring, bioactive glass ceramic (Gandolfi et al., 2016)	Coltène/Whaledent AG, Altstatten, Switzerland	2
iRoot Fast Set	HCSC	IR FS	Calcium silicates, zirconium oxide, tantalum pentoxide, calcium sulphate, calcium phosphate monobasic and filler agents[a]	Innovative Bioceramix, BC, Canada	1
iRoot SP	HCSS	IR SP	Calcium silicate, calcium phosphate, calcium hydroxide, niobium oxide and zirconium oxide[a]	Innovative Bioceramix, BC, Canada	1

(Continues)

TABLE 1 (Continued)

Material	Type	Abbreviation	Composition	Manufacturer/ Developer	Times studied
MTA Fillapex	CSS	MTA F	Paste A: salicylate resin, bismuth trioxide and fumed silica. Paste B: fumed silica, titanium dioxide, mineral trioxide aggregate and base resin (Gaudin et al., 2020)	Angelus, Londrina, PR, Brazil	4
MTA Repair HP	HCSC	MTA R	Powder: tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide and calcium tungstate. Liquid: water and polymer plasticizer (Collado-González et al., 2019)	Angelus, Londrina, PR, Brazil	1
MTA	HCSC	MTA	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, ferroaluminate tricalcium, calcium oxide, bismuth oxide[a]	Angelus, Londrina, PR, Brazil	2
Nano-ceramic Sealer	HCSS	NCS	Calcium silicates, zirconium oxide, filler, thickening agent (Lee et al., 2019)	B&L Biotech, Fairfax, VA, USA	2
NeoMTA-Plus	HCSC	N MTA	Powder: tricalcium silicate, dicalcium silicate and tantalum oxide. Liquid: Water and proprietary polymers (Rodríguez-Lozano et al., 2019a)	Avalon Biomed, Bradenton, FL, USA	1
ProRoot ES	HCSS	PR ES	Powder: calcium sulphate, dicalcium silicate, tricalcium silicate, bismuth oxide and a bit of tricalcium aluminate. Liquid (viscous aqueous solution of a water-soluble polymer) (Gaudin et al., 2020)	Dentsply Sirona, York, PA, USA	1
ProRoot MTA	HCSC	PR MTA	Powder: tricalcium silicate, dicalcium silicate, tricalcium aluminate, tetracalcium aluminoferrite, free calcium oxide, bismuth oxide (Collado-González et al., 2019)	Dentsply Tulsa, TN, USA	2
TotalFill BC RRM Putty	HCSC	TF BCR	Powder: tricalcium silicate, dicalcium silicate, zirconium oxide, tantalum pentoxide, calcium sulphate. (Rodríguez-Lozano et al., 2020a)	FKG Dentaire SA, LaChaux-de-fonds, Switzerland	1
TotalFill BC Sealer	HCSS	TF BCS	Tricalcium silicate, dicalcium silicate, zirconium oxide, calcium hydroxide[a]	FKG Dentaire SA, LaChaux-de-fonds, Switzerland	2
Wellroot ST	HCSS	WST	Calcium silicate compound, calcium sulphate dehydrate, calcium sodium phosphosilicate, zirconium oxide, titanium oxide, thickening agents (Lee et al., 2019)	Vericom, Chuncheon, Gangwon, Korea	2

Abbreviations: CSS, calcium silicate-based sealer; HCSC, hydraulic calcium silicate-based cement; HCSS, hydraulic calcium silicate-based sealer.
[a]Data extracted from the material's safety data sheet (MSDS) and/or provided by the manufacturer.

expression and mineralized nodule formation, are summarized in Table S2.

All of the studies used periodontal ligament stem cells of human origin (hPDLSCs) as their cellular sample for the *in vitro* biological assays. The modal range of cellular passages used was the 2nd-4th passages, with the lowest being the 2nd and the highest the 7th passage.

Three different groups of analyses were performed to evaluate the biological interaction between hPDLSCs and CSCs/CSSs: cytocompatibility assays, cell plasticity/differentiation assays and mineralization assays. As cytocompatibility indicators, studies performed a series of cell viability, migration/proliferation, morphology and/or adhesion assays. The assessment of cell viability was represented by three assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, Cell Counting Kit-8 or CCK8, and live/dead viability kit. To evaluate hPDLSC migration, studies performed either wound healing/scratch-wound, transwell migration or similar assays. Cell morphology and/or adhesion was qualitatively assessed

by various studies under scanning electron microscopy (SEM). Parallelly, cell cytotoxicity was evaluated by means of the measurement of cell apoptosis using Annexin-V/7-AAD staining.

To assess the plasticity/differentiation potential of hPDLSCs treated with CSCs/CSSs, studies quantified the expression of a series of osteo/odonto/cementogenic markers by means of quantitative reverse transcription polymerase chain reaction (RT-qPCR). Various studies performed an alkaline phosphatase (ALP) staining or assay to complementarily assess the increase in cellular activity and differentiation potential.

Lastly, the evaluation of the mineralization potential of CSCs/CSSs-treated hPDLSCs was represented in its totality by the quantification of mineralized nodule formation using Alizarin Red S staining (ARS).

Study results

Tables 2–4 present the significant results found by the included studies for cytocompatibility assays (i.e. cell viability and migration), osteo/odonto/cementogenic marker expression and mineralization assays; respectively. Results are presented along with the time point at which they were recorded (duration) and their significance level (as a *P*-value).

From the total of 16 CSSs assessed by the included studies, 14 exhibited significant positive results in hPDLSC cell viability, migration, differentiation and/or mineralization assays when compared to a negative control.

The most studied CSS, MTA F, exhibited significant negative results in cell viability (Collado-González et al., 2017b; Gaudin et al., 2020; Rodríguez-Lozano et al., 2017) and migration assays (Rodríguez-Lozano et al., 2017, 2019b). Complementarily, hPDLSCs cultured with MTA F showed a decreased cell viability after Annexin-V/7-AAD staining (Rodríguez-Lozano et al., 2019b) and a low spreading and adhesion under SEM (Collado-González et al., 2017b; Rodríguez-Lozano et al., 2017, 2019b). In a similar manner, ES MTA showed negative results in MTT and wound healing assays using unconditioned hPDLSC culture media as a reference (Collado-González et al., 2017a; López-García et al., 2020), and mixed results in SEM adhesion and morphology assays (positive (Lee et al., 2019) and negative (Collado-González et al., 2017a; López-García et al., 2020)). BR, however, exhibited significant mixed results in cytocompatibility assays (positive (Gaudin et al., 2020) and negative (Jing et al., 2020)), and significant positive results in migration, differentiation and mineralization assays (Gaudin et al., 2020). Additionally, BR exhibited a substantial hPDLSC spreading and attachment under SEM (Collado-González et al., 2017a).

Amongst the CSSs which were studied twice, BCS, BCHiF and TF BCS showed significant positive results in both MTT and wound healing assays when compared to a negative control. Additionally, all three CSSs showed a high cell spreading and attachment under SEM (Rodríguez-Lozano et al., 2017; 2020b; López-García et al., 2019, 2020; Sanz, et al., 2020c). Similarly, the studies assessing GFB and NCS reported positive results in cytocompatibility assays, as well as adequate cell morphology and adhesion when compared to a control (Collado-González et al., 2017a,b; Lee et al., 2019; Rodríguez-Lozano et al., 2019b). The remaining CSSs considered in the present review (BioC S, BioC I, CR, CS, PR ES, and WST), which were studied once, all exhibited significant positive results in at least one cytocompatibility assay, that is cell viability or migration (Gaudin et al., 2020; Jing et al., 2020; Lee et al., 2019; López-García et al., 2019, 2020; Sanz et al., 2020c).

From those CSSs deemed as cytocompatible, when cultured with hPDLSCs, the following additionally exhibited a significantly higher calcified nodule formation compared to a negative control: BR (Jing et al., 2020), BCS (López-García et al., 2020; Rodríguez-Lozano et al., 2020b), BCHiF (Sanz et al., 2020c; Rodríguez-Lozano et al., 2020b), TF BCS (López-García et al., 2019), BioC S (López-García et al., 2019), BioC I (Sanz et al., 2020c), CR (Jing et al., 2020) and CS (López-García et al., 2020). In a similar manner, hPDLSCs, when cultured with the following CSSs, reported an upregulation of one or more cementogenic, osteogenic and/or odontogenic markers: BR (Jing et al., 2020), BCS (López-García et al., 2020; Rodríguez-Lozano et al., 2020b), BCHiF (Sanz et al., 2020c; Rodríguez-Lozano et al., 2020b), GFB (Rodríguez-Lozano et al., 2019b), BioC I (Sanz et al., 2020c) and CS (Jing et al., 2020).

Amongst the 9 CSCs evaluated, BD was the only cement which exhibited significant positive results in cell viability and migration assays when compared to a negative control group (unconditioned culture medium; Abuarqoub et al., 2020). Studies assessing BioC R, TF BCR and IR FS reported an adequate cytocompatibility of the tested materials, exhibiting similar results to a negative control group (Abuarqoub et al., 2020; Rodríguez-Lozano et al., 2020a). All of the aforementioned cements showed a high cell adhesion and morphology (Abuarqoub et al., 2020; Luo et al., 2018; Rodríguez-Lozano et al., 2020a). Additionally, BD, BioC R, TF BCR and IR FS-treated hPDLSCs exhibited an upregulation of one or more cementogenic, osteogenic and/or odontogenic markers (Abuarqoub et al., 2020; Rodríguez-Lozano et al., 2020a). BioC R and TF

TABLE 2 Summary of the results of included studies showing significant differences between CSCs/CSSs or a CSC/CSS and a control for hPDLSC cytocompatibility assays

Author	Assay	Significant results	Duration	P value
Sanz et al., 2020c	MTT	1:1 BCHiF > −control	48 h	*
	Wound healing	1:4 BioC I > −control	48 h	*
		1:1 BCHiF > −control	48 h	*
Oh et al., 2020	CCK8	Freshly mixed CS > −control	7 d	*
Gaudin et al., 2020	MTT	1:1, 1:2, 1:4 BR >PR ES, −control >1:1, 1:2, 1:4 MTA F	24 h	*
Rodríguez-Lozano et al., 2020a	MTT	−control >1:1, 1:2 BioC R	24 h	**
		−control >1:4 BioC R	24 h	***
		−control >1:4 BioC R	48 h	*
		−control >1:1 TF BCR	72 h	*
		−control >1:4 TF BCR	72 h	**
		1:4 TF BioC R > −control	48 h	***
		1:4 TF BioC R > −control	72 h	**
	Scratch migration	−control >1:1 BioC R	24 h	*
		−control >1:1, 1:2 BioC R	48 h	*
Abuarqoub et al., 2020	MTT	0.02, 0.2, 2mg/mL BD > −control	6 d	*
		−control >20mg/mL BD	6 d	*
	Scratch migration	1:1 BCHiF > −control	24 h	**
Rodríguez-Lozano et al., 2020b	MTT	1:1 BCHiF, 1:1 BCS > −control	24 h	**
		1:2 BCHiF, 1:2 BCS > −control	24 h	*
	Scratch migration	1:1 BCHiF > −control	24 h	**
López-García et al., 2020	MTT	−control >1:1, 1:2, 1:4 ES MTA	48, 72 h	***
		−control >1:1 ES MTA	24 h	***
		CS > −control	72 h	*
		BCS > −control	72 h	**
	Wound healing	1:1BCS, 1:1 CS > −control	24 h	*
		1:1BCS > −control	48 h	*
		1:1, 1:2 BCS > −control	72 h	*
		−control >1:1 ES MTA	24, 48 h	**
		−control >1:1 ES MTA	72 h	***
		−control >1:2 ES MTA	24, 48, 72 h	**
Zheng et al., 2020	MTT	−control >MTA	24, 48 h	*
	Annexin V-FITC/PI	−control >MTA	24, 48, 72 h	*
		−control >IR SP	24, 48 h	*
Olcay et al., 2020	MTS	PR MTA >WST	1 d	*
		WST > −control	3 d	*
		PR MTA >BD, −control	7 d	*
López-García et al., 2019	MTT	−control >1:1, 1:2 BioC S	48, 72 h	***
		−control >1:1, 1:2 TF BCS	48, 72 h	***
		1:2 TF BCS > −control	48 h	*
		1:2 TF BCS > −control	48 h	**
		1:4 TF BCS > −control	72 h	***
	Scratch wound	−control >1:1 BioC S	24, 72 h	*
		−control >1:1 BioC S	48 h	**
		−control >1:1 TF BCS	48, 72 h	*

(Continues)

TABLE 2 (Continued)

Author	Assay	Significant results	Duration	P value
Rodríguez-Lozano et al., 2019b	Wound healing	−control >1:1, 1:2, 1:4 MTA F	48 h	***
		−control >1:1 MTA F	24 h	***
		−control >1:2 MTA F	24 h	**
		−control >1:4 MTA F	24 h	*
Lee et al., 2019	MTT	WST > −control	3 d	*
Jing et al. 2020	CCK8	−control >BR, CR	7 d	*
Collado-González et al., 2017b	MTT	−control >1:1, 1:2, 1:4 MTA F	24, 48, 72, 168 h	***
		−control >1:1, 1:2, 1:4 GFB, GF2	72 h	**
		−control >1:2 GF2	48 h	**
		−control >1:4 GF2	48 h	*
		1:1, 1:2, 1:4 GFB > −control	168 h	***
Collado-González et al., 2017a	MTT	−control >1:1, 1:2 ES MTA	24, 48, 72 h	***
		−control >1:4 ES MTA	48 h	**
		−control >1:4 ES MTA	72 h	***
		1:1 NCS > −control	24 h	*
	Wound healing	−control >1:1 ES MTA	24, 48 h	***
		−control >1:2 ES MTA	24 h	*
		−control >1:2 ES MTA	48 h	***
		−control >1:4 ES MTA	24, 48 h	*
		−control >1:1 NCS	24 h	***
		−control >1:1 NCS	48 h	**
		−control >1:2 NCS	24, 48 h	*
		−control >1:4 NCS	48 h	*
		1:2 BR > −control	24 h	*
		1:4 BR > −control	24 h	***
Rodríguez-Lozano et al., 2017	MTT	1:1, 1:2, 1:4 TF BCS >1:1, 1:2, 1:4 MTA F	24, 48, 72 h	***
		−control >1:1, 1:2, 1:4 MTA F	24, 28, 72 h	*
	Scratch wound	−control >1:1, 1:2, 1:4 MTA F	24, 48 h	***
		1:1, 1:2 TF BCS > −control	24 h	*
		1:4 TF BCS > −control	24 h	**

Note: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Abbreviations: >, significantly higher cytocompatibility (viability in cell viability assays and wound closure in cell migration assays); −control, negative control (hPDLSCs in unconditioned media).

BCR also showed a significantly higher mineralization potential compared to a negative control (Rodríguez-Lozano et al., 2020a).

The remaining CSCs assessed were tested together with hPDLSCs both in neutral (pH 7.4) and acidic (pH 5.2) conditions. MTA R and PR MTA showed significant positive results at lower dilutions (1:4) in both neutral and acidic conditions in cell viability assays, compared to a negative control (Collado-González et al., 2019). EC MTA and N MTA, however, exhibited a higher cell viability in an acidic environment (pH 5.2) (Rodríguez-Lozano et al., 2019a). Both of the aforementioned studies also reported high hPDLSC adhesion in both neutral and acidic conditions for the tested CSCs.

Quality assessment

The results from the quality assessment, following the 'Modified CONSORT checklist of items for reporting *in vitro* studies of dental materials' (Faggion, 2012), are presented in Table 5. The 20 studies included in the present

TABLE 3 Summary of the results of included studies showing significant differences between CSCs/CSSs or a CSC/CSS and a control for hPDLSC osteo/odonto/cementogenic marker expression using RT-qPCR

Author	Significant results	Marker	Duration	P value
Sanz et al., 2020c	1:1 BCHiF >1:1 BioC I, +control, −control	CEMP−1	7 d	***
	1:1 BioC I > −control		7 d	**
	1:1 BioC I > 1:1 BCHiF, +control, −control	CAP	7 d	***
	1:1 BCHiF < +control		7 d	***
	1:1 BCHiF > −control		7 d	**
	1:1 BCHiF > −control	ALP	7 d	***
	1:1 BCHiF >1:1 BioC I, +control		7 d	**
	1:1 BCHiF >1:1 BioC I, +control, −control	Runx2	7 d	***
	1:1 BioC I > −control		7 d	***
Zheng et al., 2020	MTA > −control	ALP	14, 21 d	*
	IR SP > −control		14 d	**
			21 d	*
	MTA > −control	OC	14 d	**
			21 d	*
	IR SP > −control		14, 21	**
	MTA, IR SP > −control	Runx2	7, 14, 21 d	*
	MTA > −control	COL1A1	14, 21 d	*
	IR SP > −control		14, 21 d	**
Oh et al., 2020	Set ES TCS >−control	Runx2	7 d	*
	Set ES TCS >Set CS	OCN	7 d	*
Rodríguez-Lozano et al., 2020a	1:1 BioC R > −control	CEMP−1	7 d	*
	−control >1:1 BioC R		14 d	*
	1:1 BioC R > −control	CAP	7 d	*
	1:1 BioC R > −control		14 d	**
	1:1 TF BCR > −control		14 d	***
	−control >1:1 BC R, 1:1 TF BCR	ALP	7 d	***
	−control >1:1 BioC R		14 d	**
	−control >1:1 TF BCR		14 d	***
	1:1 BioC R > −control	Runx2	7 d	***
	1:1 BioC R > −control		14 d	*
	1:1 TF BCR > −control		7, 14 d	***
Abuarqoub et al., 2020	2mg/mL BD > −control	ALP, OPN, OCN	21 d	*
Rodríguez-Lozano et al., 2020b	1:1 BCHiF, 1:1 BCS > −control	ALP, CEMP−1, Runx2	7 d	***
	1:1 BCHiF, −control	CAP	7 d	**
	1:1 BCS, −control		7 d	*
López-García et al., 2020	1:1 BCS, 1:1 CS > −control	ALP	3 d	***
	1:1 CS > −control		7 d	***
	1:1 BCS > −control		7 d	**
	1:1 CS > −control		14 d	*
	1:1 CS > −control		21 d	**
	1:1 BCS, CS > −control	CAP	3, 7, 14, 21 d	***
	1:1 CS > −control	CEMP−1	3, 7, 14 d	***
	1:1 CS > −control		21 d	*
	1:1 BCS > −control		7 d	**
	1:1 BCS > −control		14 d	*

(Continues)

TABLE 3 (Continued)

Author	Significant results	Marker	Duration	P value
Rodríguez-Lozano et al., 2019b	1:1 GFB, 1:1 GF2 > −control	AMBN	7 d	**
	1:1 GFB, 1:1 GF2 > −control	AMELX	7 d	*
	1:1 GF2 > −control	CAP	7 d	*
	1:1 GFB > −control	CEMP−1, BSP	7 d	*
Jing et al. 2020	BR > −control	OCN	7 d	*
Wang et al., 2018	MTA > −control	OCN, OSX, RUNX2, DSPP, DMP1, COL- I	3, 7 d	**
Luo et al., 2018	−control >BD	ALP, COL1	7,14 d	*
	−control >IR FS		14 d	*
	IR FS > −control	OCN, Runx2	14 d	*

Note: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Abbreviations: +control, positive control (hPDLSCs in osteogenic media); >, significantly higher expression; −control, negative control (hPDLSCs in unconditioned media).

review followed a similar pattern in terms of the quality (risk of bias) of the data reported in their respective papers.

The manuscripts presented an updated and concise rationale and introduction, together with clear objectives and/or hypotheses (items 1 and 2). Methodological data were presented in a structured manner, with defined study groups and measures of outcome (items 3 and 4). However, items 5–9, regarding the sample size calculation and randomization process, were not fulfilled by any of the studies, since they were nonapplicable. The statistical analyses performed were reported in all studies (item 10), and outcomes were presented along with their significance levels or confidence intervals (item 11). 12 out of the 20 studies addressed the potential limitations of their respective methodologies (item 12). Sources of funding (if any) were reported by all studies (item 13), and no reference to an available protocol was made by any of the studies (item 14). The percentage of complied items varied from 80% to 90%.

TABLE 4 Summary of the results of included studies showing significant differences between CSCs/CSSs or a CSC/CSS and a control for hPDLSC mineralization assays using ARS

Author	Significant results	Duration	P value
Sanz et al., 2020c	1:1 BCHiF, 1:1 BioC I > +control, −control	21 d	***
	1:1 BioC I > 1:1 BCHiF	21 d	*
Rodríguez-Lozano et al., 2020a	1:1 BioC R, 1:1 TF BCR > −control	21 d	**
Rodríguez-Lozano et al., 2020b	1:1 BCHiF, 1:1 BCS > −control	21 d	***
López-García et al., 2020	1:1 BCS, 1:1 CS > −control >1:1 ES	21 d	***
	1:1 BCS, 1:1 CS > +control	21 d	**
	1:1 BCS >1:1 CS	21 d	*
López-García et al., 2019	1:1 BioC S, 1:1 TF BCS > −control	21 d	**
Jing et al. 2020	BR, CR > −control	14 d	*
Wang et al., 2018	MTA >−control	14 d	**
	MTA + +control > +control	14 d	**

Note: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Abbreviations: +control, positive control (hPDLSCs in osteogenic media); >, significantly higher mineralized nodule formation; −control, negative control (hPDLSCs in unconditioned media).

TABLE 5 Quality assessment of the included studies

	Modified CONSORT checklist															
Studies	**1**	**2a**	**2b**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**%**
Sanz et al., 2020c	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Gaudin et al., 2020	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	N	Y	N	80
Rodríguez-Lozano et al., 2020a	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Abuarqoub et al., 2020	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Rodríguez-Lozano et al., 2020b	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	N	Y	N	80
López-García et al., 2020	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	N	Y	N	80
Olcay et al., 2020	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Oh et al., 2020	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Zheng et al., 2020	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	N	Y	N	80
López-García et al., 2019	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	N	Y	N	80
Lee et al., 2019	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Collado-González et al., 2019	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Rodríguez-Lozano et al., 2019a	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Rodríguez-Lozano et al., 2019b	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Jing et al. 2020	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	N	Y	N	80
Wang et al. 2018	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Luo et al., 2018	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Collado-González et al., 2017a	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	Y	Y	N	90
Collado-González et al., 2017b	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	N	Y	N	80
Rodríguez-Lozano et al., 2017	Y	Y	Y	Y	Y	-	-	-	-	-	Y	Y	N	Y	N	80

Abbreviations: %, percentage of compliance per article. Based on the checklist of items from 'Guidelines for Reporting Pre-clinical In Vitro Studies on Dental Materials' (Faggion, 2012); -, nonapplicable; N, not reported on the study; Y, reported on the study.

DISCUSSION

In the present systematic review, both calcium silicate sealers and cements of endodontic use are considered. Although their clinical application differs (i.e. CSSs are used as root canal filling materials along with a core material in RCT, and CSCs, often referred to as 'putty' biomaterials, are reserved for ES, RET or defect repair), both biomaterial subsets will be placed in direct contact with biological tissues during endodontic procedures (Torabinejad et al., 2018).

The composition and physicochemical properties of commercially available calcium silicate-based biomaterials have been extensively described, along with their favourable biological properties. However, the biological interaction between newly introduced CSCs and CSSs and DSCs is still a relevant subject of study (Donnermeyer et al., 2019; Duarte et al., 2018). Thus, the aim of this review was to present a qualitative synthesis of available *in vitro* studies assessing the biological interaction CSCs and CSSs with one of the cellular populations with which they will come into contact during their endodontic application, namely periodontal ligament stem cells.

On the methodology studies: considerations on material preparation, composition and physicochemical properties

Studies considered in the present review assessed the biological response of hPDLSCs towards one or more commercially available calcium silicate-based biomaterial compositions by means of their combined culture *in vitro*. The tested biomaterials were mixed following their respective manufacturers' instructions prior to the sample preparation, which consisted of different concentrations of freshly mixed (unset) material eluates or incubated (set) material eluates. The latter was the most common sample preparation method amongst the included studies, as shown in Table S2. Nonetheless, none of the studies provided an analysis of the biological properties of the tested materials from the perspective of sample

preparation. As reported in previous works (Costa et al., 2016; Pedano et al., 2018), the majority of *in vitro* studies on the biological interaction between calcium silicate-based materials and dental stem cells use incubated (set) material eluates as test samples. Clinically, since CSCs/CSSs are applied directly after mixing (before setting), the use of freshly mixed (unset) material eluates can better predict the initial response of dental stem cells towards them. Nevertheless, the use of incubated (set) material eluates could be useful to predict the late/long-term response after cellular renewal (Oh et al., 2020). The physicochemical differences between both types of sample preparations, such as pH (Pedano et al., 2018), may lead to different results in biological assays. Thus, future studies in the field could include both designs in order to provide an exhaustive biological profiling of the tested materials.

Various of the latter studies then additionally profiled the superficial chemical composition of the tested biomaterials under scanning electron microscopy–energy dispersive spectroscopy (SEM-EDS; Collado-González et al., 2017b; 2019; Rodríguez-Lozano et al., 2019a, 2020a,b; Sanz et al., 2020c; López-García et al., 2020), but did not assess the influence of compositional differences with their biological properties. As shown in Table 1, the assessed materials present a common basic composition: calcium and silicate compounds, a radiopacifying agent and fillers (thickening, dispersing or plasticizing agents), which can be complemented with different additives such as resins (MTA F) or gutta percha powder (GFB), amongst others. Previous evidence indicates that even small chemical variations, the inclusion of a low percentage of additives, or even differences in the radiopacifier may substantially modify the physical, chemical and biological properties and behaviour of calcium silicate-based biomaterials (Guimarães et al., 2018; Koutroulis et al., 2019; Silva et al., 2017).

Amongst the calcium silicate-based materials assessed in the present review, Portland cement-based formulations (i.e. cements: EC MTA, MTA, PR MTA; sealers: MTA F, PR ES, TF BCS) contain bismuth oxides as radiopacifying agents, whilst the remaining calcium silicate-based materials contain zirconium oxides (cements: BioC R I, BD, IR FS; sealers: BioC S, BioC I, BR, CR, CS, ES TCS, BCS, BCHiF, GFB, IR SP, NCS, TF BCS, WST). Two recent Portland cement-based formulations, however, incorporate different radiopacifiers (calcium tungstate: MTA R; tantalum oxide: N MTA). Singularly, the CSC TF BCR contains both zirconium and tantalum oxides. These differences may influence the biological properties of the aforementioned materials, but were not addressed by any of the studies included in the present review. Future research on the biological properties of calcium silicate-based and other endodontic materials could be performed from the perspective of the differences in radiopacifying agents, as performed by a recent study (Queiroz et al., 2021).

The compositional differences of the assessed materials may also account for their differences in ionic release, which was tested by various of the included studies via inductively coupled plasma mass spectrometry (ICP-MS) (Rodríguez-Lozano et al., 2019a, 2020b; Sanz et al., 2020c; López-García et al., 2020). Significant differences in calcium ion release were reported between various of the tested CSCs/CSSs: BioC I > BCHiF (p<0.05; (Sanz et al., 2020c), CS>BCS, ES MTA ($p < 0.05$; (López-García et al., 2020), BCHiF >BCS ($p < 0.05$; (Rodríguez-Lozano et al., 2020b). Calcium release favours the differentiation of dental stem cells and aids in the process of biomineralization and thus has been associated with the biological properties exhibited by calcium silicate-based materials (An et al., 2012; Rathinam et al., 2021). The ionic release of new commercial formulations of CSCs/CSSs should be further studied in order to better understand the mechanisms behind their biological properties. By establishing the role of the different ionic interactions occurring between calcium silicate-based biomaterials and surrounding tissues, new material formulations with specific additives to enhance their performance could be developed (Gandolfi et al., 2014; Kasraei et al., 2021).

On the results of studies: cytocompatibility, differentiation and mineralization potential of the tested materials

Prior to the biological assays, all of the included studies characterized the hPDLSCs subject for testing via flow cytometry or used previously characterized hPDLSCs, in order to define their mesenchymal-like phenotype. For the subsequent biological assays, hPDLSCs were cultured together with varying concentrations/dilutions of the tested biomaterials (test groups), alone in an osteogenic medium (positive control group) and/or alone in an unconditioned medium (negative control group). The negative control groups used by the included studies consisted of hPDLSCs cultured in either Dulbecco's Modified Eagle Medium (DMEM) or Minimum Essential Medium α (αMEM) together with variable concentrations of foetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). Half of the included studies included a positive control group for analysis, as shown in Table S2.

Upon comparison with a negative control group, all of the tested CSCs exhibited at least similar results in cytocompatibility assays when cultured together with hPDLSCs. Additionally, all of the CSCs assessed for hPDLSC differentiation potential resulted in an upregulation of

osteogenic, cementogenic and/or odontogenic markers, suggesting their positive influence on hPDLSCs differentiation. These results are in accordance with similar reviews in the field. A recent systematic review of *in vitro* studies assessed the cytotoxicity and bioactive properties of pulp capping-agents towards human dental pulp cells (hDPCs) and reported that resin-free calcium silicate-based cements, such as those considered in the present review, promoted cell viability and bioactivity (Pedano et al., 2020). This is also supported by another systematic review of *in vitro*, human and animal studies on the effects of silicate cements on dental pulp cells, which concluded that most CSCs are biocompatible and can promote pulp healing (Emara et al., 2018). In fact, a series of original research papers on the interaction of CSCs with dental pulp cells support the use of this group of biomaterials in vital pulp therapy (VPT) procedures from a physicochemical point of view (Gandolfi et al., 2015; Peng et al., 2011).

In a similar manner, previous systematic reviews reported positive results for CSCs in terms of cytocompatibility, activity-related marker expression and mineralization potential together with human dental pulp stem cells (hDPSCs; Sanz et al., 2019), hSCAPs (Sanz et al., 2020a), and stem cells from human exfoliated deciduous teeth (SHEDs; Sanz et al., 2020b). In all of the cases, MTA (Angelus, Londrina, Brazil), ProRoot MTA (Dentsply Tulsa) and Biodentine (Septodont) were the most studied CSCs and exhibited at least adequate cytocompatibility, upregulation of osteo/odontogenic markers and mineralization potential *in vitro* with the aforementioned DSCs.

However, this is the first systematic review to specifically assess the biological interaction between hPDLSCs and CSCs/CSSs. Dental stem cells, as a subpopulation of MSCs, share a specific mesenchymal-like phenotype, but also exhibit individual characteristics which could result in a different response to external stimuli (Huang et al., 2009). For example, hPDLSCs have exhibited a higher osteogenic potential compared to hDPSCs (Winning et al., 2019) and SHEDs (Chadipiralla et al., 2010). This justifies the need for the separate assessment and categorization of the biological behaviour of the different dental stem cell variants.

Nevertheless, the majority of the studies assessing the biological interaction between DSCs and dental biomaterials use the expression of activity-related markers as an indicator of their differentiation potential. In the present review, the following markers were studied: cementogenic markers (cementum protein 1 (CEMP-1), cementum attachment protein (CAP)), osteogenic markers (runt-related transcription factor 2 (Runx2), osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP)), odontogenic markers (ameloblastin (AMBN), amelogenin X (AMELX)) and other markers (ALP (alkaline phosphatase), colagen-1 (COL1)). Cementogenic markers are of particular interest with regard to hPDLSCs, due to their specific location within the periodontium and involvement in periodontal regeneration. CAP has been described to be involved in the recruitment and differentiation of cells during the process of cementum formation (Pitaru et al., 1993; Valdés De Hoyos et al., 2012). Complementarily, CEMP-1 is expressed in cementoblasts and cementum matrix and has been found to play an essential role in the regulation of the differentiation of periodontal cells (Hoz et al., 2012; Arzate et al., 2015). Therefore, the CSCs and CSSs which exhibited an upregulation of such genes when cultured with hPDLSCs (shown in Table 3) may enhance their cementogenic differentiation and consequently promote the repair or regeneration of periodontal defects resulting from lesions of endodontic origin (Gandolfi et al., 2011). The upregulation of osteogenic markers exhibited by CSCs and CSSs, on the other hand, supports their potential use for bone regeneration. In fact, the use of CSCs as mineral fillers in scaffolds for regenerative procedures is also under investigation (Gandolfi et al., 2018; Huang et al., 2018).

All of the studies assessing the differentiation potential of hPDLSCs using RT-qPCR measured the expression of cellular activity-related markers relative to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GADPH) at different time points, ranging from 3 to 21 days. However, only two studies reported a statistical analysis of the differences in marker expression between the different time points (Lee et al., 2019; Luo et al., 2018). ES MTA, NCS and WST-treated hPDLSCs exhibited a significant increase in the expression of *ALP from 3 to 9 days* of culture ($p < 0.05$) (Lee et al., 2019). Interestingly, ALP expression was significantly downregulated from day 7 to day 14 of culture in hPDLSCs treated with iRoot FS, and the same occurred with COL1 after treatment with iRoot FS or BD ($p < 0.05$; Luo et al., 2018). The analysis of the expression of cellular activity-related markers at individual time points provides a quantitative measurement with regard to the cellular differentiation potential, but only by analysing the change between different time points can we assess the time-dependency of the described effect. Consequently, future investigations in the field should consider performing measurements of both the static and variable expressions of cellular activity-related markers.

Regarding the assessment of the mineralization potential, it is well-established that calcium silicate-based biomaterials promote the formation of a hydroxyapatite-like superficial layer by a process of ionic interchange with surrounding fluids (Kim et al., 2015; Watson et al., 2014). Amongst the studies included in the present review, the *in vitro* assessment of the biomineralization exhibited by

hPDLSCs cultured with CSCs or CSSs was performed by Alizarin Red S staining. In general terms, those biomaterials which produced positive results in cytocompatibility assays also produced positive results in the biomineralization assays (Jing et al., 2020; López-García et al., 2019, 2020; Rodríguez-Lozano et al., 2020a,b; Sanz et al., 2020c), as shown in Tables 2 and 4.

As an additional test for hPDLSC mineralization potential, three of the included studies performed assays involving ALP staining (Jing et al., 2020; Lee et al., 2019; Luo et al., 2018). ALP acts as a marker for stem cell mineralization, and its expression is increased to produce inorganic phosphate for hydroxyapatite synthesis (Luo et al., 2018). The CSSs which exhibited positive results in ARS mineralization assays (BR and CR (Jing et al., 2020)) also exhibited significant positive results in ALP activity assays when compared to a negative control group ($p < 0.05$). Therefore, for future studies, performing both mineralization assays could be suitable to further confirm the mineralization potential of the tested biomaterials.

Strengths, limitations and future perspectives

Considering the *in vitro* nature of the assays performed by the included studies, the generalization and extrapolation of the results to the clinical setting is problematic, since the influence of a number of potential external factors is not considered. Nevertheless, *in vitro* study designs used to preliminarily define and perform a biological profiling of novel biomaterial formulations or commercially available endodontic materials, prior to the advancement towards animal studies or clinical trials (Pedano et al., 2020). Therefore, the qualitative synthesis of the studies considered in the present review provides preliminary evidence as to how CSCs and CSSs could act in direct contact with periodontal tissues during endodontic procedures.

The quality of the evidence reported in the present review was assessed by means of a modified CONSORT checklist for *in vitro* studies on dental materials (Faggion, 2012), in the absence of a specific quality assessment tool for this type of studies. The current guidelines do not offer a quality indicator, that is high/medium/low quality, based on the compliance of the proposed items. To overcome this limitation, the percentage item compliance was calculated for each study as an objective measure of their quality. All of the studies assessed presented a high percentage of item compliance, which can be interpreted as a low risk of bias. It should be noted that the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines have been recently published, in order to improve quality and transparency in reporting *in vitro* studies in experimental endodontic research (Nagendrababu et al., 2021a,b).

The assessment of the biological properties of these biomaterials in different conditions, namely the effect of pH variations (Collado-González et al., 2019; Rodríguez-Lozano et al., 2019a,b), varying temperatures (Chen et al., 2020) or different material manipulations (Domingos Pires et al., 2021), amongst others, could act as relevant topics for future investigations in the field. Parallelly, the cellular mechanisms behind the observed biological properties of CSCs and CSCs are still under investigation. Most recently, a systematic review on the involvement of signalling transduction pathways on the interaction between DSCs and CSCs has been reported (Sanz et al., 2021), where the potential involvement of MAPK, NF-κβ, Wnt/β-catenin, BMP/Smad and CAMKII signalling pathways was elucidated. Nevertheless, the exact mechanism/s behind this interaction are still unclear and require further investigation.

CONCLUSIONS

Calcium silicate-based cements and sealers, as a group of endodontic materials, exhibit adequate cytocompatibility and favour the osteo/cementogenic differentiation and mineralization potential of human periodontal ligament stem cells, as evidenced from the *in vitro* studies included in the present systematic review. Thus, within the limitations of the *in vitro* nature of the included studies, this work supports their potential use in stem cell therapy and biologically based regenerative endodontic procedures. The influence of the compositional differences, inclusion of additives, sample preparation, and varying conditions and manipulations on the biological properties of calcium silicate-based materials remain as a subject for future research.

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CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

JL Sanz and M Melo involved in conceptualization, data curation and writing – review and editing. JL Sanz involved in formal analysis, investigation, methodology and roles/writing – original draft. M Melo involved in project administration. J Guerrero-Gironés, MP Pecci-Lloret and MR. Pecci-Lloret designed the software and involved in

study validation and visualization. M Melo supervised the study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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ORIGINAL ARTICLE

Microstructural composition, ion release, and bioactive potential of new premixed calcium silicate–based endodontic sealers indicated for warm vertical compaction technique

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Abstract

Objective The aim of this study was to evaluate the microstructural composition, ion release, cytocompatibility, and mineralization potential of Bio-C Sealer ION+ (BCI) and EndoSequence BC Sealer HiFlow (BCHiF), compared with AH Plus (AHP), in contact with human periodontal ligament cells (hPDLCs).

Materials and methods The sealers' ionic composition and release were assessed using energy-dispersive spectroscopy (EDS) and inductively coupled plasma mass spectrometry (ICP-MS), respectively. For the biological assays, hPDLCs were isolated from third molars, and sealer extracts were prepared (undiluted, 1:2, and 1:4 ratios). An MTT assay, wound-healing assay, and cell morphology and adhesion analysis were performed. Activity-related gene expression was determined using RT-qPCR, and mineralization potential was assessed using Alizarin Red staining (ARS). Statistical analyses were performed using one-way ANOVA and Tukey's post hoc test ($\alpha < 0.05$).

Results The three sealers exhibited variable levels of silicon, calcium, zirconium, and tungsten release and in their composition. Both BCI and BCHiF groups showed positive results in cytocompatibility assays, unlike AHP. The BCHiF group showed an upregulation of CAP ($p < 0.01$), CEMP1, ALP, and RUNX2 ($p < 0.001$) compared with the negative control, while the BCI group showed an upregulation of CEMP1 ($p < 0.01$), CAP, and RUNX2 ($p < 0.001$). Both groups also exhibited a greater mineralization potential than the negative and positive controls ($p < 0.001$).

Conclusions The calcium silicate–based sealers considered in the present in vitro study exhibited a high calcium ion release, adequate cytocompatibility, upregulated osteo/cementogenic gene expression, and increased mineralized nodule formation in contact with hPDLCs.

Clinical relevance From a biological perspective, BCI and BCHiF could be clinically suitable for root canal filling.

Keywords Hydraulic calcium silicate–based sealers · Ion release · Periodontal ligament stem cells · Cytocompatibility · Biocompatibility · Mineralization

Introduction

Endodontic procedures require the use of materials with specific biological properties, in order to increase their predictability and improve the prognosis of the affected tooth [1]. Regarding root canal treatment, endodontic sealers will be placed in direct contact with both organic and inorganic tissue, with a substantial cellular component. Thus, these biomaterials should at least exhibit an absence of cytotoxicity towards surrounding tissues [2] and ideally promote an active biological response that favors cell survival, differentiation, and consequent tissue repair or neoformation [3].

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Within endodontic sealers, those with calcium silicate–based compositions have shown high clinical success rates in root canal treatment [4, 5] and bioactive properties in vitro [6–8]. Their ion release and interchange with tissue fluids result in the formation of a hydroxyapatite-like layer on their surface, and a consequent mineral attachment to the inorganic component of dentine [9, 10].

The complex healing process of any pre-existent periapical lesion after root canal disinfection and sealing depends on the reparative potential of surrounding cells and tissues [11]. Current investigations with regard to endodontic sealers have centered on their interaction with dental stem cells (DSCs). Since the characterization of DSCs from different local sources [12–14] and the description of their active role in reparative dentinogenesis [15, 16], properties like cytocompatibility have become essential for endodontic sealers. Among DSCs, periodontal ligament stem cells (PDLSCs) have been shown to contribute to the repair and regeneration of the periodontium and other tissues [17].

Most recently, a new hydraulic calcium silicate–based endodontic sealer (hCSS), Bio-C Sealer ION+ (Angelus, Londrina, PR, Brazil), has been released. Along with other silicate-based endodontic sealers such as EndoSequence BC Sealer HiFlow (Brasseler, Savannah, GA, USA), Bio-C Sealer ION+ is presented in a pre-mixed ready-to-use format that can be used for both cold and warm vertical obturation techniques, according to the manufacturer. Recent evidence suggests that the traditional epoxy resin–based sealer AH Plus (Dentsply, Konstanz, Germany) could also be suitable for warm techniques [18]. Differences in biomaterial composition may lead to variable ionic interactions with surrounding tissues and cellular responses [19, 20]. To the authors' knowledge, there is no evidence about the biological properties of the newly introduced Bio-C Sealer ION+, compared with traditional and well-established endodontic sealers which share its clinical indications.

Accordingly, the aim of this study was to determine the microstructural composition, ion release, and evaluate the cytocompatibility and mineralization potential of Bio-C Sealer ION+ and EndoSequence BC Sealer HiFlow, compared with AH Plus, in contact with human periodontal ligament cells (hPDLCs). The null hypothesis was that there is no difference between the tested materials in relation to their microstructural composition, ion release, cytocompatibility, and mineralization potential on hPDLCs.

Materials and methods

Cell isolation, culture, and characterization

The present in vitro study was formerly approved by the Ethics Committee from the Universidad de Murcia (ID: 2199/2018), following the Helsinki Declaration guidelines. Human PDLCs were isolated from healthy extracted third molars ($n = 10$) and cells at passages 2–4 were used for consecutive experimentation, as described previously [6]. In brief, third molars were transported in Minimum Essential Medium with Alpha modifications (α-MEM; Gibco, Invitrogen, Waltham, MA, USA) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and amphotericin B (Fungizone; Sigma-Aldrich, USA), at a temperature of 4 °C. The teeth were then rinsed thrice with phosphate-buffered saline (PBS) and their periodontal tissues were scraped from the middle and apical thirds of the root surface. Tissues were then sliced into smaller fragments and digested with collagenase type I solution (Gibco, USA) for 1 h at 37 °C. Finally, periodontal cells were seeded in α-MEM supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, USA). Prior to their use, hPDLCs were characterized using flow cytometry (FACSCalibur Flow Cytometry System; BD Biosciences, San José, CA, USA) and the high expression of the mesenchymal stem cell (MSC)–specific surface markers CD73, CD90, and CD105, and low expression of the hematopoietic markers CD34, CD45, CD14, and CD20 were confirmed [6].

Preparation of BC-Sealer ION+, EndoSequence HiFlow, and AH Plus extracts

For the in vitro assays, the hCSSs Bio-C Sealer ION+ (BCI; Angelus, Londrina, PR, Brazil) and EndoSequence BC Sealer HiFlow (BCHiF; Brasseler, Savannah, GA, USA) were used, along with the epoxy resin–based sealer AH Plus (AHP; Dentsply, Konstanz, Germany). Their respective compositions, as stated by their manufacturers, are listed in Table 1.

The materials were prepared according to their manufacturers' instructions under sterile conditions. To allow for a complete setting of the sealers, they were placed individually in 2-mm-depth and 5-mm-diameter casts with Hank's balanced salt solution (HBSS; H6648; Sigma-Aldrich, Gillingham, UK), sterilized under UV radiation for 15 min, and stored in an incubator at 37 °C, 5% CO_2, and 95% humidity for 48 h ($n = 30$). After the setting time, sample discs were incubated in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) for 24 h at the previously mentioned conditions, achieving a sample surface area/medium ratio of 1.5 cm^2/mL. Sample preparation and conditioning for in vitro experimentation were carried out in accordance with the current International Organization for Standardization (ISO) guidelines [21]. For the cell viability, migration, RT-qPCR, and mineralization assays, material extracts were filtered (0.22 μ pore size) and left undiluted, diluted to 1:2, and diluted to 1:4 ratios.

Table 1 Tested sealers

Sealer	Manufacturer	Composition	Lot number
Bio-C Sealer ION+	Angelus, Londrina, PR, Brazil	Calcium silicate, magnesium silicate, polyethylene glycol, zirconium oxide, silicon dioxide nanoparticles, potassium sulphate, calcium sulphate hemihydrate	151018
EndoSequence BC Sealer HiFlow	Brasseler, Savannah, GA, USA	Zirconium oxide, tricalcium silicate, dicalcium silicate, calcium hydroxide, fillers	(10)1802SPWF
AH Plus	Dentsply, 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

Scanning electronic microscopy and energy-dispersive spectroscopy

Two-millimeter-high and 5-mm-diameter sample discs for BCI, BCHiF, and AHP were prepared using the aforementioned process ($n = 9$), with a sample surface area/HBSS ratio of 6 cm^2/mL. After 48 h of setting in the previously described conditions, i.e., 37 °C, 5% CO_2, and 95% humidity, the discs underwent a carbon-coating process in a CC7650 SEM Carbon Coater unit (Quorum Technologies Ltd., East Sussex, UK). Then, the coated discs were examined individually under a SEM unit (Jeol 6100 EDAX; Jeol Inc., Peabody, MA, USA) attached to an EDS system (INCA 350 EDS; Oxford Instruments, Abingdon, UK) for element analysis.

Inductively coupled plasma mass spectrometry

Three sample discs with the previously described dimensions were immersed in 5-mL purified water (Milli-Q; Merck KGaA, Darmstadt, Germany) for 7 days and the proportion of calcium, iron, zirconium, silicon, and tungsten release from each sealer was determined by means of ICP-MS (Agilent 7900 ICP-MS; Agilent, Santa Clara, CA, USA).

Cytotoxicity assay (MTT)

Cell viability of hPDLCs in contact with sealer extracts was evaluated by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Cells were seeded onto 96-well plates with 180 μL of DMEM and stored for 24 h at 37 °C, 5% CO_2, and 95% humidity. 1×10^4 hPDLCs were then placed in contact with the undiluted, 1:2, and 1:4 material extracts ($n = 3$), and incubated for 24, 48, and 72 h in the same conditions. At each of the time intervals, 1 mg/mL of MTT solution was added to the hPDLC-sealer samples and incubated for a further 4 h. To solubilize the formazan crystals produced by viable cells through the reduction of MTT, 0.2 mL of dimethyl sulfoxide (DMSO) solution was added to each sample. Light absorption was then evaluated for each of the sample wells using a Synergy H1 multi-mode microplate reader (BioTek, Winooski, VT, USA) at 570 nm (Abs570), using the control group (cells cultured in unconditioned medium) as a reference, following previous evidence [22].

Cell migration assay (wound healing)

A wound healing assay was performed to assess hPDLC migration within the different material samples (undiluted, 1:2, and 1:4). Cells were seeded onto 6-well plates (2×10^5 hPDLCs per well, $n = 3$ for each dilution) and left to proliferate until confluent. Using a 200-μL pipette tip, a scratch was made on the surface of each cell monolayer, and each well was then rinsed three times with PBS to clear away any remaining cell debris. Wound healing (closure) was evaluated on an unconditioned sample (used as a control) and sealer-conditioned samples (non-diluted, 1:2, and 1:4) at 24, 48, and 72 h. Cell migration distances were assessed at three time-intervals: first 24-h period (0–24 h), second 24-h period (24–48 h), and third 24-h period (48–72 h). To account for width variations among the scratch wounds, migration rates were presented as percentage areas of relative wound closure or RWC and calculated as follows: RWC (%) = (wound closure area (pixels)/total number of pixels) × 100. Results were measured as the percentage of the total wound area at the different time points relative to the total wound area at 0 h for each respective well [23], using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell morphology and adhesion

Sample discs with the aforementioned standardized dimensions were obtained ($n = 15$) for each of the sealers and allocated into three groups ($n = 5$). 5×10^4 hPDLCs were seeded onto the surface of each disc and left to culture for 72 h. Cells were then fixed with 4% glutaraldehyde in PBS for 4 h, after which they were dehydrated via a gradual series of ethanol dilutions (30–90% v/v), air-dried, and coated with sputtered gold/palladium. Once coated, the analysis of cell morphology and adhesion was performed under SEM at × 100, × 300, and × 1500 magnifications.

Table 2 RT-qPCR primer sequence

Gene	Sequence (5′-3′)	Size (bp)
CEMP1	Forward: GGGCACATCAAGCACTGACAG Reverse: CCCTTAGGAAGTGGCTGTCCAG	165
CAP	Forward: TTTTTCTGGTCGCGTGGACT Reverse: TCACCAGCAACTCCAACAGG	142
ALP	Forward: TCAGAAGCTCAACACCAACG Reverse: TTGTACGTCTTGGAGAGGGC	177
RUNX2	Forward: TCCACACCATTAGGGACCATC Reverse: TGCTAATGCTTCGTGTTTCCA	136
GAPDH*	Forward: TCAGCAATGCCTCCTGCAC Reverse: TCTGGGTGGCAGTGATGG	117

CEMP1: cementum protein 1; *CAP*: cementum-derived attachment protein; *ALP*: alkaline phosphatase; *RUNX2*: runt-related transcription factor 2; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase. *bp*: base pairs

*Housekeeping gene

Activity-related gene expression

hPDLC differentiation was evaluated by means of the expression of activity-related genes using real-time quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). The sequences of primers for the differentiation markers assessed are presented in Table 2. hPDLCs were seeded onto 12-well plates (2×10^4 cells/well, $n = 3$) with undiluted sealer-conditioned medium and incubated for 7 days, as previously reported [22]. The undiluted sealer-conditioned medium was previously prepared by immersing sealer discs in culture medium (DMEM; Gibco, USA) for 24 h. Total RNA of hPDLCs was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA (1 μg) was reverse transcribed for first-strand complementary DNA (cDNA) synthesis using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Inc., Hercules, CA, USA). Both the total RNA extraction and cDNA synthesis were carried out following their respective manufacturers' instructions. The analysis of relative gene expression data was calculated using the $2-\Delta\Delta CT$ method [24]. hPDLCs cultured in unconditioned medium (DMEM; Gibco, USA) acted as the negative control, and an osteo/cementogenic medium (OsteoDiff media; Miltenyi Biotec, Bergisch Gladbach, Germany) acted as the positive control.

Mineralization assay (Alizarin Red staining)

Alizarin Red staining (ARS) was used to assess the mineralization potential of hPDLCs in contact with BCI, BCHiF, and AHP. Cells were seeded onto 12-well plates (2×10^4 cells/well, $n = 3$) and left to proliferate until confluent. hPDLCs were then cultured in an undiluted sealer-conditioned medium for 21 days. After the culture period, the samples were washed (PBS) and fixed with 70% ethanol for 1 h, and then stained with 2% Alizarin Red solution (Sigma-Aldrich, USA) for 30 min in the dark at room temperature. Absorbance values of the samples were measured using Synergy H1 multi-mode microplate reader (BioTek, USA) at 405 nm. hPDLCs cultured in unconditioned medium (DMEM; Gibco, USA) acted as the negative control, and OsteoDiff media (Miltenyi Biotec, Germany) acted as the positive control.

Statistical analysis

All of the in vitro assays were performed in triplicate. Data are expressed as mean ± standard deviations (SD) and were analyzed using one-way ANOVA and Tukey's post hoc test using GraphPad Prism v8.1.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was considered at $p < 0.05$.

Results

SEM-EDS analysis

Regarding the chemical composition of the surface of each sealer, BCHiF presented the highest calcium (Ca) content. BCI, on the other hand, contained the highest percentage of zirconium (Zr) and silicon (Si). Interestingly, the percentage of Ca in BCHiF was higher than that of Zr, whereas the opposite was exhibited by BCI. With reference to AHP, the main difference in terms of composition was the presence of high amounts of tungsten (W). Both hCSSs exhibited an irregular prismatic crystalline structure on their surface, as opposed to the regular surface morphology shown by AHP (Fig. 1).

ICP-MS analysis

Results of the analysis of ion release from the different endodontic sealers are presented in Table 3. BCHiF exhibited a significantly higher concentration of Zr release when compared with BCI and AHP ($p < 0.05$). Both hCSSs showed a higher release of Ca than the epoxy resin–based sealer AHP ($p < 0.05$), which was greater in BCI than in BHiF ($p < 0.05$). BCI released a significantly higher amount of Si compared with the other sealers ($p < 0.05$), whereas the highest concentration of W release was detected in AHP ($p < 0.05$).

MTT assay

After the treatment of hPDLCs with endodontic sealer extracts, a cytotoxicity assay was performed using MTT. Cells which were cultured with BCHiF and BCI extracts (undiluted, 1:2, or 1:4) showed a similar formazan production in comparison with the control group, evidencing the cytocompatibility of both materials. At 48 h, the undiluted BCHiF group showed

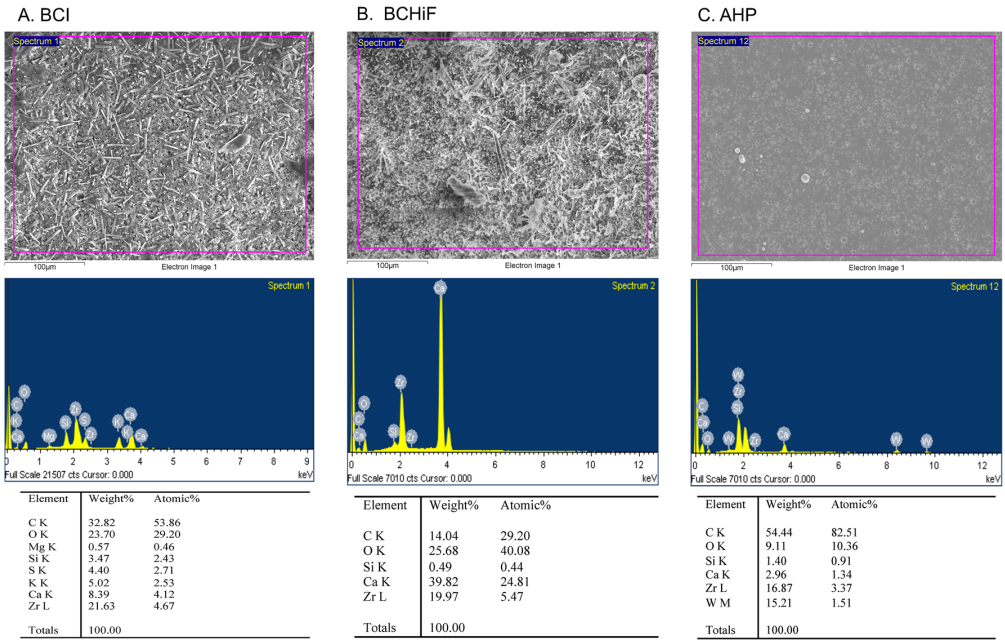

Fig. 1 SEM-EDS analysis results for BCI, BCHiF, and AHP discs. The first row presents SEM images of each sealer (scale bar: 100 μm). The second row illustrates the elemental spectra produced by the EDS system. The third row classifies the list of elements present per sealer by weight and atomic weight

a higher production than the control ($p < 0.05$). Conversely, for all AHP extracts, the relative formazan formation was significantly lower than that of the control at all end time points ($p < 0.001$), indicating the cytotoxicity of the AHP extracts towards hPDLCs. The relative formazan formation by hPDLCs having been exposed to undiluted (1:1) and diluted (1:2 and 1:4) material extracts for 24, 48, and 72 h is summarized in Fig. 2.

Wound healing assay

The migration rates of hPDLCs exposed to undiluted (1:1) and diluted (1:2 and 1:4) sealer extracts are illustrated in Fig. 3 as changes in the open wound area at 24, 48, and 72 h. In the BCI group, significant differences were found in the 1:4 diluted group at 48 h when compared with the control group ($p < 0.05$), whereas no significant differences were observed at 24 nor 72 h in any dilution. At 24 h, significant differences were detected in all dilutions of BCHiF compared with that of the control group (undiluted, $p < 0.01$; 1:2 and 1:4, $p < 0.05$), while at 48 h, significant differences were only observed in the undiluted extracts ($p < 0.05$). Regarding the AHP group, at all time-periods and dilutions, the cell migration rate was significantly lower than that of the control group ($p < 0.001$), being unable to heal the wound.

Table 3 ICP-MS results for endodontic sealer extracts

Sample	28 Si [He] Conc. (ppm)	44 Ca [He] Conc. (ppm)	56 Fe [He] Conc. (ppm)	91 Zr [He] Conc. (ppm)	182 W [He] Conc. (ppm)
BCI	109.88 ± 0.06^{AB}	157.36 ± 0.03^{AB}	< 0.000	2.36 ± 0.01^{A}	16.50 ± 0.00^{AB}
BCHiF	3.11 ± 0.00^{A}	69.64 ± 0.02^{AC}	< 0.000	4.20 ± 0.02^{AC}	52.82 ± 0.01^{AC}
AHP	5.17 ± 0.01^{B}	1.22 ± 0.00^{BC}	< 0.000	2.17 ± 0.02^{C}	4522.27 ± 0.01^{BC}

[A] Significant difference ($p < 0.05$) between BCI and BCHiF
[B] Significant difference ($p < 0.05$) between BCI and AHP
[C] Significant difference ($p < 0.05$) between BCHiF and AHP
Conc., concentration; *ppm*, parts per million

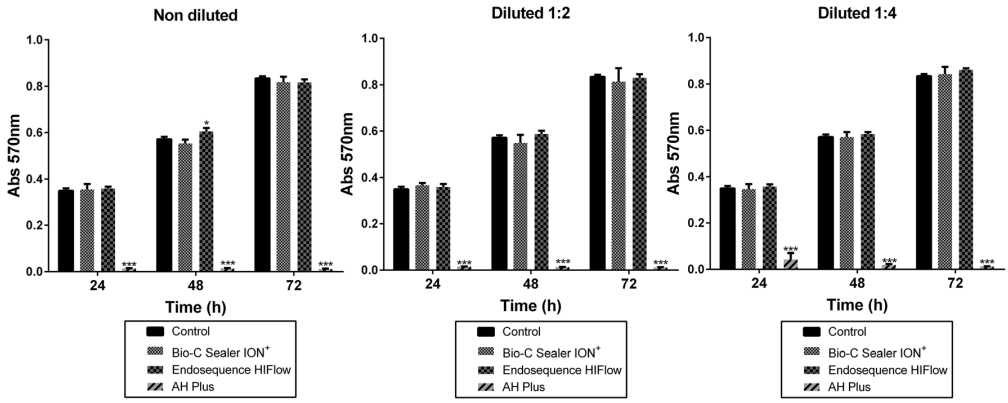

Fig. 2 MTT assay results for BCI, BCHiF, and AHP extracts on hPDLCs. Data are presented absorbance values (570 nm) at 24, 48, and 72 h of exposure of the sealer extracts to hPDLCs, compared with the control. *$p < 0.05$; ***$p < 0.001$

Cell morphology and adhesion

The analysis of hPDLC morphology and adhesion onto the surfaces of the different sealers showed the presence of a low quantity of cells and debris in the AHP group, evidencing cell death, whereas BCI and BCHiF groups showed abundant cell adhesion with a greater spread. Additionally, cells adhered to the surface of BCHiF exhibited an intense growth and elongation (Fig. 4).

RT-qPCR assay

The relative expressions of CEMP1, CAP, RUNX2, and ALP from hPDLCs, cultured with undiluted sealers for 7 days, are

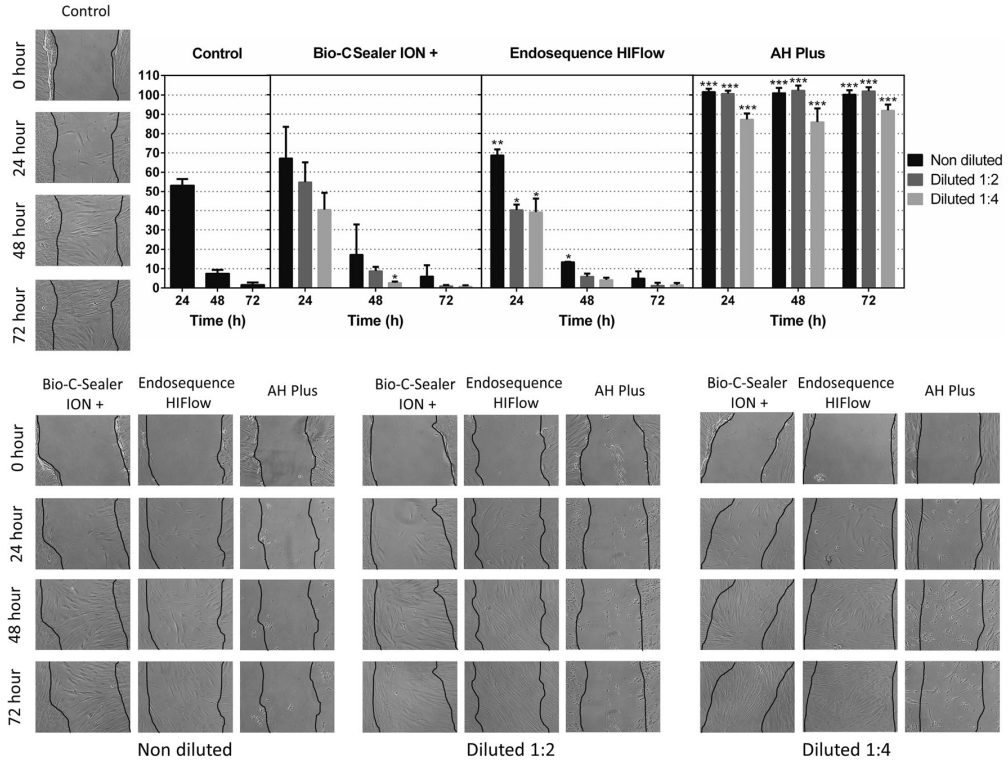

Fig. 3 hPDLCs migration rates at 24, 48, and 72 h of exposure to undiluted (1:1) and diluted (1:2 and 1:4) sealer extracts. Graphical results are presented as mean RWC percentages at each of the time points, relative to the total wound area at 0 h. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

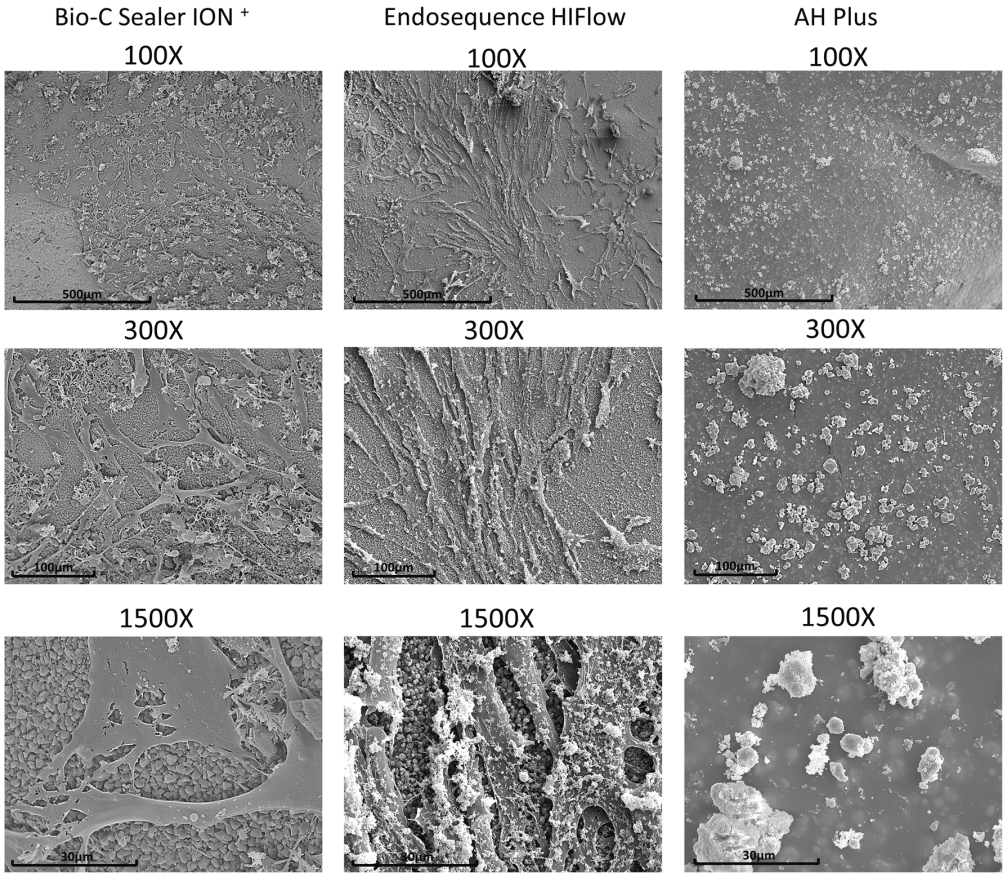

Fig. 4 SEM images of hPDLCs morphology and adhesion to sealer sample surfaces after 72 h of culture. Magnifications: × 100X, × 300, and × 1500. Scale bars: 500 µm, 100 µm, and 30 µm

summarized in Fig. 5. GAPDH was used to normalize the results. The BCHiF group showed an upregulation of CAP ($p < 0.01$), CEMP1, ALP, and RUNX2 ($p < 0.001$) compared with the negative control, while the BCI group showed an upregulation of CEMP1 ($p < 0.01$), CAP, and RUNX2 ($p < 0.001$). At the same time, cells exposed to BCHiF exhibited a higher expression of ALP ($p < 0.01$), CEMP1, and RUNX2 ($p < 0.001$) than those treated with BCI or Osteodiff (positive control). However, CAP expression was higher in the BCI and Osteodiff groups when compared with the BHiF group ($p < 0.001$). For the AHP group, RT-qPCR analysis was not performed, due to the evidenced cell death from the previous cytocompatibility assays.

Mineralization assay

The mineralization potential of the tested sealers is presented in Fig. 6. After 21 days of culture with undiluted sealer extracts, no mineralization was observed in the AHP group, showing similar rates to the untreated hPDLCs (control group). Both the BCI and BCHiF groups exhibited a greater mineralized nodule formation than the negative and positive control groups ($p < 0.001$). Likewise, cells treated with BCI showed a higher mineralization potential than those treated with BCHiF ($p < 0.05$).

Discussion

Traditionally, sealer-based obturations using hCSSs for root canal treatment were performed by means of cold compaction techniques, due to the lack of evidence on the effect of temperature and heating time on the properties of endodontic sealers [25, 26]. Recent evidence demonstrated that various sealers—with the well-established epoxy resin–based sealer AHP among them—can withstand a rise in temperature up until 100 °C and a subsequent cooldown, without suffering irreversible changes in their structure [18]. In view of this, AHP was used in the present study as a reference material to compare with two newly introduced hCSSs, as done by

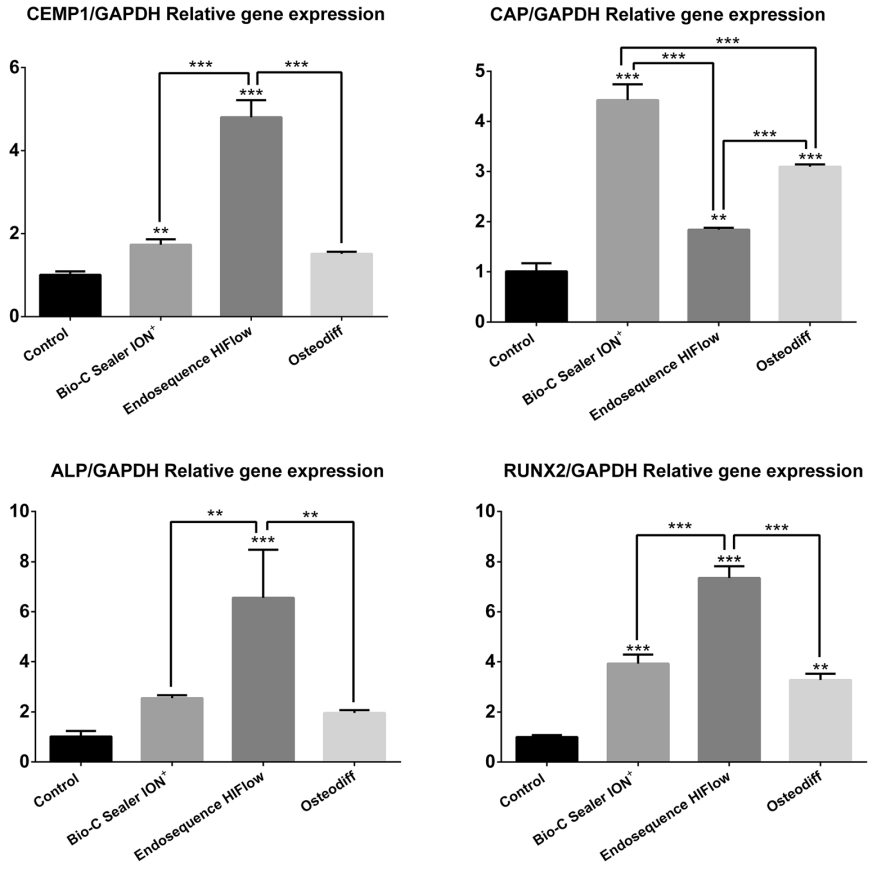

Fig. 5 RT-qPCR results for osteo/cementogenic gene expression by hPDLCs treated at 7 days of incubation with DMEM (negative control), BCI, BCHiF, or Osteodiff (postive control); relative to GAPDH expression. $^{**}p < 0.01$; $^{***}p < 0.001$

previous studies in the field [27, 28]. Both BCI and BCHiF are also indicated for warm compaction techniques according to their respective manufacturers, but their biological properties and, in particular, the biological response of hPDLCs towards them have not been described.

Accordingly, the present in vitro study aimed to outline the main clinically relevant chemical and biological properties of BCI and BCHiF, to anticipate their behavior in direct contact with the inorganic and organic tissues present within the root canal, and surrounding the root apex. The biological assays carried out comprise cell cytotoxicity, morphology, adhesion, proliferation, differentiation, and mineralization analyses, in accordance with similar studies within this framework [8, 29]. With regard to the chemical and ionic profiling of the assessed biomaterials, SEM-EDS and ICP-MS were used, respectively, as performed by various studies [6, 30].

The results from SEM-EDS and ICP-MS analyses revealed a high calcium content and ionic release in both BCI and BCHiF, as previously shown by other hCSSs: BioRoot RCS, MTA Fillapex [31]; Bio-C Sealer, TotalFill BC Sealer [32]; NeoMTA-Plus, Endocem-MTA [33]; EndoSequence BC Sealer, Ceraseal, Endoseal MTA [6]. The dissolution of calcium silicate–based biomaterials and release of their major cationic components, namely Ca^{2+}, in contact with tissue fluids, results in an ionic interchange which resolves in the formation of a superficial mineral layer [34–36]. In the present study, calcium release was higher from BCI than from BCHiF samples after 48 h of setting ($p < 0.05$). The differences in ion release may influence the clinical sealing ability or, at least, the characteristics (i.e., thickness and composition) of the mineral attachment formed to the dentin substrate [36].

For the biological assays, hPDLCs were used as cell substrates, in an attempt to extrapolate their in vitro response to hCSSs, to their clinical behavior. These cells have shown long-term survival, self-renewal, and a capability to form bone/cementum-like mineralized tissue and ligament structures with associated vasculature [37, 38]. In root canal treatment, hPDLCs can be exposed directly to sealer extrusions from the root canals, and therefore these materials should at least ensure cell survival [32, 39]. Regarding the results from the present study, hPDLCs exposed to both BCI and BCHiF independently, exhibited adequate formazan production, high proliferation, and abundant adhesion when compared with AHP and the negative control. These results act as evidence

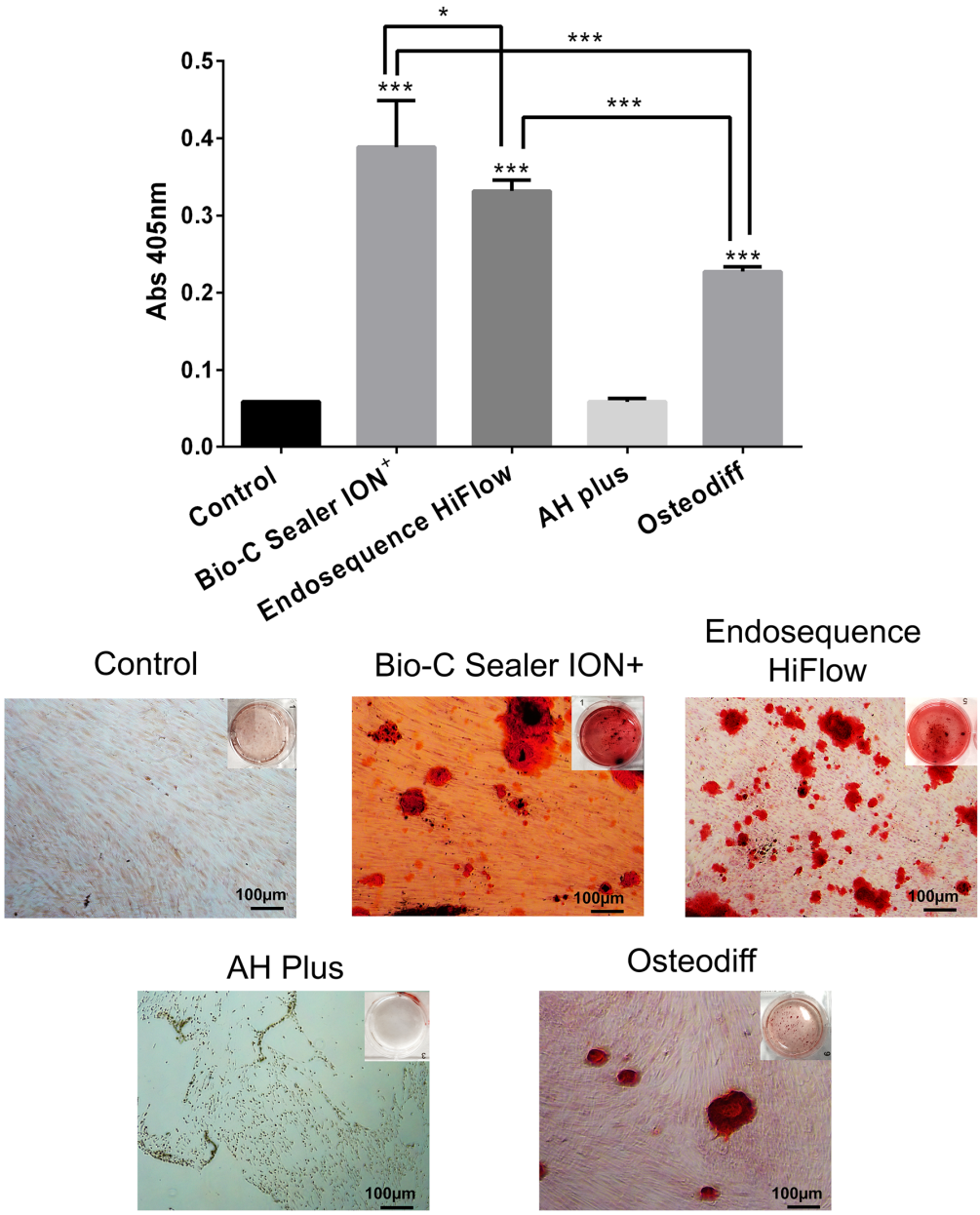

Fig. 6 hPDLC mineralized nodule formation after 21 days of culture with DMEM (negative control), BCI, BCHiF, AHP, or Osteodiff (postive control). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

of the cytocompatibility of hCSSs on hPDLCs, coinciding with previous studies in the field [6, 32, 39].

To assess the influence of sealer concentration on hPDLC viability and proliferation, various dilutions were prepared for each of the tested materials (undiluted, 1:2, and 1:4 ratios). With regard to cell viability, all BCI and BCHiF dilutions showed a similar formazan formation when compared with the control group after 48 h of incubation. In contrast, recent evidence reported that BCHiF eluates showed lower periodontal ligament fibroblast viability at higher concentrations (1:4 vs 1:8, 1:16, and 1:20 dilutions), significantly decreasing from 24 to 72 h of incubation [40]. Nevertheless, as highlighted in their study, it is expected that clinically, periapical tissues come into contact with considerably lower concentrations of the endodontic sealers and thus may exhibit a greater cytocompatibility.

Cytocompatibility assays, however, revealed negative results for the reference material used, AHP. hPDLCs incubated with the epoxy resin-based sealer extracts exhibited a significantly lower formazan production and migration than the control group in all of the sealer dilutions (undiluted, 1:2, 1:4). These results concur with those from previous in vitro studies [27, 41]. This, added to the evidenced hPDLC death on the surface of AHP sample discs observed under SEM and discouraged the inclusion of AHP on the posterior gene expression assays.

To evaluate the osteo/cementogenic differentiation of hPDLCs, the expression of a series of target genes was quantified using RT-qPCR, as follows: ALP, mediator in osteoblast activity and osteogenic potential [42, 43]; RUNX2, involved in the early stages osteogenesis as a transcriptional regulator factor [44]; CAP and CEMP1, cementoblast and cementocyte markers implicated in cementogenesis [45, 46]. hPDLCs cultured with both BCI or BCHiF exhibited a significant upregulation of osteogenic and cementogenic genes at 7 days of incubation compared with the negative control group. Likewise, both BCI and BCHiF groups showed a significant increase in mineralized nodule formation in comparison with the negative and positive control. Altogether, results indicate that both hCSSs are not only cytocompatible but also capable to induce the osteo/cementogenic differentiation and increase the mineralization potential of hPDLCs. These properties have also been reported for other hCSSs [6], supporting that they may be a shared characteristic among this group of biomaterials. In addition, recent evidence suggests that silicate-based endodontic materials may also induce the angiogenic stimulation of various DSCs [47].

Available evidence on the biological response of hPDLCs and other DSCs towards hCSSs is still limited and remains at an in vitro level, with the limitations that this may imply. The cellular behavior in controlled sealer-conditioned media shown in the present study and similar studies may provide an insight as to how these biomaterials may perform at a clinical level, but the presence of factors that may influence this behavior clinically [11] limits the extrapolation of the results. Nonetheless, to the authors' knowledge, this is the first study to assess the biological properties of the recently introduced BCI. The release of calcium ions, the cytocompatibility and upregulation of osteo/cementogenic markers, and the increased mineralization in contact with hPDLCs shown in the present study act as evidence for its potential use in root canal treatment.

Conclusions

The calcium silicate–based sealers considered in the present in vitro study exhibited a high calcium ion release, adequate cytocompatibility, upregulated osteo/cementogenic gene expression, and increased mineralized nodule formation in contact with hPDLCs.

Author contributions Investigation and methodology: Sergio López-García, Francisco Javier Rodríguez Lozano, and José Luis Sanz; supervision, visualization, conceptualization, and data curation: Adrián Lozano and Julia Guerrero-Gironés; investigation, methodology, and writing—original draft: José Luis Sanz and Francisco Javier Rodríguez-Lozano; conceptualization, formal analysis, project administration, supervision, validation, and writing—review and editing: Leopoldo Forner, Carmen Llena, and Maria Pilar Pecci-Lloret; investigation, methodology, project administration, resources, writing—original draft, and writing—review and editing: José Luis Sanz, Francisco Javier Rodríguez-Lozano, and Leopoldo Forner. All authors have read and agreed to the published version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

The study protocol was approved by the Clinical Research Ethics Committee of the Universidad de Murcia (ID: 2199/2018). Likewise, permission was obtained from the Health Department authorities to use the information contained in the CDHs, previously anonymized by one of the investigators belonging to the medical staff of the Health Department in order to protect patient confidentiality. All the information was processed in abidance with the confidentiality regulations defined under Act 15/1999 referred to as personal data protection.

Informed consent Informed consent was obtained from the parents of all individual participants included in the study.

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Comparative Biological Properties and Mineralization Potential of 3 Endodontic Materials for Vital Pulp Therapy: Theracal PT, Theracal LC, and Biodentine on Human Dental Pulp Stem Cells

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SIGNIFICANCE

This *in vitro* study supports the use of Theracal PT over its predecessor Theracal LC as a potential alternative to Biodentine for vital pulp treatment because of its improved cytocompatibility on human dental pulp stem cells.

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ABSTRACT

Introduction: The aim of this study was to assess the biological properties and mineralization potential of the new Theracal PT (Bisco Inc, Schaumburg, IL) compared with its predecessor Theracal LC (Bisco Inc) and the hydraulic silicate–based cement Biodentine (Septodont, Saint-Maur-des-Fossés, France) on human dental pulp stem cells (hDPSCs) *in vitro*. **Methods:** Standardized sample discs were obtained for each material ($n = 30$) together with 1:1, 1:2, and 1:4 material eluates. Previously characterized hDPSCs were cultured with the different materials in standardized conditions, and the following assays were performed: a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, a wound healing assay, Annexin-V-FITC and 7-AAD staining (BD Biosciences, San Jose, CA), reactive oxygen species production analysis, cell adhesion and morphology evaluation via scanning electron microscopy and immunofluorescence, quantification of the expression of osteo/odontogenic markers via real-time quantitative reverse-transcriptase polymerase chain reaction, and alizarin red S staining. Statistical significance was established at $P < .05$. **Results:** All of the tested dilutions of Theracal LC exhibited a significantly higher cytotoxicity and reactive oxygen species production ($P < .001$) and a lower cell migration rate than the control group (hDPSCs cultured in growth medium without material extracts) at all of the measured time points ($P < .001$). Both 1:4 Theracal PT and Biodentine-treated hDPSCs exhibited similar levels of cytocompatibility to that of the control group, a significant up-regulation of at least 1 odontogenic marker (Biodentine: dentin sialophosphoprotein ($P < .05$); Theracal PT: osteonectin and runt-related transcription factor 2 [$P < .001$]), and a significantly higher mineralized nodule formation ($P < .001$). **Conclusions:** The newly introduced TheraCal PT offers an improved *in vitro* cytocompatibility and mineralization potential on hDPSCs compared with its predecessor, TheraCal LC, and comparable biological properties to Biodentine. *(J Endod 2021;47:1896–1906.)*

KEY WORDS

Bioactivity; calcium silicate–based cements; cytotoxicity; resin modified; vital pulp therapy

Vital pulp therapy (VPT) comprises a series of conservative procedures that rely on the intrinsic reparatory mechanisms of the dentin-pulp complex[1]. Upon external noxae, such as trauma or carious lesions, this tissue complex will undergo a series of inflammatory stages that, if uncontrolled, could eventually lead to cellular death or necrosis[2]. During this dynamic inflammatory process, the dentin-pulp complex remains

resilient toward microbial damage, maintaining a regenerative potential even when indicative symptoms of irreversible pulpitis are present[3]. Accordingly, the maintenance of pulp vitality via VPT procedures regained interest as a more conservative alternative to root canal treatment in cases of pulpitis[4].

The removal of irritants and nonviable tissue via chemical-mechanical disinfection, added to the placement of materials as pulp cappers, has shown promising results in terms of the asymptomatic maintenance of pulp vitality and the restoration of physiological dental function[5]. These materials require a series of specific biological properties in order to provide a favorable medium for repair. Because they will be placed in indirect contact (indirect pulp capping) or direct contact (direct pulp capping, partial pulpotomy, or coronal pulpotomy) with pulp tissue, such materials should exhibit at least an adequate cytocompatibility toward its cellular component[6].

Among the cellular component of pulp tissue, human dental pulp stem cells (hDPSCs) play a crucial role in the process of pulp repair and, consequently, in VPT[7]. Since their isolation and characterization, research regarding hDPSCs revealed their mesenchymal nature and their ability to differentiate into odontoblastlike cells involved in the process of tissue neoformation[8]. Therefore, to assess the adequacy of a specific material for VPT, its cytocompatibility toward hDPSCs should be previously elucidated.

Nevertheless, materials used in VPT are expected to not only express adequate cytocompatibility but also bioactive properties and a favorable influence on cellular plasticity. Regarding bioactivity, these materials should favor the deposit of a hydroxyapatitelike superficial layer and consequently form a mineralized attachment to the dentin substrate[9]. Parallelly, a positive effect on cellular plasticity would imply favoring the osteo/odontogenic differentiation of hDPSCs[10].

The aforementioned properties have been exhibited by a subgroup of bioactive materials, namely hydraulic calcium silicate–based cements (HCSCs), in numerous *in vitro*, *ex vivo*, and animal studies[11,12]. These materials have also shown high success rates in clinical studies when used in VPT procedures[13]. Among them, Biodentine (BD; Septodont, Saint-Maur-Des-Fossés, France), a tricalcium silicate–based cement presented in a powder-liquid format, has been extensively studied. Together with the Portland cement–based material mineral trioxide aggregate (MTA), it is being used as the reference hydraulic silicate–based cement[14].

The desirable properties of silicate-based materials led to the development of new material compositions, such as resin-modified calcium silicate–based materials[15]. Among them, Theracal LC (ThLC; Bisco Inc, Schamburg, IL) was introduced as a light-curing material for VPT, combining the desirable properties of the silicate-based component and the superior handling of resin[15,16]. Since its introduction, ThLC has been extensively studied both *in vitro* and *in vivo*, exhibiting mixed results. In fact, various authors recommended limiting its use to indirect pulp capping[17–19].

Most recently, a new dual-cured resin-modified calcium silicate–based material under the name of Theracal PT (ThPT, Bisco Inc) has been introduced[20]. According to its manufacturer, it is primarily indicated for pulpotomies and can also be used for indirect and direct pulp capping. However, its biological properties have not been compared with those exhibited by HCSCs.

Accordingly, the aim of the present study was to perform an *in vitro* analysis of the biological properties and mineralization potential of ThPT compared with its predecessor ThLC and the hydraulic silicate–based cement BD on hDPSCs. The null hypothesis was established as the absence of difference between the tested materials with regard to their cytocompatibility, influence in cell plasticity, and mineralization potential on hDPSCs.

MATERIALS AND METHODS

Material Sample Preparation

Five-millimeter-diameter and 2-mm-high sample discs ($n = 30$) were obtained for the tested materials (ie, ThPT [batch no.: 2000002968], ThLC [batch no.: 2000001054], and BD [batch no.: B25359]) by means of sterile (ultraviolet radiation, 15 minutes) cylindrical rubber molds and stored in an incubator (37°C, 48 hours) to ensure complete setting. BD was mixed by following its manufacturer's instructions. Both ThPT and ThLC are presented in a premixed format and were injected into the rubber molds and photocured (1200 mW/cm^2 output for 20 seconds at a 2-mm distance) using a light-emitting diode curing light (Bluephase 20i; Ivoclar Vivadent, Schaan, Liechtenstein). Light intensity was monitored by means of a MARC Resin Calibrator (BlueLight Analytics, Halifax, Canada).

One:one, 1:2, and 1:4 material eluates were then extracted under sterile conditions as described by previous studies in the field[21]. Dulbecco modified Eagle medium (DMEM; Gibco BRL, Burlingame, CA) was used as an extraction vehicle and stored for 24 hours at 37°C and 5% CO_2 in a humid atmosphere. Following the indications of the ISO 10993-5 guidelines[22], a material surface area/medium volume ratio of approximately 1.5 cm^2/mL was obtained.

Dental Stem Cell Isolation, Culture, and Characterization

The cell extraction protocol was previously approved by the Human Research Ethics Committee from the University of Murcia (reference no. 2199/2018). Human dental pulp was obtained from the pulp chamber and root canals of extracted teeth by means of a barbed broach. Teeth were obtained from 18- to 30-year-old healthy donors and were extracted for orthodontic or periodontal reasons. Once extracted, human dental pulp was rinsed with Hank's Balanced Salt Solution (Gibco BRL) and digested using 3 mg/mL collagenase A (Sigma-Aldrich, St Louis, MO). The resultant cells were cultured in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco), 1% L-glutamine, and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Gibco BRL) and incubated under controlled conditions (37°C, 5% CO_2, humid atmosphere). For subsequent assays, cells from passage 4 onward were used.

Stem cell characterization was performed under the International Society of Cellular Therapy guidelines[23] to confirm their mesenchymal phenotype. Cell surface antigens were assessed using fluorophore-conjugated antibodies via flow cytometry (FACS Calibur Flow Cytometer; Becton Dickinson, Franklin Lakes, NJ). The following antibodies from the Human MSC Phenotyping Cocktail (Miltenyi Biotec, Bergisch Gladbach, Germany) were used: CD73-APC (clone AD2), CD90-FITC (clone DG3), CD105-PE (clone 43A4E1), CD34-PerCP (clone AC136), CD20-PerCP (clone LT20.B4), CD14-PerCP (clone TÜK4), and CD45-PerCP (clone 5B1). Additionally, the resultant characterized hDPSCs were cultured in different media (osteogenic/adipogenic/chondrogenic) (Miltenyi Biotech) to confirm their trilineage mesenchymal differentiation following the methodology from similar studies[24]. Both the mesenchymal phenotype and trilineage differentiation of the cells used in this study were confirmed by a previous study performed by our research group[20].

Cytotoxicity Evaluation (3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide Assay)

hDPSCs cultured in growth medium without material extracts (negative control group) and with 1:1, 1:2, or 1:4 ThPT, ThLC, or BD extracts (test groups) were assessed for cytotoxicity. To do so, cell metabolic activity was measured by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[25]. Material extracts were placed in direct contact with the hDPSC culture, and an MTT reagent (Sigma-Aldrich) was added for 4 hours as specified by the manufacturer's instructions; 100 μL/well dimethylsulfoxide (Sigma-Aldrich) was then added once a purple precipitate was observed. Covered plates were kept in the dark for 2–4 hours. Cell metabolic activity was assessed at 24, 48, and 72 hours of culture. The absorbance per well at 570-nm wavelength was recorded using a microplate reader (ELx800; Bio-Tek Instruments, Winooski, VT).

Cell Migration Evaluation (Wound Healing Assay)

To evaluate cell migration, hDPSCs were seeded into 24-well plates (2×10^5 cells/well) and cultured in normal growth medium for 24 hours. Then, the medium was replaced with a serum-free medium, and cells were cultured for an additional 24 hours. For the wound healing assay, a scratch wound was made using a sterilized pipette tip, and hDPSCs were exposed to the following experimental conditions: growth medium without material extracts (control group) or complete growth medium with 1:1, 1:2, or 1:4 ThPT, ThLC, or BD extracts (test groups). Images were obtained using a phase-contrast microscope at 0, 24, 48, and 72 hours. The percentage of open wound area at the different time points was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Cell Apoptosis/Necrosis Assay (Annexin-V-FITC and 7-AAD Staining)

hDPSC viability was quantified after 72 hours of culture at 37°C in complete growth medium (control) or in complete growth medium treated with 1:1, 1:2, or 1:4 dilutions of the tested material eluates. To assess cell viability, Annexin-V-FITC and 7-AAD staining (BD Biosciences, San Jose, CA) were applied according to the manufacturer's instructions. Within 1 hour of staining, specimens were analyzed by flow cytometry (FACS Calibur Flow Cytometer). Cell viability was assessed as a percentage and categorized as follows: viable (double negative), early apoptotic (Annexin-V-FITC positive, 7AAD negative), and late apoptotic and necrotic (Annexin-V-FITC negative/7-AAD positive or double positive, respectively).

Reactive Oxygen Species Release

The levels of reactive oxygen species (ROS) released by 1:1, 1:2, and 1:4 dilutions of the tested materials cultured with hDPSCs were determined by flow cytometry and compared with a negative control (hDPSCs in normal growth medium). To do so, hDPSCs were resuspended in 1 mL complete growth medium and stained with a general oxidative stress indicator—5 μmol/L CM-H_2DCFDA (Invitrogen, Carlsbad, CA)—for 30 minutes at 37°C. Then, the cells were rinsed twice and assessed by flow cytometry as described earlier.

Cell Morphology and Adhesion (Scanning Electron Microscopic Visualization)

The response (ie, morphologic variations and adhesion rates) of hDPSCs to the direct contact with the surface of the tested materials was assessed under scanning electron microscopy. The previously prepared sample discs ($n = 15$) were randomly divided into 3 groups, 1 for each material ($n = 5$). The surface of the sample discs were seeded with hDPSCs and cultured in normal growth medium for 72 hours. Then, cells were fixed with 3% glutaraldehyde (Sigma-Aldrich) in fetal bovine serum for 30 minutes. Samples were subsequently dehydrated under a graded ethanol series and treated with hexamethyldisilazane (Sigma-Aldrich) for 5 minutes. Finally, the samples underwent a gold sputter coating and visualized under a scanning electron microscope (100×, 300×, and 1500× magnifications).

Cell Cytoskeleton Staining (Immunofluorescence)

To assess any variation in cellular morphology, structure, and organization of the actin cytoskeleton of hDPSCs under exposure to the different material eluates, a qualitative description of immunofluorescence images of phalloidin-stained cells was performed. In brief, hDPSCs were seeded onto glass coverslips, left to adhere, and cultured in complete growth medium (control) or in complete growth medium treated with 1:1, 1:2, or 1:4 dilutions of the tested material eluates for 72 hours at 37°C. Then, cells underwent the following process:

(1) cells were rinsed twice using prewarmed fetal bovine serum at 37°C,

(2) they were fixed in 4% formaldehyde solution (Merck Millipore, Darmstadt, Germany) for 10 minutes,

(3) they were made permeable with 0.25% Triton X-100 solution (Sigma-Aldrich) for 5 minutes, and

(4) their cytoskeleton and nuclei were stained with AlexaFluor 594–conjugated phalloidin (Invitrogen) and 4,6-diamidino-2-phenylindole dihydrochloride (ThermoFisher Scientific, Waltham, MA), respectively. Lastly, immunofluorescence images were obtained and observed under a confocal microscope (Leica TCS SP2; Leica, Wetzlar, Germany).

Cell Osteo/Odontogenic Marker Expression (Real-time Quantitative Reverse-transcriptase Polymerase Chain Reaction)

The odontogenic differentiation of hDPSCs cultured with the tested materials was evaluated by means of the expression of odontogenic markers via real-time quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). Test groups consisted of hDPSCs (2×10^4 cells/well $n = 3$) seeded onto 12-well plates containing undiluted material-conditioned medium (1:1) and cultured for 14 days. Considering the negative results exhibited by ThLC in the cytocompatibility assays, only BD and ThPT were included in this assay. The negative control consisted of hDPSCs cultured in unconditioned culture medium (DMEM). A positive control was also included as a reference, consisting of an osteo/odontogenic medium (OsteoDiff Media, Miltenyi Biotec). The following sequence of primers for odontogenic markers were used (forward/reverse):

- Alkaline phosphatase (ALP): 5′-TCAGAAGCTCAACACCAACG-3′/5′-TTGTACGTCTTGGAGAGGGC-3′
- Collagen type 1: 5′-CCCGGGTTTCAGAGACAACTTC-3′/5′-TCCACATGCTTTATTCCAGCAATC-3′
- Osteonectin (ON): 5′-GCATCAAGCAGAAGGATA-3′/5′-AATAGTTAAGTTACAGCTAAGAAT-3′
- Dentin sialophosphoprotein (DSPP): 5′-GCATTTGGGCAGTAGCATGG-3′/5′-CTGACACATTTGATCTTGCTAGGAG-3′
- Runt-related transcription factor 2 (RUNX2): 5′-TCCACACCATTAGGGACCATC-3′/5′-TGCTAATGCTTCGTGTTTCCA-3′

The 2-ΔΔCT method was used to calculate the relative gene expression[26]. The expression of the housekeeping gene

glyceraldehyde 3-phosphate dehydrogenase was used as a reference, with the following sequence of primers (forward/reverse): 5′-TCAGCAATGCCTCCTGCAC-3′/5′-TCTGGGTGGCAGTGATGG-3′.

Cell Mineralization Assay (Alizarin Red S Staining)

Alizarin red S staining was used to analyze the formation of calcified nodules as a means to describe the mineralization potential of hDPSCs cultured with the tested materials. To do so, hDPSCs were left to proliferate in 12-well plates (2×10^4 cells/well, $n = 3$). Once confluent, cells were transferred onto undiluted material-conditioned medium and cultured for 21 days. Then, specimens were rinsed with fetal bovine serum and fixed with 70% ethanol for 1 hour. Samples were subsequently stained with 2% Alizarin Red S Solution (Sigma-Aldrich) for 30 minutes in dark conditions at room temperature and solubilized using 10% cetylpyridinium chloride monohydrate solution (Sigma-Aldrich). Finally, the absorbance was measured at 570 nm using a Synergy H1 multimode microplate reader (BioTek, Winooski, VT). The same control groups (positive and negative) used for the RT-qPCR analysis were used for this assay.

Statistical Analysis

Three separate measurements were performed for each of the dilutions (1:1, 1:2, and 1:4) of the tested materials for each assay. The homogeneity of variance and normal distribution of the data were confirmed. Consequently, a parametric analysis was performed with 1-way analysis of variance followed by the pair-wise Tukey post hoc test using Graph-Pad Prism v8.1.0 (GraphPad Software, San Diego, CA). The assay involving alizarin red S staining was statistically analyzed by 2-way analysis of variance. Data are presented as the mean ± standard deviation. Statistical significance was established at $P < .05$.

RESULTS

MTT Assay

The MTT assay results of hDPSCs exposed to different dilutions of the tested materials after 24, 48, and 72 hours of culture are presented in Figure 1*A*. All of the tested dilutions of ThLC exhibited a significantly higher cytotoxicity than the control group at all of the measured time points ($P < .001$). The same pattern was observed in the 1:1 and 1:2 ThPT-treated groups ($P < .001$). However, hDPSCs treated with 1:4 ThPT did not present significant differences with the control group after 24 and 48 hours of culture. At 72 hours of culture, a significantly higher cytotoxicity was observed ($P < .05$). Both the 1:2 and 1:4 BD-treated groups exhibited similar results to the control group at all of the measured time points without significant differences. The treatment of hDPSCs with nondiluted BD resulted in a significantly higher cytotoxicity than the control group after 48 and 72 hours of culture ($P < .001$). However, at 24 hours of culture, the difference observed was not significant.

Wound Healing Assay

The wound healing assay results of hDPSCs exposed to different dilutions of the tested materials after 24, 48, and 72 hours of culture are presented in Figure 1*B*. All of the tested dilutions of ThLC resulted in a significantly lower migration of hDPSCs compared with the control group at all of the measured time points ($P < .001$). The same occurred in hDPSCs cultured with nondiluted ThPT or BD at 48 and 72 hours ($P < .001$). On the other hand, the treatment with 1:2 and 1:4 ThPT or BD resulted in similar migration rates to the control group at all of the measured time points without significant differences. As an exception, 1:4 BD-treated hDPSCs exhibited significantly higher migration rates than the control group at 24 hours ($P < .05$).

Annexin-V-FITC and 7-AAD Staining

The mean cell viability rates of hDPSCs exposed to different dilutions of the tested materials after 72 hours of culture are presented in Table 1. The treatment with nondiluted BD or ThPT resulted in a similar percentage of viable hDPSCs (91.2% and 86.7%, respectively), whereas the 1:1 ThLC-treated group exhibited a substantially lower cell viability rate (14.7%). The same was observed with 1:2 dilutions of the tested materials as follows: BD (92.9%) > ThPT (88.4%) > ThLC (38.2%). Similar cell viability rates were obtained in the 1:4 BD- and ThPT-treated groups (92.4% and 89.1%, respectively); the 1:4 ThLC-treated group exhibited a higher percentage of viable hDPSCs compared with 1:2 and 1:1 dilutions (82.2% vs 38.2% and 14.7%, respectively) although still lower than BD and ThPT.

ROS Release

Intracellular ROS release in hDPSCs induced by the different dilutions of the tested materials is shown in Figure 2 as the percentage of CM-H_2DCFDA–positive cells compared with the control group. None of the dilutions of the BD-treated groups exhibited significant differences with the control group. However, all of the dilutions of the ThLC-treated groups exhibited a significantly higher percentage of CM-H_2DCFDA–positive cells ($P < .001$), which was lower as the material was more diluted. The ThPT-treated groups exhibited a significantly higher percentage of CM-H_2DCFDA–positive cells when nondiluted ($P < .05$), but no significant differences were found when diluted at a ratio of 1:2 or 1:4.

Scanning Electron Microscopic Visualization

Scanning electron microscopic images at 100×, 300×, and 1500× magnifications of the surface of standardized sample discs of the tested materials seeded with hDPSCs and cultured in normal growth medium for 72 hours are presented in Figure 3*A*. The ThLC samples exhibited a lack of adhered cells and debris, which indicate cellular death. However, on the ThPT samples, a moderate number of elongated cells adhered to the surface were visualized. Lastly, a high number of functionally oriented cells were observed on the surface of the BD samples.

Cell Cytoskeleton Staining

Immunofluorescence images of phalloidin–4,6-diamidino-2-phenylindole dihydrochloride staining of hDPSCs exposed to different dilutions of the tested materials after 72 hours of culture are presented in Figure 3*B*. Both BD- and ThPT-treated groups exhibited a wide spread of hDPSCs with a fibroblastlike spindle-shaped morphology and a high F-actin content. At 1:1 and 1:2 dilutions, BD and ThPT immunofluorescence images were similar to that of the control group. However, at a 1:4 dilution, a higher number of functionally oriented cells were observed in both cases. In contrast, ThLC-treated groups exhibited a scarce number of cells with an aberrant morphology at 1:1 and 1:2 dilutions and a low count of functionally oriented cells at a 1:4 dilution.

Cell Osteo/Odontogenic Marker Expression (RT-qPCR)

The expression of the previously described odontogenic markers exhibited by hDPSCs after a culture period of 14 days with the tested materials relative to the expression of glyceraldehyde 3-phosphate dehydrogenase is shown in Figure 4*A*. The ThPT-treated groups exhibited a significantly higher expression of ON and RUNX2 compared with the negative control group ($P < .001$). Alternatively, a significantly higher expression of DSPP was observed in the BD-treated groups ($P < .05$). Both of the test groups exhibited a significantly lower expression of ALP ($P < .001$), but only the BD-treated

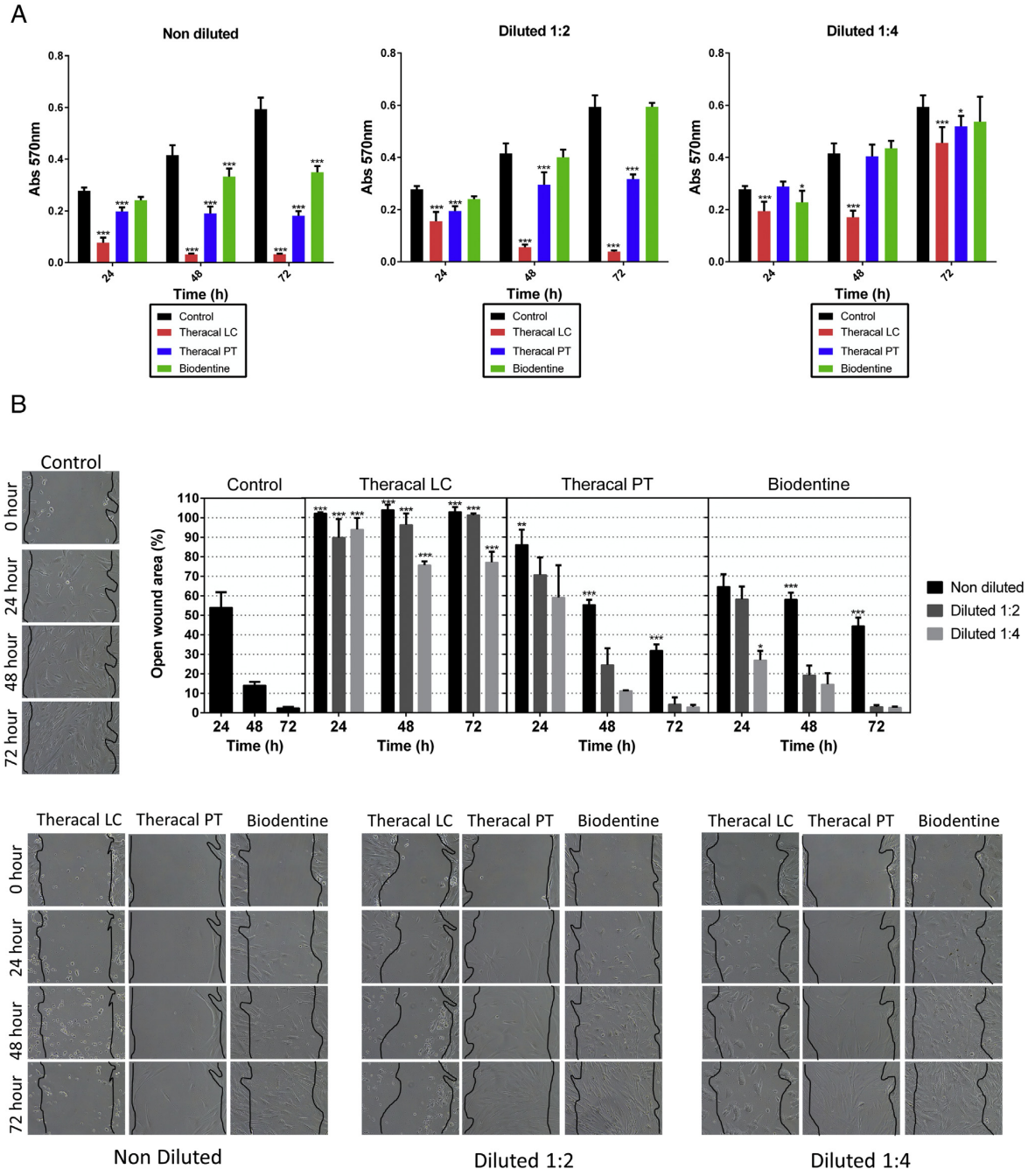

FIGURE 1 – Cytocompatibility and migration assays. (*A*) The results of the MTT assay on the cytotoxicity of 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, and BD after 24, 48, and 72 hours of culture with hDPSCs. Data are expressed as absorbance values at 570 nm compared with the control. *$P < .05$, ***$P < .001$. (*B*) The results of the wound healing assay on the migration of hDPSCs cultured with 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, or BD for 24, 48, and 72 hours. The percentages of the open wound area at the different time points are presented graphically, relative to that of the control group. *$P < .05$, **$P < .01$, ***$P < .001$.

groups showed a significantly lower expression of collagen type 1 ($P < .05$). Lastly, the reference osteogenic medium, used as a positive control, exhibited a significant up-regulation of ALP, DSPP, and RUNX2 compared with the negative control ($P < .001$).

Alizarin Red S Staining

The mineralization potential exhibited by hDPSCs after a culture period of 21 days with

TABLE 1 - Annexin-V-FITC and 7-AAD Staining Results after 72 Hours of Culture

Groups	Dilution	Percentage of viable hDPSCs			
		Q1	Q2	Q3	Q4
Control	—	0	0.04	4.62	95.3
Biodentine	1:1	0.09	2.18	6.50	91.2
	1:2	0.01	1.75	5.36	92.9
	1:4	0.01	2.11	5.47	92.4
Theracal LC	1:1	0.24	57.8	27.3	14.7
	1:2	0.03	29.4	32.4	38.2
	1:4	0.04	5.13	12.6	82.2
Theracal PT	1:1	0.10	6.81	6.37	86.7
	1:2	0.02	4.31	7.28	88.4
	1:4	0.09	0.02	5.58	89.1

hDPSCs, human dental pulp stem cells; Q1, necrotic (double positive); Q2, late apoptotic (Annexin-V-FITC negative/7-AAD positive); Q3, early apoptotic (Annexin-V-FITC positive, 7AAD negative); Q4, viable (double negative).

the tested materials, expressed as the formation of calcified nodules stained by alizarin red S staining, is shown in Figure 4*B*. Expectedly, the ThLC-treated groups exhibited a significantly lower mineralization potential than the control groups and the other test groups ($P < .001$). However, both the BD- and ThPT-treated groups showed a significantly higher mineralization potential than both the negative and positive control groups ($P < .001$). Interestingly, the difference in the mineralization potential between the BD- and ThPT-treated groups was also significant in favor of BD ($P < .001$).

DISCUSSION

In vitro assays have been widely used in the literature to characterize and establish a "biological profile" of new commercially available endodontic material compositions before the analysis of their performance in animal models or clinical trials[6]. Following this framework, the present study aimed to report the biological properties of ThPT, a new resin-modified calcium silicate–based material, compared with its predecessor, ThLC, and the well-established HCSC BD. The superficial element distribution of the tested materials has been previously described[20,27]. However, this

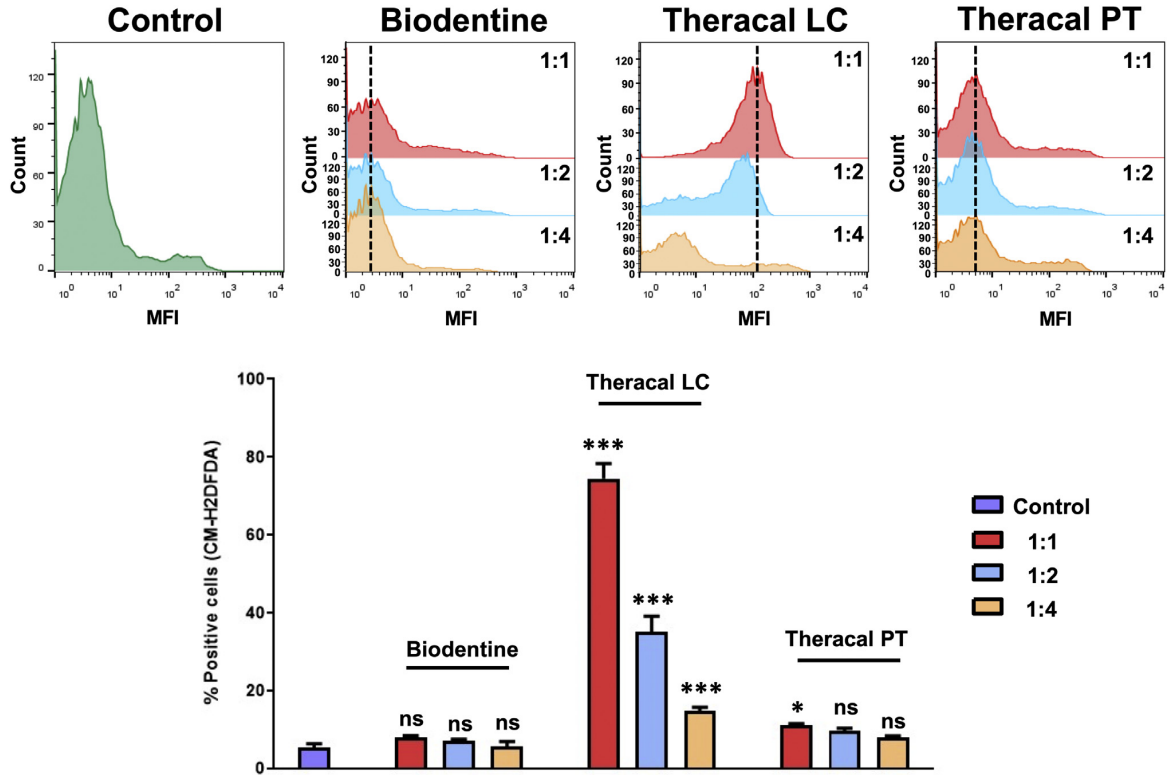

FIGURE 2 – The results of the ROS assay on the percentage of CM-H_2DCFDA–positive cells after culture with 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, or BD compared with the negative control group. *$P < .05$, ***$P < .001$. The histogram's y-axis represents the number of cells ×10e3, whereas the x-axis represents the mean fluorescence intensity (MFI) in logarithmic scale. The dashed line marks the MFI value of each material at 1:1 dilution.

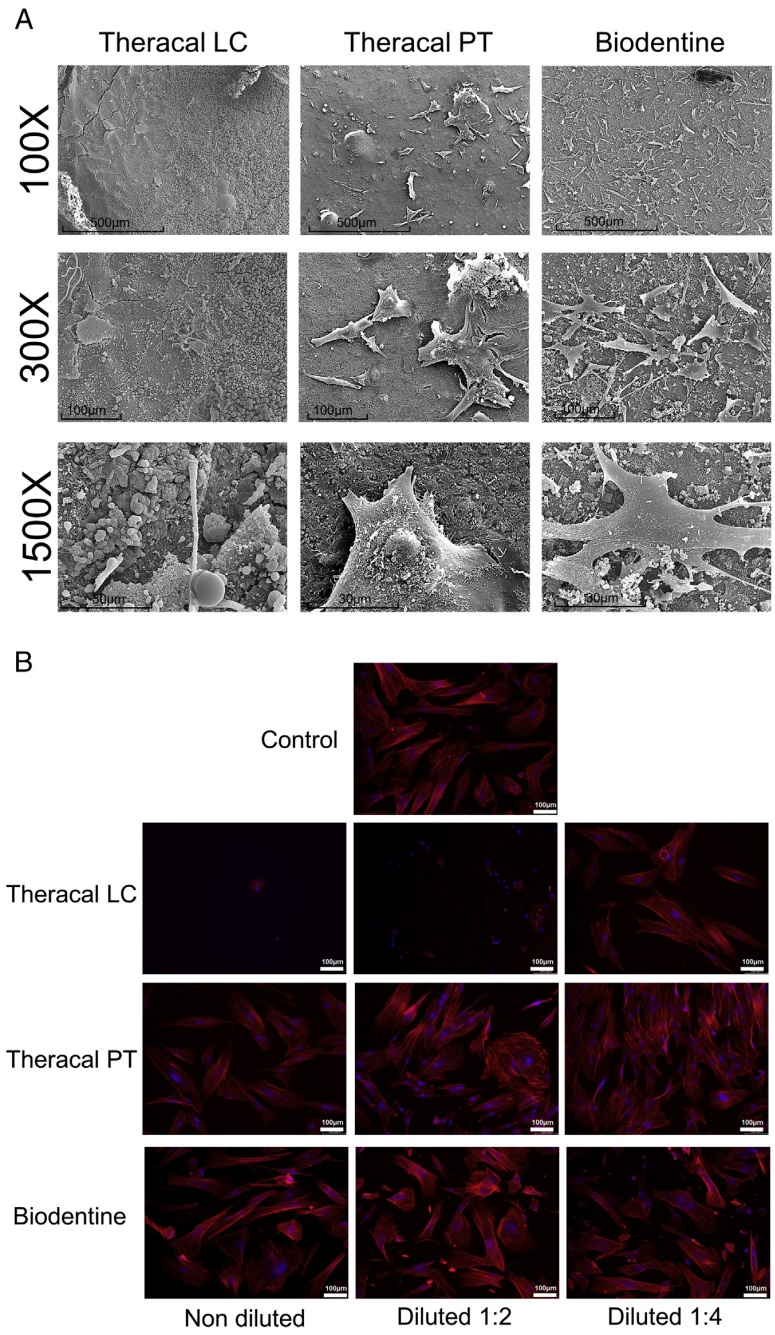

FIGURE 3 – Cell morphology and adhesion assays. (*A*) The results of the scanning electron microscopic visualization assay after 72 hours of culture with 5-mm-diameter and 2-mm-high sample discs of ThPT, ThLC, or BD compared with the negative control group. Magnifications: 100×, 300×, and 1500×. Scale bars: 500 μm, 100 μm, and 30 μm. (*B*) The results of the cell cytoskeleton staining via immunofluorescence after 72 hours of culture with 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, or BD compared with the negative control group. The images shown are representative from $n = 3$ separate experiments. Scale bar: 100 μm.

is the first study to compare the biological properties of ThPT with BD as a reference HCSC.

The comparison of this newly introduced material with ThLC and BD appears suitable because of their shared clinical indications, namely VPT procedures. The selection of BD as a reference HCSC was based on similar studies in the field[17,27], its high clinical success rates[28], and extended use among daily endodontic practice[29]. The traditional reference material, MTA, was also previously compared with ThPT by our research group in order to preliminarily establish the difference between the biological behavior of resin-modified calcium silicate–based materials and Portland cement–based materials *in vitro*[20].

As performed by similar studies[20,27], previously characterized hDPSCs were used in the present study as the target cellular population. These cells, residing within perivascular niches, are recruited into the dentin-pulp complex, both in a healthy and an inflammatory state, and induced to differentiate into odontoblastlike cells through a cascade of molecular events involved in the process of reparative dentinogenesis[2,8,30]. Therefore, by analyzing the biological response of hDPSCs toward the tested materials by means of a wide variety of *in vitro* tests that assess different cellular parameters, their clinical behavior may be potentially anticipated.

Within the biological properties of the tested materials, their cytocompatibility, as expressed by the MTT and wound healing assay and by Annexin-V-FITC and 7-AAD staining, can at least be deemed as adequate. Recurrently, the cytocompatibility of the tested materials appeared to be highest when more diluted. Both the 1:4 BD- and 1:4 ThPT-treated hDPSCs exhibited similar results in the MTT assay as the control group after 48 hours of culture as well as similar migration rates after 72 hours of culture. Furthermore, cell viability rates were the highest when treated with the highest dilution (1:4) of both materials (92.4% and 89.1%, respectively). Altogether, the results from the *in vitro* cytocompatibility assays point toward the absence of cytotoxicity of the lowest dilutions of ThPT and BD toward hDPSCs.

In contrast, all of the tested dilutions of ThLC exhibited significantly higher cytotoxicity and lower migration rates than the control group at all of the measured time points ($P < .001$) and the lowest cell viability rates among the tested materials (82.2%, 38.2%, and 14.7% at 1:4, 1:2, and 1:1 dilutions, respectively). These results are in accordance with a previous study in which the biological properties of ThLC and ThPT were compared with the Portland cement–based material MTA (Angelus, Londrina, PR, Brazil). Similar to the present study, both ThPT and MTA exhibited an absence of cytotoxicity when highly diluted (1:4), whereas a high cytotoxicity was observed in the ThLC-treated groups[20].

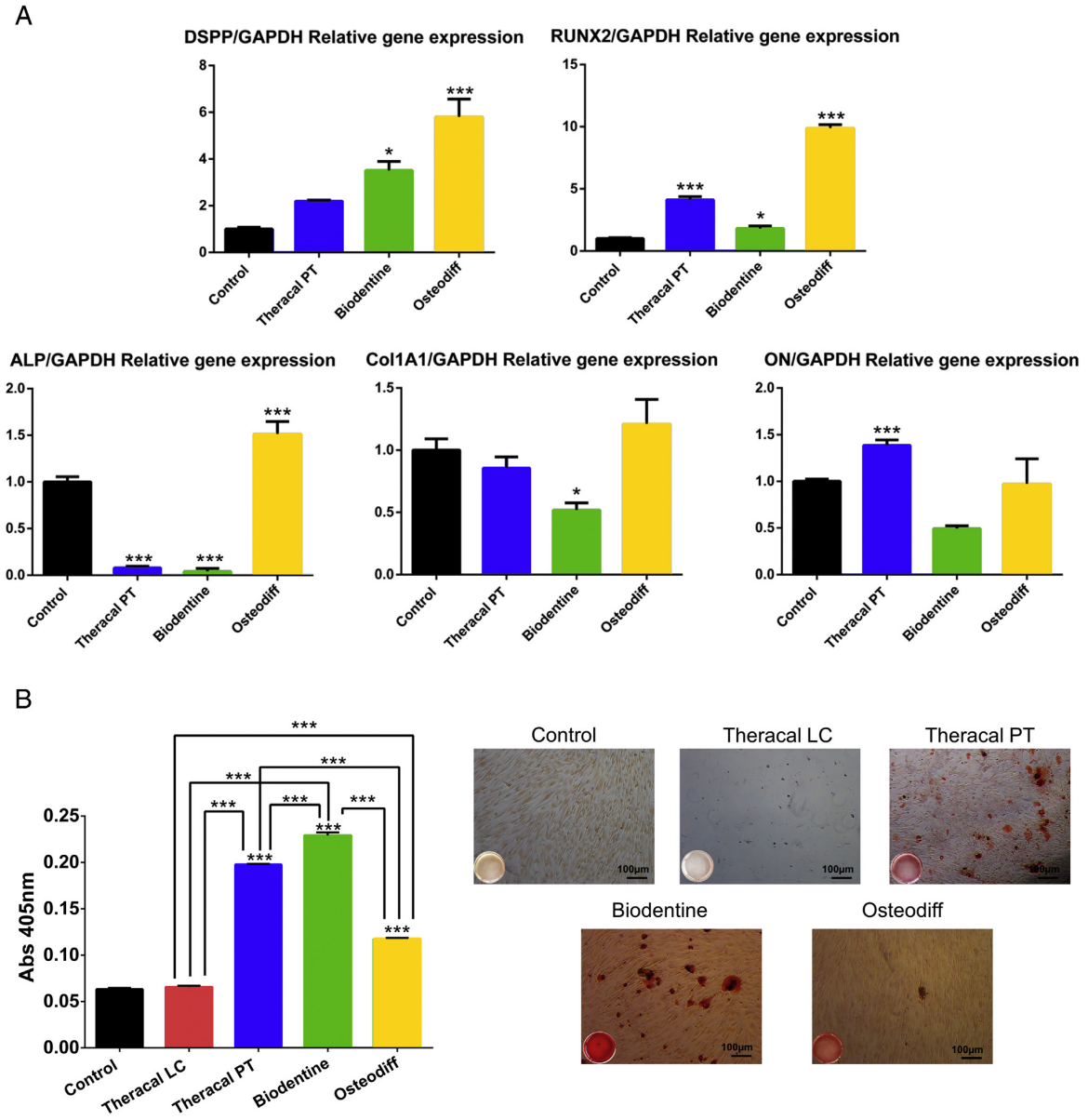

FIGURE 4 – Cell differentiation and mineralization assays. (*A*) The RT-qPCR results on hDPSC expression of osteo/odontogenic markers after 14 days of culture with 1:1, 1:2, and 1:4 eluates of ThPT or BD compared with the negative control group. Data are expressed as the mean ± standard deviation and relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. **P* < .05; ****P* < .001. (*B, left*) Alizarin red S staining results on the mineralized nodule formation of hDPSCs after 21 days of culture with 1:1, 1:2, and 1:4 eluates of ThPT, ThLC, or BD compared with the negative control group and the positive control group. ****P* < .001 (2-way analysis of variance). (*B, right*) Representative images of alizarin red S staining. An increase in the red color from the samples indicates a higher fixation to calcium deposits and therefore a higher mineralization.

Coincidental results have also been described with regard to ThLC in studies on stem cells from human exfoliated deciduous teeth[21]. Regarding BD, similar *in vitro* studies in the field have also reported its absence of cytotoxicity toward hDPSCs[31]. This is also supported by a recent clinical study on the efficacy of ThLC, BD, and MTA as pulp capping agents in which ThLC resulted in a lower success rate than BD and MTA[32].

Following this tendency, the culture with the different eluates of ThLC led to significantly higher CM-H_2DCFDA–positive hDPSCs than the control group ($P < .001$), whereas none of the dilutions of BD or 1:2 and 1:4 ThPT exhibited significant differences.

CM-H_2DCFDA is a chloromethyl derivative of H_2DCFDA, which is used as an indicator for ROS in cells[33]. Upon bacterial infection, hypoxia, or low nutrient supply, dental stem cells produce excessive levels of intracellular ROS, which result in oxidative stress. Excessive ROS may lead to the oxidation of cellular DNA, proteins, lipids, and membranes,

among others, and consequently alter the cells' architecture and integrity[34]. Therefore, the adequate levels of ROS exhibited by BD- and ThPT-treated groups further support their use in VPT procedures in which the regenerative potential of hDPSCs with an appropriate stress response is exploited.

Both of the morphologic assays, namely scanning electron microscopic visualization and immunofluorescence staining, expectedly support the aforementioned findings. The lower cytocompatibility exhibited by ThLC translates into the following observable indicators of cellular death: the presence of cell debris, the lack of cellular adhesion, and the absence of functionally oriented cells, whereas the opposite was observed in the ThPT- and BD-treated hDPSCs. The morphologic differences between the ThLC- and ThPT-treated hDPSCs observed in the present study confirm those reported in a previous study performed by our research group[20].

Altogether, the results from the cytocompatibility assays disfavored the inclusion of ThLC in the cell plasticity assay because of its negative influence on hDPSC viability, proliferation, adhesion, and morphology. The decision to exclude ThLC from this assay was further supported by previous studies in the field in which it was also excluded from a cell plasticity assay[20], it was associated with significantly lower rates of stem cells from human exfoliated deciduous teeth viability[21], the ThLC-treated hDPSCs exhibited a significantly lower ALP activity than BD and MTA[31], and it adversely affected the osteogenic differentiation of hDPSCs[35].

Regarding the evaluation of the influence of the tested materials on cellular plasticity, both the ThPT- and BD-treated hDPSCs exhibited a significant up-regulation of at least 1 odontogenic marker compared with the negative control group after a 14-day culture period. The process of reparative dentinogenesis involves a complex intercommunication of a series of cell-signaling transduction pathways, which culminate overexpression of specific markers for cell proliferation, differentiation, secretory activity, and/or inflammatory response[30]. The up-regulation of odontogenic markers expressed by both of the tested materials suggests their favorable influence on hDPSC odontogenic differentiation.

Lastly, the influence of all of the tested materials on hDPSC mineralized nodule formation was assessed via alizarin red S staining. The significantly higher mineralization potential exhibited by both BD and ThPT compared with both the negative and positive control groups highlights their bioactive properties and, consequently, their ability to potentially favor the development of a mineralized layer on their surface when placed in direct contact with pulp tissue in VPT procedures.

Altogether, considering the differences exhibited by the tested biomaterials in terms of their cytocompatibility, influence on cell plasticity, and mineralization potential on hDPSCs led to the rejection of the null hypothesis. However, the lack of evidence regarding the comparative biological properties between ThPT and HCSCs hinders the direct comparison of the results of this study with previous evidence, thus acting as a limitation of the present work. The use of standardized procedures, such as those described in the ISO 10993-5 guidelines[22], together with proven methodologies, such as the 2-ΔΔCT method to calculate relative gene expression[26], ensure that the data are reported with adequate reliability. However, the extrapolation of the results of the present *in vitro* study into the clinical setting requires further support from animal and clinical trials. Nevertheless, the *in vitro* biological properties of the tested materials described in the present work may act as a preliminary assessment of their potential biological behavior.

CONCLUSION

The newly introduced ThPT offers an improved *in vitro* cytocompatibility and mineralization potential on hDPSCs compared with its predecessor, ThLC, and comparable biological properties to the HCSC BD.

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The authors deny any conflicts of interest related to this study.

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ORIGINAL ARTICLE

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Cytocompatibility and bioactive potential of AH Plus Bioceramic Sealer: An *in vitro* study

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Abstract

Aim: To assess the cytocompatibility and bioactive potential of the new calcium silicate cement-based sealer AH Plus Bioceramic Sealer (AHPbcs) on human periodontal ligament stem cells (hPDLSCs) compared with the epoxy resin-based sealer AH Plus (AHP) and the calcium silicate cement-based sealer Endosequence BC Sealer (ESbcs).

Methodology: Standardized sample discs and 1:1, 1:2 and 1:4 eluates of the tested materials were prepared. The following assays were performed: surface element distribution via SEM–EDX, cell attachment and morphology via SEM, cell viability via a MTT assay, cell migration/proliferation via a wound-healing assay, osteo/cemento/odontogenic marker expression via RT-qPCR and cell mineralized nodule formation via Alizarin Red S staining. HPDLSCs were isolated from extracted third molars. Comparisons were made with hPDLSCs cultured in unconditioned (negative control) or osteogenic (positive control) culture media. Statistical significance was established at $p < .05$.

Results: A higher peak of Ca^2+ was detected from ESbcs compared with AHPbcs and AHP in SEM–EDX. Both AHPbcs and ESbcs showed significantly positive results in the cytocompatibility assays (cell viability, migration/proliferation, attachment and morphology) compared with a negative control group, whilst AHP showed significant negative results. Both AHPbcs and ESbcs exhibited an upregulation of at least one osteo/odonto/cementogenic marker compared with the negative and positive control groups. Both ESbcs and AHPbcs showed a significantly higher calcified nodule formation than the negative and positive control groups, indicative of their biomineralization potential and were also significantly higher than AHP group.

Conclusion: AH Plus Bioceramic Sealer exhibited a significantly higher cytocompatibility and bioactive potential than AH Plus and a similar cytocompatibility to that of Endosequence BC Sealer. Endosequence BC Sealer exhibited a significantly higher mineralization potential than the other tested sealers. The results from this *in vitro* study act as supporting evidence for the use of AH Plus Bioceramic Sealer in root canal treatment.

KEYWORDS
AH Plus Bioceramic Sealer, AH Plus sealer, bioactivity, biocompatibility, Endosequence BC sealer

INTRODUCTION

Root canal treatment involves the chemical–mechanical disinfection of the root canal system and its subsequent filling to ensure an adequate seal (Li et al., 2014). The materials placed inside the root canal should be dimensionally stable, biocompatible and present adequate handling properties (Donnermeyer et al., 2019). The most commonly used materials for such purpose are gutta-percha and root canal sealers (Kishen et al., 2016; Vishwanath & Rao, 2019).

A wide variety of root canal sealer compositions are available, such as zinc oxide-eugenol, epoxy resin and calcium silicate cement sealers (Sfeir et al., 2021). These materials differ in terms of their setting reactions, which take place by chelate formation, polymer formation by addition and hydration, respectively (Komabayashi et al., 2020). The physicochemical and biological properties of these sealers also differ (Silva et al., 2019, 2021).

Endodontic sealers are placed inside the root canal and may extrude to a variable extent during root canal treatment through the apical and/or secondary foramina into the surrounding supporting tissues (Aminoshariae & Kulild, 2020). Therefore, sealers should exhibit an adequate biocompatibility, i.e. they should not induce an adverse reaction or response from biological tissues upon contact (Ferreira et al., 2021). The same applies on a cellular level, wherein surrounding cellular populations should not experience a decrease in their viability, migration/proliferation or differentiation (da Silva et al., 2017). In other words, root canal sealers should exhibit an adequate cytocompatibility and absence of cytotoxicity.

Periodontal ligament stem cells (PDLSCs), a subgroup of dental stem cells (DSCs) with a mesenchymal phenotype (Bartold & Gronthos, 2017), are in contact with root canal sealers. These cells possess a multilineage differentiation potential and may play a crucial role in the healing process of existing periapical lesions (Gay et al., 2007; Seo et al., 2004). Consequently, the extrusion of a root canal sealer with adequate biological properties should not hinder these cells.

Previous evidence has demonstrated that resin-based or resin-containing sealers and cements often are cytotoxic toward various cell subpopulations (Collado-González, Tomás-Catalá, et al., 2017; Manaspon et al., 2021). The well-known AH Plus sealer (Dentsply DeTrey GmbH) has shown a negative effect on periodontal ligament stem cell viability, migration/proliferation and attachment in various *in vitro* studies (Oh et al., 2020; Rodríguez-Lozano et al., 2019) compared with a negative control group.

Conversely, calcium silicate cement-based sealers have adequate biocompatibility and bioactive properties when cultured together with PDLSCs (Rodríguez-Lozano et al., 2017; Zheng et al., 2020). These sealers have shown the ability to induce the precipitation of a layer of hydroxyapatite on their surface (Kim et al., 2015). which may form a mineral attachment to dentin tissue (Vallittu et al., 2018). The same term is used to describe their positive influence on cell plasticity, e.g. by favouring the osteo/odonto/cementogenic differentiation of PDLSCs which, in turn, may result in an enhanced repair process and resolution of periapical lesions (Sanz, Guerrero-Gironés, et al., 2021).

AH Plus Bioceramic Sealer (Maruchi) was introduced into the market as a pre-mixed tricalcium silicate cement-based sealer. According to its distributor (Dentsply Sirona USA), this new sealer presents a faster setting time, lower solubility, lower film thickness and higher radiopacity than the Endosequence BC Sealer (Brasseler). However, to the authors' knowledge, the biological properties of the new AH Plus Bioceramic Sealer towards PDLSCs have not been elucidated.

Accordingly, the aim of the present *in vitro* study is to assess the cytocompatibility and bioactive properties of AH Plus Bioceramic Sealer on hPDLSCs compared with the classic AH Plus and Endosequence BC Sealer.

MATERIALS AND METHODS

The manuscript of this *in vitro* study has been written in accordance with the Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021' guidelines (Nagendrababu et al., 2021a).

Preparation of material extraction mediums

Sample discs were prepared for each of the tested materials (n = 30 in total): AH Plus Bioceramic Sealer (AHPbcs), AH Plus (AHP) and Endosequence BC Sealer (ESbcs). The number of discs was based on the protocol from a previous study with similar methodology (Sanz, López-García, et al., 2021). Material data (composition, manufacturer and batch number) are listed in Table 1. Materials were placed into 5-mm diameter and 2-mm

high sterile (ultraviolet radiation, 15 min) cylindrical rubber molds with Hank's balanced salt solution (HBSS; H6648; Sigma Aldrich) and set in an incubator at 37°C, 5% CO_2 and 95% humidity for 48 hours. AHP is presented in a two-component paste/paste format. Accordingly, pastes A and B were mixed following its manufacturer's instructions. Both AHPbcs and ESbcs are presented in an injectable syringe (pre-mixed) format and were directly placed into the rubber moulds.

To simulate clinical conditions, where cells are in contact with the silicate cement-based sealers, extracts or eluates were obtained from each of the materials, following the International Standard ISO 10993-5 guidelines (ISO, 2009). The eluates of the different materials were extracted in sterile conditions, using Dulbecco Modified Eagle's Medium (DMEM) (Gibco) with 10% of foetal bovine serum (FBS) as an extraction vehicle. The extraction procedure was as follows: the tested materials were immersed in the culture medium for 24 h at 37°C in a humid atmosphere containing 5% CO_2. In accordance with the ISO standard, the ratio between the surface of the sample and the volume of the medium was 1.5 cm^2/ml. The extraction medium was collected at the end of this period and filtered through a 0.22-μm syringe filter (Merck Millipore). Thereafter, in order to study the effect of the concentration of each material, various dilutions (1:1, 1:2 and 1:4 v/v) of these extraction media were prepared using fresh complete DMEM medium (Rodríguez-Lozano et al., 2019).

Material surface element distribution: SEM-EDS analysis

The previously prepared material sample discs were selected for the analysis ($n = 5$ per material). After the incubation period, the set material discs were coated with carbon under a CC7650 SEM Carbon Coater Unit (Quorum Technologies Ltd.). The superficial element distribution of the coated discs was then individually examined in a scanning electron microscopy (SEM) unit (Jeol 6100 EDAX; Jeol Inc.) attached to an energy dispersive spectroscopy (EDS) system (INCA 350 EDS; Oxford Instruments) for the elemental analysis.

Isolation, culture and characterization of human PDLSCs

The human PDLSC (hPDLSC) extraction protocol had been approved by the Human Research Ethics Committee from *Universidad de Murcia* (ID: 2199/2018), following the Helsinki Declaration guidelines. HPDLSCs were isolated from healthy third molars from 18–30-year-old patients ($n = 10$), which had been extracted for orthodontic or periodontal reasons; with written informed consent. The molar sample size was selected in accordance with a previous study with similar methodology (Sanz, López-García, et al., 2021).

Extracted molars were immediately placed in Minimum Essential Medium with Alpha modifications (α-MEM; Gibco, Invitrogen) supplemented with 1% penicillin/streptomycin (Sigma Aldrich) and amphotericin B (Fungizone; Sigma Aldrich) and stored at 4°C. The teeth were rinsed thrice with phosphate-buffered saline (PBS) (Gibco), and the periodontal tissues were scraped from the surface of the middle and apical thirds of their roots. Periodontal tissues were sliced into smaller fragments and digested with Collagenase type I solution (3 mg/ml; Gibco) for 1 h at 37°C. The periodontal cells were seeded in α-MEM supplemented with 10% foetal bovine serum

TABLE 1 Data on the tested materials

Material	Manufacturer	Composition[a]	Batch number
AH Plus Bioceramic Sealer	Manufactured by Maruchi Distributed by Dentsply DeTrey GmbH	Zirconium dioxide (50%–75%), tricalcium silicate (5%–15%), dimethyl sulfoxide (10%–30%), lithium carbonate (<0.5%), thickening agent (<6%)	KS210728
AH Plus	Dentsply DeTrey GmbH	Paste A: bisphenol-A epoxy resin, bisphenol-F expoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments Paste B: dibenzyldiamine, aminoadamantane, tricyclodecane-diamine, calcium tungstate, zirconium oxide, silica, silicone oil	2105000678
Endosequence BC Sealer	Manufactured by Innovative Bioceramix Distributed by Brasseler	Zirconium dioxide (35%–45%), tricalcium silicate (20%–35%), dicalcium silicate (7%–15%), calcium hydroxide (1%–4%)	21001SP

[a]The concentration of each component of the tested materials is presented as a percentage by weight (WT%) within brackets. Data were extracted from the respective Material Safety Data Sheets, if available.

(FBS; Sigma Aldrich) and 1% penicillin/streptomycin (Sigma Aldrich).

Before their use in the *in vitro* experimentation, hPDLSC characterization was performed following the International Society of Cellular Therapy (ISCT) guidelines (Dominici et al., 2006), to confirm their mesenchymal nature. The process was as follows: cells were analysed under flow cytometry (FACSCalibur Flow Cytometry System; BD Biosciences), and the high expression of the mesenchymal stem cell (MSC)-specific surface markers CD73, CD90 and CD105 and low expression of the hematopoietic markers CD34, CD45, CD14 and CD20 were confirmed. This was performed in accordance with similar studies in the field (Collado-González, García-Bernal, et al., 2017; Oh et al., 2020). Additionally, the resultant characterized hPDLSCs were cultured in different media (osteogenic/adipogenic/chondrogenic) (Miltenyi Biotec) to confirm their trilineage mesenchymal differentiation. Both the mesenchymal nature and trilineage differentiation potential of the cells used were confirmed by a previous study performed by the present research group (Rodríguez-Lozano et al., 2019). For the subsequent *in vitro* experimentation, cells from passages 2–4 were used, as performed in previous similar studies (Sanz, López-García, et al., 2021).

Material cytotoxicity: MTT assay

Material cytotoxicity was assessed for the different eluates (1:1, 1:2 and 1:4) of AHP, AHPbcs and ESbcs cultured with hPDLSCs (test groups) and compared with hPDLSCs cultured in unconditioned growth medium (negative control group). This analysis was performed via a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously reported by similar studies (Rathinam et al., 2021). In brief, hPDLSCs were seeded onto 96-well plates with 180 µl of DMEM and stored for 24 h at 37°C, 5% CO_2 and 95% humidity. The material eluates were placed in the culture medium with 1×10^4 hPDLSCs ($n = 3$). An MTT reagent (Sigma Aldrich) was added for 4 h, following its manufacturer's instructions. When a purple precipitate was detectable, Dimethylsulfoxide (DMSO) (Sigma-Aldrich) was added to each well (100 µl/well), and plates were covered and kept in dark conditions for 4 h to solubilize the formazan crystals produced by viable cells, after reducing the MTT reagent. After 24, 48 and 72 h of culture, light absorbance per well was recorded by means of a microplate reader (ELx800; Bio-Tek Instruments) at 570 nm wavelength. Culture media with fresh eluates from the respective groups were replaced every 3 days.

Cell migration/proliferation: Horizontal wound healing assay

HDPLSC migration/proliferation was assessed after culture in growth medium with the eluates (1:1, 1:2 and 1:4) of AHP, AHPbcs and ESbcs and compared with the cells cultured in unconditioned growth medium (negative control group) via a wound healing assay. HPDLSCs were seeded onto 6-well plates (2×10^5 cells per well; $n = 3$ for each experimental condition) and left to proliferate until cell confluency was reached. Then, a superficial scratch wound was made on each cell monolayer using a 200-µl sterilized pipette tip, and each well was rinsed thrice to remove any remaining cell debris. Wound closure/healing was assessed for all experimental conditions in triplicate (test groups and negative control) at 24, 48 and 72 h. At each time-point, the percentage of open wound area was quantified for each of the samples by means Image J software (National Institutes of Health). Migration rates were presented as percentage areas of relative wound closure (RWC) to account for width variations amongst the scratch wounds. RWC values were calculated as follows: RWC = (wound closure area [in pixels]/total number of pixels)×100. Results are expressed as the percentage of the total wound area thrice relative to the total wound area at 0 h for each respective well.

Cell morphology and attachment: SEM visualization

Sealer discs were made using the previously described methods ($n = 5$ for each sealer). The surface of the discs was seeded with 5×10^4 hDPSCs and cultured in normal growth medium for 72 h. Cells were fixed with 4% glutaraldehyde (Sigma-Aldrich) in PBS for 4 h. The cells were dehydrated using a series of gradually increasing ethanol dilutions (30 to 90% v/v) and treated with hexamethyldisilazane (Sigma-Aldrich) for 5 min. Finally, cells were air-dried, sputter-coated with gold and palladium and examined using a SEM (Jeol 6100 EDAX; Jeol Inc.) at 100×, 300× and 1500× magnifications.

Cell osteo/cemento/odontogenic gene expression: RT-qPCR assay

The osteo/cemento/odontogenic marker expression of hPDLSCs cultured together with the materials was assessed via real-time quantitative polymerase chain reaction (RT-qPCR), as a measurement of cell differentiation. Twenty-thousand hPDLSCs per well were

seeded onto 12-well plates ($n = 3$) and incubated for 3, 7, 14 and 21 days with undiluted (1:1) sealer-conditioned medium from the two calcium silicate-cements (test groups: AHPbcs or ESbcs), in unconditioned culture medium (negative control groups) or in osteogenic differentiation medium (positive control; OsteoDiff media; Miltenyi Biotec). Culture media with fresh eluates from the respective groups were replaced every 3 days. The undiluted sealer-conditioned medium was prepared by immersing the previously conditioned standardized sealer discs in culture medium (DMEM; Gibco) for 24 h. AHP was excluded from the marker expression assay because of its negative results in the hPDLSC viability, migration/proliferation and attachment assays. Total RNA was extracted from hPDLSCs using the RNeasy Mini Kit (Qiagen). One μg of RNA was reverse transcribed for first-strand complementary DNA (cDNA) synthesis via iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Inc.). Both processes were performed following their respective manufacturers' kit instructions.

The primer sequences for the differentiation markers used for the assay were as follows (5′–3′): Cementum attachment protein or CAP (forward: TTTTTCTGGTCGCGTGGACT, reverse: TCACCAGCA ACTCCAACAGG), cementum protein 1 or CEMP1 (forward: GGGCACATCAAGCACTGACAG, reverse: CCCTTAGGAAGTGGCTGTCCAG), alkaline phosphatase or ALP (forward: TCAGAAGCTCAACACCAACG, reverse: TTGTACGTCTTGGAGAGGGC), runt-related transcription factor 2 or RUNX2 (forward: TCCAC ACCATTAGGGACCATC, reverse: TGCTAATGCTTCGT GTTTCCA), bone sialoprotein or BSP (forward: TGCC TTGAGCCTGCTTCCT, reverse: CTGAGCAAAATTAA AGCAGTCTTCA), amelogenin X or AMELX (forward: CACCCTGCAGCCTCATCACC, reverse: GTGTT GGATTGGAGTCATGG).

Differentiation marker expression was measured relative to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), with the following sequence (5′-3′): (forward: TCAGCAATGCCTCCTGCAC, reverse: TCTGGG TGGCAGTGATGG). To calculate the relative gene expression, the standardized $2-\Delta\Delta CT$ method was used (Livak & Schmittgen, 2001).

Cell mineralization/calcified nodule formation: Alizarin Red S Staining

An Alizarin Red S Staining (ARS) assay was performed to assess hPDLSC calcified nodule formation in contact with the tested sealers (AHPbcs, AHP and ESbcs), as a measurement of their biomineralization potential. Twenty-thousand hPDLSCs per well were seeded onto 12-well plates ($n = 3$) and left to proliferate until confluency was reached. The cells were then transferred into undiluted (1:1) sealer-conditioned medium and cultured for 21 days. After the culture period, the samples were rinsed with foetal bovine serum and fixed with 70% ethanol for 1 h. Then, samples were stained with 2% Alizarin Red solution (Sigma Aldrich) for 30 min in controlled conditions (dark ambient and room temperature) and solubilized using 10% cetylpyridinium chloride monohydrate solution (Sigma-Aldrich). Lastly, absorbance values of the samples were measured using Synergy H1 multi-mode microplate reader (BioTek) at 570 nm. For this assay, both a negative control (hDPSCs cultured in unconditioned growth medium [DMEM; Gibco]) and a positive control (hDPSCs cultured in osteogenic medium (OsteoDiff; Miltenyi Biotec) were used for reference.

Statistical analysis

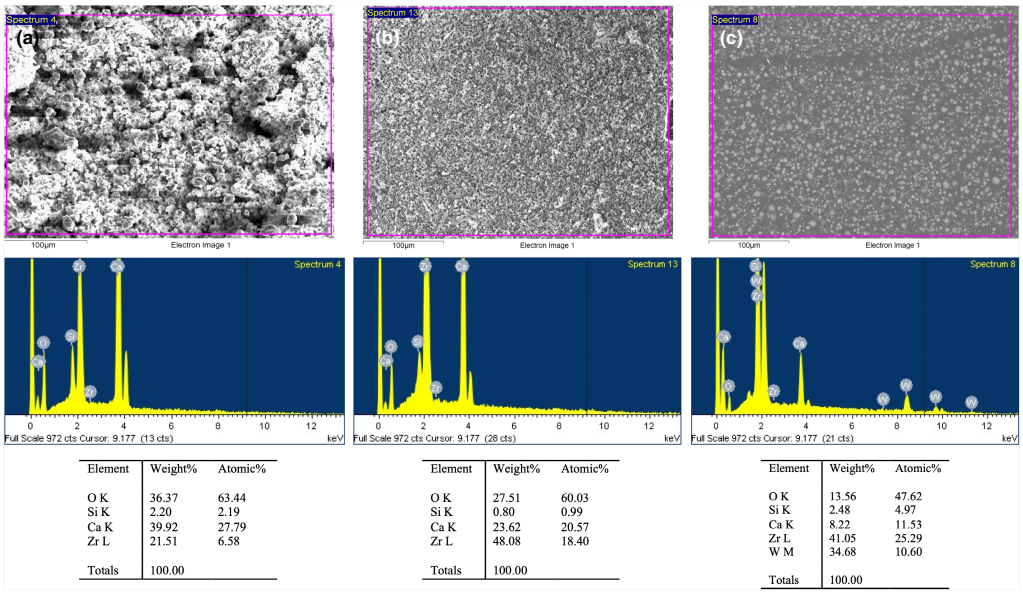
All the experimental conditions and measurements were performed in triplicate for each of the tested sealers (AHPbcs, AHP and ESbcs). Data are expressed as mean ± standard deviations (SD). The normality in the distribution of the data was previously confirmed via a Q-Q plot. Data were analysed using one-way ANOVA and Tukey's *post hoc* test using Graph-Pad Prism v8.1.0 (GraphPad Software). To perform the one-way ANOVA test, we grouped the data by time (24 h, 48 h and 72 h) and analysed them independently. Each dilution was considered an independent treatment. Statistical significance was considered at $p < .05$.

RESULTS

Data (mean and standard deviations) from the biological assays are presented in the Supplementary Material, as follows: Table S1 (MTT assay), Table S2 (wound healing assay), Table S3 (Alizarin Red S staining) and Table S4 (RT-qPCR).

SEM-EDS analysis

SEM-EDS analysis revealed the superficial element distributions of the root canal sealers (ESbcs, AHPbcs and AHP; Figure 1). ESbcs and AHPbcs displayed a superficial crystalline structure, whilst particles on the smoother AHP surface were spherical. The elements O, Si, Ca and Zr



Element	Weight%	Atomic%
O K	36.37	63.44
Si K	2.20	2.19
Ca K	39.92	27.79
Zr L	21.51	6.58
Totals	100.00	

Element	Weight%	Atomic%
O K	27.51	60.03
Si K	0.80	0.99
Ca K	23.62	20.57
Zr L	48.08	18.40
Totals	100.00	

Element	Weight%	Atomic%
O K	13.56	47.62
Si K	2.48	4.97
Ca K	8.22	11.53
Zr L	41.05	25.29
W M	34.68	10.60
Totals	100.00	

FIGURE 1 Results from the SEM-EDS analysis for the tested sealers (ESbcs [column a], AHPbcs [column b], AHP [column c]). The first row illustrates SEM images of each material (scale bar: 100 μm). The second row shows the EDS elemental spectra. The third row lists the elements present per sealer by weight and atomic weight.

were detected in all samples. Interestingly, a higher peak of Ca^{2+} was detected in ESbcs compared with AHPbcs and AHP, whereas a higher peak of zirconium (Zr) was observed in AHPbcs compared with the other material samples. Tungsten (W) was detected only in the AHP samples.

MTT assay

The MTT assay revealed an adequate cell viability from all eluates of ESbcs and AHPbcs at all the tested time points (24, 48 and 72 h of culture), similar to that of the control group; the 1:1 AHPbcs-treated cells, however, exhibited a significantly lower viability than the control group ($p < .001$). AHP-treated cells exhibited a significantly lower viability compared with the control group after 24, 48 and 72 h of culture ($p < .001$; Figure 2).

Wound healing assay

Human periodontal ligament stem cells cultured with all the eluates of AHPbcs and ESbcs exhibited similar migration to that of the control group at all time-points (24, 48 and 72 h) in the wound healing assay. Similar to the cytotoxicity assay, cells cultured with AHP exhibited a significantly lower migration compared with the control group after 24, 48 and 72 h of culture ($p < .001$; Figure 3).

SEM visualization for hPDLSCs

SEM images revealed differing hPDLSC morphologies and attachment on the surface of the root canal sealer discs (ESbcs, AHPbcs and AHP). HPDLSCs seeded onto the surface of ESbcs and AHPbcs sample discs exhibited a spindle-like elongated morphology, intense growth and spread. It should be highlighted that a higher number of attached cells were visible on the surface of ESbcs compared with that of AHPbcs. Conversely, the surface of AHP sample discs had a low quantity of cells and debris, indicating cell death (Figure 4).

RT-qPCR assay

The RT-qPCR assay for the assessment of osteo/odonto/cementogenic marker expression from hPDLSCs cultured with the tested materials (ESbcs or AHPbcs) produced a wide variety of results (Figure 5).

AHPbcs-treated cells exhibited a significantly higher early expression (3 and 7 days of culture) of ALP compared with the negative control group and a significantly higher late expression (14 and 21 days of culture) of CEMP1, CAP, ALP ($p < .01$ at 21 days), RUNX2 and BSP. Compared with the positive control group, AHPbcs-treated cells exhibited a significantly higher early expression of ALP and RUNX2 and a late expression of ALP and AMELX.

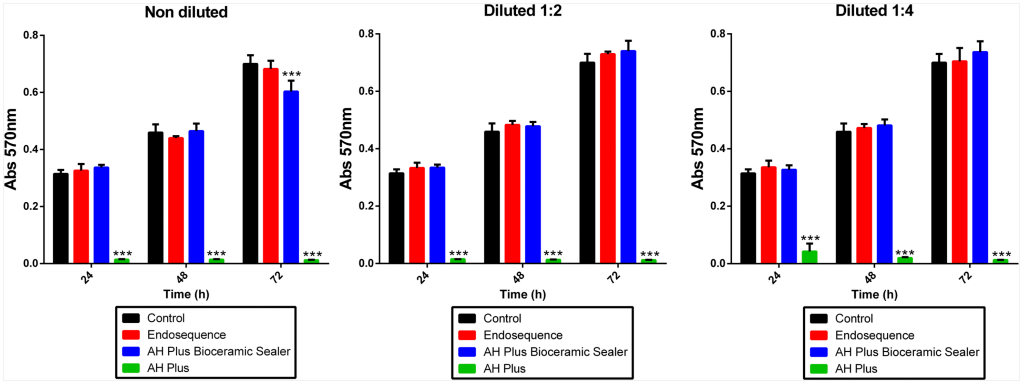


FIGURE 2 Results from MTT assay for the different eluates (1:1, 1:2, 1:4) of the tested sealers (ESbcs, AHPbcs and AHP) after 24, 48 and 72 h of culture with hPDLSCs. Data are presented absorbance values (570 nm) at the different measurement time-points, compared with the negative control group. ***$p < .001$ (One-way ANOVA analysis).

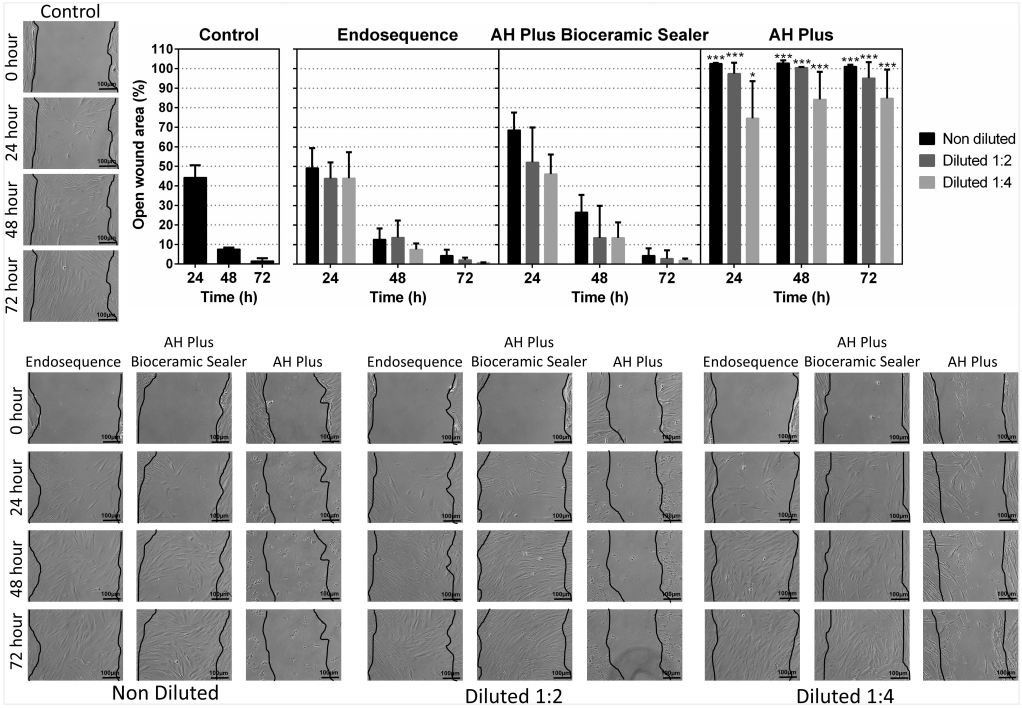


FIGURE 3 Results from the wound healing assay for the different eluates (1:1, 1:2, 1:4) of the tested sealers (ESbcs, AHPbcs and AHP) after 24, 48 and 72 h of culture with hPDLSCs. Graphical results are presented as percentages of open wound areas at the different measurement time points, compared with the negative control group. ***$p < .001$ (One-way ANOVA analysis). Images: Scale bar 100 μm.

ESbcs-treated cells exhibited a significantly higher early expression of all the tested markers compared with the negative and positive control groups, and a significantly higher late expression of CEMP1, CAP, ALP, BSP and AMELX compared with the negative control group. ESbcs-treated cells also exhibited a significantly higher late expression of ALP and BSP compared with the positive control group.

When comparing the two calcium silicate cement sealers, ESbcs-treated cells exhibited a significantly higher early expression of all the tested markers and a significantly higher late expression of ALP, BSP and AMELX. On the other hand, AHPbcs-treated cells exhibited a significantly higher late expression of CEMP, CAP and RUNX2.

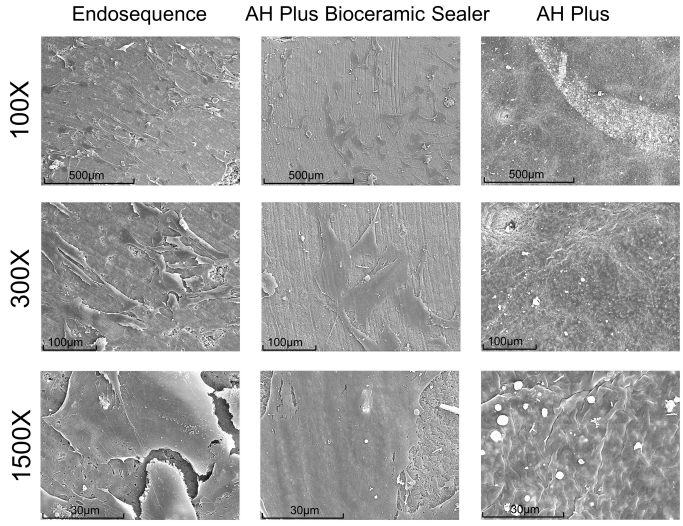


FIGURE 4 Results from the SEM visualization after 72 h of culture of hPDLSCs seeded onto the surface of the tested material sample discs (ESbcs, AHPbcs and AHP). Magnifications: 100×, 300× and 1500×. Scale bars: 500 µm, 100 µm and 30 µm.

Alizarin Red S Staining

Results from the cell mineralization assay are presented in Figure 6. Both AHPbcs and ESbcs-treated hPDLSCs exhibited a significantly higher mineralized nodule formation than the negative and positive control groups ($p < .001$). The AHP-treated hPDLSCs exhibited a significantly lower mineralization compared with the negative and positive control groups ($p < .05$ and $p < .001$; respectively). As expected, cells cultured in osteogenic medium (Osteodiff; positive control) showed a significantly higher mineralization ($p < .01$) than those cultured in unconditioned medium (negative control). ESbcs-treated cells showed a significantly higher calcified nodule formation than those treated with AHPbcs ($p < .01$).

DISCUSSION

New biomaterial formulations are constantly being introduced into the market for clinical use in the field of endodontics (Camilleri et al., 2022). Currently, calcium silicate cement-based materials are increasing in use amongst clinicians (Careddu et al., 2021). The recently introduced AH Plus Bioceramic Sealer is presented as a potential alternative to the classic AH Plus and other calcium silicate cement-based sealers such as Endosequence BC Sealer. Accordingly, the aim of the present study was to assess its cytocompatibility and bioactive properties on hPDLSCs and compare them with the aforementioned sealers.

In vitro study designs like the present one offer a consistent analysis of the main biological properties of dental materials cultured together with cellular subpopulations with which they will come into contact during their clinical use and thus may predict their clinical behaviour (Pedano et al., 2020). However, as a limitation of the present work, several variables could affect the differences between the results observed under laboratory and clinical conditions, such as variations in pH, variations in oxygen levels or the patients' immune response (Sanz, Guerrero-Gironés, et al., 2021). Nevertheless, as a strength of the present work, the use of standardized material sample preparation and biological assay procedures (ISO 10993-5, 2009) results in an increased reproducibility of the study design and consequently an increased homogeneity between studies. Lastly, following specific reporting guidelines enhances the comparability between studies with similar methodologies. In the present study, the recently introduced PRILE guidelines were followed for such purpose (Nagendrababu et al., 2021b). Accordingly, the main steps of this work have been depicted in the PRILE 2021 flowchart (Figure 7).

Biocompatibility and cytocompatibility assays are useful to confirm the positive or negative response of biological tissues and cellular populations. In the present study three cytocompatibility assays were performed: MTT assay, as a measure of cell viability; wound healing assay, as a measure of cell proliferation/migration; and SEM visualization, as a measure of cell morphology and attachment. All the cytocompatibility assays produced concordant results. Both calcium silicate cement-based sealers (ESbcs and AHPbcs) exhibited an adequate cytocompatibility compared with a negative control group. These results are in accordance with previous studies on the biological properties of calcium silicate cement-based sealers as a group of dental materials (Mann et al., 2022; Park et al., 2021; Zordan-Bronzel et al., 2019). The main components tricalcium silicate and a radiopacifying agent (zirconium oxides) have been shown to be biocompatible in previous studies (Campi et al., 2022).

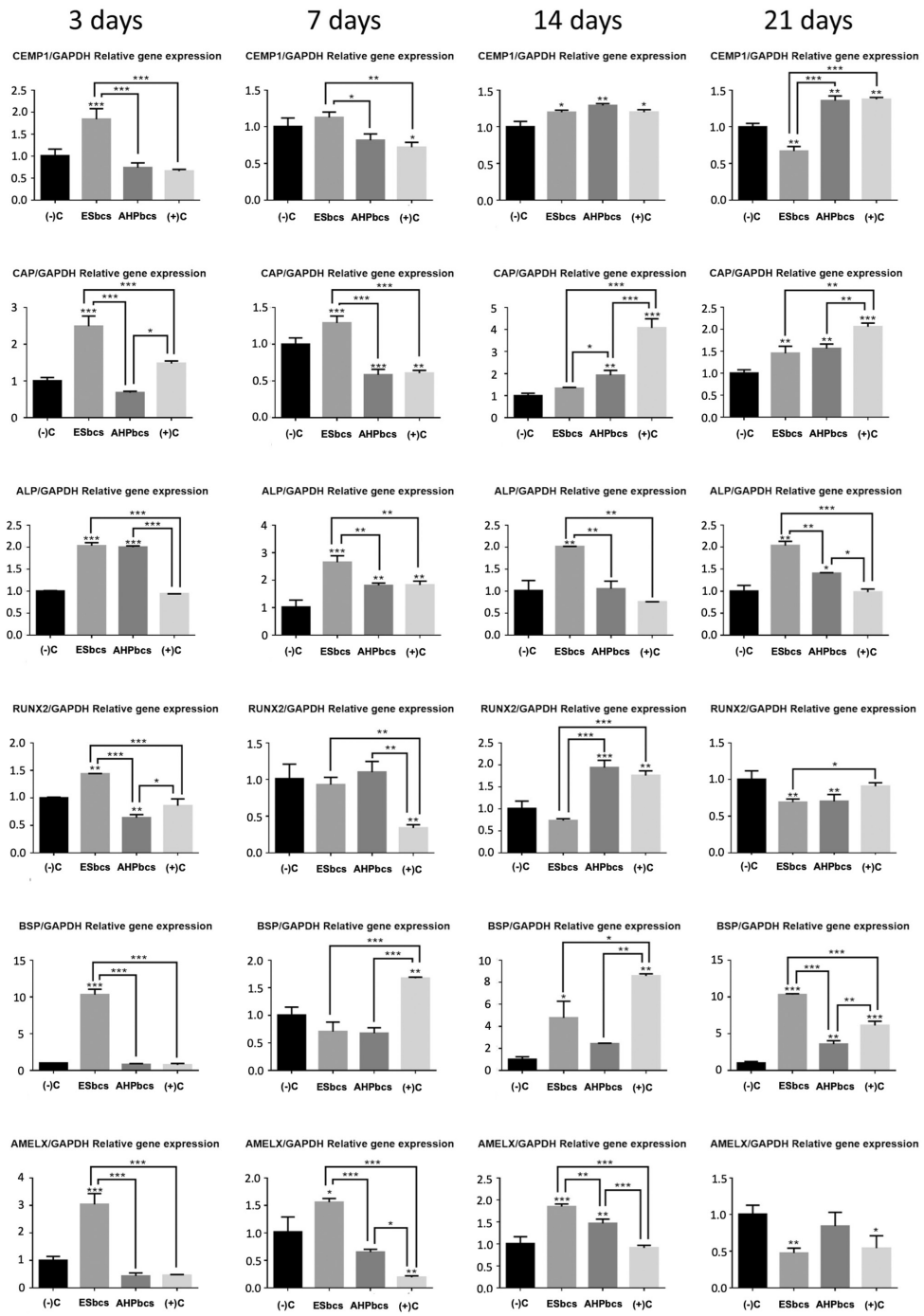


FIGURE 5 Results from the analysis of hPDLSCs osteo/odonto/cementogenic marker expression via RT-qPCR after 3, 7, 14 and 21 days of culture with DMEM (negative control), ESbcs, AHPbcs, or Osteodiff (postive control). *$p < .05$; **$p < .01$; ***$p < .001$ (Two-way ANOVA analysis). Asterisks above the bars indicate a significant difference with the negative control group. Asterisks above the lines indicate a significant difference between the groups which the line is connecting.

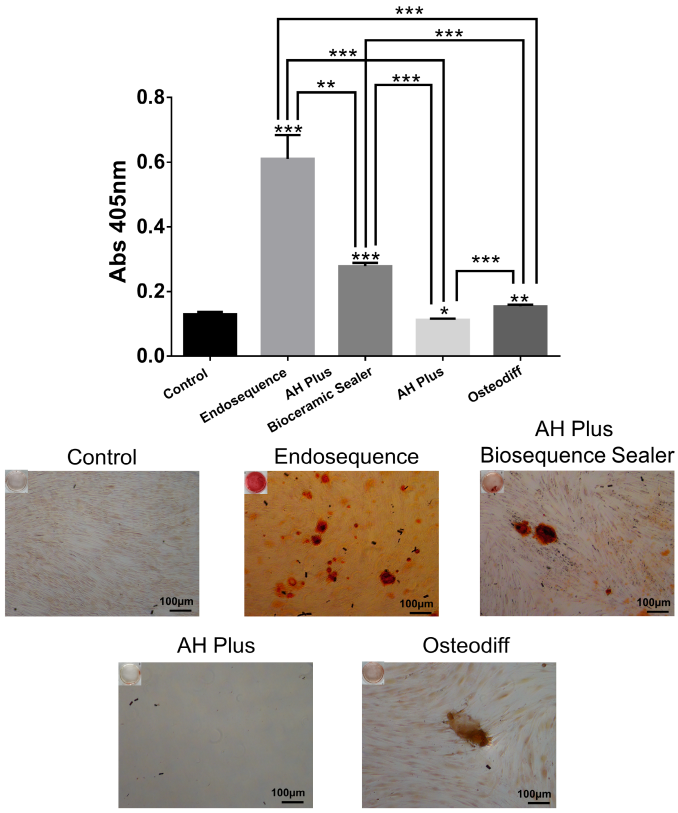


FIGURE 6 Results from the Alizarin Red S staining of hPDLSCs after 21 days of culture with DMEM (negative control), ESbcs, AHPbcs, AHP or Osteodiff (positive control). *$p < .05$; **$p < .01$; ***$p < .001$ (Two-way ANOVA analysis). Asterisks above the bars indicate a significant difference with the negative control group. Asterisks above the lines indicate a significant difference between the groups which the line is connecting.

The epoxy resin-based sealer AHP showed signs of cytotoxicity on hPDLSCs, evidenced by their significant decrease in cell viability and proliferation and aberrant morphology and attachment. These results are in accordance with previous studies on several cell subpopulations (Saygili et al., 2017; Rodríguez-Lozano et al., 2019). The resinous component in endodontic sealers or in resin-modified calcium silicate cements has been associated with the increased cytotoxicity (Bakir et al., 2022; Sanz, Soler-Doria, et al., 2021; Silva et al., 2013).

SEM is useful for the evaluation of the superficial morphology and texture of materials. Regarding calcium silicate cement-based materials, it can also be suitable for the evaluation of their hydration (Anthrayose et al., 2021). Both ESbcs and AHPbcs displayed a crystalline surfaces, unlike the epoxy resin sealer. AHPbcs exhibited a denser and more homogeneous microstructure. The hydration extent can influence the properties of cements (Camilleri, 2007) and thus may account for the differences observed in the mineralization assay.

Energy dispersive spectroscopy was used to examine the elements present on the surface of the samples. However, EDS only exhibits the distribution of elements on the sample's surface. Other complementary techniques such as X-ray diffraction (XRD) analysis or attenuated total reflection–Fourier transform infrared (ATR-FTIR) spectroscopy are needed to identify calcium hydroxide peaks and other crystalline phases in hydraulic cements after setting (Ferreira et al., 2021).

Differences in the elemental composition of the tested biomaterials may account for their differing biological properties. For example, both calcium silicate cement-based sealers disclosed tricalcium silicate in their composition, but ESbcs also incorporates dicalcium silicate. Additionally, tricalcium silicate only represents 5%–15% in weight in AHPbcs, whilst ESbcs contain 20%–35% of tricalcium silicate and 7%–15% of dicalcium silicate. Differences in the composition of calcium silicate cement-based materials affect their behaviour (Watson et al., 2014). This explains the significantly higher mineralization exhibited by ESbcs-treated cells compared with those treated with AHPbcs in the ARS assay.

As expected, both ESbcs and AHPbcs contained more calcium and oxygen than AHP. Calcium and hydroxyl ion release after hydration has been associated with the favourable biological properties of calcium silicate cement-based endodontic biomaterials (Khalil et al., 2016). The observed Ca^{2+} peak in AH Plus can be explained by the inclusion of calcium tungstate ($CaWO_4$) as a radiopacifier in its composition. The Zr observed in AHPbcs compared with

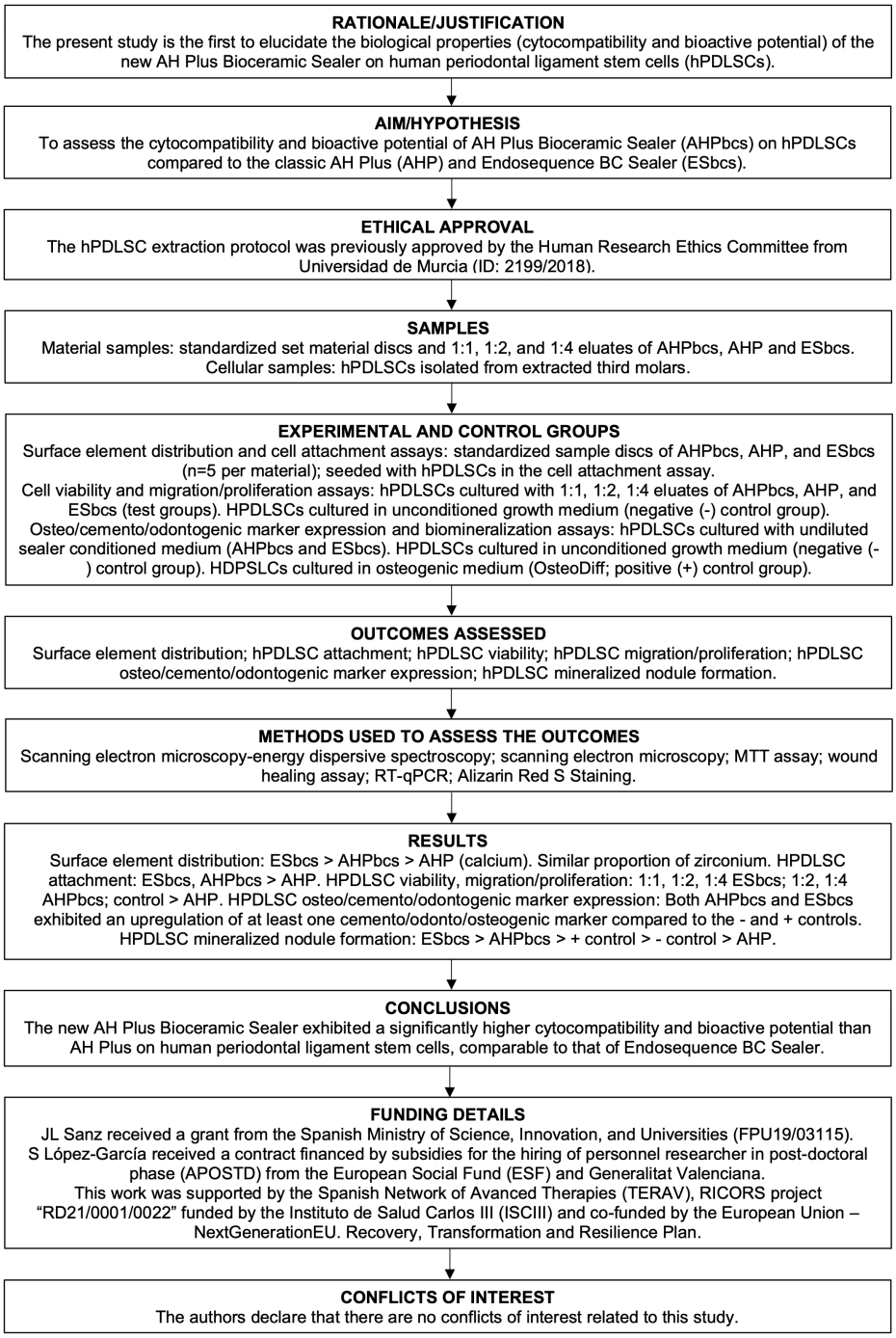


FIGURE 7 Preferred Reporting Items for Laboratory studies in Endodontology (PRILE 2021)-based flowchart.

ESbcs is supported by the differences in their percentage by weight (WT%) listed in their respective Material Safety Data Sheets (50%–70% vs. 35%–45%). The higher proportion of radiopacifier (ZrO_2) in the AHPbcs correlated with differences in in the biological properties of endodontic sealers, as others have noted (Queiroz, Torres, Rodrigues, Viola, Bosso-Martelo, Chavez-Andrade, et al., 2021b). Thus, in future research, it could be interesting to study

the biological properties of new biomaterial compositions from the perspective of the differences in radiopacifying agents and concentrations, as performed by a recent study (Queiroz, Torres, Rodrigues, Viola, Bosso-Martelo, Chavez-Andrade, et al., 2021a).

It should be highlighted that the inability to assess the influence of fillers, thickening agents, additives and/or vehicles can act as a limitation of the analysis of the biological properties of the tested materials from the perspective of the differences in their composition. The presence and proportion of these components in the composition of the tested materials are often regarded as confidential business information. For example, 1%–4% of calcium hydroxide is included as a non-confidential additive in Endosequence BC Sealer. This contributed to the observed Ca^{2+} peak in the SEM-EDS analysis. Thus, other confidential additives could explain other differences in the materials' biological properties.

Results from the RT-qPCR assay are varied but follow a general pattern. In brief, hPDLSCS cultured with both ESbcs or AHPbcs exhibited a significant upregulation of at least one cementogenic, osteogenic and odontogenic marker compared with the negative and positive control group. These markers were assessed, based on similar studies in the field (Rodríguez-Lozano et al., 2020; Zheng et al., 2020): CEMP1, CAP, ALP, RUNX2, BSP, and AMELX.

Cementogenic markers such as CEMP and CAP are important indicators of hPDLSC activity since they play a crucial role in the regeneration and repair of the periodontum. Specifically, an overexpression of CAP is seen during cell recruitment and differentiation during the formation of cementum tissue, whilst CEMP-1 is involved in the regulation of the differentiation of periodontal cells (Arzate et al., 2015; Pitaru et al., 1995). Thus, the overexpression of CEMP and CAP exhibited by ESbcs and AHPbcs-treated hPDLSCs may indicate their positive influence in cell plasticity and enhancement of the healing process of periodontal defects from lesions of endodontic origin.

BSP is a mineralized tissue-specific marker that is highly expressed during the initial formation of bone tissue and is synthesized by osteoblasts and osteoclastic-like cells in culture (Garcia et al., 2003; Ogata, 2008). ALP promotes bone formation by degrading inorganic pyrophosphate and generating inorganic phosphate, a crucial molecule in differentiation and mineralization of osteoblasts (Osathanon et al., 2009; Seltzer et al., 1962). Therefore, the overexpression of these markers exhibited by ESbcs and AHPbcs-treated hPDLSCs is a complementary indicator of the positive influence of these materials in cell plasticity and differentiation into an osteoblast-like lineage. This may reflect their potential enhancement of the process of bone tissue repair or regeneration.

RUNX2 has been reported to be essential for the later stages of tooth formation, since it is involved in the development of mineralized dental tissue (Camilleri & McDonald, 2006). Additionally, it has been reported that RUNX2 is essential for osteoblast differentiation (Bruderer et al., 2014), and that its overexpression enhances the osteogenic activity of bone marrow stromal cells (Zhao et al., 2005). AMELX encodes for amelogenin, a structural modeling protein involved in the biomineralization process of amelogenesis (Green et al., 2019). Amelogenesis results in the formation and growth of hydroxyapatite crystals (Guo et al., 2015). Thus, the upregulation of these markers adds to the evidence on the enhancement of these materials of the process of mineralized tissue formation.

An Alizarin Red S staining assay was performed as a complementary measure of the influence of the tested materials on hPDLSC mineralized tissue formation. The significantly higher calcified nodule formation exhibited by ESbcs and AHPbcs-treated cells, compared with the negative and positive controls, provided further support to their biomineralization ability. Similar results have been obtained in previous studies on other calcium silicate cement-based endodontic sealers (Rodríguez-Lozano et al., 2019; Sanz, López-García, et al., 2021). Contrarily, AHP-treated cells showed negative results on this assay, as observed in the aforementioned studies.

Altogether, the results from the cytocompatibility and bioactivity assays point towards the positive influence of ESbcs and AHPbcs on hPDLSC viability, migration, morphology, attachment, differentiation and biomineralization; and the negative influence of AHP on the same parameters. To the authors' knowledge, this is the first study to elucidate the biological properties of AHPbcs in controlled laboratory conditions. Further studies of interest in testing the material's behaviour in animal models or clinical trials.

CONCLUSION

The new calcium silicate cement-based sealer AH Plus Bioceramic Sealer exhibited a significantly higher cytocompatibility and bioactive potential than the epoxy resin-based sealer AH Plus on human periodontal ligament stem cells. The cytocompatibility of AH Plus Bioceramic Sealer was comparable with that of the calcium silicate cement-based sealer Endosequence BC Sealer. Both calcium silicate-based sealers exhibited a significantly higher bioactive potential compared with a negative control group. However, Endosequence BC Sealer exhibited a significantly higher mineralization

potential than AH Plus Bioceramic Sealer and AH Plus. The results from this *in vitro* study act as supporting evidence for the use of AH Plus Bioceramic Sealer in root canal treatment.

AUTHOR CONTRIBUTIONS

Investigation and methodology: Sergio López-García, Francisco Javier Rodríguez Lozano, José Luis Sanz; supervision, visualization, conceptualization and data curation: María Melo, Leopoldo Forner, Carmen Llena; investigation, methodology and writing—original draft: José Luis Sanz, Francisco Javier Rodríguez-Lozano; conceptualization, formal analysis, project administration, supervision, validation and writing—review and editing: Leopoldo Forner, Carmen Llena, María Melo; investigation, methodology, project administration, resources, writing—original draft, and writing—review and editing: José Luis Sanz, Francisco Javier Rodríguez-Lozano, Leopoldo Forner. All authors have read and agreed to the published version of the manuscript.

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CONFILCT OF INTEREST

The authors declare no conflicts of interest related to this study.

ETHICS STATEMENT

The cell extraction protocol was approved by the Human Research Ethics Committee from the University of Murcia (reference: 2199/2018).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Dental stem cell signaling pathway activation in response to hydraulic calcium silicate-based endodontic cements: A systematic review of *in vitro* studies

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ABSTRACT

Objective. To present a qualitative synthesis of *in vitro* studies which analyzed human dental stem cell (DSC) molecular signaling pathway activation in response to hydraulic calcium silicate-based cements (HCSCs).

Methods. A systematic electronic search was performed in Medline, Scopus, Embase, Web of Science and SciELO databases on January 20 and last updated on March 20, 2020. *In vitro* studies assessing the implication of signaling pathways in activity related marker (gene/protein) expression and mineralization induced by HCSCs in contact with human DSCs were included.

Results. The search identified 277 preliminary results. After discarding duplicates, and screening of titles, abstracts, and full texts, 13 articles were considered eligible. All of the materials assessed by the included studies showed positive results in cytocompatibility and/or bioactivity assays. ProRoot MTA and Biodentine were the modal HCSCs studied, hDPSCs were the modal cell variant used, and the most studied signaling pathway was MAPK. *In vitro* assays measuring the expression of activity-related markers and mineralized nodule formation evidenced the involvement of MAPK (and its subfamilies ERK, JNK and P38), NF-κB, Wnt/β-catenin, BMP/Smad and CAMKII pathways in the biological response of DSCs to HCSCs.

Significance. HCSCs considered in the present review elicited a favorable biological response from a variety of DSCs *in vitro*, thus supporting their use in biologically-based endodontic procedures. MAPK, NF-κβ, Wnt/β-catenin, BMP/Smad and CAMKII signaling pathways have been proposed as potential mediators in the biological interaction between DSCs and HCSCs.

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Understanding the signaling processes involved in tissue repair could lead to the development of new biomaterial compositions targeted at enhancing these mechanisms through biologically-based procedures.

Contents

1. Introduction

With the paradigm shift from conventional dental treatment to minimally invasive biologically-based procedures, vital pulp treatment (VPT) and regenerative endodontic treatment (RET) regained interest within the field of endodontics [1,2]. Relying on the reparative potential of the dentin-pulp complex, VPT aims to preserve pulp vitality by providing a bioactive medium that induces a reparatory response from the healthy and reversibly affected tissues [3]; and even from teeth diagnosed with irreversible pulpitis using the current clinical criteria [4]. The placement of biomaterials as pulp capping agents in VPT procedures (*i.e.* indirect and direct pulp capping, and partial or full pulpotomy), such as the well-established calcium hydroxide $(CaOH)_2$, results in the formation of a mineralized dentin barrier within the reversibly affected pulp tissue and the biomaterial itself [5,6].

RET, on the other hand, aims to form a pulp-like tissue by the induction of bleeding into the root canal of immature necrotic teeth, as an alternative to traditional apexification procedures [7]. This biologically-based approach has shown high success rates in terms of symptom resolution and periapical pathosis healing, and, although inconsistent, an increase in root length and thickness by mineralized tissue deposition [8]. The repair or neoformation of tissue observed in both procedures depends on a biological response mediated by dental stem cells (DSCs), a minor population of stem cells with mesenchymal characteristics present in specialized dental tissues [9].

DSCs residing within perivascular niches are recruited and induced to differentiate into odontoblast-like cells after moderate to severe dental trauma or carious lesions. This differentiation is mediated by a cascade of molecular events, in the process of reparative dentinogenesis [10]. The pulp itself acts as a source of DSCs for tissue repair *via* VPT procedures, both in a healthy state (dental pulp stem cells or DPSCs/DPCs) [11] or inflammatory state (inflammatory dental pulp stem cells or iDPSCs) [12]. In the case of RET in immature necrotic teeth, intracanal bleeding and blood clot formation provide an influx of mesenchymal stem cells (MSCs) from local tissues adjacent to the apex [13]. Stem cells are available in various nearby sources, such as the apical papilla (stem cells from the apical papilla or SCAPs) [14], periodontal ligament (periodontal ligament stem cells or PDLSCs) [15], and the inflammatory periapical tissue (inflamed periapical progenitor cells or iPAPs) [16]. Regardless of stem cell source, a bioactive environment can provide a local release of mediators and trigger signaling cascades to regulate differentiation and tissue repair [17].

Hydraulic calcium silicate-based cements (HCSCs), commonly categorized as bioceramic materials, exhibit excellent cytocompatibility [18] and bioactive properties in direct contact with DSCs [19,20], upregulating the expression of osteogenic, odontogenic, cementogenic and/or angiogenic markers, and increasing the production of mineralized deposits. These properties make HCSCs suitable candidates for their use as pulp capping agents in VPT [21] and coronal barriers in RET [22]. However, the mechanisms by which stem cell activity is favored in contact with HCSCs are yet to be fully understood [23].

The mechanism for stem cell differentiation and odontoblast secretory activity during the process of reparative dentinogenesis comprises the activation of a series of signaling transduction pathways [10]. Amongst them, the MAPK family (and its subfamilies p38, ERK and JNK), TFG-β/Smad, NF-κB, Wnt/β-catenin, and P13K/AKT/mTOR pathways seem to act as mediators in this process [24–28]. These pathways function as regulators of DSC activity, by the sequential activation of intermediary protein complexes which culminate with the translocation of transcription factors into the cell nucleus and the consequent activation of specific genes for cell proliferation, differentiation, secretory activity and/or inflammatory response [29].

Recent studies have explored the influence of HCSCs in such pathways [23], to anticipate their response in biologically based endodontic procedures. Within this field of study, the present systematic review aimed to present a qualitative synthesis of available literature consisting of *in vitro* assays which analyzed human dental stem cell molecular signaling pathway activation in response to calcium silicate-based cements.

2. Method

The present work followed the guidelines recommended by the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement [30]. Data were reported by following the structure and content dictated by the 27 items included in the statement (Supplementary Table 1). The systematic review protocol was previously registered in Open Science Framework (OSF) Registries (DOI: 10.17605/OSF.IO/D2GB8).

2.1. *Eligibility criteria*

In vitro studies assessing the implication of one or more signaling pathways in activity-related gene/protein expression and mineralization induced by HCSCs in contact with human DSCs were included. Assays comparing the aforementioned variables both between HCSCs and/or a control, and between a HCSC with or without an additive were accepted. Studies assessing only one HCSC were also accepted. Regarding stem cells, studies using any of the formerly identified and characterized dental mesenchymal stem cells [31] from human origin were eligible.

These criteria were based on the PICOS framework [32], as follows: Population/problem (P): human dental stem cells; intervention (I): culture medium conditioned with calcium silicate-based cements; comparison/control (C): unconditioned culture medium; outcome (O): signaling-pathway activation; study design (S): *in vitro* studies.

2.2. *Search strategy*

The search process, study selection, data extraction, and quality assessment were carried out by two independent examiners (J.L.S. and L.F.). In the event of any discrepancy between them, a third author was consulted (C.L).

A systematic electronic search was performed in Medline, Scopus, Embase, Web of Science and SciELO databases on January 20 and last updated on March 20, 2020, without any language or year restrictions. The search strategy was designed considering previous studies in the field and their most cited descriptors. Accordingly, the following terms were combined for each database: 'Silicate', 'bioceramic', 'molecular', 'signaling', 'pathway', 'mesenchymal stem cells' and 'dental stem cells.' Boolean operators 'AND' and 'OR' were used to annex the terms and develop the search strategy, as shown in Table 1. Additionally, the references of the resulting studies were screened to search for potentially eligible studies that did not appear in the preliminary database search.

2.3. *Study selection*

References identified using the search strategy were exported from each database into Mendeley reference manager software (Elsevier, Amsterdam, Netherlands) to check for duplicates. After discarding duplicates, a preliminary screening of record titles and abstracts was carried out according to the previously established inclusion and exclusion criteria. Studies which met the criteria were further assessed for eligibility by full-text screening.

2.4. *Data extraction*

Data synthesis from the included studies was divided into variables for study characteristics, methodology and results/outcomes. The variables recorded for study characteristics were: authors and year of publication. Methodological variables included: the stem cell variants used, HCSCs used and their concentration, the signaling pathways studied, the activity analyses performed and their duration, and the pathway-specific markers and/or inhibitors used for such analyses. Outcome variables included: the significant results found, the time at which they were recorded (duration), and their significance level. Assay-specific variables were also recorded, as follows: pathway-specific marker expression, pathway-specific inhibitor concentration and exposure time, and dental stem cell activity-related marker expression.

2.5. *Quality assessment*

Studies included in the present review were independently evaluated for inner methodological risk of bias using the 'Modified CONSORT checklist of items for reporting *in vitro* studies of dental materials' [33], assessing the fulfillment for each of the parameters or items considered in the checklist. Once recorded, the compliance percentage of each of the studies was calculated.

3. Results

3.1. *Search results and study selection*

The search identified 277 preliminary results, of which 89 were found in Medline, 87 in Scopus, 13 in Embase, and 88 in Web of Science. The search performed in SciELO database

Table 1 – Search strategy and findings per database.

Database	Search strategy	Findings
Medline	#1 (silicate) OR bioceramic	41,998
	#2 ((molecular) OR signaling) OR pathway	4,001,118
	#3 ((mesenchymal stem cells[MeSH Terms]) OR mesenchymal stem cells) OR dental stem cells	66,778
	#1 AND #2 AND #3	**89**
Scopus	#1 TITLE-ABS-KEY (silicate) OR TITLE-ABS-KEY (bioceramic)	173,871
	#2 TITLE-ABS-KEY (molecular) OR TITLE-ABS-KEY (signaling) OR TITLE-ABS-KEY (pathway)	5,421,413
	#3 TITLE-ABS-KEY (mesenchymal AND stem AND cells) OR TITLE-ABS-KEY (dental AND stem AND cells)	84,091
	#1 AND #2 AND #3	**87**
Embase	#1 silicate:ab,ti OR bioceramic:ab,ti	9,464
	#2 molecular:ab,ti OR signaling:ab,ti OR pathway:ab,ti	2,680,546
	#3 'mesenchymal stem cells':ab,ti OR 'dental stem cells':ab,ti	54,590
	#1 AND #2 AND #3	**13**
Web of Science	#1 TS=(silicate OR bioceramic)	106,934
	#2 TS=(molecular OR signaling OR pathway)	5,401,723
	#3 TS=(mesenchymal stem cells OR dental stem cells)	94,869
	#1 AND #2 AND #3	**88**
SciELO	#1 silicate OR bioceramic	634
	#2 molecular OR signaling OR pathway	19,129
	#3 mesenchymal stem cells OR dental stem cells	326
	#1 AND #2 AND #3	**0**

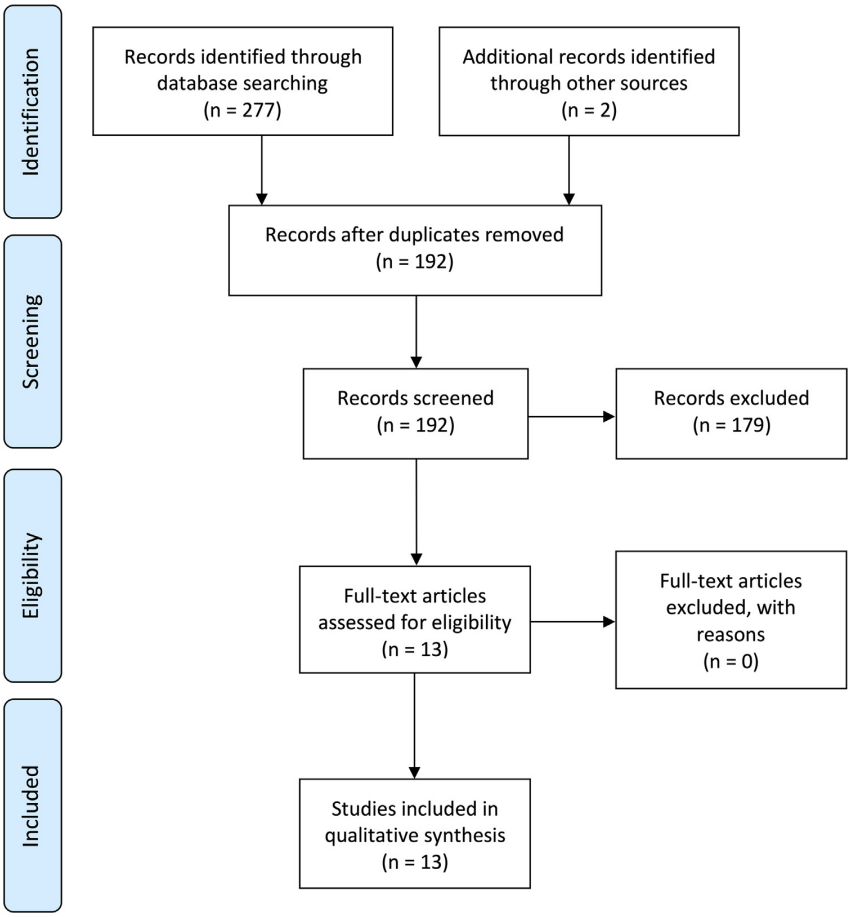

Fig. 1 – Systematic flow chart representing study inclusion. Based on the PRISMA flow diagram [30].

yielded no results. 2 additional eligible studies were found upon screening the references of the resulting studies. Duplicates were removed manually using the reference manager software, resulting in 192 records. Of these, 179 were excluded upon reading the title and abstract. The resulting 13 articles were assessed by full-text screening, and all of them were considered eligible for qualitative synthesis (Fig. 1).

3.2. *Study methodology*

Supplementary Table 2 (ST2) summarizes the methodology used by the included studies to assess signaling-pathway implication in activity-related gene/protein expression and mineralization induced by HCSCs in contact with DSCs.

Regarding the stem cells studied, DPSCs were the modal variant, used in 8 studies [34–41]. PDLSCs were used by 2 studies [42,43], as well as SCAPs [44,45], and iDPSCs were studied once [46]. All of the studies specified that the DSCs for testing were obtained from healthy donors. No specification was made regarding the donors' age, sex, o socio-demographic characteristics.

With reference to commercially-available HCSCs used, ProRoot MTA -PR MTA- (Dentsply Tulsa Dental, Tulsa, OK, USA) was the most studied, being used by 8 studies [34,38–42,44,46]. Biodentine -BD- (Septodont, Saint Maurdes-Fosses, France) was studied twice [34,38], and both Bioaggregate -BA- (Innovative Bioceramix, Vancouver, BC, Canada) and iRoot Fast Set root repair material -iRoot FS- (Dentsply Tulsa Dental, Tulsa, OK, USA) were only studied once [38,45]. The remaining studies synthesized and tested experimental calcium-silicate based compositions: $Li_2Ca_4Si_4O_{13}$ (LCS) [43], $Ca_7Si_2P_2O_{16}$ (CSP) [37], and tricalcium silicate (C3S) [35]. In addition, two additives were tested along with PR MTA: Propolis (Damyang Agriculture Technology Center, Damyang, Korea) [41], and platelet-rich fibrin -PRF- (Chonnam National University Dental Hospital, Gwangju, South Korea) [40].

As to signaling pathways, the majority of studies explored the involvement of the MAPK pathway and its subfamilies (ERK, JNK and P38) in DSC response to HCSCs. 4 studies assessed the involvement of the 3 subfamilies [34,36,38,42], while another 3 studied the ERK subfamily individually [35,41,43]. P38 was assessed individually by one study [37]. The NF-κB pathway [36,42,44,46] was assessed in 4 studies, and the Wnt/β-catenin pathway in 3 studies [39,43,45]. Lastly, the BMP/Smad pathway was assessed twice [40,45], and CaMKII signaling once [36].

In order to evaluate the influence of HCSCs on DSC signaling pathway activation, studies could follow various routes (Fig. 2):

- Quantification of pathway-specific gene expression using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and/or protein expression using western blot (Fig. 2A) [34,35,46,47,37–42,44,45].
- Quantification of activity-related gene expression using qRT-PCR and/or protein expression using western blot, after exposure to a pathway-specific inhibitor (Fig. 2B) [34,35,46,36,37,39,40,42–45].
- Quantification of mineralized nodule formation using alizarin red staining (ARS), after exposure to a pathway-specific inhibitor (Fig. 2C) [36,38–41,45,46].
- Quantification of alkaline phosphatase (ALP) activity by means of ALP activity assay kits (ALPa), or ALP staining (ALPs) to evaluate ALP deposition, after exposure to a pathway-specific inhibitor (Fig. 2D) [37,40,43,44,46].

Assays covered in Fig. 2A involved the use of pathway-specific genes and/or proteins, and those illustrated in Fig. 2B–D required the use of a pathway-specific inhibitor. To assess the involvement of the MAPK pathway, studies could target ERK, JNK and/or P38 subfamilies. For the ERK pathway, target genes used were MMP2, FN [43], and Col-1 [35,43]; and target proteins were ERK and p-ERK [34,35,38,41–43]. All of the studies assessing the ERK pathway used U0126 as an inhibitor, except for one study, which used SCH772984 [43]. For the JNK pathway, target proteins used were JNK and p-JNK [34,38,42].

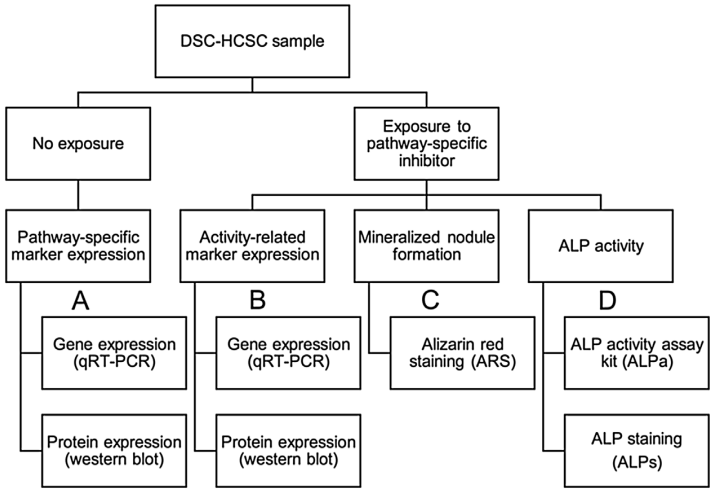

Fig. 2 – Schematic illustration representing study methodology to determine signaling pathway involvement in DSC response to HCSCs.

All of the studies assessing the JNK pathway used SP600125 as an inhibitor. For the P38 pathway, target proteins used were P38, p-P38 [34,38,42], and MEPE [37]. All of the studies assessing the P38 pathway used SB203580 as an inhibitor.

Wnt/β-catenin pathway target genes used were Wnt3a, β-catenin [39,43], and Axin2 [39]. Target proteins used were nuclear β-catenin and total β-catenin [45]. Inhibitors used varied from ICG-001 [43], XAV-939 [45], and cardamonin [39].

As for the NF-κB pathway, target proteins used included p-P65, IκBα, p-IκBα, nuclear P65 [42,44,46] and P65 [42,46]. Three studies used BMS345541 [42,44,46] as an inhibitor, and the remaining one used PDTC [36].

Target proteins used for the BMP/Smad pathway were p-Smad1/5/8 [40,45], BMP2/4, and Smad1 [40]. LDN193189 was used as an inhibitor for this pathway [40]. The study assessing CAMKII pathway used KN-93 as an inhibitor [36].

For the assays illustrated in Fig. 2B, studies quantified the expression of DSC activity-related markers, consisting of a series of osteogenic, dentinogenic, cementogenic and angiogenic genes and proteins. The following markers were used:

- DSPP (Dentin Sialophosphoprotein) [34–37,39,40,42,44–46];
- OCN (Osteocalcin) [34–36,42,44,46];
- ALP [34,39,43–45];
- DMP1 (Dentin Matrix Acidic Phosphoprotein 1) [36,37,40,42];
- Col-1 (Collagen type 1) [34,35,42,43];
- OPN (Osteopontin) [35,37,43];
- BSP (Bone Sialoprotein) [34,36,44];
- OSX (Osterix) [44,46];
- CAP (Cementum Attachment Protein), CEMP-1 (Cementum Protein 1), BMP2 (Bone Morphogenetic Protein 2), PDGF (Platelet-Derived Growth Factor), EGF (Epidermal Growth Factor) [43].

3.3. *Study results*

Table 2 summarizes the results of the included studies which produced significant differences for signaling pathway-specific marker expression. Data are presented as significant differences shown between a specific concentration of a HCSC and a control (unconditioned medium) or a HCSC with an additive, for DSC expression of one or more pathway-specific genes or proteins at a given duration of culture, and their significance level.

Table 3 presents a summary of the results of the included studies showing a significant downregulation of DSC activity-related marker expression in the presence of signaling pathway-specific inhibitors. Data is presented in a similar manner as Table 2, adding the concentration of inhibitor used and the duration of its exposure to the DSC-HCSC samples.

Table 4 outlines the results of included studies reporting a significantly lower mineralized nodule formation from the DSC-HCSC samples in the presence of a signaling pathway-specific inhibitor, using Alizarin Red staining.

Table 5 highlights the results of included studies which showed a significantly lower ALP activity from the DSC-HCSC samples in the presence of a signaling pathway-specific inhibitor, using ALP activity assays or ALP staining.

3.4. *Quality assessment*

The results of the evaluation of the quality of the evidence using the modified CONSORT checklist are presented in Table 6. The mean compliance of the included studies was 56%, with a maximum score of 64%, and a minimum score of 50%. Parameters 5–9 and 14 were not fulfilled by any of the studies. Parameters 2a, 3, 4, 10, 11 and 13 were fulfilled by all of the studies.

4. Discussion

The application of HCSCs in contemporary endodontic procedures is supported by their established bioactive properties. By the release of their major cationic components, they promote an interaction with the mineralized constituent of dentinal tissue, thereby forming a mineral attachment of hydroxyapatite-like composition [48]. Physiochemical reactions occurring between HCSCs and tissue fluids during this process of biomineralization are responsible for other significant properties, namely their biocompatibility and induction of osteo-dentinogenic activity [49,50]. To further assess this interaction, studies considered in the present review explored the implication of various signaling pathways in the increased activity and mineralized nodule formation shown by DSCs in contact with HCSCs.

To evaluate the biological response of DSCs to this group of biomaterials *in vitro*, studies can perform tests for cytocompatibility (cell viability, migration, and proliferation assays) and bioactivity (quantification of activity-related gene/protein expression using qRT-PCR/western blot, ALP activity assays, and ARS to assess mineralized nodule formation); as shown in previous systematic reviews performed by our research group [51,52]. All of the studies included in the present review carried out such tests in order to confirm the biological effects of the examined material/s towards DSCs from healthy donors prior to the assessment of the mechanism involved in the response. The analysis of such biological effects in DSCs from donors with different characteristics (*i.e.* underlying pathologies, varying ages...) could act as a relevant research topic for future studies in the field of stem cell therapy and dental biomaterials.

The same methods used to evaluate bioactivity could then be performed after treatment of the samples with a pathway-specific inhibitor. A reduction in activity-related gene expression, ALP activity and/or mineralized nodule formation after treatment with a pathway-specific inhibitor would indicate that the examined signaling pathway is involved in such biological responses. Interestingly, the majority of studies even measured the expression of pathway-specific genes/proteins after the treatment with pathway-specific inhibitors as a control [34,35,37,39–43,45], since they would evidently be downregulated if the inhibitor functioned correctly.

The upregulation of pathway-specific genes/proteins in the absence of pathway-specific inhibitors could be another way of confirming the involvement of a signaling pathway in the observed biological effects. To test this, the majority of the studies included in the review measured the difference

Table 2 – Signaling pathway-specific gene/protein expression.

Author	Cell variant	Analysis	Pathway	Significant results	Pathway marker	Duration	P value
Kim et al. [41]	hDPSCs	Western blot	ERK	PR MTA (1 mg/mL) + Propolis (10, 50 ng/mL) > PR MTA (1 mg/mL)	ERK; p-ERK	2d	P < 0.05
Zhang et al. [43]	hPDLSCs	qRT-PCR	Wnt/β-catenin	LCS (25, 37.5, 50 mg/mL) > control	Wnt3a; β-catenin	7d	P < 0.05
				LCS (6.25, 12.5, 25, 37.5, 50 mg/mL) > control	Wnt3a; β-catenin	14d	P < 0.05
			ERK	LCS (25, 37.5, 50 mg/mL) > control	MMP2; FN; Col-1	7d	P < 0.05
				LCS (12.5, 25, 37.5, 50 mg/mL) > control	MMP2	14d	P < 0.05
		Western blot	Wnt/β-catenin	LCS (25, 37.5, 50 mg/mL) > control	FN; Col-1	14d	P < 0.05
				LCS (25, 37.5, 50 mg/mL) > control	Wnt3a	7d	P<0.05
			ERK	LCS (12.5, 25, 37.5, 50 mg/mL) > control	Col-1	7d	P < 0.05
				LCS (12.5, 25 mg/mL) > control	p-ERK	7d	P < 0.05
Woo et al. [40]	hDPSCs	Western blot	BMP/Smad	PR MTA (1 mg/mL) + PRF (1.25%) > PRF (1.25%)	BMP2/4	2d	P < 0.001
					p-Smad1/5/8	2d	P < 0.05
Chen et al. [39]	hDPCs	qRT-PCR	Wnt/β-catenin	PR MTA (6.25, 12.5, 25, 50, 100 mg/mL) > control	Wnt3a; β-catenin	3, 7d	P < 0.05
					Axin2	7d	P < 0.05
				PR MTA (12.5, 25, 50, 100 mg/mL) > control	Axin2	3d	P < 0.05

d: days; n/s: not specified; >: significantly superior expression.

between the expression of pathway-specific genes/proteins in DSC samples with or without HSCSs (control), at a given duration of culture [35–37,39,43,45]. Studies assessing the influence of an additive [40,41] on the activity induced by HCSCs, also analyzed the differences between the treatment and control groups at a given duration. However, various studies reported results as time-dependent changes in pathway-specific marker expression [34,38,42,44,46], comparing the difference in the expression of markers and their phosphorylated counterparts between a series of time points. This provides a dynamic representation of marker phosphorylation, and therefore, signaling pathway activity, over the period of the culture of DSCs with HCSCs.

All of the materials assessed by the studies included in the present review showed positive results in the preliminary cytocompatibility and/or bioactivity assays, in accordance with previous studies in the field [20,53–57]. Studies then proceeded to evaluate the implication of signaling pathways on these results. Due to the heterogeneity of the experimental designs and the range of biomaterials, stem cell variants and pathways studied, we believed convenient to present a qualitative summary and an illustrative support (Figs. 34) of the significant results extracted from the included studies; in consonance with a similar review carried out within this field of study [23]. The aforementioned review aimed to present a qualitative analysis of studies which assessed the activity-related gene expression of various cells in response to tricalcium silicate cements. The present review included such analysis (shown in Fig. 2A) to assess the biological interaction between DSCs and HCSCs, along with an analysis of signaling pathway involvement (shown in Fig. 2B–D).

The most studied material (PR MTA), in direct contact with DPSCs/DPCs, produced significant positive results in assays assessing the involvement of the MAPK subfamilies JNK and P38 at a concentration of 10 mg/mL [38], and the ERK subfamily at 0.2 mg/mL [34], and 10 mg/mL [38]. The same happened with the NF-κB pathway at 2 mg/mL [42] and Wnt/β-catenin at 6.25, 12.5, 25, 50 and 100 mg/mL [39]. In contact with PR MTA, PDLSCs exhibited implication of JNK, P38, ERK and NF-κB pathways at a concentration of 2 mg/mL [42]. The NF-κB pathway was also involved in the response shown by PR MTA-treated SCAPs and iDPSCs at a concentration of 2 mg/mL [44] and 0.2 mg/mL [46], respectively. BD was studied twice, and showed significant positive results for the involvement of MAPK subfamilies ERK and JNK in the biological response shown by DPSCs, at a concentration of 0.2 mg/mL [36]. The MAPK pathway was also involved in the response shown by DPSCs for both BD and BA at 10 mg/mL [38]. The remaining commercially-available biomaterial studied, iRoot FS, was tested with SCAPs and produced significant positive results for Wnt/β-catenin implication at a concentration of 2 mg/mL [45]. From the experimental calcium silicate-based compositions studied, C3S showed an involvement of the ERK pathway at a concentration of 5 mg/mL when cultured with DPCs [35], whereas LCS exhibited an implication of the ERK and Wnt/β-catenin pathways at 25 and 50 mg/mL with PDLSCs [43]. Since the majority of studies analyzed the samples at individual durations of culture, conclusions about the time-dependence of the activation of signaling pathways shown are challenging to establish. Similarly, evidence was insufficient to provide an insight into the dose-dependence of the biological effects shown by each of the HCSCs.

Regarding the use of additives together with HCSCs, PR MTA produced significantly superior results in signaling pathway activation assays when combined with propolis and platelet-rich fibrin: at a concentration of 1 mg/mL, PR MTA showed a significantly higher expression of ERK-specific markers together with 10 and 50 ng/mL of propolis [41], and BMP/Smad-specific markers together with 1,25% platelet-rich fibrin [42]. The combination of additives with bioceramic materials appears to be a promising line of research within this field, having shown positive results in terms of the biological response from dental stem cells in various studies. For example, the incorporation of additives like gold [58], iron oxide [59], and bioactive glass [60] nanoparticles to calcium phosphate cement has shown positive biological responses from

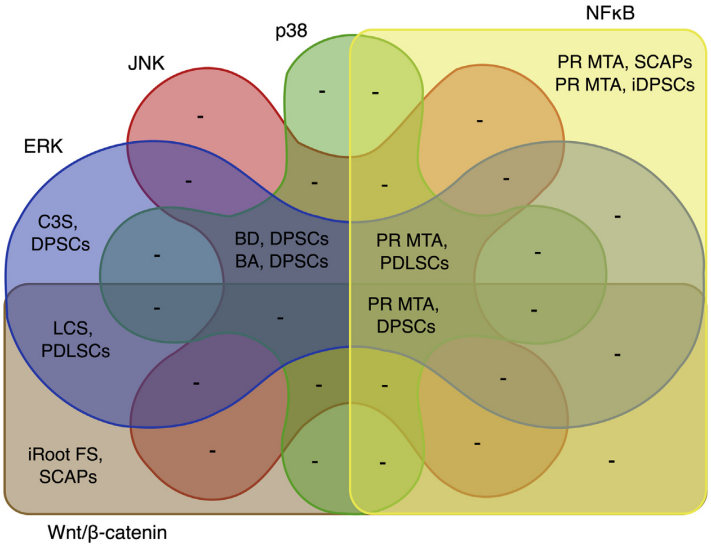

Fig. 3 – Venn diagram representing signaling pathway involvement in the biological response shown by DSCs in contact with HCSCs (based on the Venn diagram software from Bioinformatics & Evolutionary Genomics (BEG), VIB/UGENT, Belgium). Each colored set corresponds to a signaling pathway (yellow: NFκB, green: p38, red: JNK, blue: ERK, brown: Wnt/β-catenin). HCSCs and DSCs presented within one or more colored sets showed significant positive results for the corresponding signaling pathway/s in *in vitro* assays assessing their involvement i.e. NFκB, ERK, JNK, p38 and Wnt/β-catenin pathways were involved in the biological response shown by PDLSCs in contact with PR MTA. Signaling pathways which were only assessed by an individual study (BMP/Smad, CAMKII) are not included.

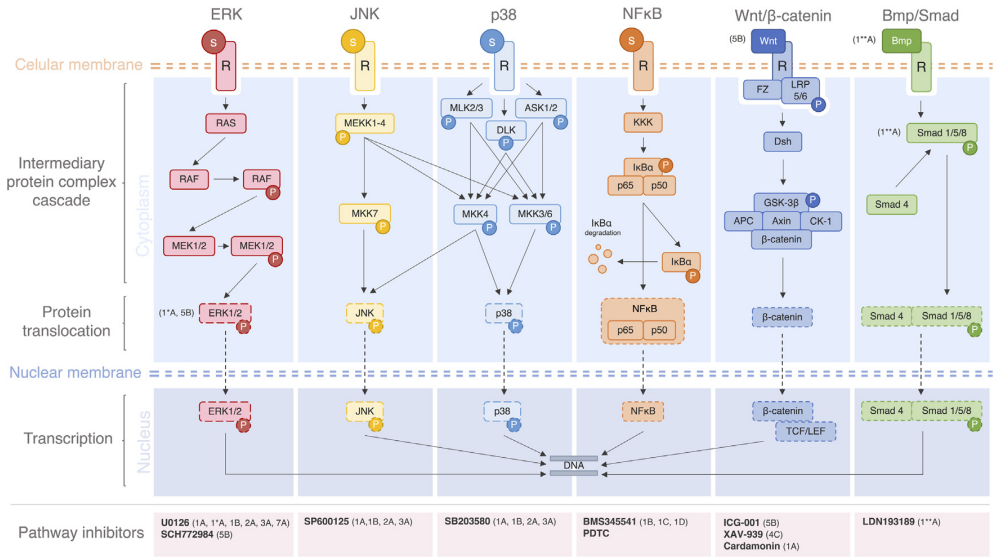

Fig. 4 – Schematic simplification of the main signaling pathways considered in the present review (based on the works by Simon et al. [24], Yoshioka et al. [25], Chang et al. [26], Shin et al. [27], Zhang et al. [28] and Da Rosa et al. [29]), their inhibitors and their involvement in DSC-HCSCs biological activity. HCSCs -represented by numbers- and DSCs -represented by capital letters- appear within brackets next to the signaling proteins which were significantly upregulated in DSC-HCSC samples when compared to a control, and the pathway-specific inhibitors which induced a significant reduction in the activity, differentiation and/or mineralization exhibited by DSC-HCSC samples. S: signaling molecule; R: receptor; P: phosphorylated. HCSCs (1: PR MTA; 1*: PR MTA + Propolis; 1: PR MTA + PRF, 2: BD; 3: BA; 4: iRoot FS; 5: LCS; 6: CSP; 7: CS3). DSCs: (A: DPSCs, B: PDLSCs; C: SCAPs; D: iDPSCs).**

Table 3 – Downregulation of activity-related gene/protein expression in the presence of signaling pathway-specific inhibitors.

Author	Cell variant	Analysis	Pathway	Inhibitor (concentration; exposure time)	Materials (concentration)	Activity marker	Duration	P value
Zhang et al. [43]	hPDLSCs	qRT-PCR	Wnt/β-catenin	ICG-001 (n/s)	LCS (25, 50 mg/mL)	OPN; ALP; CAP; Runx2; CEMP1; MMP2	7d	P < 0.05
			ERK	SCH772984 (n/s)	LCS (25, 50 mg/mL)	MMP2; FN; Col-1; PDGF; EGF; Runx2	7d	P < 0.05
		Western blot	Wnt/β-catenin	ICG-001 (n/s)	LCS (25, 50 mg/mL)	ALP; OPN; BMP2; Runx2	7d	P < 0.05
			ERK	SCH772984 (n/s)	LCS (25, 50 mg/mL)	p-P65; MMP2; p-ERK	7d	P < 0.05
Liu et al. [45]	hSCAPs	qRT-PCR	Wnt/β-catenin	XAV939 (20 μM; 24 h)	iRoot FS (2 mg/mL)	DSPP	6d	P < 0.005
						ALP	6d	P < 0.01
Wang et al. [42]	hPDLSCs	qRT-PCR	ERK	U0126 (10 μM; 7d)	PR MTA (2 mg/mL)	OCN, DMP1, DSPP, Runx2, OSX, Col-1	7d	P < 0.01
			JNK	SP600125 (10 μM; 7d)	PR MTA (2 mg/mL)	DMP1, DSPP, OSX, Col-1	7d	P < 0.01
						OCN, Runx2	7d	P < 0.05
			P38	SB203580 (10 μM; 7d)	PR MTA (2 mg/mL)	DMP1, DSPP, Col-1	7d	P < 0.01
						Runx2	7d	P < 0.05
			NF-κB	Bms345541 (10 μM; 7d)	PR MTA (2 mg/mL)	OCN, DMP1, DSPP	7d	P < 0.01
						Runx2, OSX, Col-1	7d	P < 0.05
Woo et al. [40]	hDPSCs	Western blot	BMP/Smad	LDN193189 (0.1 μmol/L; 1 h)	PR MTA (1 mg/mL) + PRF (1.25%)	DSPP	2d	P < 0.001
						DMP1	2d	P < 0.05
Chen et al. [39]	hDPCs	qRT-PCR	Wnt/β-catenin	Cardamonin (10 mmol/L; 7d)	PR MTA (12.5 mg/mL; 50 mg/mL)	ALP, DSPP, DMP-1	7d	P < 0.05
Luo et al. [36]	hDPSCs	qRT-PCR	CaMKII	KN-93 (1 μmol/L; 14d)	BD (0.2 mg/mL)	OCN, DSPP, CMP1, BSP	14d	P < 0.05
			ERK	U0126 (25 μmol/L; 14d)	BD (0.2 mg/mL)	OCN, DSPP, CMP1, BSP	14d	P < 0.05
			JNK	SP600125 (25 μmol/L; 14d)	BD (0.2 mg/mL)	OCN, DSPP, CMP1, BSP	14d	P < 0.05
Yan et al. [44]	hSCAPs	qRT-PCR	NF-κB	Bms345541 (n/s)	PR MTA (2 mg/mL)	ALP, DSPP, Runx2, OSX, OCN, BSP, TNFα, IL1α, IL1β, IL6	3d	P < 0.05
Wang et al. [46]	iDPSCs	qRT-PCR	NF-κB	Bms345541 (n/s)	PR MTA (0.2 mg/mL)	OSX, OCN, DSPP	3d	P < 0.01
Du et al. [35]	hDPCs	qRT-PCR	ERK	U0126 (20 μmol/L; 2 h)	C3S (5 mg/mL)	DSPP, OPN, Col-1	3d, 7d	P < 0.05
						OCN	7d	P < 0.05
Zhao et al. [34]	hDPSCs	qRT-PCR	ERK	U0126 (10 μM; 1 h)	PR MTA (0.2 mg/mL)	ALP, DSPP, Col-1, OCN, BSP	12 h	P < 0.05

h: hours; d: days; n/s: not specified.

human dental pulp stem cells *in vitro*. The identification of the potential signaling pathways implicated in such biological responses can provide an insight as to how additives promote the biological interactions of bioceramic materials with dental stem cells [41,42].

Quality assessment of the included studies was carried out using a modified CONSORT checklist [33], in the absence of a specific guide to evaluate *in vitro* studies of dental materials. It may be worth mentioning for future systematic reviews in the field, that a CRIS (checklist for reporting *in vitro* studies) statement is being developed [61] to allow for a uniform methodology in the evaluation of this kind of studies. The general pattern of the study sample was to report a structured abstract, clear aims or hypotheses, a detailed description of the methodology to allow for replication, and relevant conclusions. However, authors often failed to address the potential limitations of their studies in the discussion section.

Considering the mean compliance of the studies to the quality assessment parameters and the *in vitro* nature of the assays carried out, the results of the studies and, consequently, of the present review, need to be interpreted with caution; being far from applicable at a clinical level. This acts as the main limitation of this review. As an additional limitation, the methodological heterogeneity both in terms of the assays carried out and the outcomes measured by the studies, added to the small sample of available studies considered in the present review; make preforming a quantitative analysis or meta-analysis impracticable. Consequently, the results from the biological response of DSCs cultured in a HCSC-conditioned medium may provide a preliminary notion as to how such materials and cells would interact in a clinical environment, in which they would coexist with a series of factors which could alter the behavior expressed at an *in vitro* level.

The identification of the potential role of the markers expressed and signaling pathways activated by DSCs in tissue

Table 4 – Reduced mineralized nodule formation in the presence of signaling pathway-specific inhibitors.

Author	Cell variant	Pathway	Inhibitor (concentration; exposure time)	Materials (concentration)	Duration	P value
Kim et al. [41]	hDPSCs	ERK	U0126 (0.1 μM; 1 h)	PR MTA (1 mg/mL) + Propolis (50 ng/mL)	14d	P < 0.05
			U0126 (1 μM; 1 h)	PR MTA (1 mg/mL) + Propolis (50 ng/mL)	14d	P < 0.05
Liu et al. [45]	hSCAPs	Wnt/β-catenin	XAV939 (20 μM; 24 h)	iRoot FS (2 mg/mL)	4w	P < 0.01
Woo et al. [40]	hDPSCs	BMP/Smad	LDN 193189 (0.1 μmol/L; 1 h)	PR MTA (1 mg/mL) + PRF (1,25%)	7d	P < 0.05
Jung et al. [38]	hDPCs	ERK	U0126 (10 μM; 1 h)	PR MTA (10 mg/mL), BD (10 mg/mL), BA (10 mg/mL)	14d	P < 0.01
		JNK	SP600125 (10 μM; 1 h)	PR MTA (10 mg/mL), BD (10 mg/mL), BA (10 mg/mL)	14d	P < 0.01
		P38	SB203580 (10 μM; 1 h)	PR MTA (10 mg/mL), BD (10 mg/mL), BA (10 mg/mL)	14d	P < 0.01
Luo et al. [36]	hDPSCs	ERK	U0126 (25 μmol/L; 14d)	BD (0.2 mg/mL)	14d	P < 0.05
		JNK	SP600125 (25 μmol/L; 14d)	BD (0.2 mg/mL)	14d	P < 0.05
		CaMKII	KN-93 (1 μmol/L: 14d)	BD (0.2 mg/mL)	14d	P < 0.05

h: hours; d: days; w: weeks.

Table 5 – Reduced ALP activity in the presence of signaling pathway-specific inhibitors.

Author	Cell variant	Pathway	Inhibitor (concentration; exposure time)	Materials (concentration)	Duration	Significance level
Zhang et al. [43]	hPDLSCs	Wnt/β-catenin	ICG-001 (n/s)	LCS (12.5, 25 mg/mL)	14d	P < 0.05
Woo et al. [40]	hDPSCs	BMP/Smad	LDN 193189 (100 nmol/L; 1 h)	PR MTA (1 mg/mL) + PRF (1,25%)	7d	P < 0.001
Yan et al. [44]	hSCAPs	NF-κB	Bms345541 (n/s)	PR MTA (2 mg/mL)	3d	P < 0.01
Wang et al. [46]	iDPSCs	NF-κB	Bms345541 (n/s)	PR MTA (0.2 mg/mL)	3, 5d	P < 0.01

h: hours; d: days; n/s: not specified.

Table 6 – Quality assessment results.

Author	Modified CONSORT checklist															
	1	2a	2b	3	4	5	6	7	8	9	10	11	12	13	14	%
Kim et al. [41]	N	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	50
Zhang et al. [43]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Liu et al. [45]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	Y	Y	N	64
Wang et al. [42]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Woo et al. [40]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Chen et al. [39]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Jung et al. [38]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Zhang et al. [37]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Luo et al. [36]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Yan et al. [44]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Wang et al. [46]	Y	Y	Y	N	Y	N	N	N	N	N	Y	Y	N	Y	N	50
Du et al. [35]	Y	Y	Y	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	57
Zhao et al. [34]	Y	Y	N	Y	Y	N	N	N	N	N	Y	Y	N	Y	N	50

Y: reported on the article; N: not reported on the article; %: percentage of compliance per article. Based on the checklist from 'Guidelines for Reporting Pre-clinical *in vitro* Studies on Dental Materials' [33].

repair is beyond the scope of this review, and has been previously reported by various published works [10,23,29]. Studies included in the present review limited to the presentation of possible signaling pathways implicated in the biological response of DSCs to HCSCs. However, one study suggested that the calcium ions released by HCSCs could be responsible for the triggering of the phosphorylation of specific markers and the consequent activation of signaling transduction pathways [42]. Available literature also leads towards the possible solubilization and release of a reservoir of growth factors from dentinal tissue induced by HCSCs, which can contribute to the proliferation and chemotaxis of stem cells, and functional repair [5,6,62,63].

Most recently, the role of micro RNAs, a small group of non-coding RNA molecules, on DSC differentiation and signaling pathway activation has been explored [47,64,65], and could act as a possible line of research regarding the interaction of bioceramic materials and DSCs. Similar to HCSCs, calcium

silicate-based endodontic sealers (CSSs) have been subject of investigation within the field of dental stem cell research. Clinically, CSSs used in root canal treatment can extrude from the root canal system into the periapex and remain in contiguity with various cell populations. This group of biomaterials have also shown bioactive properties *in vitro* [66,67], although currently evidence is much more limited.

Nonetheless, the underlying mechanisms by which silicate-based biomaterials interact with DSCs and the dentin-pulp complex *in vivo* are still far from being fully understood and remain as a subject for future research. The authors hope that present systematic review provides an updated and structured qualitative analysis of the current state of the investigations regarding the mechanisms involved in the biological interaction of HCSCs and dental stem cells, which can serve as a reference for future studies in the field of dental biomaterial research. Mapping the cascade of molecular events and understanding the signaling processes involved in tissue repair could lead to the development of new biomaterial compositions targeted at enhancing these mechanisms through through biologically-based therapeutic procedures in the clinical setting. Regarding HCSCs specifically, new compositions with enhanced biological properties could result in an increased predictability in vital pulp therapy and regenerative endodontic procedures.

5. Conclusions

Calcium silicate cements considered in the present review elicited a favorable biological response from a variety of dental stem cells *in vitro*, thus supporting their use in biologically-based endodontic procedures. The results from *in vitro* assays measuring the expression of activity-related markers and mineralized nodule formation suggest an active role of MAPK, NF-κB, Wnt/β-catenin, BMP/Smad and CAMKII signaling pathways as potential mediators in the biological interaction between DSCs and HCSCs. The mechanisms by which HCSCs induce this response remain unclear.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dental.2021.01.025.

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