VNIVERSITAT DE VALÈNCIA

FACULTAT DE MEDICINA I ODONTOLOGIA

TESIS DOCTORAL

BIOCOMPATIBILIDAD Y BIOACTIVIDAD DE NUEVOS MATERIALES PARA ODONTOLOGÍA REGENERATIVA

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PROGRAMA DE DOCTORADO
3139 MEDICINA

Valencia, septiembre 2021

VNIVERSITAT DE VALÈNCIA Facultat de Medicina i Odontologia

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hacen constar que,

la tesis doctoral titulada "Biocompatibilidad y bioactividad de nuevos materiales para Odontología Regenerativa", presentada por el doctorando D. Francisco Javier Rodríguez Lozano 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 | Profª Drª López Ginés

Valencia, 27 de septiembre de 2021.

Esta memoria ha sido concebida como un compendio de las **cinco** publicaciones siguientes:

- **Rodríguez-Lozano, F.J.;** López-García, S.; García-Bernal, D.; Pecci-Lloret, M.R.; Guerrero-Gironés, J.; Pecci-Lloret, M.P.; Lozano, A.; Llena, C.; Spagnuolo, G.; Forner, L. In Vitro Effect of Putty Calcium Silicate Materials on Human Periodontal Ligament Stem Cells. *Appl. Sci.* **2020**, *10*, 325.
- **Rodríguez-Lozano, F.J.;** Oñate-Sánchez, R.; Gonzálvez-García, M.; Vallés-Bergadá, M.; Martínez, C.M.; Revilla-Nuin, B.; Guerrero-Gironés, J.; Moraleda, J.M.; García-Bernal, D. Allogeneic Bone Marrow Mesenchymal Stem Cell Transplantation in Tooth Extractions Sites Ameliorates the Incidence of Osteonecrotic Jaw-Like Lesions in Zoledronic Acid-Treated Rats. *J. Clin. Med.* **2020**, *9*, 1649.
- **Rodríguez-Lozano, F. J.;** López-García, S.; García-Bernal, D.; Tomás-Catalá, C. J.; Santos, J. M.; Llena, C.; Lozano, A.; Murcia, L.; & Forner, L. Chemical composition and bioactivity potential of the new Endosequence BC Sealer formulation HiFlow. *Int. Endod. J.* **2020,** *53*(9), 1216–1228.
- **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. Cytocompatibility and bioactive properties of the new dual-curing resin-modified calcium silicate-based material for vital pulp therapy. *Clin Oral Investig*. **2021**, *25*(8), 5009–5024.
- **Rodríguez-Lozano, F. J.,** Lozano, A., López-García, S., García-Bernal, D., Sanz, J. L., Guerrero-Gironés, J., Llena, C., Forner, L., & Melo, M. Biomineralization potential and biological properties of a new tantalum oxide (Ta_2O_5)-containing calcium silicate cement. *Clin Oral Investig*. **2021**, 10.1007/s00784-021-04117-x.

Otras publicaciones producidas durante la tesis doctoral:

- Guerrero-Gironés, J., Alcaina-Lorente, A., Ortiz-Ruiz, C., Ortiz-Ruiz, E., Pecci-Lloret, M. P., **Rodríguez-Lozano, F. J.,** Martínez, C. M., & Ortiz-

Ruiz, A. J. (2020). Melatonin as an Agent for Direct Pulp-Capping Treatment. *Int. J. Environ. Res. Public Health* **2020,** *17*, 1043.

- 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. Comparative Surface Morphology, Chemical Composition, and Cytocompatibility of Bio-C Repair, Biodentine, and ProRoot MTA on hDPCs. *Materials* **2020**, *13*, 2189.
- Sanz, J. L.; López-García, S.; Lozano, A.; Pecci-Lloret, M. P.; Llena, C.; Guerrero-Gironés, J.; **Rodríguez-Lozano, F. J.;** & Forner, L.. Microstructural composition, ion release, and bioactive potential of new premixed calcium silicate-based endodontic sealers indicated for warm vertical compaction technique. *Clin Oral Investig*. **2021** *25*, 1451–1462.
- Sanz, J.L.; Forner, L.; Llena, C.; Guerrero-Gironés, J.; Melo, M.; Rengo, S.; Spagnuolo, G.; **Rodríguez-Lozano, F.J.** 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. *J. Clin. Med.* **2020**, *9*, 3872.
- 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.** Melatonin Treatment Alters Biological and Immunomodulatory Properties of Human Dental Pulp Mesenchymal Stem Cells via Augmented Transforming Growth Factor Beta Secretion. *J Endod.* **2021,** 47, 424–435.
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AGRADECIMIENTOS

En primer lugar, quisiera agradecer a los doctores Forner y Llena su confianza y apoyo incondicional para realizar esta tesis. Gracias por creer en mí, y tratarme como un miembro más de vuestro equipo y casi de vuestra familia.

A la Dra. Concha López, por prestar su tiempo y grandes conocimientos para guiarme en la concepción de esta tesis.

Mi agradecimiento al profesorado y a los compañeros y a las compañeras del Máster de Endodoncia de la *Universitat de València*, Adrián Lozano, Amelia Almenar, María Melo y José Luis Sanz, que han estado siempre dispuestos a ayudarme.

Al grupo de investigación "Trasplante Hematopoyético y Terapia Celular" de la Universidad de Murcia, liderado por el Dr. D. José M. Moraleda. Muchas gracias por todos estos años en los que he formado parte de él pudiendo avanzar en el campo de la terapia celular. Con una especial mención a Sergio y David que siempre han estado presentes y colaborando activamente en cuanto a la confección y diseño de experimentos relacionados con esta tesis.

A la unidad docente de "Pacientes Especiales y Gerodontología" de la Universidad de Murcia. A Ricardo, Mª Carmen, Federico, Julia, Miguel y Pilar. Gracias por todo vuestro apoyo a seguir formándome.

A mis padres y hermanos, gracias por estar siempre ahí, y ser parte activa de mi vida.

A mi esposa, Laura. Ya sabes que eres mi alegría, m fuerza y mi ilusión. Mil gracias por entender la vida académica y apoyarme en todos mis proyectos. Y a mis pequeños Elena y Juan, que los quiero con locura.

FINANCIACIÓN

Este trabajo ha sido financiado por la Red Española de Terapia Celular (TerCel) y el Instituto de Salud Carlos III (ISCiii) (RD16/0011/0001) y el Programa Conjunto para la Investigación Biomédica en Terapias Avanzadas y Medicina Regenerativa del ISCiii y la FFIS (Fundación para la Formación e Investigación Sanitarias de la Región de Murcia).

Índice

Abreviaturas

AAE: *American Association of Endodontists*

AAOMS: *American Association of Oral and Maxillofacial Surgeons*

AHP: Cemento AH-Plus

ALP: *Alkaline phosphatase*

BCHiF: Cemento Endosequence BC Sealer HiFlow

BCS: Cemento Endosequence BC Sealer

BMSCs: *Bone marrow stromal cells*

BRONJ: *Bisphosphonate-related osteonecrosis of the jaws*

CB-RET: *Cell-based regenerative endodontic therapy*

CF-RET: *Cell-free regenerative endodontic therapy*

Col1A1: *Collagen type I alpha 1*

CPE: Células progenitoras endoteliales

CSC: Cemento de silicato cálcico

DPSCs: *Dental pulp stem cells*

DSPP: *Dentin sialophosphoprotein*

EDX: Espectroscopía de rayos X de energía dispersa

ESCs: *Embryonic stem cells*

ESE: *European Society of Endodontology*

GIC: *Glass ionomer cement*

IPS: *Induced pluripotent stem (iPS) cells*

IT: Ingeniería tisular

MSCs: *Mesenchymal stem cells*

MRONJ: *Medication-related osteonecrosis of the jaw*

MTA: *Mineral trioxide aggregate*

MTT: Bromuro de 3-(4,5- dimetiltiazol-2-ilo)-2,5-difeniltetrazol

ON: *Osteonectin*

PDLSCs: *Periodontal ligament stem cells*

qPCR: Reacción en cadena de la polimerasa cuantitativa en tiempo real

RUNX 2: *Runt-related transcription factor 2*

Ta_2O_5: Óxido de Tantalio

VEGF: *Vascular endothelial growth factor*

VTP: *Vital pulp therapy*

Introducción

1. ANTECEDENTES DEL TEMA

1.1. Ingeniería Tisular. Medicina Regenerativa

La Ingeniería Tisular (IT) es un campo que aplica los principios de la ingeniería a la Biología y a la Medicina, mediante el desarrollo de sustitutos biológicos para su posterior implantación en el organismo vivo, con el fin de restaurar, mantener o mejorar la función de diversos órganos dañados por una lesión o enfermedad (Langer and Vacanti, 1993). Aunque el origen de la disciplina de la Ingeniería Tisular data de 1980, existen precedentes documentados incluso en la época del Mesolítico (Sahakyants and Vacanti, 2020). No obstante, el primer congreso científico donde ya se usó de forma oficial el término Ingeniería Tisular fue en 1988, concretamente en California, EE.UU. (Nerem, 2010). Desde ese primer congreso, han ido proliferando congresos científicos hasta la fecha actual. A primeros de los años 90, la disciplina aumentó considerablemente su campo de actuación, con un gran avance de la industria tanto en el sector privado como en el público, saliendo al mercado diversos productos comerciales. Sin embargo, con la emergencia de las células mesenquimales, la IT se dirigió hacia la Medicina Regenerativa (Nerem, 2010).

La Medicina Regenerativa es considerada un área emergente encargada de investigar, con un enfoque translacional, la reparación, sustitución o regeneración de células, tejidos u órganos, devolviendo una función alterada debida a defectos congénitos, enfermedad, trauma o envejecimiento (Dieckmann et al., 2010). Las aproximaciones tecnológicas que alberga este campo incluyen el uso de moléculas solubles, terapia con células madre o "*stem*", terapia génica y el empleo de biomateriales que proporcionen un soporte. El concepto de "Medicina Regenerativa" fue propuesto por primera vez por Haseltine en 2001, que la define como un área compuesta tanto por investigación básica como clínica, y que abarca áreas como la IT, la terapia génica y celular, la biología del desarrollo, la biología química, bioingienería y la nanociencia (Haseltine, 2001).

La existencia del concepto de regenerar órganos o tejidos viene de antaño. Así, en el siglo VIII a. C., Hesíodo describe el mito del Titán Prometeo, que fue encarcelado y castigado por Júpiter durante 30.000 años por robar el fuego a la humanidad y a que su hígado fuera devorado diariamente por un águila llamada Ethon todo el tiempo que estuvo encarcelado, regenerándose el hígado todas las noches para así fuera devorado al día siguiente, hasta que Prometeo fue liberado por Hércules (Calderón Dorda, 2015; Rosenthal, 2003).

También Aristóteles describe en su obra la existencia de una mayor capacidad de regeneración de los animales, concretamente de las extremidades de las salamandras o astas de los ciervos, postulando lo que posteriormente se conocería como "epigénesis" (Barnes, 1753).

En el Génesis, también se describe una regeneración epigenética, "de la costilla tomada del hombre, el Señor Dios formó a la mujer y se la presentó al hombre" (Foster and Enzensberger, 2010). O el famoso "milagro del trasplante de la pierna negra" donde se describe la sustitución de una pierna con gangrena por una pierna negra sana, llevada a cabo por los santos mártires Cosme y Damián (Matthews, 1968).

En el siglo XVIII, apareció la "Teoría de la Epigénesis" de origen aristotélico, que se fundamentaba en "la forma biológica ordenada se origina a partir de la materia indiferenciada amorfa que por un proceso de ordenamiento es capaz de dar vida", con la que autores como Reaumer, Trembly y Spallanzani, promovieron diferentes investigaciones en modelos animales mediante publicaciones en torno a este postulado (Polykandriotis et al., 2010).

El siglo XIX estuvo marcado por una rápida sucesión de acontecimientos. Schleiden y Schwann describieron en 1838-1839 la Teoría Celular. En 1858, Rudolf Virchow declaró el famoso "*omnis cellula ex cellula"* y, mediante observaciones microscópicas, confirmó la teoría celular estableciendo que las células constituyen unidades fundamentales de vida capaces de dividirse (Coleman, 1984; Stocum et al., 2006).

En 1867, el patólogo alemán Julius Cohnheim postuló lo que se conoció como la “Hipótesis de Cohnheim". Sugirió que todas las células con capacidad de reparación e implicadas en la regeneración de heridas provienen de la sangre -procedentes de la médula ósea- (Wohlrab and Henoch, 1988). Finalmente, a finales del siglo XIX, Barth observó que, tras el autotrasplante de hueso en perros de caza, la gran mayoría de las células mueren y dejan un andamio para ser repobladas lentamente por nuevas células huésped y una red neovascular adecuada (Polykandriotis et al., 2010).

A partir del hallazgo del uso de células para reparar, apareció el concepto moderno de “Terapia Celular”, postulado por el Dr. Paul Niehans (1882-1971), el cual comprobó que la inyección de células de paratiroides de ternero fue capaz de reparar la lesión producida accidentalmente en una intervención quirúrgica de glándula tiroides, en la paratiroides de un humano (Paul, 1960).

No obstante, la gran eclosión de la Terapia Celular vino de la mano del Dr. Edward Donnall Thomas (1920-2012), quien pudo demostrar que la transfusión endovenosa de células procedentes de médula ósea (MO) de pacientes sanos en pacientes con enfermedades neoplásicas medulares, sometidos a tratamiento con radioterapia y agentes alquilantes, permitió que varios pacientes sanaran, repoblando la MO dañada, y que desapareciera la leucemia aguda (Thomas, 1999). Todos estos resultados dieron lugar a que se le concediera el Premio Nobel de Medicina en el año 1990.

Otra gran incógnita procedente de estas células madre fue la capacidad de división que tenían y si se mantenía ilimitadamente. De los experimentos de Alexis Carrel y de Leonard Hayflick, se postuló finalmente el denominado “Límite de Hayflick”, donde se concluyó que cada especie animal tiene un número limitado de divisiones celulares, superado el cual las células entraban en estado de apoptosis. Esta conclusión no era aplicable a células cancerígenas en las cuales se había evidenciado la ausencia de dicho “límite” (Hayflick, 2007).

Sin embargo, en los años 80 se determinó que esta característica común de capacidad ilimitada para células embrionarias y cancerígenas no le confería el

perfil maligno a las células embrionarias, ya que presentaban un cariotipo no alterado y podían ser expandidas generando líneas celulares pluripotentes con capacidad para diferenciarse a células de diferentes linajes, siendo finalmente nombradas como “células madre embrionarias pluripotenciales” (ESCs, Embrionic Stem Cells). Posteriores investigaciones permitieron obtener en 1998 un cultivo de células madre embrionarias por parte de Thompson y cols., evidenciando su capacidad ilimitada de división y ofreciendo grandes expectativas dentro de la Medicina Regenerativa (Thomson et al., 1998).

Sin embargo, el problema ético que planteaban las células embrionarias dio lugar a la búsqueda de otros tipos celulares que pudieran salvar este obstáculo. Esto contribuyó al origen de las células madre pluripotenciales inducidas (IPs). Son células no embrionarias en su origen, pero tras inducirlas a pluripotenciales se comportan de forma similar a las ESCs. Las IPs fueron descritas por primera vez en el año 2006 y supusieron un nuevo avance en la terapia celular, ya que se podría disponer de células con características similares a las ESCs, pero evitando el problema ético y la manipulación de embriones. Además, a diferencia de las ESCs, habría que destacar la posibilidad de realizar trasplantes de tipo autólogo en los pacientes, evitando así posibles problemas de rechazo (Takahashi and Yamanaka, 2006). Este descubrimiento supuso, en el año 2012, la concesión del Premio Nobel de Medicina a los investigadores J. B. Gurdon y S. Yamanaka.

Alexander Friedenstein fue el primero en evidenciar la presencia de una población de células no hematopoyéticas capaces de autorrenovación y diferenciación ósea en la médula ósea (Friedenstein, 1980). Posteriormente, otros mostraron que las células procedentes de la médula ósea aisladas según la técnica de Friedenstein, también poseían una gran capacidad de proliferación y de diferenciación en tejidos mesenquimales, por lo que Caplan utilizó el término "células madre mesenquimales" (MSC) para describirlas (Caplan, 1991). Otros estudios han considerado a las células madre mesenquimales como una población celular heterogénea en la que cada célula individual varía en su expresión génica, capacidad de diferenciación, potencial de expansión y fenotipo (Owen, 1988; Prockop, 1997). Además, todas ellas parecen no cumplir los

criterios de las células madre. Por lo tanto, se prefiere que se denominen "células estromales multipotentes" con el mismo acrónimo "MSC" (Prockop, 1997). Varios estudios han demostrado que las MSCs se pueden aislar de múltiples tejidos, como la médula ósea, la sangre del cordón umbilical, el tejido conectivo adulto, la sangre periférica, los tejidos dentales, la placenta y la membrana amniótica (Insausti et al., 2012; Moraleda et al., 2006; Rodríguez-Lozano et al., 2011).

Existen evidencia contrastada con células mesenquimales adultas que avala su uso en Medicina Regenerativa como herramienta terapéutica en enfermedades neurodegenerativas, lesiones cardiacas (regeneración de tejido infartado), enfermedades osteoarticulares, y enfermedades hematológicas (Matthes-Martin et al., 2000; Novelli et al., 2021; Peinemann et al., 2017; Tripathi et al., 2021). La plasticidad o capacidad de diferenciarse de las células adultas ha favorecido la puesta en marcha de diferentes ensayos clínicos de terapia celular incluso en el área orofacial, lo que abre una nueva posibilidad en Odontología (Gonzálvez-García et al., 2013).

En cuanto a la endodoncia, a principios de la década de 1990, se comenzó a aplicar conceptos modernos de Medicina Regenerativa para probar regeneración pulpar *in vitro* mediante la siembra de células pulpares en andamios de ácido poliglicólico sintético biodegradable (Mooney et al., 1996). Posteriormente, Nakashima y Akamine presentaron este moderno concepto de ingeniería de tejidos en la regeneración de pulpa y dentina, con énfasis en los morfógenos, células madre y los sistemas de andamiaje sintetizados (Nakashima and Akamine, 2005). Sin embargo, fue en 2007, cuando se promovió la importancia de la regeneración de los tejidos endodónticos dañados con procedimientos de base biológica (Murray et al., 2007). Actualmente, la Endodoncia Regenerativa basada en células todavía está aún en fase de ensayo clínico (Brizzi et al., 2012; Meza et al., 2019; Nakashima et al., 2017; Xuan et al., 2018). La AAE y la ESE (Galler et al., 2016; Lin et al., 2021) aún no han recomendado el autotrasplante de células madre en Endodoncia Clínica Regenerativa, porque implica aislamiento de células madre, expansión *ex vivo*, buenas prácticas de fabricación, bancos de células madre, ~~gobierno~~ cuestiones

normativas, la habilidad profesional, el personal asistente en la clínica, capacitación y costo comparativamente alto (Huang et al., 2013).

1.2. Patología dental

La patología dental de diversa etiología cursa con la destrucción de los tejidos mineralizados del diente o con la interrupción del desarrollo del mismo (según el momento evolutivo del diente). Además de un tratamiento etiológico, se requiere un tratamiento rehabilitador de las estructuras perdidas, lo cual, tradicionalmente, se ha hecho mediante la inserción de materiales ajenos al diente. En los últimos años se ha comenzado a desarrollar la perspectiva biológica de este tratamiento, a través de materiales con actividad biológica (activando respuestas osteo- o dentino-formadoras, etc.). De esta manera se busca la aplicación de técnicas de Odontología Regenerativa con el fin de resolver problemas odontodestructivos: perforaciones o resorciones radiculares y también la inducción de nuevos tejidos dentales mineralizados e, incluso, el mantenimiento de la vitalidad del diente mediante la regeneración de los tejidos dentales no mineralizados. La Endodoncia es la rama de la Odontología que se ocupa del conocimiento del complejo dentino-pulpar, tanto en su estado de salud como de su patología y de la terapéutica de ésta (Bjorndal, 2008; García Barbero, 2015).

1.3. El complejo dentino-pulpar

El interior del diente está formado por el complejo dentino-pulpar, un tejido parcialmente mineralizado (como el hueso, pero cuyas células formadoras de tejido mineralizado no se sitúan en el interior de éste, sino periféricamente), de origen mesenquimal, el cual está recubierto por el esmalte (en la zona expuesta del diente), de origen neuroectomesenquimatoso y altamente calcificado pero sin componente celular, y por el cemento (en la superficie de la raíz de los dientes), también calcificado con células (solo en su parte más apical), de origen mesenquimal. Habitualmente, se denomina “dentina” a la porción mineralizada y “pulpa” a la no mineralizada. En esta última se localizan las células productoras de dentina, los odontoblastos (Chiego and Avery, 2014; Gómez de Ferraris et al., 2019).

Las funciones establecidas para una pulpa dental sana son: la capacidad de nocicepción, como indicador de daño tisular (Cox et al., 2001), una adecuada respuesta inmunológica y por último la capacidad de neoformación dentinaria como mecanismo de respuesta frente a toxinas y microorganismos (Sangwan et al., 2013); además, cuando la formación radicular se ha completado, la pulpa vital en dientes jóvenes inmaduros, debe promover dicha finalización (Levin et al., 2020). Con relación al estado de salud de la pulpa dental, pueden surgir distintas alteraciones de origen diverso que comprometan la vitalidad de ésta. En base al tipo de proceso patológico que las definen se pueden agrupar en alteraciones inflamatorias (existe una infección subyacente) o degenerativas. Dentro de las inflamatorias tenemos la pulpitis reversible, que se manifiesta clínicamente por presentar dolor leve o moderado al frío, dulce o tacto, pero no se produce espontáneamente, siendo la curación posible. Pero incluso cuando se ha producido una exposición pulpar, con pérdida de la capa de odontoblastos, existe la posibilidad de crearse una barrera de dentina reparadora en 2-6 semanas (Andreasen et al., 1995), debido al efecto inmunomodulador de células presentes en la pulpa dental y a su capacidad de migrar y diferenciarse a odontoblastos que secretarán colágeno, y que posteriormente se mineralizará formando esa capa protectora (Jung et al., 2019). Estas células indiferenciadas o "*stem*" presentes en la pulpa dental, han sido descritas previamente por su gran capacidad de proliferación, plasticidad y mineralización, denominadas como células madre de pulpa dental -DPSCs- (Rodríguez-Lozano et al., 2011; Rodríguez-Lozano et al., 2012). Por el contrario, sin un temprano tratamiento y un incremento del estímulo, la inflamación producida puede evolucionar hacia un estado denominado irreversible. En este estadio, el tratamiento tradicional promueve la extirpación de la pulpa, obviando la capacidad reparadora/regenerativa de la misma (da Rosa et al., 2018; Diogenes et al., 2016).

1.4. Reparación Pulpar

Ante una lesión de caries que abarca esmalte y dentina, con pulpa vital aunque inflamada y sin pérdida de función, está consensuado optar por un tratamiento de protección pulpar para intentar conservar la integridad y vitalidad de la pulpa

(Hanna et al., 2020). Los requisitos principales para aplicar estos tratamientos son la ausencia de necrosis pulpar y la ausencia de una pulpitis irreversible, o lo que es lo mismo, la pulpa debe ser normal o presentar una pulpitis reversible. La ventaja fundamental es que se contribuye a alargar el periodo de supervivencia y la vitalidad del diente afectado (Kodonas et al., 2021; Meschi et al., 2020).

Es importante establecer los límites en cuanto a la reparación del tejido pulpar o la regeneración del complejo dentino-pulpar (Peters, 2014). Cuando se procede a la reparación del tejido pulpar, la estructura que aparece, no es considerada como una nueva dentina, sino que es una estructura similar a la preexistente debido a la carencia de marcadores moleculares específicos de los tejidos obtenidos o secretados, para distinguir dentina normal o reparada. Del mismo modo, las células que se cree que se han diferenciado a odontoblastos, también se denominan “similares a”. De este modo, se mantiene un criterio prudente en cuanto a la terminología usada (Bjorndal et al., 2019).

El recubrimiento pulpar directo es la terapia más utilizada para reparar la pulpa dental que ha sido parcialmente dañada por caries o trauma (Duncan et al., 2019). Se centra en eliminar los daños de la pulpa y en la reparación del tejido pulpar mediante la formación de un puente de dentina sobre el sitio del defecto, que sirve para aislar la pulpa de la cavidad bucal, protegiéndola así de futuras infecciones. En algunos casos, el recubrimiento pulpar exitoso en dientes inmaduros puede ayudar a un mayor desarrollo de los dientes, incluyendo la formación de la raíz del diente (Smith et al., 2016). Para promover la reparación del complejo dentino-pulpar, es necesario estimular las células madre de la pulpa dental (DPSCs) que residen dentro de él para dividirse y producir 'células madre hijas' destinadas a la reparación/regeneración pulpar, mientras permanecen en su lugar para mantener el nicho de células madre. Las células madre hijas recién formadas pueden luego migrar lejos del nicho celular al área del defecto y participar en la formación de nuevos puentes de dentina y reparación de tejido pulpar. Es importante resaltar que la estructura y composición del nuevo tejido remineralizado producido es incierto, siendo prudente llamar a esta cicatrización terapéuticamente producida, reparación en vez de regeneración (Zhang and Yelick, 2021).

1.5. Regeneración Pulpar

Como hemos mencionado anteriormente, el concepto de Endodoncia Regenerativa fue postulado en el año 2007 por Murray que lo definió así: procedimientos biológicos destinados a la regeneración de dentina y las estructuras radiculares, como también de células del complejo dentino-pulpar (Murray, 2012; Murray et al., 2007).

En situaciones donde existen dientes permanentes inmaduros con pulpa necrótica/periodontitis apical, tradicionalmente, se ha tratado con procedimientos de apicoformación, que utilizan hidróxido de calcio para inducir la formación de una barrera de tejido duro apical o tapones de MTA apicales antes de la obturación del conducto radicular (Rafter, 2005). Sin embargo, estos tratamientos requieren múltiples visitas y en el caso del uso de hidróxido de calcio, se puede favorecer la fractura radicular (Chala et al., 2011). Tanto el MTA como el hidróxido de calcio en la apicoformación no tienen capacidad para restaurar la vitalidad del tejido dañado en el espacio del conducto y promover la maduración radicular (engrosamiento de las paredes del conducto radicular y/o cierre apical) de los dientes permanentes inmaduros con pulpa necrótica. En el año 2001, se introdujo una nueva opción de tratamiento en Endodoncia denominada "revascularización" para tratar un diente permanente inmaduro con periodontitis apical y fístula (Iwaya et al., 2001)

El protocolo de revascularización fue propuesto en 2004 (Banchs and Trope, 2004), basado en los experimentos observados de revascularización de dientes reimplantados (Kling et al., 1986), desinfección del conducto radicular (Hoshino et al., 1996) e inducción de coágulos sanguíneos en el espacio del conducto (Nygaard-Ostby and Hjortdal, 1971). Banchs y Trope (2004) agregaron el antibiótico minociclina al utilizado por Iwaya et al. (2001) y se ha hecho conocida como pasta antibiótica triple (ciprofloxacina, metronidazol y minociclina). Además, se utilizó agregado de trióxido mineral (MTA) como barrera intracanal en lugar de cemento de ionómero de vidrio. Este protocolo ha sido ampliamente adoptado en muchos estudios posteriores en la literatura y en las consideraciones clínicas para los procedimientos regenerativos propuestos por

la AAE y la ESE (American Association of Endodontists, 2016; Galler et al., 2016).

El término "revascularización" fue utilizado por primera vez por Iwaya et al. (2001). Posteriormente, se propuso revitalización en lugar de revascularización como un término más aplicable, ya que los tejidos regenerados en el espacio del canal no eran sólo vasos sanguíneos, sino también tejidos duros y blandos (Huang and Lin, 2008). El término "Endodoncia Regenerativa" fue adoptado por la AAE en 2007 (Murray et al., 2007), basado en un concepto de ingeniería de tejidos. Aplica el concepto de la tríada de ingeniería de tejidos: células madre, andamio biomimético y factores de crecimiento bioactivos, en el espacio del conducto para regenerar el tejido pulpar dañado por infección, trauma o anomalías del desarrollo (Nakashima and Akamine, 2005). El término "revitalización" fue adoptado por la ESE (Galler et al., 2016). En la literatura endodóntica, revascularización, revitalización y Endodoncia Regenerativa se utilizan como sinónimos e indistintamente.

Sin embargo, en 2021 se ha querido todavía clarificar una serie de términos en cuanto a los procedimientos regenerativos, de tal modo, que se han diferenciado los términos siguientes: terapia regenerativa endodóntica sin células o "*cell-free regenerative endodontic therapy* (CF-RET)" *versus* terapia regenerativa endodóntica con células o "*cell-based regenerative endodontic therapy* (CB-RET)" (Lin et al., 2021). La presencia de un aporte exógeno de células es lo que difiere entre uno y otro procedimiento.

El concepto "puro" de regeneración pulpar dentro del marco de la Medicina Regenerativa hace necesario el aporte de forma exógena de un número suficiente de células madre que contribuyan a lo establecido en la triada de la IT que incorpora células, andamio y factores de crecimiento. Concepto que difiere *a priori* de los procedimientos regenerativos, revascularización o revitalización, que conlleva un aporte endógeno de células procedentes supuestamente de la papila apical, ligamento periodontal o tejidos periapicales. Además, tanto la ESE como la AAE no recomiendan el uso de trasplante autólogo de células madre para los procedimientos de Endodoncia Regenerativa (Lin et al., 2021). Es por

ello, que los procedimientos endodónticos regenerativos deben ser considerados como terapia regenerativa endodóntica sin células. De igual forma ha sido considerado el término “angiogénesis” más óptimo que el término ~~de~~ “revascularización” en cuanto a los procedimientos de Endodoncia regenerativa sin células. En el campo de la Medicina el término revascularización hace referencia al procedimiento quirúrgico para restaurar el suministro de sangre del tejido isquémico debido a bloqueo o ruptura de vasos. En el caso de la pulpa de un diente avulsionado, donde los vasos sanguíneos están cortados en el ápice, la reimplantación inmediata puede conducir a la reconexión o anastomosis de algunos vasos y el suministro de sangre puede restablecerse y, por tanto, revascularizarse (Huang and Lin, 2008). Sin embargo, Para los casos de CF-RET, la angiogénesis es el proceso de cómo los tejidos vitales se regeneran en el espacio del conducto. La revascularización no es un término óptimo para este proceso. La “neovascularización” es una expresión más adecuada para la revitalización del tejido en el conducto después de CF-RET. Evidentemente, en dientes con pulpas necróticas y periodontitis apical, las células y tejidos se destruyen por infección y no existen (AAE, 2016). Así, células procedentes de los tejidos periapicales migran hacia el interior del conducto y junto con la acción de diferentes factores de crecimiento pueden favorecer la migración de células precursoras endoteliales y células progenitoras perivasculares dentro de los tejidos, facilitando la angiogénesis, formándose nuevos vasos en el interior del conducto junto con otros tejidos (Lin et al., 2021).

1.6. Tejidos Periapicales

Las estructuras fundamentales periapicales son cemento, hueso alveolar y ligamento periodontal; estas estructuras están originadas en la capa celular interna del saco dentario, con mismo origen embriológico. El hueso que rodea el cemento y alveolo, están fuertemente adheridos entre sí por las fibras de colágeno procedentes del ligamento periodontal, y conforman el denominado complejo alveolo-dentario, responsable de resistir las fuerzas de la masticación y mantener fijo al diente en su lugar correcto (Gómez de Ferraris and Campos Muñoz, 2009).

El cemento constituye una estructura de tejido conectivo mineralizado cubriendo a la dentina en su porción radicular. Los componentes celulares del cemento son los cementocitos. La función del cemento es de ser anclaje de las fibras de colágeno del ligamento periodontal, amortiguar las fuerzas oclusales que recibe el ligamento periodontal y participar en la reparación tisular de resorciones radiculares (Gómez de Ferraris and Campos Muñoz, 2009).

El hueso alveolar, forma las apófisis alveolares, llamadas también procesos alveolares y bordes alveolares, que contienen los alveolos dentarios, que son cavidades cónicas formadas especialmente para sostener los denominados elementos dentarios. La porción del hueso alveolar que limita directamente el alveolo pertenece al ligamento de inserción que, junto al ligamento periodontal y al cemento, forma la conocida articulación (gónfosis) alveolodentaria.

El ligamento periodontal está constituido por una capa delgada de tejido conectivo, compuesto por células defensivas, resortivas, formadoras, células madre mesenquimales de ligamento periodontal y células epiteliales de Malassez. Su función radica en soportar y resistir las fuerzas de la masticación, mantener el diente en el alveolo, y actuar como receptor sensorial. En el ápice dentario se pone en contacto con el conectivo pulpar y en la coronal con el corion gingival. Esta relación es muy importante clínicamente, debido a que infecciones inicialmente originadas en esta zona podrían conectarse entre sí y extenderse a otras zonas produciendo las denominadas lesiones Endoperiodontales (Al-Fouzan, 2014).

Estos tejidos, como las células madre presentes en los mismos, están implicados en la reparación/curación de la zona tras un tratamiento de conductos o una apicectomía, donde el potencial irritante del material de obturación podría retrasar o incluso evitar la reparación tisular debido a que la irritación inherente al proceso patológico en sí mismo y la agresión por el tratamiento endodóntico, puede conllevar la pérdida variable de tejido conectiv0 (Saghiri et al., 2020). Además, La formación de nuevos vasos sanguíneos apicalmente es especialmente vital en los tratamientos de conductos ya que estudios previos han descubierto que materiales como los selladores de conductos radiculares

que han estado en contacto con los tejidos perirradiculares después de un tratamiento endodóntico pueden causar periodontitis apical (Nair et al., 1990). De ahí la búsqueda de materiales que favorezcan una Endodoncia biológica y no solamente mecánica y selladora.

1.7. Terapia celular en la osteonecrosis de maxilares secundaria a medicamentos

La AAOMS en su último documento de consenso cambió el término "osteonecrosis de maxilares asociada a bifosfonatos" (BRONJ) a "osteonecrosis de maxilares asociada a medicamentos" (MRONJ), en vista del aumento de frecuencia de osteonecrosis maxilar y mandibular relacionada no sólo a los bisfosfonatos, sino también a otros agentes antirresortivos como denosumab y otros medicamentos antiangiogénicos, como bevacizumab (Ruggiero et al., 2014). El documento de consenso de la AAOMS también modificó la definición de MRONJ establecida en 2009 (Ruggiero et al., 2009), y los pacientes susceptibles a MRONJ ahora se definen como los que presentan las siguientes características: a) un antecedente de tratamiento farmacológico antirresortivo o antiangiogénico; b) la presencia de exposición ósea intraoral o extraoral y fistulización durante más de 8 semanas sin remisión; y c) sin antecedentes de radioterapia o enfermedades con metástasis al maxilar.

El tratamiento clásico en determinadas lesiones de MRONJ es a veces insuficiente; por lo tanto, son necesarias terapias alternativas seguras y que mejoren la osteogénesis y vasculogénesis. La falta de precursores osteogénicos se encuentra en las lesiones patológicas de MRONJ con un apoyo vascular insuficiente causado por la escasez de células progenitoras endoteliales -CPE- (Fournier et al., 2002). Como es sabido, el estroma de la médula ósea contiene una variedad de células madre adultas, incluidas las células madre hematopoyéticas y células madre mesenquimales (MSC) con potencial osteogénico y condrogénico, así como células madre endoteliales que puede contribuir a la vasculogénesis y la angiogénesis, proporcionando reparación vascular (Leventhal et al., 2012). Estas células están presentes en la fracción mononuclear del aspirado de médula ósea. Sus propiedades funcionales han

sido probadas por varios estudios experimentales y clínicos utilizando implantes autólogos de células madre de médula ósea (BMSCs) para curación, reparación de la arquitectura celular y recuperación de flujo sanguíneo en tejidos lesionados e isquémicos (Sun et al., 2009).

Otro aspecto interesante de las células madre, es su efecto inmunomodulador, que podría evitar la aparición de la enfermedad. De hecho, se ha descrito el uso de diferentes fuentes de células madre para el tratamiento de la MRONJ en modelos animales y en menor frecuencia para su prevención (Kaibuchi et al., 2021; Kushiro et al., 2021). También existen estudios aislados en humanos sobre el uso de terapia celular para el tratamiento de la MRONJ (Cella et al., 2011; Gonzálvez-García et al., 2013).

1.8. Biomateriales basados en silicatos

En 1993 fue presentado en la literatura científica un nuevo biomaterial: el MTA, acrónimo en inglés de “agregado trióxido mineral” (42). Desde ese momento han proliferado los estudios sobre sus propiedades y aplicaciones clínicas. Las posibilidades que ofrece como material, no solo biocompatible, sino bioactivo, potenciaron el estudio de sus propiedades. Se trata de un material compuesto por silicatos de calcio y aluminato cálcico), junto con un agente radioopacificante, el óxido de bismuto, para facilitar su identificación radiográfica y, por tanto, su uso clínico.

A partir de él, se están desarrollando en estos momentos nuevos biomateriales de uso odontológico (especialmente de uso endodóntico), destinados al tratamiento de la patología pulpar y a la consecución de la regeneración del complejo tisular dentino-pulpar. Estos nuevos biomateriales, basados en silicatos, se caracterizan por ser biocompatibles, por no provocar, por tanto, reacciones adversas, y por interactuar con los sistemas biológicos dentales y peridentales (Camilleri, 2011). Los biocerámicos o materiales a base de silicatos se han propuesto como la nueva generación de materiales, derivados del MTA con modificaciones significativas en su composición (Drukteinis and Camilleri, 2021; Duarte et al., 2018). Según su uso y área de aplicación, estos materiales

son categorizados en intracoronales (materiales de recubrimiento pulpar y procedimientos regenerativos), intrarradiculares (selladores de conductos radiculares, cementos de tapón apical y cementos de reparación de perforaciones) y extra-radicular (materiales de obturación del extremo de la raíz y cementos de reparación de perforaciones). Además, estos materiales se clasifican en función de su composición química como derivados del cemento portland o derivados de silicato tricálcico. Así, el tipo de cemento, radioopacificante, vehículo y aditivos varían entre los diferentes tipos de cementos a base de silicato (Drukteinis and Camilleri, 2021; Pelepenko et al., 2021).

La investigación sobre estos nuevos biomateriales se centra, por una parte, en su estudio clínico (comportamiento, tasas de éxito y mejora de las condiciones de utilización -consistencia, tiempo de fraguado y otras características-) y, por otra, en conocer con detalle su biocompatibilidad con los tejidos sobre los que se aplica y su potencial bioactividad, tanto en el sentido de la regeneración pulpar como en el de la reparación tisular y reposición de tejidos mineralizados perdidos -dentina y hueso peridental- (Wongwatanasanti et al., 2018).

Esto significa que el estudio de nuevos biomateriales con bioactividad (Torabinejad et al., 2018) se encuentra en estos momentos en un lugar preferente de la investigación, básica y clínica, en el marco de la terapia regenerativa odontológica (y especialmente en el ámbito de la Endodoncia), con unas perspectivas muy interesantes que dirigirán los tratamientos pulpares a un terreno más biológico y menos mecánico.

Justificación

Diversas situaciones patológicas dentales pueden verse beneficiadas de un tratamiento que favorezca la regeneración tisular. Las resorciones radiculares, las perforaciones iatrogénicas, la patología infecciosa periapical y las lesiones traumáticas de los dientes se asocian a una pérdida de tejido óseo peridentario. Los tratamientos restauradores convencionales pueden eliminar las circunstancias etiológicas con materiales que no solo sean biocompatibles sino que sean bioactivos.

Un aspecto especial es el de la patología que afecta al complejo dentino-pulpar, la parte vital del diente. Se trata de un tejido similar al hueso (una zona mineralizada y otra no) pero con una distribución celular diferente. Este tejido se afecta fundamentalmente por causa infecciosa (caries) o traumática. La posibilidad de neoformación de tejido dentinario (su parte mineralizada) y el mantenimiento de la vitalidad pulpar es uno de los objetivos prioritarios de cualquier tratamiento dental.

La terapia vital pulpar incluye procedimientos como protección pulpar directa, indirecta y pulpotomía parcial o total. Los materiales usados en terapia vital deben tener adecuada biocompatiblidad y bioactividad para promover la actividad de las células madre dentales y la reparación pulpar en diente permanentes. Por otro lado, las células mesenquimales son capaces de proveer odontoblastos y también tienen actividad antiinflamatoria e inmunoinmoduladora, demostrada en numerosos estudios.

Como previamente se ha expuesto, la osteonecrosis mandibular es una enfermedad que conduce a una merma importante en la calidad de vida de los pacientes provocando intenso dolor y dificultad a la alimentación. Actualmente no existe ningún tratamiento efectivo. En esta situación la única opción terapéutica supone la realización de técnicas quirúrgicas muy costosas para el sistema de salud, y muy complejas. Requiere la implantación de materiales de osteosíntesis asociada al empleo de injertos libres microvascularizados, etc. Estas técnicas requieren ingresos hospitalarios de larga duración y, en ocasiones, frecuentes. Además, y considerando la edad y el estado basal de los

pacientes, tienen una baja tasa de éxitos debido a la complejidad de la técnica. El evento patológico subyacente es la necrosis ósea. Para evitar esta evolución tan tórpida, el mejor tratamiento disponible es utilizar aloinjertos óseos tras la realización del legrado del foco osteonecrótico. Esta técnica no está exenta de morbilidades, sin embargo, todo lo expuesto con anterioridad apoya la idea de que un constructo formado por células madre mesenquimales más fosfato tricálcico puede ser igual de eficaz que el injerto óseo. De este modo se evitaría la cirugía de obtención del injerto óseo libre microvascularizado. Este hecho se traduciría en un menor tiempo quirúrgico, en una menor necesidad de personal facultativo para la misma, en una menor estancia de los pacientes en el hospital y de la necesidad de cuidados postoperatorios, así como una disminución de la frecuencia de revisiones hospitalarias una vez dada de alta. También supondría un aumento de la calidad de vida de los pacientes, así como una mejora del dolor y de la alimentación.

Objetivos

2.1. Objetivo general

Evaluar la biocompatibilidad y la bioactividad de materiales, considerados bioactivos, que favorezcan la reparación/regeneración tisular en diferentes situaciones clínicas mediante ensayos in vitro y/o in vivo.

2.2. Objetivos específicos

2.2.1: Analizar *in vitro* la biocompatibilidad y la capacidad de biomineralización de dos nuevos materiales biocerámicos bioactivos indicados para obturación retrógrada y para el tratamiento de perforaciones radiculares.

2.2.2: Comprobar *in vivo* la capacidad de las células mesenquimales de médula ósea sembradas sobre *β*-fosfato tricálcico para prevenir la aparición de osteonecrosis de maxilares en un modelo de ratas zolendronizadas.

2.3.3: Determinar los efectos biológicos *in vitro* de un nuevo material sellador en base a silicato de calcio indicado para realizar obturación vertical sobre células madre del ligamento periodontal humano (hPDLSCs).

2.3.4: Verificar *in vitro* la bioactividad y biocompatibilidad de Theracal PT, Theracal LC, and MTA Angelus, materiales considerados bioactivos y usados para la terapia vital pulpar, usando células madre de pulpa dental humana (hDPSCs).

2.3.5: Evaluar *in vitro* los efectos biológicos y el potencial de biomineralización de un nuevo material que contiene óxido de tantalio (Ta_2O_5), diseñado para la terapia pulpar vital o para la reparación de perforaciones (NeoMTA 2), en comparación con las propiedades correspondientes del NeoMTA Plus y del Bio-C Repair.

Diseño experimental

El diseño experimental realizado para abordar los objetivos específicos planteados en esta Tesis Doctoral, se muestra en las Figuras 1-5 a continuación:

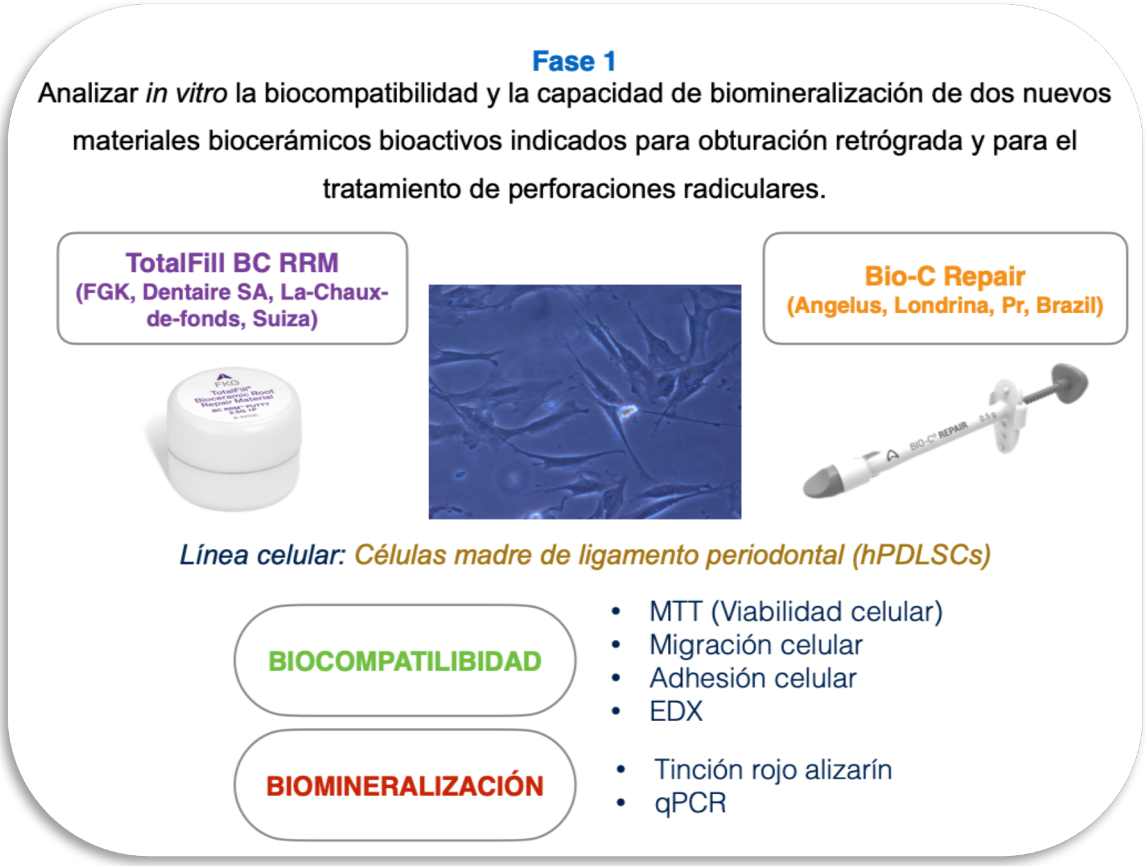

Figura 1. Fase 1 planteada para alcanzar el objetivo 1 propuesto.

Figura 2. Fase 2 planteada para alcanzar el objetivo 2 propuesto.

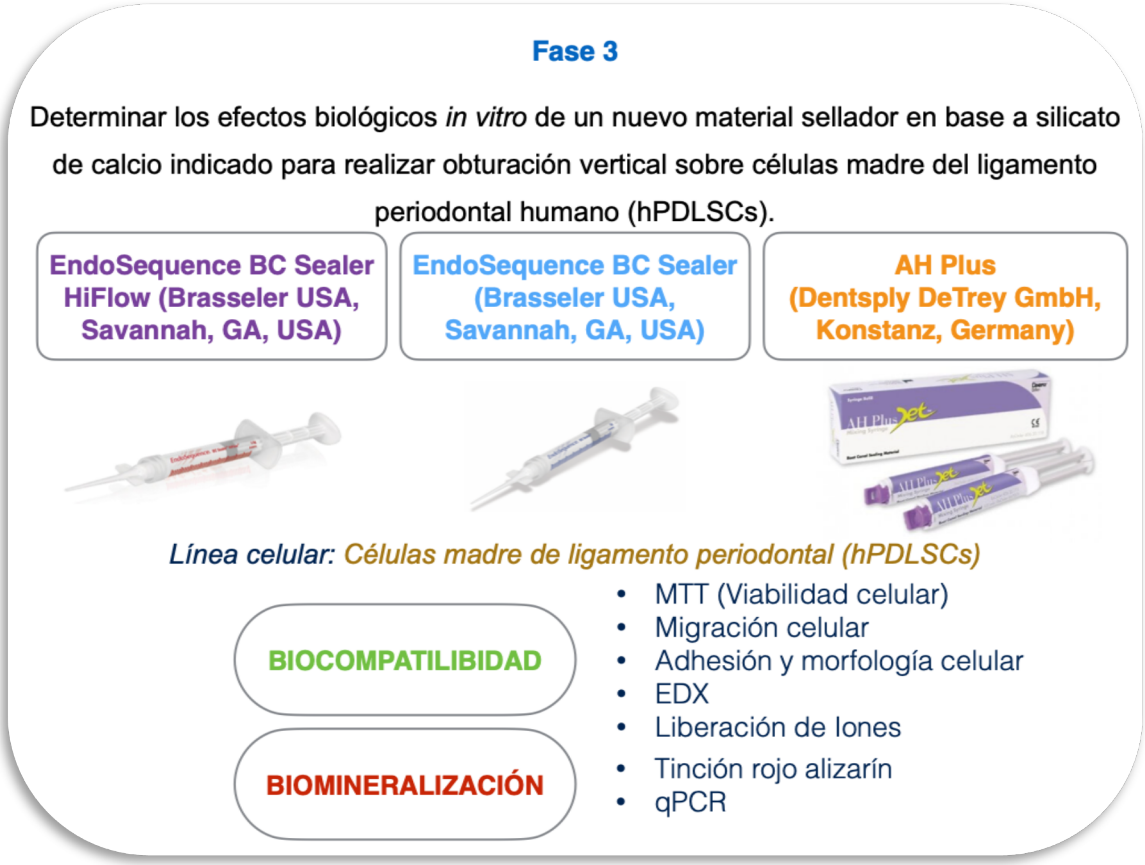

Figura 3. Fase 3 planteada para alcanzar el objetivo 3 propuesto.

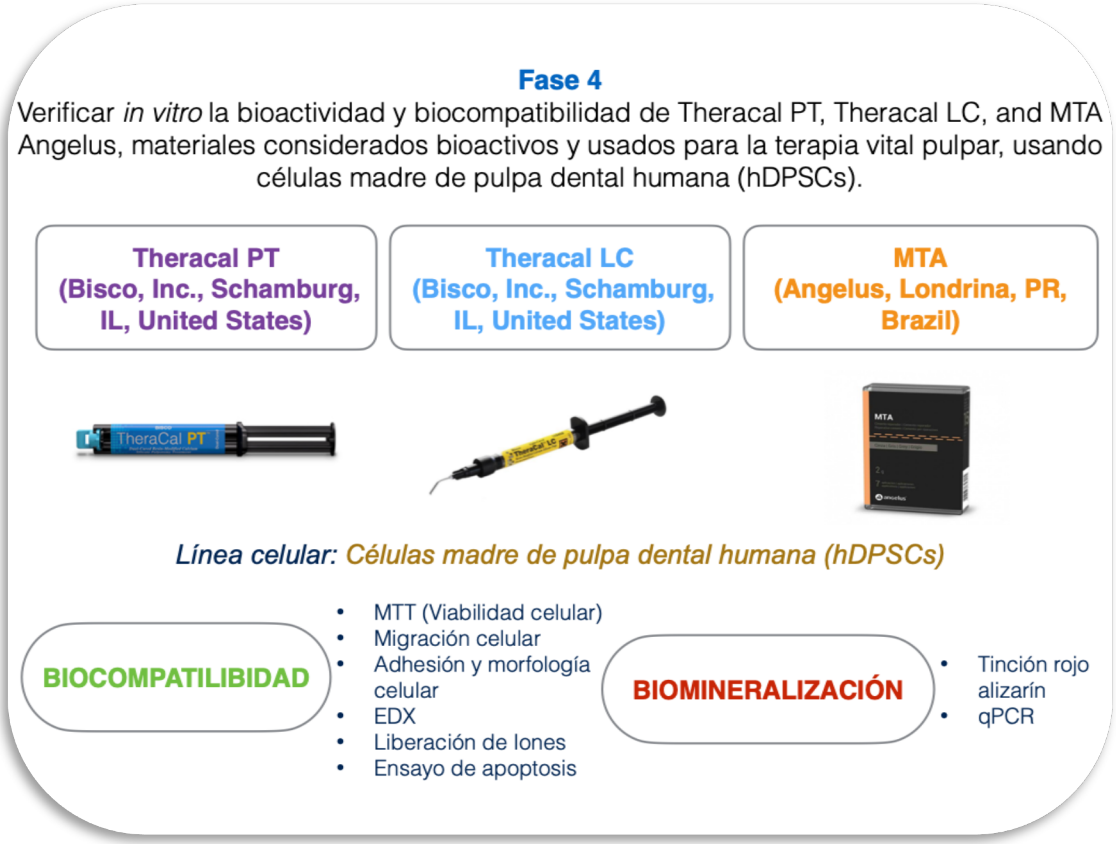

Figura 4. Fase 4 planteada para alcanzar el objetivo 4 propuesto.

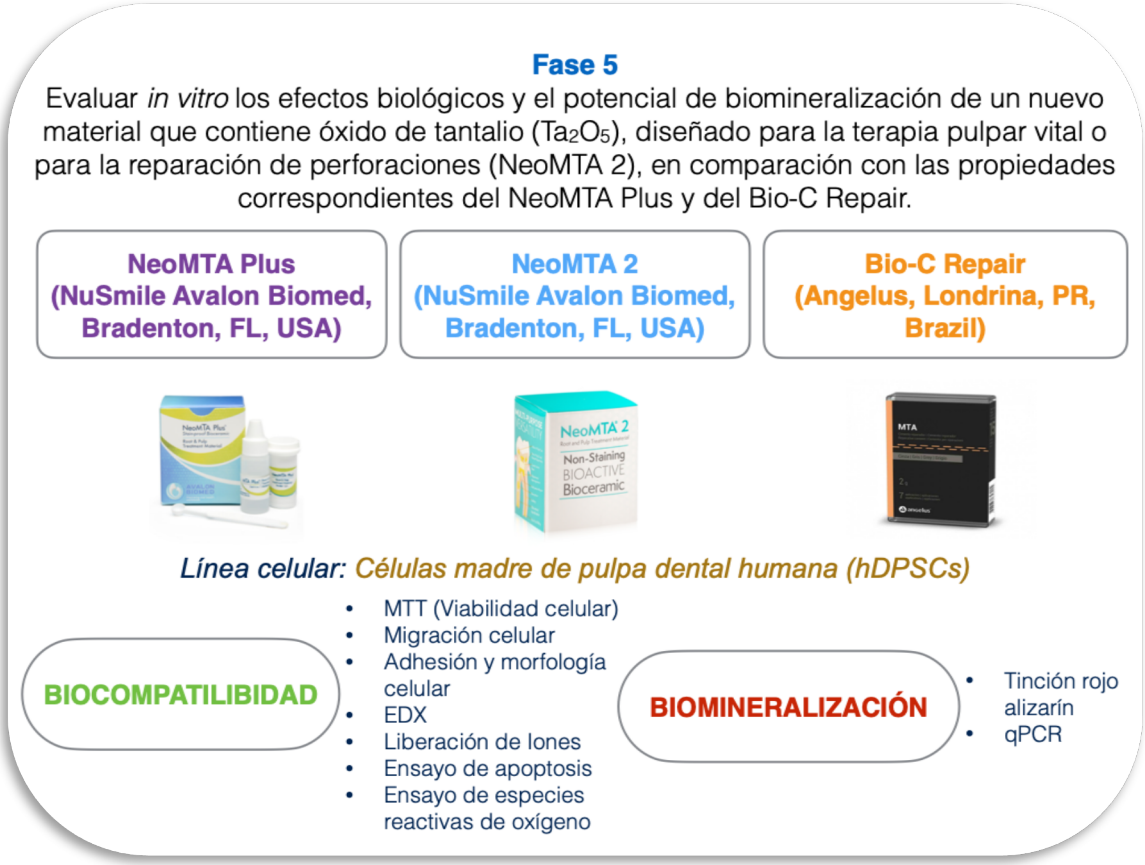

Figura 5. Fase 5 planteada para alcanzar el objetivo 5 propuesto.

Resultados Y Discusión

A continuación, se muestra el resumen de los resultados y discusión, de acuerdo con los objetivos descritos anteriormente y las publicaciones adjuntas en anexos. La metodología y bibliografía de la misma se mencionará sucintamente, ya que se encuentra en cada uno de los artículos, aunque se añadido alguna cita reciente no incluida en los artículos publicados previamente.

ARTÍCULO 1

Revista: Applied Sciences
Factor impacto (2020): 2.679
Ámbito: ENGINEERING, MULTIDISCIPLINARY
Rango: 38/91 (Q2)
Título: In Vitro Effect of Putty Calcium Silicate Materials on Human Periodontal Ligament Stem Cells

En la publicación número 1 quedan recogidos los resultados del trabajo realizado para alcanzar el siguiente objetivo:

Objetivo 1: Analizar *in vitro* la biocompatibilidad y capacidad de biomineralización de dos nuevos materiales biocerámicos bioactivos indicados para obturación retrógrada y perforaciones radiculares.

Como ya se mencionó en la introducción, se han desarrollado nuevos materiales bioactivos aplicados dentro del campo de la Odontología. Concretamente, para el campo de la cirugía apical, también se han desarrollado nuevos materiales para obturar de forma retrógrada. Estos materiales entran en contacto con tejidos vitales y facilitan la biomineralización y la reparación apical (Torabinejad et al., 2018). En esta publicación se realizó una comparación, desde un punto de vista biológico, de dos materiales considerados bioactivos, Bio-C Repair (Angelus, Londrina, Brasil) y TotalFill BC RRM (FGK Dentaire SA, La-Chaux-de-fonds, Suiza) y que además presentaban dentro de sus aplicaciones clínicas, la de ser un material para retro-obturación. Para el estudio se usaron células madre de ligamento periodontal humano (hPDLSCs) debido a su presencia a nivel apical, siendo una línea adecuada para intentar representar estudios *in vitro* (Akbulut et al., 2016). Las pruebas experimentales fueron la viabilidad celular, la migración

y la adhesión celular. Además, se realizaron qPCR y ensayo de mineralización para evaluar el potencial de biomineralización de estos cementos. La viabilidad celular y la migración celular de Bio-C Repair y de TotalFill BC RRM fueron similares al control sin diferencias estadísticamente significativas, excepto a las 72 h, usando TotalFill BC RRM, que fue ligeramente menor ($p<0,05$). Ambos materiales exhibieron una excelente adherencia celular y morfología de las células sobre estos materiales. Además, en cuanto a la biomineralización, ambos cementos promovieron la diferenciación osteogénica y cementogénica de las hPDLSCs. Estos resultados sugieren que Bio-C Repair y TotalFill BC RRM son materiales biológicamente apropiados para ser usados como material de obturación a retro.

El uso de células madre o multipotenciales en estudios *in vitro* con materiales bioactivos favorece el análisis de la bioactividad de los mismos. Una de las definiciones de bioactividad es el efecto celular provocado por la liberación de iones de estos materiales facilitando la biomineralización (Vallittu et al., 2018). Para ello, son necesarias células que tengan la capacidad de diferenciarse a células similares a osteoblastos, cementoblastos y odontoblastos, con capacidad de favorecer la mineralización (Rodriguez-Lozano et al., 2019b). Por ello se han usado células madre de ligamento periodontal.

Debido al poco tiempo que tienen estos materiales en el mercado, la presencia de artículos que tratan aspectos de biocompatiblidad y bioactividad de estos materiales específicos es limitada. Usando fibroblastos L929 (ratón), se comparó la biocompatibilidad y la biomineralización de Bio-C Repair frente a MTA, obteniendo que Bio-C Repair presentaba una biocompatiblidad e inducía una biomineralización similar al MTA (Benetti et al., 2019). Otro estudio del grupo de Camilleri, hizo una comparación múltiple con diferentes materiales, entre los que se encontraba el TotalFill BC RRM, y que mostró altas tasas de viabilidad celular tanto en fibroblastos de ratón como en células humanas de pulpa dental (Koutroulis et al., 2019). Una de las limitaciones que más encontramos en la literatura es que el uso de materiales y líneas celulares es muy dispar, lo que dificulta una buena comparación.

ARTÍCULO 2

Revista: Journal of Clinical Medicine

Factor impacto (2020): 4.241

Ámbito: MEDICINE, GENERAL & INTERNAL – SCIE

Rango: 39/169 (Q1)

Título: Allogeneic Bone Marrow Mesenchymal Stem Cell Transplantation in Tooth Extractions Sites Ameliorates the Incidence of Osteonecrotic Jaw-Like Lesions in Zoledronic Acid-Treated Rats.

En la publicación número 2 quedan recogidos los resultados del trabajo realizado para alcanzar el siguiente objetivo.

Objetivo 2: Comprobar *in vivo* la capacidad de las células mesenquimales de médula ósea sembradas sobre *β*-fosfato tricálcico para prevenir la aparición de osteonecrosis de maxilares en un modelo de ratas zolendronizadas

La terapia celular es un tratamiento de la Medicina Regenerativa que persigue obtener células madre para, posteriormente, usarlas clínicamente en pacientes con el fin de regenerar o reparar células o tejidos dentro del organismo (Blanquer et al., 2012). Es por ello que el desarrollo de medicamentos de terapia avanzada, entre los que se encuentran los medicamentos o productos de terapia celular, se encuentran en auge (Ortiz-Arrabal et al., 2021). De ahí la búsqueda de alternativas a enfermedades donde la terapia tradicional no soluciona todos los casos. En concreto, en esta publicación lo que se pretendió fue comprobar si el producto de terapia celular compuesto por células mesenquimales de médula ósea sembradas sobre fosfato tricálcico era capaz de prevenir la aparición de la osteonecrosis de los maxilares en un modelo de ratas a la que se le había administrado ácido zolendrónico. No se observaron, ni clínica ni histológicamente, signos de osteonecrosis en aquellas ratas que habían sido tratadas con fosfato tricálcico y células mesenquimales de médula ósea; mientras que en el grupo control (suero salino y fosfato tricálcico), se observaron signos de osteonecrosis en un 33% de los casos.

Con el fin de comprobar si el efecto regenerador era directamente favorecido por nuestras células, se obtuvieron células de médula ósea de ratas macho las cuales se sembraron y posteriormente se inocularon en ratas hembra. Tras finalizar el periodo de estudio se sacrificaron y mediante PCR se detectó el cromosoma Y de las células inoculadas, de tal modo que pudimos concluir que el efecto preventivo está íntimamente relacionado con la presencia de BM-MSCs.

El uso de células mesenquimales para la prevención o el tratamiento de la osteonecrosis maxilar, se está estudiando cada vez más. De hecho, hace unos años se comenzó con aproximaciones en humanos (Gonzalvez-Garcia et al., 2013) y recientemente ha habido un incremento de estudios, principalmente en animales (Kaibuchi et al., 2021). Con células procedentes de grasa y plasma rico en plaquetas en el mismo modelo que el que usamos, se obtuvieron resultados similares a los nuestros, de tal modo que células mesenquimales procedentes de grasa resultaron ser útiles también para la prevención de la osteonecrosis de los maxilares (Barba-Recreo et al., 2015). Además, también se puso en valor la infusión de células mesenquimales procedentes de cordón umbilical, que en un modelo animal de osteonecrosis asociada a bifosfonatos (BRONJ) promovieron el cierre de tejidos blandos y la menor incidencia de BRONJ (Yang et al., 2021). Incluso se describió la administración local de células dentales para la prevención de la BRONJ. Concretamente, con DPSCs pudo comprobarse en un modelo animal un mayor cierre de mucosa y neoformación, además de una gran presencia de muchas células positivas para el factor de crecimiento endotelial vascular (VEGF), implicadas en la angiogénesis (Kushiro et al., 2021).

ARTÍCULO 3

Revista: International Endodontic Journal

Factor impacto (2020): 5.264

Ámbito: DENTISTRY, ORAL SURGERY & MEDICINE – SCIE

Rango: 10/91 (Q1)

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

En la publicación número 3 quedan recogidos los resultados del trabajo realizado para alcanzar el siguiente objetivo específico.

Objetivo 3: Determinar los efectos biológicos *in vitro* de un nuevo material sellador basado en silicato de calcio e indicado para realizar obturación vertical sobre células madre del ligamento periodontal (hPDLSCs).

Los selladores basados en silicato de calcio pertenecen a la clase de selladores biocerámicos bioactivos que durante el proceso de fraguado favorecen la precipitación de fosfato de calcio, que puede estimular la bioactividad por la formación de un tejido con composición química y estructura cristalina similares a las propiedades de un material de apatita (Khalil et al., 2016). Además, otras reacciones como las reacciones de hidratación que promueven la formación de hidróxido de calcio son responsables de las propiedades biológicas de estos selladores, ya que la liberación de calcio (Ca^{2+}) y los iones de hidroxilo (OH^-) estimulan la formación de tejido mineralizado y contribuyen a la reparación y/o regeneración de tejidos periapicales (Primus et al., 2019).

En esta publicación, se ha buscado evaluar los efectos biológicos de Endosequence BC Sealer HiFlow (BCHiF) en comparación con su predecesor Endosequence BC Sealer (BCS) y un sellador del conducto radicular basado en resina epóxica, AH-Plus (AHP), en un entorno de laboratorio. A partir de eluatos obtenidos de cada material se evaluaron los efectos biológicos que provocaban sobre las hPDLSCs. Los resultados de nuestro estudio evidenciaron que desde un punto de vista biológico estos materiales resultan apropiados para ser usados clínicamente, ya que promueven la adhesión, la viabilidad y la migración celular,

además estimulan, sin aditivos, la mineralización, aspecto importante en la reparación tisular.

En cuanto a la metodología usada, el uso de eluatos o eluidos es un proceso estandarizado para evaluar los efectos biológicos de nuevos materiales, además representa una herramienta sencilla, económica y reproducible, avalada por su uso en ensayos de citotoxicidad, estando recogidas en una serie de normas internacionales establecidas (ISO, 2009). Estudios previos dentro del campo de la odontología siguen estos mismos criterios para determinar los efectos biológicos producidos en los nuevos materiales (Wu et al., 2015).

La sobreexpresión de genes cementogénicos/osteoblásticos también corrobora la bioactividad de estos materiales, ya que inducen la diferenciación de estas células en presencia de estos materiales. Este efecto parece estar claramente relacionado con la composición de estos materiales, ya que liberan grandes cantidades de calcio (Zordan-Bronzel et al., 2019) y además regulan la actividad de la fosfatasa alcalina, implicada en la mineralización y en la diferenciación. Por otra parte, e incidiendo en esta cuestión, recientemente se ha descrito que BCS es de los pocos materiales que promueve *in vivo* la formación de apatita (Belal et al., 2021). Con lo que se evidencia que los sellados biocerámicos no solo sellan mecánicamente el conducto si no, que también lo hacen desde un punto de vista biológico acelerando la curación apical (Sanz et al., 2021a).

ARTÍCULO 4

Revista: Clinical Oral Investigations

Factor impacto (2020): 3.573

Ámbito: DENTISTRY, ORAL SURGERY & MEDICINE – SCIE

Rango: 21/91 (Q1)

Título: Cytocompatibility and bioactive properties of the new dual-curing resin-modified calcium silicate-based material for vital pulp therapy.

En la publicación número 4 quedan recogidos los resultados del trabajo realizado para alcanzar el siguiente objetivo específico.

Objetivo 4: Verificar *in vitro* la bioactividad y la biocompatibilidad de Theracal PT, Theracal LC, y MTA Angelus, materiales considerados bioactivos y usados para la terapia vital pulpar, usando células madre de pulpa dental (hDPSCs).

La búsqueda de nuevos materiales que faciliten la reparación pulpar es de gran interés en el campo de la Endodoncia (Pedano et al., 2020). Los cementos de silicato cálcico (CSC) han ganado impulso para su uso en procedimientos de terapia vital pulpar VPT (Li et al., 2020).

En esta publicación se analizó la bioactividad y la biocompatibilidad de un nuevo biomaterial compuesto por silicato y resina (Theracal PT), comparándolo con MTA y con el material predecesor del primero, el Theracal LC. Dado que su indicación es terapia vital pulpar, la línea celular usada se constituyó con células madre de la pulpa dental (DPSCs). Nuestros resultados mostraron mejor viabilidad y migración celular en el grupo del Theracal PT que en el grupo del Theracal LC. De igual modo hubo una mayor sobreexpresión de genes odontogénicos y mineralización en el grupo Theracal PT corroborando su mayor bioactivad. Sin embargo, en general, los valores fueron menores que en el grupo del MTA.

Los CSC son una clase de materiales que incluyen silicatos tricálcicos, silicatos dicálcicos, cementos hidráulicos de silicato de calcio y materiales "biocerámicos". Los resultados clínicos han demostrado un éxito constante con

estos materiales y el agregado de trióxido mineral (MTA) es uno de los silicatos tricálcicos más estudiado y que se usa ampliamente. Cuando se utilizan MTA y otros CSC para procedimientos de VPT en dientes permanentes con pulpitis irreversible sintomática o asintomática, las tasas de éxito oscilan entre el 85-100% a los 1-2 años (American Association of Endodontists, 2021; Linsuwanont et al., 2017). Sin embargo, cabe destacar que el hidróxido de calcio, los cementos de ionómero de vidrio (GIC) y los materiales basados en resina demuestran un rango de éxito más bajo, que varía del 43% al 92% (Bakhurji, 2020; Taha and Khazali, 2017).

Como ya se ha comentado previamente, en el presente estudio se utilizaron hDPSCs previamente caracterizadas como población celular diana. Estas células, que residen en nichos perivasculares, se reclutan en el complejo dentino-pulpar, tanto en un estado saludable como inflamatorio, y son inducidas a diferenciarse en células similares a los odontoblastos a través de una cascada de eventos moleculares involucrados en el proceso de la dentinogénesis reparadora (Sanz et al., 2021b; Smith et al., 2016). Por lo tanto, al analizar la respuesta biológica de las hDPSCs hacia los materiales probados, mediante una amplia variedad de pruebas in vitro que evalúan diferentes parámetros celulares, se puede anticipar potencialmente su comportamiento clínico.

Una gran limitación que encuentra nuestro estudio es la falta de investigaciones acerca del TheraCal PT, debido a que es un material muy reciente y solo existe un artículo más donde se investigan sus efectos y se comparan con los de otros materiales (Sanz et al., 2021c). En cambio, sí que hay evidencia sobre su precursor, el Theracal LC, que ya solamente se recomienda para protección pulpar indirecta, debido a la toxicidad que ha demostrado sobre células pulpares (Bakhtiar et al., 2017; Giraud et al., 2019; Jeanneau et al., 2017).

ARTÍCULO 5

Revista: Clinical Oral Investigations

Factor impacto (2020): 3.573

Ámbito: DENTISTRY, ORAL SURGERY & MEDICINE – SCIE

Rango: 21/91 (Q1)

Título: Biomineralization potential and biological properties of a new tantalum oxide (Ta_2O_5) - containing calcium silicate cement.

En la publicación número 5 quedan recogidos los resultados del trabajo realizado para alcanzar el siguiente objetivo específico.

Objetivo 5: Evaluar *in vitro* los efectos biológicos y el potencial de biomineralización de un nuevo material, que contiene óxido de tantalio (Ta_2O_5), diseñado para la terapia pulpar vital o para la reparación de perforaciones (NeoMTA 2), en comparación con NeoMTA Plus y Bio-C Repair.

Como hemos comentado anteriormente, la composición química es otra forma de clasificar a estos materiales biocerámicos (Drukteinis and Camilleri, 2021). La presencia de radioopacificantes como es el óxido de tantalio en vez del óxido de bismuto ha sido propuesta en algunos cementos con el fin de evitar la posible discoloración que se producía a causa del óxido de bismuto (Rodriguez-Lozano et al., 2019a). Sin embargo, la evidencia sobre si este cambio afecta a la biocompatibilidad de estos materiales es limitada. De ahí que esta publicación se propusiera evaluar cambios en la biocompatibilidad y biomineralización de NeoMTA 2 comparándolo con su precursor NeoMTA Plus y con otro cemento cuyo radiopacificante es el óxido de zirconio (Bio-C Repair).

Siguiendo la coherencia que anteriormente se ha descrito en otras publicaciones en cuanto a la metodología, con una perspectiva traslacional e intentado simular las condiciones clínicas bajo un entorno de laboratorio, se realizaron diferentes análisis con el fin de alcanzar el objetivo planteado.

NeoMTA 2 mostró una liberación de calcio significativamente mayor en comparación con los otros materiales ($p<0,05$). Cuando se cultivaron hDPSCs en presencia de los diferentes extractos, todos los grupos exhibieron tasas de migración y viabilidad de hDPSCs similares en comparación con las células no tratadas (control). Se observó una adherencia de las células en todas las superficies de los materiales, sin diferencias significativas. Las hDPSCs tratadas con NeoMTA 2 mostraron una sobreexpresión de los genes ALP, Col1A1, RUNX2 ($p <0,001$), ON y DSPP ($p<0,05$), y mostraron el mayor potencial de mineralización en comparación con otros grupos ($p<0,001$). Finalmente, los eluatos más concentrados de estos materiales, especialmente de NeoMTA Plus y de NeoMTA 2, promovieron una mayor producción de especies reactivas de oxígeno (ROS) en las hDPSCs en comparación con las células Bio-C Repair y control ($p<0,001$), aunque estos niveles de ROS no dieron como resultado un aumento de la muerte celular.

La evaluación de estos materiales se realizó mediante la incubación de células cultivadas con varias diluciones de los materiales (1:1, 1:2 y 1:4). Esto se hizo para simular diferentes condiciones clínicas en las que se aplicarían los materiales, ya que pueden ser colocados en espesores de dentina remanente de 0,01 a 0,25 mm (recubrimientos pulpares indirectos) o en cavidades con exposición pulpar (recubrimiento pulpar directo) y la concentración del material que llega al tejido pulpar viable puede variar (Santos et al., 2021). En otros estudios con materiales para la terapia vital pulpar es habitual usar diluciones con el fin de simular la situación clínica real (Benetti et al., 2019; Lv et al., 2016; Pedano et al., 2018).

En cuanto a la liberación de iones, NeoMTA 2 se asoció con la mayor liberación de iones de calcio de todos los materiales probados ($p<0,05$). Se ha descrito que la liberación de Ca estimula la formación de hidroxiapatita, y también la actividad de la fosfatasa alcalina, esencial en el proceso de mineralización (Gandolfi et al., 2014).

La capacidad de mineralización se ha identificado como un indicador de diferenciación odontoblástica exitosa (Kodonas et al., 2012). Las hDPSCs

mostraron formación de nódulos de calcio después de 21 días de cultivo en presencia de materiales liberadores de iones y en el medio de inducción osteogénico, como lo indica la tinción con rojo de alizarina. Siguiendo nuestra línea, estudios previos han mostrado que la presencia de tantalio y circonio promueve la diferenciación osteo / odontogénica de hDPSCs (Abou ElReash et al., 2021). Así, estudios anteriores demostraron que iRootBP Plus y NeoMTA, que contienen Ta_2O_5, promueven la mineralización y la formación de puentes dentinarios *in vitro e in vivo* (Liu et al., 2015; Tanomaru-Filho et al., 2017). El mayor potencial de mineralización exhibido por NeoMTA 2 frente a su precursor, el NeoMTA Plus, podría estar relacionado con la mayor proporción de tantalita y la presencia de diferentes polímeros en su composición. Nuevamente, la ausencia de más publicaciones sobre NeoMTA 2, que podrían haber permitido la comparación con otros trabajos, representa la mayor limitación de nuestra publicación.

Conclusiones

De los resultados obtenidos y dando respuesta a los objetivos planteados para la presente tesis doctoral se pueden establecer las conclusiones que siguen.

1. Los materiales bioactivos empleados en el presente trabajo de investigación no sólo no producen efectos adversos a nivel celular/tisular, sino que, además, favorecen la curación, siendo aptos, desde un punto de vista biológico, para ser considerados clínicamente.

2. Los materiales Bio-C Repair y TotalFill BC RRM putty son materiales biocompatibles y favorecen la mineralización de las hPDLSCs, estando su uso indicado en perforaciones radiculares y en tratamientos quirúrgicos periapicales como material de relleno.

3. La administración local del producto de terapia celular formado por células mesenquimales de médula ósea sembradas sobre un andamio bioactivo de fosfato tricálcico previene la aparición de la osteonecrosis de los maxilares asociada a medicamentos, favoreciendo la neoformación ósea.

4. Tras analizar los efectos biológicos provocados en las hPDLSCs por parte del sellador Endosequence BC Sealer HiFlow, basado en silicato cálcico, se establece que se trata de un material apropiado para ser usado clínicamente, ya que promueve la adhesión, la viabilidad y la migración celular, estimulando, además, sin aditivos, la mineralización, aspecto importante en la reparación tisular.

5. El material Theracal PT indicado para terapia vital pulpar, es un material dual que mejora las propiedades biológicas de su precursor, y desde un punto de vista bioactivo, promueve la sobreexpresión de genes odontogénicos, así como favorece la formación de depósitos cálcicos, fenómeno clave en la formación de puentes dentinarios.

6. La presencia en la composición química de óxido de tantalio en el nuevo material para terapia vital pulpar NeoMTA 2, no altera sus propiedades

biológicas, sino que, comparándolo con su precursor, el NeoMTA Plus o con Bio-C Repair, presenta una mayor liberación de iones cálcicos, que favorecen la diferenciación osteo-/odontogénica de las hDPSCs y la capacidad de mineralización.

No obstante, son necesarios más estudios tanto *in vitro* como *in vivo* que verifiquen lo obtenido con todos los materiales bioactivos que han sido planteados en este trabajo de investigación.

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Anexos

applied sciences

MDPI

Article

In Vitro Effect of Putty Calcium Silicate Materials on Human Periodontal Ligament Stem Cells

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Received: 24 November 2019; Accepted: 23 December 2019; Published: 1 January 2020

Abstract: New bioactive materials have been developed for retrograde root filling. These materials come into contact with vital tissues and facilitate biomineralization and apical repair. The objective of this study was to evaluate the cytocompatibility and bioactivity of two bioactive cements, Bio-C Repair (Angelus, Londrina, Pr, Brazil) and TotalFill BC RRM putty (FGK, Dentaire SA, La-Chaux-de-fonds, Switzerland). The biological properties in human periodontal ligament stem cells (hPDLSCs) that were exposed to Bio-C Repair and TotalFill BC RRM putty were studied. Cell viability, migration, and cell adhesion were analyzed. Moreover, qPCR and mineralization assay were performed to evaluate the bioactivity potential of these cements. The results were statistically analyzed using ANOVA and the Tukey test ($p < 0.05$). It was observed that cell viability and cell migration in Bio-C Repair and TotalFill BC RRM putty were similar to the control without statistically significant differences, except at 72 h when TotalFill BC RRM putty was slightly lower ($p < 0.05$). Excellent cell adhesion and morphology were observed with both Bio-C Repair and TotalFill BC RRM putty. Both cements promoted the osteo- and cementogenic differentiation of hPDLSCs. These results suggest that Bio-C Repair and TotalFill BC RRM putty are biologically appropriate materials to be used as retrograde obturation material.

Keywords: retrograde filling materials; biocompatibility; apical surgery; periodontal ligament stem cells; endodontic

1. Introduction

In our daily practice, it is common to find in some problematic cases with lesions such as external or internal resorption, iatrogenic perforations, or periapical lesions that have a doubtful prognosis especially when there is a radiolucent area adjacent to the perforation site, and therefore it is necessary to perform apical surgery with a retrograde root filling [1,2].

Periapical surgery is the last option to save a tooth before performing the extraction when a periapiapical lesion cannot be managed with conventional endodontic retreatment. To achieve the treatment, the material is placed in the root-end cavity sealing the entire area [3–5]. This area is where the stem cells from the periodontal ligament (PDLSCs) are located and are in direct contact with the retrograde root filling material [6,7]. The interaction between the PDLSCs and the material should improve the regeneration of the area; several studies have proven that it favors the healing of the cementum, alveolar bone, gingiva, and periodontal ligament [8,9]. Therefore, it is crucial to conduct cytocompatibility studies of these calcium silicate-based materials and because it is a complicated and challenging treatment, we need the properties of the materials to be optimal [10].

Over the years, the most commonly used materials to perform a retrograde root filling have been mineral trioxide aggregate (MTA), Biodentine, amalgam, super ethoxy benzoic acid (super EBA). However, new materials are appearing on the market, such as calcium silicate materials [2].

Calcium silicate-based materials with numerous properties are appearing on the market that makes them ideal for these cases [11]. Calcium silicate materials are cements with a formulation based on MTA-like material or with minor modifications. Different cements have been studied in the last years and have demonstrated their numerous advantages [12,13]. So far, there is no specific material that meets all the necessary properties (antimicrobial, physical, chemical, and ease of handling) for these situations [14–16]. It is important to note that one material with all these requirements would cause an improvement in the prognosis of these treatments.

Bio-C Repair (Angelus, Londrina, Pr, Brazil) (BCR) is available in an individual syringe and TotalFill BC RRM putty (FGK, Dentaire SA, La-Chaux-de-fonds, Switzerland) (TFB) is available in a jar; both have an excellent consistency to facilitate handling unlike MTA or amalgam, and according to the manufacturer, are bioactive, antibacterial, nonstaining, and promote healing. Some tests were used to check whether these substances are cytotoxic [17].

This study aims to compare the cytotoxic effects and the bioactivity potential of two premixed materials used in retrograde root filling surgery such as BCR and TFB with PDLSCs.

2. Material and Methods

2.1. Isolation of Human Periodontal Ligament Stem Cells (hPDLSCs)

The Ethical Committee of the University of Murcia approved the present study (ID: 2199/2018). Wisdom molars (n = 10) were extracted and transported to the laboratory in Minimum Essential Medium Alpha (α-MEM) (Gibco, Invitrogen, Carlsbad, CA, USA) solution containing 1% antibiotics (Sigma-Aldrich, St. Louis, MO, USA) and fungi zone maintained at 4 °C. Next, after washing three times with PBS, the periodontal tissues were scraped from the middle and the apical part of the root surface and were cut into small fragments with surgical blades. The fragment tissues were digested with an enzymatic solution, Collagenase type I (Gibco, Gaithersburg, MD, USA) during 1 h at 37 °C. Then, periodontal cells were seeded in Minimum Essential Medium Alpha with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Culture medium was replaced every three days. Cells at passages 2 to 4 were used for subsequent experiments.

2.2. Flow Cytometric Characterization

The expression of cell surface markers was detected using fluorescence-activated cell sorting (FACS) (Calibur Flow Cytometer, BD Biosciences, San José, CA, USA). Flow cytometry was used to analyze the immunophenotype of cells at passage 3. Briefly, hPDLSCs (2×10^5) were trypsinized, washed with PBS, and then incubated for 15 min at 4 °C with monoclonal antibodies conjugated with fluorescent dyes. The following antibody cocktails were used: MSC-positive cocktail (CD90, CD105, and CD73) and MSC-negative cocktail (CD34, CD14, CD20, and CD45) (MiltenyiBiotec, Bergisch Gladbach, Germany). Results were evaluated using FlowJo software (FlowJo LLC, Ashland, OR, USA).

2.3. Sample Extracts

BCR and TFB were tested (Table 1). Under aseptic conditions, discs of these cements (diameter 5 mm and height 2 mm) were prepared and stored in Minimum Essential Medium Alpha to achieve setting. Following the recommendations of ISO 10993-5 (Biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity) and ISO 10993-12 (Part 12: Sample preparation and reference materials), cements extracts were collected and diluted in free-serum culture medium (1:1, 1:2, and 1:4) [18].

Table 1. Materials tested.

Materials	Manufacturer	Composition
BIO-C Repair	Angelus, Londrina, Brasil.	**Powder**: Calcium Silicate (Ca_2SiO_4), Calcium Oxide(CaO), Zirconium Oxide(ZrO_2), Iron Oxide ($Fe_2O_{3)}$, Silicon Dioxide (SiO_2) and Dispersing Agent
TOTALFILL BC RRM Putty	Innovative BioCeramix Inc. Burnaby, Canada	**Powder**: Tricalcium Silicate(Ca_3SIO_4), Dicalcium Silicate (Ca_2SiO_4), Zirconium Oxide (ZrO_2), Tantalum Pentoxide (Ta_2O_5), Calcium Sulfate ($CaSO_4$).

2.4. Cytotoxicity Evaluation

The methyl thiazolyltetrazolium (MTT) (3-(4, 5-dimethylthiazol- 2-yl) -2, 5-diphenyltetrazolium bromide) assay (Sigma) was conducted to detect the metabolic activity, as described previously [19]. Briefly, hPDLSCs were cultured in 96-well plates (2000 cell/well) and treated differentially according to the experimental design (1:1, 1:2, and 1:4 extracts) for 72 h. Cells without extracts served as control. In accordance with the manufacturer's instructions (Sigma) the MTT reagent was added to the wells for 4 h. When the purple precipitate was obviously noticeable, dimethyl sulfoxide (DMSO) (100 µL/well) was added to create the formazan dye soluble. The cover was removed, and the light absorption in each well was evaluated by spectrophotometer (Synergy H1, BioTek, Winooski, VT, USA) at 570 nm (Abs 570).

2.5. Scratch Migration Assay

To assess the effect of different cement extracts on hPDLSCs migration, scratch migration assay was performed. 2×10^5 hPDLSCs/well were seeded onto six-well plates (n = 3) and proliferated to achieve confluency. A scratch was made with a 200 µL pipette tip, and each well was washed three times to remove cell debris using PBS. The wound closure was evaluated at 24 h, 48 h, and 72 h. The wound closure was evaluated using photographs and ImageJ software (National Institutes of Health, Bethesda, MD, USA) to quantify the wound area at three time points after the scratch. The width of the scratches was measured during the following three periods: 0 to 24 h (first period), 24 to 48 h (second period), and 48 to 72 h (third period). Cells without extracts served as control.

2.6. Scanning Electronic Microscopy

For the scanning electron microscope (SEM) analysis, root repair cements were mixed and transferred to previously prepared molds that were 2 mm thick and 5 mm in diameter. Then, discs were subdivided into two groups (n = 3). After three days of cell culture on the discs, cells were fixed with 4% glutaraldehyde in PBS for four hours and dehydrated, air-dried, and sputter-coated with palladium-gold. Finally, cell morphology was evaluated using 100× and 300× magnifications. In addition, chemical compositions and morphological properties of root repair cements were carried out by energy-dispersive X-ray spectroscopy (EDX).

2.7. RT-qPCR Gene Expression Analysis

To evaluate the expression of cementoblastic/osteoblastic-related genes (ALP, CEMP-1, and CAP), 2×10^4 hPDLSCs/well were seeded onto twelve-well plates (n = 3) and stimulated with

undiluted extracts of BCR and TFB, for seven days. Cell cultures without material extracts served as a negative control and cells cultured with an optimized differentiation medium to generate osteoblasts from human mesenchymal stromal cells (StemMACS OsteoDiff Medium; MiltenyiBiotec, Bergisch Gladbach, Germany) served as a positive control. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 1 µg of RNA by using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad) following the manufacturer's instructions. Changes in gene expression were calculated by the 2-ΔΔCT method. The primers used in this study are listed in Table 2.

Table 2. Primers for qPCR analysis.

GADPH	**Forward** 5′ TCAGCAATGCCTCCTGCAC 3′ **Reverse** 5′ TCTGGGTGGCAGTGATGGC 3′
CEMP1	**Forward** 5′ GGGCACATCAAGCACTGACAG 3′ **Reverse** 5′ CCCTTAGGAAGTGGCTGTCCAG 3′
CAP	**Forward** 5′ TTTTTCTGGTCGCGTGGACT 3′ **Reverse** 5′ TCACCAGCAACTCCAACAGG 3′
ALP	**Forward** 5′ TCAGAAGCTCAACACCAACG 3′ **Reverse** 5′ TTGTACGTCTTGGAGAGGGC 3′
RUNX2	**Forward** 5′ TCCACACCATTAGGGACCATC 3′ **Reverse** 5′ TGCTAATGCTTCGTGTTTCCA 3′

2.8. Alizarin Red Assay

Mineralization capacity of root repair cements was evaluated using Alizarin red staining. 2×10^4 hPDLSCs /well were seeded onto twelve-well plates (n = 3) and proliferated to achieve confluency. Then, cells were stimulated with undiluted extracts of BCR and TFB for 21 days. Cells cultured without extracts served as negative control and cells cultured with OsteoDiff media (MiltenyiBiotec) served as a positive control. At the end of the experimental period, the cells were washed with PBS and fixed for 1 h using 70% ethanol. Then, they were incubated with 2% Alizarin Red solution (Sigma AB, Malmö, Sweden) at room temperature in the dark for 30 min. Finally, the absorbance value at 550 nm was measured using the microplate reader.

2.9. Statistical Analysis

Data were presented as the mean ± standard deviation (SD). All analyses were carried out using Graph-Pad Prism (version 8.1.0, GraphPad Software, San Diego, CA, USA). Normal data with equal variance was analyzed using one-way analysis of variance (ANOVA) and Tukey´s test. Significance was defined when $p \leq 0.05$. All assays were performed at least three times.

3. Results

3.1. Characterization of hPDLSCs Immunophenotype

FACS analysis showed a mesenchymal phenotype of cells isolated from periodontal tissues. There was a high expression of CD90, CD105, and CD73 (>95%), and low expression of cell markers CD45, CD34, CD14, and CD20 (<5%) (Figure 1).

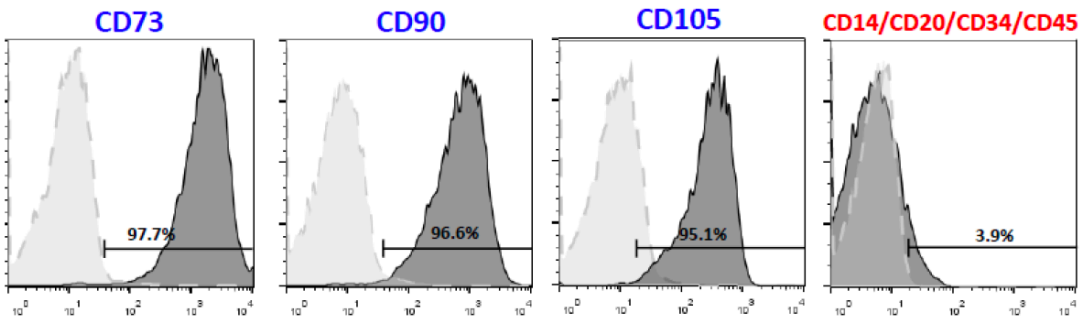

Figure 1. Fluorescence-activated cell sorting (FACS) analysis. Positive expression from cells isolated from periodontal ligament tissue (<95%) of mesenchymal stem cells markers CD90, CD105, and CD73 and negative expression for cells markers CD34, CD45, CD14, and CD20 (<5%).

3.2. Cytotoxicity Evaluation

At 24 h, extracts of Bio-C Repair reduced the viability of hPDLSCs with significant differences as compared with the untreated cells (control) ($p < 0.001$), while TFB exhibited similar cell viability to that of the control. At 48 h, both cements showed adequate cell viability rates, except 1:4 TFB that increased cell viability ($p < 0.001$). Finally, TFB showed slight differences as compared with the control group ($p < 0.05$) after 72 h of incubation, whereas BCR did not suffer any significant variation of cell viability rates as compared with untreated cells group (Figure 2).

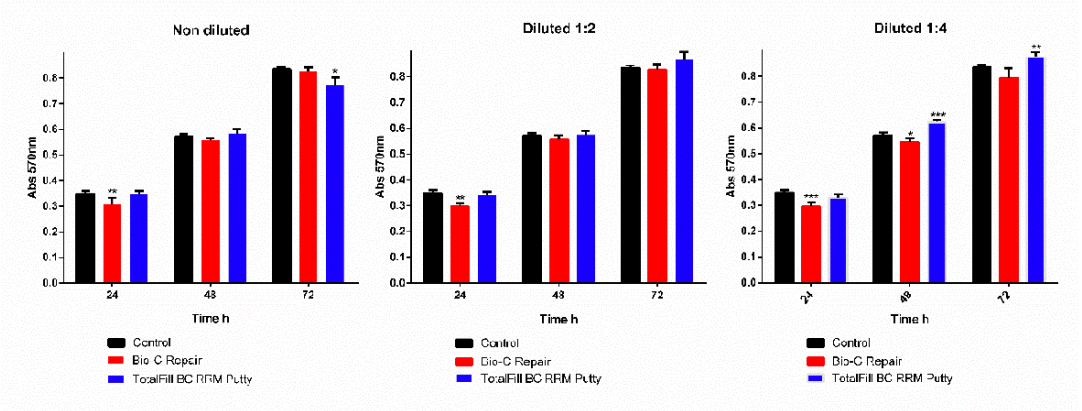

Figure 2. Cytotoxicity assay. Evaluation of human periodontal ligament stem cells (hPDLSCs) treated with Bio-C Repair and TotalFill BC RRM putty eluates. Data shown are the mean (± SD) of three different experiments. Cytotoxic differences observed are shown as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, respectively.

3.3. Scratch Migration Assay

At all time periods and all dilutions studied, cell migration rates in the TFC group were similar to that of the untreated cells, and no detectable differences were found (Figure 3). In the BCR, at 24 h in the nondiluted group, significant differences were found (** $p < 0.01$) and at 48 h significant differences were found; meanwhile, no statistical difference were revealed at 72 h in any dilution as compared with the control group wound closure. These results indicate that both cements had similar migration values to those of the control.

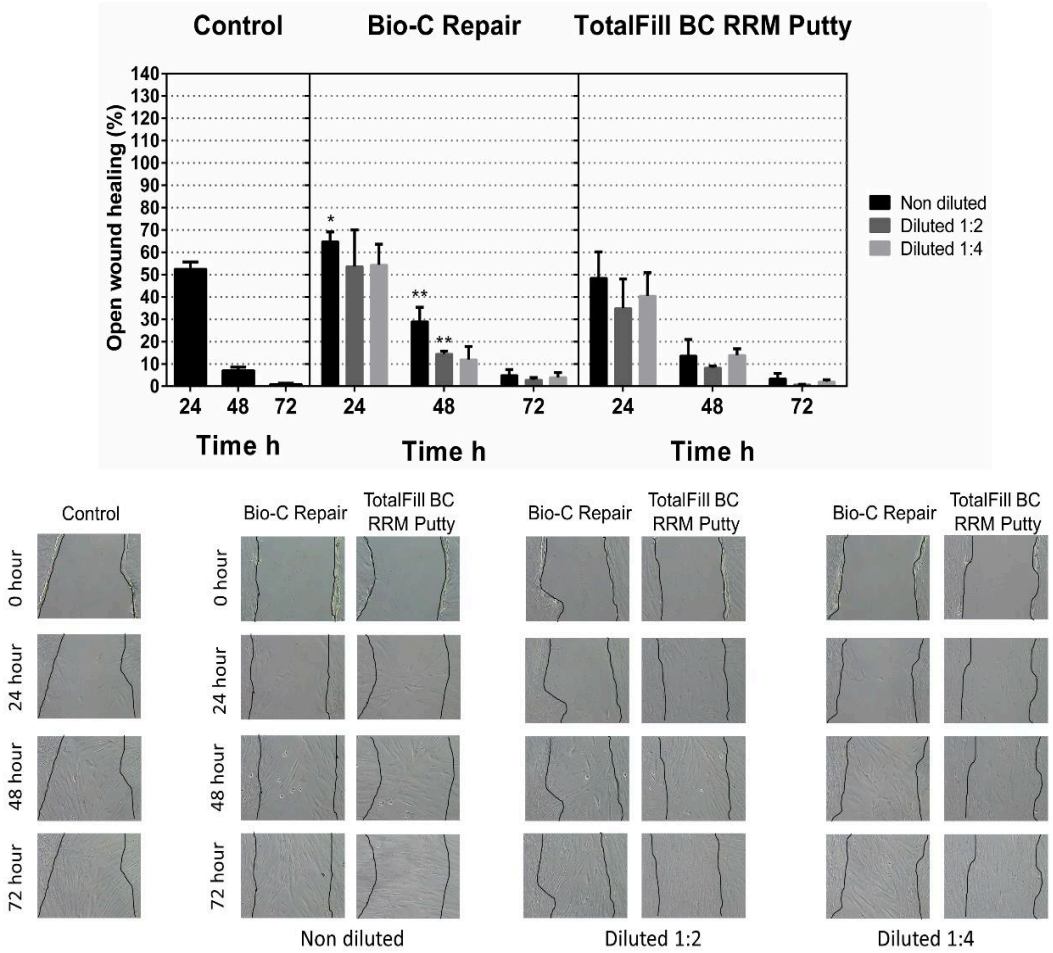

Figure 3. In vitro scratch migration assay. The wound closure was evaluated at 24 h, 48 h, and 72 h, in the presence of different material extracts, or without extracts (control group). Data are expressed as fold of the control group (considered as 100%). Cell migration differences are shown as * $p < 0.005$, ** $p < 0.01$.

3.4. Scanning Electronic Microscopy

Abundant cells were observed in the surface of both cements (Figure 4). The morphology of these cells in contact with these materials suggested an active adhesion interaction with the surface since we can observe multiple prolongations and a flattened morphology. The EDX analysis provided the qualitative semi-quantitative elemental composition of each surface´s material, which are represented in Figure 5. BCR and TFB showed the same elemental composition. C, O, and Si showed different % concentration in both cements. With respect to Ca^{2+}, TFB contained a higher % of Ca^{2+} and Zr as compared with BCR. On the other hand, the % of Si in BCR was higher as compared with TFB.

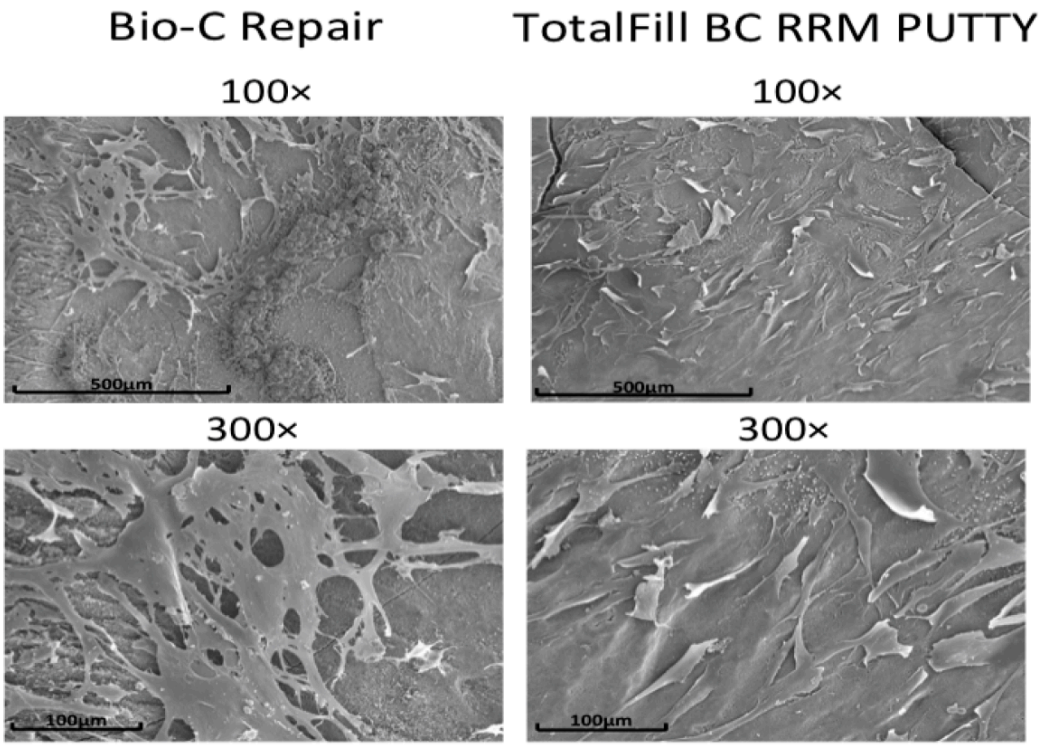

Figure 4. Cell attachment analysis. Adhesion of the stem cells from the periodontal ligament (PDLSCs) on Bio-C Repair (BCR) and TotalFill BC RRM putty (TFB) at 72 h. Scale bar: 100 µm and 500 µm.

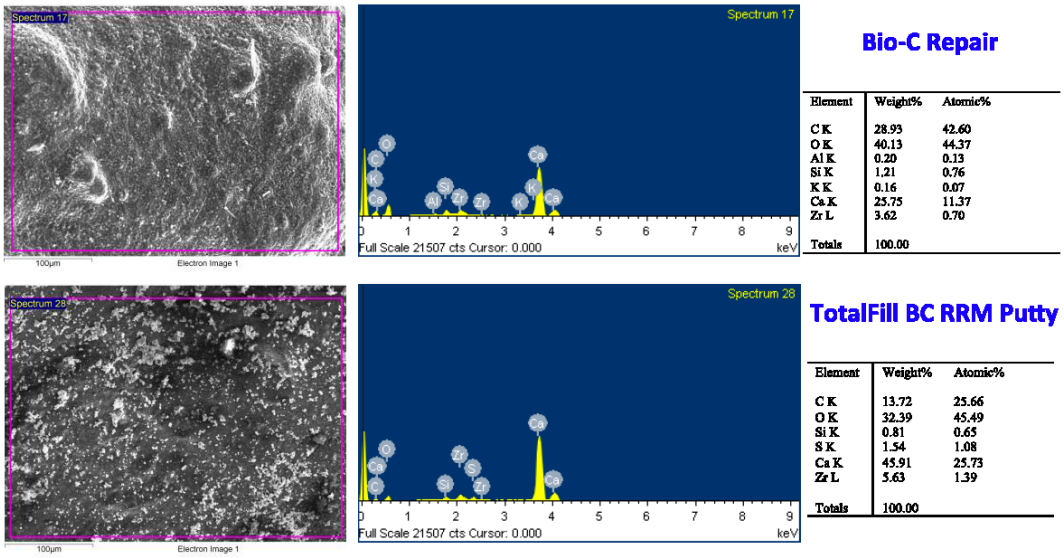

Figure 5. Scanning electron microscopy with energy-dispersive X-ray analysis (SEM-EDX) evaluation. Surface properties and composition of BCR and TFB. SEM image (**left column**), EDX spectra (**middle column**), and table of elements (**right column**). Scale bar represents 100 µm for all images.

3.5. qPCR Analysis

At day seven, CAP, CEMP-1, and RUNX2 expression were higher in Osteodiff and BC-Repair groups as compared with TFB and the negative control group ($p < 0.001$) and also the expression of CAP

and RUNX2 were higher in BCR and TFB as compared with the groups Osteodiff and negative control groups, in different significances (Figure 6) at day 14. GAPDH was used to normalize the results.

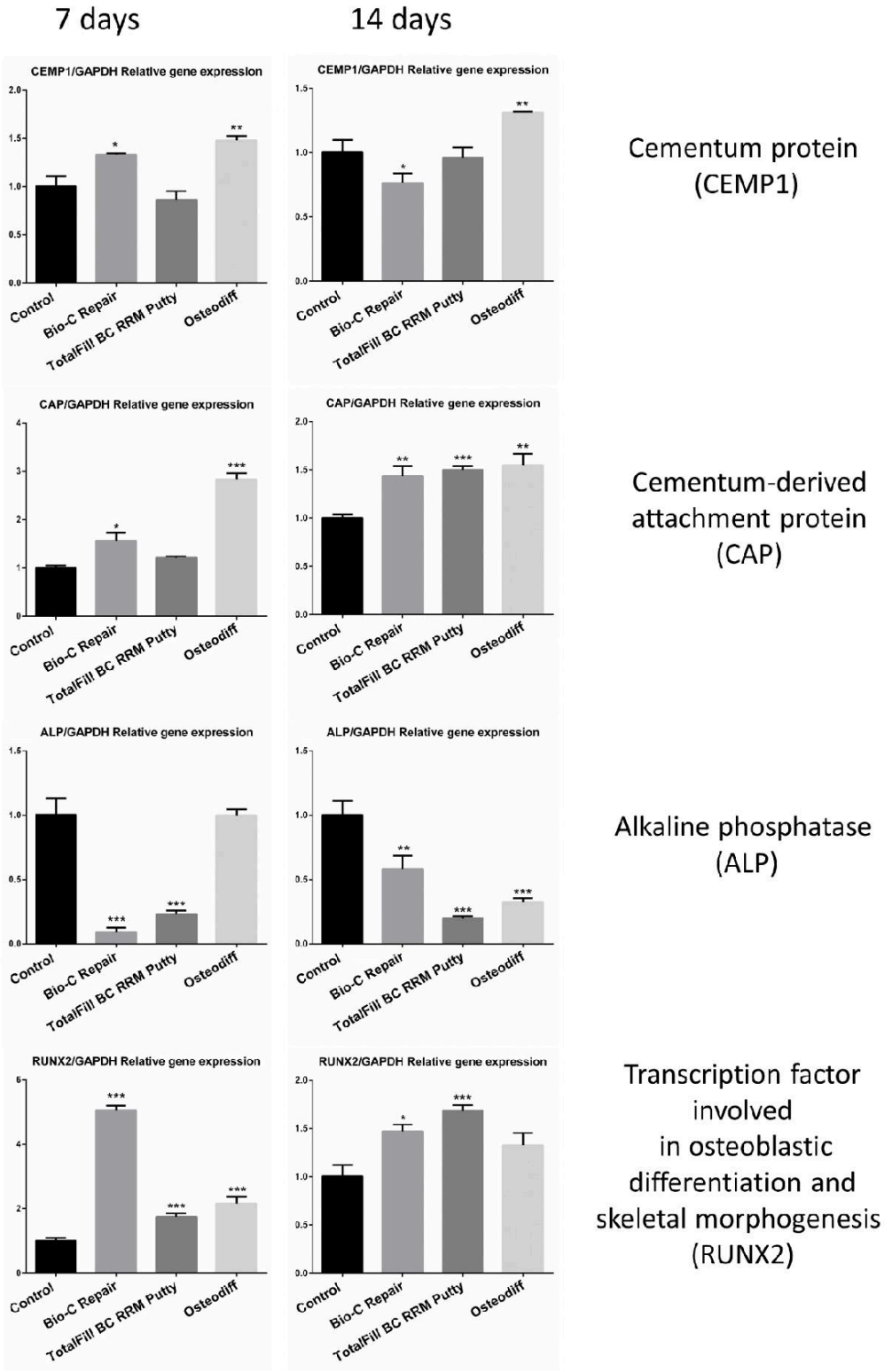

Figure 6. qPCR analysis. Gene expression profiles of hPDLSCs treated with undiluted materials showing expression of ALP, CEMP, RUNX2, and CAP genes. Values indicated with a * represent significant differences between the groups. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3.6. Mineralization Assay

As shown in Figure 7, TFB and BCR promoted significantly more calcium deposits than the negative control ($p < 0.01$). However, Osteodiff (positive control) exhibited more mineralization capacity than root repair cements ($p < 0.001$).

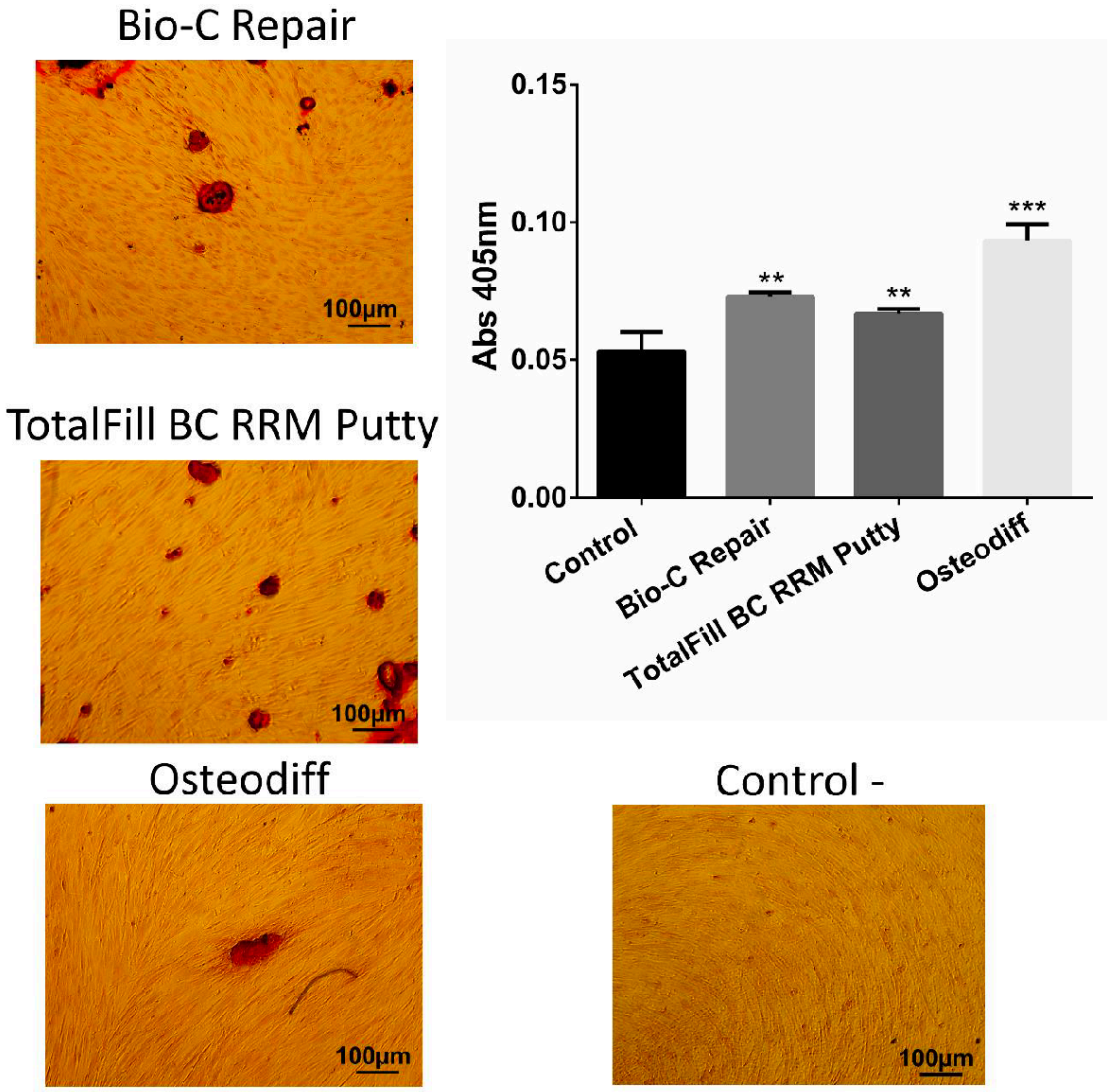

Figure 7. Mineralization capacity of root repair cements. Alizarin Red staining to evaluate the bioactivity potential of BCR and TFB. Differences observed are shown as ** $p < 0.01$ and *** $p < 0.001$.

4. Discussion

The appearance of different materials to treat dental perforation or to perform apical surgery facilitates our choice of options. It is necessary to know the properties of the materials to determine which one is better for each type of problem [20].

In our study, we compared the cytocompatibility and bioactivity potential of these two new bioactive materials, BCR and TFB, with PDLSCs. We chose this cell type because, in the indications of these materials, PDLSCs have contact with the periodontal ligament which are at the root-end cavity when periapical surgery is done or when iatrogenic perforation is repaired it is next to the periodontal ligament [10,21]. Other studies have used dental pulp stem cells with direct pulp capping materials [22], or osteoblast cells when they have also studied endodontic cements [23,24]. Therefore, the clinical situation is crucial for the choice of cell line.

We used the MTT test due to its ease of performing and its accuracy [25]. In addition, the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay is commonly accepted for in vitro cytotoxic studies. TFB and BCR showed similar results in terms of cell viability as compared with a control group. In line with this work, previous reports have described similar results, also using the MTT test with TotalFill BC sealer, which has an almost identical composition, except it changes consistency [25,26]. Benetti et al. [27] also reported that BCR is a biocompatible material that induces mineralization in vivo. Unsurprisingly, our results evidenced that both cements were safe and did not promote adverse effects on hPDLSCs.

Cellular migration is essential for an apical healing after a retrograde root filling, or after repair of a furcal iatrogenic perforation because it helps in calcified tissue formation [28]. In our study, both materials allow cell migration; this may be due to calcium ions since they favor migration [29]. The SEM-EDX assay showed a high amount of Ca^{2+} ions in both cements, especially in TFB, which could help increase the pH of the area and have a positive influence on healing [30]. Our results are consistent with other studies that have shown the migration capacity of the root repair materials. For example, iRoot FS (Innovative Bioceramix, Vancouver, BC, Canada) showed cell migration rates higher than ProRootMTA (Dentsply, Tulsa, OK, USA) [28], or Biodentine (Septodont, St Maur des Fosses, France) has been evidenced by anti-inflammatory properties and favored fibroblast migration to the injury site [31]. Nevertheless, the detailed mechanism of how premixed and putty calcium silicate materials favors cell migration is unknown.

The connection between cells and biomaterial is essential for cell differentiation, and cell attachment has been associated with actin stress fibers [32,33]. Both premixed material´s surfaces favored cell adhesion. In our study, hPDLSCs displayed a well interconnected characteristic fibroblastic morphology. This result is congruent with another report that showed that calcium silicate materials facilitated cell attachment and promoted cell proliferation [34]. Furthermore, a biological seal on the root repair materials can be achieved by the interactions between materials and hPDLSCs.

Bioactivity potential is defined as the cellular effects induced by ions or active substances from biomaterials [35]. Our results showed overexpression of CEMP-1, CAP, and Runx2 with BCR on day seven. These genes are involved in the osteo- and cementogenic differentiation of stem cells, and consequently in apical healing. CEMP-1 is related to migration, proliferation, and differentiation of periodontal ligament cells into cementoblast-like cells. CAP promotes maturation and deposition of the mineralized extracellular matrix. Runx2 is considered a transcriptional regulation factor, closely associated with the early stage of osteogenesis [36,37]. Previous data have shown the overexpression of these genes in the presence of bioactive endodontic materials [38]. In agreement with our results, Lee et al. [39] showed overexpression of osteogenic markers in the presence of other bioactive cements such as ProRootMTA (Dentsply, Tulsa, USA), Biodentine, and Bioaggregate (Innovative Bioceramix, Vancouver, BC, Canada). Our group also demonstrated that GuttaFlow Bioseal (Coltène/Whaledent AG, Altstatten, Switzerland), a bioactive silicon material, induced AMELX, AMBN, CAP, and CEMP1 overexpression, and reduced ALP expression in hPDLSCs [40].

Alizarin Red assay was assessed to evaluate mineralization capacity of the root repair cements; both cements produced more calcium deposits than the negative control, confirming the inductive effect of both root repair materials on the mineralization and differentiation of hPDLSCs. Previous reports have shown that putty calcium silicate materials induced mineralization [25,27]. Calcium silicates are the main components of both cements (Table 1) which justifies the result obtained.

The number of studies on these new calcium silicate-based materials is meager, and it would be interesting to develop studies on mechanical, sealing, and cytotoxicity properties.

5. Conclusions

These results suggest that BCR and TFB are biologically appropriate materials to be used as root repair material or root-end filling.

Author Contributions: Investigation and methodology, S.L.-G. and D.G.-B.; supervision and visualization; conceptualization and data curation, M.R.P.-L., M.P.P.-L. and J.G.-G.; investigation, methodology and writing-original draft, A.L., C.L.; conceptualization, formal analysis, project administration, supervision, validation and writing—review & editing, L.F. and G.S.; investigation, methodology, project administration, resources, writing-original draft and writing—review & editing, F.J.R.-L. All authors have read and agreed to the published version of the manuscript.

Funding: The Spanish Net of Cell Therapy (TerCel), RETICS subprograms of the I+D+I 2013-2016 Spanish National Plan, and project "RD16/0011/0001" funded by the Instituto de Salud Carlos III to JMM and co-funded by the European Regional Development Fund supported this work.

Conflicts of Interest: The authors declare that there is no conflict of interest related to this article.

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Allogeneic Bone Marrow Mesenchymal Stem Cell Transplantation in Tooth Extractions Sites Ameliorates the Incidence of Osteonecrotic Jaw-Like Lesions in Zoledronic Acid-Treated Rats

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Received: 25 April 2020; Accepted: 26 May 2020; Published: 31 May 2020

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Abstract: Medication-related osteonecrosis of the jaw (MRONJ) is defined as the exposed necrotic bone involving the maxillofacial structures in bisphosphonate treated patients, and the pathophysiology of this disease remains unclear. The aim of this study was to assess the effects of the allogeneic transplantation of bone marrow-derived mesenchymal stem cells (BM-MSCs) in a model of Wistar mice with induced MRONJ disease. BM-MSCs from five male Wistar rats were characterized and cultured on β-tricalcium phosphate (β-TCP) granules. Thirty female Wistar rats were injected intraperitoneally with zoledronic acid and afterwards upper jaw molars were extracted. The animals were randomized to receive: Group 1: 1×10^6 BM-MSCs/β-TCP construct in the alveolar socket; and Group 2: Saline solution/β-TCP construct. A clinical and histological analysis was performed. Nested polymerase chain reaction (PCR) was assessed to verify the presence of transplanted male rat cells in the female recipient jaws. Clinical and histological findings evidenced that none of the animals in Group 1 exhibited uncovered sockets or bone exposure associated to MRONJ, whereas we detected 33% of MRONJ cases in Group 2. In addition, male rat cells were detected in the maxillae site four weeks after transplantation in the BM-MSCs-group. Allogeneic BM-MSCs in extractions sites ameliorates MRONJ incidence in zoledronic acid-treated rats compared to non-MSC treatments.

Keywords: bone marrow mesenchymal stem cells; zoledronic acid; osteonecrosis; jaw; MRONJ

1. Introduction

In 1906, necrosis of jaw was described among those people working in the matchstick industry, an occupational disease provoked by inhalation of white phosphorus vapor and lack of the appropriate security measures. The incidence of these disorder, called "phossy" jaw almost disappeared after the ban on the use of phosphorus in matchsticks at the convention held in Bern (Switzerland) [1].

Nevertheless, a reappearance of this disease was noticed and reported in 2003, when osteonecrosis of the jaw was identified as an adverse side effect of pamidronate and zoledronate treatment [2]. These bisphosphonates—and other type of anti-resorptive drugs used for preventing osteoclast-mediated bone resorption—have been widely used to treat primary osteolytic bone pathologies such as Paget disease, osteoporosis, multiple myeloma and metastatic bone disease [3].

Medication-related osteonecrosis of the jaw (MRONJ) is defined as the exposed necrotic bone involving the maxillofacial structures that fails to heal after eight weeks. It has a great impact in the oral and maxillofacial clinical area and the pathophysiology of this disease remains unclear to date. Possible pathophysiologic mechanisms involved in MRONJ include the delay of bone remodeling [4,5], direct toxicity to the mucosal barrier [6,7], an altered angiogenesis that could lead to avascular necrosis [8,9], and an altered function of macrophages and T cells that would facilitate subsequent infections [10].

The aim of the treatment of patients at risk to development MRONJ should be based on prevention [11], being as once the disease is established, therapeutic interventions are not uniformly successful and have been controversial in recent years [12]. Lately, new regenerative medicine strategies such as cell therapy using mesenchymal stem cells (MSCs) for bone regeneration have arisen as a promising treatment [13–16].

MSCs are adult progenitor cells distributed in every organ and tissue with capacity to differentiate to adipocytes, osteoblasts and chondrocytes [17]. They have shown promising results in cell architecture repair, wound healing and recovery of local blood flow in damaged and ischemic tissues [18]. In addition, MSCs secrete multiple cytokines and trophic factors with immunomodulatory and anti-inflammatory properties [13,16], and also, they lack human leukocyte antigen (HLA)-II expression and a limited expression to HLA-I, that make them suitable for allogeneic transplantation. Remarkably, MSCs can be easily obtained under local anesthesia from various anatomic sites such as bone marrow, adipose tissue, dermis, neural tissue, periodontal ligament and dental pulp [19–21], all of which are reasonably non-invasive procedures. Furthermore, ex vivo culture and expansion is feasible and not difficult.

To date, few studies have tested the beneficial properties of MSCs for MRONJ prevention and treatment, including their systemic infusion [13,16,22] or local application [23]. Some of these studies in different animal models generated encouraging clinical results, but more research is needed prior to the clinical application of this therapeutic approaches. In this study, we aim to assess the effects of the allogeneic transplantation of bone marrow-derived-MSCs (BM-MSCs) in a proven model of Wistar mice with induced MRONJ disease.

2. Experimental Section

Animals used in this study were obtained from the Animal Facility Research Support Unit at the University of Murcia (Spain) (REGA ES300305440012). The Bioethics Committee at the University of Murcia (A1320141001) approved the experimental protocol. The entire study was conducted according to the European Union guidelines for animal experimentation (EU/63/2010).

In this quantitative and experimental research study, a total of 35 Wistar rats (30 female and 5 males; 250–300 g; 8–12 weeks old) were included. Rats were kept in clean cages (five animals per cage) with litter bedding and 12-h light cycle. Normal diet and water were provided ad libitum.

2.1. BM-MSCs Isolation, Characterization and Culture on Scaffolds

Donor healthy male Wistar rats (n = 5) were anesthetized and sterilized using intraperitoneal injection of sodium thiopental (50 mg/kg) using 75% ethanol for 20 min. After removing the

femurs under sterile conditions, cells were flushed out with phosphate buffer saline (PBS) with penicillin/streptomycin. Bone marrow mononuclear cells were isolated by Ficoll density gradient centrifugation over Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA), plated in 175 cm^2 culture flask at 1.5×10^5 cells/cm^2 in DMEM low glucose (Gibco, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10 fetal bovine serum (Gibco, Thermo Fischer Scientific, Waltham, MA, USA), 1% L-glutamine (Lonza, Basel, Switzerland), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza, Basel, Switzerland) (complete culture medium) and incubated at 37 °C and 5% CO_2. BM-MSCs from passages 3 were used in all experiments.

Immunophenotype characterization of BM-MSCs were analyzed by flow cytometry using a FACSCanto flow cytometer (Beckton Dickinson, San Jose, CA, USA) after staining with fluorochrome-conjugated monoclonal antibodies specific for markers CD73 (clone 5F/B9, Beckton Dickinson, San Jose, CA, USA), CD90 (clone HIS51, eBioscience, San Diego, CA, USA), CD105 (clone 8A1, Abcam, Cambridge, UK), CD34 (clone ICO-115, Abcam, Cambridge, UK) and CD45 (clone OX1, eBioscience, San Diego, CA, USA).

A commercially available bone graft substitute (granules) was used: synthetic β-tricalcium phosphate (β-TCP) (Odoncer, Teknimed, L'Union, France) with size of 0.5–1.0 mm, 50% porosity and pore size between 100–1000 µm. This dimension was appropriate for the specific subcutaneous/intramuscular implantation. Under aseptic conditions in the laminar flow hood, the sterile β-TCP granules were pre-moistened in complete medium for 30–60 min. For cell seeding in the study group, BM-MSCs were detached from the culture flasks by trypsinization, centrifuged at 400 g and then re-suspended in complete culture medium.

To assess the continuing effect of β-TCP on the behavior of BM-MSCs in terms of cell adherence and growth, study periods of 24 h and 7 and 15 days were established. Then, BM-MSCs were directly seeded onto β-TCP granules at a density of 5×10^4 cells/mL. In the control group, β-TCP granules were pre-moistened with complete culture medium without BM-MSCs. After 24 h, 7 and 15 days of culture, the cell-scaffold constructs were fixed with PBS and 3% glutaraldehyde in 0.1 M cacodylate buffer for 1.5 h at 4 °C. Then, they were rinsed again and dehydrated via a graded series of ethanol (30–90% v/v). Final drying was performed by the critical-point method (CPDO2 Balzers Union, Balzers, Liechtenstein). Before observation with a scanning electronic microscope (SEM) (JEOL-6100, Oxford Instruments, Abingdon, United Kingdom), samples were mounted on stubs and sputtered gold/palladium coated.

2.2. Experimental Design

Rats were considered as animal model for bisphosphonate-related osteonecrosis of the jaws due to is bigger size more suitable for manipulations, extractions and implant placement than mice [24,25]. All female animals (n = 30) received zoledronic acid (ZA) (Zometa® 0.05 mg/mL (Teva Pharmaceutical Industries, Petaj Tikva, Israel)), at a dose of 0.1 mg/kg body weight. The rats were weighted before every experimental phase to properly dose the administered drugs, as well as for controlling weight gain or loss during the study. The medication was administered by intraperitoneal injection three times per week, for nine weeks in accordance with previous studies [23,26].

The rats were randomly assigned to the following groups:

Group 1 consisted of 15 female rats that received ZA + implantation of 1×10^6 allogeneic BM-MSCs/β-TCP construct.

Group 2 (control) consisted of 15 female rats that received ZA + implantation of PBS/β-TCP construct.

Extractions of the three right upper molars in each animal were performed in the eighth week of treatment. One hour before the procedure, dipyrone was applied subcutaneously (160 mg/kg). The rats were weighed and then intraperitonially injected with 100 mg/kg of ketamine (Ketavet 100, Gellini Farmaceutici Spa, Peschira Borromea, Milan, Italy), in combination with 10 mg/kg of xylazine (Rompun, Bayer AG, Leverkusen, Germany) for general anesthesia. The three molars were dislocated and removed with infant forceps number 1. In addition to the exodontia, it was also carried out a bone cut (osteotomy) in the surgical cavity, to insert the scaffolds. For the fixation of

the scaffold in the cleft, animals were treated with a buccal mucoperiosteal flap to cover the alveolar bone post-extractions. Light subperiosteal debridement and advancement of the buccal mucosa were performed. Subsequently, gingival borders were sutured with 6–0 Nylon thread and washed with polyvinylpyrrolidone-iodine, covering the defect without tension. Tramadol at a dose of 0.075 mg/kg body weight was administrated subcutaneously each day for the first 3 post-operative days.

2.3. Macroscopic Analysis

Changes in wound healing of tooth extraction site was performed at 4 weeks post-extraction, because of the faster healing period reported in rats compared to humans [27]. Clinical analyses were assessed by the senior investigator and a maxillofacial surgeon in a lighted room as previously published [28]. The following parameters were analyzed: exposed bone, level of wound healing, infection and degree of inflammation. A scale from 0–3 was used, 0 representing favorable healing and absence of clinical signs of osteonecrosis and 3 representing severe signs of impaired healing and osteonecrosis.

2.4. Histological Observation

Maxillary bone samples were fixed in 4% paraformaldehyde for 48 h at 4 °C. Specimens were decalcified with 10% ethylenediaminetetraacetic acid (EDTA) solution at room temperature (RT) for 4–6 weeks. Then, samples were dehydrated in an ascending series of ethanol solution and finally embedded in paraffin. Serial sections (3–4 μm) were obtained and stained with hematoxylin and eosin (HE) staining according to protocol. These pictures were recorded using a light microscope (Olympus, Tokyo, Japan).

Immunohistochemical analysis for osteocalcin expression was performed with an indirect-ABC immunohistochemical procedure was performed by using a commercial kit (Dako EnVision Flex (Agilent, Santa Clara, CA, USA). Briefly, after deparaffination, rehydration, demasking antigen and peroxidase blocking treatments, sections were incubated overnight with a polyclonal anti-osteocalcin antibody (Abcam, Cambridge, UK, dilution 1:200) and with the labeled polymer anti-rabbit for 20 min. Sections were revealed with 3–3′ diaminobencidine (DAB) and hematoxylin counterstained. Positive reaction was identified as a dark-brown precipitated, with an osteoblastic intracytoplasmic pattern.

For immunohistopathologic examination, the following parameters were taken into account: (1) degree of osteonecrosis as presence of 8–10 adjacent empty lacunae in alveolar bone, according with previously reports [29,30]; (2) inflammatory infiltrate; (3) degree of fibrosis; (4) neoformed bone; (5) neovascularization; (6) osteocalcin expression; and (7) presence of osteoclasts. Evaluation was performed in a semi-quantitative scale as follows: absence (value 0), mild (value 1), moderate (value 2), severe (value 3) and very severe (value 4); except for evaluation of neoformed bone that was as follows: absence (value 0), 5–25% (value 1), 25–50% (value 2) and >50% (value 3). Lastly, the presence of osteoclasts was represented as the average numbers of osteoclasts per 10-high power fields (×400).

2.5. Nested Polymerase Chain Reaction (PCR) Protocol for Y Chromosome-Specific DNA Detection in Rat Decalcified Jaw Samples

Nested polymerase chain reaction (PCR) was assessed to determine the presence of transplanted male rat cells in the female recipient jaws using the sex-determining region of the Y chromosome (SRY) as a quantification marker. DNA was extracted from formalin-fixed, paraffin-embedded rat jaws sections using QiAamp DNA FFPE tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Then, xylene was added to four 5-μm-thick sections of FFPE samples to remove paraffin. Tissue was digested with proteinase K at 56 °C for 1 h. After washing, DNA was eluted with distilled water. The extracted DNA was quantified by absorbance at 260 nm and its purity was evaluated by the absorbance ratio at 260/280 nm with a NanoDrop-2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Detection of rat transcribed testis specific protein (TSPY) gene, a Y-chromosome exclusive gene, was performed by two consecutive PCRs. The product of the first PCR was used

as a template in the second PCR. Both PCRs were carried out by using 35 cycles of 94 °C for 30 s, 60 °C annealing temperature for 45 s and elongation at 72 °C for 40 s. Reactions were performed using 1-U/µL Taq DNA polymerase (Roche Diagnostics, Basel, Switzerland) using the supplier's buffer (1×: 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH 8.3), 0.2 mM mix dNTPs and 1 µM of each primer. The primer sequence was as follow: TSPY-Fwd: ATTCCGGGAACTGGTACTCC; and TSPY-Rev: AGGGGTACCCAATCTTCCAC. The final PCR product was run on an 2% agarose gel. Molecular weight marker 0.5 µg/lane of 100 bp DNA Ladder (Invitrogen, Molecular Probes, Eugene, CA, USA) was used.

2.6. Statistical Analysis

Data were analyzed using the Graph-Pad Prism (version 8.1.0, GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test for parametric values and Mann–Whitney U test for nonparametric values were used. Data are presented as the mean ± standard deviation. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Isolation, Characterization and Culture on Scaffold

After isolation, the average time required for cell adhesion was 48–72 h. Isolated cells evidenced a radiative or spindle shape and the polar growth under a microscope. Bone marrow cells contained heterogeneous cell population in shape, among which MSCs were no more than 50%. After one month of culture, a homogeneous population of stromal cells was evidenced.

To verify the mesenchymal immunophenotype, BM-MSCs were characterized by flow cytometry. Cells expressed levels of specific MSC-surface markers, such as CD73, CD90 and CD105 and lacked the expression of the non-specific markers of MSCs (hematopoietic): CD34 and CD45 (Figure 1).

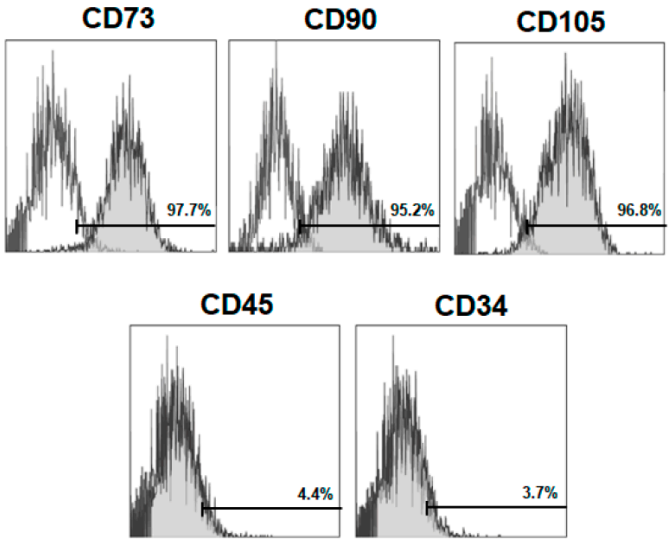

Figure 1. Rat bone marrow-derived-MSCs (BM-MSCs) express the typical mesenchymal stem cells (MSCs) markers CD73 (97.7%), CD90 (95.2%) and CD105 (96.8%), whereas expression of CD45 and CD34 were low or negative. Specific antibodies staining (light gray histograms) were compared with their corresponding control isotypes (white histograms). Values inside histograms represent percent of positive cells for each specific marker.

BM-MSCs adhesion and growth on β-TCP granules was performed by scanning electron microscopy (SEM) micrographs. After 24 h, few BM-MSCs were detected adhered to β-TCP granules.

Importantly, at 7 days, it was observed that cell density was increased and covered the biomaterial, exhibiting multiple extensions that anchored the cells to the β-TCP granules. After 14 days of culture, multilayered cultures of BM-MSCs adhered to the biomaterial, were evidenced. Furthermore, irregular-shaped particles and numerous extracellular matrix components and were observed on the surface of the cells (Figure 2).

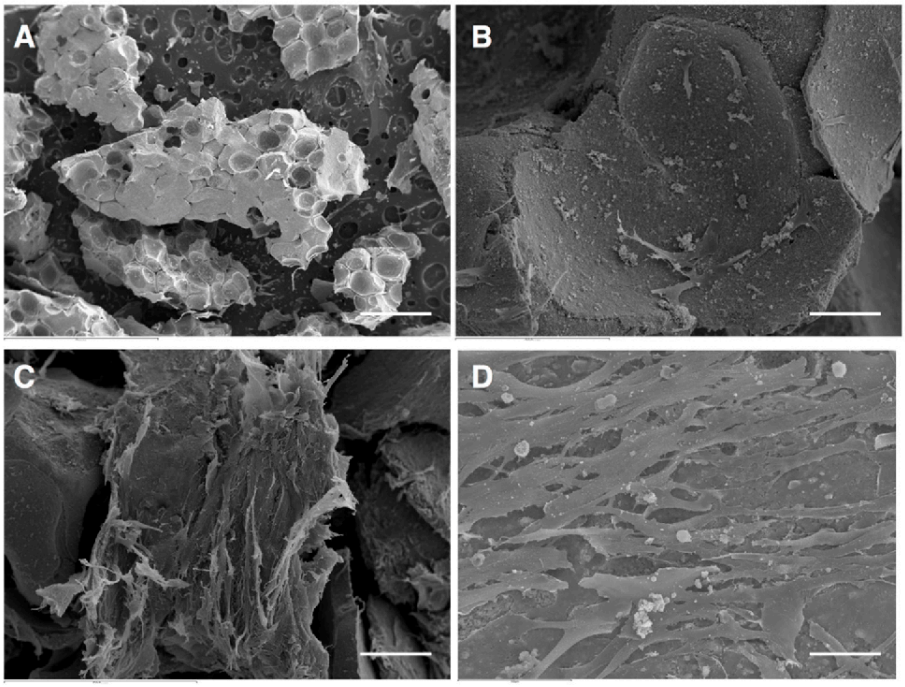

Figure 2. Scanning electron microscopic photomicrographs showing BM-MSC morphologic features and attachment and growth on β-tricalcium phosphate (β-TCP) scaffolds. (**A**) Morphology of β-TCP scaffolds without cells; (**B**) few cells were detected adhered to β-TCP granules after 24 h of culture; (**C**) abundant cells well adhered to β-TCP granules and active adhesive interactions with the scaffold surface was observed at 7 days; (**D**) multilayered cultures of BM-MSCs adhered to the β-TCP granules was observed at 14 days. Scale bar: 100 μM.

3.2. Clinical Visualization of MRONJ

After 4 weeks of tooth extraction, none of the animals in Group 1 (i.e., rats that received ZA + implantation of 1×10^6 allogeneic BM-MSCs/β-TCP constructs) evidenced bone exposure or uncovered sockets associated to MRONJ (Figure 3A). In contrast, 33% of the animals in Group 2 (i.e., rats that received ZA + implantation of saline/β-TCP constructs) showed osteonecrotic jaw-like lesions such as mucosal ulcerations at the teeth extraction site and frequent exposure of necrotic bone areas (Figure 3B).

3.3. Histological Analysis

Microscopic examination of maxillary bone samples after HE staining revealed that animals of the control group treated only with the β-TCP scaffolds (i.e., w/o BM-MSCs) displayed typical histopathologic signs of osteonecrosis, i.e., formation of granulation tissue, inflammatory cell infiltrates, fibrosis and sequestra (Figure 4A, top images). Conversely, BM-MSC + β-TCP scaffold treated-group of animals showed new bone formation areas and a concomitant substantially reduced degree of osteonecrosis 4 weeks after BM-MSC transplantation (Figure 4A, bottom images).

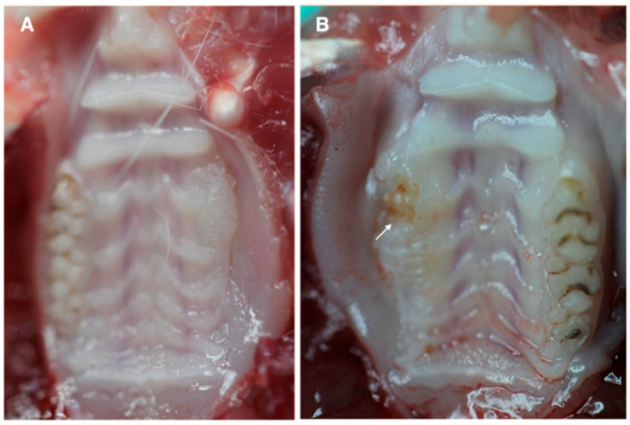

Figure 3. Tooth extraction sites in ZA-treated rat mandibles 4 weeks after being transplanted with (**A**) BM-MSC + β-TCP scaffold or (**B**) control saline + β-TCP scaffold. The last group displayed osteonecrotic lesions such as mucosal ulcerations and exposure of necrotic bone areas.

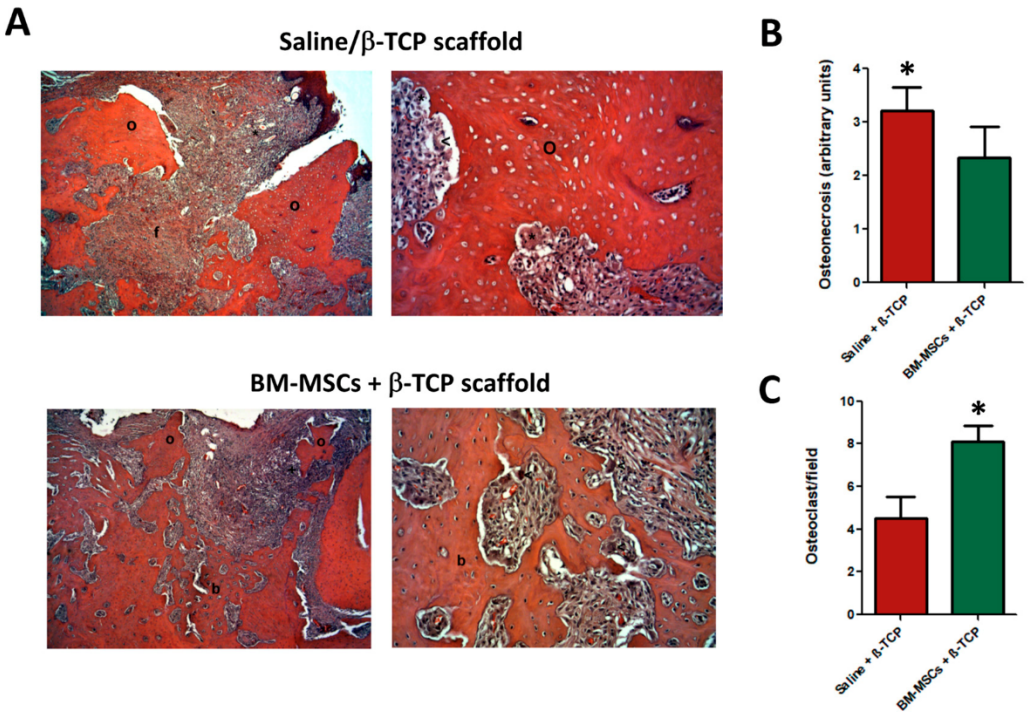

Figure 4. Representative images of HE staining of maxillary bone after induction of osteonecrosis in control animals transplanted only with saline + β-TCP scaffold, (**A**, top images) and those treated with BM-MSCs + β-TCP scaffold (**A**, bottom images) 4 weeks after treatment. Left images magnification: ×100; Right images magnification: ×400; (**B**) control animals displayed a higher degree of osteonecrosis than BM-MSC-treated counterparts (* $p < 0.05$) whereas osteoclast number per high-power field at 400× was significantly augmented in (**C**) the BM-MSC-treated group (* $p < 0.05$). Symbols: o: osteonecrotic areas; f: fibrotic areas; b: bone neoformation.

Using a semi-quantitative scale for histological analysis, we showed that control animals displayed a higher degree of osteonecrosis than BM-MSC-treated counterparts ($p < 0.05$) (Figure 4B). Importantly, osteoclasts number, i.e., bone cell population that mediates bone resorption, was significantly increased in the BM-MSCs-treated group samples ($p < 0.05$), while the necrotic zone of the alveolar bone in the control group harbored lower osteoclast density (Figure 4C).

Histomorphometrical analysis demonstrated that only the BM-MSC-treated group showed a significant increased bone neoformation compared to control bone samples ($p < 0.001$) (Figure 5A). This new tissue contained abundant osteocytes and osteoblast cells in its lining surface (Figure 4A, bottom images). By contrast, the presence of necrotic bone or sequestra with loss of osteocytes from their lacunae was evident in control bone samples (Figure 4A, top images).

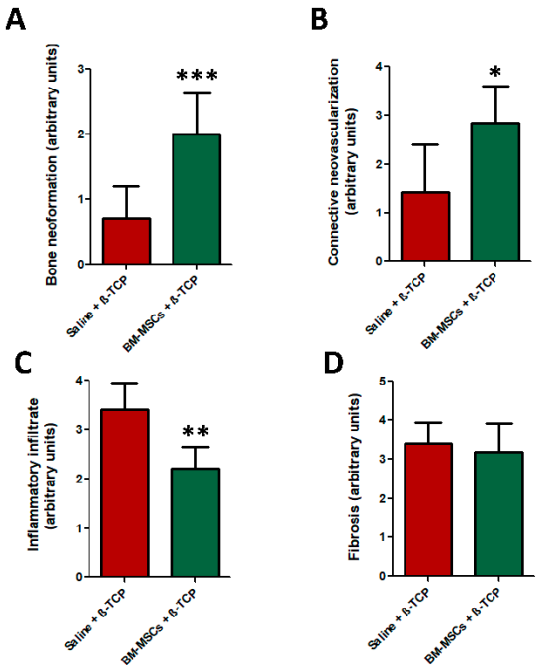

Figure 5. Evaluation of the effect of BM-MSC mediated bone neoformation, connective tissue neovascularization, inflammatory infiltrate and degree of fibrosis. (**A**) Maxillary bone of BM-MSC + β-TCP-treated animals showed a significant degree of bone neoformation; (**B**) connective tissue neovascularization and (**C**) significant lower inflammatory infiltrate, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, respectively; (**D**) however, the degree of fibrosis was not significantly different between both experimental groups.

In addition, the effects of MSC-therapy on neovascularization of the connective tissue of tooth extraction sockets, degree of inflammatory infiltrate and degree of fibrosis were also analyzed. BM-MSCs-group samples displayed a significant increased number of blood vessels and vessel surface area compared to the control group (Figure 5B), as well as lower amounts of inflammatory cell infiltrates ($p < 0.01$). Regarding the degree of fibrosis, no significant differences between both experimental groups were detected (Figure 5D).

Regarding osteocalcin expression, BM-MSCs-treated group showed an evident higher expression of osteocalcin positive cells compared to control group ($p < 0.001$), thus evidencing the higher degree of bone neoformation previously addressed (Figure 6A–C).

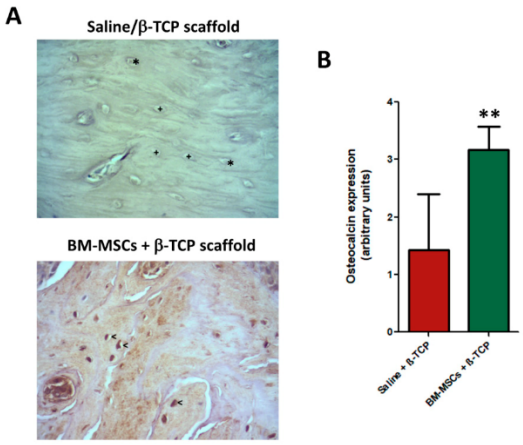

Figure 6. Representative images of immunohistochemical expression of osteocalcin of maxillary bone after induction of osteonecrosis in control animals transplanted only with saline + β-TCP scaffold, (**A**, top image) and those treated with BM-MSCs + β-TCP scaffold (**A**, bottom image) 4 weeks after treatment. While in control animals the empty lacunae (+) and osteocytes (*) are negative, osteocytes from BM-MSC-treated bone strongly expressed osteocalcin (<). ABC immunohistochemical procedure anti-osteocalcin were performed. Magnification: ×400; (**B**) number of osteocalcin positive cells were significantly more abundant, ** $p < 0.01$.

3.4. Y-Chromosome Detection

Finally, bone marrow stromal cells, that were initially isolated from male rats, were detected in the maxillary site of transplanted female rats 4 weeks after transplantation in the BM-MSCs-treated group by detecting the sex-determining region of the Y-chromosome, while, as expected, there were no detected cells in the saline + β-TCP group (Figure 7).

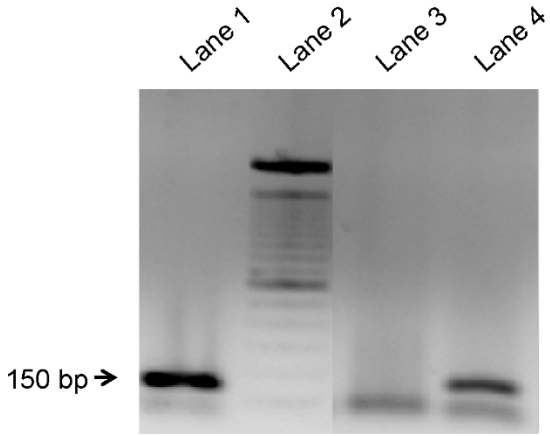

Figure 7. Representative analysis of nested polymerase chain reaction (PCR) for defining the presence of transplanted male rat BM-MSCs in the female recipient maxillary using the sex-determining region of the Y chromosome as a quantification marker. PCR amplification products were 150 bp. Lane 1: positive control (liver cells from male rats); Lane 2: 100 bp DNA ladder; Lane 3: maxillary cells from female rats transplanted with saline + β-TCP scaffold; Lane 4: maxillary cells from female rats transplanted with BM-MSCs + β-TCP scaffold.

4. Discussion

The prevention or treatment of MRONJ disease in patients receiving high doses of intravenous anti-resorptive drugs are still an unmet clinical need for maxillofacial surgeons. The estimated incidence of MRONJ in patients treated with intravenous ZA or other bisphosphonates for their different clinical applications ranges 0.8–12% [31], but evidence that explains its etiology remain inconclusive. Several therapeutic and preventive protocols for MRONJ have been proposed including conservative measures such as 0.12% chlorhexidine mouthwashes and antibiotics during the initial stages or the surgical approach that includes resection and reconstruction of the maxilla, aiming to prevent infections and/or inflammation and to decrease the stage of the disease. Other therapeutic alternatives have also been developed, including laser, ozone therapy, teriparatide, hyperbaric oxygen therapy, use of autologous platelet concentrates or MSC-based therapies [15,32]. However, BM-MSCs have not yet been proposed as an effective preventive treatment. In our study, we demonstrated that transplantation of allogeneic BM-MSCs in combination with β-TCP scaffolds in tooth extractions sites significantly ameliorates MRONJ in ZA-treated rats compared to those transplanted with only the scaffold.

To date, dental extractions were considered to be the most common risk factor for developing MRONJ and patients with other concomitant oral inflammatory diseases (e.g., periodontitis or dental abscesses) are at increased risk for developing MRONJ [32]. Previous experimental studies of MRONJ suggest that inflammation and oral infections contribute to its pathogenesis [33]. In this regard, there is increasing evidence that cell–cell interactions between MSCs and different immune cell populations, i.e., T cells, B cells, macrophages or dendritic cells, as well as MSC secretion of distinct immunomodulatory molecules or trophic factors, are likely to play role in the beneficial effects of MSC in inflammation-related diseases [34,35].

Previous reports have utilized dogs, mice and minipigs as animal models in MRONJ. However, most studies have utilized rats because they are a clinically relevant, inexpensive and easily replicable animal model with a high frequency MRONJ development after bisphosphonates treatment [23,24,26,36,37]. Furthermore, there are animal models able to develop MRONJ with and without tooth extraction. In these studies, histological examination in the animals injected with bisphosphonates which underwent dental extractions showed wide osteonecrotic areas; however, animals injected with bisphosphonates without tooth extraction only displayed periodontal inflammation [38]. Hence, unilateral extraction of three right molars were performed to trigger MRONJ development, where invasive dental procedures are the number one precipitating the main event for osteonecrosis appearance in bisphosphonate-treated human patients [39].

Autologous BM-MSCs have been utilized in patients with MRONJ lesions after necrosectomy, although the number of studies is very limited [14,15,40]. However, no reports were conducted to analyze the role of allogeneic BM-MSCs transplants to reduce the pathologic consequences of MRONJ. Although bone marrow harvesting is a non-invasive procedure, the medical conditions of the patients (i.e., bone fragility) could make obtaining autologous cells difficult, so we used allogeneic BM-MSCs.

In this study, we observed that animals with ongoing MRONJ and treated only with the β-TCP scaffolds (i.e., w/o BM-MSCs) showed evident histopathologic signs of osteonecrosis that were characterized by the formation of granulation tissue, an abundant inflammatory cell infiltrate, a decreased bone neoformation after tooth extraction, appearance of fibrotic tissue and low connective tissue neovascularization. Conversely, when these β-TCP scaffolds were cellularized with BM-MSCs, these cells were able to significantly reverse some of these osteonecrotic-related histopathologic events. Conspicuously, our results evidenced that necrotic zone of the alveolar bone in control animals was often located adjacent to inflammatory cells infiltrates. In this regard, some authors have also reported a correlation between necrosis and inflammation in MRONJ [13]. Although BM-MSC transplantation did not ameliorate the degree of fibrosis, BM-MSCs decreased the inflammatory infiltrate level, increased bone neoformation and neovascularization and importantly, gave rise to the presence of an abundant number of osteoblasts (osteocalcin positive cells) and osteoclasts, a histopathologic finding closely

related with an active bone remodeling process [41]. In the same line with our results, Mergoni et al. [42] concluded that high expression of osteocalcin is associated with an intense osteoblast activity and bone healing.

Taken together, different cell therapy approaches using mesenchymal stem cells can offer effective and safe therapeutic alternatives for preventing MRONJ development in all those patients who must be treated with anti-resorptive drugs for conditions such as osteoporosis or other metabolic/neoplastic bone diseases.

5. Conclusions

In summary, allogeneic BM-MSCs implanted in extractions sites ameliorates MRONJ incidence in zoledronic acid-treated rats. Further studies are necessary to confirm the rationale for in vivo cell therapy using MSCs to prevent or treat MRONJ disease in human patients.

Author Contributions: Conceptualization, R.O.-S., J.M.M. and D.G.-B.; Data curation, R.O.-S. and D.G.-B.; Formal analysis, R.O.-S. and J.M.M.; Funding acquisition, J.M.M. and D.G.-B.; Investigation, F.J.R.-L., M.G.-G., M.V.-B., C.M.M., B.R.-N., J.G.-G. and D.G.-B.; Methodology, F.J.R.-L., M.G.-G., M.V.-B., C.M.M., B.R.-N. and J.G.-G.; Project administration, R.O.-S., J.M.M. and D.G.-B.; Resources, D.G.-B.; Software, D.G.-B.; Supervision, F.J.R.-L., R.O.-S., J.M.M. and D.G.-B.; Validation, R.O.-S., J.M.M. and D.G.-B.; Visualization, F.J.R.-L., J.M.M. and D.G.-B.; Writing—original draft, F.J.R.-L., R.O.-S., M.G.G., M.V.-B., C.M.M., B.R.-N., J.G.-G., J.M.M. and D.G.-B.; Writing—review & editing, R.O.-S., J.M.M. and D.G.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by FIS EC07/90762 Grant and the Spanish Net of Cell Therapy (TerCel) provided by Carlos III Institute of Health (ISCIII) (PI13/02699 and EC11-009) together with the Junction Program for Biomedical Research in Advanced Therapies and Regenerative Medicine from ISCIII.

Conflicts of Interest: The authors declare that they have no conflict of interest in this study.

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ARTÍCULO 3

INTERNATIONAL ENDODONTIC JOURNAL

doi:10.1111/iej.13327

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

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Abstract

Rodríguez-Lozano FJ, López-García S, García-Bernal D, Tomás-Catalá CJ, Santos JM, Llena C, Lozano A, Murcia L, Forner L. Chemical composition and bioactivity potential of the new Endosequence BC Sealer formulation HiFlow. *International Endodontic Journal*, **53**, 1216–1228, 2020.

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

Methodology Eluates of EndoSequence BC Sealer HiFlow (BCHiF) (Brasseler USA), EndoSequence BC Sealer (BCS) (Brasseler USA) and AH Plus (AHP) (Dentsply DeTrey GmbH, Konstanz, Germany) were placed in contact with hPDLSCs. The characterization of the chemical elements of the root canal sealers was assessed using scanning electron microscopy and energy-dispersive X-ray analysis (SEM-EDX). Inductively coupled plasma-mass spectrometry (ICP-MS) was used to determine the ion release of the sealers. MTT assay and wound healing techniques were used to determine cell viability and migration, respectively. Cell morphology and cell attachment were assessed using a direct contact technique of hPDLSCs onto the surface of the sealers and analysed by SEM. The bioactivity potential was carried out with the Alizarin Red and qPCR testing methods. The statistical differences were evaluated using one-way ANOVA and Tukey's test ($P < 0.05$).

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

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

Keywords: bioactivity potential, cytotoxicity, endodontic sealers, Endodontics, ion release.

Received 23 December 2019; accepted 8 May 2020

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Introduction

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

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

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

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

New root canal hydraulic sealers continuously develop in order to combine proper sealing and bioactive properties. Endosequence BC Sealer (BCS; Brasseler USA, Savannah, GA, USA) is a premixed ready-to-use injectable calcium silicate-based material developed for root canal filling and sealing (Hess *et al.* 2011), with suitable physicochemical properties that harden in the presence of moisture as found in wet locations such as dentinal tubules (Candeiro *et al.* 2012). The formulation of Endosequence BC Sealer has been modified into Endosequence BC Sealer HiFlow (BCHiF; Brasseler USA) to obtain a suitable calcium silicate-based sealer to use in warm canal filling techniques. This new sealer, according to the manufacturer, has a lower viscosity when heated and is more radiopaque than its predecessor. However, there is no information on its biological properties.

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

Materials and methods

Sealer eluates

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

All three sealers were mixed under aseptic conditions and following the manufacturers' indications. Each sealer was placed in preformed moulds of 2-mm

height and 5-mm diameter, sterilized by using ultraviolet radiation for 15 min and stored for 48 h in an incubator at 37 °C, 5% CO_2 and 95% humidity to achieve complete setting ($n = 30$), immersed in Hank's balanced salt solution (HBSS; Koutroulis *et al.* 2019). After this time, specimen discs were stored within the culture medium (DMEM) for 24 h at 37 °C, 5% CO_2 and humid atmosphere. This procedure was carried out following the International Organization for Standardization (ISO) guideline 10993-12. The ratio of the specimen surface area was 1.5 cm^2 mL^{-1} (ISO 10993-5). Prior to using these extracts in the MTT assay, migration and Alizarin Red experiments, the extracts were filtered through a 0.22 μm pore size mesh and prepared undiluted, diluted 1:/2 and diluted 1:/4.

Isolation and culture of hPDLSCs

The study was approved by the Ethical Committee of the University of Murcia (ID: 2199/2018). Third molars ($n = 10$) were extracted and transported to the laboratory in Minimum Essential Medium Alpha (α-MEM; Gibco, Invitrogen, Carlsbad, CA, USA) solution containing 1% antibiotics (Sigma Aldrich, St. Louis, MO, USA) and fungizone maintained at 4 °C. Next, after washing three times with PBS, the periodontal tissues were scraped from the middle and the apical part of the root surface and were cut into small fragments with surgical blades. The fragment tissues were digested with an enzymatic solution (Collagenase type I (Gibco)) during 1 h at 37 °C. Then, periodontal cells were seeded in Minimum Essential Medium Alpha (α-MEM; Gibco) with 10% foetal bovine serum (Sigma) and 1% penicillin/streptomycin (Sigma). Culture medium was replaced every 3 days. Cells at passages 2 4 were used for subsequent experiments (Rodríguez-Lozano *et al.* 2017). The expression of cell surface markers was detected using FACS (Calibur Flow Cytometer, BD Biosciences, San José, CA, USA). Flow cytometry was used to analyse the immunophenotype of cells at passage 3. Briefly, hPDLSCs (2×10^5) were trypsinized, washed with PBS and then incubated for 15 min at 4 °C with monoclonal antibodies conjugated with fluorescent dyes. The following antibody cocktails were used: MSC-positive cocktail (CD90, CD105 and CD73) and MSC-negative cocktail (CD34, CD14, CD20 and CD45) (Miltenyi Biotec, Bergisch Gladbach, Germany). Results were evaluated using FlowJo software (FlowJo LLC, Ashland, OR, USA).

Scanning electronic microscopy and energy-dispersive X-ray analysis

Samples of BCS, BCHiF and AHP were shaped into 5 mm in diameter and 2-mm high using sterile rubber moulds and were immersed in HBSS in a ratio of 6 cm^2 mL^{-1} and stored at 37 °C for 24 h. Then, discs were coated with carbon in a CC7650 SEM Carbon Coater unit (Quorum Technologies Ltd, Laughton, UK) and each sample was examined using a scanning electron microscope (SEM; Jeol 6100 EDAX, Peabody, MA, USA) connected to a secondary electron detector for energy-dispersive X-ray analysis (EDX; Oxford INCA 350 EDX, Abingdon, UK) by using computer-controlled software (INCA energy version 18, Inca Oxford Instruments, Abingdon, UK).

Table 1 Tested materials

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

Assessment of inductively coupled plasma-mass spectrometry of sealer extracts

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

Cell viability assay

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

Cell migration assay

To assess the effect of different sealers extracts on hPDLSCs migration, scratch migration assay was performed. 2×10^5 hPDLSCs/well were seeded onto six-well plates ($n = 3$) and proliferated to achieve confluency. A scratch was made with a 200 μL-pipette tip, and each well was washed three times to remove cell debris using PBS. The wound closure was observed in the absence (control group) or presence of the different sealers' eluates (1 : 1; 1 : 2 and 1 : 4). The migration analysis of the scratched area was observed at 24, 48 and 72 h. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to measure the percentage of wound area closed/open after 24, 48 or 72 h relative to the total wound area measured at 0 h in the same well. Migration distances were analysed separately during periods 0 24 h (migration during first 24 h period), 24 48 h (during second 24 h period) and 48 72 h (during third 24 h period). A 'relative wound closure' area (RWC) was calculated (RWC [-%] = wound closure area [pixel] × 100 [%]/x [pixel] in order to avoid any scratch width variation.

Cell morphology and cell adhesion analysis

Fifteen discs of 2-mm height and 5-mm diameter of the three sealers were obtained and subdivided into three groups ($n = 5$). A total of 5×10^4 hPDLSCs were directly added to each disc's surface and cultured for 72 h. Then, cells were fixed with 4% glutaraldehyde in PBS for 4 h and dehydrated, air-dried and sputter-coated with gold/palladium. Finally, cell morphology was evaluated using 100× and 300× magnifications by SEM.

RT-qPCR gene expression analysis

To evaluate the expression of cementoblastic/osteoblastic-related genes (ALP, CEMP-1 and CAP), 2×10^4 hPDLSCs/well were seeded onto twelve-well plates ($n = 3$) and stimulated with undiluted extracts of endodontic sealers during 7 days. For this purpose, six discs were immersed in culture medium for 24 h. Medium without extracts served as negative control and an optimized differentiation medium to generate cementoblast/osteoblasts-like cells, OsteoDiff media (Miltenyi Biotec, Bergisch Gladbach, Germany), as a positive control. Total RNA was prepared using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 1 μg of RNA by using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad) following the manufacturer's instructions. Changes in gene expression were calculated by the 2-ΔΔCT method. Primer sequences for human genes encoding cementum protein 1 (CEMP1), cementum-derived attachment protein (CAP), alkaline phosphatase (ALP), Runt-related transcription factor 2 (RUNX2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows (forward/reverse): CEMP1 (5′-GGGCACATCAAGCAC TGACAG-3′/5′-CCCTTAGGAAGTGGCTGTCCAG-3′); CAP (5′-TTTTTCTGGTCGCGTGGACT-3′/5′-TCACCAGCAAC TCCAACAGG-3′); ALP (5′-TCAGAAGCTCAACACCA ACG-3′/5′-TTGTACGTCTTGGAGAGGGC-3′); RUNX2 (5′-TCCACACCATTAGGGACCATC-3′/5′-TGCTAATGC

TTCGTGTTTCCA-3′); GAPDH (5′-TCAGCAATGCCTC CTGCAC-3′/5′-TCTGGGTGGCAGTGATGG-3′).

Alizarin red staining

Mineralization capacity of endodontic sealers was evaluated using Alizarin red staining. 2×10^4 hPDLSCs/well were seeded onto twelve-well plates ($n = 3$) and proliferated until achieving confluency. Then, cells were stimulated with undiluted extracts of BCS, BCHiF and AHP during 21 days. Medium without extracts served as negative control and OsteoDiff media (Miltenyi Biotec) as a positive control. At the end of the experimental period, the cells were washed with PBS and fixed for 1 h using 70% ethanol. They were then incubated with 2% Alizarin Red solution (Sigma AB, Malmö, Sweden) at room temperature in the dark for 30 min. Finally, the absorbance value at 550 nm was measured using the microplate reader.

Statistical analysis

Data were presented as the mean ± standard deviation (SD). All analyses were carried out using GraphPad Prism (version 8.1.0; GraphPad Software, San Diego, CA, USA). Normal data with equal variance were analysed using one-way analysis of variance (ANOVA) and Tukey's test. Significance was defined when $P < 0.05$. All assays were performed at least three times.

Results

Characterization of hPDLSCs immunophenotype

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

Scanning electronic microscopy and energy-dispersive X-ray analysis

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

Assessment of inductively coupled plasma-mass spectrometry of sealer extracts

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

MTT assay

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

Cell migration assay

At all period times and all dilutions studied, cell migration rates in the BCS group were similar to that

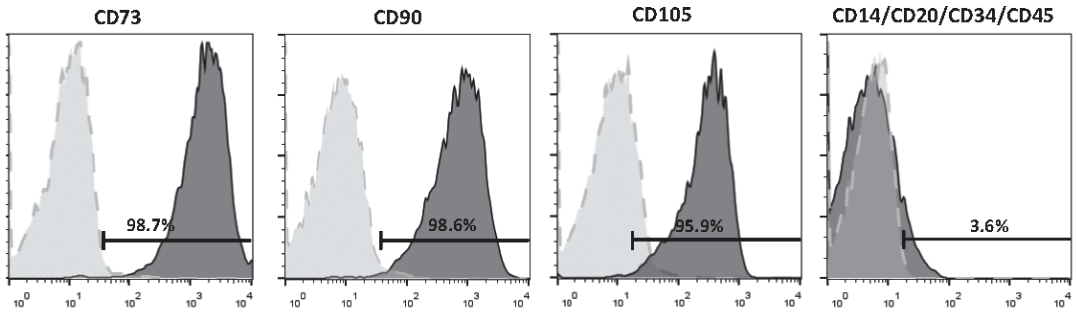

Figure 1 Flow cytometric characterization. FACS analysis showed high expression (>95%) of MSCs specific surface markers (CD73, CD90 and CD105) and low expression (<5%) of CD34, CD45, CD14 and CD20.

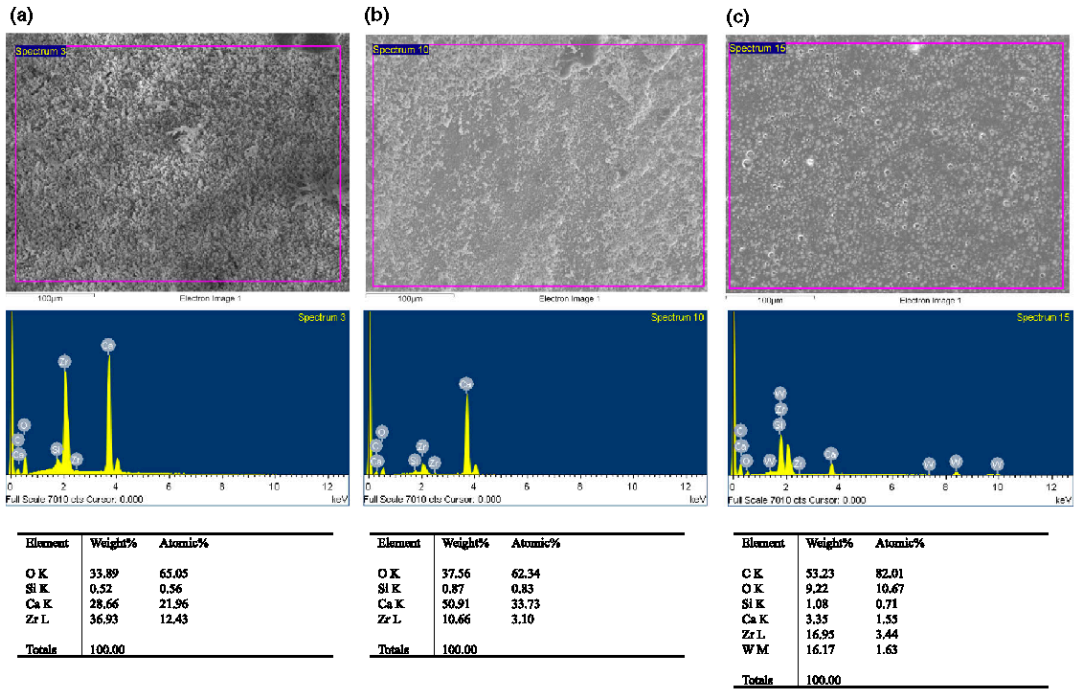

Figure 2 EDX analysis. Evaluation of the chemical composition (spectra) and the element distribution (elemental mapping) of BCHiF (*Column a*), BCS (*Column b*) and AHP (*Column c*) conducted with energy-dispersive X-ray spectroscopy. BCHiF contains higher % of Zr than BC Sealer. The % of Ca^{+2} in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

of the control group and no detectable differences were found (Fig. 4). In the BCHiF group, only at 24 h in the nondiluted group, significant differences were found ($P < 0.01$) meanwhile, no significant differences were revealed at 48 nor 72 h in any dilution when compared with the control group wound closure. On the contrary, at all period times and all dilutions, the AHP group exhibited significant differences ($P < 0.001$), being unable to heal the wound when compared with the control group (without extracts). These results indicate that both BC Sealers had similar migration values to those of the control.

Table 2 Assessment of ICP-MS of endodontic sealer eluates

Sample name	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]
AH Plus	2,08 ± 0.02^{B}	1,79 ± 0.00^{AB}	<0.000	1,24 ± 0.00^{AB}	4267 ± 0.00^{AB}
Endosequence BC Sealer	8,09 ± 0.00^{BC}	67,22 ± 0.02^{B}	<0.000	1,55 ± 0.02^{BC}	120,87 ± 0.00^{BC}
Endosequence BC Sealer Hiflow	1,91 ± 0.01^{C}	63,53 ± 0.00^{A}	<0.000	3,85 ± 0.00^{AC}	50,65 ± 0.00^{AC}

Uppercase A indicates significant difference ($P < 0.05$) between AH Plus and Hiflow.
Uppercase B indicates significant difference ($P < 0.05$) between AH Plus and Endosequence BC Sealer.
Uppercase C indicates significant difference ($P < 0.05$) between Hiflow and Endosequence BC Sealer.

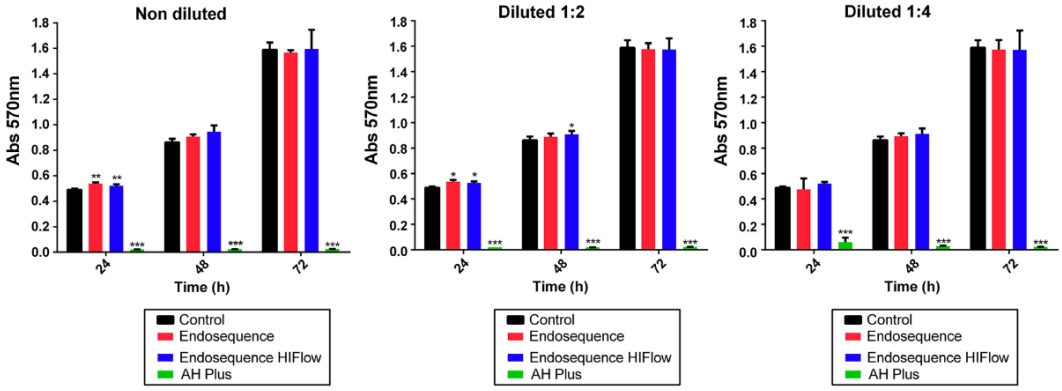

Figure 3 MTT assay. Determination of cell viability was carried out by an MTT assay. After the contact of hPDLSCs with the three sealers extracts in all dilutions at 24, 48 and 72 h, the absorbance results are shown in these graphics. Absorbance values were significantly different from to the control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

Cell morphology and cell attachment analysis

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

qPCR analysis

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

Alizarin red staining

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

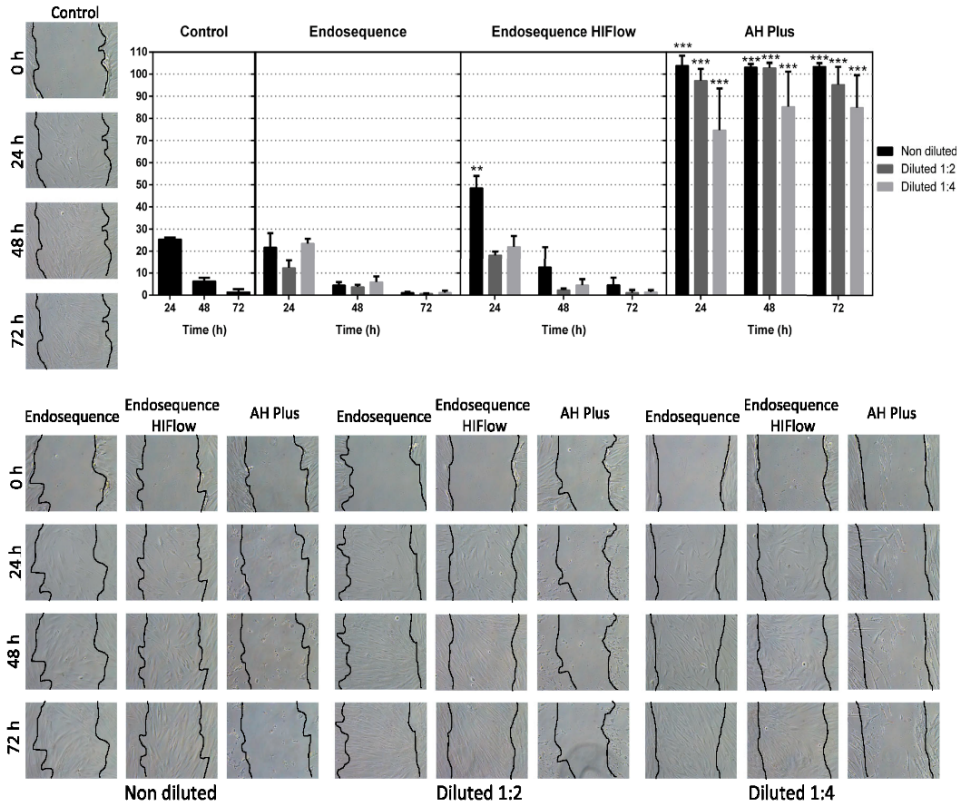

Figure 4 Scratch migration assay. The closure of the space created in the wound healing technique after the contact of the eluates of all three sealers with the hPDLSCs after 24, 48 and 72 h is represented in this figure by a composition of the photographs analysed by the Image J program and representation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

Discussion

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

Since the biological properties of this new sealer have not been tested yet, in this laboratory study, the cytocompatibility and bioactivity potential of BCS, and the new calcium silicate sealer formulation, BCHiF were analysed. AHP was chosen as reference material as it is one of the most commonly used and investigated root filling cements (Santos *et al.* 2019). In general, excellent cytocompatibility was observed with BCHiF, as well as with BCS. The results revealed that AHP had lower cytocompatibility when compared to the control group and with the other tested materials. These results are consistent with previous studies (Candeiro *et al.* 2015, Graunaite *et al.* 2018, Benetti *et al.* 2019).

As stated in the latest review of the ISO 7405:2018, prior to conducting cytotoxicity studies as part of the biocompatibility tests, material characterization is required before biological testing is performed. In this study, following other published

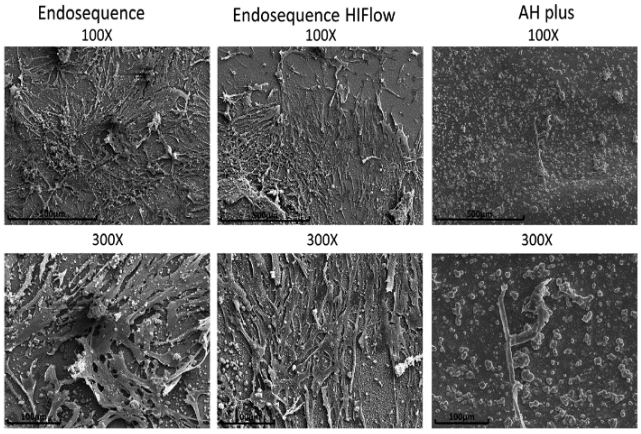

Figure 5 SEM analysis. Photomicrographs showed hPDLSCs cultured on the discs' surface of BCS, BCHiF and AHP for a 72 h period time. Photomicrographs show hPDLSCs fully adhered to the surface's disc of BCS and BCHiF, with a polyhedral shape extended, and displaying dendritic extensions. No cells were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

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

The biological reaction of cells in contact with these materials can be evaluated by a cell migration assay (Yarrow *et al.* 2004). In the present study, cell migration rates with BCS were similar to those with the control group, meanwhile with AHP, hPDLSCs were unable to migrate in order to close the wound ($P < 0.001$). These same migration results are shown in a study conducted by Seo *et al.* (2019) with human dental pulp stem cells. hPDLSCs exposed to BCHiF extracts revealed no significant differences with those in contact with BCS nor the control group.

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

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

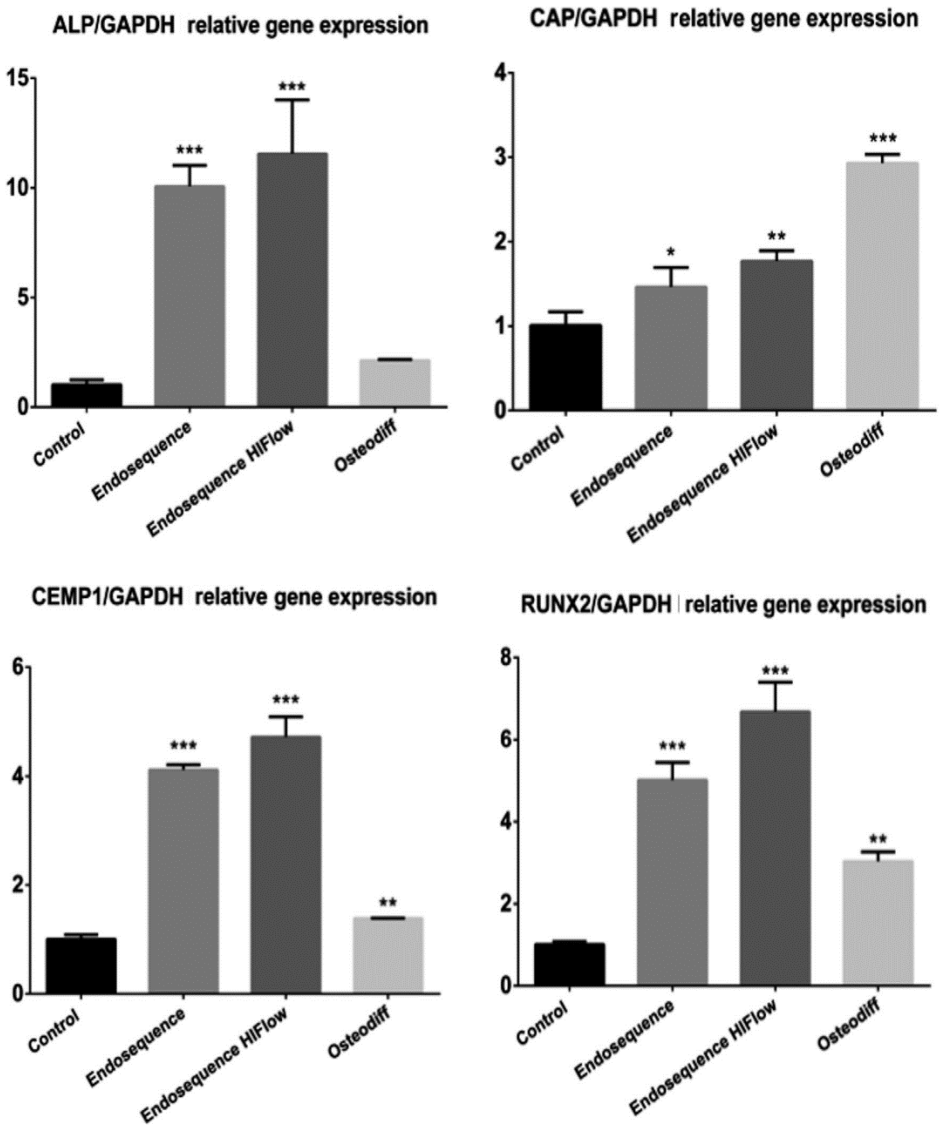

Figure 6 RT-qPCR gene expression analysis. Gene expression profiles of hPDLSCs treated with the test materials showing expression of ALP, CEMP, RUNX2 and CAP genes. Values indicated with a * represent significant differences between the groups. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, respectively).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

expressing cells, significant gene expression up-regulation of osteogenic genes and mineralization potential. Unsurprisingly, in the study, both BCHiF and BCS promoted greater osteo/cementogenic genes expression than the control group. Interestingly, BC sealer groups exhibited greater mineralization capacity than the Osteodiff group (positive control), with more visible calcium deposits. On the contrary, there was no mineralization in the cells treated with AHP as this material is associated with extensive cell death. These results may be related to the significantly higher calcium release observed for both calcium silicate-based sealers (Zordan-Bronzel *et al.* 2019). Although this seems to be counterintuitive, it is possible that the high alkalinity of the calcium silicate-based sealer media can up-regulate alkaline phosphatase activity and enhance mineralization (Wu *et al.* 2018). Moreover, the main component of both calcium silicate-based

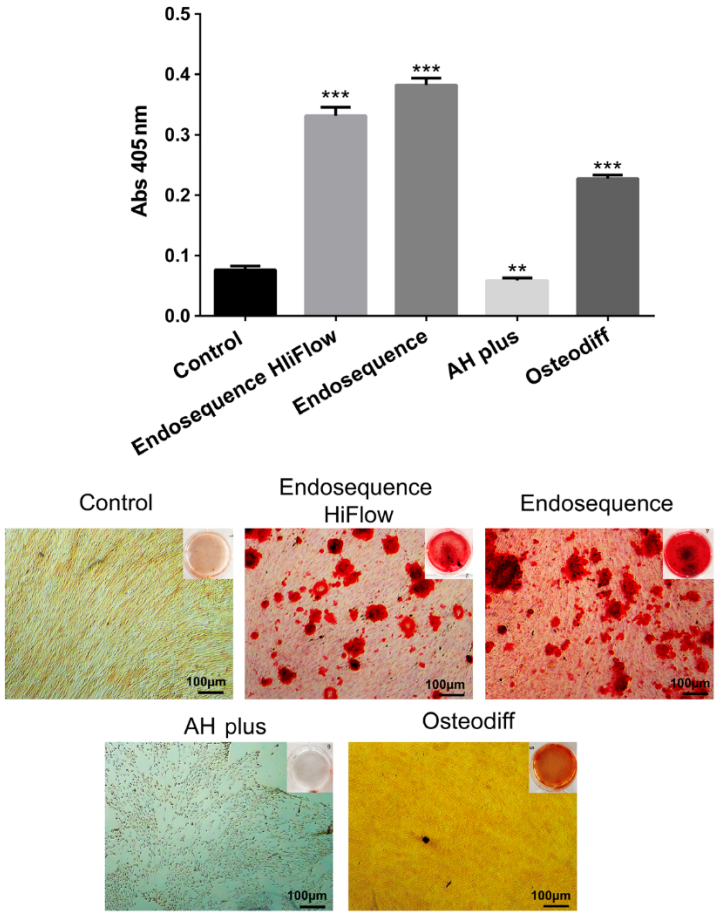

Figure 7 Mineralization assay. Alizarin Red staining to evaluate the bioactivity potential of BCS, BCHiF and AHP. Values indicated with an asterisk (*) represent significant differences between the groups. (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, respectively). One-way ANOVA and Tukey's *post hoc*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

sealers is calcium silicates, whose mineralization capacity has previously been reported (Zordan-Bronzel *et al.* 2019), which is in line with the present results. Based on this mineralization boost provided by the exposure of hPDLSCs to BC sealer, it can be speculated that clinically this can induce hard tissue deposition by periodontal ligament cells in the areas of contact with the sealer, reduce the size of the root canal portal of exit and improve the biological seal.

Conclusions

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

Acknowledgements

This work was carried out in the facilities of The Institute of Biosanitary Research of Murcia and supported by the Spanish Net of Cell Therapy (TerCel) provided by Carlos III Institute of Health (ISCiii).

Conflict of interest

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

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ARTÍCULO 4

Clinical Oral Investigations (2021) 25:5009–5024
https://doi.org/10.1007/s00784-021-03811-0

ORIGINAL ARTICLE

Cytocompatibility and bioactive properties of the new dual-curing resin-modified calcium silicate-based material for vital pulp therapy

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Received: 31 October 2020 / Accepted: 22 January 2021 / Published online: 27 February 2021

Abstract

Objective The aim of the present study was to evaluate the in vitro biocompatibility of Theracal PT, Theracal LC, and MTA Angelus, considered as bioactive materials used for vital pulp treatment, on human dental pulp stem cells (hDPSCs).

Materials and methods Human dental pulp stem cells (hDPSCs) were isolated from third molars, and material eluates were prepared (undiluted, 1:2, and 1:4 ratios). The hDPSC cytotoxicity, adhesion, morphology, viability, and cell migration were assessed. The mineralization nodule formation was determined by Alizarin red S staining (ARS). The odonto/osteogenic differentiation potential was assessed by osteo/odontogenic marker expression real-time qPCR. The chemical composition and ion release of the vital pulp materials were determined by energy dispersive X-ray (EDX) and inductively coupled plasma-mass spectrometry (ICP-MS), respectively. Statistical differences were assessed by ANOVA and Tukey's test ($p < 0.05$).

Results The three vital pulp materials showed variable levels of calcium, tungsten, silicon, and zirconium release and in their chemical composition. Cytocompatibility assays revealed higher hDPSC viability and migration rates when treated with Theracal PT than with Theracal LC. The lowest cell adhesion and spreading were observed in all Theracal LC-treated groups, whereas the highest were observed when treated with MTA. Theracal PT and MTA promoted the upregulation of DSPP and RUNX2 gene expression ($p < 0.05$). After 21 days, both MTA Angelus and Theracal PT–treated cells exhibited a significantly higher mineralized nodule formation than the negative control ($p < 0.05$).

Conclusions This study demonstrates the favorable in vitro cytocompatibility and bioactive properties of the recently introduced Theracal PT and the well-established MTA Angelus on hDPSCs, as opposed to Theracal LC. More studies, including in vivo animal testing are suggested before these new formulations might be used in the clinical setting.

Clinical relevance Theracal PT is a new material that could be clinically suitable for vital pulp therapy. Further studies considering its biocompatibility and bioactivity are necessary.

Keywords Tricalcium silicate materials · Vital pulp therapy · Cytotoxicity · Mineral trioxide aggregate · Bioactivity · Human dental pulp stem cells

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Introduction

Dental pulp cells can be exposed to a number of different potentially harmful stimuli, such as dental caries, trauma, or even iatrogenic factors; which can compromise their survival. The dentin-pulp complex presents a number of physiological defensive mechanisms, which are encompassed within the term reparative dentinogenesis [1, 2]. Thus, the maintenance of pulp vitality is the ultimate goal of biologically based minimally invasive therapeutic approaches. Within this framework, vital pulp treatment (VPT) has gained interest among daily dental practice, including procedures which range from indirect and direct pulp capping to partial and full pulpotomy [3–5].

Previous studies have reported the ability of human dental pulp stem cells (hDPSCs) to regenerate dentin, measured by the expression of odontogenic genes in vitro and/or dentin bridge formation in vivo in the process of dentinogenesis [6, 7]. Under favorable circumstances, hDPSCs are thought to differentiate into odontoblast-like cells and produce reparative dentin, which is the goal of VPT. However, the characteristics and extent of the tissue repair resulting from VPT depend on the clinical situation of the affected tooth and the type of materials used for vital pulp therapy. In fact, one of the ideal requirements of vital pulp materials is to induce and modulate the healing process and repair of the dentin-pulp complex [8, 9].

Traditionally, the material considered as the gold standard for the treatment of pulp exposures was calcium hydroxide, until studies demonstrated the superior clinical performance of calcium silicate-based materials, e.g., mineral trioxide aggregate (MTA) [1, 10]. Both materials have similar mechanisms of action, but calcium silicate–based cements induce the formation of a mineralized barrier with a higher uniformity and thickness, and elicit lower inflammatory responses and pulp tissue necrosis. Due to their favorable biological, physical, and mechanical properties, new versions of calcium silicate–based materials have been developed for their use as vital pulp materials [11]. These materials can enhance the process of mineralized-tissue formation by inducing the differentiation of precursor cells into mineral-secreting cells [12]. They also stimulate odontoblast-like differentiation and the secretion of growth factors and modulators of tissue repair [13].

Theracal LC (Bisco, Inc., Schamburg, IL, US) is a light-curable resin–modified tricalcium silicate–based material with easy handling, greater release of calcium ions compared to MTA and Dycal (Dentsply, York, PA, USA), but with controversial biological properties, which hinder its recommendation as pulp capping materials [14, 15]. Recently, Theracal PT (Bisco, Inc., Schamburg, IL, USA), a new dual-cured, resin-modified calcium silicate material designed for VPT, has been presented for clinical use. According to its manufacturer, this material maintains tooth vitality by acting as a barrier to protect the dentin-pulp complex. Due to its recent introduction to the market, to the authors' knowledge, there are no studies regarding this material for VPT.

Studies assessing cytotoxicity act as the preliminary analysis of the biological responses of a variety of dental materials. The advantage of these studies is that they present a controlled design for the evaluation of different properties of the materials, while evaluating their possible health risks [16, 17].

Accordingly, this study aimed to evaluate the bioactivity and biological properties of Theracal PT, and to compare these properties to those of MTA (Angelus, Londrina, PR, Brazil) and Theracal LC. The null hypothesis was that there is no difference between the tested materials in relation to their cytocompatibility and bioactivity potential on hDPSCs.

Materials and methods

Preparation of vital pulp material extracts

MTA (Angelus, Londrina, PR, Brazil), Theracal LC (Bisco Inc., Schamburg, IL, USA) and Theracal PT (Bisco, Inc., Schamburg, IL, USA) were prepared following their respective manufacturers' instructions into 5-mm diameter and 2-mm high sterile cylindrical rubber molds, sterilized under ultraviolet irradiation for 15 min and stored in an incubator at 37 °C, 5% CO_2, and 95% humidity, for 48 h to achieve complete setting. TheraCal LC (Bisco) was light-cured with a LED curing light (Bluephase 20i, Ivoclar Vivadent, Schaan, Liechtenstein) at an output of 1200 mW/cm^2 for 20 s with a 2-mm light-curing distance; the intensity of the light was measured using a Marc Resin Calibrator (BlueLight Analytics, Halifax, Canada). Their complete compositions are described in Table 1 obtained from the data sheet available at the respective manufacturers' websites.

In accordance with the International Organization for Standardization (ISO), the eluates of the different materials were extracted in sterile conditions, using DMEM culture medium as an extraction vehicle. The extraction procedure was performed as follows: the materials were stored in the culture medium for 24 h at 37 °C in a humid atmosphere containing 5% CO_2 with agitation. The ratio of material surface area to medium volume was set at approximately 3 cm^2/mL in accordance with the guidelines of the International Organization for Standardization 10993-12 [18]. Finally, serial dilutions of the extraction medium were prepared at 1/1, 1/2, and 1/4 ratios [19].

Ion release of vital pulp material extracts

Specimens (n = 3) were prepared from each vital pulp material. The ion release of each material in deionized water (Milli-Q; Merck KGaA, Darmstadt, Germany) was analyzed using inductively coupled plasma-optical emission spectrometry

Table 1 Manufacturers and compositions of the tested materials

Materials	Manufacturer	Composition	Lot number
MTA Angelus	Angelus, Londrina, PR, Brazil	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, calcium tugstate	101752
Theracal LC	Bisco Inc. Shaumburg, IL, USA	Calcium oxide, calcium silicate particles (type III Portland cement), strontium glass, fumed silica, barium sulphate, barium zirconate and resin containing bisphenol A-glycidyl methacrylate (Bis-GMA) and polyethylene glycol dimethacrylate (PEGDMA)	2000001054
Theracal PT	Bisco Inc. Shaumburg, IL, USA	SG-Mix cement, Bis-GMA, barium zirconate, ytterbium fluoride, initiator	2000002968

(ICP-MS; Agilent 7900, Stockport, UK). The proportion of aluminium (Al), silicon (Si), sulfur (S), calcium (Ca), strontium (Sr), barium (Ba), and tungsten (W) released from each material was analyzed at 1 day in triplicate, and the elements were calibrated with pure deionized water.

Scanning electronic microscopy and energy-dispersive spectroscopy

Disks of each cell-free material (n = 9) were immersed in Ca^{2+}/Mg^{2+}-free Hank's balance salt solution (HBSS; Gibco, Gaithersburg, MD, USA) at 3 cm^2/mL and stored at 37 °C for 24 h. Once set, the disks underwent a carbon-coating process in a CC7650 SEM Carbon Coater unit (Quorum Technologies Ltd, East Sussex, UK). Then, the element distribution was analyzed using a scanning electron microscope with an energy-dispersive X-ray spectrometer (SEM-EDX, JSM-610LV; JEOL, Tokyo, Japan). A qualitative analysis was conducted for the surface element distribution.

Isolation and culture of hDPCs

Cells were obtained from impacted third molars (n = 10) from 10 healthy subjects (18–30 years old). The human dental pulp (hDP) isolation protocol was approved by the Human Research Ethics Committee from the University of Murcia (protocol ID: 2199/2018). hDP was obtained from the pulp chamber and root canals using a barber broaches. After extraction, hDP was thoroughly rinsed with HBSS, and subjected to collagenase-A digestion (3 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C. Then, cells were maintained in Dulbecco's Modified Eagle Medium (DMEM Gibco BRL, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% L-glutamine, 100-U/ml penicillin, and 100-μg/ml streptomycin (Gibco). Finally, cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere. For subsequent experiments, hDPCs were used from culture passage 2 up to 6.

Characterization of hDPSCs

Cultured cells were identified following the guidelines of the International Society of Cellular Therapy (ISCT) to confirm their mesenchymal stem cell phenotype [20]. The surface antigens of the hDPSCs were analyzed using antibodies conjugated to fluorophores under flow cytometry. The antibodies used were CD73-APC (clone AD2), CD90-FITC (clone DG3), CD105-PE (clone 43A4E1), CD34-PerCP (clone AC136), CD19-PerCP (clone LT20.B4), CD14-PerCP (clone TÜK4), and CD45-PerCP (clone 5B1) (Human MSC Phenotyping Cocktail, Miltenyi Biotec, Bergisch Gladbach, Germany). Flow cytometry analysis was performed using a FACS Calibur Flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Furthermore, hDPSCs were cultured in osteogenic, adipogenic and chondrogenic medium (Miltenyi Biotech) to evaluate the in vitro trilineage mesenchymal differentiation, as previously described [21].

Cytotoxicity assay

Cytotoxicity was assessed by analyzing the mitochondrial activity by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The extracts were placed in contact with the cell culture and cell metabolic activity was measured at 24 h, 48 h and 72 h of culture, as described by Sequeira et al. [22]. Cells cultured in growth medium w/o any extract were used as the negative control. According to the manufacturer's instructions, the MTT reagent (Sigma-Aldrich) was added to the wells for 4 h. When the purple precipitate was visibly noticeable, dimethylsulfoxide (DMSO) (Sigma-Aldrich) was added to solubilize the formazan dye (100 μl/well). Covered plates were kept in the dark for 2–4 h. Finally, the absorbance at 570-nm wavelength in each well was measured in a microplate reader (ELx800; Bio-Tek Instruments, Winooski, VT, USA). Each experimental condition was performed in triplicate and analyzed in three independent experiments.

Cell migration

Cell migration was evaluated using an in vitro wound healing assay. Cells were seeded onto 12-well plates (2×10^4 hDPSCs per well, $n = 3$ for each dilution) and left to proliferate until confluent. The culture medium was then replaced with serum-free culture medium and cultured for an additional 24 h. Then, a scratch was created using a sterilized-pipette tip and the cells were exposed to complete growth medium alone (control) or complete growth medium containing 1:1, 1:2, or 1:4 dilutions of the tested materials, and imaged using a phase-contrast microscope at 0, 24, 48, and 72 h. The percentage of open wound area was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA). The data are expressed as the mean of three independent experiments ± standard deviation (SD).

Cell adhesion evaluation

To test the effect of surface chemistry of the different cements on cell adhesion and growth, scanning electronic microscopy (SEM) was used. Sample discs with the aforementioned standardized dimensions were obtained ($n = 15$) for each of the materials and allocated into three groups ($n = 5$). hDPSCs were directly seeded onto the material´s surface and cultured in normal growth medium. After 72 h, cells were fixed with 3% glutaraldehyde (Sigma-Aldrich) in PBS for 30 min. The specimens were then dehydrated using a graded ethanol series, followed by hexamethyldisilazane (Sigma-Aldrich) treatment for 5 min. The samples were gold sputter–coated and observed under SEM using × 100, × 300, and × 1500 magnifications.

Cell cytoskeleton staining

Phalloidin staining was used to analyze possible changes in cell morphology and in the actin cytoskeleton structure and organization of hDPSCs cultured with the different material eluates. Briefly, cells were seeded on glass coverslips, allowed to adhere, and cultured in complete growth medium alone (control) or in complete growth medium containing 1:1, 1:2, or 1:4 dilutions of the different eluates for 72 h at 37°C. Then, hDPSCs were rinsed twice with prewarmed PBS at 37 °C, fixed in 4% formaldehyde solution (Merck Millipore, Darmstadt, Germany) for 10 min, permeabilized with 0.25% Triton X-100 solution (Sigma-Aldrich) for 5 min, and rinsed 3 times with PBS. Cell cytoskeleton and nuclei were then stained with AlexaFluor™594-conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (ThermoFisher Scientific, Waltham, MA, USA), respectively. Finally, immunofluorescence images were observed under a Leica TCS SP2 confocal microscope (Leica, Wetzlar, Germany). Each experimental condition was carried out in triplicate for each VTP material and analyzed in three independent experiments.

Apoptosis/necrosis assay

To evaluate hDPSC viability after exposure to the different materials, hDPSCs were cultured in complete growth medium alone (control) or in complete growth medium containing 1:1, 1:2, or 1:4 dilutions of the different eluates for 72 h at 37 °C. Cell viability was assessed by Annexin-V-FITC and 7-AAD staining (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions. Samples were analyzed in a FACS Calibur Flow cytometer (Becton Dickinson) within 1 h of staining. Finally, the percentages of viable (double negative), early apoptotic (Annexin-V-FITC positive, 7AAD negative), and late apoptotic and necrotic (double positive and Annexin-V-FITC negative/7-AAD positive respectively) cells were determined. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments.

Odontogenic marker expression (RT-qPCR)

The induction of odontogenic differentiation of hDPSCs cultured with undiluted material-conditioned medium was evaluated by analyzing the expression of a series of odontogenic markers by real-time quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). The sequences of primers for the odontogenic markers used were as follows (forward/reverse): alkaline phosphatase (*ALP*): 5′- TCAGAAGC TCAACACCAACG-3′/5′-TTGTACGTCTTGGA GAGGGC-3′; collagen type 1 (*Col1A1*): 5′-CCCGGGTT TCAGAGACAACTTC-3′/5′- TCCACATGCTTTAT TCCAGCAATC-3′; osteonectin (*ON*): 5′-GCATCAAG CAGAAGGATA-3′/5′-AATAGTTAAGTTACAGCTAA GAAT-3′; dentin sialophosphoprotein (*DSPP*): 5′-GCATTTGGGCAGTAGCATGG-3′/5′-CTGACACA TTTGATCTTGCTAGGAG-3′; runt-related transcription factor 2 (*RUNX2*): 5′-TCCACACCATTAGGGACCATC-3′/5′-TGCTAATGCTTCGTGTTTCCA-3′; and Bone Sialoprotein Progenitor (BSP): 5′-TGCCTTGAGCCTGC TTCCT-3′/ 5′-CTGAGCAAAATTAAAGCAGTCTTCA-3′. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene to quantify and normalize the results, with the following primers sequences (forward/reverse): 5′-TCAGCAATGCCTCCTGCAC-3′/5′-TCTGGGTGGCAGTGATGG-3′.

hDPSCs (2×10^4 cells/well $n = 3$) were seeded onto 12-well plates with the material-conditioned medium (1:1) and cultured for 7 days. The analysis of relative gene expression data was calculated using the $2^{-\Delta\Delta CT}$ method. Cells cultured in unconditioned medium (DMEM) acted as the negative

control, and an osteo/odontogenic medium (OsteoDiff media; Miltenyi Biotec) acted as the positive control.

Mineralization assay (Alizarin red S staining)

Alizarin Red S staining was used to assess the mineralization potential of hDPSCs in contact with Theracal PT, Theracal LC, and MTA Angelus. Cells were seeded onto 12-well plates (2×10^4 cells/well, $n = 3$) and left to proliferate until confluent. Then, hPDLSCs were cultured in undiluted material-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 S solution (Sigma Aldrich) for 30 min in the dark at room temperature. The staining was solubilized with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich, MO, USA) solution and the absorbance was measured at 570 nm using a spectrophotometer. hDPSCs cultured in unconditioned medium (DMEM) acted as the negative control, and OsteoDiff media (Miltenyi Biotec) acted as the positive control.

Statistical analysis

All of the in vitro assays were performed in triplicate, and analyzed in three independent experiments. After confirming the homogeneity of variance and normal distribution of the data, we performed one-way ANOVA followed by pair-wise Tukey's post hoc test using Graph-Pad Prism v8.1.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean ± standard deviations (SD). Statistical significance was considered at $p < 0.05$.

Results

Ion release

The analysis of the ion release demonstrated by ICP-MS is presented in Table 2. The results revealed that silicon (Si) ion release was higher in Theracal PT and Theracal LC compared to MTA Angelus ($p < 0.05$), while barium (Ba) and tungsten (W) ion release was significantly increased in MTA ($p < 0.05$). Calcium (Ca) ion release was significantly lower in MTA Angelus compared to Theracal PT and Theracal LC ($p < 0.05$).

SEM-EDS analysis

The qualitative analysis of the chemical elements on the specimens' surfaces was comparable to the material composition according to the manufacturers' information, as shown in Fig. 1 and Table 1. MTA showed the highest Ca content. Theracal PT, on the other hand, exhibited the highest percentage of zirconium (Zr) and Si. Interestingly, the percentage of Si in Theracal PT was higher than Theracal LC and MTA Angelus. However, fluoride (F) was not detected in Theracal PT. Additionally, the SEM-EDS analysis disclosed other elements not mentioned in the manufacturers' information: Aluminium (Al) was found in Theracal LC and Theracal Pt and W was found in Theracal LC and Theracal PT.

hDPSC characterization

The results of flow cytometry showed that isolated hDPSCs were CD73 (99.9%), CD90 (99.7%), and CD105 (99.9%) positive; and negative for CD34, CD45, CD14, and CD20 (2.5%) (Fig. 2a). The percentages are presented relative to the proper expression of mesenchymal stem cell surface markers. Furthermore, hDPSCs exhibited ability to differentiate into osteoblasts, chondroblasts, and adipocyte-like cells (Fig. 2b).

Cytotoxicity assay

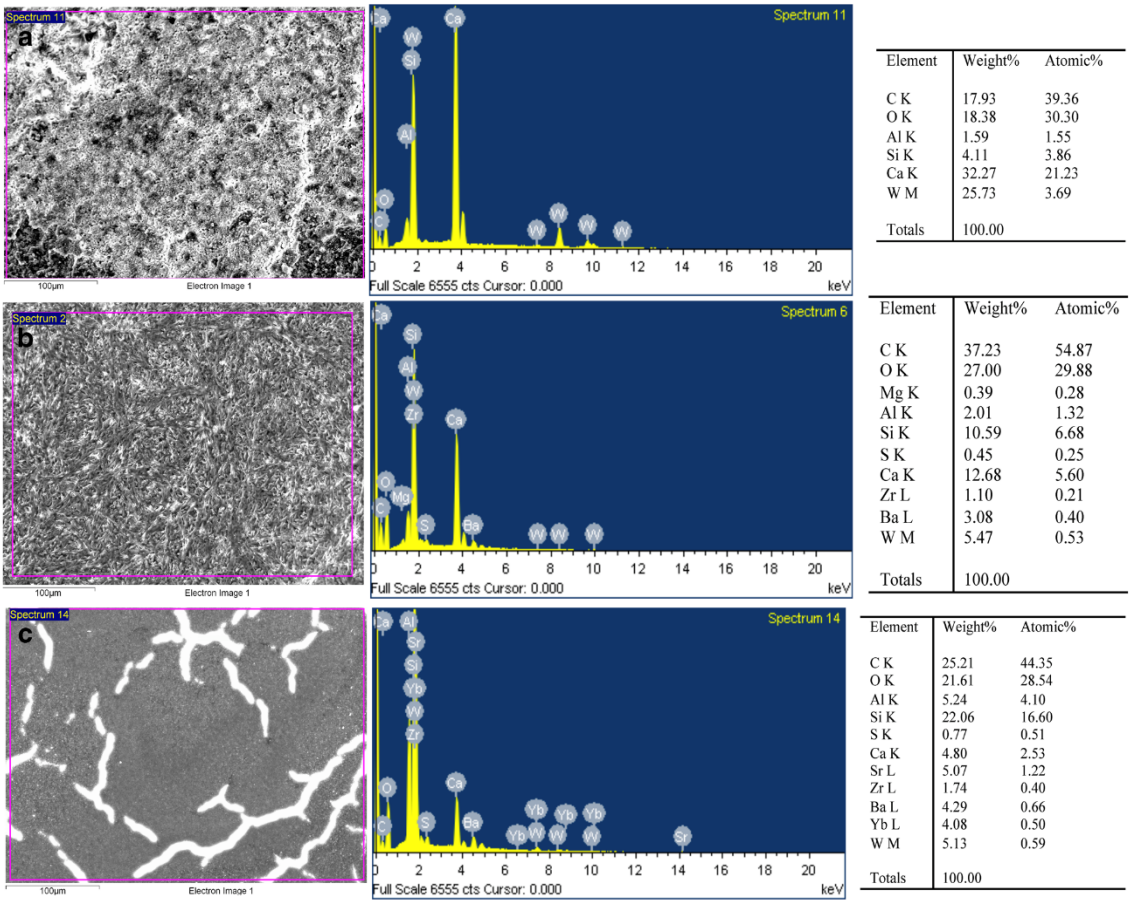
hDPSCs were exposed to several dilutions of Theracal PT, Theracal LC and MTA Angelus for 72 h, and cell viability was determined using an MTT assay (Fig. 3). Theracal LC showed a significant reduction in cell viability compared to the negative control in all concentrations and time-points ($p < 0.001$), whereas MTA Angelus exhibited no significant

Table 2 ICP-MS analysis of vital pulp materials ($n = 3$)

Sample name	Al Conc. [ppm]	Si Conc. [ppm]	S Conc. [ppm]	Ca Conc. [ppm]	Sr Conc. [ppm]	Ba Conc. [ppm]	W Conc. [ppm]
MTA Angelus	20.65 ± 0.02^{AB}	6.34 ± 0.00^{AB}	$< 0.000^{AB}$	8.56 ± 0.00^{AB}	413.48 ± 0.01^{AB}	2682.96 ± 0.04^{AB}	4267 ± 0.00^{AB}
Theracal PT	$< 0.000 \pm 0.00^{AC}$	20.25 ± 0.02^{AC}	17.00 ± 0.02^{AC}	15.33 ± 0.02^{AC}	897.60 ± 0.03^{AC}	2.91 ± 0.03^{A}	$120{,}87 \pm 0.00^{AC}$
Theracal LC	68.89 ± 0.03^{BC}	14.76 ± 0.00^{BC}	63.58 ± 0.02^{BC}	670.56 ± 0.00^{BC}	$106434{,}83 \pm 0.00^{BC}$	1.68 ± 0.00^{B}	$50{,}65 \pm 0.00^{BC}$

Uppercase A (^{A}) indicates significant difference ($p < 0.05$) between MTA Angelus and Theracal PT. Uppercase B (^{B}) indicates significant difference ($p < 0.05$) between MTA Angelus and Theracal LC. Uppercase C (^{C}) indicates significant difference ($p < 0.05$) between Theracal PT and Theracal LC

ppm parts per million, *Conc.* concentration



Element	Weight%	Atomic%
C K	17.93	39.36
O K	18.38	30.30
Al K	1.59	1.55
Si K	4.11	3.86
Ca K	32.27	21.23
W M	25.73	3.69
Totals	100.00	

Element	Weight%	Atomic%
C K	37.23	54.87
O K	27.00	29.88
Mg K	0.39	0.28
Al K	2.01	1.32
Si K	10.59	6.68
S K	0.45	0.25
Ca K	12.68	5.60
Zr L	1.10	0.21
Ba L	3.08	0.40
W M	5.47	0.53
Totals	100.00	

Element	Weight%	Atomic%
C K	25.21	44.35
O K	21.61	28.54
Al K	5.24	4.10
Si K	22.06	16.60
S K	0.77	0.51
Ca K	4.80	2.53
Sr L	5.07	1.22
Zr L	1.74	0.40
Ba L	4.29	0.66
Yb L	4.08	0.50
W M	5.13	0.59
Totals	100.00	

Fig. 1 SEM-EDS analysis results for MTA Angelus (**a**), Theracal LC (**b**), and Theracal PT (**c**) disks (n = 9). The first column presents SEM micrographs of each materials (scale bar: 100 µm). The second column illustrates the EDS plots with correspondent peaks detected. The third column classifies the list of elements present per materials by weight and atomic weight

differences in cell viability. Theracal PT 1:1 and 1:2 showed a discrete cell viability compared to the control, while in the 1:4 dilution, no difference was found compared to the control group in the first time-points (24 and 48 h).

Migration assay

An in vitro wound healing assay was performed to evaluate cell migration. Cells cultured in medium without any material eluate were employed as the negative control. 1:1 and 1:2 Theracal LC displayed a statistically significant decreased cell migration after 24, 48, and 72 h of culture ($p < 0.001$), while no significant differences were found between 1:4 Theracal LC and the negative control (Fig. 4). Cell migration rates exhibited by hDPSCs cultured with undiluted Theracal PT were significantly lower at 24, 48, and 72 h compared to the control group ($p < 0.001$), while this inhibitory effect was not so evident with Theracal PT 1:2 and 1:4. Regarding MTA Angelus, the cell migration rate was similar than that of control group at all time-periods and dilutions (Fig. 4).

Cell adhesion

The analysis of cell adherence and morphology of hDPSCs on the surfaces of the different material specimens showed the presence of few cells and debris in the Theracal LC samples, evidencing cell death, whereas abundant and functionally oriented cells were exhibited by MTA. Theracal PT samples exhibited a moderate growth of elongated cells (Fig. 5).

Cell cytoskeleton staining

In the MTA group, at all dilutions, a high number of well-adhered and spread cells with a fibroblastic spindle-shaped

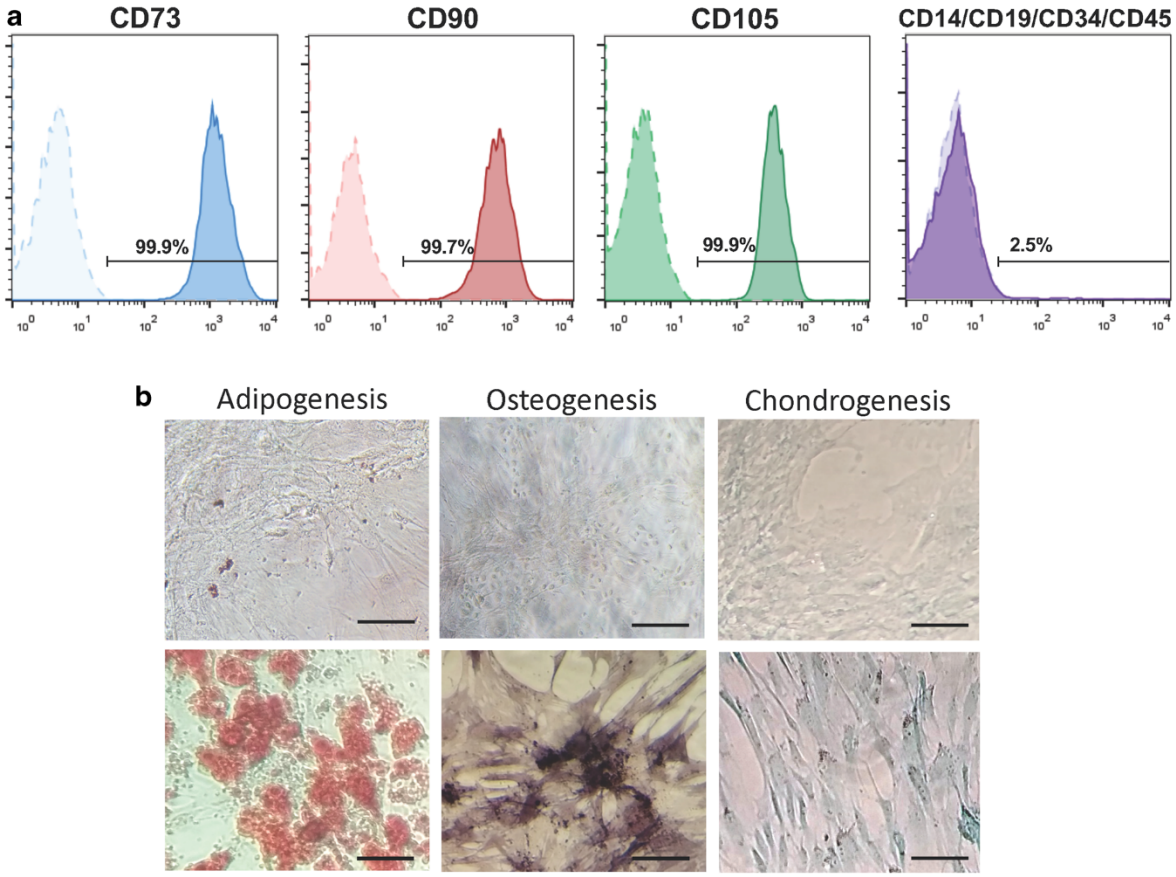


Fig. 2 Characterization of hDPSCs (**a**) and in vitro trilineage mesenchymal differentiation (**b**). Scale bar: 100μm

morphology and increased F-actin content were observed, similar to that observed on the cells from the negative control group (Fig. 6). 1:1 and 1:2 Theracal LC showed lower numbers of attached cells, some of them exhibiting an aberrant

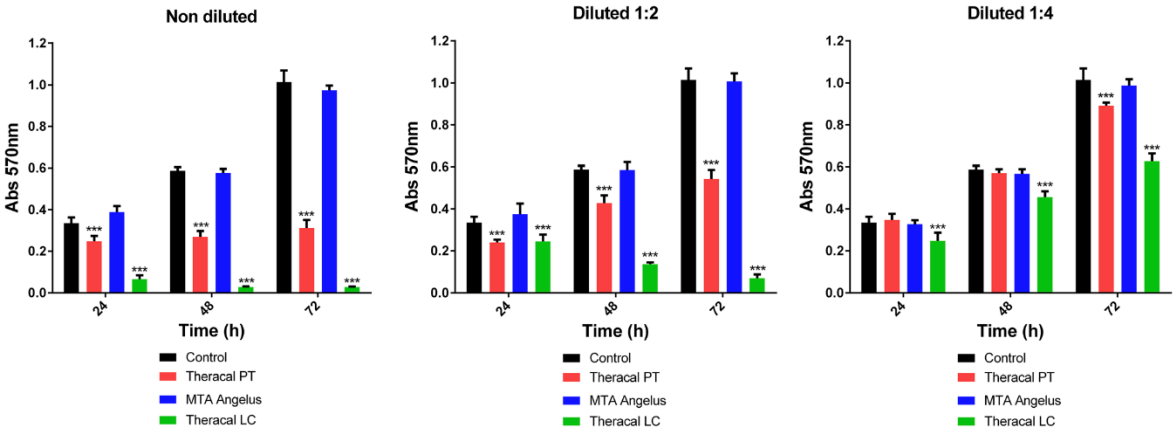


Fig. 3 MTT assay. In vitro cytotoxicity of hDPSCs after exposure to extracted medium prepared from MTA Angelus, Theracal LC, and Theracal PT. Data are presented absorbance values (570 nm) at 24, 48, and 72 h of exposure of the material eluates to hDPSCs, compared to the control. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

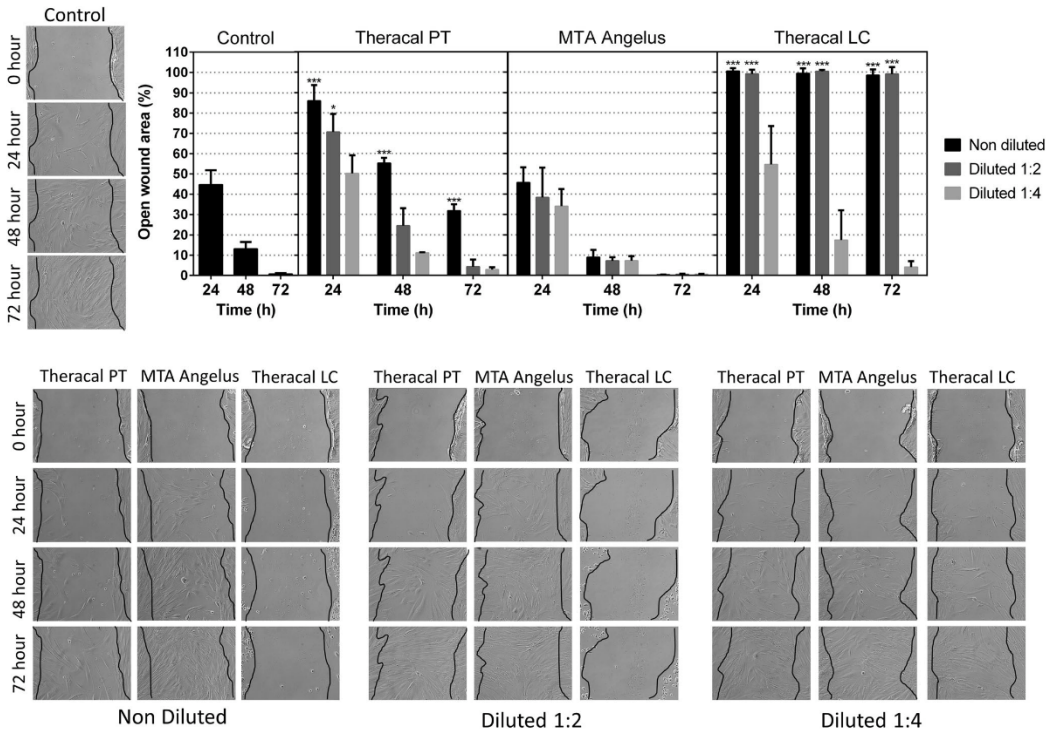


Fig. 4 Cell migration was evaluated using a scratch assay. Cells were exposed to undiluted (1:1) and diluted (1:2 and 1:4) eluates from materials. The control condition was cells maintained in normal growth medium. Graphical results are presented as mean RWC percentages at each of the time points, relative to the total wound area at 0 h. Asterisk designates significant differences compared with the control. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

morphology, while Theracal LC 1:4 specimens exhibited a similar number of cells with well-evident actin cytoskeleton compared to the control group. Conversely, cells from the Theracal PT specimens showed a favorable growth and similar well-organized F-actin filaments compared to the control group, especially at 1:2 and 1:4 dilutions (Fig. 6).

Apoptosis/necrosis assay

The apoptosis/necrosis rate in each group was obtained by flow cytometry. As shown in Fig. 7, undiluted materials displayed the following percentage of viable cells: MTA Angeluls (90.5%) > Theracal PT (80.5%) > Theracal LC (10.5%). At 1:2 dilutions all materials displayed a high percentage of viable cells (> 89%), except Theracal LC (21.1%). Similarly, at 1:4 dilution, all materials displayed a similar biocompatibility compared to the control medium, exhibiting more than 89% of viable cells, excluding Theracal LC (64.6%). These findings were in agreement with those obtained in the MTT and migration assays.

RT-qPCR assay

The mRNA expression of odonto/osteogenic markers was evaluated at day 7 after culturing hDPSCs with the different materials (Fig. 8). GAPDH expression was used to normalize the results. Due to the in vitro toxicity exhibited by Theracal LC in the aforementioned assays, this material was not employed in the cell differentiation assays. The MTA Angelus group displayed an upregulation of ALP, DSPP, RUNX2 ($p < 0.001$), ON, Col1A1 ($p < 0.01$), and BSP genes ($p < 0.05$) compared to the negative control, while the Theracal PT group showed an upregulation of DSPP and RUNX2 ($p < 0.001$). Interestingly, the expression of DSPP and RUNX2 was significantly different in the Osteodiff group (positive control) compared to the negative control at day 7. However, a marked decrease in the expression Col1A1 and ALP was observed.

Mineralization assay

Calcium deposition, as a final product of the odonto/osteogenic differentiation process, was assessed by using

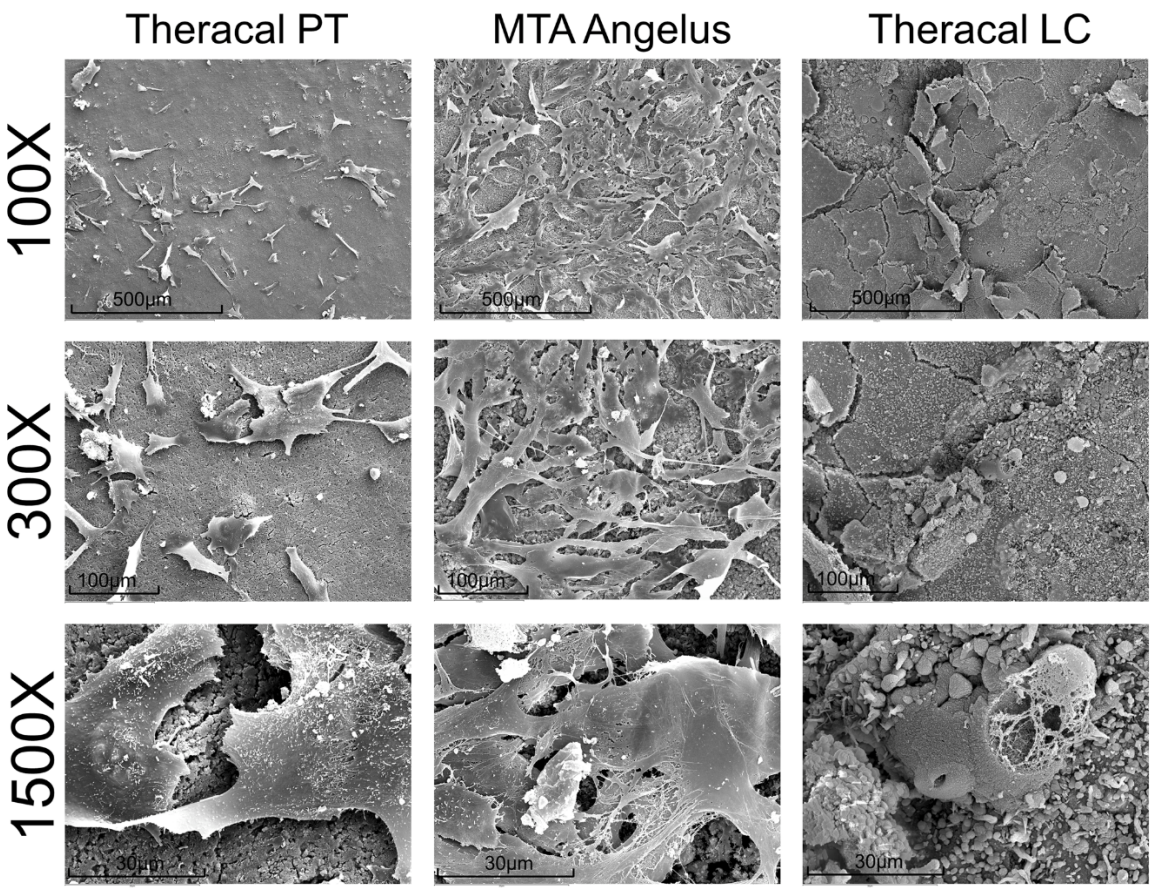


Fig. 5 Sample discs with the aforementioned standardized dimensions were obtained ($n = 15$) for each of the materials and allocated into three groups ($n = 5$). Representative SEM micrographs illustrate the adhesion of hDPSCs directly seeded on Theracal PT, MTA Angelus and Theracal LC. Magnifications: × 100, × 300, and × 1500. Scale bars: 500 µm, 100 µm, and 30 µm

Alizarin Red S staining on day 21 (Fig. 9). There were significant differences between the tested groups, with Osteodiff (positive control) exhibiting the highest amounts of calcium deposition ($p < 0.001$). In contrast, Theracal LC exhibited the lowest amounts of mineralized nodules. MTA Angelus and Theracal PT showed higher amounts of mineralized nodules when compared to the negative control ($p < 0.001$).

Discussion

The present study investigated the effects of the three vital pulp materials on the viability, morphology, migration, adhesion, osteo/odontoblastic differentiation, and mineralization potential of hDPSCs. It is crucial that the material placed in direct contact with the pulp tissue during VPT procedures presents, at least, an adequate biocompatibility and ideally, a bioactive effect. A lack of biocompatibility will result in an intense irritation to the pulp tissue, which can irreversibly compromise its defensive mechanisms and in turn, its vitality [23–25]. For this reason, mesenchymal stem cells from dental pulp where used as the target cells for the cytotoxicity analyses.

A high number of quantitative and qualitative assessments of in vitro and in vivo cytotoxicity highlight the effectiveness of vital pulp materials [26]. These studies were generally performed to gain an increased understanding of the biological mechanisms involved in tertiary dentin formation. However, the limitations of those reports were the types of target cells used, the duration of the exposure to the tested materials, and the formulation of such materials [4]. The biological properties of well-established vital pulp materials have been extensively described among available literature. However, to our knowledge, this the first study assessing Theracal PT.

ICP-MS and SEM-EDS assays showed calcium content and ion release in all materials, as previously reported for other vital pulp materials: NeoMTA Plus (Avalon Biomed, Houston, TX, USA), MTA Repair HP (Angelus), or

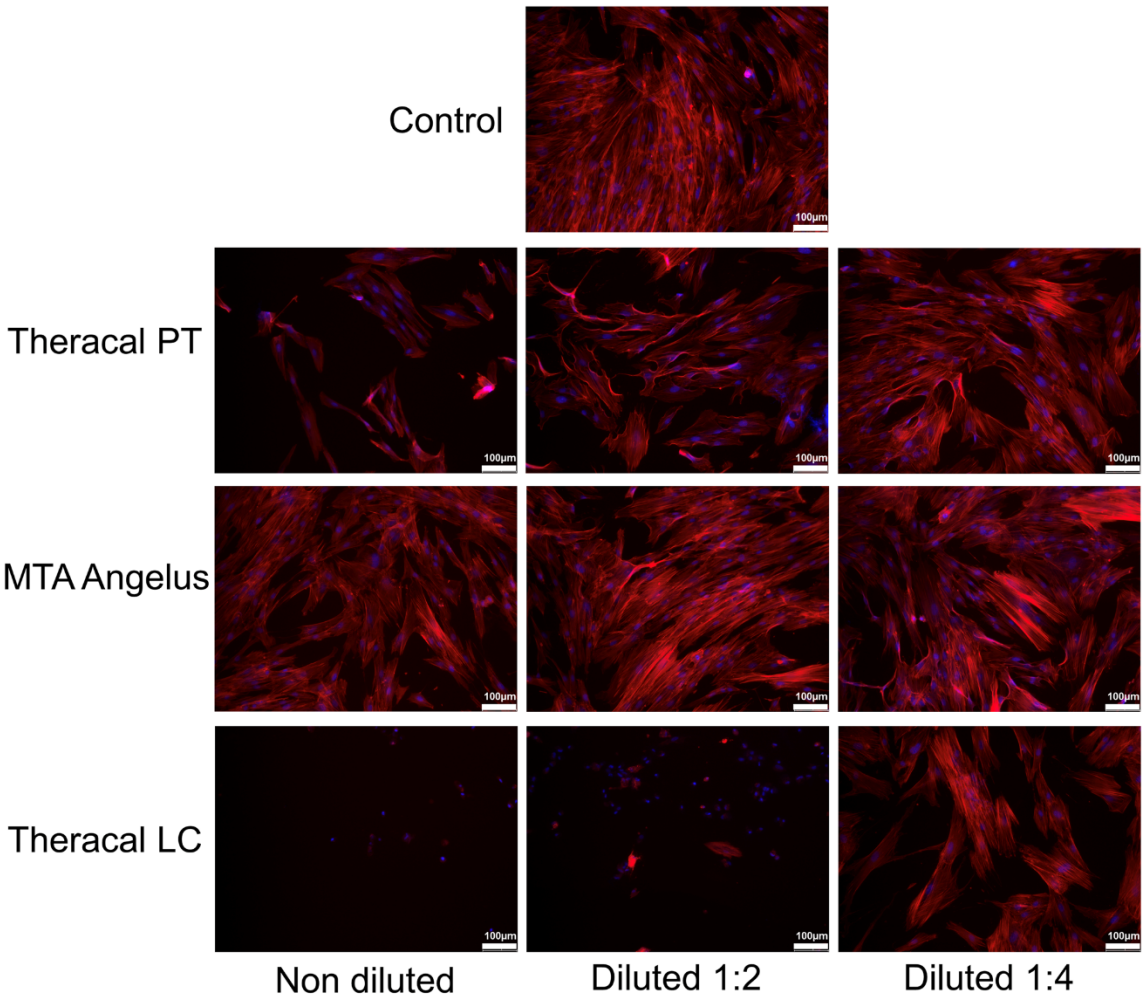


Fig. 6 Analysis of cell morphology changes, in the actin cytoskeleton structure and organization on hDPSCs after treatment with Theracal PT, MTA Angelus and Theracal LC by confocal fluorescence microscopy. F-actin fibers were stained with AlexaFluor™ 594-conjugated phalloidin (red), whereas cell nuclei were counterstained with DAPI (blue). Confocal fluorescence microscopy images shown are representative from $n = 3$ separate experiments. Scale bar: 100 µm. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

Biodentine (Septodont, Saint-Maur-des-Fossés, France) [27]. Furthermore, ytterbium (Yb) was detected in Theracal PT. It has been described that the incorporation of Yb as a radiopacifying agent for calcium silicate–based cements does not alter their physicochemical and biological properties, and preserves their bioactive potential [28]. Also, Si release was higher from Theracal PT than from Theracal LC samples after setting ($p < 0.05$). Previous studies demonstrated that Si ions from silicate bioceramics stimulated the osteogenic differentiation of mesenchymal stem cells [29]. Thus, the differences in ion release may influence the biological effects or, at least, the characteristics of the mineral attachment formed to the dentin substrate.

The materials used for VPT, in addition to possessing antiinflammatory properties, play an important role in the proliferation, migration, and mineralization of dental pulp stem cells [30]. In the present study, the MTT assays revealed that Theracal LC negatively affected hDPSC viability at all extraction medium concentrations, whereas this effect was lower in the Theracal PT group. Unsurprisingly, MTA group exhibited a higher cell viability compared to the other materials and the control. These findings were in agreement with previous reports demonstrating that Theracal LC exhibited a cytotoxic effect [15, 31].

It is well known that bioactive materials release substances that could potentially delay or enhance the healing process

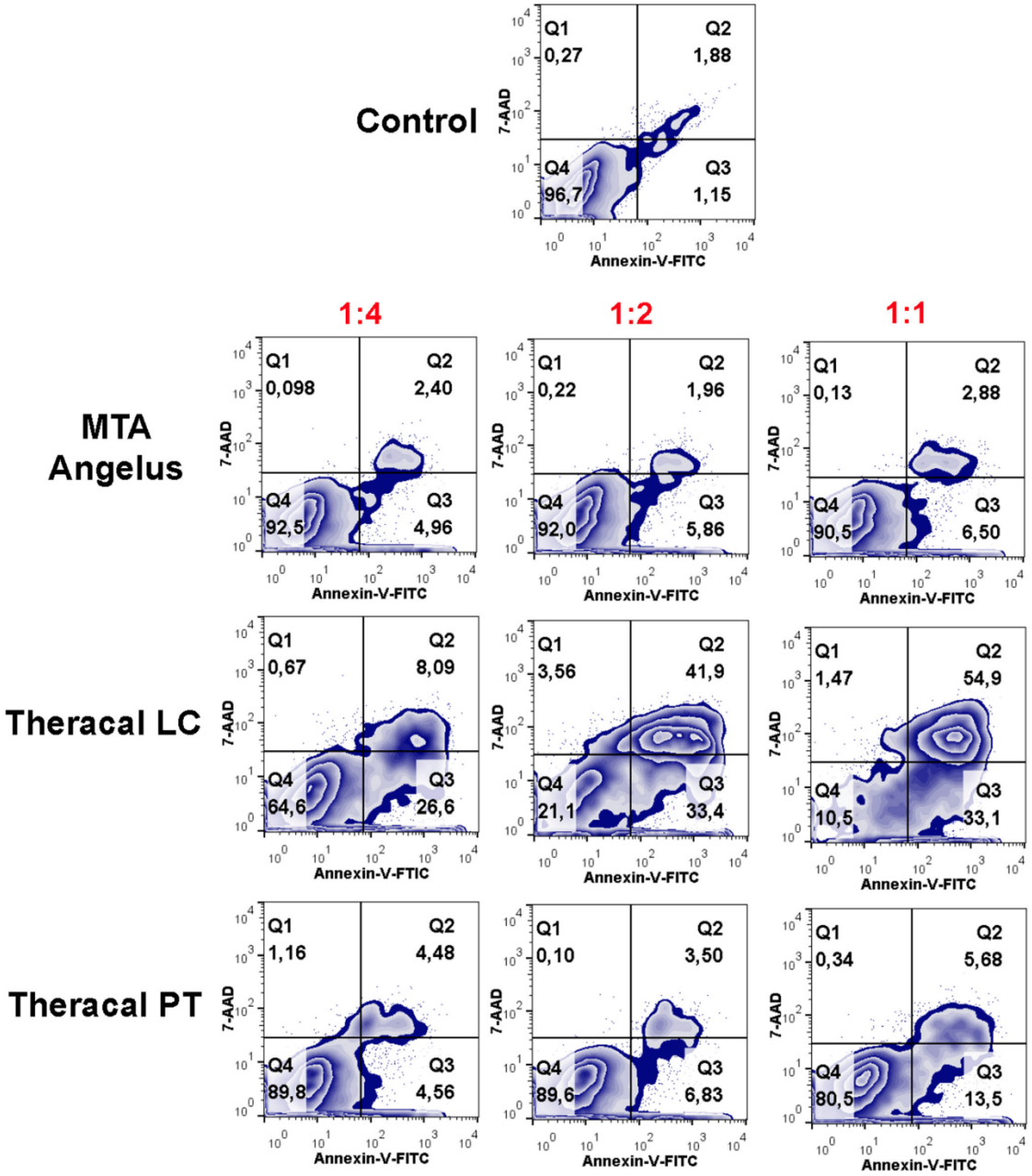


Fig. 7 Flow cytometry analysis of cell apoptosis and necrosis induced by the different vital pulp material extracts on hDPSCs by annexin V-PE/7-AAD staining. Numbers inside dot plots represent percentages of live (Q4 quadrants), early apoptotic (Q3 quadrants), and late apoptotic and necrotic cells (Q1 and Q2 quadrants). Dot plots shown are representative from $n = 3$ separate experiments

[32]. For this reason, wound healing assays were performed in order to preliminarily predict how the coordinated migration of hDPSC would occur during pulp inflammation or after injury. The marked decrease in cell migration in the Theracal LC–treated group could be due to the effect of this material on cell viability. Nevertheless, no significant differences were observed in 1:2 and 1:4 Theracal PT groups, evidencing an optimal biological response with these

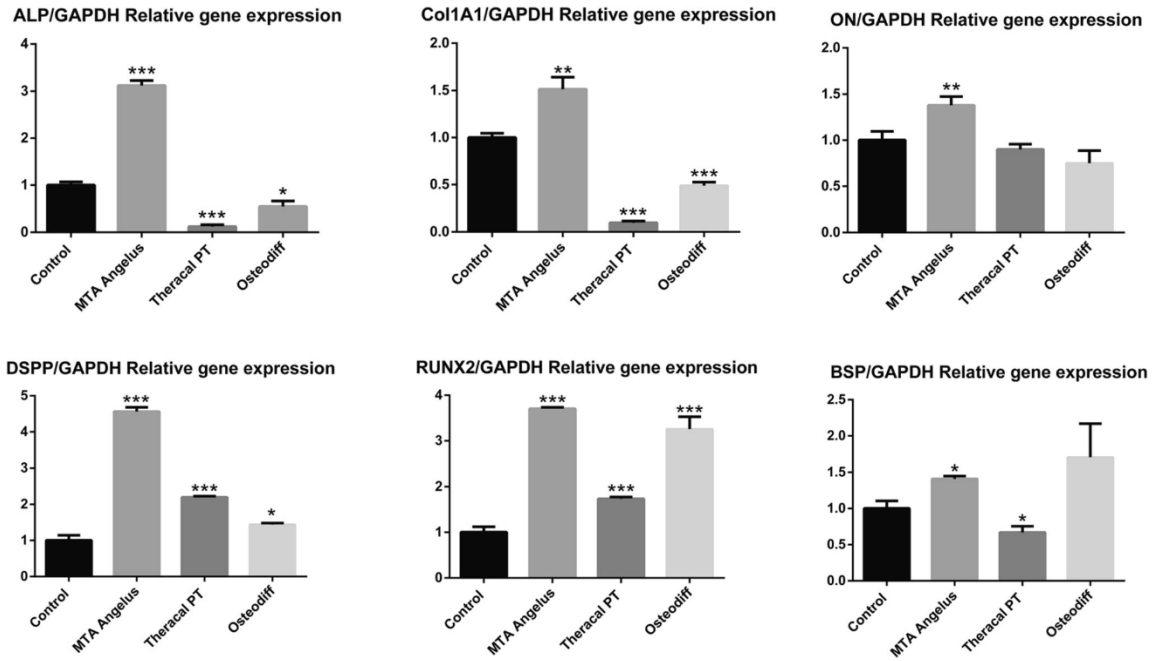


Fig. 8 The expression of osteo/odontogenic genes expression detected by RT-qPCR. Data are expressed as mean ± SD; relative to GAPDH expression. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

dilutions. The cell migration results correlated with the cell attachment and spreading results, as expected, since cells require an adequate attachment and spreading on a surface for subsequent migration. Low cell attachment and F-actin fibers content and an aberrant morphology were evidenced in the 1:1 and 1:4 Theracal LC–treated group as observed by phalloidin staining and SEM. Hence, their migration would also be affected.

Apoptosis/necrosis assay evidenced a reduced number of viable cells in Theracal LC–treated group. This phenomenon was less evident in Theracal PT–treated group. It has been speculated that the cytotoxicity of Theracal LC may be due to remaining unpolymerized resin monomers which leads to their leaching [25, 33]. Furthermore, other authors have demonstrated that cured Theracal LC released specific additives such as ethyl-4-(dimethylamino) benzoate and camphorquinone [34]. Primary human pulp fibroblasts exposed to camphorquinone evidenced increased reactive oxygen species production [35, 36]. Taken together, it can be suggested that the light curing additives released from Theracal LC may induce cell death by increasing the production of reactive oxygen species.

To evaluate the potential effects of the tested materials for VTP on the osteo/odontoblastic differentiation of hDPSCs, expressions of ALP (alkaline phosphatase), BSP (bone sialoprotein), Col1A1 (collagen type I alpha 1), DSPP (dentin sialophosphoprotein), ON (osteonectin), and RUNX2 (runt-related transcription factor 2) genes were used as markers of different phases of this process. As mentioned previously, the marked cytotoxicity exhibited by Theracal LC resulted in its exclusion from the differentiation assays, since no gene upregulation was to be expected. MTA exhibited an upregulation of all of the tested markers. A similar outcome was observed in a previous study, in which the cytotoxicity and osteogenic potential of MTA and Theracal LC, among other materials, was assessed together with hDPSCs [37]. Theracal LC exhibited no upregulation of osteo/odontogenic markers, while MTA showed a significant upregulation of ALP, OCN (osteocalcin), BSP, DSPP, and DMP1 (Dentin matrix acidic phosphoprotein 1) at 7 days when compared to a control ($p < 0.05$). In contrast with our study, however, MTA did not induce an upregulation of RUNX2.

In addition, both MTA and Theracal PT–cultured cells showed a significant upregulation of DSPP and RUNX2 genes at day 7. Since, to the authors' knowledge, this is the first study to assess the biological properties of Theracal PT, these results cannot be compared with other studies. Nevertheless, the upregulation of RUNX2, a key transcription factor for osteoblast differentiation [38], and DSPP, involved in the process of mineralization of dentin [39], highlights the potential influence of both of the assessed materials on hDPSC osteo/odontogenic differentiation.

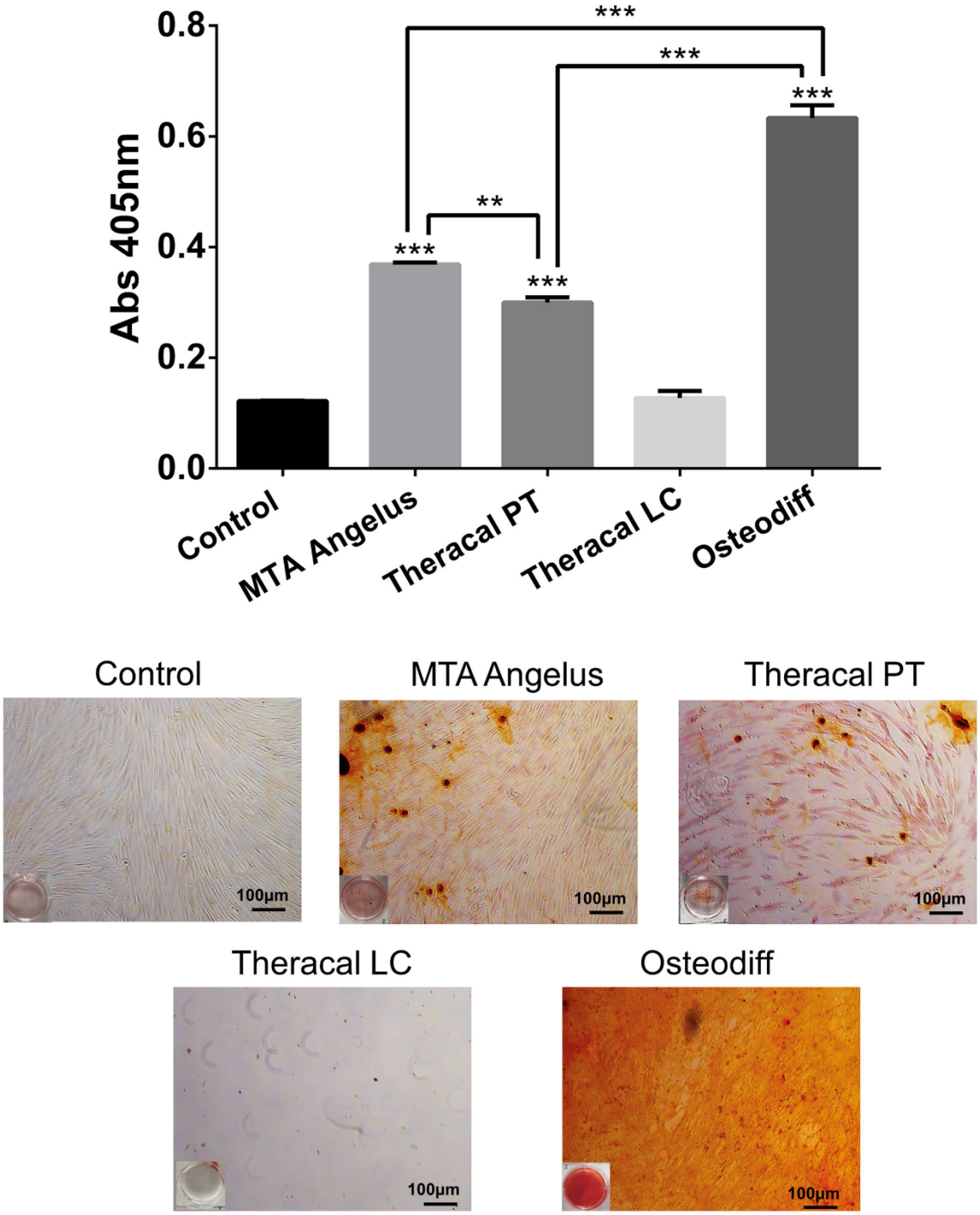


Fig. 9 Calcium deposition, as a final product of the odonto/osteogenic differentiation process, was assessed by using Alizarin Red S staining on day 21. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

Regarding the mineralization potential of the tested materials for VPT, a similar pattern was observed. hDPSCs treated with Theracal LC exhibited the lowest calcified nodule formation, and both MTA and Theracal PT–treated cells showed a significant mineralization potential. These results are in accordance with previous studies: in direct contact with stem cells from human exfoliated deciduous teeth (SHEDs), MTA showed a higher mineralization potential than Theracal LC [40]; and significantly higher mineralized nodule formation compared to a negative control when cultured with human dental pulp cells (hDPCs) [41]. Again, no available evidence was found regarding Theracal PT. Nonetheless, the results from the mineralization assays from the present study highlight its comparable mineralization potential to that exhibited by MTA. In VPT procedures, the materials used should possess the ability to induce the superficial mineralization of the viable pulp tissue to form a calcified bridge. However, in order to preserve the correct functioning of the pulp tissue, its diffused calcification should be avoided, as it will lead to pulp inflammation and loss of vitality, and eventually to treatment failure [42].

Altogether, the biological properties and bioactive potential of hydraulic calcium silicate–based cements, such as those assessed in the present study, have been evidenced by numerous studies [5]. Still, the rapid introduction of new material compositions into the market calls for an updated biological and chemical-mechanical profiling of each new composition before its clinical use. This highlights the importance and relevance of in vitro studies as a preliminary approach to the assessment of new materials. However, results from these assays should be interpreted with caution, since the behavior of the tested materials could be potentially influenced by a number of external factors in the clinical setting.

Conclusions

This study demonstrates the favorable in vitro cytocompatibility and bioactive properties of the recently introduced Theracal PT and the well-established MTA Angelus on hDPSCs, as opposed to Theracal LC. More studies, including in vivo animal testing are suggested before these new formulations might be used in the clinical setting.

Funding This work was supported by the Spanish Network of Cell Therapy (TerCel), RETICS subprograms of the I+D+I 2013-2016 Spanish National Plan, project "RD16/0011/0001" funded by the Instituto de Salud Carlos III to JMM and cofunded by the European Regional Development Fund.

Declarations

Conflict of interest The authors declare 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 University of Murcia (procedure number: 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 personal data protection.

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

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ARTÍCULO 5

Clinical Oral Investigations
https://doi.org/10.1007/s00784-021-04117-x

ORIGINAL ARTICLE

Biomineralization potential and biological properties of a new tantalum oxide (Ta_2O_5)–containing calcium silicate cement

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Received: 22 April 2021 / Accepted: 1 August 2021

Abstract

Objective The present study evaluated the biological effects and biomineralization potential of a new tantalum oxide (Ta_2O_5)–containing material designed for vital pulp therapy or perforation repair (NeoMTA 2), compared to NeoMTA Plus and Bio-C Repair.

Material and methods Human dental pulp stem cells (hDPSCs) were exposed to different eluates from NeoMTA Plus, NeoMTA 2, and Bio-C Repair. Ion release from each material was determined using inductively coupled plasma-optical emission spectrometry (ICP-MS). The biological experiments performed were MTT assays, apoptosis/necrosis assays, adhesion assays, migration assays, morphology evaluation, and reactive oxygen species (ROS) production analysis. Biomineralization was assessed by Alizarin red S staining. Finally, osteo/odontogenic gene expression was determined by real-time quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparison test.

Results NeoMTA 2 displayed a significantly higher calcium release compared to the other materials ($p < 0.05$). When hDPSCs were cultured in presence of the different material eluates, all groups exhibited similar hDPSC viability and migration rates when compared to untreated cells. Substantial cell attachment and spreading were observed in all materials' surfaces, without significant differences. hDPSCs treated with NeoMTA 2 displayed an upregulation of ALP, Col1A1, RUNX2 ($p < 0.001$), ON, and DSPP genes ($p < 0.05$), and showed the highest mineralization potential compared to other groups ($p < 0.001$). Finally, the more concentrated eluates from these materials, specially NeoMTA Plus and NeoMTA 2, promoted higher ROS production in hDPSCs compared to Bio-C Repair and control cells ($p < 0.001$), although these ROS levels did not result in increased cell death.

Conclusions The new tantalum oxide (Ta_2O_5)–containing material shows an adequate cytocompatibility and the ability to promote biomineralization without using chemical osteogenic inducers, showing great potential as a new material for vital pulp therapy.

Clinical relevance NeoMTA 2 seems to be a promising material for vital pulp therapy. Further studies considering its biocompatibility and biomineralization potential are necessary.

Keywords Bioactivity · Ion-releasing materials · NeoMTA · Vital pulp therapy biomineralization

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Published online: 12 August 2021

Introduction

The main objective of the dental pulp is to provide vitality to the tooth, since it is responsible for nourishing it by providing sensitivity, and responding to different stimuli such as pressure [1]. Certain dental injuries such as caries or trauma, if uncontrolled, could eventually lead to cellular death or necrosis. However, the pulp tissue, when affected in a reversible manner, presents an intrinsic reparative potential. Accordingly, the maintenance of pulp vitality by means of vital pulp therapy (VPT) procedures regained interest as a more conservative alternative to root canal treatment in cases of pulpitis [2]. Thus, this therapy can be defined as a restorative dental treatment and includes direct and indirect pulp capping, and pulpotomy, which can be partial or total [3].

Human dental pulp stem cells (hDPSCs) are considered a multipotential undifferentiated cell population with self-renewal capacity and the ability to repair dentin by dentin bridge formation [4]. These cells show a fibroblast-like morphology with superior proliferative ability compared to human bone marrow–derived mesenchymal stem cells. It has been shown that hDPSCs can differentiate into ectodermal-, mesodermal-, and endodermal-derived cells. Hence, these cells are being proposed as an alternative cell source for various reparative applications [5, 6].

Mineral trioxide aggregate (MTA) is considered the gold standard for vital pulp treatment due to its bioactivity, biocompatibility, hydrophilicity, and low solubility [7]. It is also reported to induce dental pulp proliferation, ion release, and hard tissue formation [8]. However, MTA has shown some disadvantages such as difficulty in handling, low compressive strength due to its porous matrix, and prolonged setting time that leads to washout in presence of excess moisture. To overcome the limitations in the clinical performance of MTA, new ion-releasing endodontic materials such as NeoMTA 2 (NuSmile Avalon Biomed, Bradenton, FL, USA) or Bio-C Repair (Angelus, Londrina, PR, Brazil) have been recently marketed [9].

Bio-C Repair is a new ready-for-use material with the same applications as MTA and has the ability to release calcium ions [10]. This new ion-releasing material contains zirconium oxide, calcium silicate, iron oxide, calcium aluminate, calcium oxide, silicon dioxide, and dispersing agent in its composition. In addition, previous reports have shown that this material exhibits similar biological effects and mineralization potential to MTA-based materials [9]. NeoMTA 2 is the second generation of root and pulp treatment materials whose predecessor was NeoMTA Plus (NuSmile Avalon Biomed, Bradenton, FL). Both materials are composed of a new tricalcium silicate–based material, with tantalum oxide (Ta_2O_5) as a radiopacifying agent instead of bismuth oxide to overcome its well-known discoloration potential [11, 12]. It is mixed with a water-based gel that results in superior handling properties. The mixing powder-to-liquid ratio can be varied depending on the indication for use: thin consistency as a sealer or thick consistency as a vital pulp material or perforation repair material [13]. Due to its recent introduction to the market, the effects of NeoMTA 2 on dental pulp cells are not fully understood in terms of biocompatibility or the expression profile of genes related to biomineralization.

A preliminary requirement for new materials is to establish the biological responses of cells exposed to them in vitro. In vitro tests are traditionally used as first-line testing to evaluate material properties including potential cytotoxicity [14].

Therefore, the objectives of this study were to analyze the biomineralization potential and biological effects of this new tantalum oxide (Ta_2O_5)–containing material (NeoMTA 2) and to compare these properties to those exhibited by NeoMTA Plus and Bio-C Repair. The null hypothesis of this study was that all ion-releasing endodontic materials exhibit a similar biomineralization potential and cytocompatibility.

Materials and methods

Tested materials and elution preparation

The tested materials in this study were as follows: NeoMTA Plus (NuSmile Avalon Biomed, Bradenton, FL, USA), NeoMTA 2 (NuSmile Avalon Biomed), and Bio-C Repair (Angelus, Londrina, PR, Brazil) (Table 1). Specimens of 2 mm diameter and 1 mm height were prepared and left undisturbed to set at 37 °C in 5% CO_2 environment and 95% relative humidity for 48 h. Once set, the surfaces of the specimens were exposed for 20 min to ultraviolet light to ensure sterility. The grouping is depicted in Table 1. Each group was evaluated for cytotoxicity according to ISO 10,993–12 [15]. Final concentrations were 1/1, 1/2, and 1/4.

Ion release analysis

Test specimens with the aforementioned dimensions ($n=3$) were prepared and stored at 37 °C in 100% humidity for 24 h. Each specimen was suspended in 5 mL deionized water for 1 day and the solution collected was analyzed using inductively coupled plasma-optical emission spectrometry (ICP-MS; Agilent 7900, Stockport, UK). The proportions of aluminum (Al), silicon (Si), sulfur (S), calcium (Ca), strontium (Sr), barium (Ba), and tungsten (W) released from each material were calibrated with pure deionized water. Analyzes were performed independently in triplicate ($n=3$).

Table 1 The grouping of materials

Materials	Manufacturer	Composition	Composition
NeoMTA Plus	NuSmile Ltd (Avalon Biomed). 3315 West 12th Street Houston, TX 77,008 USA	**Powder**: Tricalcium silicate (Ca_3SiO_5), dicalcium silicate (Ca_2SiO_4), tantalum oxide (Ta_2O_5), and minor amounts of calcium sulfate ($CaSO_4$) and tricalcium aluminate ($Ca_3Al_2O_6$) **Liquid**: Water (H_2O) and proprietary polymers	2,019,091,001
NeoMTA 2	NuSmile Ltd (Avalon Biomed). 3315 West 12th Street Houston, TX 77,008 USA	**Powder**: Tricalcium silicate (Ca_3SiO_5), dicalcium silicate (Ca_2SiO_4), tantalum oxide (Ta_2O_5), and minor amounts of calcium sulfate ($CaSO_4$) and tricalcium aluminate ($Ca_3Al_2O_6$) **Liquid**: Water (H_2O) and proprietary polymers different from above	2,020,051,501
NeoMTA 2 has more tantalite and different polymers than NeoMTA Plus			
Bio-C Repair	Angelus, Rua Waldir Landgraf, Barrio Lindóia, Londrina, Brasil	Calcium silicate (Ca_3SiO_5), calcium aluminate ($CaAl_2O_4$), calcium oxide (CaO), zirconium oxide (ZrO_2), iron oxide (Fe_2O_3), silicon dioxide (SiO_2), and dispersing agent	531,522

Surface characterization

The surface and morphology analyses of the different materials were performed using SEM-coupled energy-dispersive spectroscopy (SEM–EDS, JSM-610LV, JEOL, Tokyo, Japan). Specimen disks of each material ($n=9$) were prepared, and after setting time at 37 °C and 95% humidity, the disks underwent a carbon-coating process in a CC7650 SEM Carbon Coater unit (Quorum Technologies Ltd, East Sussex, UK). Finally, SEM micrographs were registered, and a qualitative analysis was performed for the surface element distribution.

Isolation, culture, and characterization of human dental pulp stem cells

Cells were obtained from impacted third molars ($n=12$) extracted for orthodontic reasons from patients aged 18–26 years old with prior informed consent using guidelines approved by the Institutional Committee of the University of Murcia (protocol ID: 2199/2018).

The extracted teeth were rinsed once in saline (0.9% w/v sodium chloride) and several times in sterile PBS, and then immersed in 1% povidone iodine for 2 min and 0.1% sodium thiosulfate in PBS for 1 min, and washed again in sterile PBS. Teeth were then vertically split to expose the dental pulp. The pulp tissue was gently extirpated and placed into an enzymatic bath containing collagenase type I (Gibco, Life Tech, NY, USA) at 37 °C for 40 min to digest the tissue and liberate the cells. Then, isolated cells were washed with PBS, filtered through 40-µm nylon cell strainers (BD Biosciences, San Jose, CA, USA), and cultured in DMEM supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland), 1% GlutaMAX™ (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific) (complete growth medium) at 37 °C and 5% CO_2. Cell immunophenotype was performed by fluorescence-activated cell sorting analysis using specific antibodies for human CD14, CD20, CD34, CD45, CD73, CD90, and CD105, as described previously [15].

Mitochondrial viability assay

hDPSC viability in presence of ion-releasing materials was performed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) assays. Briefly, 5×10^3 cells per well were seeded in 96-well plates, allowed to attach for 24 h, and treated with several dilutions (1/1, 1/2, and 1/4) for 24, 48, and 72 h. Then, the medium was replaced with 5 mg/mL MTT under standard culture conditions for 4 h. In these assays, mitochondrial viability was measured by converting the MTT tetrazolium salt to a colored formazan compound by mitochondrial dehydrogenases. Finally, the absorbance was determined at 570 nm using a microplate reader (ELx800, Bio-Tek Instruments, Winooski, VT, USA) after cell lysis in 0.4 M hydrochloric acid solution in isopropanol (200 µl per well). Three independent experiments were performed for each sample and condition [15].

Cell migration

The migratory ability of hDPSCs cultured with ion-releasing material eluates was determined using in vitro wound healing assays. hDPSCs were seeded into 12-well plates (2×10^4 hDPSCs per well), and a vertical scratch was created using a 200-µL sterile-pipette tip. Microscopy images were then taken at 0, 24, 48, and 72 h and analysis was performed using ImageJ software (National Institutes of Health,

Bethesda, MD, USA) [15]. Each experimental condition was carried out in triplicate for each material and analyzed in three independent experiments.

Cell attachment

hDPSCs were cultured on the surface of the materials for 72 h. Afterwards, the culture medium was removed and cells were washed with PBS solution. Then, the adherent cells on the specimens were fixed with 3% glutaraldehyde for 30 min at 4 °C. The specimens were dehydrated using hexamethyldisilazane (Sigma-Aldrich, St. Louis, MO, USA) and varying concentrations of ethanol at room temperature. After fixation, the cell attachment was observed using scanning electron microscopy (SEM) after gold sputtering on the samples. Images were taken at × 100, × 300, and × 1500 magnifications [16]. Each experimental condition was carried out in triplicate for each material and analyzed in three independent experiments.

Immunocytochemistry

Immunocytochemistry was performed to evaluate possible alterations in hDPSC morphology after exposure to undiluted ion-releasing materials. Briefly, hDPSCs were grown on coverslips for 72 h at 37 °C. Then, hDPSCs were fixed in 4% formaldehyde solution (Merck Millipore, Darmstadt, Germany) for 10 min and blocked with 5% bovine serum albumin (Sigma-Aldrich) for 30 min. The coverslips were stained with AlexaFluor™ 594–conjugated phalloidin (Invitrogen, Carlsbad, CA, USA) or phosphate-buffered saline in the control group. Afterwards, the nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the coverslips were analyzed under a confocal microscope (Leica, Wetzlar, Germany) [16]. Each experimental condition was carried out in triplicate for each material and analyzed in three independent experiments.

Apoptosis/necrosis assays and ROS production analyses

Cell viability and reactive oxygen species (ROS) production after exposure to the different material eluates were analyzed by annexin-V/7-AAD and the general oxidative stress indicator CM-H_2DCFDA staining, respectively [17]. Briefly, hDPSCs were treated with complete culture medium alone (w/o any eluate) (control group) or with complete medium supplemented with different dilutions of the materials (1/1, 1/2, or 1/4) for 72 h at 37 °C. Afterwards, cells were washed and stained with FITC-conjugated annexin-V and 7-AAD (Immunostep, Salamanca, Spain) for 15 min at r/t, or with 5 μM CM-H_2DCFDA (Molecular Probes, Eugene, OR, USA) for 30 min at 37 °C. Finally, samples were acquired in a BD LSRFortessa™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and percentages of live and apoptotic/necrotic cells or CM-H_2DCFDA-positive cells were analyzed with FlowJo software (FlowJo LLC, Ashland, OR, USA). Each experimental condition was carried out in triplicate for each material and analyzed in three independent experiments.

Gene expression analysis

The genetic expression of human alkaline phosphatase (*ALP*), collagen type 1 (*Col1A1*), dentin sialophosphoprotein (*DSPP*), osteonectin (*ON*), runt-related transcription factor 2 (*RUNX2*), and bone sialoprotein progenitor (*BSP*) was determined by quantitative polymerase chain reaction (qPCR) from cells treated with discs of the different material eluates after 14 days ($n = 3$) as described previously [16]. Briefly, cells were harvested and total RNA was isolated (Purelink RNA Mini Kit, Invitrogen, Thermo Fisher Scientific). Following that, cDNA synthesis was performed (iScript RT Supermix, Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression values obtained by qPCR analysis for each gene compared to human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. Cells cultured with basal growth media were used as negative control, and cells treated with a commercial differentiation media (StemMACS OsteoDiff Media, Miltenyi Biotec, Bergisch Gladbach, Germany) acted as the positive control. Each experimental condition was carried out in triplicate for each sample and analyzed in three independent experiments.

Alizarin Red S staining

To identify whether hPDSCs produced mineralized nodules in vitro in presence of NeoMTA Plus, NeoMTA 2, and Bio-C Repair eluates, Alizarin Red S staining was used according to previous studies [18]. Briefly, hPDSCs (2×10^4 cells/well) were cultured in undiluted tested materials for 21 days. After the culture period, the culture medium was removed, and a 4% paraformaldehyde solution was added to each well for fixation for 15 min. The solution was removed, and the cells were washed with PBS for 10 min. Then, hPDSCs were stained with 2% Alizarin Red S solution (Sigma-Aldrich) (pH 4.1) for 15 min at r/t. The staining was solubilized with 10% cetylpyridinium chloride monohydrate (Sigma-Aldrich) solution and the absorbance of the eluted stains was read at 570 nm using a spectrophotometer. hDPSCs cultured in unconditioned medium (DMEM w/o any eluate) and OsteoDiff media were used as the negative and positive controls, respectively. Each experimental condition was carried out in

triplicate for each material and analyzed in three independent experiments.

Statistical analysis

All data represented the mean ± standard deviations (SD) of at least three independent culture experiments. Data were tested for normal distribution by the Kolmogorov–Smirnov test, and group comparisons were analyzed by one-way ANOVA, and differences between means were compared by Tukey's multiple comparison test. The data obtained were analyzed using GraphPad Prism v8.0.2 (GraphPad Software, San Diego, CA, USA). p-values < 0.05 were considered statistically significant.

Results

Ion release

The results from ion release analysis are shown in Table 2. In general terms, NeoMTA 2 exhibited a higher release of Ca^{2+} (23.03 ± 0.02) compared to NeoMTA Plus (9.04 ± 0.01) and Bio-C Repair (15.01 ± 0.00) ($p < 0.05$), while strontium (Sr) and silicon (Si) ion release were significantly increased in Bio-C Repair ($p < 0.05$). In contrast, NeoMTA Plus was associated with the highest release of sulfur (S) and tungsten (W).

SEM–EDS analysis

The EDX analysis of the chemical elements on the surface of the specimens was comparable to the material composition according to the manufacturers' information, as shown in Fig. 1 and Table 1. EDX analysis of NeoMTA 2 displayed a higher peak of Ca^{2+} than NeoMTA Plus and Bio-C Repair. Peaks of Zr^{+} and Si^{4+} were also observed in Bio-C Repair due to the presence of ZrO_2 and silica, while tantalum (Ta^{5+}) was detected in NeoMTA 2 and NeoMTA Plus. Additionally, the SEM–EDS analysis disclosed other elements which were not mentioned in the manufacturers' information: aluminum (Al^{3+}) and sulfur (S^{2-}) were found in NeoMTA 2 and NeoMTA Plus, while aluminum (Al^{3+}) alone was detected in Bio-C Repair.

Mitochondrial viability assay

After 72 h of culture, the exposure of hDPSCs cells to the material eluates did not significantly affect mitochondrial metabolism (Fig. 2). Only with undiluted materials, a significant reduction in cell viability was evidenced, compared to the negative control in the first time point (24 h) ($p < 0.05$, $p < 0.01$).

Migration assay

Cell migration ability was monitored by wound healing assays. hDPSCs cultured in basal growth medium served as the negative control. No significant differences were found between any of the ion-releasing materials and the negative control at 24 and 48 h (Fig. 3). Only at 24 h, undiluted NeoMTA 2 promoted a higher cell migration than the control group ($p < 0.05$). These results confirmed that all materials allowed an adequate cell migration.

Cell attachment

The cellular attachment and morphology can be affected by exposure to cytotoxic agents and directly reflects cell injuries. The analysis of cell attachment of hDPSCs on the surfaces of the different ion-releasing materials was observed after 3 days of culture. Abundant well-adhered and functionally oriented cells were observed in all materials, suggesting no cytotoxic effect (Fig. 4).

Cell cytoskeleton staining

All ion-releasing materials exhibited positive phalloidin staining with a fibroblastic spindle-shaped morphology and increased F-actin content, indicating the contractile property of hDPSCs. A similar phenomenon was observed on the cells from the negative control group (Fig. 5).

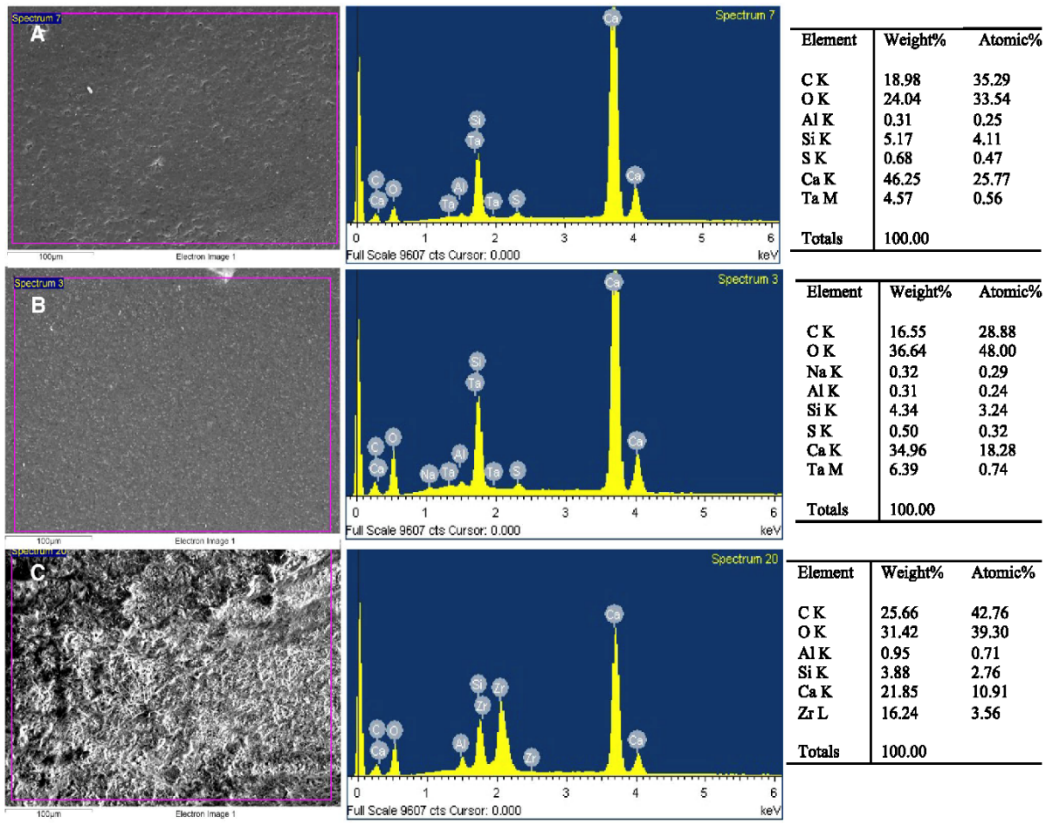
Table 2 Assessment of ICP-MS of ion-releasing materials

	29 Si [He]	34 S [He]	42 Ca [He]	88 Sr [He]	137 Ba [He]	181 Ta [He]	182 W [He]
Sample name	Conc. [ppm]	Conc. [ppm]	Conc. [ppm]	Conc. [ppm]	Conc. [ppm]	Conc. [ppm]	Conc. [ppm]
NeoMTA Plus	6.75 ± 0.00^{AB}	7.80 ± 0.01^{AB}	9.04 ± 0.01^{AB}	425.48 ± 0.01^{AB}	5.23 ± 0.04^{A}	0.12 ± 0.02^{AB}	59.66 ± 0.00^{AB}
NeoMTA 2	2.40 ± 0.02^{AC}	1.79 ± 0.01^{AC}	23.03 ± 0.02^{AC}	212 ± 0.01^{AC}	3.57 ± 0.03^{C}	0.48 ± 0.02^{BC}	1.86 ± 0.00^{AC}
Bio-C Repair	19.57 ± 0.00^{BC}	3.64 ± 0.02^{BC}	15.01 ± 0.00^{BC}	928 ± 0.03^{BC}	10.77 ± 0.00^{AC}	$< 0.000 \pm 0.000^{AC}$	8.52 ± 0.00^{BC}

Uppercase A indicates significant difference ($p < 0.05$) between NeoMTA Plus and Bio-C Repair

Uppercase B indicates significant difference ($p < 0.05$) between NeoMTA Plus and NeoMTA 2

Uppercase C indicates significant difference ($p < 0.05$) between NeoMTA 2 and Bio-C Repair



Element	Weight%	Atomic%
C K	18.98	35.29
O K	24.04	33.54
Al K	0.31	0.25
Si K	5.17	4.11
S K	0.68	0.47
Ca K	46.25	25.77
Ta M	4.57	0.56
Totals	100.00	

Element	Weight%	Atomic%
C K	16.55	28.88
O K	36.64	48.00
Na K	0.32	0.29
Al K	0.31	0.24
Si K	4.34	3.24
S K	0.50	0.32
Ca K	34.96	18.28
Ta M	6.39	0.74
Totals	100.00	

Element	Weight%	Atomic%
C K	25.66	42.76
O K	31.42	39.30
Al K	0.95	0.71
Si K	3.88	2.76
Ca K	21.85	10.91
Zr L	16.24	3.56
Totals	100.00	

Fig. 1 SEM–EDS analysis results for NeoMTA 2 (**A**), NeoMTA Plus (**B**), and Bio-C Repair (**C**) disks ($n=9$). The first column presents SEM micrographs of each material (scale bar: 100 µm). The second column illustrates the EDS plots with the correspondent peaks detected. The third column classifies the list of elements present per material by weight and atomic weight

Apoptosis/necrosis and ROS production assays

The cell viability after exposition to several dilutions of each material (1/1, 1/2, and 1/4) was analyzed by annexin-V/7-AAD staining by flow cytometry. After 72 h of culture, hDPSCs in presence of 1/4 dilutions of each material did not show significant levels of apoptosis or necrosis compared to control cells. Only undiluted extracts (1/1) caused a slight decrease in cell viability (~ 10%) (Fig. 6). Moreover, as shown in Fig. 7, percentages of CM-H_2DCFDA-positive cells were progressively decreasing from 1/1 to 1/4 dilutions of each material compared to control cells, especially for tantalum oxide (Ta_2O_5)–containing materials (NeoMTA Plus and NeoMTA 2). Among them, CM-H_2DCFDA-positive cells after NeoMTA 2 treatment were significantly increased at every dilution compared to the same dilutions of the other materials and control cells.

RT-qPCR assay

To evaluate the effect of ion-releasing materials in promoting odontogenic gene expression of hDPSCs, cells were cultured up to 14 days with either control negative medium, osteogenic inducing medium (Osteodiff), or medium containing ion-releasing materials. As shown in Fig. 8, hDPSCs treated with NeoMTA 2 displayed an upregulation of *ALP*, *Col1A1*, *RUNX2* ($p<0.001$), *ON*, and *DSPP* genes ($p<0.05$) compared to the negative control, while Bio-C Repair and NeoMTA Plus groups showed only upregulation of *ON* ($p<0.05$) and *RUNX2* ($p<0.001$) respectively. Finally, when hDPSCs were cultured in an osteogenic inducing medium (Osteodiff group), upregulated expression of *DSPP*, *RUNX2* ($p<0.001$), and *ALP* ($p<0.01$) was observed when compared to the negative control at day 14.

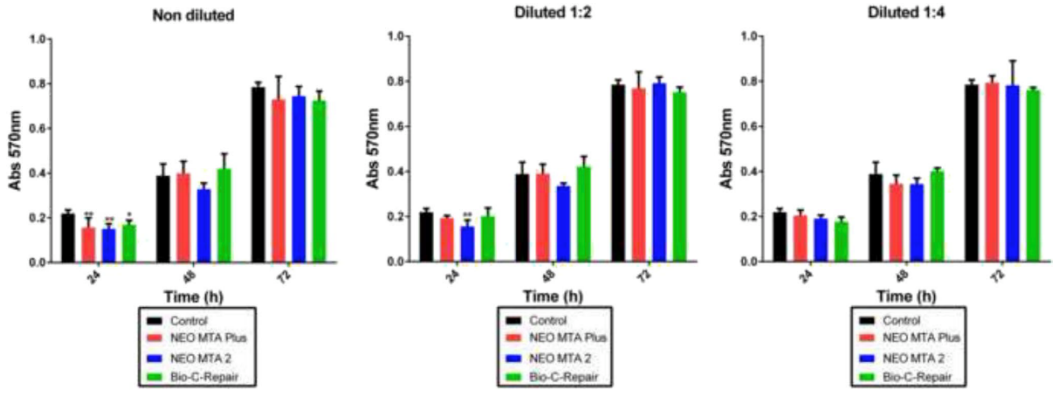


Fig. 2 Mitochondrial viability assay. In vitro cytotoxicity of hDPSCs after exposure to extracted medium prepared from Neo MTA Plus, Neo MTA 2, and Bio-C Repair. Data are presented as absorbance values (570 nm) at 24, 48, and 72 h of exposure of the material eluates to hDPSCs, compared to the control. $*p<0.05$; $**p<0.01$; $***p<0.001$. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

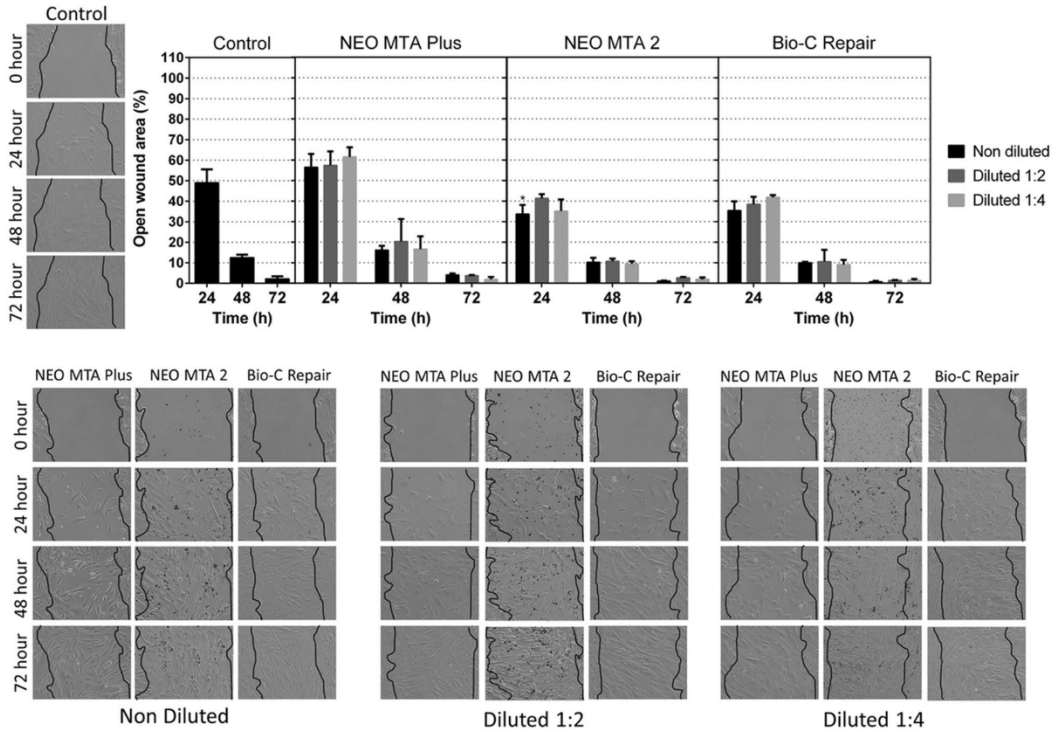


Fig. 3 Migration was evaluated using wound healing assays. Cells were exposed to undiluted (1/1) and diluted (1/2 and 1/4) eluates from materials. The control condition consisted of cells maintained in normal growth medium. Graphical results are presented as mean relative wound closure (RWC) percentages at each of the time points, relative to the total wound area at 0 h. Asterisks designate significant differences compared to the control. $*p<0.05$; $**p<0.01$; $***p<0.001$. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

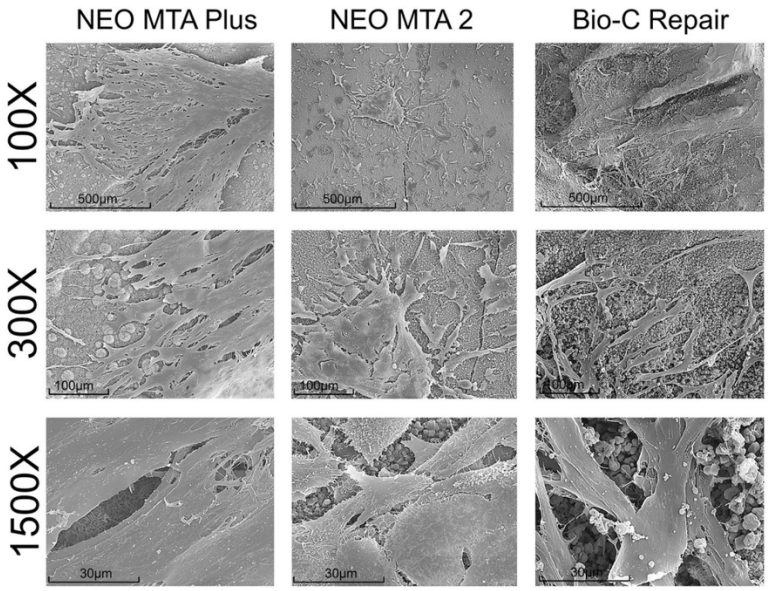


Fig. 4 Cell attachment. Sample disks with the aforementioned standardized dimensions were obtained ($n = 15$) for each of the materials and allocated into three groups ($n = 5$). Representative SEM micrographs illustrate the adhesion of hDPSCs directly seeded on Neo MTA Plus, Neo MTA 2, and Bio-C Repair. Magnifications: × 100, × 300, and × 1500. Scale bars: 500 µm, 100 µm, and 30 µm

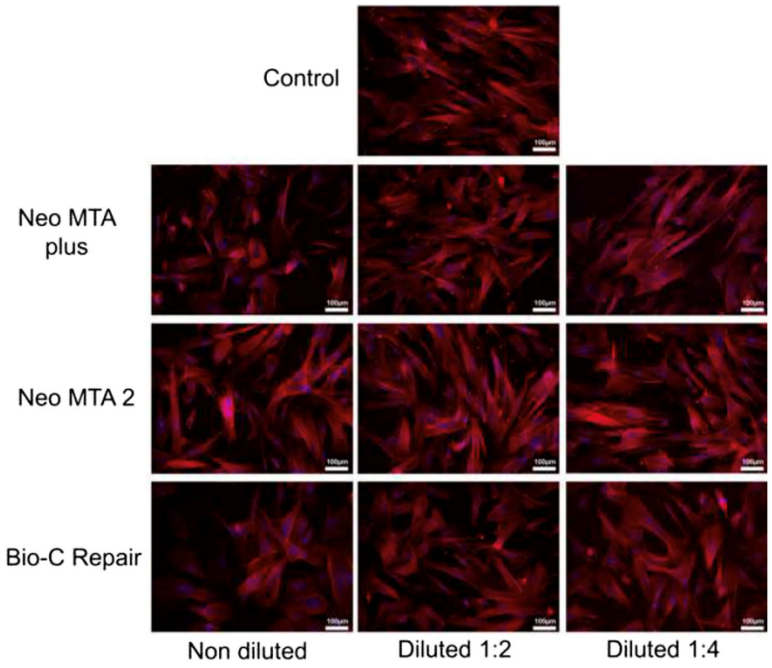


Fig. 5 Analysis of changes in cell morphology, actin cytoskeleton structure, and organization on hDPSCs after treatment with NeoMTA Plus, NeoMTA 2, and Bio-C Repair by confocal fluorescence microscopy. F-actin fibers were stained with AlexaFluor™ 594–conjugated phalloidin (red), whereas cell nuclei were counterstained with DAPI (blue). Confocal fluorescence microscopy images shown are representative from $n = 3$ separate experiments. Scale bar: 100 µm. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

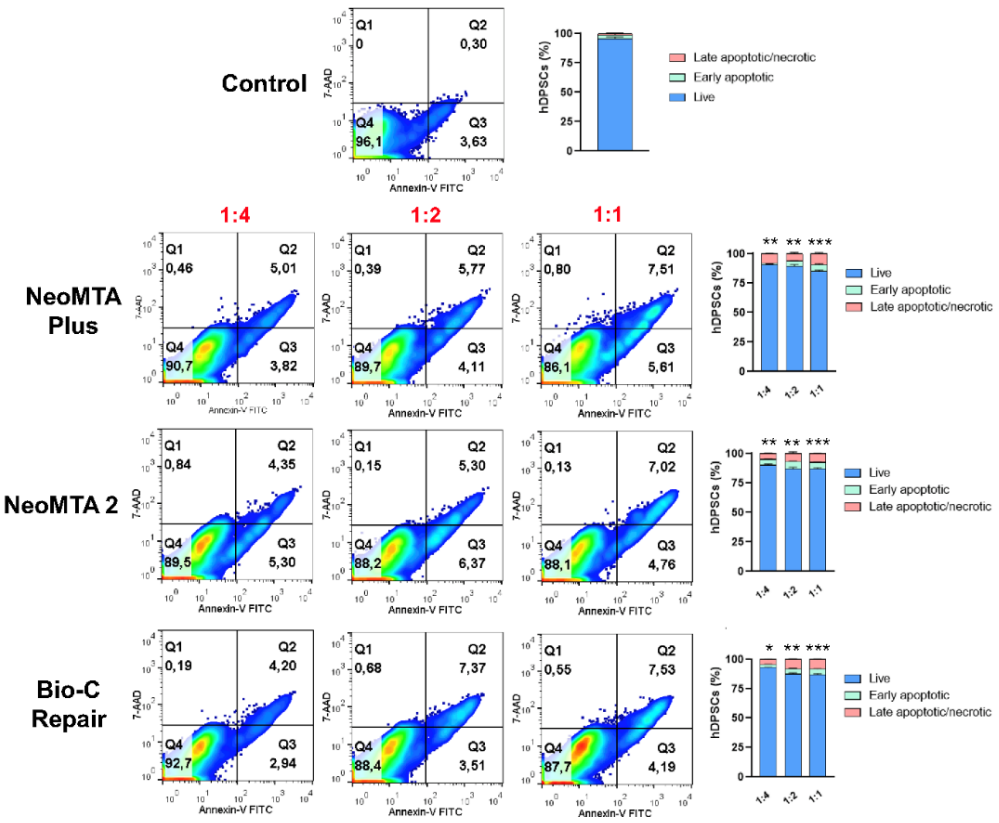


Fig. 6 Flow cytometry analysis of cell apoptosis and necrosis induced by the different vital pulp material extracts on hDPSCs by annexin V-PE/7-AAD staining. Numbers inside representative dot plots represent percentages of live (Q4 quadrants), early apoptotic (Q3 quadrants), and late apoptotic and necrotic cells (Q1 and Q2 quadrants). Bar graphs show mean ± SD from $n = 3$ separate experiments. Percentages of live cells were significantly decreased compared to the control, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, respectively

Mineralization assay

The potential for calcium deposition of ion-releasing materials was identified by using Alizarin Red S staining on day 21 (Fig. 9). All hDPSCs treated with the undiluted materials and in the osteogenic induction medium exhibited deposition of calcium nodules. There were significant differences between the tested groups, with NeoMTA 2 showing the highest mineralization potential ($p < 0.001$). In contrast, the control group containing hDPSC cells cultured in the basal culture medium without any materials did not show mineralized nodule deposition.

Discussion

The purpose of this study was to analyze the cytocompatibility and biomineralization ability of the three ion-releasing materials. In vital pulp therapy, materials play an important role in the proliferation, differentiation, and calcium nodule deposition of hDPSCs [19]. Moreover, successful vital pulp therapy also depends on the placement of a bioactive and biocompatible material which promotes human tooth-pulp cells to create a reparative dentin barrier to protect the pulp tissue from external noxious agents [7, 20, 21]. For this reason, human dental pulp stem cells were used as the

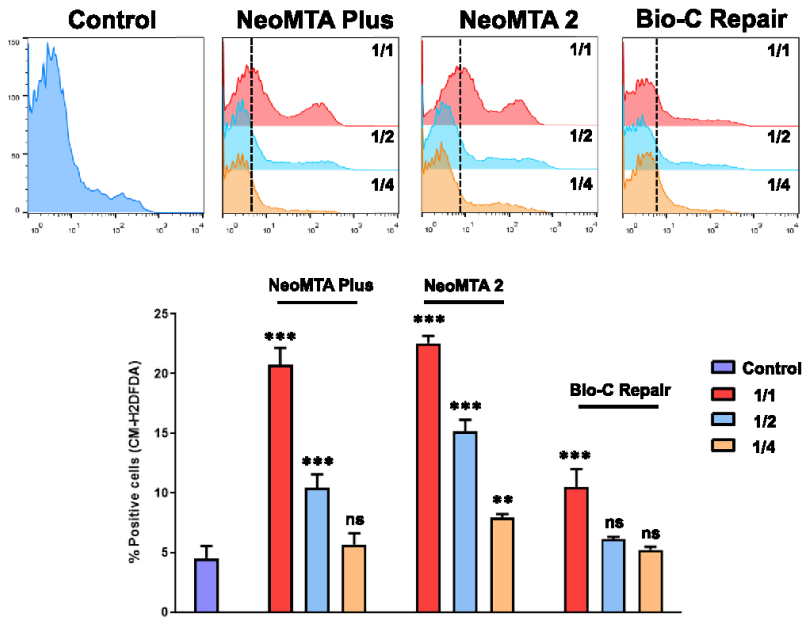


Fig. 7 Reactive oxygen species (ROS) production. CM-H_2DCFDA staining was used to evaluate intracellular ROS production in hDPSCs exposed to ion-releasing materials. Bar graph showing percentages of positive cells for ROS production in each experimental condition is shown and represented as mean ± SD from $n = 3$ separate experiments. Percentages of CM-H_2DCFDA-positive cells were significantly increased compared to the control, *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, respectively

target cells for the laboratory tests. Furthermore, immortalized cells are genetically modified and may exhibit clinically inappropriate toxic responses to compounds [22].

Some studies on cytotoxicity of ion-releasing materials have been previously reported in human cells, for example, apical papilla [23] and endothelial cells [24], while others have been tested on animal cells [25]. However, to our best knowledge, the potentially toxic effects of NeoMTA on human stem cells remain unknown. In addition, the evaluation of these materials was performed via incubation of the cultured cells with several dilutions of the materials (1:1, 1:2, and 1:4). This was done to simulate the clinical conditions in which the materials will be applied, since they are placed on remaining dentin thicknesses of 0.01 to 0.25 mm or in cavities with pulp exposure and the concentration of material which reaches the viable tissue may vary [23, 26].

Regarding ion release, NeoMTA 2 was associated with the higher release of calcium ions from all of the tested materials ($p < 0.05$). It has been described that Ca^{2+} release stimulates hydroxyl apatite formation and release of alkaline phosphatase and bone morphogenetic protein 2, which are important in the mineralization process [27]. Both SEM–EDS and ICP-MS assays exhibited Ca^{2+} release and calcium content in all materials, as previously described for NeoMTA Plus and Bio C-Repair [28, 29]. Moreover, tantalum (Ta^{5+}) was detected both in NeoMTA 2 and NeoMTA Plus. A recent report described that the incorporation of Ta^{5+} as an alternative radiopacifier does not cause discoloration and preserves acceptable values of radiopacity [30]. Also, Si^{4+} release was detected in all ion-releasing materials after setting ($p < 0.05$). Other than the release of Ca^{2+} ions, other ions, namely silicon dioxide, aluminosilicates, and CaO, are also released during hydration and could have also enhanced the cellular differentiation and proliferation [31]. The differences in ion release may also influence the role of calcium silicates in upregulating the expression of genes related to mineralization by hDPSCs [32].

The mitochondrial viability assay revealed that after 24 h, undiluted materials and 1:2 NeoMTA 2 slightly affected the mitochondrial metabolism of hDPSCs. This finding may be related to the initial dissociation of Ca^{2+} during the first 24 h. In fact, excessive intracellular accumulation of Ca^{2+} may lead to mitochondrial dysfunction, alteration of cytoskeleton organization, and activation of catabolic enzymes [33]. However, both the MTT assay results and flow cytometry results after annexin-V/7-AAD staining showed that hDPSCs were not affected by any of the tested material concentrations after 48 h and 72 h of culture, compared to the control ($p > 0.05$). Besides, significant oxidative effects were not evidenced, especially with NeoMTA 2 and NeoMTA Plus. These results suggest that these materials did not exhibit an apparent cytotoxic effect after 48 h and 72 h. These findings are consistent with previous reports showing that NeoMTA Plus and Bio-C Repair elutes were not cytotoxic [28, 34].

Cell migration is a crucial factor in angiogenesis, and it has been described that released substances could potentially delay or enhance the angiogenesis and healing process [35]. For this reason, scratch assays were performed in

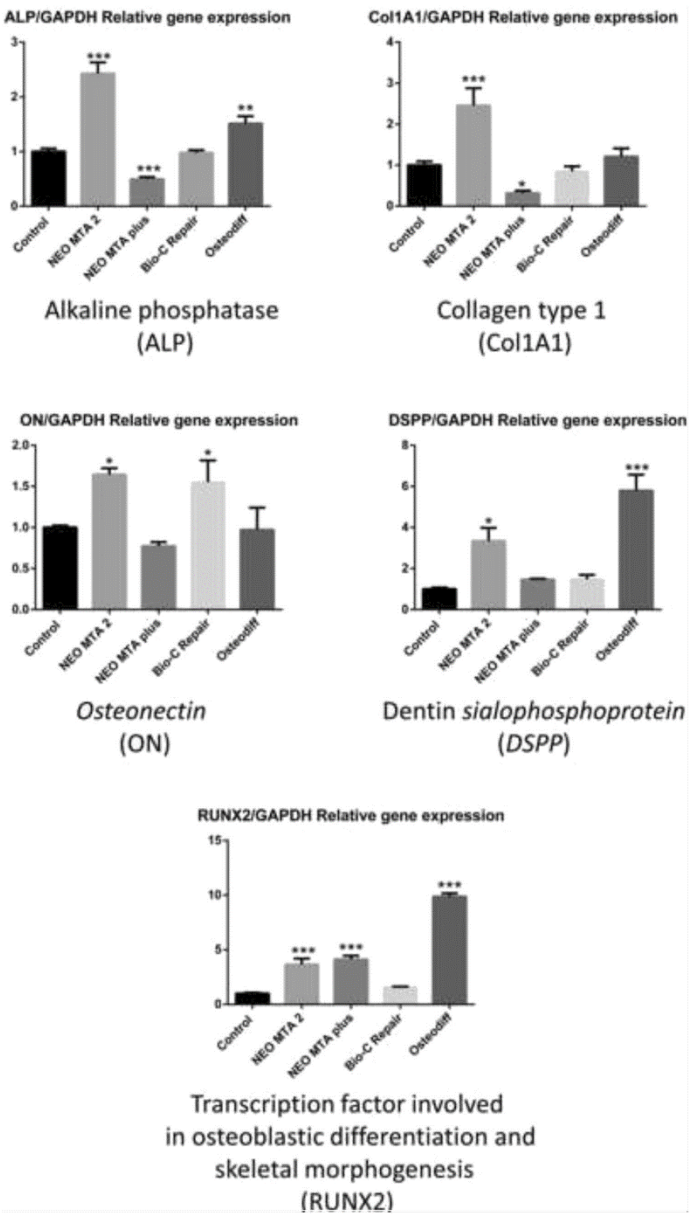


Fig. 8 The expression of osteo/odontogenic genes detected by RT-qPCR. Data are expressed as mean ± SD and relative to *GAPDH* gene expression. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

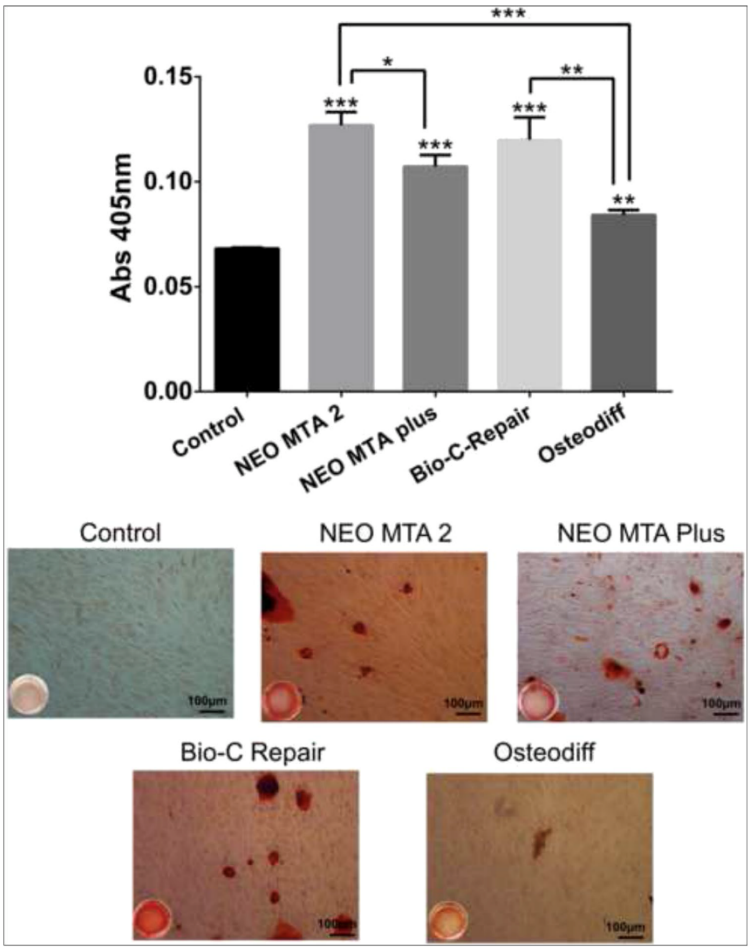


Fig. 9 Calcium deposition, as a final product of the odonto/osteogenic differentiation process, was assessed by using Alizarin Red S staining on day 21 (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Each experimental condition was performed in triplicate for each VPT material and analyzed in three independent experiments

order to preliminarily predict how the coordinated migration of hDPSC would occur during pulp inflammation or after injury. In agreement with our observations on the accumulation of MTT formazan assay, no important differences were apparent between the groups, exhibiting an optimal migration speed with these tested ion-releasing materials. In fact, cell migration rates were the highest when treated with the highest dilution (1:4) of NeoMTA 2 at 24 h ($p < 0.05$). A similar tendency for cell migration was observed by Mestieri et al. who also found an increased cell migration with ion-releasing materials, corroborating our findings [36]. The cell migration results correlated with the observed cell adhesion, explained by the fact that cellular morphology can be affected by exposure to cytotoxic agents and directly reflects cell injuries [37]. Furthermore, changes in cellular morphology have been considered a direct indicator in assessing cytotoxicity [38]. In this study, hDPSCs adopted a spread morphology and adequate attachment when exposed to ion-releasing materials. Hence, these results suggest that the tested materials did not affect cell attachment and migration.

Our results showed that a new tantalum oxide (Ta_2O_5)–containing material (NeoMTA 2) increased the expression of osteo/odontogenic-related genes after 14 days compared to the untreated control. ALP is an enzyme which is present in the osteoblast membrane and participates in bone matrix synthesis and mineralization [39]. The increase in this enzyme's activity is considered an early marker of stem cell differentiation in pre-osteoblasts and osteoblasts [40]. The early expression of osteogenic-related markers

seems to be consistent across studies when pulp cells are treated with calcium silicate–based cement [4, 41]. Previously, high gene expression of osteocalcin (OCN) was observed in human dental pulp cells exposed to BioAggregate, iRoot BP Plus, and MTA for 7 days [42] and positive expression of osteopontin (OPN) protein was observed at an early time point in the dental pulp of dogs treated with MTA and Biodentine [43]. The differences in the expression profiles may be explained by the fact that OCN and OPN markers are expressed in late polarizing odontoblasts and secretory odontoblasts while DSPP and DMP-1 expression is higher in terminally differentiated and secretory or functional odontoblasts [44]. However, one limitation of this study is the lack of direct comparability of its results due to the absence the studies on the differentiation potential of NeoMTA 2.

The capacity for calcium deposition has been identified as an indicator of successful odontoblastic differentiation [45, 46]. hDPSCs showed deposition of calcium nodules after 21 days of culture in the presence of ion-releasing materials and in the osteogenic induction medium, as indicated by the staining with Alizarin Red S. Also, the control groups containing hDPSCs cultured in the basal medium and without extracts did not present mineralized nodules. It has been reported that the presence of tantalum and zirconium promotes the osteo/odontogenic differentiation of hDPSCs [4]. Previous studies showed that iRootBP Plus and NeoMTA Plus, which contain Ta_2O_5, promote mineralization and the formation of reparative dentine bridges in vitro and in vivo [47, 48]. The higher mineralization potential exhibited by NeoMTA 2 than his predecessor NeoMTA Plus may be related to the higher proportion of tantalite and different polymers. Again, no available evidence on mineralization potential was found regarding NeoMTA 2. However, future laboratory studies are necessary to understand the exact mechanisms of the induction of osteogenic differentiation and deposition of mineralized matrix stimulated by new ion-releasing materials. Also, physicochemical properties as radiopacity, flowability, or setting time should be considered in future studies.

Conclusions

Within the limitations of this in vitro study, it can be concluded that NeoMTA 2 promotes cell viability, cell migration, and cell attachment, and induces the odonto/osteogenic differentiation of hDPSCs without using chemical osteogenic inducers.

Funding Open Access funding provided thanks to the CRUECSIC agreement with Springer Nature. This work was supported by the Spanish Network of Cell Therapy (TerCel), RETICS subprograms of the I+D+I 2013–2016 Spanish National Plan, project "RD16/0011/0001" funded by the Instituto de Salud Carlos III and co-funded by the European Regional Development Fund.

Declarations

Ethics 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 University of Murcia (procedure number: 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 personal data protection.

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

Conflict of interest The authors declare no competing interests.

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