TESIS DOCTORAL

AVANCES EN EL USO DEL PLASMA RICO EN FACTORES DE CRECIMIENTO EN LA REGENERACIÓN PERIODONTAL

MARÍA TROYA ESTAVILLO

VITORIA, 2018

Universidad del País Vasco
Euskal Herriko Unibertsitatea

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Universidad del País Vasco

Euskal Herriko Unibertsitatea

FACULTAD DE FARMACIA

Departamento de Farmacia y Ciencias de los Alimentos

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uirmi University Institute for Regenerative Medicine and Oral Implantology

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A mis padres

"This is for the ones who stand
For the ones who try again
For the ones who need a hand
For the ones who think they can"

Comes and goes (In Waves), Greg Laswell

ABREVIATURAS

ADN: ácido desoxirribonucleico.

ADP: adenosín difosfato.

AEMPS: Agencia Española de Medicamentos y Productos Sanitarios.

ANG-1: angiopoietin-1 (angiopoyetina-1).

ATP: adenosín trifosfato.

BPs: bifosfonatos.

BRONJ: bisphosphonate-related osteonecrosis of the jaw (osteonecrosis de los maxilares inducida por bifosfonatos).

CD14: cluster of differentiation 14 (clúster de diferenciación 14).

CTAP-3: connective tissue-activating peptide III (proteína activadora de tejido conjuntivo).

CTGF: connective tissue growth factor (factor de crecimiento de tejido conectivo).

DFSCs: dental follicle mesenchymal stem cells (células madre del folículo dental).

EGF: epidermal growth factor (factor de crecimiento epidérmico).

FBS: fetal bovine serum (suero bovino fetal).

FDA: Food and Drug Administration (Administración de Alimentos y Medicamentos).

FGF: fibroblast growth factor (factor de crecimiento fibroblástico).

FP-A: fibrinopéptido A.

FP-B: fibrinopéptido B.

FPP: farnesil pirofosfato.

FPPS: farnesil pirofosfato sintasa.

GBD: global burden of disease (carga mundial de enfermedad).

GGPP: geranil-geranil pirofosfato.

GMSCs: gingival mesenchymal stem cells (células madre derivadas del tejido gingival).

GP: glicoproteína.

GPP: geranil pirofosfato.

hDPSCs: human dental pulp stem cells (células madre humanas de pulpa dental).

HGF: hepatocyte growth factor (factor de crecimiento hepático).

hMSCs: human mesenchymal stem cells (células madre mesenquimales humanas).

IGF: insulin-like growth factor-1 (factor de crecimiento insulínico).

IKK: I kappa B kinase (I κ B quinasa).

IL: interleuquina.

IPP: isopentenil pirofosfato.

IRAK: interleukin-1 receptor-associated kinase (quinasa asociada al receptor de la interleuquina 1).

ISCT: International Society for Cellular Therapy (Sociedad Internacional de Terapia Celular).

I κ B: NF- κ B inhibitor (inhibidor de NF- κ B).

LBP: lipopolysaccharide binding protein (proteína de unión a LPS).

L-PRP: plasma rico en plaquetas y leucocitos.

LPS: lipopolisacáridos.

MD2: myeloid differentiation protein 2 (proteína de diferenciación mieloide 2).

MMPs: matrix metalloproteinases (metaloproteasas de matriz).

MRONJ: medication related osteonecrosis of the jaw (osteonecrosis de los maxilares inducida por medicación).

MyD88: myeloid differentiation primary response 88 (factor de diferenciación mieloide 88).

NF- κ B: factor nuclear kappa B.

PAMP: pathogen-associated molecular patterns (patrón molecular asociado a patógenos).

PBP: proteína básica de las plaquetas.

PDGF: platelet derived growth factor (factor de crecimiento derivado de plaquetas).

PDLSCs: periodontal ligament stem cells (células madre del ligamento periodontal).

PF4: platelet factor 4 (factor plaquetario-4).

PMPs: proteínas microbicidas plaquetarias.

PRGF: plasma rico en factores de crecimiento.

PRP: plasma rico en plaquetas.

RANK: receptor activator of nuclear factor κ B receptor (activador del factor nuclear kappa B).

RANKL: receptor activator for nuclear factor κ B ligand (ligando de unión al receptor activador del factor nuclear kappa B).

RANTES: regulated on activation, normal T cell expressed and secreted (quimioquina de regulación por activación expresada y secretada por los linfocitos T).

ROS: reactive oxygen species (especies reactivas del oxígeno).

SA-β-gal: senescence-associated beta-galactosidase (beta-galactosidasa asociada a la senescencia).

SCAP: stem cells of the apical papilla (células madre de papila apical).

SHED: stem cells from human exfoliated deciduous teeth (células madre de pulpa dental de diente de leche).

TAK1: transforming growth factor beta-activated kinase 1 (quinasa 1 activada por TGF-β).

TGF-β: transforming growth factor beta (factor de crecimiento transformante beta).

TLRs: Toll-like receptors (receptores tipo Toll).

TNF: tumor necrosis factor (factor de necrosis tumoral).

TRAF-6: tumor necrosis factor receptor (TNFR)-associated factor 6 (factor 6 asociado al receptor del TNF).

TSP-1: thrombospondin 1 (trombospondina 1).

Tβ-4: timosina beta 4.

VEGF: vascular endothelial growth factor (factor de crecimiento endotelial vascular).

vWF: factor de Von Willebrand.

ZA: zoledronic acid (ácido zoledrónico).

ÍNDICE

PUBLICACIONES CIENTÍFICAS

La presente tesis doctoral se basa en las siguientes publicaciones originales, a las que se hace referencia en el texto mediante números romanos (I-VI).

Las publicaciones I-III son revisiones científicas que se integran en el apartado de "Introducción". Las publicaciones IV-VI son trabajos experimentales que se incluyen en los apartados de "Diseño experimental y Resultados" y "Discusión".

I. Anitua E, Troya M, Zalduendo M, Tejero R, Orive G. **Progress in the use of autologous regenerative platelet-based therapies in implant dentistry.** *Curr Pharm Biotechnol*. 2016; 17(5): 402-413. Factor de impacto: 1,802.

II. Anitua E, Troya M, Zalduendo M. **Progress in the use of dental pulp stem cells in regenerative medicine.** *Cytotherapy*. 2018; 20(4): 479-498. Factor de impacto: 3,993.

III. Anitua E, Troya M, Zalduendo M, Orive G. **Personalized plasma-based medicine to treat age-related diseases**. *Mater Sci Eng C Mater Biol Appl*. 2017; 74: 459-464. Factor de impacto: 4,164.

IV. Anitua E, Zalduendo M, Troya M, Padilla S, Orive G. **Leukocyte inclusion within a platelet rich plasma-derived fibrin scaffold stimulates a more pro-inflammatory environment and alters fibrin properties.** *PLoS One*. 2015; 10(3):e0121713. Factor de impacto: 3,057.

V. Anitua E, Zalduendo M, Troya M, Orive G. **PRGF exerts a cytoprotective role in zoledronic acid-treated oral cells.** *Clin Oral Invest*. 2016; 20(3): 513-521. Factor de impacto: 2,207.

VI. Anitua E, Zalduendo M, Troya M. **Autologous PRGF technology for isolation and *ex vivo* expansion of human dental pulp stem cells for clinical translation of cell therapy.** Enviado para su consideración.

RESUMEN

RESUMEN

El envejecimiento global de la población está aumentando de manera acelerada. Estos cambios demográficos conllevan un aumento en la prevalencia de enfermedades crónicas y degenerativas relacionadas con la edad y asociadas a un incremento en la ingesta de medicamentos. En la medida en que las necesidades sociales y sanitarias de la sociedad varían, la presión por desarrollar nuevas alternativas aumenta. En los últimos años, el creciente conocimiento de los procesos biológicos implicados en la regeneración tisular ha favorecido el camino para desarrollar nuevas terapias con el objetivo final de promover y acelerar dicha regeneración [1,2]. En este contexto, la medicina personalizada está emergiendo como una opción terapéutica prometedora que se basa en la compleja singularidad de cada paciente ofreciendo tratamientos a medida [3].

La compleja estructura del periodonto y la interacción estructural de múltiples tejidos blandos y duros convierte a la regeneración periodontal en un proceso único. Las enfermedades periodontales se caracterizan por afectar a la composición e integridad de todas esas estructuras involucradas en el periodonto cuyo funcionamiento sólo se alcanza mediante la integración estructural de todos sus componentes. La periodontitis está considerada como la condición inflamatoria crónica más común en los humanos, cuya prevalencia aumenta con la edad [4]. La carga mundial como consecuencia de la morbilidad, costes e impacto socioeconómico es notoriamente elevada [4]. Por el contrario, la patología de la osteonecrosis de los maxilares inducida por bifosfonatos (BRONJ) es relativamente poco común, sin embargo, presenta importantes repercusiones tanto para la calidad de vida del paciente como para los recursos médicos [5,6]. El tratamiento terapéutico para estas patologías sigue siendo controvertido y aún no se ha establecido una terapia estándar para estas enfermedades [7]. Teniendo en cuenta las actuales limitaciones de los enfoques terapéuticos disponibles, los esfuerzos en investigación se están dirigiendo hacia la ingeniería de tejidos. Este enfoque alternativo, se centra en superar las deficiencias de las modalidades de tratamiento convencionales para restaurar la arquitectura y funcionalidad originales. El uso efectivo y pertinente de la ingeniería de tejidos requiere la integración de tres componentes esenciales: una población de células progenitoras multipotentes, moléculas de señalización y matrices biocompatibles [8]. En este sentido, la terapia con células madre representa una herramienta emergente y

prometedora por su gran potencial terapéutico en la medicina regenerativa [9]. Las células madre de pulpa dental se obtienen mediante métodos de aislamiento no invasivos, representando así una fuente valiosa de fácil acceso y con propiedades biológicas especiales de células madre mesenquimales y células madre de la cresta neural [10,11]. Dado que la cantidad de células madre presentes en un determinado tejido es reducida, se requiere una expansión *ex vivo* para producir una dosis adecuada con el fin de lograr resultados terapéuticos [12]. El suero bovino fetal se ha utilizado durante mucho tiempo como un suplemento de cultivo celular estándar ampliamente aceptado tanto para investigación como para uso clínico [13]. Sin embargo, cuestiones como la limitada disponibilidad, el riesgo de contaminación por patógenos, los problemas éticos relacionados con el bienestar animal o la inducción de reacciones inmunológicas en el huésped [14,15], han llevado a explorar otras alternativas para proporcionar productos de terapia celular seguros, regulados y efectivos para los pacientes [16,17]. Actualmente, se están explorando numerosos productos autólogos derivados de la sangre humana como posibles alternativas al uso del suero bovino fetal [18-21].

En los últimos años, los derivados plaquetarios han surgido como nuevas herramientas para ser utilizadas por sus propiedades en la reparación tisular [22]. En este sentido, la tecnología del plasma rico en factores de crecimiento (PRGF) es pionera en el uso autólogo de factores de crecimiento, proteínas y biomateriales derivados del plasma y de las plaquetas con fines curativos [23,24]. La versatilidad de esta tecnología le ha permitido ser aplicada con seguridad y eficacia en diferentes campos de la biomedicina (ortopedia, oftalmología, cirugía oral y maxilofacial y dermatología [24,25]), así como alternativa al uso de productos xenogénicos en la expansión celular *ex vivo* [26].

Por todo ello, mediante esta tesis se pretende realizar un trabajo de investigación que evalúe *in vitro* el potencial terapéutico de la tecnología autóloga PRGF en el tratamiento de dos patologías orales: periodontitis y BRONJ, y su aplicabilidad en terapias celulares, con objeto de estimular la regeneración tisular.

INTRODUCCIÓN

1 PERIODONTO

El periodonto es un tejido especializado que rodea y da soporte a los dientes, proporcionando el apoyo necesario para conservar su función manteniéndolos en el maxilar y los huesos mandibulares [27]. La compleja estructura del periodonto (figura 1) compuesto por encía, ligamento periodontal y los tejidos mineralizados: cemento y hueso alveolar, convierte al proceso de regeneración periodontal en un proceso complejo y único donde todos sus componentes deben ser restituidos [27,28]. El correcto funcionamiento del periodonto sólo se alcanza mediante la correcta interacción e integridad estructural de todos sus componentes, cada uno de los cuales presenta una ubicación, arquitectura tisular y composición bioquímica y celular diferente [29]. La región periodontal es un microambiente altamente dinámico que está sometido a una remodelación constante debido a los frecuentes y elevados niveles de estrés mecánico que soporta y a las condiciones inflamatorias presentes en los tejidos afectados por la enfermedad periodontal [30]. Por tanto, es importante comprender que cada uno de estos componentes periodontales tiene sus propias y especializadas funciones y que sin embargo, funcionan juntas como una sola unidad [29,31].

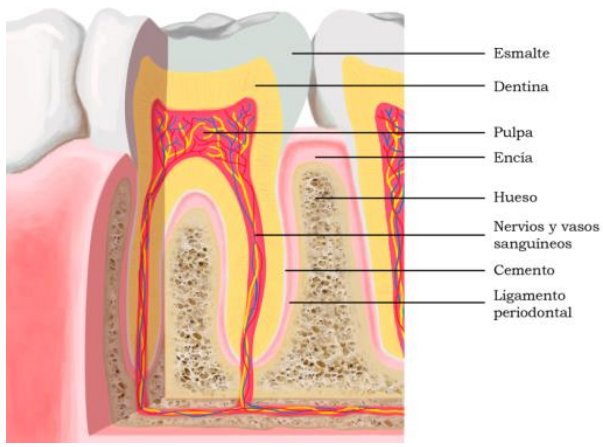

Figura 1. Anatomía del diente. Adaptada con permiso de Anitua *et al* [32].

1.1 Encía

La encía es la fibromucosa que cubre el hueso alveolar y la raíz del diente hasta la unión cemento-esmalte a la altura de la corona, proporcionando un sellado que protege a todos los tejidos de soporte. Está constantemente expuesta a estímulos traumáticos, mecánicos, térmicos y bacterianos [33]. Anatómicamente, la encía se divide en tres regiones diferentes: encía marginal, encía interdental y encía insertada. Histológicamente, se compone de dos elementos distintos: el epitelio y el tejido conectivo subyacente (figura 2). El epitelio es predominantemente celular, por el contrario el tejido conectivo es menos celular y está compuesto principalmente por una red integrada de proteínas estructurales, factores de crecimiento, minerales, lípidos y agua. Estos dos componentes son responsables de orquestar las respuestas más tempranas asociadas con el desarrollo de enfermedades como la gingivitis y la periodontitis [29] .

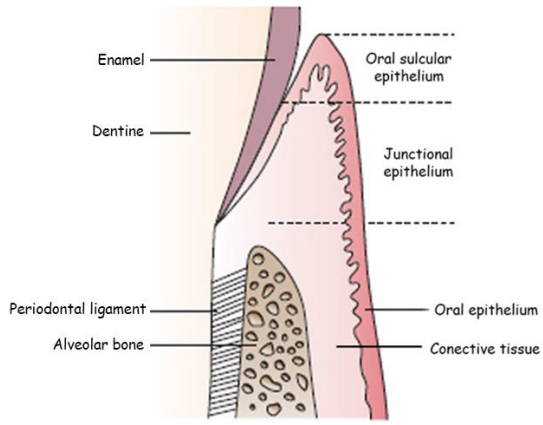

Figura 2. Anatomía del diente y composición de la encía. Adaptado de https://pocketdentistry.com/1-anatomy-of-the-periodontium/

1.1.1 Epitelio gingival

El epitelio gingival puede clasificarse en tres tipos de epitelios diferentes basados en su ubicación y composición: oral, sulcular y de unión. Estos tres epitelios difieren ultraestructuralmente y fenotípicamente en la expresión de diferentes citoqueratinas y marcadores de superficie celular [34,35]. Los queratinocitos, son el tipo celular más

ampliamente distribuido en el epitelio oral y sufren de muerte celular continua para ser reemplazados por la maduración progresiva de las células proliferantes y diferenciadoras de las capas subyacentes. Otros tipos de células, que constituyen el epitelio de la mucosa oral, incluyen las células de Langerhans, las células de Merkel, los melanocitos y las células inflamatorias [8].

- Epitelio oral

Epitelio estratificado, escamoso y queratinizado compuesto por cuatro capas: el estrato basal, el estrato espinoso, el estrato granuloso y el estrato córneo. Está ubicado en la superficie y presenta un espesor medio de entre 0,2 y 0,3 mm. La interfaz entre el tejido epitelial y el conectivo está delimitada por extensiones y depresiones que forman las llamadas "crestas de Rete". Este epitelio proporciona resistencia al desgaste y desplazamiento provocados por las fuerzas masticatorias [29,35].

- Epitelio sulcular

Epitelio de transición entre el epitelio oral y el de unión y el revestimiento del surco gingival, espacio virtual entre la superficie del diente y el epitelio sulcular [36]. La estructura celular y composición de este tipo de epitelio es muy similar a la del epitelio oral siendo estratificado y paraqueratinizado [29].

- Epitelio de unión

Este epitelio media la unión de la encía con los dientes a través de hemidesmosomas y la lámina basal interna, mientras que se une al tejido conectivo mediante la lámina basal externa. Es un epitelio estratificado no queratinizado que difiere de los otros dos tipos de epitelios tanto en el origen como en la morfología celular, proliferación y diferenciación. Es una estructura con una elevada tasa de renovación y altamente permeable, lo que le permite una cierta extravasación de neutrófilos en el líquido gingival como protección natural contra la acumulación de placa dental en la superficie externa del diente. Esta elevada permeabilidad también le convierte en una ruta preferencial para la entrada de bacterias desde el surco hacia el tejido conectivo, representando junto con el epitelio sulcular uno de los espacios cruciales para el inicio y desarrollo de las enfermedades

periodontales. Así mismo, proporciona una vía idónea para el movimiento bidireccional de sustancias entre el tejido conectivo y la cavidad oral [29,35-37].

Por tanto, el epitelio gingival desempeña funciones clave de protección y defensa para mantener la integridad de los tejidos periodontales subyacentes. La exposición continua a estímulos bacterianos y mecánico-traumáticos conduce a la diversidad cualitativa y cuantitativa en la composición molecular de la encía. Para mantener la homeostasis, las células epiteliales son metabólicamente activas, capaces de reaccionar a estímulos externos sintetizando una red de factores de crecimiento, moléculas de adhesión, péptidos antimicrobianos y otra serie de proteínas que regulan diversos mecanismos moleculares [29,38].

1.1.2 Tejido conectivo gingival (I)

Los fibroblastos gingivales son el tipo de célula más común en el tejido conectivo gingival. Las principales funciones de estos fibroblastos son sintetizar y mantener los componentes de la matriz extracelular así como participar activamente en la defensa inmune, ya que su exposición a patógenos ocurre desde etapas muy tempranas de la enfermedad periodontal [39-41]. Estas células tienen la capacidad de responder a las señales no sólo de forma paracrina, sino que también pueden sintetizar y secretar una serie de factores de crecimiento, citoquinas y productos metabólicos que dirigen la actividad celular de una manera autocrina [29]. Los fibroblastos del tejido conectivo gingival aunque representan una población celular heterogénea se caracterizan por presentar una elevada tasa de renovación tisular, un alto potencial de regeneración y capacidad para regular la inflamación así como para reparar las heridas con escasa evidencia de cicatrización, simulando a los tejidos fetales [39,42]. El colágeno tipo I es el principal componente de la matriz extracelular del tejido conectivo gingival. Otras proteínas como el colágeno tipo III, IV, V, proteoglicanos y fibronectina también están presentes [29,35]. El tejido conectivo de la encía también es rico en vasos sanguíneos, nervios y otras células específicas de los sistemas inmunoinflamatorios y vasculares [29,43]. El tejido conectivo gingival representa

además una fuente de fácil acceso y mínimamente invasiva para la obtención de células madre, así como de células madre pluripotentes inducidas [42,44,45].

1.2 Cemento

El cemento es un tejido mineralizado que cubre toda la superficie de la raíz y que debido a su posición intermedia, forma la interfaz entre la dentina de la raíz y el ligamento periodontal. Posee muchas características en común con el tejido óseo. Sin embargo, es un tejido avascular, carente de inervación, que no experimenta remodelación continua, aumentando su espesor a lo largo de la vida [33,46,47].

Una de las principales funciones del cemento es proporcionar la unión del diente al hueso alveolar mediante la inserción de las fibras del ligamento periodontal a la superficie de la raíz. Así mismo, el cemento desempeña un papel crucial en el mantenimiento de la relación oclusal y evita la resorción de la raíz durante la remodelación del periodonto, protegiendo la integridad de la superficie radicular [35,46,48]. Sin embargo, el cemento sigue siendo un tejido relativamente poco estudiado. Se clasifica en base a la presencia o ausencia de células y en la naturaleza extrínseca o intrínseca de las fibras de colágeno. Se distinguen principalmente tres tipos de cemento:

- Cemento celular de fibras intrínsecas

El cemento celular es relativamente grueso y a menudo contiene tanto fibras extrínsecas como intrínsecas que cubren la mitad apical de la raíz. Se denomina celular debido a la presencia de cementoblastos, células formadoras de cemento y cementocitos, células envueltas en lagunas que se asemejan a los osteocitos del hueso [8]. Desempeña un papel clave en el mantenimiento de la posición del diente y en el proceso de reparación aunque no presenta una función directa en la fijación del diente [46,49,50].

- Cemento acelular de fibras extrínsecas

Cubre la mitad cervical de la raíz y su grosor aumenta con la edad. Ostenta la exclusiva función de anclar la raíz al ligamento periodontal. La matriz de fibras de cemento extrínseco acelular consiste en una franja densa de fibras cortas de colágeno que se

implantan en la matriz dentinaria. Cuando se vuelven alargadas y, finalmente, continuas con las principales fibras del ligamento periodontal, se llaman fibras de Sharpey [46,50].

- Cemento celular mixto estratificado:

Predomina en las regiones interradiculares y apicales de las raíces y consiste en una mezcla de fibras extrínsecas entremezcladas con fibras de la matriz intrínseca circundante [35,50].

Como ocurre con otros tejidos calcificados, la matriz inorgánica del cemento está formada principalmente por cristales de hidroxiapatita. La matriz orgánica restante está compuesta por colágeno, glicosaminoglicanos y glicoproteínas [48,51]. El principal componente orgánico es el colágeno tipo I que desempeña funciones estructurales durante el proceso de biomineralización [46]. El colágeno tipo III también está presente aunque en proporciones mucho menores. El cemento es un tejido que está sometido a fuerzas compresivas y por lo tanto el porcentaje de glicosaminoglicanos es elevado. Los principales mucopolisacáridos presentes en el cemento humano son el ácido hialurónico, el dermatán sulfato y el condroitín sulfato [51]. La sialoproteína y la osteopontina son dos glicoproteínas fosforiladas y sulfatadas que también se encuentran presentes en el cemento. Estas proteínas permanecen unidas a la matriz de colágeno y contienen secuencias de adhesión bien reconocidas RGD (arginina-glicina-ácido aspártico) que se dirigen a receptores de integrinas específicos promoviendo la migración y adhesión celular [46,48,51].

Hasta el momento no existen proteínas específicas para caracterizar el cemento. El factor de crecimiento derivado del cemento y la proteína de fijación del cemento han sido claros candidatos, sin embargo y aunque en menor proporción, se han detectado en otros tejidos [46,50,52].

1.3 Ligamento periodontal (I)

El ligamento periodontal es un tejido conectivo vascular y altamente celular que conecta el cemento con el hueso alveolar circundante. La inserción de las fibras del ligamento

periodontal se realiza, tanto en el hueso como en el cemento, mediante las fibras de Sharpey [53-55]. Además de proporcionar la fijación del diente, el ligamento periodontal proporciona estabilidad mecánica transmitiendo, absorbiendo y disipando las cargas mecánicas. Así mismo, aporta nutrientes al diente y junto con la encía establece una barrera protectora contra los patógenos de la cavidad oral. El ligamento periodontal también desempeña un papel regulador en los procesos de propiocepción que rigen la masticación y juega un papel significativo en el mantenimiento y renovación de los tejidos periodontales adyacentes [8,35,53,55-57].

Está compuesto por poblaciones celulares heterogéneas, que incluyen fibroblastos, osteoblastos, cementoblastos, osteoclastos, macrófagos, restos epiteliales de Malassez, células endoteliales y células neuronales, entre otras [8,53,55,57,58]. El ligamento periodontal también contiene una población de células madre mesenquimales [54,59,60]. Sin embargo, el tipo celular predominante es el fibroblasto [56,57,61]. El componente extracelular está compuesto principalmente por fibras de colágeno tipo I con una contribución menor de colágenos tipo II, III, V, VI y XII. Otros componentes adicionales de la matriz extracelular incluyen proteoglicanos, fibronectina, tenascina y fibras de oxitalán [8,53,55,57].

La orientación espacial compleja de las fibras principales es esencial para la función del ligamento periodontal, ya que permite el soporte tridimensional y la protección contra las fuerzas multidireccionales de la masticación y otros movimientos orales [57]. La integridad del ligamento periodontal se debe principalmente a la elevada tasa de renovación que presenta [35,57,58].

1.4 Hueso alveolar (I)

El hueso alveolar es la parte del hueso maxilar y mandibular que rodea los dientes y forma los alveolos dentarios. La presencia y mantenimiento del hueso alveolar es dependiente del diente [35,62]. Es un tejido dinámico, comparable a otros tejidos óseos del cuerpo, que se forma y reabsorbe continuamente en respuesta a los requisitos funcionales. Por tanto, el hueso alveolar está sujeto a una remodelación continua y rápida asociada a la erupción dental y, posteriormente, a las demandas funcionales de la masticación. La capacidad del

hueso alveolar para someterse a una remodelación rápida también es importante para la adaptación posicional de los dientes, sin embargo, puede ser perjudicial para la progresión de la enfermedad periodontal [63]. El proceso fisiológico de remodelación ósea se controla a través de un complejo sistema compuesto por tres proteínas: la osteoprotegerina, el receptor activador del factor nuclear kappa B (RANK) y el ligando de unión al receptor activador del factor nuclear kappa B (RANKL), todas ellas pertenecientes a la superfamilia del factor de necrosis tumoral (TNF). Este intrincado sistema promueve la interacción entre los diferentes tipos celulares para favorecer el equilibrio entre la formación o resorción ósea [64,65].

El hueso alveolar en realidad se compone de dos elementos. El primero es el proceso alveolar del maxilar y la mandíbula. Esta estructura ósea laminar se forma para albergar los brotes de los dientes en desarrollo y, una vez erupcionados, las raíces de los dientes. Proporciona soporte estructural para la dentición y se compone de una capa externa de hueso cortical y una región interna de hueso esponjoso [66,67]. El segundo tipo de hueso es hueso alveolar propiamente dicho, que es la porción de hueso que recubre el alvéolo dental. Proporciona una zona de fijación para el ligamento periodontal y su diente asociado. Es decir, proporciona el espacio de fijación para las fibras de Sharpey del ligamento periodontal. Estas fibras de colágeno se organizan en haces y se calcifican dentro del hueso para proporcionar una unión fuerte entre el diente y el hueso. Esta porción de hueso alveolar se denomina hueso fasciculado debido a la presencia de estos haces de fibras. La porción restante del hueso alveolar propiamente dicho es un hueso laminar que está perforado por numerosos pequeños agujeros que permiten que los nervios y vasos sanguíneos del proceso alveolar lleguen a los tejidos del ligamento periodontal. Este hueso perforado a menudo se conoce como placa cribosa [67].

El hueso alveolar se compone de diferentes tipos celulares [53,62,63]:

- Osteoblastos

Son las células secretoras más activas del hueso, y por tanto, principales responsables de la producción de la matriz orgánica. Tras la maduración, los osteoblastos pueden sufrir

apoptosis, quedar atrapados en la matriz como osteocitos o permanecer en la superficie del hueso como células de revestimiento óseo.

- Osteocitos

Son osteoblastos atrapados en la matriz, ocupando espacios denominados lagunas.

- Células de revestimiento óseo

Las células de revestimiento óseo cubren la mayoría de las superficies óseas quiescentes en el esqueleto adulto. La transición de los osteoblastos a las células del revestimiento óseo implica una serie de cambios morfológicos y funcionales que culminan en una disminución de la secreción de proteínas.

- Osteoclastos

Son las células responsables de la resorción del hueso. El acoplamiento de la resorción ósea con la formación de hueso constituye uno de los principios fundamentales por los cuales el hueso es remodelado a lo largo de la vida.

La composición de la matriz extracelular del hueso alveolar es similar al resto de tejidos óseos del organismo. El colágeno tipo I constituye el principal componente de la matriz orgánica que representa aproximadamente un tercio de la matriz extracelular ósea. La matriz orgánica contiene además proteínas no colágenas que incluyen osteocalcina, osteonectina, osteopontina, fibronectina y sialoproteína ósea entre otras [53,63,68]. Los iones de calcio y fosfato predominan en la fase inorgánica que se combinan para formar los cristales de hidroxiapatita. Junto con el colágeno, las proteínas de la matriz no colágenas forman una plataforma para la deposición de hidroxiapatita, esta asociación es responsable de la rigidez y resistencia típicas del tejido óseo [63,68].

2 PULPA DENTAL (II)

En las últimas décadas el campo emergente de la medicina regenerativa ha progresado de manera significativa gracias al descubrimiento de un creciente número de células madre

adultas. Hasta el momento, se han descrito diferentes tipos, incluidas las células madre embrionarias, células madre somáticas adultas, entre las que se incluyen las células madre mesenquimales, hematopoyéticas y endoteliales, y las células madre pluripotentes inducidas derivadas artificialmente de células somáticas diferenciadas en adultos [69,70]. Existe poca controversia en torno a las células madre adultas humanas ya que no están asociadas con aspectos éticos a diferencia de las células madre embrionarias. La terapia con células madre representa una herramienta prometedora por su potencial terapéutico para la medicina regenerativa y otras aplicaciones biomédicas [71].

La médula ósea y el tejido adiposo son fuentes convencionales de células madre mesenquimales, pero los protocolos altamente invasivos de recolección celular junto con el considerable riesgo de morbilidad han promovido la búsqueda de tejidos alternativos [72,73]. En este sentido, la cavidad oral ha atraído la atención de los científicos de manera significativa ya que alberga una gran variedad de células madre adultas. Las células madre dentales (figura 3) abarcan las células madre de pulpa dental (DPSCs), células madre de pulpa dental de diente de leche (SHED), células madre de papila apical (SCAP), células madre del folículo dental (DFSCs), células madre del ligamento periodontal (PDLSCs), y células madre derivadas del tejido gingival (GMSCs) [59,74-77].

Las células madre de pulpa dental, con un acceso quirúrgico sencillo se postulan como una seria alternativa a las fuentes convencionales. La no invasividad de los métodos de aislamiento de DPSCs en comparación con otras fuentes de tejido adulto hace que estas células sean una fuente valiosa de células madre mesenquimales para la reparación y regeneración de tejidos.

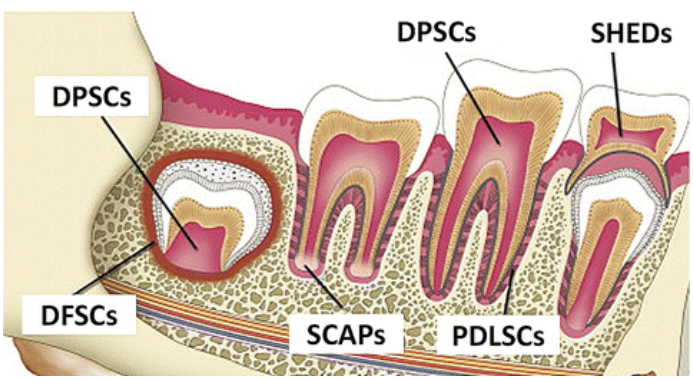

Figura 3. Tejidos dentales a partir de los cuales se pueden aislar diferentes poblaciones de células madre. Adaptada con permiso de Egusa *et al.* [70].

La pulpa dental se encuentra en la llamada "cámara pulpar" de cada diente y contiene una población heterogénea de células representada por fibroblastos, células endoteliales, neuronas, odontoosteoprogenitores, células inflamatorias e inmunes [78,79]. Las DPSCs fueron aisladas por primera vez por Gronthos *et al.* [80] en el año 2000, a partir del tejido de la pulpa de terceros molares o muelas del juicio. Las DPSCs humanas son células de origen ectodérmico que se originan durante el desarrollo del diente de células ectodérmicas que migran desde el tubo neural a la región oral y finalmente se diferencian en células mesenquimales [78,79,81]. Esta característica les confiere propiedades biológicas especiales tanto de células madre mesenquimales como de células madre de la cresta neural. Estas células presentan una alta tasa de proliferación, una baja inmunogenicidad y exhiben gran plasticidad para la diferenciación a múltiples linajes celulares [78-80].

En 2006, la Sociedad Internacional de Terapia Celular (ISCT) propuso tres criterios mínimos para definir a las células madre mesenquimales humanas (hMSCs) [82]. En primer lugar, las hMSCs deben adherirse al plástico cuando se mantienen en condiciones de cultivo estándar. En segundo lugar, las hMSCs deben expresar (≥ 95%) los marcadores CD105, CD73 y CD90, y carecer de expresión (≤ 2%) de CD45, CD34, CD14 o CD11b, CD79α o CD19 y HLA-DR. En tercer lugar, las hMSCs deben tener la capacidad para diferenciarse *in vitro* a osteoblastos, adipocitos y condroblastos. En el caso de las DPSCs no existe un marcador específico para identificarlas. De hecho, la población de DPSCs es heterogénea y consiste en subpoblaciones mixtas con diferentes propiedades fenotípicas y biológicas [11,83]. Los perfiles de expresión de marcadores difieren entre los estudios y no todas las DPSCs expresan todos los marcadores descritos ni en el mismo porcentaje [78]. Esta heterogeneidad en la población de DPSCs es en parte responsable de la alta diversidad de antígenos expresados. Las condiciones de cultivo celular y la composición del medio pueden también interferir con el patrón de expresión de la superficie celular. Además de la expresión específica de antígenos propuesta por la ISCT, se han determinado otros marcadores de superficie en la caracterización de las DPSCs (Tabla 1).

ISCT	Mesenquimales	Troncales	Neuronales	Otros
CD73	CD13	OCT-3/4	Nestina	CD40
CD90	CD29	SSSEA4	β -III tubulina	CD120a
CD105	CD44	NANOG	S100	CD261
	CD146		Notch1	CD262
	CD166		CD271	CD264
			Sinaptofisina	CD266
				Integrina alfa-4
				Integrina alfa-6
				Integrina alfa-10
				CD121a
				CD130
				CD213a1
				CD217
				CDw210b

Tabla 1: Resumen de los marcadores positivos para las DPSCs [83-90]. Reproducida con permiso de Anitua *et al* [32].

La aplicación de células madre se basa en su autorenovación a largo plazo y su capacidad para diferenciarse a nuevas células maduras y especializadas que faciliten el reemplazo y la regeneración de los tejidos. Las células madre humanas de pulpa dental muestran una gran plasticidad para la diferenciación multilinaje incluso hacia linajes diferentes a los mínimamente establecidos por la ISCT (Tabla 2).

Diferenciación	Inductores	Confirmado por
Osteogénica ([91-94])	▪ Dexametasona ▪ Ácido ascórbico ▪ β -glicerofosfato	▪ Rojo alizarín ▪ Fosfatasa alcalina ▪ Colágeno tipo I ▪ Osteocalcina ▪ Osteonectina ▪ Osteopontina ▪ Osterix ▪ RUNX2
Adipogénica ([95-97])	▪ Insulina ▪ Dexametasona ▪ Indometacina ▪ 3-isobutil-1-metilxantina	▪ Rojo aceite O ▪ Receptor γ activado por el factor proliferador de peroxisomas ▪ Transportador de glucosa tipo 4 ▪ Proteína de unión de ácidos grasos 4 ▪ Lipoproteinlipasa
Condrogénica ([95,98-100])	▪ ITS ▪ Dexametasona ▪ Ácido ascórbico ▪ L-prolina ▪ Piruvato sódico ▪ TGF-β 3	▪ Azul alcián ▪ Safranina O ▪ Azul de toluidina ▪ Agrecán ▪ Proteína de la región Y determinante del sexo (SRY) ▪ Colágeno tipo II ▪ Colágeno tipo X

Tabla 2. Inductores y marcadores para la diferenciación multilinaje mínimamente establecida por la ISCT. Adaptada con permiso de Anitua *et al* [32].

Dado su origen en la cresta neural, las DPSCs muestran también un potencial neuroregenerativo significativo. Se ha demostrado que se diferencian a múltiples tipos celulares del linaje de la cresta neural, como células neuronales, neuronas dopaminérgicas, oligodendrocitos y células de Schwann [101-104]. También se ha

demostrado la capacidad de las DPSCs para diferenciarse a células endoteliales [105,106], cardiomiocitos [107], linaje de células hepáticas [107-109], linaje de células pancreáticas [110,111] y células de músculo liso de la vejiga [111].

Las DPSCs debido a su multipotencia, pueden proporcionar una fuente alternativa de células para la ingeniería de tejidos en múltiples campos médicos [32], entre los que cabe destacar (figura 4):

- Defectos orales y maxilofaciales.
- Regeneración neuronal.
- Cardiopatía isquémica y angiogénesis.
- Regeneración corneal
- Diabetes.
- Diferenciación hepática.

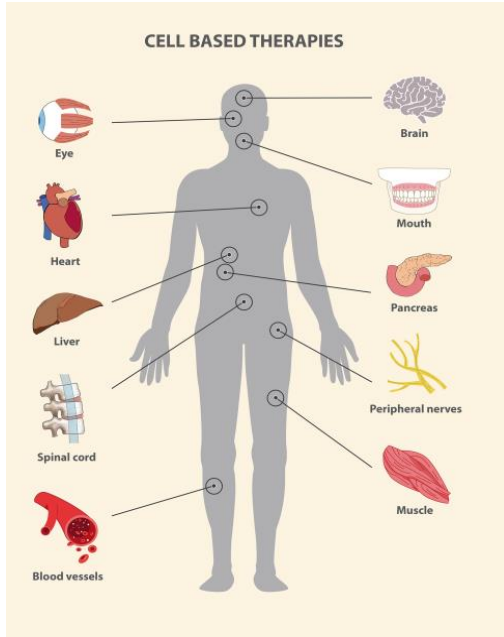

Figura 4. Potencial regenerador de las DPSCs. Reproducida con permiso de Anitua *et al* [32].

El establecimiento de biobancos de células madre de pulpa dental representa una estrategia terapéutica para el almacenamiento de muestras para futuras aplicaciones. Las células madre somáticas muestran una reducción significativa en su potencial de troncalidad al aumentar la edad del donante. El hecho de que las DPSCs puedan obtenerse mediante procesos mínimamente invasivos a partir de tejidos descartados de manera rutinaria, así como la baja inmunogenicidad e inmunosupresión exhibida, lleva a

pensar que incluso biobancos de DPSCs alogénicos proporcionarían un excelente enfoque, una vez superadas las barreras legales correspondientes [112].

3 ENFERMEDADES DENTALES

El envejecimiento de la población mundial está provocando un aumento en el número de personas que conviven con secuelas de lesiones y enfermedades. Los cambios en el perfil epidemiológico impulsados por los cambios socioeconómicos contribuyen a su vez a un aumento continuado en los años vividos con dichas discapacidades [113]. Las enfermedades bucales son las enfermedades más prevalentes en el ser humano. En el año 2010, se estimó que el coste económico global debido a dichas enfermedades ascendió a más de 400 billones de euros [4,8,114,115]. Los enfoques terapéuticos actuales son incapaces de lograr una regeneración periodontal fiable y predecible. Como consecuencia de las actuales limitaciones de los tratamientos convencionales, las investigaciones han comenzado a centrarse en la ingeniería tisular. Desde una perspectiva biológica, debe tenerse en cuenta la naturaleza compleja del periodonto y la interacción estructural de múltiples tejidos blandos y duros. La regeneración periodontal exige la utilización efectiva y pertinente de la ingeniería de tejidos que requiere la integración de tres componentes básicos: una población de células progenitoras multipotentes, biomoléculas inductivas para estimular el crecimiento y desarrollo de procesos celulares y tisulares y un andamio de matriz extracelular conductiva como soporte estructural y molecular [8].

3.1 Periodontitis

La enfermedad periodontal es una de las enfermedades más frecuentes en humanos [4,8,33]. Se distinguen dos formas de enfermedad periodontal, la gingivitis y la periodontitis. La primera se define como la inflamación de la encía y se limita al compartimento de los tejidos blandos del epitelio gingival y del tejido conectivo. La segunda, es la periodontitis, que se define como una enfermedad inflamatoria crónica que afecta a los tejidos que dan soporte al diente con una pérdida de inserción progresiva y

destrucción ósea [116]. Sus principales características clínicas incluyen la formación de bolsas periodontales, la pérdida de la unión del tejido conectivo, la resorción ósea alveolar y la inflamación gingival [117,118]. La periodontitis es la principal causa de pérdida de dientes en la población adulta a nivel mundial y su prevalencia aumenta con la edad [4,119]. Factores tanto genéticos como ambientales (tabaquismo, estrés, alimentación…) juegan un papel clave en la patogénesis de esta enfermedad [4,120]. La periodontitis se ha asociado además con múltiples trastornos sistémicos, que incluyen enfermedades cardiovasculares, cerebrovasculares, diabetes y artritis reumatoide [8,120,121].

Un reciente informe sobre la carga mundial de las enfermedades (GBD, 1990-2010 [115]) indica que la periodontitis severa es la sexta enfermedad más prevalente en el mundo, de un total de 291 enfermedades y afecciones investigadas, con una prevalencia global del 11,2% y alrededor de 743 millones de personas afectadas [4,115,122]. La periodontitis tiene efectos socioeconómicos significativos y se estima que el coste global por productividad en el 2010 fue de 54 billones de dólares americanos / año [4,115,122]. La actualización en 2015 de dicho informe ha destacado que las enfermedades bucodentales se encuentran clasificadas entre las 10 principales causas a nivel mundial de años vividos con discapacidad [123]. A pesar de la relevancia clínica de estas patologías, la conciencia mundial sobre la salud periodontal sigue siendo baja.

3.1.1 Patogénesis

El ecosistema microbiano de la cavidad oral es el hábitat de una multitud de especies bacterianas y virales. En una situación no patológica existe una relación estable entre los microorganismos residentes de la biopelícula y la respuesta inmune inflamatoria de los huéspedes a esa biopelícula [124]. Un desequilibrio en la microbiota oral residente facilita la aparición de enfermedades periodontales. En el caso de la periodontitis se ha constatado un cambio en la composición bacteriana del surco gingival, pasando de un predominio de microorganismos Gram positivos, facultativos y fermentativos a predominar Gram negativos, anaerobios y quimioorganotróficos [118,119]. La periodontitis se describe como una alteración polimicrobiana de la homeostasis del huésped. Las bacterias *Porphyromonas gingivalis*, *Tannerella forsythia* y *Treponema denticola* se encuentran entre

las bacterias clave implicadas en la patología de la enfermedad periodontal [117,125]. Estas tres especies bacterianas han sido designadas como periopatógenos del "complejo rojo" por mostrar una fuerte asociación con la enfermedad [126]. *Porphyromonas gingivalis* es una bacteria anaerobia Gram negativa considerada como el principal agente etiológico que contribuye a la periodontitis crónica [43,118,127]. *P. gingivalis* produce varios factores de virulencia como lipopolisacáridos (LPS), fimbrias, proteasas, gingipaínas, cápsulas y vesículas de membrana externa [118,120]. El lipopolisacárido es uno de los principales factores de virulencia de este patógeno. Constituye un componente importante de la membrana externa bacteriana, siendo esencial para la viabilidad de la mayoría de las bacterias Gram negativas, ya que juega un papel crucial en la integridad de la membrana externa como una barrera de permeabilidad, protegiendo así a las bacterias de las moléculas tóxicas [119]. En general, el LPS bacteriano consiste en un polisacárido distal denominado antígeno O, un oligosacárido central o "núcleo" no repetitivo y un dominio hidrofóbico conocido como lípido A (o endotoxina) (figura 5). El lípido A, el componente más interno, es la región biológica activa de LPS que causa una respuesta inmune altamente innata a través de los receptores del huésped [118,119,128].

El huésped responde al desafío microbiano produciendo una amplia gama de citoquinas proinflamatorias, quimioquinas y metaloproteasas de matriz (MMPs) que participan en la destrucción del tejido periodontal. Estas señales quimioatrayentes reclutan neutrófilos, macrófagos y linfocitos que amplifican la respuesta inflamatoria en el periodonto infectado. La interleuquina-1β (IL-1β), la interleuquina-6 (IL-6), la interleuquina-8 (IL-8) y el factor de necrosis tumoral alpha (TNF-α) son esenciales en el desarrollo de reacciones inflamatorias y están implicados tanto en la respuesta inmune local como sistémica a los antígenos bacterianos [40,43,127,129].

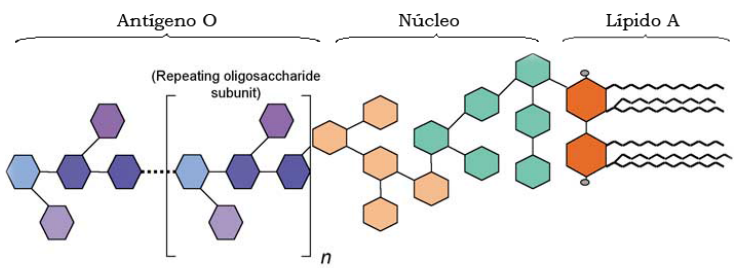

Figura 5. Estructura general de un lipopolisacárido de una bacteria Gram negativa. Adaptada con permiso de Erridge *et al.* [128].

3.1.2 Mecanismo de acción

La señalización a través de los receptores tipo Toll (TLRs) se considera la interfaz más importante entre el huésped y los patógenos. Las vías de señalización del TLR inician rutas de señalización complejas tras el reconocimiento de LPS bacterianos mediante un complejo macromolecular que implica el clúster de diferenciación 14 (CD14), la proteína de diferenciación mieloide 2 (MD2) y el receptor de tipo Toll 4 (TLR4) [119,130]. Los LPS forman agregados o micelas en soluciones acuosas debido a su cadenas hidrófobas, por ello, la transferencia de una molécula de LPS al complejo TLR4 / MD2 constituye uno de los pasos críticos en el reconocimiento de un patrón molecular asociado a patógenos (PAMP) de bacterias Gram-negativas [131]. Las proteínas accesorias LBP (proteína de unión a LPS) y CD14 median el reconocimiento de los LPS así como su eficiente transferencia al complejo TLR4 / MD2. La unión de los LPS al complejo TLR4 / MD2 induce la dimerización del complejo TLR4 / MD2, que recluta proteínas adaptadoras como el factor de diferenciación mieloide 88 (MyD88) en niveles posteriores. Al unirse al TLR, el factor MyD88 recluta quinasas de la familia IRAK (quinasa asociada al receptor de la interleuquina 1) a través de su dominio muerte. Las proteínas IRAK interactúan con TRAF-6 (factor 6 asociado al TNF), permitiendo que TRAF-6 active a TAK1 (quinasa 1 activada por TGF-β). El factor nuclear kappa B (NF-κB) se encuentra secuestrado en el citoplasma celular como un precursor inactivo que forma un complejo con la proteína inhibidora IκB. TAK1 fosforila el complejo IKK (IκB quinasa), activándolo. Una vez que el complejo IKK está activado destruye al inhibidor de NF-κB (IκB), fosforilándolo. Una vez fosforilado, el inhibidor IκB es modificado por un proceso llamado ubiquitinación, que le lleva a ser degradado posteriormente por una estructura celular llamada proteosoma. Este proceso permite la activación del factor de transcripción NF-κB que se transloca al núcleo para unirse a las regiones promotoras de genes pro-inflamatorios para finalmente activar su transcripción [128,131,132] (figura 6).

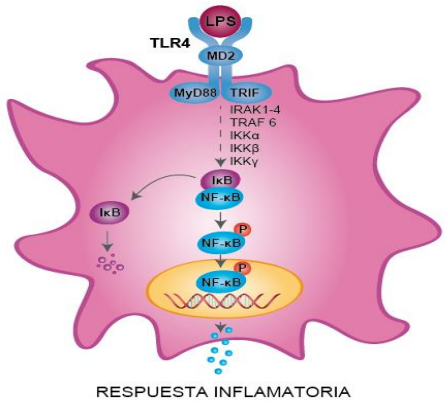

Figura 6. Ilustración de la vía de señalización de los receptores tipo Toll. Adaptada con permiso de Padilla *et al.* [133].

En la actualidad no existen tratamientos regenerativos eficaces contra la periodontitis. Las principales terapias actuales se centran en controlar los síntomas y detener la progresión de la enfermedad más que en la regeneración de los tejidos perdidos. El hecho de que la enfermedad periodontal presente una alta tasa de recurrencia y se prevea un aumento con el envejecimiento poblacional, insta a desarrollar nuevas medidas terapéuticas que puedan derivarse de la ingeniería de tejidos con la utilización de células madre como principal candidato para alcanzar el objetivo final de la regeneración periodontal [54,57].

3.2 Osteonecrosis de los maxilares inducida por bifosfonatos

Los bifosfonatos (BPs) son análogos sintéticos, con una estructura similar al pirofosfato inorgánico, que se obtienen mediante la sustitución de una molécula de oxígeno por una de carbono, entre dos fosfatos. Poseen una gran afinidad por la hidroxiapatita que les confiere una gran capacidad para depositarse en el tejido óseo, por lo que son administrados en pacientes con patologías en las que es necesario disminuir la resorción ósea (osteoporosis, hipercalcemia maligna, metástasis óseas de tumores originados principalmente en la mama, el pulmón y la próstata, mieloma múltiple o enfermedad de Paget, entre otras)[7,134,135]. Estos compuestos impiden la actividad osteoclástica, generando no sólo una reducción de la resorción ósea, sino también un aumento de la

densidad y la masa ósea y una disminución de la tasa de fracturas en los pacientes con enfermedades asociadas. Otro de sus efectos, es la acción antiangiogénica mediante la inducción de la apoptosis de las células endoteliales [136].

Estos medicamentos se dividen en dos grupos principales: bifosfonatos que contienen nitrógeno y bifosfonatos que no lo contienen. Actualmente, en la práctica clínica se utilizan los bifosfonatos nitrogenados, mucho más potentes que sus homólogos no nitrogenados [136,137]. Los BPs son administrados tanto por vía oral (alendronato, risedronato...) como por vía intravenosa (principalmente pamidronato y ácido zoledrónico) [132,138,139]. Son compuestos de gran polaridad, por lo que su biodisponibilidad por vía oral es muy baja (<1%). Tras su administración oral, los bifosfonatos se absorben en todo el tracto gastrointestinal, pero principalmente en el intestino delgado y el estómago. La administración oral está limitada por su baja biodisponibilidad, que exige altas dosis, y por su toxicidad gastrointestinal (principalmente esofagitis y diarrea). Por el contrario, los BPs intravenosos requieren una dosificación menos frecuente y no suelen presentar efectos adversos gastrointestinales [140,141]. Sólo aproximadamente el 50% del fármaco absorbido se incorpora al hueso, mientras que el resto se elimina a través del riñón sin metabolizar [141,142]. Una vez absorbidos por el tejido óseo, pueden persistir durante aproximadamente 10 años en los tejidos esqueléticos, ya que son pobremente metabolizados por los enzimas biológicos [143].

Aunque los BPs son muy efectivos en reducir la pérdida ósea, el dolor y otras manifestaciones clínicas esqueléticas, pueden inducir efectos adversos como la osteonecrosis de los maxilares inducida por bifosfonatos (BRONJ) (figura 7). La primera serie de casos de osteonecrosis de los maxilares fue descrita por Marx [144] en 2003, quien reportó una serie de 36 casos de exposición ósea maxilar o mandibular por bifosfonatos.

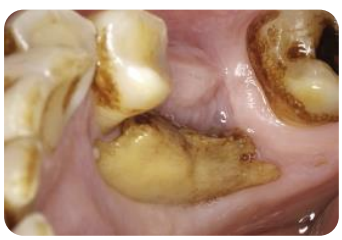

Figura 7. Aspecto intraoral de la lesión de BRONJ, donde se aprecia gran cantidad de hueso necrótico expuesto al medio oral. Reproducida con permiso de Anitua E. *et al.* [145].

3.2.1 Definición de BRONJ

En el año 2014, la Asociación Americana de Cirujanos Orales y Maxilofaciales recomendó cambiar la nomenclatura de osteonecrosis de los maxilares inducida por bifosfonatos (BRONJ) por el término osteonecrosis de los maxilares inducida por medicación (MRONJ) [146]. Este cambio fue justificado por el creciente número de casos de osteonecrosis de los maxilares asociados con otras terapias antirresortivas y antiangiogénicas. Estas dos nuevas clases de fármacos utilizados son los inhibidores del ligando RANK (activador del receptor del factor nuclear-kB) y los antiangiogénicos [143,147,148]. El Denosumab es el inhibidor del ligando RANK, que al igual que los BPs ha sido desarrollado para inhibir la actividad osteoclástica en patologías con una alta resorción ósea. Los medicamentos antiangiogénicos incluyen los medicamentos bevacizumab y sunitinib que inhiben la formación de nuevos vasos sanguíneos al interrumpir la cascada de señalización de la angiogénesis [143,147].

Para poder considerar que un paciente presenta MRONJ deben estar presentes todas las características siguientes [146]:

1. Tratamiento actual o previo con agentes antirresortivos o antiangiogénicos.
2. Hueso expuesto o hueso que puede ser explorado a través de una fístula intraoral o extraoral en la región maxilofacial que persiste durante más de ocho semanas.
3. No tener antecedentes de radioterapia en los maxilares o enfermedad metastásica evidente en los maxilares.

3.2.2 Patofisiología

Aunque la etiología de MRONJ no se ha dilucidado por completo, se han propuesto varios mecanismos potenciales [143,149-151].

- Supresión del recambio óseo
- Supresión de la angiogénesis
- Infección/Inflamación
- Toxicidad de tejidos blandos
- Disfunción inmune

3.2.3 Mecanismo de acción

A nivel molecular, los bifosfonatos que contienen nitrógeno inhiben la actividad del farnesil pirofosfato sintasa (FPPS), un enzima regulador clave en la vía del mevalonato [143,152,153] (figura 8). Esta vía es una ruta biosintética responsable de la producción de colesterol, otros esteroles y lípidos isoprenoides. La inhibición del enzima FPPS bloquea la síntesis de algunos de estos lípidos isoprenoides, como el farnesil pirofosfato (FPP) y el geranil-geranil pirofosfato (GGPP), lo que a su vez evita la prenilación y activación de las proteínas de señalización GTPasas (Ras, Rho, Rac, Rab y Cdc42) [148,154,155]. Estas proteínas desempeñan papeles críticos en el crecimiento y la diferenciación celular, la reorganización del citoesqueleto, la expresión génica y la supervivencia celular [148,152,154]. La inhibición de enzimas a lo largo de la ruta del mevalonato puede perjudicar el proceso de prenilación y conducir a la pérdida de la función de las pequeñas GTPasas. Estas pequeñas GTPasas son esenciales para la actividad de resorción ósea y la supervivencia de los osteoclastos. Por lo tanto, la inhibición del FPPS es un mecanismo central por el cual los bifosfonatos nitrogenados inhiben la resorción ósea [154,155].

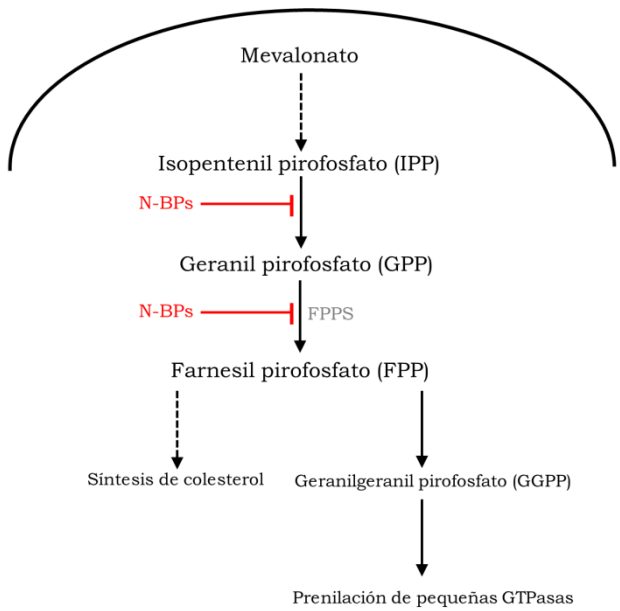

Figura 8. Esquema simplificado del mecanismo de acción de los bifosfonatos nitrogenados.

3.2.4 Factores de riesgo

A medida que la población envejece, el número de pacientes con osteoporosis y cáncer que necesitan terapias antirresortivas y antiangiogénicas también lo hace y con ello el período de administración de estos medicamentos, lo que dará lugar a una mayor incidencia de MRONJ [143]. A pesar de ser relativamente poco común, la osteonecrosis de los maxilares inducida por medicación presenta importantes repercusiones tanto para la calidad de vida del paciente como para los recursos médicos [5,6]. Existe una gran disparidad en la tasa de incidencia entre los diferentes estudios que puede ser atribuida a variaciones en el régimen de tratamiento, a la propia naturaleza del estudio y a la gran variedad de factores de riesgo que existen [156].

Por tanto, el riesgo de padecer esta patología depende de diferentes factores:

1. Duración de la terapia

El nivel de riesgo de desarrollar MRONJ está asociado con la dosis, la potencia y la duración de los medicamentos utilizados. La osteonecrosis de los maxilares inducida por bifosfonatos es inducida con mayor frecuencia por bifosfonatos intravenosos que por orales, siendo el ácido zoledrónico el bifosfonato más potente que también presenta la mayor incidencia de BRONJ [148,151,156,157].

2. Cirugía intraoral

La cirugía dentoalveolar, especialmente las extracciones dentales, se considera uno de los principales factores de riesgo para desarrollar MRONJ [148,151,158].

3. Localización anatómica

Los BPs tienen una acción sistémica, por lo que la osteonecrosis podría ocurrir en cualquier hueso. Sin embargo, este tipo de osteonecrosis sólo ocurre en los maxilares y no en otras áreas [159]. Esta peculiaridad parece estar relacionada con la elevada tasa de renovación ósea del hueso alveolar y su susceptibilidad a la infección bacteriana [143,160]. El maxilar inferior (mandíbula) es la localización más frecuente para esta patología cuya prevalencia es el doble que en el maxilar superior [139,147,151,161].

4. Uso de esteroides

Los glucocorticoides no solo activan los osteoclastos, sino que también inhiben los osteoblastos por lo que su uso prolongado induce osteoporosis. El uso combinado de glucocorticoides y BPs aumenta el riesgo de padecer MRONJ ya que el uso de corticosteroides suprime la actividad celular inflamatoria e inmune y retrasa la cicatrización de las heridas [139,143,146,159].

5. Enfermedad oral concomitante

La enfermedad dental inflamatoria preexistente, como la enfermedad periodontal o la patología periapical, es un factor de riesgo bien reconocido [139,146,148].

6. Factores genéticos

Se ha descrito que ciertos polimorfismos de nucleótido único (SNP) están asociados con el desarrollo de MRONJ. La mayoría de estos SNP se encuentran en regiones de genes asociadas con el recambio óseo, la formación de colágeno o ciertas enfermedades óseas metabólicas [139,146,159].

7. Otros

Además de los factores ya descritos, la edad [139,151,162], el tipo de cáncer [139,146] y la diabetes [139,163] han sido descritos como factores sistémicos que aumentan el riesgo de MRONJ.

3.2.5 Tratamientos

El tratamiento terapéutico para los pacientes con MRONJ sigue siendo controvertido y aún no se ha establecido una terapia estándar para esta enfermedad [7]. Hasta la fecha, el tratamiento terapéutico se ha centrado principalmente en el tratamiento sintomático y preventivo [5,164]. El objetivo principal de la terapia debe ser eliminar el dolor y controlar la progresión de la infección ósea y la necrosis, a fin de preservar la calidad de vida. Cualquier tratamiento médico o quirúrgico de MRONJ debe equilibrarse con la terapia

oncológica en pacientes con metástasis osteolíticas y con el riesgo de fractura en pacientes con osteoporosis [165].

Entre los protocolos terapéuticos propuestos existen terapias quirúrgicas y no quirúrgicas. Las no quirúrgicas incluyen, entre otros, el cese de la terapia antirresortiva o antiangiogénica, el uso a largo plazo de antimicrobianos locales, antimicrobianos sistémicos o ambos, oxígeno hiperbárico, terapia con ozono, terapia con láser de baja intensidad, teriparatida, concentrado autólogo de plaquetas, y transplantes alogénicos de células madre mesenquimales [5,145,149,164-166].

4 REGENERACIÓN TISULAR, PLASMA RICO EN PLAQUETAS (PRP) Y PLASMA RICO EN FACTORES DE CRECIMIENTO (PRGF)

La pérdida de órganos y tejidos como consecuencia de enfermedades ha motivado el desarrollo de terapias que pueden regenerar los mismos y disminuir la dependencia de los trasplantes. La medicina regenerativa, representa un campo interdisciplinario que aplica principios de ingeniería y terapia celular para promover la regeneración, pudiendo potencialmente restaurar tejidos y órganos lesionados [167]. Este campo de la medicina abarca numerosas estrategias pero todas ellas comparten un denominador común: optimizar la capacidad de regeneración intrínseca del propio organismo. Desde que la ingeniería de tejidos y la medicina regenerativa surgieron como una industria hace aproximadamente dos décadas, varias terapias han recibido autorización o aprobación por parte de la Administración de Alimentos y Medicamentos (FDA) de Estados Unidos y están disponibles comercialmente [167]. Caben destacar Carticel como el primer producto biológico aprobado por la FDA en el campo ortopédico, que utiliza condrocitos autólogos para el tratamiento de defectos focales del cartílago articular. laViv, que implica la inyección de fibroblastos autólogos para mejorar la apariencia de las arrugas del pliegue nasolabial o Epicel que consiste en queratinocitos autólogos para quemaduras graves. También se han aprobado terapias basadas en células alogénicas como es el caso de Gintuit que consiste en queratinocitos y fibroblastos alogénicos cultivados en matriz de colágeno bovino. La incorporación de factores de crecimiento en determinados biomateriales representa un enfoque que también ha sido explotado. En este sentido,

Regranex es un gel que contiene el factor de crecimiento recombinante humano derivado de plaquetas (rhPDGF-BB) para administración tópica en úlceras diabéticas. Recientemente, también han sido aprobadas las primeras terapias génicas de inmunoterapia antitumoral conocidas comercialmente por los nombres de Kymriah y Yescarta [168].

En el contexto de la ingeniería de tejidos, las terapias biológicas brindan nuevas oportunidades para desarrollar una medicina personalizada donde las terapias autólogas están emergiendo de manera significativa. Estas terapias, al ser autólogas, proporcionan nuevas ventajas en el entorno clínico: (i) el donante está disponible de inmediato, (ii) no se requiere inmunosupresión, (iii) no se produce rechazo, (iv) se elimina la enfermedad del injerto contra el huésped, (v) se suprimen los riesgos asociados a la transmisión de enfermedades y (vi) en general, no presentan conflictos éticos [169]. El plasma rico en plaquetas se incluye dentro de estos tipos de terapias autólogas. Derivado de la propia sangre del paciente, el plasma rico en plaquetas es una técnica asequible y mínimamente invasiva [2]. Los productos enriquecidos con plaquetas se han convertido en relevantes en las últimas décadas y constituyen un foco creciente de estudio experimental y clínico en el contexto de la regeneración de tejidos [170]. Aunque las plaquetas son ampliamente reconocidas por tener un papel crítico en la hemostasia primaria y trombosis, el aumento de la evidencia experimental y clínica las sitúa como moduladoras relevantes de otros procesos fisiopatológicos, incluidos la inflamación, la respuesta inmune y la regeneración tisular [171-173]. Estos fenómenos están mediados por la liberación de factores de crecimiento, citoquinas y moduladores de la matriz extracelular que promueven secuencialmente (i) la revascularización del tejido dañado mediante la inducción de la migración, proliferación, diferenciación y estabilización de las células endoteliales en nuevos vasos sanguíneos; (ii) la restauración del tejido conectivo dañado a través de la migración, proliferación y activación de fibroblastos; y (iii) la proliferación y diferenciación de células madre mesenquimales en tipos de células específicas del tejido. Por todas estas razones, los derivados plaquetarios se están utilizando en diferentes campos de la medicina regenerativa para el tratamiento de distintas patologías clínicas [170].

4.1 Plaquetas

Las plaquetas son pequeños fragmentos anucleares derivados de la fragmentación de sus células precursoras, los megacariocitos, en la médula ósea. Tienen un promedio de 2 a 5 μm de diámetro y 0.5 μm de grosor y una vida media de aproximadamente 7-10 días [174,175]. La producción de plaquetas es un proceso complejo que requiere la diferenciación de células madre hematopoyéticas (HSC) en progenitores especializados y su interacción organizada con el microambiente de la médula ósea y las citoquinas hematopoyéticas. Existe un suministro constante de plaquetas a lo largo de la vida de un individuo, ya que el cuerpo humano es capaz de producir y eliminar diariamente 10^{11} plaquetas para mantener el recuento plaquetario en estado estacionario. Ambos procesos deben estar estrictamente controlados, siendo la trombopoyetina uno de sus principales reguladores, respaldando la supervivencia, proliferación y diferenciación de los precursores plaquetarios [176].

La estructura de la plaqueta puede dividirse básicamente en una zona periférica, una zona sol-gel (citoplasma), una zona de orgánulos y una zona de sistemas de membrana [177,178] (figura 9).

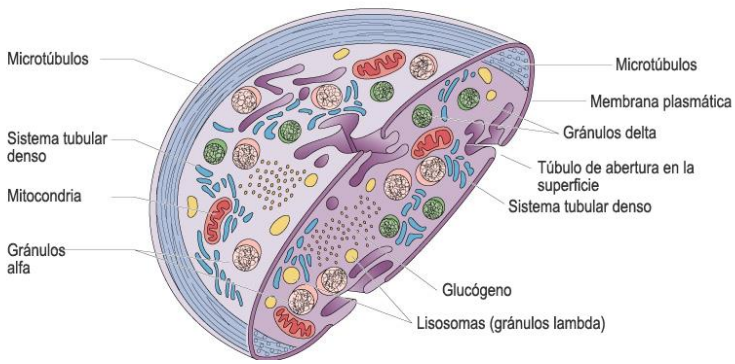

Figura 9. Estructura básica de la plaqueta.
http://medicosenformacion8.tripod.com/10_010.jpg

4.1.1 Orgánulos

Las plaquetas contienen tres tipos principales de orgánulos secretores: los gránulos α, los gránulos densos (gránulos δ) y los lisosomas. Además, las plaquetas contienen mitocondrias simples, que son importantes para su metabolismo energético, glicosomas, cadenas y agregados electrón-densos e inclusiones tubulares [177,178].

- Gránulos α:

Los gránulos α son los gránulos más abundantes de las plaquetas humanas (50-80 por plaqueta) y contienen un repertorio proteico que incluye una variedad de moléculas con actividad biológica [22,178]. En las plaquetas en reposo, estos gránulos están separados entre sí por los filamentos de actina citoplasmáticos [177,178]. Tras la activación liberan numerosas proteínas de adhesión y factores inflamatorios e inmunomoduladores que fomentan el reclutamiento y la activación de las células inflamatorias, la secreción de quimioquinas y la diferenciación celular. También participan en la defensa del huésped al proporcionar proteínas antimicrobianas. Además los gránulos α de las plaquetas contienen una serie de factores de crecimiento que son trascendentales en la reparación tisular [171,177,179,180].

- Gránulos densos:

Los gránulos densos de las plaquetas humanas son más pequeños que los gránulos α, menores en número (3-8 por plaqueta) y tienen una alta variabilidad morfológica [177,178]. Su característica más distintiva es una estructura esférica opaca a los electrones que está separada de la membrana circundante por un espacio aparentemente vacío [177,178]. Estos pequeños gránulos contienen adenosín trifosfato (ATP), adenosín difosfato (ADP), calcio, magnesio, pirofosfato, serotonina, dopamina e histamina [22,177,181].

- Lisosomas:

La función de los lisosomas plaquetarios en la fisiología de la hemostasia es desconocida. Su contenido comprende hidrolasas ácidas, elastasas, fosfatasas ácidas, catepsinas D y E, proteínas de membrana asociadas a lisosomas y CD63 [22,177,181,182].

4.1.2 Vía de activación de las plaquetas

En condiciones fisiológicas, las plaquetas circulan muy cerca de las paredes vasculares, pero están protegidas de la activación prematura por la monocapa endotelial sana que proporciona una "barrera" natural a la trombosis, liberando una serie de inhibidores como el óxido nítrico y la prostaciclina [174,181,183]. Las plaquetas se activan cuando la continuidad de la capa endotelial se interrumpe y la matriz subendotelial subyacente queda expuesta, o si la inflamación perturba el endotelio [183]. Las plaquetas humanas pueden ser activadas por numerosos agonistas a través de diferentes vías [177].

En términos generales la activación de las plaquetas puede dividirse en tres etapas superpuestas: iniciación, extensión y perpetuación [184,185]. Las plaquetas circulantes son normalmente activadas por componentes de la matriz como el colágeno y el factor de Von Willebrand (vWF) que quedan expuestos tras una alteración en la integridad en la superficie vascular. La clave de estos eventos es la presencia, en la superficie de las plaquetas, de receptores que pueden unirse al colágeno (integrina $\alpha 2\beta 1$ y glicoproteína (GP) VI) y al vWF (GPIb-IX-V y $\alpha IIb\beta 3$) y de ese modo iniciar la señalización intracelular [172,175,179,185-187]. La formación de una monocapa de plaquetas después de la exposición de colágeno y vWF es suficiente para iniciar la formación del tapón de plaquetas. Sin embargo, y debido a que el tapón de plaquetas formado durante la hemostasia primaria es todavía inestable y frágil, aproximadamente una docena de factores de coagulación que circulan en el torrente sanguíneo en un estado inactivo se activan rápida y secuencialmente en una llamada "cascada de coagulación", que conduce a la producción de trombina que posteriormente convertirá el fibrinógeno en fibrina [174,183,186]. El siguiente paso requerido es la extensión del tapón de plaquetas en el que se reclutan y activan plaquetas circulantes adicionales y adquieren la capacidad de unirse entre sí, en un proceso denominado agregación plaquetaria**.** Este reclutamiento de plaquetas adicionales es posible gracias a la acumulación local de agonistas que son secretados por las plaquetas, como ADP y tromboxano, y por la generación local de trombina, cuya formación se acelera en la superficie de las plaquetas activadas [172,179,185]. Los receptores que median esta respuesta son miembros de la superfamilia de receptores acoplados a proteínas G [184]. Cada uno de estos agonistas puede activar en las plaquetas la fosfolipasa C, causando un aumento en la concentración

citosólica de Ca^{2+} que a su vez estimula la secreción de los gránulos y un cambio dramático de forma [184,188]. La reorganización citoesquelética permite que las plaquetas previamente discoides se aplasten y se adhieran más de cerca a la pared expuesta del vaso, aumentando su superficie disponible para la adhesión entre sí y a la matriz extracelular. Estos agonistas "secundarios" perpetúan y amplifican la señal de la agregación plaquetaria. Después de la activación y agregación plaquetaria en respuesta a una lesión vascular, se deben producir procesos que consoliden la estabilidad del trombo que se forma [184,185]. En este sentido, el fibrinógeno juega un papel fundamental en el anclaje de las plaquetas agregadas al sitio de la lesión vascular. Es decir, las interacciones cohesivas entre las plaquetas, críticas para la formación del tapón hemostático, se mantienen mediante una variedad de interacciones moleculares, de las cuales la más esencial es la unión del fibrinógeno (y vWF) a la integrina α IIb β 3 activada [184,189,190].

Una vez que se completa su función en la hemostasia, los coágulos se deben romper y eliminar, y el tejido dañado que rodea los coágulos de fibrina debe repararse. Las células endoteliales vasculares intactas alrededor de los coágulos producen el activador tisular del plasminógeno. Este activador cataliza la conversión de plasminógeno a plasmina, la principal enzima responsable de la descomposición del coágulo (fibrinólisis) [174].

Durante y después de la hemostasia, las plaquetas activadas liberan una variedad de gránulos citoplásmicos, contenido lisosómico, micropartículas y exosomas, y estos además actúan como depósitos para la liberación inmediata o sostenida de factores de crecimiento, proteínas adhesivas, citoquinas y otras moléculas de señalización que juegan un papel importante en la regulación de la reparación tisular [179].

4.1.3 Funciones fisiológicas de las plaquetas

Además de mediar la hemostasia primaria y la trombosis, las plaquetas participan de forma crucial en la reparación tisular, en la modulación de reacciones inflamatorias, en diversas respuestas inmunitarias y en la defensa antimicrobiana del huésped, todo ello a través del conjunto de sustancias presentes en su secretoma y del elevado número de receptores que están presentes en su superficie.

- Inflamación:

Evidencias recientes demuestran que las plaquetas contribuyen al inicio y extensión del proceso inflamatorio. Tras la inflamación o lesión vascular, las plaquetas que se adhieren al endotelio promueven el reclutamiento de células inflamatorias, orquestando así la respuesta inflamatoria [182,183]. Las plaquetas median la respuesta inflamatoria tanto expresando receptores de adhesión que facilitan las interacciones con células endoteliales y leucocitos, como secretando una amplia gama de quimioquinas [182]. Las interacciones adhesivas generalmente dan como resultado la activación mutua y la propagación del fenotipo inflamatorio de cada célula. Estas interacciones transitorias con neutrófilos y células endoteliales están mediadas por la proteína P-selectina, que es una glicoproteína de membrana integral expresada por plaquetas, células endoteliales, macrófagos y placas ateroscleróticas [171,182]. La P-selectina se expresa en la superficie de las plaquetas activadas y las células endoteliales activadas. Además de mediar el reclutamiento de células inflamatorias, las plaquetas son una fuente importante de quimioquinas y citoquinas proinflamatorias y de factores inmunomoduladores [172,177]. Estos mediadores inducen el reclutamiento, la activación, la secreción de quimiocinas y la diferenciación de otras células vasculares y hematológicas [171].

- Reparación tisular y angiogénesis:

Las plaquetas regulan los mecanismos fundamentales involucrados en el proceso de regeneración tisular, modulando la quimiotaxis, proliferación y diferenciación de células progenitoras. [191-193]. Las plaquetas activadas liberan toda una gama de quimioquinas que promueven el reclutamiento, la adhesión y proliferación de células madre adultas, incluidas las células progenitoras positivas para CD34, las células madre mesenquimales, las células progenitoras del músculo liso y las células progenitoras endoteliales. El reclutamiento regulado de estas células madre adultas hacia células lesionadas puede ser, por lo tanto, un mecanismo sustancial para ejercer respuestas celulares regenerativas [193].

El proceso de formación de nuevos vasos sanguíneos o angiogénesis en el tejido dañado es otro mecanismo fundamental para la recuperación de la función tisular, el cual también se encuentra sustancialmente regulado por las plaquetas [193]. Tras su activación, las plaquetas liberan una gran cantidad de factores de crecimiento que potencialmente promueven (VEGF, PDGF, angiopoyetina-1…) o inhiben (PF-4, TSP-1…) la angiogénesis e influyen en la reactividad de las células sanguíneas en la inflamación y reparación de los tejidos. Además, las plaquetas son capaces de inducir la diferenciación de células progenitoras circulantes en células endoteliales maduras y funcionales, proceso que parece estar mediado por el factor derivado del estroma [192,194].

- Inmunidad y defensa antimicrobiana:

Las plaquetas son componentes clave de la respuesta inmune innata y adaptativa y desempeñan un papel significativo en la defensa del huésped contra microorganismos patógenos. Los gránulos α de las plaquetas contienen proteínas con propiedades microbicidas directas, un grupo al que se hace referencia colectivamente como proteínas microbicidas plaquetarias (PMPs) [174,182,188]. La función primaria de la inmunidad innata es reconocer patrones moleculares asociados a patógenos (PAMP) a través de receptores de reconocimiento de patrones. Para implementar esta función, las plaquetas expresan varios receptores tipo Toll, que pertenecen a una familia de receptores de reconocimiento de patógenos conservados evolutivamente, así como otro tipo de receptores, incluidos los receptores de fragmento cristalizable y los receptores de proteínas G [171,174,195,196]. La activación de estos receptores conduce a la expresión de una plétora de citoquinas, quimioquinas y moléculas de superficie celular que no sólo inician y perpetúan la hemostasia, sino que también sirven para alertar al sistema inmune e inducir el reclutamiento de leucocitos en el tejido lesionado [183,188].

4.2 PRP y PRGF (III)

El potencial terapéutico, ya descrito, de las plaquetas ha dado lugar a un desarrollo de productos basados en estas células sanguíneas y en su capacidad para promover la

regeneración tisular, tanto *in vitro* como *in vivo*. En los últimos años, los geles de plaquetas, el plasma rico en plaquetas (PRP), los factores de crecimiento derivados de plaquetas y otros derivados han surgido como tecnologías atractivas para ser utilizadas por sus propiedades en la reparación tisular [22].

El plasma rico en plaquetas se define básicamente como una porción de la fracción de plasma obtenida de la propia sangre del paciente que tiene una concentración de plaquetas por encima del valor basal de la sangre. El objetivo final de estas terapias es facilitar, optimizar y acelerar la capacidad innata del cuerpo de reparar tejidos mediante el suministro progresivo de factores de crecimiento y otras proteínas autólogas (tabla 3) por la matriz tridimensional (3D) de fibrina obtenida del plasma rico en plaquetas y necesaria para respaldar la regeneración tisular. Estas tecnologías se están empleando con frecuencia en investigación y aplicación clínica, sin embargo se debe tener en cuenta que existen numerosos protocolos para la preparación de PRPs con múltiples productos disponibles comercialmente. La mayoría comparte una secuencia común de pasos básicos: (i) extracción de sangre por venopunción simple, (ii) centrifugación sanguínea para obtener la fracción plasmática de concentración de plaquetas y (iii) activación de plaquetas para liberar factores de crecimiento de la matriz temporal de fibrina [197]. Sin embargo, todos estos productos varían ampliamente en su composición y concentración ya que no existe un consenso con respecto al uso o no de anticoagulantes, las condiciones de centrifugación, el método para la activación plaquetaria, la concentración óptima de plaquetas o la inclusión o no de leucocitos. Se introduce además una variabilidad adicional cuando se considera la aplicación clínica, incluyendo el volumen y número de dosis, el almacenamiento temporal, el momento de tratamiento y el intervalo de redosificación. Todas estas variables conducen a diferentes productos con diferente potencial biológico que carecen de aspectos de estandarización clínica adecuados [2,22,179,197,198].

La inclusión o no de leucocitos en el PRP es uno de los temas actualmente más controvertidos. La respuesta inflamatoria es necesaria para la curación de las heridas, ya que evita la infección y contaminación y estimula la proliferación, revascularización y remodelación de la matriz extracelular. Sin embargo, la inflamación crónica no resuelta en el lugar de la herida favorece la cicatrización sobre la regeneración del tejido y puede conducir al desarrollo de fibrosis o cicatrización patológica, afectando a la función normal

del tejido. Por lo tanto, la resolución temprana de la respuesta inflamatoria es un proceso crítico para el desarrollo óptimo de estrategias de regeneración [170,199-201]. En este sentido, la contribución de los leucocitos en el PRP se considera actualmente como un arma de doble filo. Los glóbulos blancos contienen sustancias microbicidas y enzimas que podrían contribuir a prevenir infecciones, sin embargo también poseen otras sustancias que podrían inducir inflamación, alterar la matriz extracelular y dañar las células involucradas en la curación de heridas [170]. Además, una reciente revisión sistemática [202] ha puesto de manifiesto que no existe suficiente evidencia para atribuir el efecto microbicida a la presencia de leucocitos en el PRP.

Históricamente, la base del tratamiento con plasma rico en plaquetas se ha centrado en maximizar los factores de crecimiento que se encuentran en los gránulos de las plaquetas para promover un ambiente anabólico en el lugar de la lesión. Los neutrófilos contienen varios tipos de gránulos que contienen numerosas citoquinas (colagenasa, gelatinasa, lisozima, elastasa, serprocidina y mieloperoxidasa). Los linfocitos T poseen varias interleuquinas, incluidas la IL-2, 4, 5, 6, 13, 17, 21 y 22; interferón-γ (IFN-γ) y factor de necrosis tumoral-α (TNF-α). El contenido en citoquinas de los linfocitos B es menor pero similar al de los linfocitos T (IL-6, IL-8, TNF-α e IL-1β)[203]**.** Por lo tanto, los leucocitos contienen y producen citoquinas biológicamente activas que son principalmente de naturaleza catabólica o inflamatoria, incluidas las MMPs que degradan el colágeno, las especies reactivas del oxígeno y otras proteasas que pueden influir en el resultado clínico de la aplicación de la tecnología PRP. [203]. Varios estudios [203-206] han mostrado una correlación positiva entre la concentración de plaquetas y la expresión génica anabólica y entre los glóbulos blancos y la expresión génica catabólica. Un exceso de leucocitos podría sobrepasar la capacidad de los factores de crecimiento para modular las citoquinas proinflamatorias [206].

Es necesario destacar, que la inclusión de leucocitos en el PRP va acompañada de la adición de un número considerable de glóbulos rojos. El papel de los eritrocitos en el tratamiento con PRP es en gran parte desconocido, ya que no existen suficientes datos sobre los efectos específicos de este componente sanguíneo. Sin embargo, un estudio reciente realizado por Braun et al. [207] demostró que tanto el tratamiento con PRP enriquecido en leucocitos, como el tratamiento con un concentrado de glóbulos rojos

promovieron la muerte celular de sinoviocitos y la producción de mediadores proinflamatorios. Estos datos sugieren que los eritrocitos pueden ser nocivos, particularmente cuando la terapia con PRP se aplica mediante inyecciones intraarticulares para la degeneración del cartílago y artrosis.

La terapia autóloga de plasma rico en plaquetas proporciona además matrices naturales, biodegradables y temporales con una amplia gama de propiedades mecánicas que se combinan con una liberación sostenida de proteínas y factores de crecimiento (tabla 3) para inducir a las células hospedadoras a reemplazar el tejido perdido estructural y funcionalmente. Los factores de crecimiento son reguladores clave de los principales procesos regenerativos tisulares, como la migración celular, la proliferación, la diferenciación, la angiogénesis y la biosíntesis de matriz extracelular y ejercen papeles destacados en la modulación de la etapa inflamatoria y el reclutamiento celular en el área dañada [180,208,209]. Los factores de crecimiento modulan sus efectos a través de la unión a receptores específicos de la superficie celular. Tras esta unión, la vía de transmisión de señales implica una compleja serie de eventos como segundos mensajeros, fosforilación de proteínas, expresión génica y síntesis de proteínas [210]. Múltiples factores de crecimiento pueden compartir mecanismos, mientras que el mismo factor de crecimiento puede enviar diferentes mensajes dependiendo de la célula y el tipo de receptor al que se una.

CATEGORÍA	PROTEÍNA	FUNCIÓN
Factores de crecimiento tróficos	TGF-β	Control de la proliferación, control del sistema inmune, diferenciación mesenquimal, inflamación, angiogénesis, reepitelización y regeneración del tejido conectivo, estimulación de la síntesis de componentes de la matriz extracelular, formación de tejido de granulación, contracción de la herida.
	CTGF	Formación de tejido de granulación, reepitelización, formación de matriz y remodelación.
	IGF	Quimiotaxis, migración celular, proliferación celular y agente antiapoptótico.
	EGF	Proliferación celular, diferenciación y

		supervivencia, reepitelización.
	FGF	Proliferación celular, migración celular, neovascularización y formación de tejido de granulación.
	HGF	Proliferación celular, angiogénesis, morfogénesis, antiinflamatorio, agente anti-fibrogénico, potente agente mitogénico y mitogénico epitelial.
	PDGF	Actividad mitogénica y quimiotáctica, reepitelización.
Reguladores de la angiogénesis	VEGF	Estimulación de las fases tempranas de la angiogénesis, migración y proliferación de células endoteliales.
	PF4	Inhibición de la migración, proliferación y angiogénesis de las células endoteliales, inhibición de la citoquina proangiogénica IL-8, inhibición de los reguladores positivos de la angiogénesis (VEGF, bFGF) mediante la unión a sus receptores, estimulación de la coagulación, quimioatracción para neutrófilos, fibroblastos y monocitos, probable papel en la inflamación y reparación de heridas.
	TSP-1	Regulador crítico de la angiogénesis vía antagonista de VEGF, inhibición de la proliferación y migración de células endoteliales. Inducción de apoptosis de células endoteliales.
	ANG-1	Maduración y estabilidad de los vasos sanguíneos.
	Endostatina	Inducción de la apoptosis de células endoteliales mediante la inhibición de la ciclina D1, uniéndose a receptores celulares que median en actividades antiangiogénicas.
Proteínas adhesivas	vWF	Detector de daño en la pared del vaso e iniciador de la hemostasia primaria, mediador de la formación de tapones de plaquetas mediante adhesión en el sitio de la lesión.
	Fibrinógeno y fibronectina	Agregación plaquetaria a través de las integrinas

		αIIbβ3, $\alpha_5\beta_1$ y αVβ3.
	Vitronectina	Regulación de la proteolisis iniciada por la activación del plasminógeno, unión a las integrinas αIIbβ3 y αVβ3 de las plaquetas, anclaje de las células a la matriz extracelular, coordinación de la migración y la señalización de las células sanguíneas y vasculares.
	TSP-1	Unión a CD36, que da como resultado la activación plaquetaria, principal activador de TGF-β1.
	Laminina	Unión a plaquetas a través de la integrina α6β1. Modulación del comportamiento celular. Diferenciación, migración y estabilidad del fenotipo. Inhibición de la apoptosis.
Agentes antiinflamatorios	HGF	Interrupción de la actividad de transactivación del NF-κB, bloqueo de la producción celular de PGE2 y expresión de ciclooxigenasa. Disminuye la producción de IL-6 (inflamatoria) y aumenta la de IL-10 (antiinflamatoria).
	IGF	Inhibición de la quinasa IκB-α.
	PDGF-BB	
Agentes antimicrobianos	Trombocidinas	Propiedades bactericidas y fungicidas.
	PMPs (PF-4, RANTES, PBP, CTAP-3, Tβ-4, FP-A, FP-B)	Proteinas asesinas de microorganismos patógenos y mediadoras de la quimiotaxis de los fagocitos.
Biomoduladores del dolor	Endocanabinoides (Anandamida, 2-araquidonilglicerol, Palmitoiletanolamida, Oleiletanolamida)	Actividad antinociceptiva.

Tabla 3. Principales factores de crecimiento y moléculas bioactivas presentes en el plasma rico en plaquetas. Reproducido con permiso de Anitua *et al.* [41].

La versatilidad de esta tecnología autóloga le permite ser aplicada en diferentes campos de la biomedicina como la ortopedia, oftalmología, cirugía oral y maxilofacial y dermatología [211,212]. Así mismo, esta terapia biológica está comenzando a ser utilizada como alternativa al uso de productos xenogénicos como suplemento de medio de cultivo para la expansión *ex vivo* y posterior trasplante de células madre en terapia celular e ingeniería de tejidos [13,22,213]. Hasta ahora el suero bovino fetal (FBS) había sido el suplemento común más utilizado en el cultivo celular y la ingeniería de tejidos [13]. Sin embargo, su uso en la terapia celular humana alberga serias preocupaciones, como el riesgo de contaminación viral, bacteriana, fúngica y priónica, la variación entre lotes y la posible inducción de rechazo inmunitario de las células trasplantadas en el huésped [69,214]. Las células progenitoras mesenquimales se producen con baja frecuencia en los tejidos y, en general, tienen que propagarse *ex vivo* para lograr una dosis adecuada para la aplicación clínica [13]. Esto requiere el establecimiento de pautas de cultivo estandarizadas para el aislamiento y la expansión de hMSCs que muestren una mínima variabilidad en sus características inherentes [14]. Por lo tanto, existe una clara necesidad de identificar nuevas alternativas para la expansión de MSCs para la correcta fabricación de productos celulares terapéuticos bajo los actuales marcos normativos y criterios de buena fabricación [16,72]. Medios químicamente definidos o productos sanguíneos humanos, como el lisado plaquetario, el suero humano o el plasma rico en plaquetas, están siendo evaluados como alternativas viables al uso del FBS en el cultivo de células madre [19,215-219].

El plasma rico en factores de crecimiento (PRGF) pertenece a los citados PRPs. Esta tecnología es pionera en el uso de plaquetas autólogas [23], se obtiene por centrifugación simple y se activa mediante cloruro cálcico. Se caracteriza por una concentración específica de plaquetas, que conduce a un efecto biológico óptimo y por la ausencia de leucocitos, evitando así fomentar la inflamación [211,220]. Desde un punto de vista terapéutico, existen dos procesos básicos relacionados con el potencial de esta tecnología. El primero, se centra en la liberación, por parte de las plaquetas, de cientos de proteínas y factores de crecimiento que estimulan activamente la regeneración tisular. El segundo se refiere a la formación de una matriz tridimensional de fibrina que retiene y posteriormente

libera parte de esos factores de crecimiento, actuando a su vez como una matriz provisional para las células [24]. Pero lo que realmente hace que la tecnología PRGF sea diferente de otros productos enriquecidos con plaquetas es su versatilidad. Esta tecnología autóloga proporciona seis formulaciones diferentes dependiendo del grado de coagulación y activación de las muestras para poder ser utilizadas en diferentes aplicaciones clínicas [25,41,221,222]. Las formulaciones incluyen (figura 10):

- PRGF líquido: preparación inyectable que se activa en el momento de uso. Utilizado en cirugía y para bioactivar las superficies de implantes dentales (figura 10A).
- Coágulo de PRGF: matriz tridimensional capaz de retener los factores de crecimiento para asegurar su disponibilidad durante las diferentes fases de la regeneración tisular. Además puede combinarse con diferentes biomateriales para perfeccionar sus propiedades (figura 10B).
- Membrana de PRGF: membrana de fibrina elástica y suturable. Se obtiene al final del proceso de retracción de la fibrina (figura 10C).
- Sobrenadante de PRGF: utilizado como colirio convencional en oftalmología y como suplemento de medios de cultivo (figura 10D).
- Gel de PRGF: gel autólogo e inyectable obtenido a través de una etapa de desnaturalización térmica del plasma combinada con una reticulación de fibrina autóloga. Esta formulación con potencial regenerador es capaz de mantener el volumen proporcionando relleno en aplicaciones estéticas (figura 10E).
- Serum tópico de PRGF: serum tópico autólogo obtenido a través de la combinación de una etapa de desnaturalización térmica del plasma con sobrenadante. Dirigido especialmente a patologías dermatológicas de larga duración y aplicaciones estéticas (figura 10F).

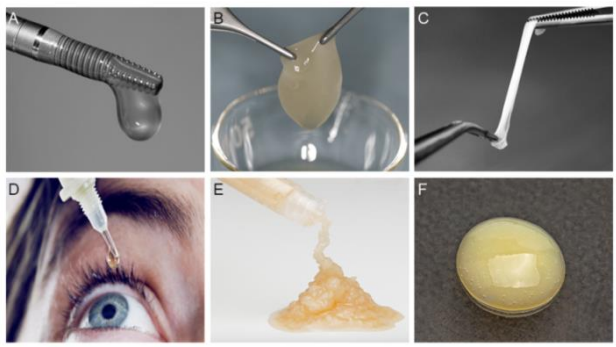

Figura 10. Formulaciones de la tecnología autóloga PRGF. Adaptada con permiso de Anitua *et al.* [25].

La seguridad y eficacia de la tecnología autóloga PRGF se ha demostrado extensamente en diferentes campos médicos, incluyendo la odontología, implantología oral, ortopedia, medicina deportiva, tratamiento de úlceras y oftalmología, entre otros [24,25,211,223-225] (figura 11).

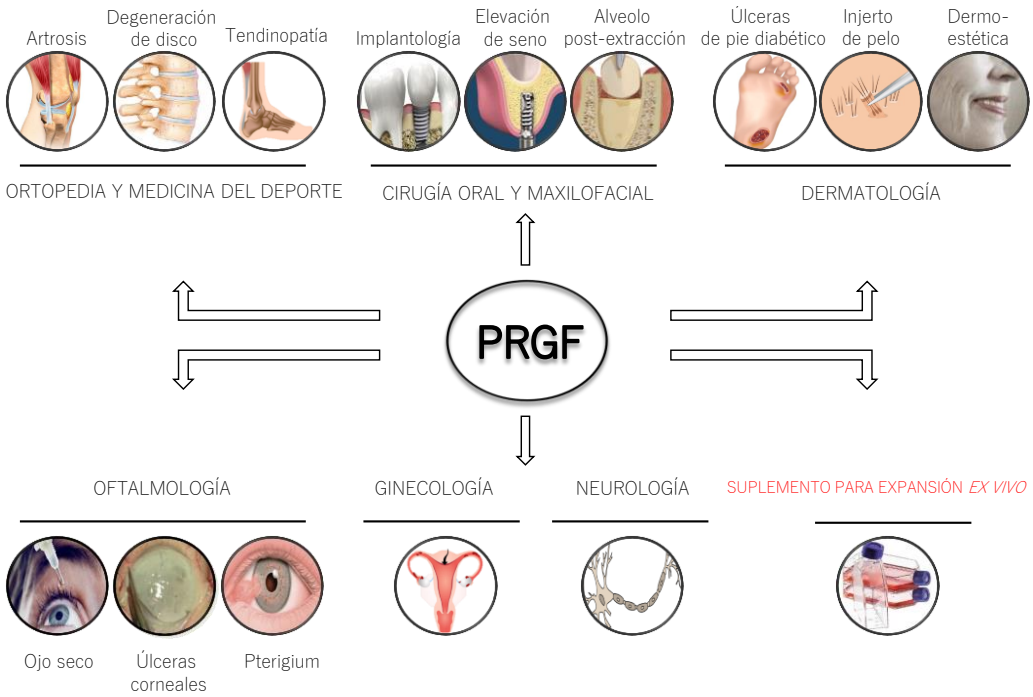

Figura 11. Principales aplicaciones de la tecnología PRGF.

4.2.1 Marco legal

El 23 de mayo de 2013, la Agencia Española de Medicamentos y Productos Sanitarios (AEMPS) publicó la resolución por la que se estableció la clasificación del uso terapéutico no sustitutivo del plasma autólogo y sus fracciones, componentes o derivados, como medicamento de uso humano para atender necesidades especiales [226]. Sin embargo, en ese documento también se especifica que no debe ser considerado como un

medicamento de producción industrial ni de terapia avanzada. De hecho, la AEMPS considera que el PRP es un medicamento de uso humano que se puede utilizar al amparo del artículo 5 de la Directiva 2001/83/CE del Parlamento Europeo y del Consejo de 6 de noviembre. Dicha Directiva establece que los Estados miembros podrán, de acuerdo con la legislación vigente y con vista a atender necesidades especiales, excluir de las disposiciones de dicha Directiva a los medicamentos elaborados de acuerdo con la prescripción de un facultativo reconocido y que los destine a un paciente individual bajo su responsabilidad personal directa [226].

El uso de plasma autólogo y sus fracciones, componentes o derivados debe hacerse siempre sujeto a prescripción de médicos, odontólogos o podólogos. Así mismo, la resolución de la AEMPS del 23 de mayo de 2013 establece las garantías mínimas exigibles para el uso de este tipo de productos [226]:

- Garantías de calidad
- Garantías de eficacia
- Garantías de trazabilidad
- Garantías de farmacovigilancia
- Garantías de información

(I) Progress in the use of autologous regenerative platelet-based therapies in implant dentistry

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Progress in the use of autologous regenerative platelet-based therapies in implant dentistry

Eduardo Anitua[1]*, María Troya[2], Mar Zalduendo[2], Ricardo Tejero[2], Gorka Orive[1]*

[1]Foundation Eduardo Anitua, Jacinto Quincoces, 39; 01007 Vitoria, Spain

[2]Laboratory of Regenerative Medicine, Biotechnology Institute (BTI), Vitoria, Spain

Corresponding authors:

Gorka Orive.

E-mail: gorka.orive@fundacioneduardoanitua.org

Eduardo Anitua.

E-mail: eduardoanitua@eduardoanitua.es

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Abstract

The field of medicine is rapidly moving towards the development of personalized treatments and non-invasive tools as to achieve a more predictable and optimal regeneration. In this sense, the goal of periodontal healing is to arrest the disease progression and functionally regenerate all the tissues that comprise the periodontium. The latter implies a well-orchestrated interaction among oral cells, growth factors and extracellular matrix. Although several procedures are performed in an attempt to regenerate lost periodontal tissue, outcomes are not always predictable. Growth factors represent a class of biologically active polypeptides that have a critical role in the healing process. Its use provides a new paradigm to understand the regenerative medicine. The use of platelet-rich plasma (PRP) products as a local source and delivery system of autologous growth factors has emerged recently. Among them stands PRGF for its remarkable stimulatory effect on oral tissue regeneration, making it a very safe and successful tool with a great value on Dentistry.

1. Introduction

The design and optimization of new therapies that may help to limit or circumvent conditions that compromise homeostasis and normal tissue function is advancing rapidly. Some of the ideal approaches proposed to improve tissue repair or regeneration are based on cell therapy, gene therapy and/or tissue engineering using newly created three-dimensional tissues [1]. The general principles of these tissue-regeneration strategies comprise a network of interactions among cell populations, scaffolds and signaling molecules to mimic natural wound healing [2-4]. These ideal therapies are still in their infancy and their pathway towards clinical regulatory acceptance is challenging. Careful consideration of the cost-effectiveness of translating these therapies from bench to bedside is of paramount importance to avoid major disappointments and the future flooding of the field of regenerative medicine.

The scientific community is nowadays witnessing strides in proposing new therapeutic alternatives that use human fluids, and especially blood, as raw material to fabricate and modulate biomaterials and therapies that can be used to promote tissue regeneration [5,6]. Human plasma and human platelets contain the necessary ingredients to create

morphogen rich solutions that provide significant cell signaling biomolecules to modulate cell behavior and to stimulate the main stages of the healing process [2,7]. In fact, the growth factor secretion by platelet α-granules confers them multiple roles in tissue regeneration beyond the basic function of maintaining homeostasis [8]. In addition, the same agents have the ability to create naturally formed biomaterials such as fibrin, which will act as a temporary scaffold and a localized and prolonged protein-release system [3]. The last few years have seen an increasing number of studies evaluating and testing human-blood based formulations. These attempts have range from oral and maxillofacial surgery to other medical disciplines including dermatology, traumatology and sports medicine [8,9]. Unfortunately, many of the studies lack basic and fundamental knowledge and most of molecular mechanisms driving tissue repair and regeneration are missing. In this review, we aim to describe the basic research behind one of the pioneering technology, that is, plasma rich in growth factors, both on different cell phenotypes of the oral cavity and on dental implants. The most innovative applications of PRGF will be discussed together with the challenges and future remarks.

2. The power of Plasma and platelets: growth factors

Although several procedures are performed in an attempt to regenerate periodontal tissue in dental surgeries, outcomes are not always predictable. Platelet-derived growth factors are biologically active proteins capable of coordinating and regulating several cellular and molecular processes of periodontal healing. Its relevant role in tissue regeneration suggests them as a new therapeutic alternative [10,11]. The use of platelet-rich plasma (PRP) technology as an autologous source of growth factors and proteins is gaining increasingly significant interest as a therapy to improve tissue healing with minimal complications [12-14]. There are a great variety of these autologous products that differ with regard to the preparation protocol, number of platelets, utilization of anticoagulant, inclusion of leukocytes, and use of activators that in turn, may lead to very different biological efficacy. The pioneer in these types of autologous therapies is the Plasma rich in growth factors (PRGF) technology which is mainly characterized by the absence of leukocytes and therefore by not promoting the inflammatory condition [14-16]. It is a predictable technique that uses calcium chloride for platelet activation and provides four different potential formulations to be employed in diverse clinical applications [15,17].

One of them, liquid PRGF can be an injectable preparation that is activated at the time of use. In addition of dental surgeries, it can be used to implant surface bioactivation [18]. PRGF scaffold, is a 3-dimensional matrix with a stability greater than 8 days at the end of which is capable of retaining almost 30% of the growth factor content to ensure the availability of these plasma and platelet proteins during the different phases of the tissue regeneration [16,19]. In addition, this formulation can be combined with different biomaterials to improve its properties such as autologous or demineralized bone or collagen among others [20]. Third preparation, PRGF supernatant that contains plasma proteins and platelet releasate, besides being applied successfully in Ophthalmology, it can be also used to supplement cell culture medium [21]. Finally, an elastic and suturable fibrin membrane can be obtained at the end of the fibrin scaffold retraction, which is an excellent tool to seal the postextraction tooth sockets [22].

Recently, a randomized controlled clinical trial, which involved sixty patients with indication of a simple one molar extraction, has shown the extraction socket healing enhancer effect of PRGF [23]. On the other hand, the advantages of the use of different PRGF formulations in different surgical techniques to achieve sinus floor augmentation [24] and horizontal bone increase also have been described [25,26].

As mentioned above, PRGF contains growth factors and other proteins, which have been shown to have positive effects on wound healing, among which it's worth highlighting those listed in Table 1. Besides the several molecules involved in tissue regeneration, other proteins related to both the acute-phase response signaling pathway and lipid metabolism, which participate in the wound healing process, are present in PRGF [27].

Category	Protein	Function	References
Trophic growth factors	TGF-β	Control of proliferation, control of immune system, mesenchymal differentiation, inflammation, angiogenesis, reepithelization and connective tissue regeneration, promoter of ECM components synthesis, granulation tissue formation, wound contraction.	[28-33]
	CTGF	Granulation tissue formation, reepithelization, matrix formation and remodeling.	[33-35]
	IGF	Chemotaxys, cell migration, cell proliferation,and antiapoptotic agent..	[7,11,36]
	EGF	Cellular proliferation, differentiation and survival, reepithelization.	[33,37,38]
	FGF	Cell proliferation, cell migration, neovascularization and, formation of granulation tissue.	[11,28,39]
	HGF	Cell proliferation, angiogenesis, morphogenesis, anti-inflammation, anti-fibrogenic agent, potent epithelial mitogenic and chemotactic agent .	[40-42]
	PDGF	Mitogenic and chemotactic activity. reepithelization.	[11,28,33]
Regulators of angiogenesis	VEGF	Promotion of the early events in angiogenesis, endothelial cell migration and proliferation.	[7,28,33]
	PF4	Inhibition of the endothelial cells (EC) migration, proliferation and	[43-45]

		angiogenesis, inhibition of the pro-angiogenic cytokine IL-8,,inhibition of the positive angiogenesis regulators (VEGF, bFGF) binding to their receptors, promotion of the coagulation, chemoattraction for neutrophils, fibroblasts and monocytes, probably intervention on inflammation and wound repair.	
	TSP-1	Critical regulator of angiogenesis via antagonism of VEGF, inhibition of the endothelial cell proliferation and migration. Induction of endothelial cell apoptosis.	[46-50]
	ANG-1	Blood vessel maturation and stability.	[7,51]
	Endostatin	Induction of endothelial cell apoptosis by inhibiting cyclin D1, binding to cell receptors which mediate anti-angiogenic activities.	[8,52,53]
Adhesive proteins	VWF	Vessel wall damage sensor and initiator of primary hemostasis, mediator of platelet plug formation via adhesion at the site of injury.	[54-57]
	Fibrinogen and fibronectin	Platelet aggregation through integrins $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_V\beta_3$.	[57-59]
	Vitronectin	Regulation of proteolysis initiated by plasminogen	[58,60,61]

		activation, binding to platelet αIIbβ3 and αVβ3 integrins, anchorage of cells to the extracellular matrix, coordinating migration and signaling of blood cells and vascular cells.	
	TSP-1	Binding to CD36, resulting in platelet activation, the major activator of TGF-β1.	[49,58,59]
	Laminin	Binding to platelets via integrin $\alpha_6\beta_{1.}$, modulation of cell behavior;, differentiation, migration, and phenotype stability, inhibition of apoptosis.	[58,59,62]
Anti-inflammatory agents	HGF	Disrupting NF-κB-transactivating activity., block of the cellular production of PGE_2 and the expression of COX proteins, decrease production of inflammatory IL-6 and increase the anti-inflammatory IL-10.	[40,63-65]
	IGF	Inhibition of IκB-α kinase.	[66]
	PDGF-BB		
Antimicrobial agents	Thrombocidins	Bactericidal and fungicidal properties	[67-69]
	PMPs (PF-4, RANTES, PBP, CTAP-3, Tβ-4, FP-A, FP-B)	Directly killer of the pathogenic microorganisms and mediator of the phagocyte chemotaxis..	[8,70,71]
Biomodulators of pain	Endocannabinoids (Anandamide, 2-arachidonoylglycerol, Palmitoylethanolamide, Oleoylethanolamide)	Anti-nociceptive activity.	[72]

3. Biological applications of PRGF on human oral primary cells

The complex structure of the periodontium, which consists of gingival soft connective tissue, periodontal ligament and mineralized tissues, makes the periodontal wound healing a unique process [73,74]. Indeed, proper functioning of the periodontium is achieved only through structural integrity and interaction between its components. Therefore, it is important to understand that each of these periodontal components has very specialized functions [15,75,76].

The aim of periodontal therapy is to regenerate all the periodontal components to return tissues to their original form and function [75,77]. In this sense, the use of PRGF technology represents a new approach to provide local concentrations of growth factors to stimulate wound healing and tissue regeneration. Therefore, the capacity of this technology to promote periodontal regeneration was evaluated on primary human oral cells.

3.1. Gingival fibroblasts and PRGF

Gingival fibroblasts are the most abundant resident cells in periodontal tissue and they play an important role in maintaining the structural integrity of the tissue as well as in modulating host defense against pathogens in the oral cavity.[78-81] They are characterized by having one of the fastest tissue turnover rates in body and a scarless wound healing [81,82]. Gingival connective tissue also represents an easily accessible source of stem cells as well as of induced pluripotent stem cells (iPS) [80,82-84]. Therefore, not only are gingival fibroblasts critical during periodontal repair but they can also offer a therapeutic potential for extra-oral applications.

The potential of PRGF on primary human gingival fibroblasts to promote the main cellular events, including adhesion, proliferation, migration, differentiation and matrix synthesis has been assessed [85]. Both PRGF fractions (F2+3 and F3) were evaluated, exerting similar *in vitro* biological effects (Fig. 1C and 2A). The results obtained confirmed that this autologous technology stimulates these consecutive but overlapping events of the wound healing in gingival fibroblasts. This is due to the PRGF growth factors content such as VEGF, HGF, PDGF-AB, EGF, TGF-β, IGF-I…, [85-90] since those proteins participate in almost all cell processes, as it has been described above. On the other hand, this

technology was also able to promote the synthesis of VEGF and HGF by gingival fibroblasts (Fig. 3A), which in turn, and together with the growth factors contained in the PRGF, stimulates the biological processes already mentioned.

The synthesis of procollagen type I and hyaluronic acid (Fig. 3A) was also increased when gingival fibroblasts were cultured with the PRGF technology. These two proteins are among the main constitutes of the extracellular matrix (ECM). The ECM is a dynamic key component that provides support and regulates several processes such as growth and wound healing and acts as a reservoir and distributor of a wide range of growth factors and proteins [91-94]. In addition, this complex network is also involved in cell signaling [91,92]. The fact that fibroblasts are the major source of the ECM proteins makes especially significant that PRGF was able to stimulate those cells to express these essential matrix proteins.

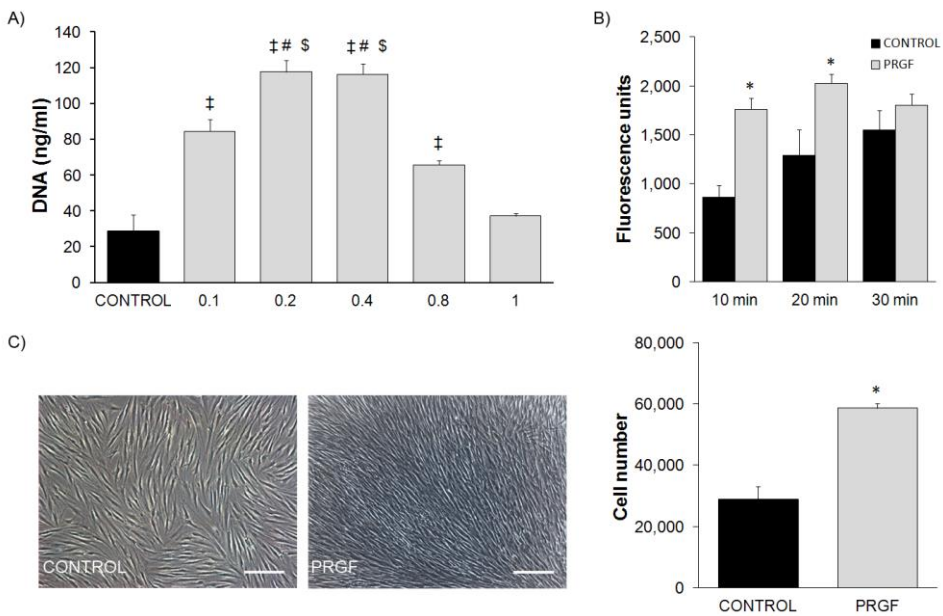

Figure 1. *In vitro* effect of the PRGF technology on the periodontium cells. A) Dose-dependent effect of PRGF on osteoblasts proliferation. B) PRGF effect on periodontal ligaments cell adhesion to a collagen type I matrix. C) Effect of PRGF on gingival fibroblast proliferation after 72 hours. *Statistically significant differences between PRGF and control ($p<0.05$). ‡Statistically significant differences relative to control and 100% dose ($p<0.05$). # Statistically significant differences relative

to 80% dose. $ Statistically significant differences relative to 10% dose. Scale bars: 200 μm.

Wound contraction and remodeling of granulation tissue involve the differentiation of fibroblasts into myofibroblasts, which are classically defined by the expression of the contractile protein α-smooth muscle actin (α-sma) [95,96]. However, the persistence of these myofibroblasts is responsible for fibrosis due to an excessive ECM deposition that can lead to pathological condition, impairing the normal function of the tissue [34,96].

The major mediator responsible for that myofibroblast transformation is TGF-β1 [97-99]. In general, oral mucosa is similar to fetal skin that displays scarless wound healing [81,82]. Low levels of TGF-β 1 and a mild and short inflammatory reaction have been found in fetal wounds [34,81,100]. However, a number of situations, including the administration of several drugs, hereditary gingival fibromatosis, and chronic inflammation lead to gingival overgrowth [101,102]. The autologous PRGF technology has shown to be able to inhibit and revert TGF-β1-induced α-sma expression of gingival fibroblasts (Fig. 2C) [85]. Moreover, the same effect has been reported for PRGF in other primary human cells including, keratocytes, conjunctival fibroblasts [90], as well as in an *in vivo* model of cornea wound healing [103].

As it happens in other cell events of the regeneration process, the key to explain the effect of PRGF on TGF-β1-induced myofibroblasts is its growth factors content. HGF and bFGF, both growth factors present in PRGF, play an important protective role in reducing scar formation via inhibition of TGF-β1-induced expression of myofibroblasts. Smad signaling is the canonical pathway responsible for TGF-β1-induced upregulation of fibrosis [104] HGF is known to inhibit Smad signaling by preventing the phosphorylation of Smad 2 and Smad 3 and its subsequent translocation into the nucleus, by upregulating the inhibitory protein Smad7 expression [105-107] bFGF in turn, blocks that pathway by the activation of ERK/MAP kinase pathway that also inhibits the translocation of Smad into the nucleus [108,109].

Taken together, these findings suggest that PRGF technology could be an interesting alternative to promote gingival regeneration.

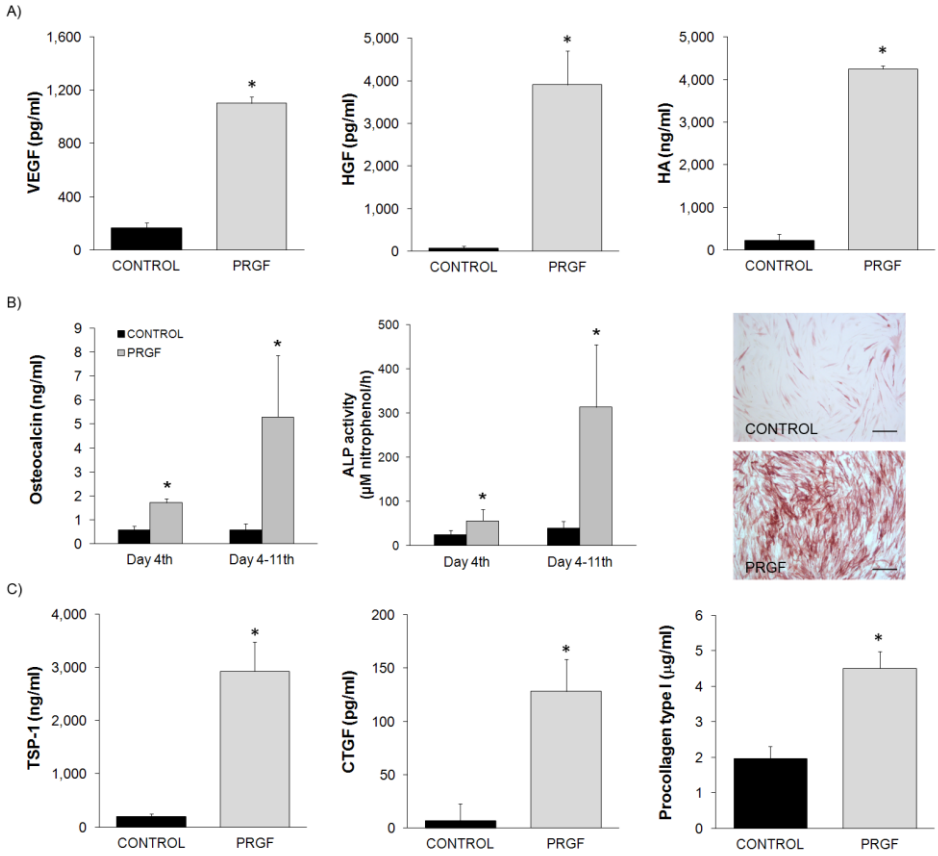

Figure 3: Effect of PRGF on the secretion of several biomolecules by the periodontium cells. A) Effect of PRGF on VEGF, HGF and HA secretion by human gingival fibroblasts after 72 hours. B) Effect of PRGF on both osteocalcin secretion and ALP activity by human alveolar osteoblasts. C) Effect of PRGF on TSP-1, CTGF and procollagen type I secretion by human periodontal ligament cells, after 72 hours. *Statistically significant differences between PRGF and control ($p<0.05$).

3.2. Periodontal ligament and PRGF

The periodontal ligament (PDL) is a soft connective tissue located between the cementum and the alveolar bone and is derived from cells present in the dental follicle [81,110]. PDL cells are responsible for the formation and maintenance of the PDL but they also play an

important role in supporting teeth, in the tooth nutrition and in repairing and regenerating the adjacent damaged tissues, as they are characterized by a rapid turnover and a high capacity for renewal and repair [111-113].

The PDL contains a heterogeneous cell population including, fibroblasts, osteoblasts, cementoblasts, osteoclasts, macrophages, cell rest of Malassez and neural elements [4,73,113,114]. PDL also contains a population of multipotent postnatal stem cells capable of differentiating into adipocytes, chondrocytes, cementoblast-like cells and connective tissue rich in collagen type I [111,114-118]. PDL fibroblasts are predominant and they represent a heterogeneous population of cells with both connective and bone tissue properties [73,112,119]. They are also suitable for reprogramming to obtain iPS cells [112,120]. The assessment of the PRGF potential in this component of the periodontium is essential since gingival and periodontal ligament fibroblasts display a different cell phenotype [81,121].

Human primary periodontal ligament fibroblasts obtained from patients undergoing simple extractions of non-impacted wisdom teeth were used to evaluate the effect of PRGF [88]. As in the case of gingival fibroblasts, this autologous technology was also able to stimulate the biological processes of proliferation and migration of the PDL fibroblasts. In addition to this, the pro-angiogenic factor VEGF, the anti-angiogenic and anti-inflammatory factor TSP-1, the anti-inflammatory and antifibrotic factor HGF and the multifunctional factor CTGF, were increasingly synthesized in the presence of PRGF (Fig. 3C). The main ECM component of the periodontal ligament, collagen type I, was also stimulated by PRGF treatment (Fig. 3C). This molecule is involved in the development, physiological function and regeneration of the periodontium as it provides a scaffold for the periodontal ligament cells to attach, spread and proliferate [77,122]. The interaction between cells and extracellular matrix components modulates cell behavior and may influence the main cellular events of tissue regeneration. The integrins are a large family of transmembrane adhesion receptor heterodimers that are composed of an α and a β subunit. These proteins mediate cell-ECM and cell-cell adhesions. In addition to their role in cell adhesion, integrins are also involved in cell signaling events regulating processes such as cell differentiation, cell proliferation, cell motility and cell survival [91,123].

PRGF stimulates the adhesion of periodontal ligament fibroblasts to a collagen type I matrix (Fig. 1B) although reduces the expression of the α2 integrin in these cells [88]. Although the results appear to be contradictory, they are not. In the process of wound healing is imperative that cell adhesion occur as soon as possible to ensure that the rest of the processes also occur actively to maintain the dynamic state of the cell. However, this dynamic state also involves optimal adhesion levels. In fact, integrin-mediated adhesions are dynamic structures that must be created and disassembled during migration and cell division [124]. The cells cultured with PRGF are also in a constant movement to proliferate and migrate, while non-stimulated cells remain static, and so the α2 integrin expression might be greater in the last ones. In fact, cell adhesion is critical for cell migration to occur and it has been demonstrated that cell migration speed depends on the expression and activation levels of integrins [125-128]. It has been already reported that cell motility was inversely correlated to the expression of the collagen receptors α1 and α2 integrins [124,129].

Therefore, PRGF technology promotes a dynamic cell state that is essential for wound healing to happen.

3.3. Alveolar bone and PRGF

Alveolar bone, also known as the “alveolar process”, includes sockets, which are designed to accommodate the roots and lower part of the teeth. It consists of an external cortical plate of compact bone, a central cancellous trabecular bone, and bone lining the alveolus (alveolar bone proper) [75,130]. The cribiform structure of alveolar bone proper allows for a connection to the neurovascular structures.

The bone is constantly remodeled by the osteoblast and osteoclast activity. Collagen, glycoproteins and proteoglycans are synthesized by osteoblasts and then mineralized to produce the bone matrix. When these cells have layed down osseous tissues they become trapped within the tissue and are termed osteocytes [131]. Contrarily, the bone matrix resorption is achieved by the action of several osteoclast enzymes such as acid phosphatase, cathepsins, and matrix metalloproteinases. The physiologic bone remodeling process is controlled by the balance of the trimolecular complex composed of

osteoprotegerin (OPG), RANK (receptor activator of nuclear factor kappa beta), and RANKL (osteoprotegerin ligand). This trimolecular complex functions as receptors and ligands and belongs to the superfamily of tumor necrosis factor (TNF) [132]. Whereas this bone tissue remodelation process is the same as at other anatomical localization [133], alveolar bone is particularly fragile and labile and it is in constant state of change, since the replacement of old bone by new bone is a normal physiologic process. So, besides the remodelation, in response to some stimulus or physical force, alveolar bone may change in three-dimensional size or shape.

Bone defects are usually caused by pathological processes including extensive trauma and infection processes. In the dentistry clinical practice many cases of bone deficit requiring the use of reconstructive surgery are often found. It is therefore, a great number of strategies have been developed to achieve bone tissue in specific locations in addition to improve the periodontal tissue regeneration process. However, the vast majority exhibits risks and limitations that may compromise their efficacy [134]. The personalized autologous PRGF technology efficacy is evidenced in several medical fields [9,24] and so, we decided evaluate their potential to improve the alveolar bone tissue regeneration process. For this purpose, primary human alveolar bone cells (PHO) were isolated from patients undergoing oral surgery and characterized, and several cellular events that are implicated in the bone regeneration process were evaluated [89]. Three plasma preparations (platelet-poor plasma (PPP), F2+F3 fractions of platelet-rich plasma (PRGF F2+F3) and F3 fraction of platelet rich plasma (PRGF F3) were assayed on the bone cells positive for alkaline phosphatase (ALP) activity, osteocalcin and osteopontin markers.

After a dose-dependent proliferation assay, the 20 percentage of plasma preparation was chosen for the following experiments, since the highest proliferation values were obtained when PHO cells were treated with either 20% or 40% of the any of the platelet-rich plasma preparations and no significant differences were found between this two percentages (Fig. 1A).

Bone regeneration requires the osteoblastic activity directed by a complex network of growth factors (GFs) and proteins. In fact, osteoblast cells are sensitive to these biomolecules chemical gradients, are able to reach the site that demands repair and/or

remodeling and deposit the premineralized matrix. Some of these signaling GFs, including TGF-β1, PDGF, bFGF, BMPs, IGF, and VEGF [135,136], are constituents of PRGF.

Results from this experimental study confirmed that PRGF technology improved all main processes related to the bone tissue regeneration. The PHO cell treatment with any fraction of PRGF stimulated the proliferation, migration and chemotaxis (Fig. 2B) events and induced the synthesis of key angiogenic (VEGF and HGF) growth factors. Recently, an anti-inflammatory effect has been attributed to HGF due to the decrease of COX-1 and COX-2 proteins described after HGF and PRP treatments in *in vitro* and *in vivo* experiments on tendon tissue [65]. Results shown in this study are consistent with previous works using platelet-released supernatants. In this way, different research groups led by Creeper [137], Celotti [138] and Mooren [139], among others, already observed a positive effect of platelet-rich plasma on bone cell proliferation, migration and chemotaxis. On the other hand, a great stimulation of the synthesis of several extracellular matrix (EM) components (procollagen and osteocalcin) as well as a significant increase of the ALP activity were also detected in alveolar bone cells after the PRGF treatment (Fig. 3B). In the same way, an increment of certain markers of the osteoblastic activity supported by cocktails of platelet-released factors has been described [140-142].

Taking together all these findings, we could conclude that PRGF technology is consolidated as a safe therapy that promotes bone tissue regeneration.

4. Implant surfaces and PRGF

Implant-based oral therapies allow predictable and long-lasting solutions for an increasing number of dental reconstructions. Although other materials have been explored, titanium-based implants are today's gold standard since the discovery of titanium osseointegration, i.e. the capability of titanium to bind bone [143]. Research at the titanium oxide interface has allowed the production of increasingly preforming implants. The most established surface modifications concern its physicochemical characteristics [144,145] but more recently, researchers have investigated on surface modifications with elements of the extracellular matrix. Unlike the classical passive-integration approach, this new approach

seeks to promote an active interplay between the implant surface and the surrounding biomolecules and tissues [146].

Pioneering in this field, PRGF was employed to bioactivate implant surfaces [5]. The general effects of PRGF in bone-tissue have already been presented. Briefly, platelet activation leads to growth factor release, which increases the local concentration of pro-angiogenic and chemotactic signals. Secreted growth factors stimulate osteoblastic cell migration, proliferation and differentiation that become adhered to the polymerized fibrin matrix [89]. The lack of leukocytes in the PRGF preparation contributes to diminishing the inflammatory phase. PRGF is autologous, which levels the wide inter-subject variability and avoids over stimulation risks found with other growth factor approaches [147,148]. Two are the main biological mechanisms intervening at PRGF-bioactivated implant surfaces. First, platelets adsorption and activation by calcium agonists. Second, the formation of a surface-bound tridimensional fibrin network with embedded platelets.

Platelet activation with calcium and concomitant fibrin matrix formation at implant surfaces permits the sustained release of growth factors over time, establishing thereby a chemical gradient for osteogenic cells and other relevant biomolecules from the surfaces. The roughness of currently available implant surfaces permits the initial anchorage of the fibrin network and prevents its detachment upon retraction, assuring in this way the prevalence of the cell signaling molecules at the surface during physiological release and until complete fibrinolysis [149,150]. The ultimate degradation of the fibrin matrix puts an end to this process with the release of the matrix-attached growth factors.

The lifespan of this provisional matrix is thus a key step. Calcium activation leads to native thrombin formation, which suffices to trigger the fundamental steps of the coagulation cascade. The use of exogenous thrombin as an activator, in addition to calcium, has been investigated [18,151-153]. Activation with calcium alone is preferred because the addition of exogenous thrombin, usually from bovine origin, led to a less dense fibrin matrix and the inhibition of release of some growth factors.

The presence and state of platelets, as well as the formation of the fibrin network on titania translates into differences in the protein composition, conformation and orientation [154]. Therefore, cells interacting with these surfaces would receive very particular

biochemical and morphological stimuli, which may determine their functional fate. More importantly, knowing the initial composition of the interface we start to understand the mechanisms of regeneration and, possibly, gain control over its evolution.

Further research on these interfaces has led to the recent development of a chemically modified implant surface specially suited to activate the coagulation cascade at implant surfaces [155]. This modification consists in calcium ions, which not only trigger the aforementioned specific effects on PRGF but also establish electrostatic bridges between the negatively charged oxide surface and the acidic residues of many proteins involved in biomineralization [156]. Calcium modified surfaces induce as well an increased level of platelet adsorption and activation on titania [157].

Implants modified with calcium are capable of advancing osseointegration due to the multiple roles of calcium in bone-tissue regeneration, specially as a major component of the highly calcified osseointegration layer that binds the titanium oxide to bone [158]. Adding PRGF, we showed recently that osseointegration improved significantly even at 2 weeks post implantation in a rabbit femoral condyle model [159]. These results are in agreement with the modulation of the early stages of healing induced by activated PRGF. Briefly, calcium at implant surfaces promotes the adhesion and activation of platelets within the first seconds of PRGF-implant surface contact. Platelet activation and aggregation leads to a progressively more dense coating and the endogenously produced thrombin cleaves the fibrinogen fibrinopeptides to form fibrin. Fibrin stabilization takes place profiting from the platelet aggregates and at around 15 minutes the provisional matrix is completely formed and laterally stabilized at the implant surface. Implants bioactivated in this way have shown significant improvements in osseointegration in several animal and human models [16,155,160,161].

In summary, the combination of PRGF with chemically modified implants capable of triggering the coagulation cascade implies a new approach driven by the importance of regulating the early healing steps. In contrast to other approaches including exogenous biomolecules, this autologous approach circumvents immune-related risks and can be easily applied in daily practice to significantly improve the results of implant-based therapies.

5. Concluding remarks

Tissue engineering or regenerative medicine is an emerging multidisciplinary field that is revolutionizing the way we know today the concept of regeneration. In this sense, the use of PRGF technology for periodontal regeneration purpose represents a new approach to provide local concentrations of growth factors to stimulate wound healing and tissue regeneration. This therapy is able to positively stimulate the main stages of the regenerative process on each of the three main components of the periodontium. PRGF has been shown to be safe and effective in many different medical applications.

Nevertheless, further research is necessary to elucidate many molecular mechanisms and interactions that remain unresolved and that can improve its biological potential as well as provide new therapeutic indications.

Abbreviations

ANG-1: angiopoietin 1.
bFGF: basic fibroblast growth factor.
COX: cyclooxygenase.
CTAP-3: connective tissue activating peptide 3.
CTGF: connective tissue growth factor.
EGF: epidermal growth factor.
FGF: fibroblast growth factor.
FP-A: fibrinopeptide A.
FP-B: fibrinopeptide B .
HGF: hepatocyte growth factor.
IGF: insulin-like growth factor.
IκB-α:
IL-10: interleukin 10.
IL-6: interleukin 6.
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.
PBP: platelet basic protein.
PDGF: platelet-derived growth factor.
PDGF-BB: platelet-derived growth factor BB.
PF-4: platelet factor 4.
PGE_2: prostaglandin E2.
PMPs: platelet microbicidal proteins.
RANTES: regulated on activation in normal T cell expressed and secreted.
TGF-β: transforming growth factor beta.
TSP-1: thrombospondin-1.
Tβ-4: thymosin β-4.
VEGF: vascular endothelial growth factor.
VWF: von Willebrand factor.

Conflict of interest

The authors declare the following competing financial interest(s): EA is the Scientific Director and MT, MZ and RT are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF technology.

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(II) Progress in the use of dental pulp stem cells in regenerative medicine

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Progress in the use of dental pulp stem cells in regenerative medicine

Eduardo Anitua[1,2*], María Troya[1,2*#], Mar Zalduendo[1,2#]

1 BTI - Biotechnology Institute, Vitoria, Spain

2 University Institute for Regenerative Medicine and Oral Implantology - UIRMI (UPV/EHU-Fundación Eduardo Anitua)

# These authors contributed equally to this work.

* Corresponding authors:

Eduardo Anitua
eduardoanitua@eduardoanitua.com

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Cell culture, clinical applications, dental pulp stem cells, DPSCs, scaffold, tissue engineering.

ABSTRACT

The field of tissue engineering is emerging as a multidisciplinary area with a promising potential in order to regenerate new tissues and organs. This approach requires the involvement of three essential components: stem cells, scaffolds and growth factors. Up to date, dental pulp stem cells have received special attention because they represent a readily accessible source of stem cells. Their high plasticity and multipotential capacity to differentiate into a large array of tissues can be explained by its neural crest origin which supports applications beyond the scope of oral tissues. Many isolation, culture and cryopreservation protocols have been proposed which are known to affect cell phenotype, proliferation rate and differentiation capacity. The clinical applications of therapies based on dental pulp stem cells demand the development of new biomaterials suitable for regenerative purposes that can act as scaffolds to handle, carry and implant stem cells into patients. Currently, the development of xeno-free culture media is emerging as a standardization way of improving safe and reproducibility. The present review aims to

describe the current knowledge of dental pulp stem cells, going in depth into different key aspects related to the characterization, establishment, maintenance and cryopreservation of primary cultures and their involvement in the multilineage differentiation potential. The main clinical applications based on these stem cells and their combination with several biomaterials will also be deeply covered in this review.

INTRODUCTION

The general principles of tissue engineering involve three essential components, such as identification of appropriate cells, development of three-dimentional (3D) scaffolds, and inductive morphogenic signals to regenerate tissues and restore normal organ function [1,2]. In this framework, research is focusing on the use of stem cells as the starting point in tissue engineering.

Different stem cell types have been described in the literature so far including embryonic stem cells (ESCs), adult somatic stem cells (mesenchymal, haematopoietic and endothelial stem cells), and the induced pluripotent stem cells (iPSCs), artificially derived from adult differentiated somatic cells [3,4]. There is little controversy surrounding human adult stem cells as they are not associated with ethical concerns unlike embryonic stem cells. Stem cell therapy represents a promising tool with a focus on their therapeutic potential for regenerative medicine and other biomedical applications [2]. Bone marrow and adipose tissue are conventional sources of mesenchymal stem cells (MSCs), but the highly invasive cell collection protocols together with the considerable risk of donor site morbidity have promoted the search for alternative tissues [5,6]. Dental pulp stem cells (DPSCs), with an easier surgical access, are a great stem cell alternative. The non-invasiveness of DPSCs isolation methods compared to other adult tissue sources makes those cells to be a valuable source of MSCs for tissue repair and regeneration.

Dental pulp is located in the central pulp cavity of each tooth called “pulp chamber” and contains a heterogeneous population represented by fibroblasts, endothelial cells, neurons, odontoosteoprogenitors, inflammatory and immune cells [7,8]. DPSCs were first isolated in 2000 by Gronthos et al. [9] from the pulp tissue of third molars. Human DPSCs (hDPSCs) are ectodermal-derived stem cells that originate during tooth development from

ectodermal cells that migrate from the neural tube to the oral region and finally they differentiate into mesenchymal cells [7,8,10]. This feature confers them special biological properties of MSCs and neural crest stem cells. Therefore, dental pulp is enclosed into the dental cavity surrounded by mineralized dentin generating a kind of sealed niche that preserves it from environmental differentiation stimuli and keeps stem cells in the adult tissue [11]. These cells are responsible for the maintenance and repair of the periodontal tissue, they have a high proliferation rate, a low immunogenicity and exhibit plasticity for multi-lineage differentiation [7-9]. In fact, they are known to differentiate into various cell lineages such as osteoblasts, chondrocytes, adipocytes, odontoblasts, neural cells and myocytes among others [8,12,13].

In tissue engineering, the selection of a suitable scaffold and its interaction with stem cells is also critical. Biomaterials that act as a scaffold for tissue repair and reconstruction should be able to support cell adhesion and ingrowths while mimicking target tissue and supporting angiogenesis. These biodegradable 3D-scaffolds incorporate biological signals that allow them to act as a bioactive platform in order to precisely control stem cell behavior [14-17].

To the extent that tissue engineering industry is being established in the clinical setting, new and encouraging questions arise. Animal derived components have traditionally been widely used in stem cell cultures for over a century. However, it harbours some concerns for human cell therapy such as the risk of viral, bacterial, fungal and prion contamination and the possible induction of immune rejection of the transplanted cells into the host [3,18]. For these reasons, the development of xeno-free cell culture protocols able to properly expand stem cells without affecting their differentiation also represent a real challenge to generate cell-based products that meet good manufacturing standards [5,19].

The aim of this review is to summarize the current knowledge of dental pulp stem cells with special focus on the characterization, establishment, maintenance and cryopreservation of primary cultures and their involvement in the multilineage differentiation potential. The main clinical applications based on these stem cells together with the different scaffold biomaterials needed to achieve damaged tissue regeneration are also deeply covered in this review.

DENTAL PULP STEM CELLS vs. OTHER STEM CELLS

Stem cells (SCs) are the good cell candidates to be used in regenerative medicine due to their biological characteristics. SCs are undifferentiated cells that have the capacity of shelf-renewal besides the potential of differentiation into mature specialized cells [20]. In relation with their differentiation potential, SCs can be classified into totipotent pluripotent, multipotent and unipotent cells.

Multipotent adult stem cells (ASCs), also referred to as somatic stem cells, are found in quiescent and undifferentiated state in a great number of tissues. ASCs begin to self-renew as well as to differentiate into specialized cells after tisular damage or in homeostasis maintenance. ASCs utilization in tissue engineering techniques would optimize the response after transplantation since their autologous origin would avoid the expected rejection.

The best-known example of multipotent ASC is the hematopoietic stem cell (HSC) which can be readily harvested from bone marrow and umbilical cord blood (UCB) [21,22]. Moreover, MSCs can be isolated from a large variety of fetal and extraembrionic tissues and a great number of tissues from children and adults [23]. MSCs are capable of differentiating into various mesodermal cell lineages and their hypoimmunogenic and immunosuppressive characteristics confer them extremely suitability for even allogenic cell transplantation [24-26]. MSCs obtained from bone marrow (BMMSCs) have been widely used in experimental studies but due to the accompanying pain and morbidity in their obtaining process, alternative sources are being sought, including the placenta, the human umbilical cord and the amniotic fluid. In this sense, DPSCs are becoming a successful and promising source of stem cells for tissue engineering therapies due to their low cost and great accessibility versus the costly and invasive techniques required for other ASCs isolation. Thus, DPSCs can be obtained without adverse effects on the health of pulp tissue of permanent teeth, habitually from third molars, supernumerary or orthodontically unnecessary teeth [7,27,28].

Dental pulp is derived from ectodermal cells that grow at the periphery of the neural tube and after migrating to the oral region differentiate into cells of the mesenchymal phenotype, therefore some authors have described them as “ectomesenchyma” [11]. Due

to its origin, stem cells derived from dental pulp can differentiate into mesodermal and non-mesodermal tissue cells among which osteoblasts, adipocytes, chondrocytes and myocytes, as well as neuronal, endothelial cells, hepatocytes and melanocytes are included [29-33]. Furthermore, DPSCs do not express the major histocompatibility complex class II antigen on their surface and holds immunoregulatory properties being able to induce activated T-cell apoptosis [25,34]. Several soluble factors and cytokines secreted by DPSCs could be immunomodulator candidates among which prostaglandin E2, transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), interleukin 6 (IL-6), interleukin 10 (IL-10), nitric oxide and Fas ligand could be included [35,36]. These immunomodulatory factors may have a profound effect on clinical cell therapy by T-lymphocyte function inhibition and up-regulation of T cell regulatory stimulating immune tolerance.

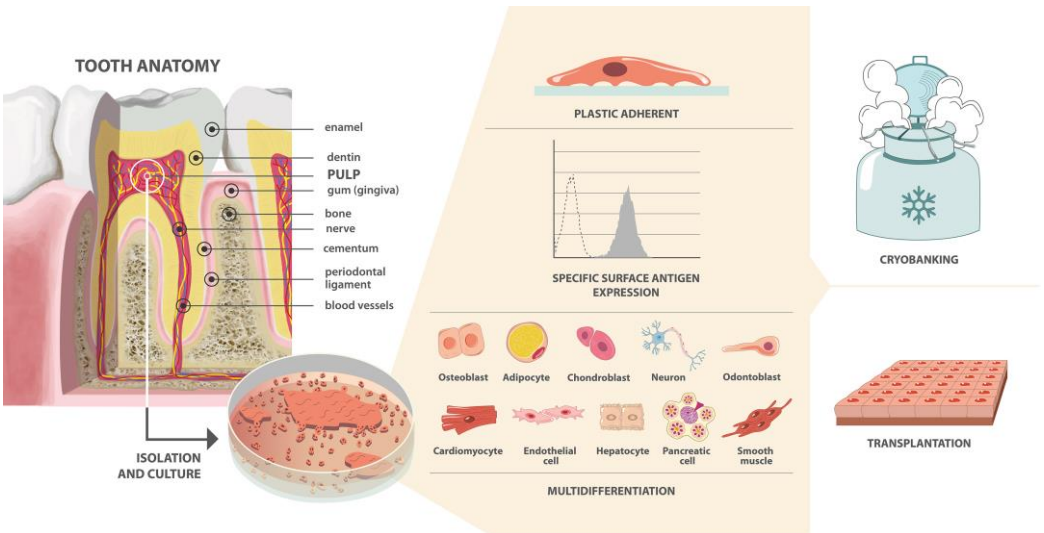

Figure 1. Schematic representation of specific characteristics of DPSCs.

ISOLATION, CULTURE AND CRYOPRESERVATION OF DENTAL PULP STEM CELLS

In 2006, The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed three minimal criteria to define human MSCs

(hMSCs) [37]. These criteria, defined for research purposes, involved adherence to plastic, specific surface antigen expression and multipotent differentiation potential (Fig. 1). Moreover, stem cells exhibit a great clonogenic capacity and proliferation potential.

Several investigators have described various methods for stem cells isolation from human dental pulp obtained from adult teeth. The two most common protocols are enzymatic dissociation and explants method. In the first case, after accessing the pulp tissue, an enzymatic cocktail is used to digest tissue fragments and to obtain a unicellular solution which will be seeded in culture flasks. In this protocol, several enzymatic formulations and approaches for digestion have been described [38,39]. Using the explant method or outgrowth, 1-2 mm^3 of pulp tissue fragments are directly placed in culture flasks and cells grow out after naturally dissociating themselves from the pulp [40]. There is no consensus on whether one isolation protocol is more successful than the other. Moreover, several authors suggest that both the enzymatic digestion and the outgrowth method could be applied to obtain a suitable DPSC population with no significant differences [41,42]. However, the explant method is more in accordance with Good Manufacturing Practice (GMP) as no additional enzymes are required and thus less manipulation and lower cost is associated [5]. Other less commonly used isolation methodologies should also be considered such as diverse combinations of the two aforementioned protocols [43] and immunoselection methods [44] among others. In addition, some techniques for obtaining a greater enrichment on stem cells have been also used [45].

Although a comprehensive review of DPSC isolation by tooth type has not been performed, the age of donor was found to be a significant factor [15,46,47]. Recently, Wu W. et al. conducted an experimental work comparing DPSC lines derived from children, adolescents, adults and elderly donors [48]. Association among isolation efficacy, increase in duplication time and the greater number of apoptotic cells with the propagation have been described in cultures derived from aged donors. Moreover, DPSCs derived from aged teeth displayed a decrease on their differentiation capacity.

As for the culture method, several studies have been carried out in order to optimize the process to obtain a sufficient number of DPSCs for regenerative medicine therapy. Different culture medium formulations have been tested including several basal media and

growth supplements and also chemically defined media too [49]. Up to now, fetal bovine serum (FBS) has been used as the gold standard for cell culture. However, several concerns related to safety risks issues and batches variation have led to explore different alternatives to meet regulatory and good manufacturing criteria [5,19]. As a consequence, many studies have investigated the possibility to isolate, expand and differentiate hDPSCs in serum-free culture media [18,49,50]. In fact, human blood products, such as platelet lysate, human serum or platelet-rich plasma, have been postulated as potential alternatives to FBS in the culture of DPSCs [51,52] and other MSCs [53-56]. In addition to this, the variability of other factors such as the substrate area, the density of cell seeding and the physical-chemical environment may impact on the heterogeneity of DPSCs described in the literature [57,58]. Regarding the chemical microenvironment of cell culture, it should not be forgotten that the tooth O_2 physiological levels ranged from 3% to 6% [7]. On the contrary, the ambient oxygen tension in conventional cell-cultures ranges from 18% to 21%. It has been hypothesized that the presence of low oxygen tension in stem cell niches offers advantages related with undergoing a lower degree of oxidative stress. In addition, the activation of molecular pathways such as Oct-4 and Notch signaling, which are involved in the stemness regulation, has been shown in hypoxia [59,60]. Furthermore, several studies have described a significantly increased in *in vitro* proliferative lifespan and a decrease in the differentiation capacity of MSCs by regulating hypoxia-inducible factor 1 mediated expression of different genes [61,62]. Recently, several authors have described that hypoxia delayed phenotypic changes in MSCs associated to long-term culture without altering their immunophenotypic features [63,64]. Bressan Werle et al. reviewed the hypoxia effects on DPSCs biological characteristics and described that increased proliferation potential was not observed in these cells unlike other stem cells [65]. Nevertheless, oxygen tensions used in the evaluated studies are considered as severe hypoxia or short course assays, so interpretation deserves caution as these parameters could affect the outcomes. On the other hand, few studies have investigated the effect of hypoxia on DPSCs' differentiation potential and stemness marker expression.

An important requirement for stem cell therapy is the considerable *in vitro* expansion required to obtain the enough number of cells for therapeutic use. For this purpose, stem

cell culture during long time becomes necessary. Cellular senescence represents a crucial role in lifespan-limitation since it is usually accompanied by altered cellular behavior. Senescence is characterized by a reduced proliferation potency, telomere shortening, changes in morphology and expression of senescence-associated genes such as senescence-associated β-galactosidase [66,67]. Hence, some researchers have reported different *in vitro* strategies to achieve hMSC culture improvements [68].

Finally, a reliable and effective storage method is required to preserve viable and functional DPSCs for future clinical applications. Due to the fact that somatic stem cells show a significant reduction in their stemness potential with increasing donor age, and that DPSCs can be obtained by minimally invasive process from routinely discarded biological tissues, the establishment of DPSC biobanks represents a challenging therapeutic strategy for the storage of samples for future applications [69]. Furthermore, the low immunogenicity and immunosuppression exhibited by DPSCs leads to believe that even allogenic biobanks would provide an excellent therapeutic approach after overcoming the corresponding legal barriers [70]. Cryopreservation of either DPSCs cultures or whole teeth has been performed [71,72]. However, a very limited number of studies regarding the optimization of cryopreservation method have been carried out. In this sense, Woods et al. assessed three cryoprotective agents using different final concentrations [72] and determined that dimethyl sulfate (Me_2SO) at a concentration between 1 and 1.5M was the best cryopreservative. Another proposed protocol is the magnetic freezing which uses a magnetic field and ensures a low temperature distribution without freezing occurring [73]. In any case, the optimal cryopreservation method should preserve the stemness characteristics of DPSCs in addition to the highest viability while following the GMP standards.

MOLECULAR MARKERS OF DENTAL PULP STEM CELLS

According to the ISCT, most MSCs (≥ 95%) should positively express CD105 (endoglin), CD73 (5'-ectonucleotidase) and CD90 (Thy-1) but do not express (≤ 2%) CD45 (a pan-leukocyte marker), CD34 (marks primitive hematopoietic progenitors and endothelial cells), CD14 or CD11b (both antigens prominently expressed on monocytes and macrophages),

CD79α or CD19 (markers of B cells) and HLA-DR (human leukocyte antigen) surface antigens [37].

DPSC populations have been also characterized by the expression of cell surface antigens using antibody-based methods [74,75] and following the standard criteria established for the ISCT. There is no distinct specific marker for identifying DPSCs. In fact, DPSC populations are heterogeneous and consist of mixed subpopulations with different phenotypic and biological properties [44,74]. The marker expression profiles differ among studies and not all the DPSCs express all markers described neither in the same percentage [7]. This heterogeneity of the dental pulp stem cell population is partly responsible for the high diversity of the antigen expression displayed. However, cell culture conditions and medium composition may interfere with the cell surface expression pattern. For example, the percentage of serum used may condition the stem cells proteome and their differentiation potential [7,27,58,75,76].

Aside from the specific antigen expression proposed by the ISCT, numerous other surface markers have been studied in the characterization of DPSCs (Table 1). Several authors have reported the expression of a range of other mesenchymal stem cells markers including CD13 (aminopeptidase N), CD29 (integrin beta-1 or fibronectin receptor beta subunit), CD44 (an integral cell membrane glycoprotein), CD146 (the melanoma cell adhesion molecule) and CD166 (activated-leucocyte cell adhesion molecule) [29,74,76-79]. The expression of the stemness factors OCT3/4 (octamer binding transcription factor-3/4), SSEA4 (Stage-Specific Embryonic Antigen-4) and NANOG (homeobox transcription factor) was also found in DPSCs [13,78,80-82]. Furthermore, expression of nestin, β-III tubulin, S100, Notch1, CD271 and synaptophysin, all of them specific neural markers, has also been reported [13,76,83]. Niehage et al [75] identified, by label-free mass spectrometry, new cell surface proteins from the DPSC proteome. Tumor necrosis factor receptor superfamily proteins (CD40, CD120a, CD261, CD262, CD264, and CD266), some integrins (alpha-4, alpha-6, and alpha-10), and interleukin receptors (CD121a, CD130, CD213a1, CD217, and CDw210b) were some of these new identified proteins. DPSCs are also negative for the following hematopoietic markers: CD117 (mast/stem cell growth factor receptor), CD133 (prominin 1), CD34 (hematopoietic progenitor cell antigen) and CD45 (protein tyrosine phosphatase, receptor type, C) [7,29,75,78,84,85].

ISCT	Mesenchymal	Stemness	Neural	Others
CD73	CD13	OCT3/4	Nestin	CD40
CD90	CD29	SSEA4	β-III tubulin	CD120a
CD105	CD44	NANOG	S100	CD261
	CD146		Notch1	CD262
	CD166		CD271	CD264
			Synaptophysin	CD266
				Integrin alpha-4
				Integrin alpha-6
				Integrin alpha-10
				CD121a
				CD130
				CD213a1
				CD217
				CDw210b

Table 1. Summary of the positive markers for DPSCs.

MULTILINEAGE DIFFERENTIATION

The classical application for stem cells is based on their long-term self-renewal and the ability to differentiate into new mature specialized cells to facilitate replacement and regeneration of tissues. In particular, the ISCT propose as a third minimal criteria to define hMSC that must differentiate *in vitro* into osteoblasts, adipocytes and chondroblasts [37]. Human dental pulp stem cells show multilineage differentiation even in other directions than those minimally established by the ISCT (Table 2).

The osteogenic potential of DPSCs has been well documented in several studies. Osteogenic differentiation has been extensively induced by dexamethasone, L-ascorbic acid and β-glycerol phosphate supplementation [86-90]. Alizarin red S staining usually allows to confirm the matrix mineralization and calcium deposition after induction. To further verify osteogenesis, the expression of bone-specific proteins such as alkaline phosphatase, collagen type I, osteocalcin, osteonectin, osteopontin, osterix and runt-related transcription factor 2 (RUNX2) has also been confirmed [50,86,87,91-94]. Runx2 is a highly conserved transcription factor known as one of the most important regulator of osteoblast and odontoblast differentiation [95,96]. Expression of Runx2 activates

osteoblast-specific genes during the early stages of osteoblast differentiation and initiates mineralization [96,97]. DPSCs can also differentiate into odontoblasts to further regenerate pulp-dentin complex tissues. Odontoblastic differentiation induction is performed by the same osteoblastic inducers and as previously mentioned. The transcription factor Runx2 also control odontoblast differentiation by activating the dentin specific gene, dentin sialophosphoprotein (DSPP), responsible for encoding dentin sialoprotein and dentin phosphoprotein [98]. Mineral deposition, increase alkaline phosphatase activity and upregulated expression of DSPP, dentin matrix protein 1 (DMP1), bone sialoprotein and osteocalcin have also demonstrated the ability of DPSCs to differentiate into odontoblasts [98-101].

DPSC have also been reported to differentiate into adipogenic cell lineage. Insulin, dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine (IBMX) have been used to induce the differentiation [91,102-104]. Cells that undergo adipogenesis are positive for the Oil Red O staining for lipid droplets. The adipogenic phenotype is usually further confirmed by the expression of several adipogenic markers such as peroxisome proliferator-activated receptor γ, glucose transporter type 4, fatty acid binding protein 4 and lipoprotein lipase [42,94,102,103,105,106].

Several studies have reported the ability of DPSCs to differentiate into chondroblasts. The acquirement of a chondrogenic lineage phenotype is mediated by several compounds including ITS (insulin, transferrin and selenium), dexamethasone, L-ascorbic acid, L-proline and sodium pyruvate [18,42,107,108]. Additional transforming growth factor-β3 (TGF-β3) is used for chondrogenic induction [42,47,103,108]. Proteins of the TGF-β superfamily play a remarkable role in all phases of chondrogenesis (condensation, proliferation, extracellular matrix deposition and finally terminal differentiation). These proteins influence the processes of differentiation and de-differentiation in the cartilage [109-111]. Glycosaminoglycans are detected by alcian blue, safranin O or toluidine blue staining. Induced DPSCs express other chondrogenic lineage specific proteins such as aggrecan, SRY (sex determining region Y)-box 9, type II collagen and type X collagen [18,91,102,107].

Given their neural crest origin, DPSCs show a significant neuro-regenerative potential. They have been shown to differentiate into multiple neural crest-lineage cell types, such as neuron-like cells, dopaminergic neurons, oligodendrocytes and Schwann cells [112-115]. A wide range of neural differentiation protocols are available, without a clear consensus about the ideal neural induction approach. These procedures involve different factors and supplements according to differentiation phases. Most proteins used to induce neuronal differentiation include B27 supplement, epidermal growth factor and basic fibroblast growth factor [113,116,117]. Neurogenic maduration is achieved through the addition of nerve growth factor (NGF), neurotrophin-3 (NT-3), cyclic adenosine monophosphate, retinoic acid, IBMX, forskolin, sonic hedgehog and brain-derived neurotrophic factor (BDNF) among others [113,116,118,119]. Successful differentiation is evaluated by the expression of several neural markers including neuronal nuclei, microtubule associated protein 2, neural cell adhesion molecule, growth associated protein 43, glial fibrillary acid protein, synapsin I, neuron specific class III beta-tubulin, myelin basic protein, neuronal differentiation 1, paired box protein 6 and sex determining region Y-box 1 and 2 [113,115,118,119]. Functional neural phenotype has also been confirmed by the patch-clamp analysis of the voltage-dependent sodium and potassium channels [113,118]. Many authors have emphasized the role of DPSCs' secretome. A wide variety of neurotrophic factors like NGF, NT-3, BDNF and glial cell line-derived neurotrophic factor have been reported to be secreted from DPSCs [114,120,121]. These neurotrophic factors play an important role in neuronal growth, survival and differentiation. Thus, the secretion of these pivotal factors suggests that DPSCs exert their neurogenerative and neuropotective properties through a paracrine mechanism.

The plasticity of DPSCs allows them to differentiate towards other directions. Hepatic differentiation of human DPSCs has been successfully demonstrated by the expression of several hepato-specific markers, albumin and cytokeratin 18 among others [32,117,122]. Positive expression of the platelet endothelial cell adhesion molecule (CD31) and the von-Willebrand factor has shown the potential of DPSCs to differentiate into endothelial cells [123,124]. The ability of DPSCs to differentiate into cardiomyocytes [117], pancreatic cell lineage [125] and bladder smooth muscle cells [126] has also been reported.

Recently, great efforts are being made in order to improve the therapeutic potential of stem cells and to optimize MSCs-based tissue engineering methods. For example, the recent progress in molecular biology has allowed pluripotent stem cells differentiation to be modulated via genetic manipulation. In this sense, the genetic engineering option has contributed to enhance DPSC osteogenic and odontoblastic differentiation [127-129]. Furthermore, implementation of this gene delivery strategy might contribute to personalized medicine by adapting to the specific needs of each patient, as long as safe and effective protocols are developed.

	Inducers	Confirmed by
Osteogenic differentiation	Dexamethasone L-ascorbic acid β-glycerol phosphate	Alizarin red S Alkaline phosphatase Collagen type I Osteocalcin Osteonectin Osteopontin Osterix RUNX2
Odontoblastic differentiation	Dexamethasone L-ascorbic acid β-glycerol phosphate	RUNX2 Alkaline phosphatase Dentin sialophosphoprotein Dentin sialoprotein Dentin phosphoprotein Dentin matrix protein 1 Bone sialoprotein Osteocalcin
Adipogenic differentiation	Insulin Dexamethasone Indomethacin 3-isobutyl-1-methylxanthine	Oil Red O Peroxisome proliferator-activated receptor γ Glucose transporter type 4 Fatty acid binding protein 4 Lipoprotein lipase
Chondrogenic differentiation	ITS Dexamethasone L-ascorbic acid L-proline Sodium pyruvate	Alcian blue Safranin O Toluidine blue Aggrecan SRY

	TGF-β 3	Type II collagen
		Type X collagen
Neural differentiation	B27 supplement	Neuronal nuclei
	EGF	Microtubule associated protein 2
	bFGF	Neural cell adhesion molecule
	NGF	Growth associated protein 43
	NT-3	Glial fibrillary acid protein
	Cyclic adenosine monophosphate	Synapsin I
	Retinoic acid	Neuron specific class III beta-tubulin
	3-isobutyl-1-methylxanthine	Myelin basic protein
	Forskolin	Neuronal differentiation 1
	Sonic hedgehog	Paired box protein 6
	BDNF	Sex determining region Y-box 1
		Sex determining region Y-box 2

Table 2. Summary of the main DPSCs' differentiations.

DENTAL PULP STEM CELLS AND SCAFFOLDS

The use of 3D scaffolds is key when implementing tissue-engineered approaches. The role of an ideal scaffold is not limited to a mechanical bioinert platform for cell support but it extends to a bioactive and dynamic environment that regulates cellular functions and intracellular communication in order to replicate the native extracellular matrix (ECM) [130]. Scaffolds must be specific for each tissue to be regenerated. Appropriate selection of the biomaterial components is vital in determining cellular response and fate. Nevertheless, there are several common features for most of them. The ideal scaffold must be biocompatible to avoid inflammatory responses and the byproducts should be non-toxic. The biomaterial must be biodegradable and the biodegradation rate should coincide with the regeneration rate of the new tissue being formed. Ideally, the scaffold should have mechanical properties according to those of the tissue to be regenerated and the pore architecture should also be suited to promote cell migration and proliferation, as well as, vascularization to allow diffusion of nutrients, oxygen and wastes. In addition, surface modification enables scaffolds to immobilize several biological active molecules

such as ECM-derived peptides, growth factors and polysaccharides to improve biocompatibility and cellular function. [131-135].

Scaffold composition

Polymers

Natural

Natural polymers such as collagen, chitosan, elastin, fibrin, alginates, hyaluronic acid and amniotic membrane are biologically active and exhibit really good biocompatibility properties. However, these compounds may display poor mechanical properties and difficulty to control the degradation rate [136-141].

Synthetic

Several synthetic polymers have been used including polylactic acid (PLA), polyglycolic acid (PGA), polyethylene glycol (PEG), and polycaprolactone (PCL) among others. This type of polymers are versatile, more controllable and predictable but with a reduced bioactivity [131,137,142,143].

Besides this classification, during the last decades, the use of an important class of water-swollen, cross-linked polymeric forms known as hydrogels has significantly expanded due to their high resemblance of living soft tissues. Hydrogels can be produced by natural, synthetic or hybrid polymers. Bioactive hydrogels are obtained by the incorporation of several ECM-derived molecules that favors protein binding and the subsequent signaling pathway in order to achieve a complete biomimetic ECM [132,144,145]. Collagen fibers are one of the most popular natural polymer-based hydrogel [146,147]. PEG, in turn, is the most widely used synthetic polymer to form hydrogels due to its versatility, low toxicity and limited immunogenicity [148-150].

Ceramics

Ceramic scaffolds such as hydroxyapatite (HA), tricalcium phosphate (TCP), biphasic calcium phosphate (BCP), calcium silicate and bioactive glass are widely used for bone

regeneration. They are chemically and structurally similar to the native bone, with high mechanical stiffness and resistance to deformation [131,136,151,152].

Composites

Hybrid composites are one of the most promising biomaterials. They consist of a combination of several materials (co-polymers, polymer-polymer blends or polymer-ceramic composites) to achieve a more functional scaffold, overcoming the limitations of any of the constituents alone [136,153-156]

Techniques for scaffolds fabrication

In the last decade, several methods have been developed for the fabrication of 3D scaffolds. They can be divided into two main categories: conventional techniques and rapid prototyping.

Conventional techniques

The available technologies within this group include electrospinning, freeze-drying, solvent casting and particulate leaching, gas foaming, melt molding and phase separation. These conventional techniques do not allow an exhaustive control either of the porosity or the internal architecture of the scaffold. In addition, the presence of organic solvents in the manufacturing process limits the scaffold biocompatibility [134,136,157].

Rapid Prototyping techniques

Rapid prototyping techniques have been recently developed to overcome the limitations of the conventional ones. They are also known as solid free form fabrication or additive manufacturing and include fused deposition modeling, selective laser sintering, stereolithography, 3D printing and bioprinting. Scaffolds are achieved by deposition of overlying layers. These techniques require the use of computer model such as computer-aided design and manufacturing (CAD/CAM), computed tomography (CT) and magnetic resonance imaging (MRI). These methods enable a better control of the architecture, mechanical properties and biocompatibility of the scaffold [134,136,158-160].

In particular, bioprinting or direct cell writing is a novel emerging technology that allows printing of living cells in order to create 3D structures with or without scaffold support, that is, bioprinting enables fabrication of devices from bioinks [161]. Cell-laden hydrogels, decellularized extracellular matrix and cell aggregates are the main types of bioinks used in bioprinting technology [162,163]. According to its working mechanism, bioprinting is based on inkjet, laser or extrusion techniques [164]. This promising technology provides highly complex, customized and tailor-made constructs with the suitable properties to restore tissue and organ biological function [165-168].

Scaffolds for 3D cultures of DPSCs

Tissue engineering strategies require the interaction among stem cells, morphogens and scaffolds to develop functional tissue products resembling the native niche. Up to date, numerous biomaterials have been used in combination with different stem cells for that purpose and dental pulp stem cells, as a source of valuable stem cells, have been no exception.

Only in the last couple of years several biomatrices including fibroin-based biomaterials [169], poly(lactic-co-glycolic acid) microscaffolds [90], self-assembling peptide hydrogels [170], polyethylene glycol diacrylate-hyaluronan-gelatin hydrogels [148], chitosan-collagen calcium-aluminate biomembrane [171] or chitosan-intercalated organic montmorillonite/poly(vinyl alcohol) fibrous scaffolds [172] have been studied *in vitro* in combination with DPSCs. These combinations have shown to support cell viability, biocompatibility and differentiation as well as to offer promising tools for neural tissue engineering applications, and bone and dentin successful regeneration.

In addition to this, many studies have also been performed to determine the *in vivo* performance of several bioengineered constructs of DPSCs and different biomaterials. In this sense, Atalayin et al. [173] used three scaffolds (copolymer of L-lactide and DL-lactide (PLDL), copolymer of DL-lactide (PDL) and HA/TCP) to transplant hDPSCs into the backs of mice in order to evaluate the odontogenic regeneration. Regarding the enamel-dentin transcript expressions, DSPP, DMP1 and phosphate regulating endopeptidase homolog X-linked, the three materials enhanced the odontogenic matrix formation. In another recent work, gelatin methacrylate (GelMA)-encapsulated hDPSCs

were described as a relevant application to regenerate pulp-like tissues in root segments [174]. Root segments injected with GelMA-encapsulated hDPSCs revealed an increase collagen deposition, a highly cellularized pulp-like tissue, cell attachment to the inner dentin surface and cellular extensions into the dentin tubules. Chamieh et al [139] demonstrated the potential of DPSC-loaded-dense collagen gel scaffolds to improve the bone healing process in craniofacial bone defects. The authors reported an increase in bone density, matrix turnover and type I collagen, ALP and tartrate-resistant acid phosphatase expression when DPSC-seeded scaffolds were used in rat calvarial critical-size defect model. In another study in a rabbit calvarial model [175], hDPSCs combined with modified melt stretching and multilayer deposition (mMSMD) PCL–BCP scaffolds enhanced bone regeneration. This combination revealed an excellent osteogenic differentiation thus enhancing the formation of bone-like tissue, instead of dentin like tissue. These recent examples demonstrate the regenerative potential of DPSCs and bioactive platforms combination.

DENTAL PULP STEM CELLS POTENTIAL IN TISSUE ENGINEERING

Langer and Vacanti defined tissue engineering as an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [176]. In this sense, the creation of a medical device requires accessible sources of cells and biocompatible materials that can also carry signaling molecules. DPSCs results in a specific cell type that could contribute to the regeneration of numerous tissues due to the peculiar development pathway that give rise to the dental pulp; therefore, it could be applied in several medical fields (Fig. 2 and Table 3).

Oral and maxillofacial defects

Maintenance of dental pulp function is critical for the teeth homeostasis, thus root canal therapy, which becomes necessary after infection of pulp by caries or trauma, could lead to the loss of the dental piece. In early studies, Gronthos et al. demonstrated that transplantation of *in vitro* expanded DPSCs formed a dentin-pulp complex [9,177]. The tissue regeneration capability of DPSCs was further examined by transplantation using

human dentin as a carrier [178] where they generated a reparative dentin-like structure directly on the surface of human dentin. These results showed the possibility of using DPSCs in tooth repair by *in vivo* transplantation of stem cells or *in vitro* culture on biodegradable scaffolds and subsequent transplantation *in vivo*. A stimulated reparative dentin formation was achieved by applying a 3D pellet culture system of pulp cells with growth/differentiation factors. Moreover, Nakashima's group demonstrated complete *in situ* pulp regeneration with neurogenesis and vasculogenesis in an adult canine model of pulpectomy after autogenous transplantation of DPSCs with stromal cell-derived factor-1 [179]. Similarly, Iohara et al. achieved regenerated pulp tissue after autologous transplantation of DPSCs in combination with granulocyte-colony stimulating [180]. Recently, safety, potential efficacy and feasibility of human DPSCs autologous transplantation in pulpectomized teeth were assessed by Nakashima et al. in a pilot clinical study [181]. No adverse events or toxicity were demonstrated and complete pulp regeneration was achieved after 24 weeks in the five patients studied.

In the case of entire pulp tissue lost, numerous material platforms have been tested. In this sense, Galler et al. evaluated the proliferation and differentiation of DPSCs after their transplantation into PEGylated fibrin scaffolds [182]. Elongated and fibroblast-like DPSCs produced collagen and showed some increased expression of proteins that are essential for development of hard tissues as the teeth. In another approach, Qu and Liu designed nano-fibrous gelatin-silica bioactive glass (NF-gelatin-SBG) hybrid scaffolds that mimicked the nano-structured architecture and chemical composition of natural ECM. The SBG-induced expression of several odontogenic differentiation markers of hDPSCs resulted significantly higher in these hybrid scaffolds, with ECM secretion and mineral deposition [183]. More recently, Ravindran et al. designed ECM scaffolds obtained by lysing DPSCs that were cultured in collagen/chitosan hydrogel matrices [184]. The subcutaneous implantation of DPSCs resulted in the formation of dental pulp-like tissue with dentin markers expression. Subcutaneous transplantation of predifferentiated DPSCs with odontogenic/cementogenic medium resulted in hard tissue closer to dentin generation [92].

Several investigations have demonstrated the importance of a direct vascular supply to achieve the novo pulp regeneration. For example, Kodonas et al. seeded porcine DPSCs

onto collagen or poly (DL-lactic acid-co-glycolic acid) and implanted these constructs into the jawbones of adult minipigs [185]. Even though newly formed organic matrix of pre-dentin was deposited on the root canal walls, necrotic tissue at the central area of some implant was observed.

Successful reconstruction of bony defect is a difficult task in craniofacial surgery. The gold standard treatment consists in the autogenous bone utilization alone or in combination with other autologous biomaterials [186,187]. However, the ectomesenchymal origin of DPSCs gives them an osteogenic differentiation profile resulting in an emerging alternative option. Giuliani et al. [188] seeded human DPSCs onto a collagen sponge scaffold and assessed the stability and quality of the regenerated bone and vessel network 3 years after the grafting intervention. Fully compact bone with high matrix density was described in the regenerated tissue from the graft sites. This result could mean an implant stability increase or improvement in the response to mechanical and chemical agents. Other constructs of DPSCs and scaffolds have been assayed for calvarial defects in animal models with successfully generation of bone as already mentioned in the "Dental pulp stem cells and scaffolds" section [139,175]. Consequently, it could be stated that an alternative option to bone tissue engineering has emerged.

Neural regeneration

Numerous neurological disorders such as Alzheimer's disease, Parkinson's disease, neurodegenerative disease, stroke, spinal cord injury and peripheral nerve injury are characterized by the degeneration of neurons and consequently loss of their functions. As previously mentioned, hDPSCs derive from the neural crest, therefore they exhibit high expression of several neural crest developmental genes and neural crest-related genes. In the last years, several authors have demonstrated the neural differentiation potential of hDPSCs in *in vitro* studies using different approaches and methods. Regarding Parkinson's disease, a chronic neurodegenerative disease which is caused by the loss of dopaminergic neurons in the substantia nigra, the potential of hDPSCs to differentiate into dopaminergic neurons under appropriate conditions have been evaluated [189,190]. In 2014, Chang et al. reported an arrested proliferation and the acquisition of a phenotype resembling mature

neurons of a greater proportion of DPSCs after neuronal differentiation, with high expression of nestin and other mature neuron cells markers like βIII-tub.

On the other hand, Schwann cells play a major role in the regeneration axon of peripheral nervous system. In this sense, DPSCs cultured under particular conditions resulted in stem cell population expressing nestin, CD271 and SOX10, which are well-known markers for neural crest cells [191]. After four weeks of glial differentiation induction, differentiated stem cells were thin, bipolar and spindle shaped and express S100β.

Recently, the ability of single cell-derived clonal cultures of murine DPSCs to differentiate *in vitro* into immature neuronal-like and oligodendrocyte-like cells has been reported [192]. In this study, several clones of DPSCs were isolated and differences in the expression of early stage neural markers were identified. Young et al. stated that differentiation into immature neuronal-like cells was achieved only from clones with high levels of nestin expression.

The neuroregenerative potential of DPSCs has been also validated by several *in vivo* studies. For example, Askari et al. took a step forward in looking for new strategies in the repair of different demyelination diseases [112]. In their work, isolated human DPSCs were transfected with the human Olig2 gene as a differentiation inducer for oligodendrogenic pathway and later, they were transplanted into a mouse model with local sciatic damage. 2-6 weeks after transplantation, recovery of behavioral reflexes was achieved. In 2015, Spyridopoulus work team described the first evidence of the therapeutic potential of DPSCs in the peripheral nerve regeneration when, after the injection of DPSCs, the morphological and functional recovery of transected intercostals nerves was achieved [193].

Regarding spinal cord injuries, Zhang et al. designed chitosan scaffolds that significantly induced the increase of BDNF, glial cell line-derived neurotrophic factor, NGF-β and NT-3 [194]. Marked recovery of hind-limb locomotor functions was also achieved after transplantation of DPSCs together with chitosan scaffolds in a rat model with a spinal cord injury. Moreover, survival of transplanted DPSCs expressing nestin and Sox2 markers within the normal brain and injured spinal cord of rats was described [119]. In other preliminary studies, improvement of hypoxic-ischemic brain damage after intraventricular

injection of DPSCs in neonatal rats was described by Fang et al. [195]. Transcranial magnetic stimulation is an alternative therapy which is utilized in several neurological disorders. Recently, a study about the appropriate intensity to promote a favorable environment for DPSCs implantation, proliferation and differentiation was conducted by More et al. [196].

Treatment of ischemic disease and angiogenesis

Vasculogenesis is a potential treatment for ischemic disease. Vessel development consists in very complex and dynamic processes including basement membrane and ECM degradation, endothelial cell proliferation and migration and tube formation and maturation into functional blood vessels. The potential of DPSCs sub-populations for the treatment of myocardial infarction and ischemia have been evaluated in several studies.

Already in 2008, Gandia et al. evaluated the capacity of DPSCs in cardiac repair in a rat model with induced myocardial infarction [197]. Four weeks after the intramyocardially injection of stem cells, the animals showed an improvement in cardiac function correlated with a reduction in infarct size and greater angiogenesis. Even more, cardiac-specific markers expression was achieved after co-culture of DPSCs with neonatal rat cardiomyocytes [198].

The secretion of numerous pro- and anti-angiogenic factors, such as vascular endothelial growth factor, chemotactic protein-1, plasminogen activator inhibitor-1 and endostatin, has been described in cultures of DPSCs in absence of any differentiating stimulus [199]. Furthermore, DPSCs were able to significantly induce endothelial cells migration *in vitro* and blood vessel formation *in vivo*, being their angiogenic capacity higher when compared to that of bone marrow and adipose tissue derived cells [200,201]. Aksel et al. recently showed the vascular network fast formation by human and swine DPSCs seeded onto matrigel after angiogenic induction [202]. In this context, subcutaneously coinjection of DPSCs with human umbilical vein endothelial cells into matrigel in mice was assayed and microvessel like structures had been observed at 7 day post-injection [203]. These studies provide evidence of the suitability of DPSCs for the treatment of pathologies associated to inadequate angiogenesis.

Corneal regeneration

The loss of retinal ganglion cells (GGC) is the first pathological event in the majority of retinal degenerative diseases. Therefore, enhancing viability and function of these cells would be a major goal through basic and translational researches in ophthalmology. As previously mentioned, a great potential of DPSC to differentiate into neuronal lineages has been described. Moreover, recent experiments have demonstrated the expression of neuronal and retinal ganglion cell marker genes expression by differentiated DPSCs, cultured in a three-dimensional fibrin network. [204]. On the other hand, paracrine-mediated benefits have been tested in *in vitro* and *in vivo* experiments. In 2013, Mead et al described the secretion of DPSCs of multiple neurotrophins that could be responsible for promoting the neuroprotection of axotomized retinal ganglion cells (RGC) and neuritogenesis/axogenesis [205]. In this comparative study, higher expression of NGF and BDNF was found in DPSCs compared to BMSCs. One year later, the same authors confirmed that the intravitreally transplanted DPSCs promoted survival of RGC mediated by multifactor paracrine and neurite growth [206].

Several conditions such as genetic disorders, contact lens-induced keratopathy and iatrogenic multiple ocular surgeries, cause the total limbal stem cell deficiency (LSCD) which often results in persistent corneal defect that predisposes to corneal opacification. Advances in tissue culture and bioengineering have allowed the cornea functional reconstruction but the problem of graft rejection needs to be solved. DPSCs differentiation could provide a new source of limbal epithelial stem cells according to Kushnerev's recent investigations in which DPSCs were delivered to the surface of debrided epithelium of human corneas using contact lenses [207]. DPSCs differentiation into corneal epithelial progenitors was achieved in association with cytokeratin 12 expression, and the establishment by DPSCs of a barrier to avoid cornea invasion by the conjunctiva was also described. Additionally, *in vitro* expression of keratocan and keratin sulfate proteoglycans, both keratocytes proteins, has been also described after DPSCs differentiation [208]. DPSCs capacity to generate engineered corneal stromal-like constructs for potential use in regenerative therapies was evidenced. Corneal stromal extracellular matrix production by DPSCs was demonstrated after their injection into mouse corneal stroma. In these *in vivo* studies DPSCs synthesized type I collagen and keratocan without affecting corneal

transparency or inducing immunological rejection. In 2016, Mead et al. showed the highest efficacy of DPSCs efficacy as a neuroprotector of RGCs compared with other stem cells in a rat model with glaucoma induced by elevated intraocular pressure [209]. DPSCs prevented RGCs from death and protected their axons, thus preserving their function.

Treatment of diabetes

Diabetes is one of the most common chronic endocrinal diseases associated with pancreatic islet cell dysfunction. Both type 1 and 2 could be managed by transplantation of pancreatic islet cells. In this sense, the potential of DPSCs differentiation into islet-like cell aggregates has been explored. Cavernale et al. [210] described the beginning of the islet-like structures formation after 7 days of differentiation. The expression of genes related to pancreatic β-cell development and function, such as pancreatic and duodenal homebox-1 and insulin, were reported in the subsequent days of differentiation. The ability of these stem cells to differentiate into insulin-producing cells was confirmed in a separate study [211]. Blood and urine glucose levels were reduced in induced hyperglycemic mice after DPSCs transplantation [212] in association with an increase in pancreatic islets and insulin production. In addition, an improvement in renal function was also achieved as confirmed by the normalization of urea and proteinuria. On the other hand, the efficacy of DPSC transplantation for the treatment of diabetic polyneuropathy with an improvement in nerve conduction velocity has been demonstrated [213] and, more recently, with the achievement of a modulation of the subpopulation ratio of macrophages M1/M2 that favors the anti-inflammatory phenotype [214].

Hepatocyte differentiation

Acute liver failure is a refractory disease of which prognosis is extremely poor if not treated using liver transplantation. However, chronic donor shortage, post-operative severe complications, cost-effectiveness and ethical issues always limit its application. In this sense, DPSCs may be a promising cell resource for liver regenerative medicine. The differentiation potential of DPSCs into hepatocyte-like cells has also been demonstrated [32,81]. Furthermore, *in vivo* therapeutic effects of the combination of melatonin and human DPSCs significantly suppressed liver fibrosis and restored alanine transaminase, aspartate transaminase and ammonia levels in liver fibrosis mice model [215]. More

recently, Kim et al. [216] proposed the combination of hDPSCs transplantation with juglone to effectively treat liver fibrosis.

Miscellaneous

DPSCs may provide, due to their multipotency, an alternative source of cells for tissue engineering in multiple medical fields. The *in vitro* ability of human DPSCs to differentiate into bladder smooth muscle cells has been reported [126]. Regeneration of new end bulbs and creation of multiple differentiated hair fibers have also been described after DPSCs transplantation into surgically inactivated hair follicles [217]. Additionally, transplantation of DPSCs cultured with basic fibroblastic growth factor in immunocompromised mice revealed the formation of bone, cartilage and adipose tissue [218]. A recent study showed that mature tendon-like tissue was formed after DPSC and polyglycolic acid scaffold construct transplantation under mechanical loading conditions in a mouse model [219]. All these studies explore the potential of DPSCs as a therapy for several biomedical applications.

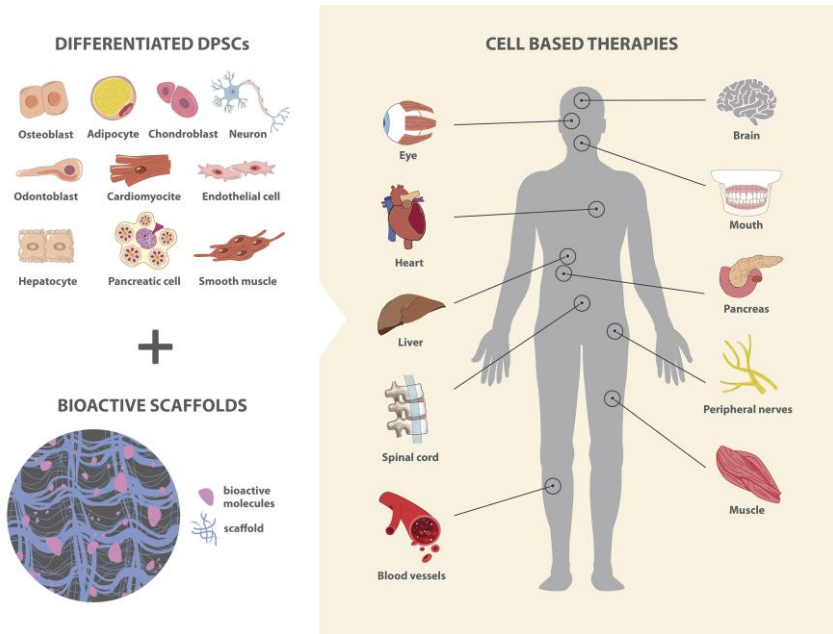

Figure 2. Illustration representing the regenerative potential of DPSCs based therapies.

Medical Field	Application	Evidence		
		In vitro	*In vivo*	
			Animal experimentation	Human
Oral and maxillofacial defects	Dentin/pulp-like complex generation	[220]	hDPSCs transplantation into mice [9,92,178] Minipigs [185]	
	Complete pulp regeneration		Dogs [179,180]	DPSCs transplantation in humans, pilot study [181]
	Osteogenesis	[182]		
	Bone regeneration		Rats [139], hDPSCs into rabbit model [175]	hDPSCs transplantation to repair human mandible defects [188]
Neural regeneration	Differentiation into dopaminergic neurons Differentiation into Schwann cells	[115,189] [191]		
	Glial differentiation	[192]	hDPSCs transplantation into a mouse model of local sciatic damage [112]	
	Peripheral nerve regeneration		Pigs [193]	
	Neural tissue injuries repair		hDPSCs transplantation into brain and spinal cord injury rat models [119,194]. Intraventricular hDPSCs injection on hypoxic-ischemic damaged brain in rats [195]	
	Others		Effects of transcranial magnetic stimulation on the surviving of	

			intracranial injected hDPSCs in rats [196]	
Treatment of ischemic disease and angiogenesis	Cardiac repair		Intramyocardially injection of hDPSCs in a rat model of myocardial infarction [197]	
	Differentiation into cardyomiocytes	[198]		
	Angiogenesis induction	[199]		
	Neurovascularization		Intramusculary injection of hDPSCs in a murine model of hindlimb ischemia and hDPSCs transplantation into a rat model of cerebral ischemia [200,201]	
	Vascular network formation	[202]	hDPSCs subcutaneously injection in mice [203]	
Corneal regeneration	Differentiation into retinal ganglion-like cells	[204]		
	Neuroprotection and axon regeneration promotion	[206]	Rat DPSCs intravitreally transplanted after a surgically induced optic nerve crush injury [205]	
	Repair and regeneration of the human corneal epithelium	hDPSCs transferred onto epithelium debrided corneas [207]		
	Corneal stromal regeneration		hDPSCs injection into mouse corneal stroma [208]	
	Protection from RGC loss and RGC function preservation		hDPSCs transplanted into the vitreous of glaucomatous rat eyes [209]	
Treatment of diabetes	Differentiation into insulin-producing	[210,211]		

	cells			
	Control of diabetes complications		DPSCs transplantation into diabetes type 1 mice [212] and rat [213,214] models	
Hepatic Regeneration	Hepatocyte differentiation	[32,81]		
	Treatment of liver cirrhosis		hDPSCs transplantation into a induced liver fibrosis mice model [215,216]	
Miscellaneous	Differentiation into bladder smooth muscle cells	[126]		
	Regeneration of new end bulbs and creation of multiple differentiated hair fibers		Human and rat DPSCs transplantation into nude mice [217]	
	Generation of bone, cartilage and adipose tissues		hDPSCs transplanted into subcutaneous tissue of mice [218]	
	Potential application in tendon tissue engineering		hDPSCs implanted subcutaneously in nude mice under mechanical loading conditions [219]	

Table 3. Overview of the main evidences of DPSCs potential in tissue engineering.

FUTURE PERSPECTIVES

Dental pulp is a promising source of stem cells, with a multifaceted differentiation capacity along with a non-invasive collection technique that can be performed after routine teeth extraction. As a consequence, DPSC-based therapy is being currently exhaustively explored. However, as for other MSCs, there are still many challenges to face. For example, further research towards optimal standardization of isolation and culture

protocols following GMPs is still needed with the main commitment to replace components of animal origin. Specific markers that clearly discriminate dental pulp stem cells are also of greatly importance. Regarding cell banking procedures, both cryopreservation media and methods must be established to maintain the properties and viability of these cells for future use. In this sense, the creation of histocompatibility allogenic biobanks would offer an innovative and attractive strategy to guarantee efficient storage for future treatments overcoming immunological barriers.

The successful development of a therapeutic medicine for its use in tissue engineering involves combining stem cells, scaffolds and signaling molecules. In this context, the design of an appropriate bioactive material involves a better understanding of the molecular mechanisms implicated in the stem cells-biomaterials interactions. 3D bioprinting is an emerging concept with a promising future that is already yielding promising results in the present. In fact, 3D bioprinting allows the manufacture of customized and highly complex high constructs. However, very few studies have been conducted that combine hDPSCs and 3D bioprinting techniques, even though this approach requires easy- to-expand and non-immunogenic cells readily available.

In summary, DPSCs therapy represents a new remarkable approach for the efficient management of disease. The in vitro and in vivo results support the conduction of more clinical trials to finally overcome the current limitations and support the clinical use of DPSCs under regulatory guidelines.

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CONFLICT OF INTEREST

The authors declare the following competing financial interests: E.A. is the Scientific Director of and M.T. and M.Z. are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology.

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(III) Personalized plasma-based medicine to treat age-related diseases

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Personalized plasma-based medicine to treat age-related diseases

Eduardo Anitua[1,2#], María Troya, Mar Zalduendo, Gorka Orive[1,3,4#]

[1]*Eduardo Anitua Foundation for Biomedical Research, Vitoria, Spain*

[2]*BTI-Biotechnology Institute, Vitoria, Spain*

[3]*NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country, School of Pharmacy, Vitoria, Spain.*

[4]*Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria, Spain*

#Corresponding author:

Dr. Gorka Orive

E-mail: gorka.orive@bti-implant.es

Dr. Eduardo Anitua

E-mail: eduardoanitua@eduardoanoitua.com

Abstract:

As social and health needs are changing, new challenges to develop innovative alternatives arise to address unmet medical needs. Personalized medicine is emerging as a promising and appealing therapeutic option. The use of patient´s own plasma and platelets as therapeutics is providing new avenues in the treatment of acute and chronic tissue injuries by promoting tissue repair and regeneration. Plasma and platelet-based therapies mimic the physiological repair process by releasing autologous growth factors and creating a natural, biodegradable and transient scaffold that acts as transient matrix. This review summarizes the recent advances and challenges in the field of personalized plasma-based medicine and its potential to treat age-related diseases.

Keywords: Platelet rich plasma, autologous therapies, personalized medicine, tissue regeneration, scaffold, growth factors.

1. Introduction

The global aging of the population is increasing at an accelerating rate. These demographic changes are leading to an increased prevalence of age-related degeneration and chronic diseases. Traditional medicine is trying to solve all these problems. In fact, successful organ transplantation (allografts) is considered among one of the major milestones of medicine of the last decades. However, success of transplantation is limited by shortage in the supply of transplantable organs and by the potential organ rejection.

As social and health needs are changing, the pressure to develop new alternatives, designed to address unmet medical needs, increases. Over the last years, the gradual understanding of the biological processes involved in wound healing has paved the way for developing new regenerative therapies with the ultimate goal of promoting and accelerating tissue regeneration [1,2]. In this context, personalized medicine is emerging as a promising and appealing therapeutic option. It is based in the complex uniqueness of each patient offering a tailor-made treatment for improving the management of patients [1,3]. Already in ancient Greece, the father of medicine, Hippocrates, mentioned the importance of personalizing the medicine: "*it is more important to know what sort of person has a disease than to know what sort of disease a person has*".

Autologous plasma-based therapies may be a form of personalized medicine. These therapies employ human-based cells or tissues, may or not manipulate them outside the body, and reintroduce them into the same donor [4]. This emerging field, by being autologous, provides new advantages in the clinical setting: (i) the donor is immediately available, (ii) no immunosupression is required, (iii) no rejection occurs, (iv) elimination of graft versus host disease, (v) risks associated to disease transmission are eliminated and (vi) in general, does not present ethical conflicts [5].

Platelet rich plasma is included within these types of autologous therapies. It is based on properly selecting, from human blood, a pool of cells and growth factors, together with fibrin forming proteins to create a three-dimensional (3D) scaffold, necessary to support tissue regeneration. As derived from patient's own blood, platelet rich plasma is an affordable and minimally invasive technique [2].

In this review we describe the potential of plasma-based medicine and in particular platelet rich plasma as an autologous therapy. We provide insight about their biological effects as well as some pivotal preclinical and clinical applications.

2. Mimicking the healing process

Following any tissue injury, one of the main priorities of the organism is the restoration of tissue integrity and function. For this purpose, the process of wound healing goes through a sequence of continuous, overlapping and precisely programmed phases involving rapid hemostasis, controlled inflammation, cell migration to the injury site and subsequent cell proliferation and differentiation, and finally, formation and remodeling of extracellular matrix (ECM) [6-8]. During the healing process some remarkable events must be specially mentioned.

2.1. Fibrin scaffold

Polymerized fibrin that is structured from soluble fibrinogen, is the principal component of blood clots, and provides a provisional scaffold which enables formation of a temporary matrix in the wound bed. Fibrinogen is a key protein for both hemostasis and homeostasis. In fact, fibrinogen assembles into the ECM at sites of tissue damage, where it may be involved in cell type-specific mechanisms of wound repair [9]. Its contribution largely depends on the interactions between specific-binding sites on fibrin(ogen), pro-enzymes, clotting factors, enzyme inhibitors, and cell receptors as well as on the structural composition of fibrin [10]. Fibrinogen circulates in normal human plasma in a high and low molecular weight form (HMW and LMW, respectively). This fibrinogen heterogeneity strongly influences coagulation rate, fibrin structure and endothelial cell behavior. HMW fibrin matrices display a porous and malleable network with thicker fibers whereas the thinner fibers bundles of LMW fibrin matrix form a dense structure. Moreover, HMW fibrins stimulate endothelial cell proliferation and tube formation more than LMW matrices do. These differences could be exploited for therapeutic applications [11,12].

2.2. Growth factors

The aggregated platelets, which are trapped in the provisional fibrin matrix, release multiple growth factors that exert outstanding roles in modulating the inflammatory stage and cell recruitment to the damaged area (Table 1). In addition, these bioactive molecules are key regulators of the main tissue regenerative processes such as cell migration, proliferation, differentiation, angiogenesis and ECM biosynthesis. Growth factors modulate their effects through binding to specific receptors on the target cell surface. Following this binding, signal-transduction pathway involves a complex array of events such as second messengers, protein phosphorylation, gene expression and protein synthesis [13]. Multiple growth factors may share mechanisms while the same growth factor may deliver different messages depending on the cell and receptor type they bind to.

Growth factor	Molecular weight (kDa)	Types	Receptors	Mechanisms of activation and signaling	Main function
EGF [14,15]	6.4	-	EGFR (ErbB1)	Receptor tyrosine kinases	Cell growth, proliferation, differentiation and survival.
FGF [16,17]	7-38	FGF1-14, FGF16-23	FGFR1, FGFR2, FGFR3, FGFR4	Receptor tyrosine kinases	Cell proliferation, migration, differentiation, angiogenesis and survival.
PDGF [18,19]	32-35	-AA, -BB, -AB, -CC, -DD	PDGF-α and PDGF-β	Receptor tyrosine kinases	Chemotactic, cell proliferation and extracellular matrix production.
TGF-β [20,21]	25	-β 1, -β 2, -β 3	TGF β RI and TGF β RII	Serine/threonine kinase receptors.	Wound healing, angiogenesis and immune supression promotion. Differentiation. Extracellular matrix production, and wound contraction.
VEGF [22,23]	34-42	-A, -B, -C, -D and	VEGFR-1, VEGFR-2 and	Receptor tyrosine kinases. Receptors	Endothelial cell proliferation and

		placental growth factor	VEGFR-3. Neuropilins (Nrp-1 and Nrp-2).	for semaphorins.	migration.

Table 1. Main growth factors involved in human tissue regeneration [24-26].

2.3. The active role of cells

After hemostasis and inflammatory stages, damaged tissue restoration process is intensified. This process is carried out by several phenotypes through different procedures. Tissue-specific adult stem cells gain special importance in the response to regulatory signals from the injured tissue [27]. In addition, bone marrow-derived stem cells significantly contribute to tissue regeneration by providing trophic factors that modulate the local environment and by promoting angiogenesis, reepithelialization and granulation tissue formation [28]. Stem cell behavior is profoundly modulated by different signals such as cell-cell interactions and the ECM, components all of them of the stem cell niche, a dynamic structure that includes immune cells during inflammation and wound healing process [29]. It is also necessary to mention the role of macrophages during the inflammatory response and tissue repair. Considered more than immune cells, macrophages have the ability of acquiring distinct functional phenotypes in response to the microenvironmental cues. Traditionally, two main phenotypes have been identified: the classically activated macrophages (M1) that produce many inflammatory cytokines, reactive oxygen species and nitrogen intermediates and the alternatively activated macrophages (M2) that are characterized by low levels of pro-inflammatory cytokines and high IL-10 expression. M1 supports pathogen killing and drives the inflammatory response whereas M2 sustains tissue remodeling. Both phenotypes also differ in the metabolic pathway, since glycolytic pathway is involved in M1 polarization, whereas fatty acid oxidation occurs in M2. Macrophages show different activation states, therefore, a continuum of phenotypes is expected to occur at the wound site. This macrophage plasticity suggests a complex molecular system that enables them to play multiple roles in inflammation and regeneration [30-32].

2.4. ECM biosynthesis

ECM presents a unique composition and topology that is generated through a dynamic and reciprocal, biochemical and biophysical dialogue between various inner cellular components and the evolving microenvironment [33]. Moreover, the ECM is a highly dynamic structure which molecular components are subjected to post-translational modifications generating the particular biochemical and mechanical properties of each organ [34]. Far from being just an inert supportive structure, the ECM, by anchoring cell and growth factors, directs cell fate in a well-orchestrated manner [29]. Cell-ECM interactions are primarily mediated by integrins, the main cell adhesion receptors, which in turn, control growth factor signaling pathways in a synergistically manner [35]. The emerging field of tissue engineering seeks to develop biomaterials that closely resemble the characteristics and functions of the native ECM.

Since fibrin glue was originally described in 1970, artificial acellular scaffolds have been considered as a promising field in tissue regeneration therapies due to their ability to create and maintain an space for tissue growth, to provide mechanical stability and to also support cell adhesion and migration [36,37]. However, synthetic polymers, as well as their degradation products, can activate the complement cascade of inflammatory response [38]. On the contrary, the fibrin scaffolds derived from plasma rich in growth factor (PRGF), a particular platelet rich plasma, are biocompatible matrices and contain a plethora of biologically active substances provided by platelets that actively participate in the regenerative process [39]. Platelet rich plasma reabsorbable matrices combine the sealant properties with the ability to gradually release the main promoters of tissue regeneration [40]. In this sense, the kinetic of GFs delivery from platelet rich plasma scaffolds have been already described [41]. Thus, it has been probed that after a quick delivery of GFs (PDGF-AB, VEGF, HGF and IGF-I), the release is maintained more than a week, being the GF retention by platelet rich plasma fibrin of almost 30% on the 8th day (Figure 1).

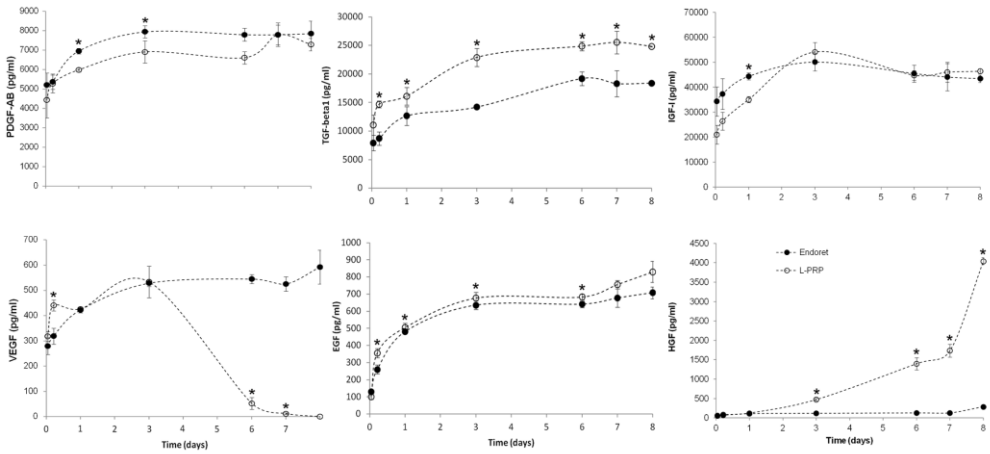

Figure 1. PDGF-AB, TGF-β1, IGF-I, VEGF, EGF and HGF release from PRGF and L-PRP fibrin scaffolds during 8 days. Reproduced, with permission from [41].

3. Autologous Platelet Rich Plasma therapies

Biological therapies provide new opportunities for developing personalized medicine. Platelet-rich plasma is included within the autologous therapies based on the reparative ability of platelets. Not long ago it was thought that the role of platelets was limited to maintaining tissue hemostasis. However, recent evidences suggest that platelets participate in a wide range of physiological processes, including the immune response, inflammation and wound healing through the large number of substances present in their secretome and the high amount of receptors that are present in their surface [42,43]. In fact, high-throughput proteomic approaches have identified over 300 proteins released by human platelets upon activation [44]. Following activation, proteins, cytokines, exosomes and microparticles are released from platelets after rapid translation of pre-existing mRNA. This process delivers bioactive mediators and also modifies the platelet surface, which provides them with the ability to modulate the function of many cells which is essential for

tissue repair. This ability of platelets beyond hemostasis confers them a great ability to be applied in multiple medical fields [43,45].

Basically, platelet rich plasma is defined as a portion of plasma fraction obtained from the patient's own blood having a platelet concentration above baseline. The ultimate goal of these therapies is to facilitate, optimize, and accelerate the body's innate ability to repair tissues by the progressive delivery of autologous growth factors and proteins by the platelet rich plasma scaffold (Figure 2). Despite the great number of possible protocols for platelet rich plasma preparation with multiple commercially available products, most share a common sequence of basic steps: (i) blood collection by simple venipuncture, (ii) blood centrifugation to obtain the platelet concentration plasma fraction and (iii) activation of platelets to release growth factors from the temporary scaffold [46]. Nevertheless, there is no consensus regarding the conditions of centrifugation, the method for platelet activation, the optimal concentration of platelets, or the inclusion or not of leukocytes. All of these variables lead to different products with different biological potential [2,46]. Therefore, PRGF is a platelet rich plasma obtained by a single centrifugation and in which activation process calcium chloride is used. This personalized technology is characterized by a specific platelet concentration that leads to an optimal biological effect and by the absence of leukocytes thus avoiding the promotion of inflammation. Due to its great therapeutic potential, this pioneering technology is widely used in several medical fields [47], and was firstly used worldwide in 2003 in the area of orthopaedics [48].

Cells, growth factors and scaffolds are the three components considered as essential for tissue regeneration. These autologous therapies provide natural, biodegradable and transient scaffolds with a wide range of mechanical properties that combine with a sustained release of proteins and growth factors to induce host cells to replace the lost tissue structurally and functionally. Additionally, platelet rich plasma fibrin matrix provides anti-inflammatory factors and anti-bacterial peptides avoiding the inflammatory response as well as bioactive factors that play important roles in regulating cellular processes such as mitogenesis, chemotaxis, differentiation and metabolism [49,50]. On the other hand, both the highly organized structure that confers to platelet rich plasma scaffold rigidity, porosity and tolerance properties and its content in several proteins such as fibrin,

fibronectin and vitronectin, favor the migration and adhesion of cells that are actively involved in the regeneration process [51].

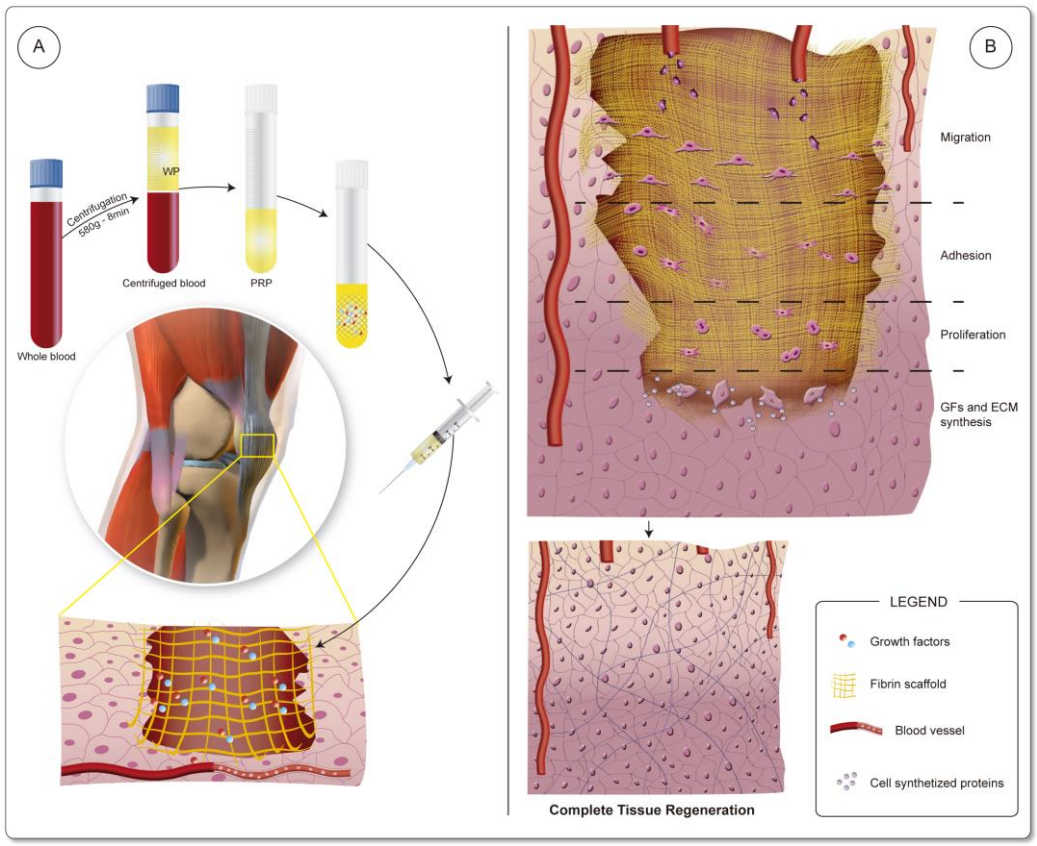

Figure 2.. Illustration representing the regenerative potential of PRGF, a particular platelet rich plasma. A) PRGF obtaining to be applied in several injured tissues. B) Wound healing main cell processes positive modulated by the temporary scaffold and growth factors provided by PRGF preparations.

The multiple formulations that can be obtained from platelet rich plasma autologous therapies confer this approach a great versatility to be applied in different medical fields, including orthopedic, sports medicine, bone reconstruction, tissue engineering, maxillofacial surgery, periodontal regenerative medicine and ophthalmology [52] (Table 2). Additionally, platelet rich plasma technology is beginning to be used in autologous cell

therapies for ex vivo expansion and subsequent transplantation of stem cells as an alternative to the use of xenogeneic products [53].

	Pathologies	Outcome	Administration
Orthopedic [48,54-56]	Achilles tendinopathy, anterior cruciate ligament (ACL) reconstruction, osteoarthritis, lateral epicondylitis, patellar tendon healing, osteochondral lesions, plantar fasciitis, hip fracture, rotator cuff repair, knee arthroplasty.	Pain reduction, improvement of functional status and joint stiffness, significant improvement in several scores (VAS, AOFAS, FAAM, WOMAC…).	Injection.
Dentistry [57-63]	Temporomandibular disorders, extraction socket, sinus elevation, horizontal bone defect, bone ridge expansion, bisphosphonate related osteonecrosis of the jaw (BRONJ).	Pain reduction, improve temporomandibular joint mobility, ridge thickening, inflammation decrease, enhance healing process, superior bone density, enhance vascularization, and regeneration of osseous and epithelial tissues.	Injection, fibrin.
Ophtalmology [64-67]	Dry eye, graft versus host disease, Sjögren's syndrome, persistent epithelial defects, corneal ulcers, macular holes, ocular surface pathologies associated with LASIK surgery.	Improvement or disappearance of symptoms, reduction of photophobia and inflammation, improvement in the resolution of keratitis punctata, increase in the lacrimal volume production, improvement in the tear break-up, re-epithelization, ocular pain decrease, visual acuity improvement.	Eyedrops, fibrin scaffold, injection.
Dermatology [68-72]	Androgenetic alopecia, diabetic and other chronic ulcers, aesthetic dermatology, vitiligo.	Increase in the follicular density, reduction in hair loss, hair growth stimulation, prevention of dermal papilla apoptosis, hair density increase, enhancement of the	Injection, spray, fibrin topical application.

		re-epithelialization process, increase healing rate, repigmentation improvement.	
Others	The versatility of this autologous technology is opening up new fields of application where the use of this biological therapy may be an alternative to current treatments (peripheral neuropathies [73], gynecological issues [74]...).		

Table 2: Main clinical applications for autologous platelet rich plasma.

4. Concluding remarks and future perspectives

Personalized medicine and specifically autologous therapies represent promising new medical technologies that offer the potential to reduce risks and increase safety using proteins and cells from the own patient. The concept is to transform the way in which medicine is practice to optimize and enhance the body's innate ability to repair tissues in order to improve medical outcomes.

The last decades have witnessed an explosion in the use of platelet rich plasma products for tissue regeneration purposes. Efficacy and safety have been demonstrated in a wide range of medical fields. Nevertheless, there is still a long way to go in regulatory and standardization terms. The goal to be achieved in the coming years should be a more predictable and efficient treatment by reducing the variability of preparation and administration protocols thus leading to completely different biological effects and to misinterpretation of results.

Most successful treatments in regenerative medicine take advantage of the synergistic effects of combining therapies. In the last years, stem cells have adopted a relevant role in this field. Therefore, an integrated strategy that combines the autologous technology of platelet rich plasma and the emerging use of stem cells may represent a new challenge to develop new applications.

Abbreviations

AOFAS: American Orthopaedic Foot and Ankle Society.

ECM: Extracellular matrix.

EGF: Epidermal growth factor.

EGFR: Epidermal growth factor receptor.

FAAM: Foot and Ankle Ability Measure.

FGF: Fibroblast growth factor.

FGFR: Fibroblast growth factor receptor.

GFs: Growth factors.

HGF: Hepatocyte growth factor.

IGF: Insuline-like growth factor.

PDGF: Platelet derived growth factor.

TGF-β: Transforming growth factor-beta.

TGF β R: Transforming growth factor-beta receptor.

VAS: Visual analogue scale.

VEGF: Vascular endothelial growth factor.

VEGFR: Vascular endothelial growth factor receptor.

WOMAC: Western Ontario and McMaster Universities Osteoarthritis.

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OBJETIVOS

HIPÓTESIS

La tecnología autóloga PRGF ejerce un efecto antiinflamatorio y citoprotector en modelos *in vitro* de enfermedades orales: periodontitis y BRONJ. Además, la tecnología PRGF puede usarse como alternativa al suero bovino fetal para el cultivo, expansión y diferenciación de células madre de pulpa dental con objeto de favorecer la regeneración periodontal.

OBJETIVOS

1. Evaluar las propiedades morfológicas y biomecánicas de la fibrina de PRGF en un modelo inflamatorio de periodontitis *in vitro*.

2. Estudiar el efecto biológico del PRGF en la proliferación y en el estado inflamatorio de fibroblastos gingivales y osteoblastos alveolares en dicho modelo experimental de periodontitis.

3. Evaluar el efecto de la inclusión de leucocitos en las propiedades mecánicas y biológicas del PRGF en condiciones inflamatorias.

4. Determinar el efecto biológico de la tecnología PRGF sobre la proliferación celular, apoptosis e inflamación de fibroblastos gingivales y osteoblastos alveolares en un modelo *in vitro* de osteonecrosis de los maxilares inducida por bifosfonatos.

5. Evaluar el uso del PRGF como sustituto del suero bovino fetal para el aislamiento, expansión, diferenciación y criopreservación de células madre de pulpa dental para su posterior traslación clínica como terapia celular autóloga en la regeneración tisular.

DISEÑO EXPERIMENTAL Y RESULTADOS

(IV) Leukocyte inclusion within a platelet rich plasma-derived fibrin scaffold stimulates a more pro-inflammatory environment and alters fibrin properties

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Leukocyte inclusion within a platelet rich plasma-derived fibrin scaffold stimulates a more pro-inflammatory environment and alters fibrin properties

Eduardo Anitua[1,2], Mar Zalduendo[2], María Troya[2], Sabino Padilla[2], Gorka Orive[1,*]

[1]*Foundation Eduardo Anitua, Vitoria, Spain*

[2]*Laboratory of Regenerative Medicine, Biotechnology Institute (BTI), Vitoria, Spain.*

[*]Corresponding authors:

E-mail: gorka.orive@ehu.es

Abstract

One of the main differences among platelet-rich plasma (PRP) products is the inclusion of leukocytes that may affect the biological efficacy of these autologous preparations. The purpose of this study was to evaluate whether the addition of leukocytes modified the morphological, biomechanical and biological properties of PRP under normal and inflammatory conditions. The release of pro-inflammatory cytokines from plasma rich in growth factors (PRGF) and leukocyte-platelet rich plasma (L-PRP) scaffolds was determined by enzyme-linked immunosorbent assay (ELISA) and was significantly increased under an inflammatory condition when leukocytes were included in the PRP. Fibroblasts and osteoblasts treated with L-PRP, under an inflammatory situation, underwent a greater activation of NF κ B pathway, proliferated significantly less and secreted a higher concentration of pro-inflammatory cytokines. These cellular events were assessed through Western blot and fluorimetric and ELISA methods, respectively. Therefore, the inclusion of leukocytes induced significantly higher pro-inflammatory conditions.

Keywords: leukocytes, white blood cells, platelet rich plasma, NF κ B, inflammation.

Introduction

Over the last few years, translational medicine has emerged as a new trend in medical practice, which aims to promote the rapid clinical translation of a wide range of therapeutic options and to receive the feedback of basic research results [1]. One of the hottest topics in translation medicine deals with those therapies focused on enhancing and promoting tissue repair and regeneration [2]. Repair, restoration and healing of lost tissue are unique among clinical treatments due to the large number of patients suffering from tissue damage and injuries. Progress in this field is pertinent as it may help to reduce the burden on the world's health systems and address the need of tissue replacement [3].

The use of patient-derived formulations and therapeutics is gaining the attention of the scientific community. Human plasma and especially human platelets contain a wide range of proteins and growth factors that have been demonstrated to promote tissue repair and regeneration in many different injured tissues [4-14]. In addition, the coagulation of plasma leads to the formation of a three-dimensional fibrin matrix that can provide transient space for the key tissue-forming cells at the same time that acts as a controlled protein delivery system [2,13]. These and other advances have promoted the research and use of platelet rich plasma products as a biomolecules delivering system [15] for wound healing, tissue regeneration and tissue engineering [16].

There are however, several concerns related to the use of platelet rich plasma products that need to be addressed. First, how these preparations exert their effects in inflammatory conditions. To our knowledge, most of the studies evaluating the biological potential of platelet rich plasma products do not mimic tissue inflammatory conditions in which most of these therapies are used. Second, how the particular protein and cell composition of these products can affect their final therapeutic outcomes. In fact, while some platelet rich plasma products contain only platelet-enriched plasma, others concentrate leukocytes, red cells and other pro-inflammatory cells. As a consequence, the composition and biological properties of these products might be potentially different. It has been demonstrated that leukocyte concentration is negatively correlated with matrix gene expression and positively correlated with catabolic gene expression in tendon and ligament tissues [17]. Moreover, white blood cells (WBC) contain numerous pro-inflammatory interleukins and extracellular

matrix degrading enzymes [18]. Third, only few studies have determined the role and properties of the fibrin scaffold formed with each type of platelet rich plasma product.

In the present study, we have carried out a complete evaluation of how including leukocytes (L-PRP) or not in plasma rich in growth factors (PRGF), a well-characterized platelet rich plasma therapy, may alter both protein and cytokine release and fibrin scaffold properties. Leukocyte containing and leukocyte free PRGFs were prepared and evaluated in non-inflammatory and inflammatory conditions that mimic a well-known chronic inflammatory disease such as periodontitis. The latter is caused by *Porphyromonas gingivalis* [19] and affects periodontal tissue due to the release of proteolytic enzymes [20,21].

Material and methods

The study was performed following the principles of the Declaration of Helsinki, as revised in 2008 and after approval from the Foundation Eduardo Anitua Institutional Review Board.

Preparation of PRP (PRGF) products

Blood from a total of 6 patients was collected into 9-mL tubes with 3.8% (wt/v) sodium citrate, after written informed consent was provided (approved by our Institutional Review Board). The following products were generated immediately from the collected blood: plasma rich in growth factors (PRGF) and Leukocyte-PRP (L-PRP). PRGF was prepared according to manufacturer's instructions (Endoret Dentistry, BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). Briefly, samples were centrifuged at 580 *g* for 8 min at room temperature. For each tube, the 2 ml of plasma just above the buffy coat was collected in each donor. Leukocyte-PRP was prepared by the addition of the buffy coat into the platelet richest fraction. Platelets and leukocytes counts were performed on samples of blood, PRGF and L-PRP with a hematology analyzer (Micros 60, Horiba ABX, Montpelier, France).

Structure of fibrin net from both PRP products

The different fibrin scaffolds were prepared by activating different volumes of PRGF and L-PRP with calcium chloride (Endoret Dentistry) at 37 ºC for 1 hour (50 µL of calcium

chloride for each mL of PRGF/L-PRP). After clot formation, the morphological analysis of PRGF and L-PRP fibrin scaffolds was performed with scanning electron microscopy (SEM). Briefly, the clots were rinsed with phosphate-buffered saline and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 4 h and washed in cacodylate-sucrose buffer. Then, the samples were post-fixed with osmium tetroxide for 1 h, washed again and dehydrated with serial concentrations of ethanol. Samples were critical point-dried (Tousimis Autosamdri 814), sputter-coated with 5 nm gold (Edwards E306A) and subsequently examined in a scanning electron microscope (Hitachi S-4800) [22].

Mechanical properties of PRGF and L-PRP fibrin scaffolds

The mechanical properties of PRGF and L-PRP fibrin scaffolds were evaluated. Two mL of each formulation and 100 μL of calcium chloride were added to the devices. Once the clot was formed, the devices were held on the mechanical testing station (858 Mini Bionix II, MTS, USA) with a loading cell of 10N, and de-moulded, leaving the fibrins ready to perform the assays. The mechanical testing station stretched the fibrin scaffolds at a constant speed of 2mm/s. Maximum elongation at failure was determined.

Culture of PRGF and L-PRP on inflammatory and non-inflammatory conditions

Two mL of PRGF or L-PRP of each of the six donors were added to each well of a 6-well cell culture plate and incubated with 100 μL of calcium chloride for 20-30 min at 37ºC until clot formation. Thereafter, half of the scaffolds of both PRGF and L-PRP were incubated with 2 mL of Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco-Invitrogen, Grand Island, NY, USA) in the absence (normal condition) or presence (inflammatory condition) of 10 μg/ml of LPS from *Porphyromonas gingivalis* (*P.g.*-derived LPS) (InvivoGen, San Diego, CA, USA) at 37ºC with 5% CO_2 for 72h. The remaining scaffolds were incubated with 2 mL of osteoblast basal medium (ObM) (Sciencell Research Laboratories, Carlsbad, CA, USA), also under both normal and inflammatory conditions. After that, the conditioned media by the scaffolds was collected and centrifuged at 460 g for 10 min at room temperature. The supernatant was stored at -80ºC for assay of protein levels.

Quantification of cytokines and growth factors by ELISA

The levels of interleukin 1 beta (IL-1β), interleukin 8 (IL-8) (Invitrogen), tumor necrosis factor-α (TNF-α, interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), platelet-derived growth factor AB (PDGF-AB) and angiopoietin-1 (R&D Systems, Minneapolis, MN, USA) on the conditioned media of both PRGF and L-PRP clots under both normal and inflammatory conditions, were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocol.

MMP-1 activity assay

In order to determine the MMP-1 activity on the conditioned media of both PRGF and L-PRP clots under both study conditions, a MMP-1 colorimetric drug discovery kit (Enzo Life Sciences, Lausen, Switzerland) was used with some modifications to the manufacturer's protocol. A standard curve for MMP-1 was performed. The enzyme activity was determined 1 minute after the reaction was started.

Cell treatment with PRGF and L-PRP

Primary human gingival fibroblasts and primary human alveolar osteoblasts, obtained from patients during routine surgical procedures and dental implant surgery, respectively, were isolated as previously described [23,24] and were used to assess the effect of both PRGF and L-PRP releasates under inflammatory conditions. Written informed consent from those patients and approval by the Foundation Eduardo Anitua institutional review board were obtained.

Fibroblasts were routinely cultured in DMEM/F12 supplemented with 2 mM glutamine and 50 μg/mL gentamicin (Sigma-Aldrich, St. Louis, MO). Osteoblasts were cultured in ObM supplemented with 50 μg/mL gentamicin. Both cultured media were supplemented with 15% fetal bovine serum FBS (Biochrom AG, Leonorenstr, Berlin, Germany).

PRGF and L-PRP scaffolds from one donor were stimulated with 10 μg/mL of *P.g.*-derived LPS for 72 h, as previously described (Figure 1). The molecule releasates from the scaffolds incubated with LPS will be identified as PRGF+LPS and L-PRP+LPS.

The cell incubation experiments were performed as follows: Cells were pretreated with 10 μg/ml of *P.g.*-derived LPS in the appropriate culture medium for 72 hours. The pretreated cells were then cultured with the corresponding culture medium and 10 μg/ml of *P.g.*-derived LPS supplemented with either 70% PRGF+LPS or 70% of L-PRP+LPS. The treatment time was 24 h for Western blot analysis, 72 h for proliferation assay, and 24h and 72h for cytokine secretion assay.

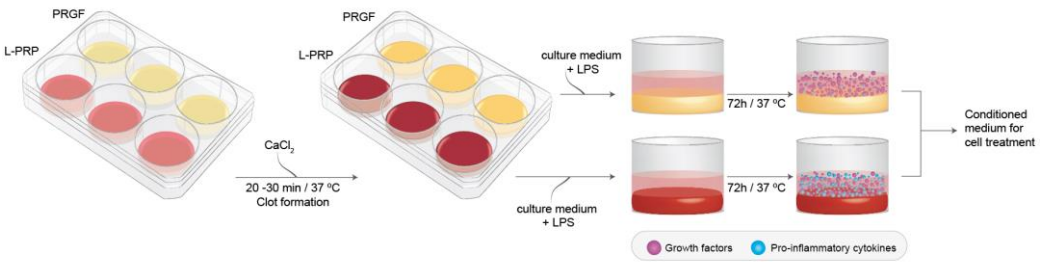

Figure 1. Illustrative representation of the cell treatment experimental procedure with PRGF and L-PRP releasates under inflammatory conditions.

Western blot analysis

After treatment, both fibroblasts and osteoblasts were washed with PBS and lysed with mammalian protein extraction reagent supplemented with protease and phosphatase inhibitors (Pierce Biotechnology, Bonn, Germany). Lysates were clarified by centrifugation at 14000 g for 10 min, and the supernatants were then collected. Cell lysates were concentrated using centrifugal filters (Amicon ultra-0.5 (3k), Chemicon-Millipore, Billerica, MA, USA) and the protein concentration was determined with the BCA assay (Pierce Biotechnology). TGX stain-free gels were used for protein electrophoresis (Bio-Rad Laboratories, Munich, Germany). The membranes were blocked using 5% non-fat dry milk in tris(hydroxymethyl) aminomethane-buffered saline containing 0.1% polysorbate surfactant (TBST) for 1 hour at room temperature. Then the membranes were incubated overnight at 4ºC with the corresponding primary antibodies, nuclear factor-κB p65 (NF-κB p65), phosphorylated nuclear factor-κB p65 (pNF-κB p65) and inhibitor-κBα (IκBα) (Cell signaling Technology, USA). Membranes were then washed several times with

TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Cell signaling Technology) accordingly, for 1 hour. The blots were washed again and developed by chemiluminescence with substrate Immun-Star HRP substrate, (Bio-Rad Laboratories) using an image analyzer (Chemidoc image analyzer, Bio-Rad Laboratories). The Stain-Free technology was used as loading control method [25].

Cell Proliferation assay

Cell proliferation was evaluated by CYQUANT cell proliferation assay (Molecular Probes-Invitrogen, Grand Island, NY, USA). Briefly, the treatments were removed and the wells were washed carefully with phosphate-buffered saline (PBS). Then, the microplates were frozen at -80ºC until assayed, to allow cell lysis. After thawing the plates at room temperature, wells were incubated with RNase A (1.35 Ku/ml) diluted in cell lysis buffer for 1 hour at room temperature. Then, 2x GR dye/cell-lysis buffer was added to each sample well, mixed gently and incubated for 5 minutes at room temperature, protected from light. Sample fluorescence was measured with a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies, Bad Wildbad, Germany). A DNA standard curve was included in each assay.

Cell-stimulated cytokine expression

ELISA was performed to analyze the concentration of pro-inflammatory cytokines secreted by human gingival fibroblasts and alveolar osteoblasts after treatment with the conditioned media from both PRGF and L-PRP. Briefly, the conditioned media was collected and levels of IL-1β, TNF-α, IL-6 and IL-8 were measured by ELISA kits (R&D Systems). The concentrations of these proteins were determined according to the manufacturer's instructions.

Statistical Analysis

The results were presented as mean ± standard deviations. Single-factor analysis of variance (ANOVA) and Kruskal-Wallis test for independent samples was used, as appropriate, to test the differences among groups. $P < 0.05$ was considered to indicate statistical significance.

Results

Composition and structure of fibrin scaffolds from PRGF and L-PRP

Cell content of PRGF and L-PRP preparations was measured (Figure 2A). No statistically significant differences were found in platelet counting. However, leukocyte concentration in L-PRP was 3.1 ± 0.3-fold higher than in blood while almost no white cell was detected in PRGF (0.2 ± 0.1 x10^3/ μl) (Figure 2B).

The fibrin scaffolds obtained with each type of platelet rich plasma showed different appearance and consistency when handled. PRGF fibrin scaffold was yellowish and easier to handle than the reddish one prepared from L-PRP (Figure 2C). In fact, the latter was easily broken after manipulation. Ultra structural analysis of both fibrin scaffolds showed the alteration of the fibrillar cross-linking due to the presence of cellular elements including leukocytes and erythrocytes in the L-PRP (Figure 2C).

Characterization of the PRGF and L-PRP-scaffold releasates

The different fibrin scaffolds were cultured at 37 ºC either in non-inflammatory (only culture medium: normal conditions) or inflammatory (LPS-containing) conditions. After 72 hours, several growth factors (GFs) involved in different phases of the regeneration process and the most relevant interleukins and pro-inflammatory molecules were measured. Under normal conditions, the IL-1 β concentration in the PRGF releasate was 0.0 ± 0.4 pg/ml while it was higher in the L-PRP releasate (13 ± 8.5 pg/mL). However, under inflammatory conditions, these difference increased drastically, being the IL-1 β concentration in the PRGF releasate of 111 ± 87 pg/mL and 22390 ± 16745 pg/mL in the L-PRP supernatant (Figure 3A). The presence of TNF-α and IL-6 cytokines in the PRGF and L-PRP releasates was almost non-detectable in normal conditions. However, once again, those levels were significantly higher in the case of L-PRP releasate under inflammatory conditions. TNF-α and IL-6 levels in PRGF were 66 ± 74 pg/mL and 896 ± 1131 pg/mL respectively, while in the case of L-PRP they were 1481 ± 835 pg/mL and 222100 ± 72878 pg/mL respectively (Figure 3B and C). Furthermore, a statistically significant higher IL-8 delivery from L-PRP scaffolds was measured in both analyzed conditions with respect to the PRGF scaffold releasates (129 ± 139 pg/mL *vs* 3535 ±

1791 pg/mL and 5415 ± 5207 pg/ml *vs* 2065000 ± 1067263 pg/ml, for PRGF and L-PRP under normal and inflammatory conditions, respectively) (Figure 3D).

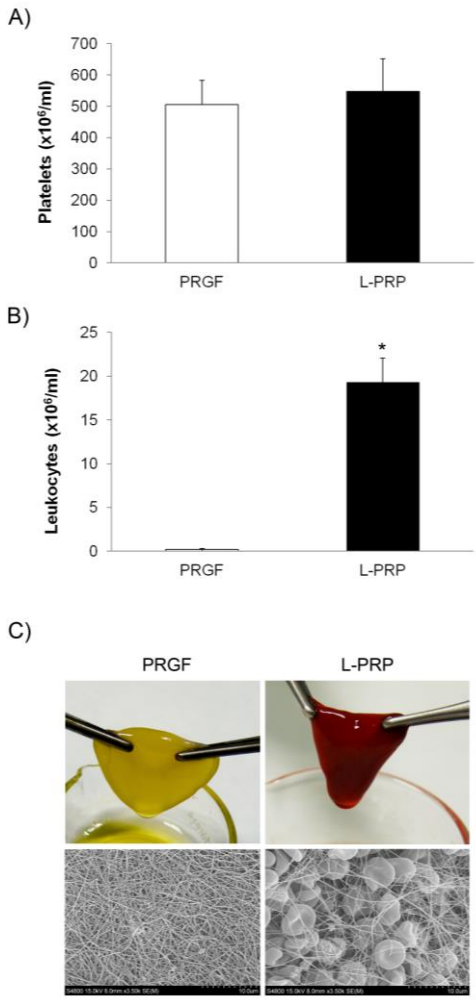

Figure 2. Composition and structure of fibrin net from both PRP products. (A) Concentration of platelets in PRGF and L-PRP preparations. (B) Leukocyte content in PRGF and L-PRP preparations. (C) Macroscopic appearance and ultraestructural composition of PRGF and L-PRP scaffolds.

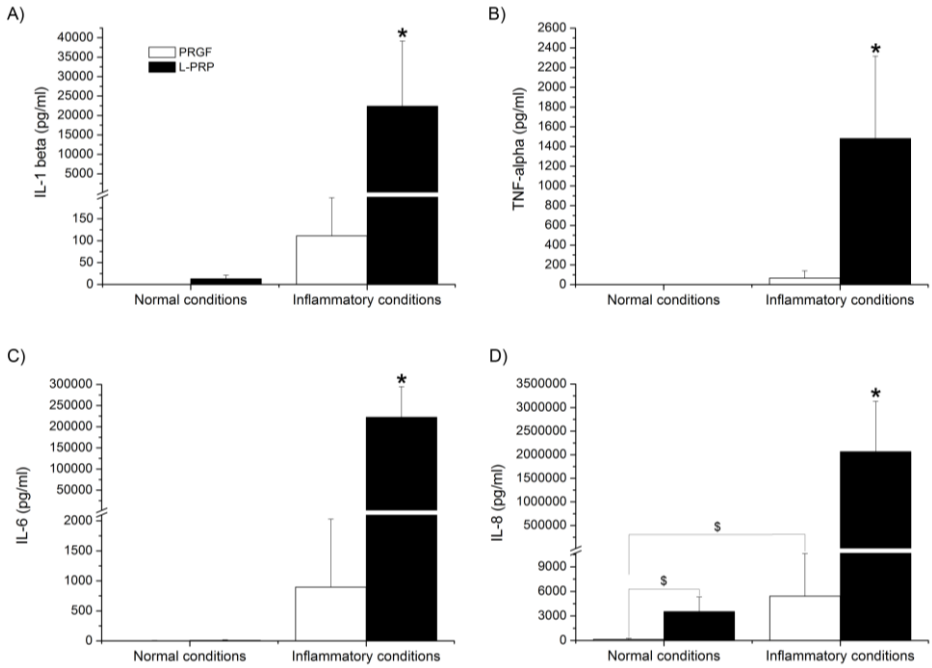

Figure 3. Characterization of the PRP scaffold releasates. Concentration of different cytokines, (A) IL1-beta, (B) TNF-alpha, (C) IL-6 and (D) IL-8 in the PRGF and L-PRP releasate under both normal and inflammatory conditions.*Statistically significant differences respect to the other treatments ($p < 0.05$). $Statistically significant differences respect to the PRGF treatment under normal conditions ($p < 0.05$).

Release of PDGF and two additional proteins with important roles in angiogenesis, Angiopoietin-1 and VEGF, were analyzed in the different releasates. In the case of PDGF-AB and angiopoietin-1, no statistically significant differences were found between PRGF and L-PRP releasates either in normal or inflammatory condition (Figure 4A and C). Nevertheless, VEGF delivery from L-PRP scaffold was significantly higher than the PRGF one, but only in the absence of inflammation (88 ± 82 pg/ml *vs* 502 ± 432 pg/ml). Interestingly, under inflammatory conditions, no VEGF was detectable in the L-PRP releasate whereas VEGF levels were maintained in the case of PRGF releasates (Figure 4B).

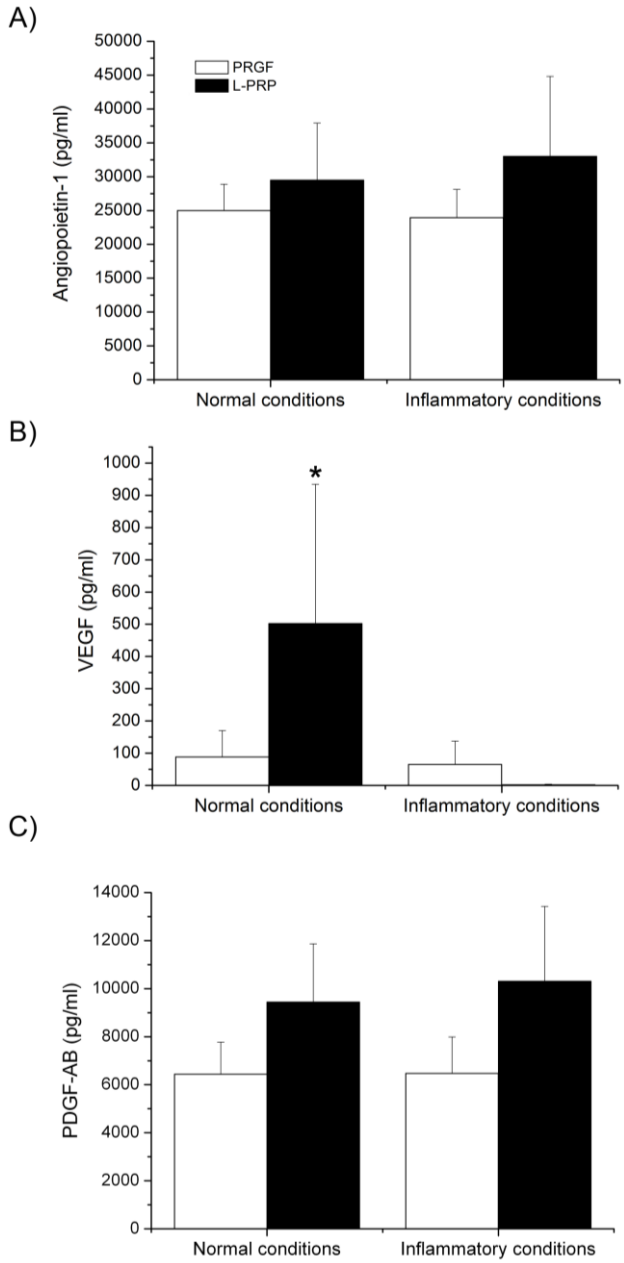

Figure 4. Characterization of the PRP scaffold releasate. Determination of different proteins concentration. (A) Angiopoietin-1 (B) VEGF and (C) PDGF-AB in the PRGF and L-PRP releasate under both normal and inflammatory conditions.*Statistically significant differences respect to the other treatments ($p < 0.05$).

PRGF and L-PRP-derived fibrin scaffolds from each of the six donors cultured with culture medium were maintained in the absence (normal condition) or presence (inflammatory condition) of LPS for 72h. PRGF fibrin scaffold preserved its stability under both normal and inflammatory conditions, while in the case of L-PRP, the fibrin scaffold were altered due to the presence of LPS (Figure 5A). In fact, half of the L-PRP fibrin scaffolds which were incubated with LPS appeared highly deteriorated after 72 hour of treatment, as it is shown in figure 5A.

MMP-1 activity was measured in the different releasates under normal and inflammatory conditions. A statistically significant higher enzyme activity was detected in the L-PRP scaffold conditioned medium under both study conditions (586 ± 128 mU/µl respect to 1168 ± 401 mU/µl for PRGF- and L-PRP-releasates under normal conditions and 745 ± 96 mU/µl respect to 1315 ± 257 mU/µl for PRGF- and L-PRP-releasates under inflammatory conditions). The detected enzyme activity in the L-PRP-releasates under inflammatory conditions was also statistically superior than the one detected in the PRGF-releasates under normal conditions (Figure 5B).

In addition, the mechanical properties of the different fibrin scaffolds were determined. For this purpose, the maximum elongation at failure was determined after stretching fibrins at a constant speed of 2mm/s. The maximum elongation achieved with the PRGF scaffolds was higher than with L-PRP ones (6.1 ± 1.7 cm *vs* 4.4 ± 1.3 cm) (Figure 5C and D).

Evaluation of cell inflammatory NF κ B pathway

Primary human gingival fibroblasts and alveolar osteoblasts were treated with conditioned media by PRGF and L-PRP scaffolds under inflammatory conditions for 24 hours. After extracting and concentrating the protein content of the treated cells, I κ B- α , p-NF κ B and NF κ B synthesis were quantified by western blotting technique. In both of the two phenotypes included in the study, a decrease in I κ B- α expression was found when cells were incubated with the L-PRP releasate compared with the ones incubated with PRGF releasates (5.1 ± 0.0 *vs* 2.9 ± 0.4 and 15.6 ± 2.1 vs 13.7 ± 2.3 for PRGF and L-PRP in gingival and bone cells, respectively) (Figure 6A y B). On the contrary, a statistically significant increase in the p-NF κ B/NF κ B ratio was observed when cells were treated with L-PRP+LPS releasate when compared with PRGF+LPS treatment (2.1 ± 0.7 *vs* 10.0

± 2.0 and 1.0 ± 0.1 *vs* 1.5 ± 0.3 for PRGF and L-PRP and in gingival and osteoblast cells respectively) (Figure 6C and D).

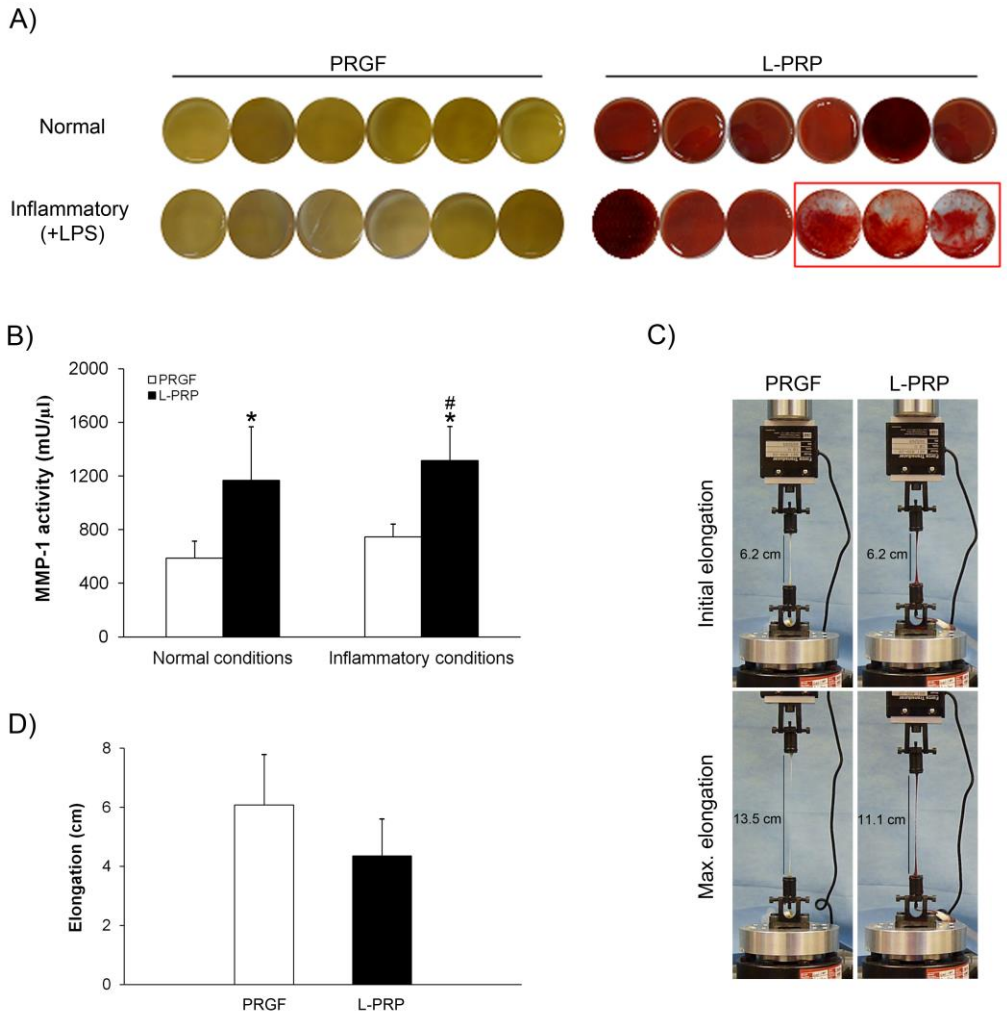

Figure 5. Analysis of the integrity and the mechanical properties of PRP scaffolds. (A) Comparison of PRP scaffolds appearance after 72 hours incubations under normal and inflammatory conditions. In half of the L-PRP matrix a great degradation was observed. (B) MMP-1 activity in PRGF and L-PRP releasates was determined under both normal and inflammatory conditions. (One U = 100 pmol/min at 37ºC, 100 µM thiopeptolide). (C) Mechanical testing station where the maximum elongation at failure of the PRP scaffolds was determined. (D) Maximum elongation of PRP scaffolds.

*Statistically significant differences between L-PRP and PRGF under normal conditions and between L-PRP and PRGF under inflammatory conditions ($p < 0.05$). #Statistically significant differences respect to the PRGF treatment under normal conditions ($p < 0.05$).

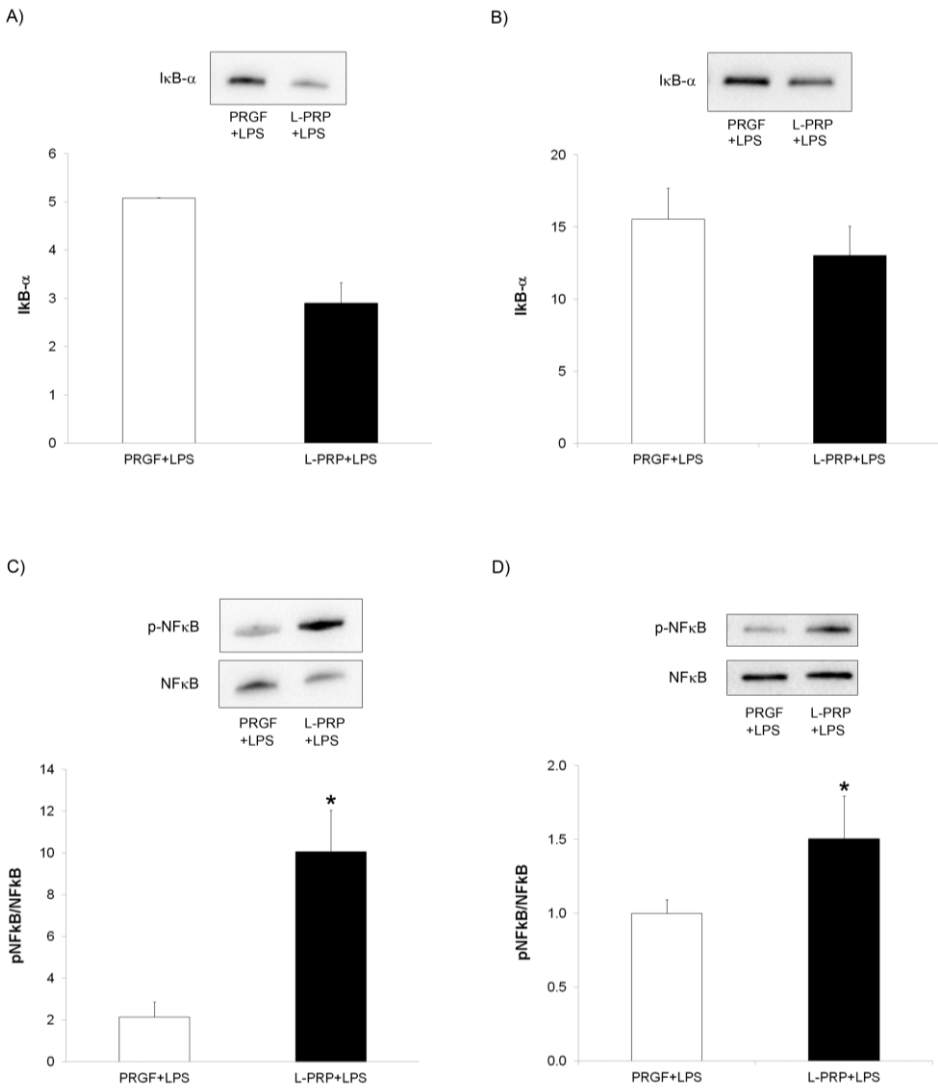

Figure 6. Analysis of the inflammatory mediators' expression by western blot. Cells were treated with PRGF+LPS and L-PRP+LPS releasates for 24 hours under inflammatory conditions and I κ B- α

and p-NF κ B/NF κ B ratio were determined. (A) I κ B- α expression in gingival fibroblasts. (B) I κ B- α expression in alveolar osteoblasts. (C) p-NF κ B/NF κ B determination in gingival fibroblasts. (D) p-NF κ B/NF κ B determination in alveolar osteoblasts. *Statistically significant differences respect to the PRGF+LPS treatment ($p < 0.05$).

Cell proliferation assay

Fibroblasts and osteoblasts were incubated with PRGF+LPS and L-PRP+LPS releasates for 72 hours and in LPS presence. Some morphological changes were observed after the treatment with L-PRP+LPS scaffold releasate, especially in the case of gingival fibroblasts. As it shown in figure 7A, cells laid out in clusters and became brighter and with a thinner cell body. In addition, a statistically significant decrease in DNA concentration was detected in gingival fibroblast and osteoblast cultures incubated with L-PRP+LPS conditioned medium under inflammatory conditions compared to the ones detected after PRGF+LPS releasate treatment (84 ± 20 *vs* 39 ± 11 and 72 ± 10 *vs* 60 ± 4 for PRGF and L-PRP in gingival and bone cell cultures, respectively) (Figure 7B and C).

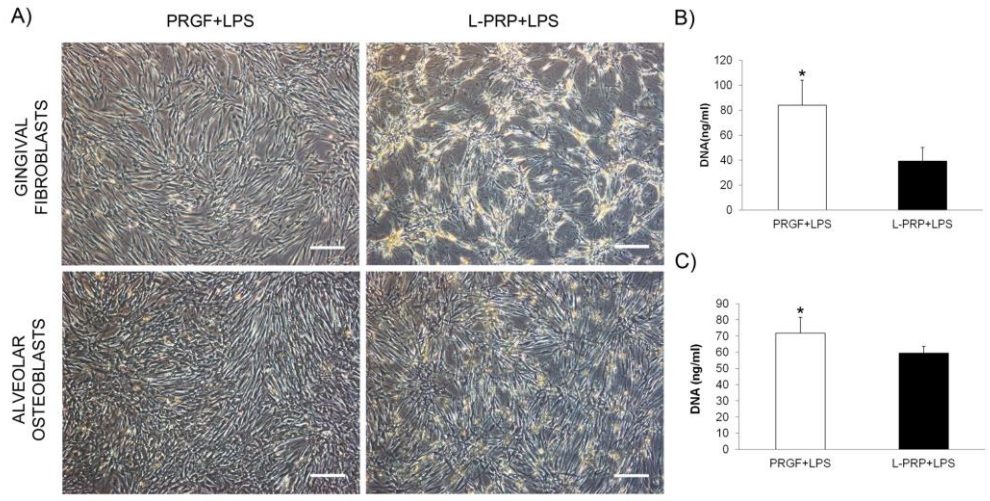

Figure 7. Effect of the releasates of PRGF+LPS and L-PRP+LPS on the proliferation of fibroblasts and osteoblast cells. (A) Morphological alterations in cultures due to 72 hour treatment with PRP releasates under inflammatory conditions. Scale bar: 300 μm. (B) Gingival fibroblast proliferation after treatment. (C) DNA quantification of alveolar osteoblast proliferation after treatment. *Statistically significant differences respect to the L-PRP+LPS treatment ($p < 0.05$).

Cell-derived cytokine expression

After treating gingival and bone cells for 24 and 72 hours with PRGF+LPS and L-PRP+LPS, the conditioned media was collected and the levels of IL-1β, TNF-α, IL-6 and IL-8 were measured. The concentration of all the pro-inflammatory cytokines was significantly enhanced after treating the cells with L-PRP+LPS releasate (Figure 8).

As it is shown in figure 8A (24 h treatment), gingival fibroblasts stimulated with PRGF released 6 ± 2 pg/mL of IL-1β while they released up to 41492 ± 3629 pg/mL when stimulated with L-PRP. Gingival cell expression of TNF-α was also significantly increased with L-PRP (500 ± 36 pg/mL) compared to PRGF (0 ± 3 pg/mL). A similar trend was observed for IL-6 and IL-8 expression. Cell culture with L-PRP significantly increased the amount of the proteins (244833 ± 87279 pg/mL for IL-6 and 1583333 ± 111430 pg/mL for IL-8) compared to those obtained with PRGF (80992 ± 23878 pg/mL and 110492 ± 7569 pg/mL for IL-6 and IL-8, respectively). A similar trend was observed after treating the cells for 72 h with PRGF or L-PRP (Figure 8B).

In a second set of experiments, the effects of the conditioned media on primary osteoblasts cells were evaluated (Fig 8C and 8D). Culturing the cells with the L-PRP media provoked the dramatic increase of the expression of the four cytokines. Interestingly, the use of a leukocyte containing PRP enhanced osteoblasts-derived release of IL-1β more than 700 times (Figure 8C). This striking increase in the synthesis of pro-inflammatory cytokines due to the leukocyte inclusion in the PRP-scaffold was also observed for the remaining three proteins. Osteoblast cell expressions of TNF-α (1152 ± 53 pg/mL), IL-6 (154333 ± 52495 pg/mL) and IL-8 (1259667 ± 124582 pg/mL) were significantly higher than those observed after PRGF treatment TNF-α (-3 ± 9 pg/mL), IL-6 (40050 ± 10300 pg/mL) and IL-8 (41056 ± 14340 pg/mL). A similar trend was observed after treating the cells for 72 h with PRGF or L-PRP (Figure 8D).

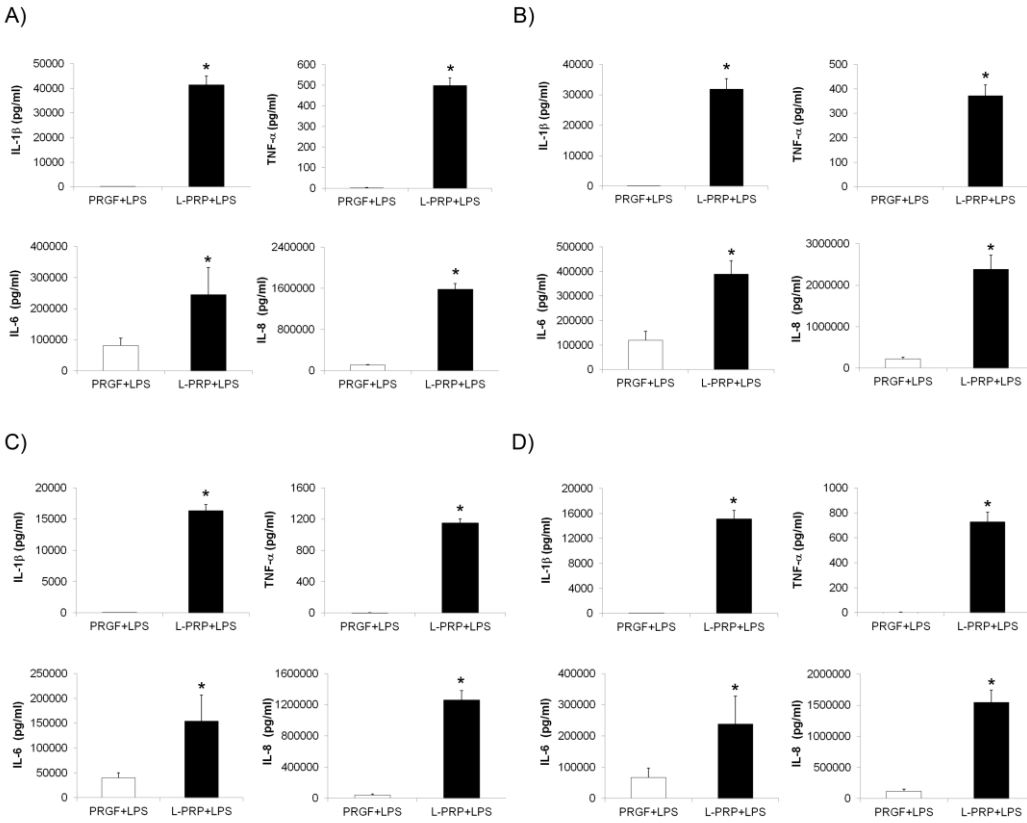

Figure 8. Pro-inflammatory cytokine synthesis due to cell treatment with PRP releasates. Gingival and bone cells were incubated with PRGF+LPS and L-PRP+LPS releasates under inflammatory conditions for 24 and 72 hours. IL-1β, TNF-α, IL-6 and IL-8 synthesis was determined by ELISA techniques. (A) Treatment of gingival fibroblast during 24 hours. (B) Gingival fibroblast response to 72 hour treatment. (C) Treatment of alveolar osteoblasts during 24 hours. (D) Bone cells response to 72 hour treatment. *Statistically significant differences respect to the PRGF+LPS treatment ($p < 0.05$).

Discussion

Periodontal disease is the most prevalent chronic inflammatory condition in humans. It is characterized by the destruction of the tooth-supporting structures, namely, soft tissue, alveolar bone, periodontal ligament and cementum [26-28]. *Porphyromonas gingivalis* is a gram-negative anaerobic bacterium that is considered to be the major etiologic agent of periodontitis. Lipopolysaccharide, a major component of the outer membrane of the gram-negative bacteria, is known to be the major virulent factor in periodontitis, as it is a potent stimulator of inflammatory mediators [26,29,30].

In the last few decades, there is growing interest in developing new cost-effective therapeutic alternatives for tissue repair and regeneration. Assuming the scarcity of human organs and tissues, there is interest in promoting new approaches that help to restore tissue homeostasis after inflammation and injury. The field of platelet rich plasma, that is, the use of patient's own proteins, growth factors and biomaterials is gaining much interest not only due to its autologous origin, but also because it is a not expensive approach with a clear translation pathway to the clinics.

In the present paper, we have addressed one of the most important concerns about PRPs; how the composition of leukocytes may impact on the final biological and mechanical properties, which are key for final therapeutic outcomes of the approach. In addition, to our knowledge this is one of the few studies that investigates the effect of PRP composition under normal and inflammatory conditions.

According to our results, the inclusion of leukocytes within PRP affects negatively the mechanical properties of the fibrin scaffolds and stimulates a more pro-inflammatory environment that is directly related with an increased cell-inflammatory condition and a reduced cell proliferation response. These effects may delay or impede a correct tissue repair or regeneration process.

The inclusion of leukocytes within platelet rich plasma products has always been a matter of debate. The deleterious effects of leukocytes have been widely described [31-34]. In contrast to platelets, white blood cells are mainly considered to contain and produce inflammatory cytokines.[31,32,35,36] The results obtained in the present study confirm

that leukocyte free PRGF is more predictable as its properties (growth factor release and fibrin scaffold integrity) are almost preserved from non-inflammatory to inflammatory conditions [27,37]. In our experiments, only the IL-8 released was found to be statistically significant different under normal conditions between PRGF and L-PRP scaffolds, being much higher in the case of L-PRP (Figure 3). However, under inflammatory conditions, the levels of IL-1β, IL-6, IL-8 and TNF-α released by L-PRP fibrin scaffolds were statistically much higher with respect to PRGF (Figure 3). Moreover, in the case of PRGF, no differences were found in protein release between normal or inflammatory conditions, except for IL-8.

Although cytokines play an important role in infection and inflammation, excessive cytokine expression may lead to tissue destruction [38,39]. Inflammatory cytokines such as IL-6 and IL-8 are believed to be the main pathological mediators in periodontal diseases. IL-6 may activate osteoclasts, promoting bone loss and causing bone resorption. At the same time, this cytokine stimulates matrix metalloproteinases, therefore increasing matrix degradation. IL-8 in turn, may induce neutrophil chemotaxis and activation [40-42]. Meanwhile, TNF-α and IL-1β have also a significant role in bone loss, playing a central role in inflammatory reaction [28,43]. In fact, IL-1β is an essential pro-inflammatory cytokine that induces the infiltration of inflammatory cells [44].

Apart from the increased enzyme activity of MMP-1 in the L-PRP-releasates under both normal and inflammatory conditions, in the latter, half of the L-PRP fibrin scaffolds were degraded (Figure 5A and B). Matrix metalloproteinases (MMPs), a family of proteolytic enzymes, are responsible for degrading the main components of the extracellular matrix. MMP-1 is a collagenase that is involved in extracellular matrix breakdown during periodontitis. The production of this enzyme is stimulated by several growth factors and cytokines such as IL-1β, TNF-α and IL-6 [42,45].

Several important differences were also observed after determining the release of growth factors from PRGF and L-PRP scaffolds. In normal conditions, the concentration of VEGF released by L-PRP scaffolds was higher than that released by PRGF scaffolds. However, under inflammatory conditions no VEGF was detected in the L-PRP fibrin-conditioned medium. The latter may be a limitation since VEGF is necessary in wound healing causing

inflammation as it promotes the early events in angiogenesis [46,47]. It is probable that activated leukocytes under inflammatory conditions are using the VEGF released mainly by the platelets from L-PRP.

Fibrin is the biological transient scaffold that it is firstly formed at injury's sites and it is commonly used in tissue engineering. The mechanical properties and biodegradation of fibrin scaffolds are essential for their role as a provision matrix in tissue regeneration approaches and as a protein delivery system [2,48-50]. Viscoelastic properties largely depend on fibrin clot structure. Here, we have reported that leukocyte addition to PRP alters the clot structure and decreases the maximum elongation of the scaffold. Higher elongations indicate a greater elasticity which involves, more malleable, easier to handle and stronger fibrins to fulfill the required function as a biomaterial that can be used as a specific shape scaffold in tissue engineering [48]. PRGF scaffold has already been used in several applications, such as the treatment of ulcers, wound closure, tissue engineering or even combined with others materials [37].

Interestingly, LPS stimulates inflammatory cells, such as neutrophils, macrophages but also resident cells such as fibroblasts and osteoblasts. Therefore, cell responses to the treatment with both types of PRP under inflammatory conditions were also evaluated. Toll-like receptor 4 (TLR4) is the main receptor in the cellular response to LPS. Ligation of these receptors initiates a cascade of events that leads to the activation of transcription factors, including nuclear factor-κB (NF-κB), that eventually induce the production of pro-inflammatory cytokines [19,30,51]. Our findings revealed that the inhibitor-κBα (IκBα expression was increased when gingival fibroblasts and alveolar osteoblasts were cultured with PRGF+LPS under inflammatory conditions (Figure 6). IκBα is considered to have an anti-inflammatory role as it maintains the NF-κB in the cytosol in an inactivated state [52,53]. These results are consistent with those observed for the p-NFκB/NFκB ratio. L-PRP+LPS induced a significantly higher phosphorylation of NF-κB p65 in both cell types (Figure 6). NF-κB p65 phosphorylation at Ser536 regulates nuclear localization, protein-protein interactions, activation of gene expression and transcriptional activity [53,54]. Moreover, IL-1β and TNF-α are cytokines that activate the NFκB pathway [52,53]. Therefore, the pro-inflammatory environment generated by L-PRP scaffolds may be behind of this increase in the activation of NFκB after cell treatment

with L-PRP+LPS. This pro-inflammatory milieu and the greater activation of NF κ B after treating the cells with L-PRP+LPS had also a negative effect on cell proliferation. Both gingival fibroblasts and alveolar osteoblasts proliferated significantly less when treated with L-PRP+LPS than when treated with PRGF+LPS (Figure7).

Finally, treatment with L-PRP+LPS also stimulated pro-inflammatory cytokine synthesis in both cell types compared with treatment with PRGF+LPS (Figure 8). This cell response was observed both at 24h and 72h. The excess of those pro-inflammatory proteins can lead to the destruction of the tissue, as previously described. This increase is related to the enhanced activation of NF κ B pathway after the treatment with L-PRP+LPS. In fact, NF κ B activation results in the production of those cytokines, which in turn, can amplify and increase the inflammatory response, thus keeping the NF κ B pathway active, establishing a positive feedback response. In the wake of this activation, the excessive pro-inflammatory cytokine release after L-PRP+LPS incubation may exacerbate the inflammatory response by stimulating the recruitment of new inflammatory cells to the injury site. This microenvironment may be conducive to a non-resolving inflammation that leads to a tissue fibrotic condition [55,56].

Conclusions

We have shown that under inflammatory conditions, white blood cells inclusion in PRP increases the delivery of pro-inflammatory cytokines, as well as stimulates the resident cells to produce a greater amount of the afore mentioned proteins. The NF κ B pathway is also stimulated in oral cells treated with L-PRP+LPS scaffold releasate under inflammatory conditions. Fibrin scaffolds mechanical properties are poorer and degradation of the fibrin mesh is promoted after inclusion of leukocytes with the PRGF. In summary, the inclusion of leucocytes within PRGF negatively affects the mechanical properties of the fibrin scaffolds and stimulates a more pro-inflammatory environment that is directly related with an increased cell-inflammatory condition and a reduced cell proliferation response, which ultimately may be detrimental for tissue regeneration.

Conflict of Interests

Some of the co-authors (EA, MT, MZ and SP) are scientists at BTI Biotechnology Institute, a dental implant company that fabricates dental implants.

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(V) PRGF exerts a cytoprotective role in zoledronic acid-treated oral cells

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PRGF exerts a cytoprotective role in zoledronic acid-treated oral cells

Eduardo Anitua*†, Mar Zalduendo†, María Troya†, Gorka Orive*

*Foundation Eduardo Anitua, Jacinto Quincoces, 39; 01007 Vitoria, Spain
† Laboratory of Regenerative Medicine, Biotechnology Institute (BTI), Vitoria, Spain
Corresponding author:
Gorka Orive.
E-mail: gorka.orive@ehu.es

Abstract

Objectives: Bisphosphonates-related osteonecrosis of the jaw (BRONJ) is a common problem in patients undergoing long-term administration of highly potent nitrogen-containing bisphosphonates (N-BPs). This pathology occurs via bone and soft-tissue mechanism. Zoledronic acid (ZA) is the most potent intravenous N-BP used to prevent bone loss in patients with bone dysfunction. The objective of this *in vitro* study was to evaluate the role of different ZA concentrations on the cells from human oral cavity, as well as the potential of plasma rich in growth factors (PRGF) to overcome the negative effects of this BP.

Material and Methods: Primary human gingival fibroblasts and primary human alveolar osteoblasts were used. Cell proliferation was evaluated by means of a fluorescence-based method. A colorimetric assay to detect DNA fragmentation undergoing apoptosis was used to determine cell death and the expression of both NF-κB and pNF-κB were quantified by western blot analysis.

Results: ZA had a cytotoxic effect on both human gingival fibroblasts and human alveolar osteoblasts. This BP inhibits cell proliferation, stimulates apoptosis and induces inflammation. However, the addition of PRGF suppresses all these negative effects of the ZA.

Conclusions: PRGF shows a cytoprotective role against the negative effects of ZA on primary oral cells.

Clinical relevance: At present there is no definitive treatment for bisphosphonates-related osteonecrosis of the jaw (BRONJ), being mainly palliatives. Our results revealed that PRGF has a cytoprotective role in cells exposed to zoledronic acid, thus providing a reliable adjunctive therapy for the treatment of BRONJ pathology.

Keywords: Zoledronic acid, bisphosphonates, oral cells, apoptosis, inflammation, plasma rich in growth factors.

Introduction

Bisphosphonates (BPs) are synthetic analogues of pyrophosphate that bind strongly to hydroxyapatite in the bone matrix and prevent bone loss due to their ability to inhibit osteoclast function. BPs are currently the first-line treatment for osteoporosis, metastatic bone cancer and Paget's disease [1,2]. These drugs can be divided into two major groups: nitrogen-containing (N-BPs) and non-nitrogen-containing bisphosphonates (non N-BPs). The former interferes with the mevalonate pathway by inhibiting farnesyl pyrophosphate synthase, the main enzyme of this pathway, whereas the latter are metabolized into compounds that replace the terminal pyrophosphate moiety of ATP, forming a nonfunctional molecule that competes with adenosine triphosphate (ATP) [3,4]. BPs are administered either intravenously (IV) (mainly pamidronate and zoledronic acid) or orally (alendronate and risedronate among others) [5] Zoledronic acid (ZA) is one of the most frequently used and potent intravenously nitrogen-containing bisphosphonates [5,6].

Bisphosphonates-related osteonecrosis of the jaw (BRONJ) is a side effect in patients undergoing long-term administration of highly potent N-BPs. In fact, it has been reported that incidence of BRONJ ranges from 0.8-12% of patients receiving IV bisphosphonates [7]. First described in 2003,[8] this condition is defined as exposed necrotic bone (indicating soft-tissue recession) in the mandible or maxilla for a period of at least 8 weeks, without radiation to the head or neck in the patient's history [1,5,7].

Most of the studies about BRONJ are focused on bone tissue [9,10]. However, oral soft tissue is extremely important, as its rupture and breakdown is necessary for the

progression of this disease. Recently, it has been observed that ZA induces a cytotoxic response in fibroblasts isolated from the gingiva and periodontal ligament [9].

The use of autologous growth factors has been proposed as an interesting alternative to the prevention and treatment of BRONJ [11-13]. Assuming that BRONJ is characterized by a delayed oral wound healing, the use of a cocktail of proteins and growth factors [14,15] might mitigate some of the negative effects of bisphosphonates and promote key biological step including cell proliferation, migration and cell viability.

Platelet rich plasma consists on a biological approach that uses patient´s own growth factor for promoting tissue repair and regeneration [16,17]. The technology of plasma rich in growth factors (PRGF) is the pioneering technology for the use of autologous proteins from human plasma and platelets for healing purposes [18] and its application has shown numerous benefits in many different fields [19].

In this study we tested the hypothesis that ZA are cytotoxic to human gingival fibroblasts and alveolar osteoblasts and that PRGF can mitigate and even overcome the negative effects of this BP in these primary cells.

Material & Methods

Cell culture

The study was performed following the principles of the Declaration of Helsinki, as revised in 2008. All experiments were performed using primary human gingival fibroblasts and primary human alveolar osteoblasts isolated as previously described [20,21]. Two primary cell culture of each phenotype were employed in all the experiments. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1 volume) (Gibco-Invitrogen, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS) (Biochrom AG, Leonorenstr, Berlin, Germany), 2 mM glutamine and 50 μg/mL gentamicin (Sigma-Aldrich, St Louis, MO, USA). Osteoblasts were cultured in osteoblast basal medium (ObM) (Sciencell Research Laboratories, Carlsbad, CA, USA) supplemented with 15% FBS and 50 μg/mL gentamicin. All the cells were maintained at 37ºC in a humidified,

5% CO_2 atmosphere. Only cells between the fourth and the sixth passages were used in the experiments.

Preparation of plasma rich in growth factors (PRGF)

Blood from one donor was collected into 9-mL tubes with 3.8% (wt/v) sodium citrate, after written informed consent was provided (approved by our Institutional Review Board). Samples were centrifuged at 580 *g* for 8 min (Endoret Dentistry, BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) at room temperature. The 2 mL of plasma just above the buffy coat was collected. Platelets and leukocytes counts were performed with a hematology analyzer (Micros 60, Horiba ABX, Montpelier, France). Plasma preparations were incubated with the activator (Endoret Dentistry) at 37ºC for 1 hour. The PRGF supernatants were collected by aspiration after centrifugation at 3000 *g* for 15 min at 4ºC. Finally, the obtained supernatant was filtered, aliquoted and stored at -80ºC until use.

Screening of ZA concentrations for proliferation assay

Zoledronic acid (ZA) (Sigma-Aldrich) was the bisphosphonate selected for the study. Fibroblasts and osteoblasts were seeded at a density of 10000 cells/cm^2 on 96-well optical-bottom black plates. The corresponding culture media were supplemented with zoledronic acid at final concentrations of 0.1, 1, 2, 3, 4, 5 and 10 μM or 0.1, 1, 5, 10, 12.5, 15, 17.5 and 50 μM for fibroblasts or osteoblasts, respectively. Treatments were maintained for 24, 48 and 96h. Control cells were incubated without the drug. All samples were assayed on triplicate. CYQUANT cell proliferation assay (Molecular Probes-Life technologies, Grand Island, NY, USA) was used according to the manufacturer's protocol.

Cell proliferation assay

The following ZA concentrations were used for the proliferation assay: 5 μM ZA and 25 μM ZA for gingival fibroblasts and alveolar osteoblasts respectively. Both fibroblasts and osteoblasts were plated at a density of 8000 or 10000 cells/cm^2, respectively, on 96-well optical-bottom black plates. Oral cells were cultured for 48h in the corresponding culture medium and ZA concentration according to their phenotype. Two experimental conditions were tested: 2% FBS+ZA and 20% PRGF+ZA. Cell proliferation of six replicates of each

sample was measured with the CYQUANT cell proliferation assay (Molecular Probes-Life technologies) according to the manufacturer's instructions.

Screening of ZA concentrations for apoptosis assay

DNA fragmentation in fibroblasts and osteoblasts undergoing apoptosis was detected with Cell Death detection ELISA^{PLUS} kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Oral cells were seeded at a density of 15000 cells/cm^2 on 24-well plates. Cells were treated with either normal culture medium (control) or with culture medium supplemented with zoledronic acid at final concentrations of 1, 2, 3, 4, 5, 7.5, 10, 12.5, 15 and 20 μM or 1, 5, 10, 15, 20, 25, 50 and 100 μM for fibroblasts or osteoblasts, respectively. Oral cells were exposed to the bisphosphonate treatments for 48 h on duplicate.

Cell apoptosis assay

After 48h incubation of primary oral cells (15000 cells/cm^2) with either ZA (10 μM or 25 μM ZA, for gingival fibroblasts or osteoblasts, respectively) and 2%FSB or ZA and 20%PRGF, DNA fragmentation of cells undergoing apoptosis was detected with Cell Death detection ELISA^{PLUS} kit (Roche Diagnostics) according to the manufacturer's protocol. Samples were analyzed on triplicate. Additionally, cell viability after 48h with the above mentioned treatments was assessed using the LIVE/DEAD® Cell Imaging Kit (Molecular Probes-Life technologies) according to the manufacturer's protocol. Cells were evaluated by fluorescence microscopy.

Analysis of Inflammation by Western blot

The quantification of two molecules involved in the NF-κB pathway was performed by Western-blot to assess the inflammatory potential of ZA on oral cells, and the ability of PRGF to mitigate or reverse those effects. Oral cells were seeded at a density of 20000 cells/cm^2 and treated with either 2% FBS, 2% FBS+ZA or 20% PRGF+ZA in the corresponding culture medium for 48h (gingival fibroblasts) or 6h (alveolar osteoblasts). The ZA working concentrations were: 10 μM ZA for fibroblasts and 25 μM ZA for bone cells.

After treatment, cells were washed with phosphate buffered saline (PBS), and lysed in mammalian protein extraction reagent supplemented with protease and phosphatase inhibitors (Pierce Biotechnology, Bonn, Germany). Lysates were clarified by centrifugation at 14000g for 10 min, and the supernatants were then collected. Following protein quantification with the BCA assay (Pierce Biotechnology), equal amounts were loaded on TGX stain-free gels (Bio-Rad Laboratories, Munich, Germany), underwent electrophoresis and were transferred to PVDF membranes. The membranes were then blocked with 5% non-fat dry milk in tris (hydroxymethyl) aminomethane-buffered saline containing 0.1% polysorbate surfactant (TBST) for 1 hour at room temperature (Bio-Rad Laboratories). Blots were incubated overnight at 4ºC with the corresponding primary antibodies, anti-nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and anti-phosphorilated NF-κB (pNF-κB p65) (Cell signaling Technology Inc, Danvers, MA, USA), and with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Cell signaling Technology) for 1 hour at room temperature. Bands were visualized by chemiluminescence with Supersignal™ West Pico substrate (Thermo Scientific-Pierce Biotechnology, Rockford, USA). An image analyzer (Chemidoc image analyzer) (Bio-Rad Laboratories) was used to detect the signal. The Stain-Free technology was used as loading control method [22].

Results

Statistical Analysis

The results were presented as mean ± standard deviations. Data were compared either by ANOVA and the Tamhane or Bonferroni-corrected post hoc tests or by the t-test. Significance was assigned at the $p < 0.05$ level.

Screening of ZA concentrations for the proliferation assay

A dose-response and time-course assays on both cell phenotypes was performed to select the appropriate ZA concentration and treatment duration for the proliferation assay. Gingival viability was not altered with none of the tested ZA concentrations in the 24-hour treatment. Nevertheless, concentrations starting from 4 μM induced a statistically significant decrease in DNA quantification after 48 hours of treatment (83 ± 14, 68 ± 14

and 48 ± 7 ng/mL for 4, 5 and 10 μM ZA respectively) and 96 hours of treatment (74 ± 2, 66 ± 8 and 26 ± 8 ng/mL for 4, 5 and 10 μM ZA respectively) (Fig. 1a).

We found statistically significant differences in osteoblast DNA quantification after cell incubation with ZA in the three treatment times. This effect was achieved with ZA concentrations starting from 12.5 μM in the 24-hour treatment (55 ± 8, 51 ± 5, 57 ± 10 and 54 ± 6 ng/mL of DNA for 12.5, 15, 17.5 and 50 μM ZA respectively), and starting from 10 μM in longer time incubations (Fig. 1b).

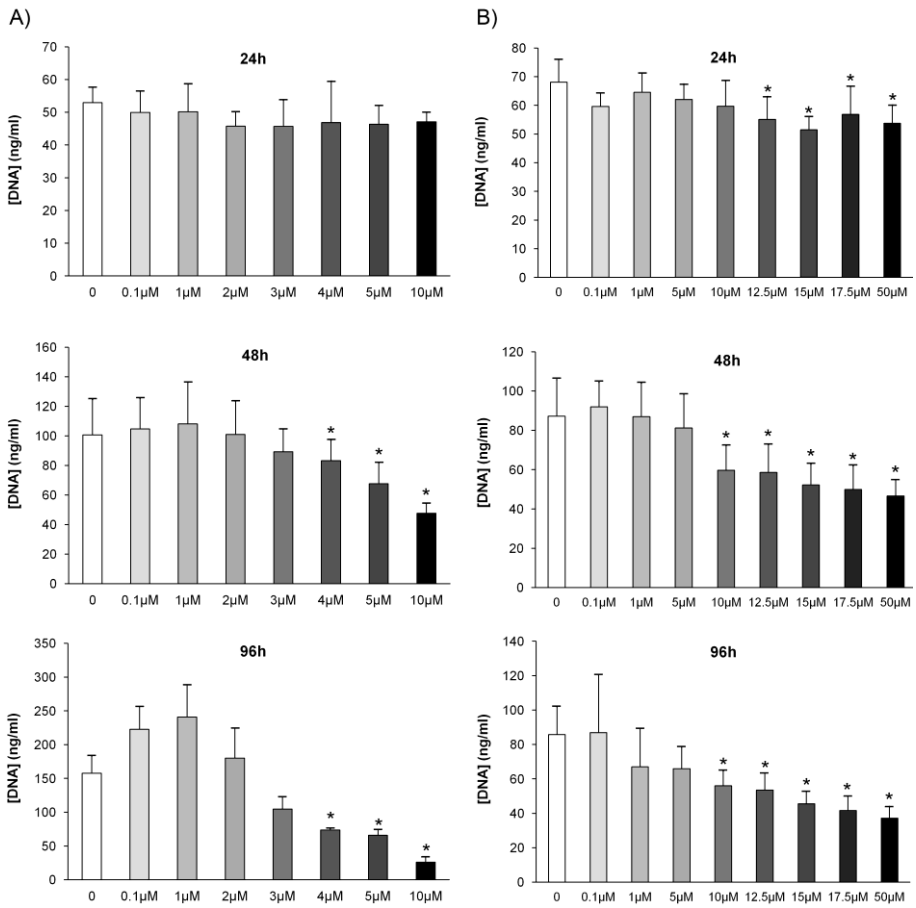

Fig. 1 Dose-dependent cytotoxic effects on cell proliferation. (a) Effect on gingival fibroblasts. (b) Effect on alveolar osteoblasts. *Statistically significant differences compared to the treatment without zoledronic acid ($p < 0.05$).

Cell proliferation assay

We tested the effects of PRGF on cell proliferation in the presence of bisphosphonates. For this purpose, gingival fibroblasts and alveolar osteoblasts were incubated with 5 μM and 25 μM ZA, respectively, during 48 hours, since cell viability was decreased in more than 30% in both situations

Considerable changes in cell morphology were induced by ZA (Fig. 2a, b). In all the cases, cells became bright, although this was more pronounced in bone cell cultures. After treatment with ZA, cell appearance suggested some kind of cellular damage (nuclei surrounded by long and very thin cytoplasmic extensions), while after PRGF treatment, oral cells seemed spindle and healthier. The cytotoxic effect of ZA on oral cells was modulated by the presence of PRGF in the culture medium. A statistically significant higher DNA concentration was detected in the cultures treated with ZA and PRGF when compared to the cultures treated with ZA alone (51 ± 4 versus 22 ± 5 and 64 ± 2 versus 52 ± 6 ng/mL in gingival fibroblasts and alveolar osteoblasts, respectively) (Fig. 2c, d).

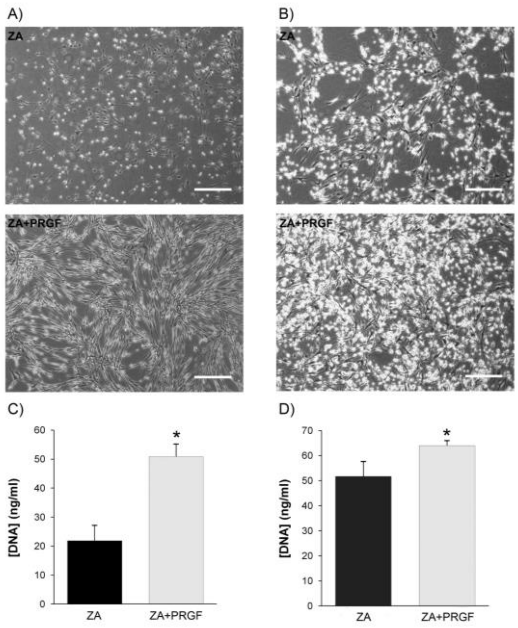

Fig. 2 Effect of PRGF addition on the proliferation of oral cell cultures treated with zoledronic acid. (a) Evaluation of morphological changes in gingival fibroblasts after 48h of treatment. (b) Evaluation of morphological changes in alveolar osteoblasts after 48h of treatment. (c) Proliferation of gingival fibroblasts after 48h with the corresponding treatments. (d) Proliferation of alveolar osteoblasts after 48h with the corresponding treatments. Scale bar: 400 μm. (ZA: treatment with zoledronic acid. ZA+PRGF: treatment with zoledronic acid + PRGF). *Statistically significant differences compared to to the ZA treatment ($p < 0.05$).

Screening of ZA concentrations for the apoptosis assay

First, we tested different increasing concentrations of ZA during 48 h on both cell phenotypes with the aim of determining the appropriate drug concentration to carry out the apoptosis assays. A statistically significant higher level of gingival fibroblasts apoptosis was detected for cells treated with ZA concentrations starting from 10 μM. Thus, 1.31 ± 0.19, 1.76 ± 0.06, 1.68 ± 0.08 and 2.21 ± 0.17 absorbance units (a.u.) were measured for 10, 12.5, 15 and 20 μM ZA, respectively, compared to 0.14 ± 0.05 a.u. obtained for the control condition (Fig. 3a). In the case of bone cells, statistically significant higher values of optical density, corresponding to higher degrees of apoptosis, were detected for concentrations of 20, 25, 50 and 100 μM (0.22 ± 0.00, 0.83 ± 0.07, 1.73 ± 0.17, and 2.36 ± 0.10 a.u., respectively) versus to the control condition (0.13 ± 0.01 a.u.) (Fig. 3b).

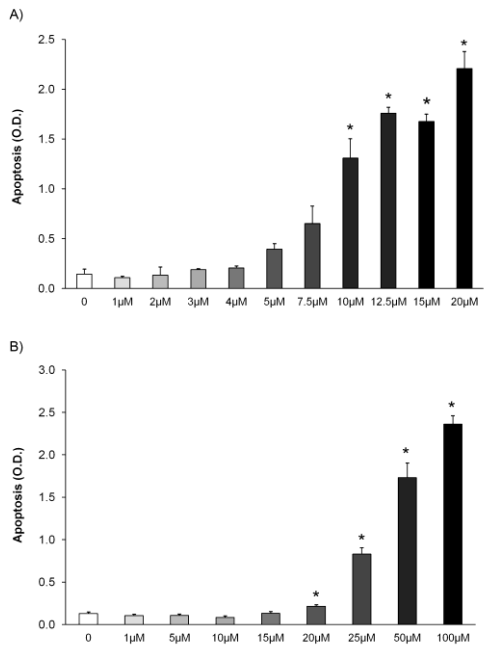

Fig. 3 Dose-dependent cytotoxic effects on cell apoptosis. (a) Effect on gingival fibroblasts. (b) Effect on alveolar osteoblasts. *Statistically significant differences compared to the treatment without zoledronic acid ($p < 0.05$).

Cell apoptosis assay

In view of the results obtained in the previous screening, we decided to treat gingival fibroblasts with 10 μM ZA and alveolar osteoblasts with 25 μM ZA, in both cases for 48h. The qualitative determination of the ratio live/dead cells is showed in figures 4a and 4b, where live and dead cells are shown as green and red, respectively. Quantitative techniques showed a statistically significant decrease in the number of apoptotic cells when fibroblasts and bone cells were treated with ZA in the presence of PRGF compared to cultures receiving the drug alone (0.25 ± 0.10 versus 0.04 ± 0.02 and 0.82 ± 0.07 versus 0.54 ± 0.08 a.u, in the case of gingival fibroblasts and alveolar osteoblasts, respectively) (Fig. 4c, d).

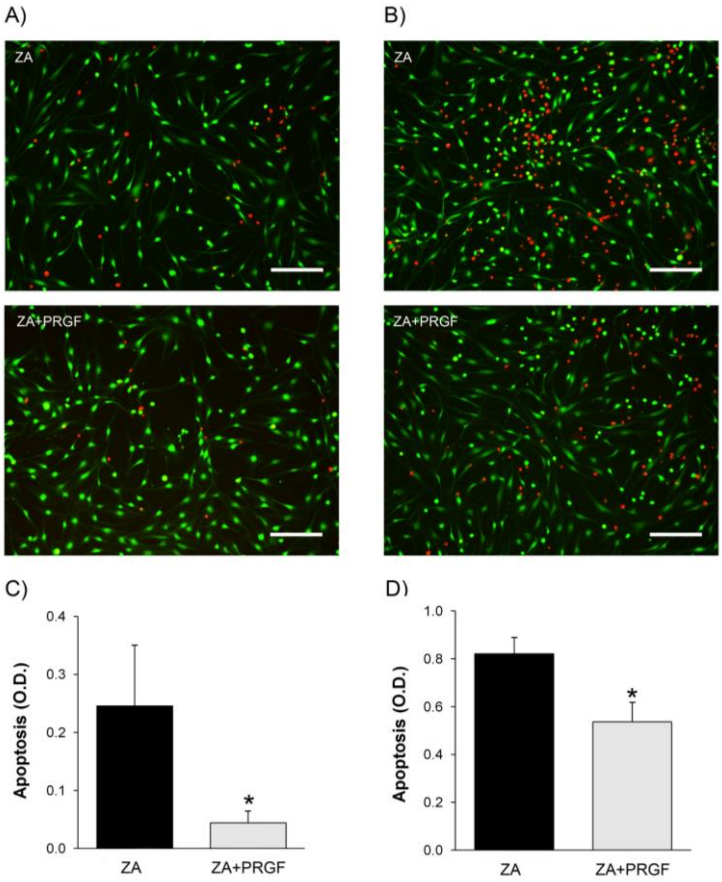

Fig. 4 Effect of PRGF addition on the apoptosis of oral cell cultures treated with zoledronic acid. (a) Fluorescence images of gingival fibroblasts after 48h of treatment. (b) Fluorescence images of alveolar osteoblasts after 48h of treatment. Live cells and dead cells. (c) Apoptosis in gingival fibroblasts after 48h with the corresponding treatments. (d) Apoptosis in alveolar osteoblasts after 48h with the corresponding treatments. Scale bar: 200 μ m. (ZA: treatment with zoledronic acid. ZA+PRGF: treatment with zoledronic acid + PRGF). *Statistically significant differences compared to the ZA treatment ($p < 0.05$).

Analysis of Inflammation by Western blot

The potential capacity of PRGF to reverse the inflammatory effect of ZA on oral cells was assayed by evaluating the main effector molecules in the NF-κB way. Results show that cell incubation with ZA induced a statistically significant increase of the pNF-κB/NF-κB ratio in both cell phenotypes. In the case of non-stimulated gingival cells, the value of this ratio increased from 1.48 ± 0.54 to 4.06 ± 0.36 after ZA addition to the culture medium. A similar response was observed in alveolar osteoblasts (0.68 ± 0.19 and 1.88 ± 0.35, for the non-stimulated cultures and ZA incubation conditions, respectively) (Fig. 5c, d). However, the addition of PRGF to the culture medium reduced the deleterious effect of this bisphosphonate. In this way, the pNF-κB/NF-κB ratio recovered the value obtained in control cultures for both oral cell phenotypes. This ratio decreased from 4.06 ± 0.36 to 1.57 ± 0.13 and from 1.88 ± 0.35 to 0.80 ± 0.22 in the case of gingival and bone cells, respectively.

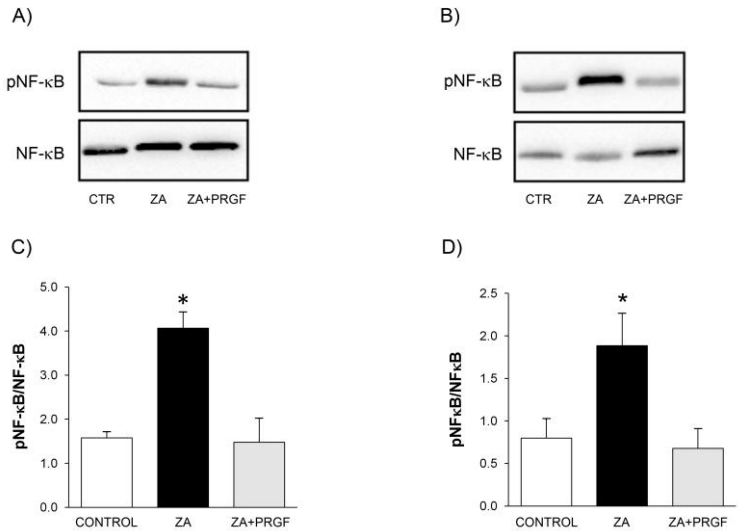

Fig. 5 Effect of PRGF addition on the expression of inflammatory mediators of oral cell cultures treated with zoledronic acid. (a) Images obtained by western blot of the NF-κB and p-NF-κB bands in gingival fibroblasts. (b) Images obtained by western blot of the NF-κB and p-NF-κB bands in alveolar osteoblasts. (c) Quantification of p-NF-κB/ NF-κB ratio in gingival fibroblasts. (d)

Quantification of p-NF-κB/ NF-κB ratio in alveolar osteoblasts (CONTROL (CTR): treatment without zoledronic acid. ZA: treatment with zoledronic acid. ZA+PRGF: treatment with zoledronic acid + PRGF). *Statistically significant differences compared to the control and ZA+PRGF treatments ($p < 0.05$).

Discussion

With the ageing population and the time life expectancy increasing, diseases such as osteoporosis and cancer are expected to increase, which in turn may imply the intake of different drugs with several side effects. Zoledronic acid is the most potent intravenous N-BP used to prevent bone loss in patients with bone dysfunction, with the osteonecrosis of the jaw (ONJ) as one of its main serious adverse effects [2]. The release of N-BPs accumulated in the jaws may also damage the surrounding oral soft tissues, reflecting that BRONJ pathology occurs via bone and soft-tissue mechanism [6,23].

The *in vitro* results presented herein show that ZA exerts a dose-dependent cytotoxic effect on proliferation and survival of human primary gingival fibroblasts and alveolar osteoblasts (Fig.1 and 3). The ability of ZA to inhibit cell growth and to promote cell death has already been described [10,23,24]. Oral fibroblasts were more susceptible than osteoblasts to ZA toxicity. In fact, a dose of 4 μM and 10 μM of ZA was sufficient to cause an adverse effect on gingival fibroblast proliferation and survival, respectively. However, in osteoblasts, ZA had to be twice concentrated to produce similar cytotoxic effects. These results may be consistent with the fact that BPs are stored in the bone and slowly released to the immediate microenvironment thus affecting local gingival fibroblasts [1,23,25], which might involve that bone exposure to ZA concentrations is several-fold higher than the ones receiving gingival fibroblasts.

PRGF is a source of growth factors including hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), transforming growth factor beta-1 (TGFβ1) and vascular endothelial growth factor (VEGF), many of which are known to stimulate cell proliferation, osteogenesis and cell survival [14,26]. Our results indicate a cytoprotective effect of PRGF in cells exposed to ZA. The use of autologous proteins and growth factors may limit the side effects of the treatment with BPs such as ZA, promoting cell proliferation and reducing apoptosis of the bisphosphonate treated oral cells (Fig. 2 and 4). Similar anti-

apoptotic effects of PRGF technology have been previously reported on a mouse model of Alzheimer's disease [27,28].

N-BPs have various inflammatory side effects that prevent wound healing in the oral cavity and lead to a persistent ONJ [23,29,30]. Inflammation is primarily a physiological and beneficial process that is also capable of injuring normal tissues [31]. However, chronic inflammation can lead to chronic non-healing wounds result in scarring and fibrosis. In fact, non-resolving inflammation is a major driver of disease [32]. The NF-κB pathway activates the transcription of many pro-inflammatory mediators. Deregulation of this pathway leads to persistent increases in the expression of several effectors associated with the perpetuation of inflammation [33].

According to our results, ZA stimulated the phosphorylation of NF-κB on both gingival fibroblasts and osteoblasts (Fig. 5). Our findings also revealed that PRGF prevented the inflammatory effect of ZA on oral cells. In fact, both cell types treated with ZA and PRGF showed a significant decrease in the activation of NF-κB p65, restoring the values obtained in absence of BPs (Fig. 5). Some of the growth factors present in this autologous technology might be the responsible for these anti-inflammatory properties of the PRGF. For example, it has been reported that HGF has the ability to suppress the inflammatory response by disrupting the transcription factor NF-κB signaling [34,35]. There are other growth factors such as insulin-like growth factor (IGF-1) and PDGF-BB that may also work as anti-inflammatory agents by suppressing interleukin-1β-induced NF-κB signaling [36].

Taking into account all the effects derived from the PRGF treatment, it seems reasonable to believe that this autologous plasma preparation could be involved in several processes due to its great complexity. Since N-BPs inhibits the mevalonate pathway, future research will make it possible to evaluate the potential PRGF direct interaction with this pathway. Regarding this matter, the isoprenoid geranylgeraniol (GGOH) has been reported as a possible therapeutic approach due to its capacity to reverse the negative effects of bisphosphonates by inhibiting the above mentioned pathway [37], therefore it could be very interesting to evaluate the combination of both treatments in further experimental research.

At present there is no definitive treatment for BRONJ, with prevention playing a significant role. In fact, treatments are mainly palliative with the aim of relieving the main symptoms of the disease. Antibiotics, hyperbaric oxygen, wound debridement, surgical resections, sequestrectomy, temporary BP treatment cessation are the predominant therapies used to manage this pathological condition [38,39]. Recently, it has been reported the clinical use of PRGF as an adjunctive therapy for the treatment of BRONJ, thus improving wound healing, enhancing vascularization and osseous and epithelial tissues regeneration [11,12,40,41]. The in vitro findings obtained in this study may be behind of these good clinical data.

In summary, the continuous release of ZA from bone tissue and the effects that this BP produce on the proliferation, apoptosis and inflammation of both bone and soft tissue, prevent the spontaneous resolution of wound healing. Here, we show that PRGF exerts a cytoprotective role that reduces or limits the ZA-triggered negative effects on oral cells and that are behind the BRONJ pathology. Nevertheless, further research is needed to elucidate the exact mechanisms and growth factors implied in the cytoprotective function of PRGF against BP-induced side effects.

Compliance with Ethical standards

Funding: This study was funded by Pharmascreen Saiotek project (Basque Government).

Conflict of interest: The authors declare the following competing financial interest(s): EA is the Scientific Director and MZ and MT are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology.

Ethical approval: The study was performed following the principles of the Declaration of Helsinki, as revised in 2008 and after approval from the Foundation Eduardo Anitua Institutional Review Board.

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

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(VI) Autologous PRGF technology for isolation and ex vivo expansion of human dental pulp stem cells for clinical translation of cell therapy

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Enviado

Autologous PRGF technology for isolation and *ex vivo* expansion of human dental pulp stem cells for clinical translation

Eduardo Anitua[1,2], Mar Zalduendo[1†] and María Troya[1†]

[1]BTI—Biotechnology Institute, Vitoria, Spain, and [2]University Institute for Regenerative Medicine and Oral Implantology UIRMI, UPV/EHU—Fundación Eduardo Anitua, Vitoria, Spain

[†]These authors contributed equally to this work.

Corresponding author:

Eduardo Anitua
eduardo@fundacioneduardoanitua.org

ABSTRACT

This study investigated the use of the autologous technology of plasma rich in growth factors (PRGF) as a human-based substitute to fetal bovine serum (FBS) in the culture of human dental pulp stem cells (hDPSCs). hDPSCs cultures isolated and maintained with PRGF showed a significantly higher number of cells per explant than FBS cultures. Cell proliferation, migration, osteogenic mineralization and adipogenic differentiation were found to be significantly higher in PRGF than FBS. The use of either FBS or PRGF did not show significant differences in cell senescence or in cell viability after freezing. The autologous PRGF technology could be a suitable and safer substitute for FBS as a culture medium supplement for clinical translation of cell therapy.

Keywords: Dental pulp stem cells (DPSCs), plasma rich in growth factors (PRGF), non-animal alternatives.

INTRODUCTION

Stem cell therapy has emerged as an alternative approach for the repair of damaged or lost tissues in a wide variety of diseases. In particular, mesenchymal stem cells (MSCs) are the most promising candidates among adult stem cells due to their accessibility and lack of ethical concerns [1-3]. They are typically obtained from many organs and tissues including, bone marrow, adipose tissue, brain, skin, teeth, skeletal muscle and heart [2,4,5]. Among these tissues, dental pulp is a cranial neural crest derived tissue enclosed into a dental cavity surrounded by mineralized dentin [6]. In 2000, Gronthos et al. [7] isolated for the first time, human dental pulp stem cells (hDPSCs) from the pulp tissue of third molars. hDPSCs have an easy surgical access as they can be collected from discarded permanent teeth and harvested following non-invasive isolation methods thus, representing a valuable source of readily accessible stem cells with special biological properties of MSCs and neural crest stem cells [8-10]. Since MSCs occur scarcely in tissues, an *ex vivo* expansion following good manufacturing practices (GMP) guidelines is required to yield a suitable dose in order to achieve therapeutic outcomes [2,11,12]. Currently, many experimental concerns are raised for human cell therapies related to the establishment of standardized xeno-free culture protocols in order to improve safe and reproducibility [13].

Fetal bovine serum (FBS) has long been employed as a widely accepted standard cell culture supplement for both research and clinical use [14]. However, the use of this animal-derived product entails several safety and regulatory concerns. FBS poses a great batch to batch variability, hence hampering reproducibility and making standardization of the production process difficult. Another issue is the risk of contamination with harmful pathogens such as viruses, mycoplasms or prions among others. In addition, limited availability, ethical issues regarding animal welfare or the induction of host immunologic reactions have also been raised [12,13,15-17]. To address all these risks, regulatory authorities, industry and the research community demand suitable alternatives to provide safe, regulated and effective cell therapy products to patients [18]. Currently, many autologous or allogenic human blood-derived products such as human serum [19,20], platelet rich plasma [21], platelet lysate [14,22-24] or umbilical cord blood serum [25,26] are being explored as potential FBS replacement.

In this sense, the technology of plasma rich in growth factors (PRGF) is the pioneering technology for the use of autologous growth factors, proteins and biomaterials from human plasma and platelets for healing purposes [27,28]. PRGF is a platelet rich plasma mainly characterized by the absence of leukocytes thus avoiding the promotion of inflammation. In addition, calcium chloride is used to activate PRGF rather than bovine thrombin, thus leading to a more biocompatible and safe technology. Multiple formulations can be obtained from this autologous technology [29,30] which confers a great versatility to be widely applied in different medical fields (maxillofacial surgery, dermatology, ophthalmology and orthopaedics [28,31]), as well as to be used for *ex vivo* stem cell expansion [32].

The development of standardized xeno-free culture protocols that maintain cell viability, phenotype and the potential of differentiation, represents one of the main milestones of cell therapies for clinical translation. In this study, the use of PRGF as a human-based substitute to FBS in the isolation, expansion, senescence, cryopreservation and differentiation potential of hDPSCs populations has been investigated.

MATERIAL & METHODS

Cell isolation and culture

The study was performed following the principles of the Declaration of Helsinki, as revised in 2013. Normal impacted wisdom teeth were obtained from 14 to 16 year old healthy patients, after written informed consent of donor parents was given. Three primary human dental pulp stem cells (hDPSC) cultures were isolated using the explant method which is briefly described below. Tooth surfaces were cleaned and mechanically fractured around the cementum-enamel junction. After gently separating, the pulp tissue from the crown and the root was minced into fragments of 1-2 mm^3 that were placed into 6-well plates and cultured in Dulbecco's modified Eagle's medium (D-MEM)/F-12 (1:1 volume) (Gibco-Invitrogen, Grand Island, NY, USA) with 2 mM glutamine, 50 µg/mL gentamicin and 2.5 µg/mL amphotericin B (Sigma-Aldrich, St. Louis, Missouri, USA) (from here on referred as isolation medium) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Leonorenstr, Berlin, Germany). Cells growing out from the pulp explants were maintained

at 37ºC in a humidified, 5% CO_2 atmosphere and medium was changed every 2-3 days. When reaching 70% – 80% confluence, cells were detached with animal origin-free trypsin-like enzyme (TrypLE select enzyme) (Gibco-Invitrogen, Grand Island, NY, USA) and cryopreserved or sub-cultured for further experiments. Cell viability was assessed by trypan blue dye exclusion. Since then, cell cultures were maintained with isolation medium without amphotericin (onwards, culture medium) supplemented with 10% FBS. Except for senescence assays, cells between third and sixth passages were used in the experiments.

hDPSCs characterization

hDPSC populations before passage 4 were characterized by flow cytometric analysis by the expression of cell surface antigens following the standard criteria established for the ISCT [33]. Concisely, detached cells were placed in 5 ml tubes (2.5 x 10^5 per antibody) and washed twice with stain buffer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After finishing the blocking time, cells were incubated with fluorescinated antibody in the same blocking buffer at 4 ºC for 1 hour and protected from light. Antibodies against CD73, CD90, CD14, CD19, CD45 and IgG1 (all of them FITC-conjugated), CD105, CD34 and IgG1 (APC-conjugated) and HLA-DR and IgG2a (PE-Cy5- conjugated) were used to phenotypic identification. All antibodies were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). After washing twice with stain buffer, hDPSC were fixed in 1% paraphormaldehyde in stain buffer and they were preserved in this solution and protected from light until their analysis by flow cytometry. The expression profile was analyzed by a Gallios flow cytometer (Beckman-Coulter, High Wycombe, Buckinghamshire,UK).

Preparation of plasma rich in growth factors (PRGF)

After written informed consent was provided, blood from 5 donors was collected into 9-mL tubes with 3.8% (wt/v) sodium citrate. Citrated venous blood was centrifuged at 580 *g* for 8 min (Endoret Dentistry, BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) at RT and the plasma column just above the buffy coat was collected. Platelets, erythrocytes and leukocytes counts were performed with a hematology analyzer (Micros 60, Horiba ABX, Montpelier, Occitania, France). Plasma preparations were activated following the manufacturer's instructions and PRGF supernatants were collected by aspiration after

centrifugation at 1000 *g* for 10 min at RT. After being filtered, obtained PRGF supernatants were stored in aliquots at -80ºC until use.

PRGF effectiveness in hDPSC isolation

To evaluate the efficiency of PRGF supernatant in the isolation process, primary hDPSC cultures were obtained from normal impacted wisdom teeth from 5 healthy patients (16 – 20 years of age), after written informed consent was provided. The protocol described in "*Cell isolation and culture"* section was followed except for that only one fragment of pulp tissue was placed into each well of 48-well plate. Therefore, eight pulp tissue pieces of similar size from each donor were achieved and maintained in culture medium supplemented with 10% FBS (4 fragments) or 10% PRGF (the other 4 fragments). In this last case, primary cultures were incubated with PRGF obtained from different donors.

Explant cultures were followed up in order to determine the day that cells were observed for the first time in each well and the number of explants from which cells grew out. When hDPSCs cultures of one of the eight wells reached subconfluence stage, cells from all wells were harvested and counted in a hemocytometer.

Analysis of the migration potential

Passage 3 human DPSCs obtained from 2 donors were seeded at high density (21,000 cell/cm^2) in culture inserts (Ibidi GmbH, Planegg/Martinsried, Munich, Germany) previously placed on a 24-well plate. Inserts comprise two cell growth areas and a gap between them. Cell cultures were maintained with culture medium supplemented with 10% FBS until confluence. Insert devices were then carefully removed and two cell monolayers, leaving a cell-free gap of 500 ± 50 μm, were created. Cells were washed with phosphate-buffered saline (PBS) and incubated with culture medium supplemented with either 10% FBS or 10% PRGF from three donors and in triplicate. After 24 hours, treatments were removed and cells nuclei were stained with 1/500 Hoechst 33342 (Molecular Probes-Thermo Fisher Scientific, Waltham, Massachusetts, USA) in PBS for 10 minutes. Phase contrast images of the central part of the gap were captured after removing the insert devices just before adding the treatments, and phase contrast and fluorescence images of the same area were captured after 24 hours of incubation using a

digital camera (Leica DFC300 FX, Leica Microsystems, Wetzlar Hesse, Germany) coupled to an inverted microscope (Leica DM IRB). The gap area was measured and migratory cells were counted using the Image J Software (NIH, Bethesda, Maryland, USA). Results were expressed as the number of cells migrated per square millimeter of area.

Cell proliferation assay

Cell proliferation was evaluated by Cyquant cell proliferation assay (Molecular Probes-Thermo Fisher Scientific). hDPSCs at passage 3 from two primary cultures were seeded on 96-well optical-bottom black plates at a density of 4,000 cells/cm^2 and they were maintained with cultured medium supplemented with either 10% FBS or 10% PRGF from three different donors for 24, 48, 72 and 96 hours. Five replicates of each primary cell culture for each time and treatment were assayed according to manufacturer's instructions. Briefly, after removing the treatments, wells were carefully washed with PBS. To promote cell lysis, the microplates were then frozen at -80ºC until assayed. After thawing the plates, RNase A (1.35 Ku/ml) were added into wells and cell lysates were incubated with enzymatic solution for 1 hour at room temperature. Then, the same volume of 2x GR dye/cell-lysis buffer was added to each sample and incubated at room temperature for 5 minutes with gently agitation and protected from light. Sample fluorescence was measured with a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies GmbH&Co, Bad Wildbad, Baden-Wurtemberg, Germany). Results were transformed into DNA concentration using a DNA standard curve, which was included in each assay.

Trilineage differentiation

Osteogenic differentiation

Osteogenic differentiation was induced on 2 hDPSCs primary cultures. Cells at passage 3 were seeded on 48-well plates at a density of 4,000 cells/cm^2 and maintained in culture medium with 10% FBS. After reaching 50% - 70% confluence, the medium was switched to different treatments, using the reagents supplied in the *Human Mesenchymal Stem Cell Functional Identification Kit* (R&D Systems, Minneapolis, Minnesota, USA) and following manufacturer's instructions. To compare the osteogenic differentiation potential of FBS

versus PRGF, cells were incubated with culture medium supplemented with 10% FBS or 10% PRGF from three donors, plus 1x osteogenic supplements containing dexamethasone, ascorbate-phosphate and β-glycerolphosphate. The culture medium was changed every 3-4 days and all treatments were assayed in triplicate.

After 28 days, mineralization was visualized by Alizarin red staining of calcium deposits. Briefly, cells were fixed with 4% paraphormaldehyde in PBS for 30 minutes at RT. After washing twice with ultrapure water, cells were incubated with 1.4% alizarin red solution (pH 4.1 ± 0.1) (Millipore Corporation, Burlington, Massachusetts, USA) for 5 minutes. Finally, cells were washed three times with ultrapure water. Before and after staining, phase contrast images of cell cultures were captured using a digital camera (Leica DFC300 FX) coupled to an inverted microscope (Leica DM IRB).

To quantify calcium deposition by differentiated cells, cetilpyridinium chloride (Sigma-Aldrich) was added into wells and incubated in gently agitation until the monolayer was colorless. After achieving a complete elution, 100 μl of this solution from each well were transferred into wells of 96-well plates. Absorbance at 540 nm was measured in an iEMS Reader (Thermo Labsystems Thermo Fisher Scientific). Measurements were made in duplicate.

Adipogenic differentiation

For adipogenic differentiation, cells at passage 3 from 2 hDPSCs primary cultures were seeded on 96-well optical-bottom black plates and on 24-well plates at a density of 10,000 – 15,000 cells/cm2, respectively. Cells were maintained in culture medium with 10% FBS until reaching 100% confluence Then, the medium was replaced with the different treatments analyzed using the reagents supplied in the Human Mesenchymal Stem Cell Functional Identification Kit. Cells were incubated for 35 days with Minimum Essential Medium Eagle-Alpha Modification (αMEM) (Sigma-Aldrich) with 2 mM glutamine, 50 µg/mL gentamicin, 1x adipogenic inductors (containing hydrocortisone, isobutylmethylxanthine and indomethacin) and supplemented with 10% FBS or 10% PRGF

as appropriate, being the latter obtained from three donors. The culture medium was changed every 3-4 days and all treatments were assayed in triplicate.

After adipogenic differentiation in 24-well plates, the intracellular accumulation of neutral lipids was confirmed by HCS LipidTOX red staining. Briefly, after fixing cells with 4% paraphormaldehyde in PBS, 1:100 LipidTox solution (Molecular Probes-Thermo Fisher Scientific) was added and incubated for 30 min. Cells nuclei were stained with 1/500 Hoechst 33342 in PBS for 10 minutes. Fatty acid-binding protein 4 (FABP4) was also detected in adipogenic differentiated hDPSCs using reagents that were included in the differentiation kit (Human Mesenchymal Stem Cell Functional Identification Kit.) and following the manufacturer's indications. Finally, Hoechst 33342 was used to stain cells nuclei following the previously described protocol. After adipogenic differentiation in 96-well optical-bottom black plates, total cellular concentrations of triglycerides were determined by a coupled enzyme assay (Adipogenesis Assay Kit, Sigma-Aldrich) in accordance with the manufacturer's instructions. The resulting fluorimetric products were measured with a fluorescence microplate reader (Twinkle LB 970).

Chondrogenic differentiation

Chondrogenic differentiation was induced in 2 hDPSC primary cultures by using the "pellet culture" technique that was described in the Human Mesenchymal Stem Cell Functional Identification Kit. In brief, 2.5 x 105 cells at passage 5 were placed in each 15 ml-tube and pelleted into micromasses by centrifugation. Then, 0.5 ml of treatment was added into them. Pellets were incubated with culture medium supplemented with ITS (insulin, transferring, selenious acid, bovine serum albumin and linoleic acid), chondrogenic supplements (dexamethasone, ascorbate-phosphate, proline, pyruvate and recombinant transforming growth factor beta 3) and with or without 10%PRGF from three different donors. The culture medium was changed every 2-3 days. Four replicates of treatments and controls were assayed.

After 42 days of culture, one pellet of each treatment was harvested, fixed in 4% paraformaldehyde, dehydrated with ethanol, cleared with xylene substitutes and finally embedded in paraffin. Chondrogenic differentiation was confirmed by alcian blue staining of 5 μm thickness sections followed by nuclear fast red counterstaining.

To quantitatively assess the success of the chondrogenic differentiation,, an assay kit for measurement of sulfated glycosaminoglycans (sGAGs) (AMS Biotechnology (Europe) Limited, Abingdon, Oxfordshire, UK) was used in the remaining pellets. For this purpose, the pellets were digested with an enzymatic solution containing 300 μg/ml of papain during 1 hour and 20 minutes at 60 ºC, following manufacturer's instructions for the papain digestion procedure. Finally, samples were transferred into a 96-well microplate, and assayed in duplicate. After adding the same volume of 1,9 dymethylmethylene blue (DMB) dye, absorbance was measured at 540 nm in an iEMS Reader.

In the three differentiation assays, hDPSCs cultured without differentiation inducers in the corresponding medium were included as negative controls.

Cellular senescence assays

To compare the effect of PRGF versus FBS on the cellular senescence, passage 4 hDPSCs were cultured in parallel until passage 11 with culture medium supplemented with either 10% FBS or PRGF obtained from three donors. For this purpose, primary cultures obtained from 2 donors were included. Senescence-associated β-galactosidase activity and the relative amounts of telomerase were measured in the cultured cells of that replicative age.

For senescence-associated β-galactosidase activity detection, cells were seeded on 48-well plates at a density of 10,000 cells/cm^2. After 24 hours, the *Senescence cells histochemical sataining kit* (Sigma-Aldrich) was used to identify blue-stained senescent cells following manufacturer's instructions. The different cellular conditions of the two hDPSC primary cultures were assayed on triplicate.

40,000 cells/cm^2 hDPSCs that were maintained from passage 4 to passage 11 with either FBS or PRGF and hDPSCs at passage 4 were seeded on 48-well plates. After culturing for

24 hours, the colorimetric cell-based ELISA CytoGlow™ kit (AssaybioTech, Fremont, California, USA) was used to detect telomerase protein, following manufacturer's indications. Briefly, after fixing, cells were incubated with the anti-telomerase antibody overnight at 4ºC and then for 1.5 hours with the HRP-conjugated anti-mouse IgG antibody. A colored solution was generated after the incubation with the substrate which absorbance was measured in an iEMS Reader (OD_{450}). Results were normalized with the crystal violet cell staining of those same wells, proportional to cell counts. For this purpose, the die was extracted with a SDS solution and absorbance at 540 nm was measured (OD_{540}). The measured OD_{450} readings were normalized using OD_{540} values via the proportion OD_{450} / OD_{540}. Cell conditions were assayed in triplicate.

hDPSCs cryopreservation

The effectiveness of the cryopreservation medium was assessed by the evaluation of two key aspects: the percentage of living cells immediately after thawing and the percentage of adherent cells in culture after 24 hours from defrosting. The effect of PRGF versus FBS in these two processes was tested. With this intention, hDPSCs obtained from 3 donors and between 4th and 5th passage were included in the assay.

In brief, subconfluent cultures were harvested and identical volume (125 μl) and cell concentration (285 cells/μl) were gradually frozen with different cryopreservation media consisting of 40% culture medium, 50% FBS or PRGF obtained from three donors and 10% dimethyl sulfoxide (DMSO). Four replicates of cryopreserved cells with each freezing medium were stored in liquid nitrogen. After 24 hours and 1 month, 2 cryovials were thawed at 37 ºC for 1 min. Firstly, 3 aliquots of the frozen cells suspension were reserved for living cell counting by trypan blue dye exclusion in a hemocytometer. Living and dead cells were counted and results were expressed by percentage of living cells. Identical remaining volumes of cells were immediately seeded into 24-well plates and maintained in culture medium supplemented with 10% FBS for 24 hours. Then, hDPSCs were detached and the number of adherent cells was counted. Taking into account the seeding volume, results were expressed by percentage of living cells that were able to adhere.

Statistical Analysis

The results were presented as mean ± standard deviations. After checking the normal distribution (Shapiro Wilk test) and homoscedasticity (Levene test), differences were analyzed using either the t-Student or the analysis of variance (ANOVA) test, with Bonferroni-corrected post hoc test, as appropriate. In those cases in which parametric tests were not applicable, significant differences were analyzed using Mann-Whitney or Kruskal-Wallis tests. Significance was assigned at the $p < 0.05$ level.

RESULTS

Immunophenotypic characterization

Isolated dental pulp stem cells exhibited a spindle-shaped morphology in culture (fig. 1 A and B). Flow cytometric analysis was conducted to confirm the mesenchymal surface marker expression. The DPSCs cultures were positive for MSCs markers CD73 (99.9 ± 0.1%), CD90 (99.6 ± 0.3%) and CD105 (99.5 ± 0.6%). Cells were also negative for CD14 (0.4 ± 0.1%), CD19 (0.6 ± 0.1%), CD34 (0.6 ± 0.1%), CD45 (0.2 ± 0.1%) and HLA-DR (1.0 ± 0.1%) (fig. 1C and D).

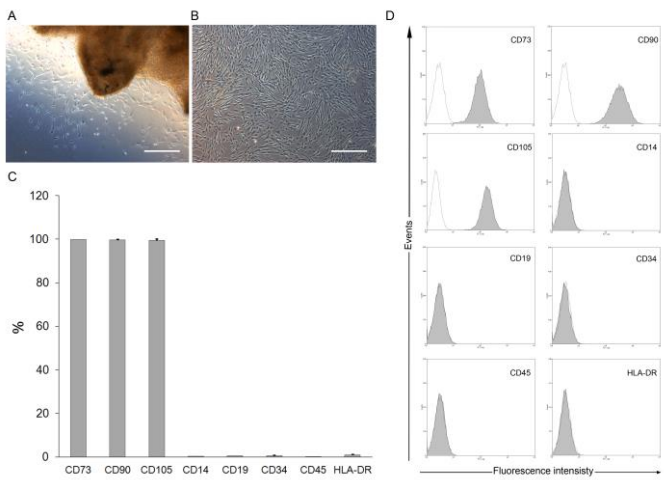

Figure 1. Isolation and culture of hDPSCs (A and B respectively). Immunophenotype analysis of primary hDPSCs by flow cytometry (C and D). Staining with isotype controls (open histograms) and with specific antibodies (filled histograms). Scale bars = 500 μ m.

Comparative isolation

Isolation capabilities of both supplemented media were evaluated. No significant differences were found regarding the number of days that the cells took to leave the explants (13 ± 5 days and 11 ±3 days for FBS-supplemented medium and PRGF-supplemented medium, respectively). There were also no differences in the percentage of explants from which cells were obtained. However, dental pulp cultures supplemented with PRGF showed a significant increase in the number of cells that were obtained per explant (9458 ± 7370 cells/explant and 19520 ± 5534 cells/explant for FBS and PRGF-supplemented medium, respectively) (fig. 2).

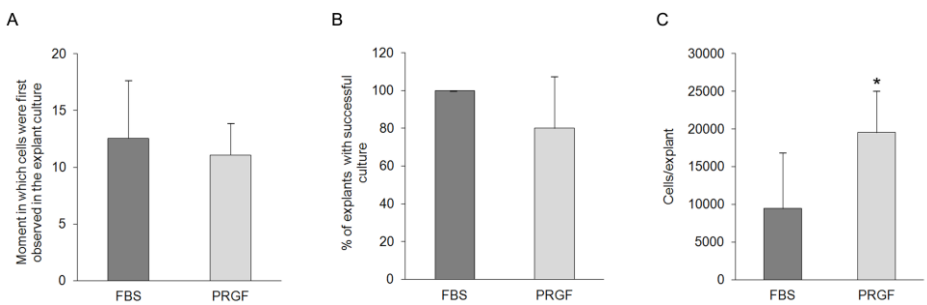

Figure 2. Comparative evaluation of the PRGF versus FBS effectiveness in the hDPSCs isolation process. Graphics represent the day in which cells were observed for the first time in the explants culture (A), the percentage of explants from which cells emerged (B), and the number of cells counted per well at the end of the culture time. *Statistically significant differences compared to FBS group ($p<0.05$).

Proliferation and migration assay

Cell proliferation analysis of primary hDPSCs cultures was performed to determine the influence of the supplement protocols. There was no significant difference in cell proliferation after treatment with both culture supplements (FBS and PRGF) for 24 and 48h. However, after 72 and 96h, statistically significant differences were observed. hDPSCs cultured with PRGF-supplemented medium showed a statistically higher DNA concentration than hDPSCs cultured with FBS (133 ± 18 ng/ml *vs.* 158 ± 23 ng/ml and 211 ± 38 ng/ml *vs.* 314 ± 64 ng/ml for FBS and PRGF-supplemented medium for 72

and 96h, respectively). Plateau phase was not reached within the experimental assay time (fig. 3A). Figure 3B shows the migratory capacity of hDPSCs after treatment with both supplements for 24h. PRGF-supplemented medium enhanced significantly cell migration 2.5 ± 0.6-folds with respect to cells cultured with FBS as supplement (fig. 3B).

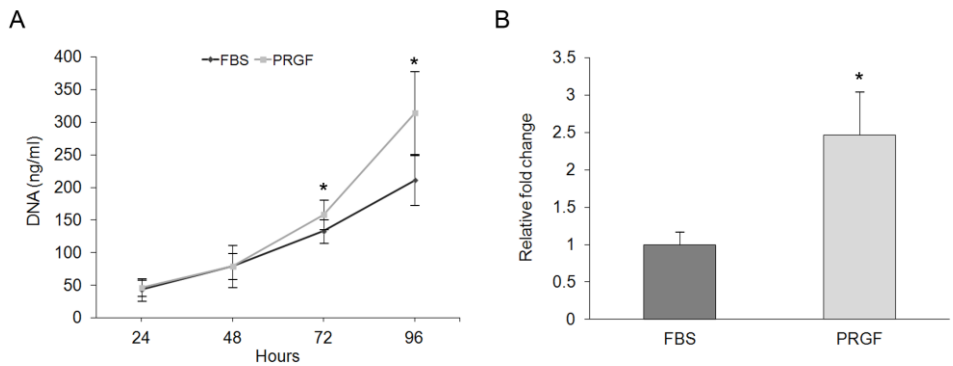

Figure 3. Proliferation (A) and migration (B) of primary hDPSCs cultured with either FBS or PRGF-supplemented medium. *Statistically significant differences compared to FBS group ($p<0.05$).

Trilineage differentiation

hDPSCs cultured with differentiation media and supplemented with either FBS or PRGF demonstrated differentiation into the three lineages, as confirmed by positive alizarin red S (osteogenic), LipidTOX and FABP4 (adipogenic) and alcian blue staining (chondrogenic) (fig. 4B, 4E and 4G, respectively). Not-induced cells were negative for the aforementioned staining (data not shown).

Osteogenic differentiation

An *in vitro* osteogenic differentiation assay was performed for four weeks to determine the effects of both culture supplements (FBS and PRGF). Visible differences were observed in the extracellular matrix mineralization (fig. 4A). The alizarin red S staining confirmed that mineral deposition was more prominent in the hDPSCs cultured with the osteogenic medium supplemented with PRGF (fig. 4B). Alizarin red elution revealed that calcium deposits significantly increased when hDPSCs were cultured with the osteogenic medium

supplemented with PRGF up to 1.731 ± 0.742-folds more than when cultured with FBS-supplemented medium (fig. 4C).

Adipogenic differentiation

hDPSCs were induced towards the adipogenic lineage. After 35 days of treatment, the neutral lipid stain LipidTox revealed the formation of cytoplasmic lipid droplets with both culture supplements (FBS and PRGF) (fig. 4D). Immunohistochemical staining also detected FABP4 expression in both treatments (fig. 4E). Quantitative analysis by a fluorimetric assay showed a significantly higher triglycerides concentration in hDPSCs cultured with the adipogenic medium supplemented with PRGF with respect to the cells cultured with FBS as supplement (2.29 ± 1.03-folds) (fig.4F).

Chondrogenic differentiation

An *in vitro* chondrogenic differentiation assay was performed for 42 days to determine the effects of PRGF addition. The alcian blue staining confirmed the chondrogenic induction with differentiation media both with and without PRGF addition (fig. 4G). No significant differences were detected in the quantitative analysis of sGAGs in hDPSCs cultured with the chondrogenic medium supplemented with PRGF with respect to the cells cultured without PRGF as supplement (1.89 ± 1.65 and 1.00 ± 0.33, respectively) (fig. 4H).

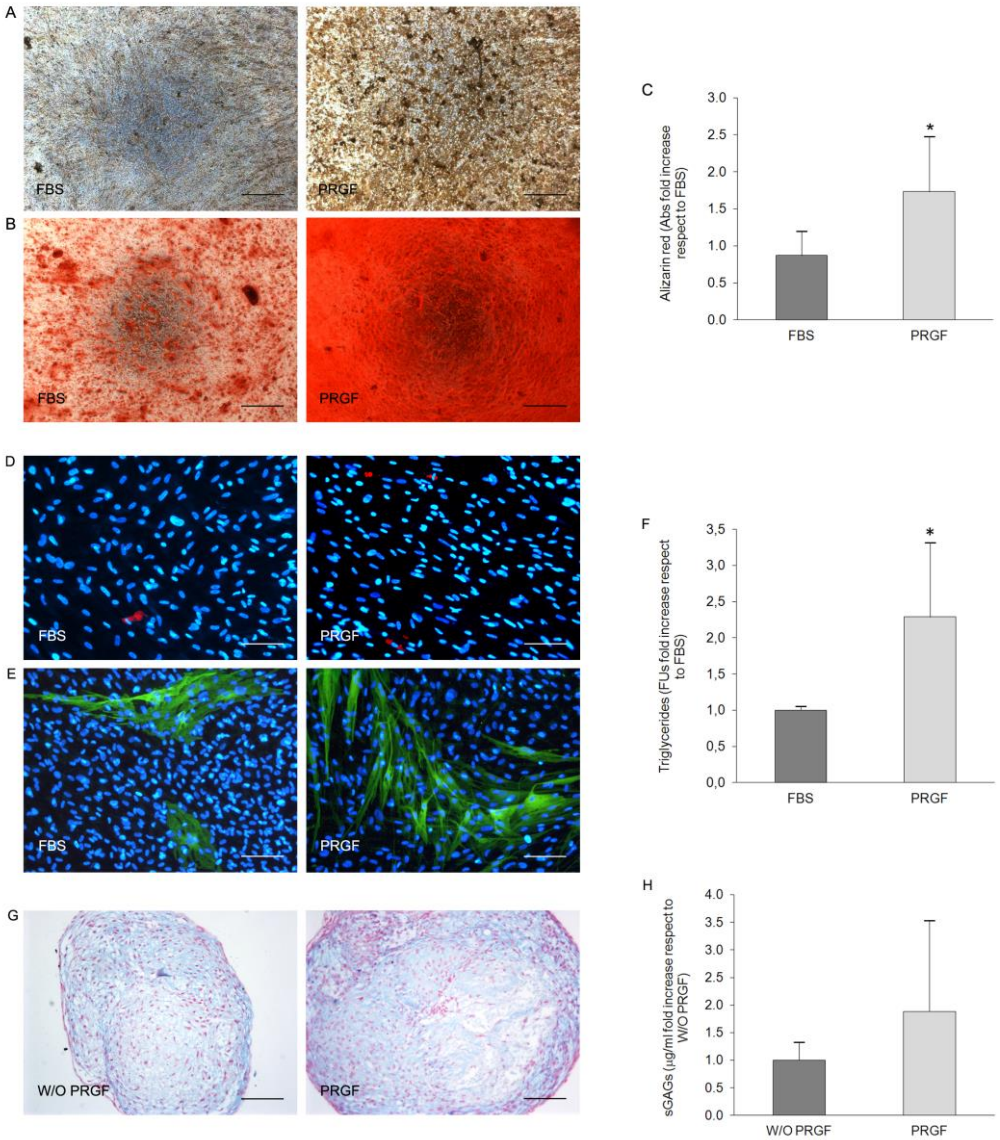

Figure 4. Multineage differentiation capacity of hDPSCs: osteogenic (A, B and C), adipogenic (D, E and F) and chondrogenic (G and H) differentiations. Phase contrast images showing the appearance of the mineralized matrix obtained after 28 days of culture with osteogenic inductors and FBS or PRGF supplements (A). Alizarin red staining of the calcium deposits (B). Alizarin red elution with cetilpyridinium chloride to quantify calcium deposition by differentiated cells (C). *Statistically significant differences compared to FBS group ($p<0.05$). HCS LipidTOX red staining of the

intracellular accumulation of neutral lipids after adipogenic differentiatiation (D). FABP4 detection by immunofluorescence in the hDPSCs differentiated toward the adipogenic lineage with FBS or PRGF supplements (E). Total cellular concentrations of triglycerides determined in hDPSCs after 35 days of adipogenic differentiation (F). *Statistically significant differences compared to FBS group ($p<0.05$). Alcian blue staining of hDPSCs pellet sections after 42 days of chondrogenic differentiation (G). sGAGs measurement in papain digested pellets to quantitatively assess the level of the chondrogenic differentiation (H). Scale bars = 400 μm (A, B and G) and 100 μm (D and E).

Cell senescence

At early passages all DPSCs were morphologically similar with a high proliferation rate. Conversely, as cells were increasing in passages, proliferation decreased and changes in cellular size and morphology were detected. High passage cells were larger with a flat and stellate appearance. In addition, senescence-associated β-galactosidase (SA-β-galactosidase) staining revealed low levels of cell senescence in hDPSCs at fourth passage. However, a significant increase in the number of positive cells for SA-β-galactosidase were observed in hDPSCs at eleventh passage regardless of the supplement used (fig. 5B and C). Moreover, these results were in line with those obtained after telomerase quantification. Significantly higher telomerase values were detected in hDPSCs at fourth passage with respect to hDPSCs values at eleventh passage cultured with either FBS or PRGF (1.845 ± 0.247 *vs.* 1.072 ± 0.384 and 0.936 ± 0.098 for hDPSCs at fourth passage and hDPSCs at eleventh passage cultured with FBS-supplemented medium and PRGF-supplemented medium, respectively) (fig. 5A). In fact, no significant differences were observed between the cells maintained until passage 11 with culture medium supplemented with either FBS or PRGF.

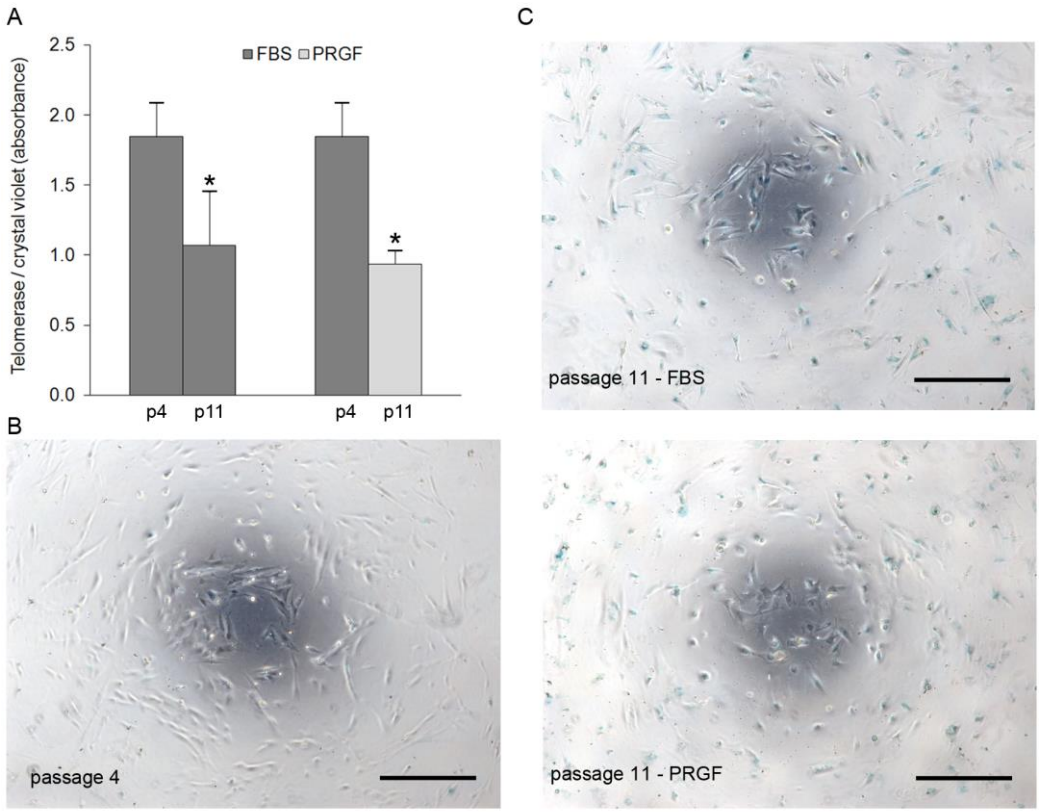

Figure 5. Effects of prolonged *in vitro* culture expansion on primary hDPSCs. Telomerase activity at both early (p4) and later passages (p11) (A). Representative images of senescence associated β-galactosidase (SA-β-gal) staining (B and C). Scale bars = 500 μm. *Statistically significant differences compared to hDPSCs at passage 4 ($p<0.05$).

Cryopreservation

Both FBS and PRGF were also investigated as a supplement for hDPSCs cryopreservation. Cells were stored up to one month prior to recovery and evaluation. Upon thawing, no differences were detected in the mean hDPSC viability, as determined by trypan blue staining. After 24h of freezing, the percentage of live cells was 76 ± 15% and 77 ± 16% for FBS and PRGF-supplemented medium, respectively. Viability did not decrease with the freezing time with none of the supplements (79 ± 10% and 86 ± 6% for FBS and PRGF-supplemented medium, respectively, after 1 month of storage in liquid nitrogen) (fig. 6A).

After viability determination, cells were cultured overnight to assess the ability of cells to adhere after cryopreservation. No statistically significant differences were detected in the percentage of adherent cells regardless of time and freezing supplement (76 ± 13% *vs.* 70 ± 21% and 99 ± 19% *vs.* 77 ± 26% for FBS and PRGF-supplemented medium and 24h and 1 month of cryopreservation, respectively) (fig. 6B).

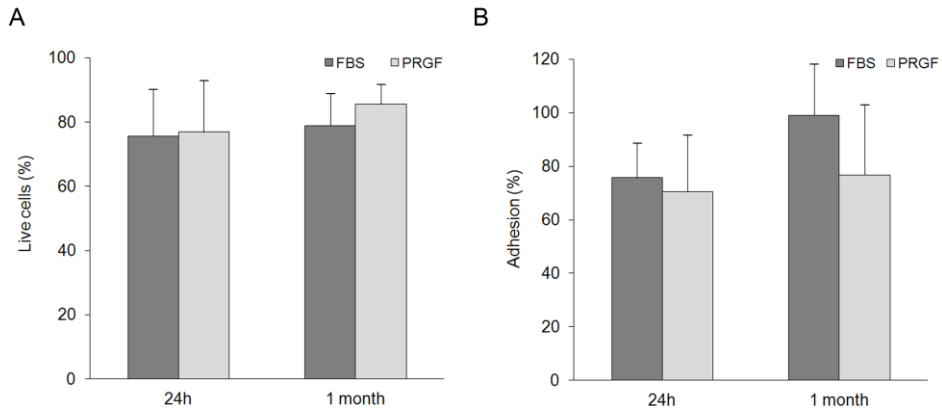

Figure 6. Effect of both culture supplements (FBS and PRGF) on cryopreservation for at least 1 month. Determination of cell viability (A) and cell adhesion (B) after thawing and plating, respectively.

DISCUSSION

In recent years, mesenchymal stem cell-based therapies have been developed to improve surgical techniques aimed at repairing human tissues. In this context, hDPSCs represent a valuable source of these progenitor cells due to the noninvasive nature of the isolation methods and to their high plasticity and multipotential capabilities [34]. Administering therapeutically meaningful numbers of hDPSCs in patients requires extensive *in vitro* cell propagation while preserving their functional properties. One of the main challenges to achieve a safer therapy is the use of animal-derived free products to avoid undesirable complications. In this sense, the objective of the present work is to assess the use of PRGF as a FBS substitute in the culture media for isolation, expansion, stimulation of the specific phenotype differentiation and finally, cryopreservation of hDPSC cells.

In previous studies, donor age-related biological properties have been described [35,36]. To avoid possible divergences in the different primary hDPSCs cultures responses non attributable to the culture medium supplements, similar aged donors were selected. On the other hand, impacted teeth were chosen to minimize the risk of pulp tissue contamination by oral microorganism, enabling to skip additional steps to samples disinfection with chemicals. In addition to this, in line with good manufacturing practice guidelines, the explant method was used for cell isolation as the obtained cells are considered "minimally manipulated" by the Food and Drug Administration. Moreover, this technique would be easier, faster, safer and cheaper than enzymatic dissociation.

There are multiple studies comparing the safety and efficacy of platelet rich plasma (PRP) versus FBS as a stem cell culture media supplement for *ex vivo* procedures. Among the most recent ones it should be mentioned the promising PRGF technology as an alternative to the actual xenogeneic supplementation in the cell therapy for corneal diseases [37]. The results of that study are in line with those obtained in the present work. Our study showed that PRGF supplement could be used as FBS substitute in the culture medium to successfully isolate hDPSCs since no differences were found in the percentage of explants from which cells grew out. Differences were neither detected in the moment in which cells were first observed in the explant culture. Conversely, the number of cells per explant that were recovered when PRGF was included in the culture medium was significantly higher than in the case of FBS. This fact was in agree with the outcomes obtained in the proliferation assay. Our results correlate with previous works in which PRPs were proved like suitable substitutes for FBS for long-term maintenance of hDPSCs [38,39]. In fact, Muraglia et al., confirmed an increased cell proliferation of several primary cell cultures promoted by PRP-derived supplements, as well as stem cell cultures maintained their biological stemness properties [24]. Similar effects have been described in dental pulp stem cells cultures after treating with PRPs [39,40]. In particular, the stimulation of cell proliferation by PRGF was also described in stem cells obtained from several sources like bone marrow, eye or adipose tissue [37,41,42]. PRGF offers an autologous source of biologically active molecules being able to synergistically stimulate not only proliferation but also cell motility. In this respect, an increase of 2.5 folds in the number of migratory cells per mm^2 was achieved in the presence of PRGF in comparison with the same percentage

of FBS in the culture medium. Supporting these results, a highly increased cell migration capability of epidermal stem cells incubated with PRP has been recently observed [43], the same effect has been described in adipose-derived stem cells after treating with PRGF [42].

The PRGF potential to stimulate hDPSCs differentiation was assessed as a quality control of the *in vitro* expanded cells. Osteogenic, adipogenic and chondrogenic differentiation were accomplished. Moreover, osteogenic and adipogenic differentiation capability was even improved in the presence of PRGF compared to FBS. As chondrogenic differentiation can be achieved in absence of FBS [44-46], the addition of PRGF was assessed to determine a possible improvement in this differentiation capability. Regardless of PRGF inclusion, results showed that no statistically significant differences were found in the potential of chondrogenic differentiation.

Numerous studies have also confirmed the contribution of PRPs in the differentiation potential of several human stem cells phenotypes to be applied in different regenerative therapies. In this sense, adipose-derived stem cell (ASC) differentiation into myofibroblast-like cells [47] as well as towards chondrogenic and osteogenic phenotypes [48,49] have been induced with differentiation culture medium containing PRP. Another clear example is the chondrogenic induction of bone marrow-derived stem cells (BMSCs) by PRP [50]. Takahashi et al. [41] recently described an increased alkaline phosphatase activity with a stronger Alizarin red S staining in BMSCs when PRGF was added.

Concerning to dental pulp stem cells, the potential of PRP to improve the differentiation processess has been assessed in multiple experimental works. Vasanthan et al., already showed the expression of several hepatic markers after substitution of FBS by PRP derivates in the differentiation culture medium of dental pulp stem cells cultures [51]. Similarly, the efficacy of PRP on promoting osteogenic differentiation of dental pulp stem cells has been proved [22,39,40]. Recently, Otero et al. have quantified a higher DPSCs osteogenic induction by PRP in comparison with other differentiation reagents [52].

Another possible risk to keep in mind with respect to stem cell therapies is the cellular alteration that could be observed because of expanding cell cultures for a long time, in particular, those related to mitotic arrest. One of the main causes for cellular senescence

has been attributed to progressive telomere shortening [53,54]. Detectable amounts of telomerase in hDPSCs cultures have been quantified in our assays, but probably they are not sufficient to fully maintain telomere length in order to avoid the decrease of proliferation capability and cellular senescence. Moreover, lower amounts of this protein have been detected in hDPSCs at late passages. According to these results, higher telomere lenghts in DPSCs with high proliferative capacity compared to DPSCs with low proliferative rate have been recently described by Alraies et al. [19,55]. In our cellular senescence assays, we did not found differences between high passages DPSCs which were maintained with PRGF or those cultured with FBS as culture medium supplement. Taking into account the improvement in the proliferation capacity of hDPSCs when PRGF is added to the culture medium, it would not be necessary to expand cells until advanced passages for obtaining the appropriate number of cells.

In the case of hDPSCs are not to be used immediately, cryopreservation could be chosen as another option. There are previous reports on human DPSCs cryopreservation for different periods of time using DMSO as cryoprotectant agent [56,57]. Results in the present work confirmed that the use of either PRGF or FBS in the freezing medium, did not show differences with respect to the cell viability after thawing. Moreover, their capability to adhere to culture flasks was neither altered. In this sense, Alsulaimani et al. showed the successful cryopreservation of human DPSCs maintaining their stemness properties for 2 years but using FBS in the freezing medium [58].

Taking all these results together, PRGF represents a promising alternative to FBS for isolate, culture and cryopreservate stem cells from dental pulp. Even more, PRGF would be a potent stimulator of hDPSCs capacities to proliferate, migrate and differentiate, aimed at a specific therapeutic therapy.

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CONFLICT OF INTEREST

The authors declare the following competing financial interests: E.A. is the Scientific Director of and M.Z. and M.T. are scientists at BTI Biotechnology Institute, a dental implant company that investigates in the fields of oral implantology and PRGF-Endoret technology.

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DISCUSIÓN

DISCUSIÓN (IV, V y VI)

La periodontitis es una de las enfermedades inflamatorias crónicas más ubicuas que compromete la integridad de los tejidos que dan soporte al diente, incluyendo la encía, el ligamento periodontal y el hueso alveolar. Se caracteriza por una respuesta inflamatoria e inmune del huésped a una infección bacteriana que conduce finalmente a la destrucción de los tejidos periodontales [227-229]. Existen cada vez más evidencias que sugieren la asociación de la periodontitis con otros tipos de enfermedades inflamatorias sistémicas como la aterosclerosis, la diabetes o la artritis reumatoide [227-230].

La microbiota oral bacteriana incluye más de 700 especies diferentes, con aproximadamente 400 de ellas encontradas en la placa subgingival [228]. Por lo tanto, es muy probable que la periodontitis no provenga de un patógeno individual sino de la sinergia polimicrobiana y la disbiosis que perturba la homeostasis del tejido periodontal [227,229]. Entre las más de 400 especies bacterianas que viven en la cavidad oral, un complejo bacteriano llamado "complejo rojo" compuesto por *Porphyromonas gingivalis, Treponema denticola y Tannerella forsythia* se ha asociado fuertemente con esta patología [231]. En este contexto, *P. gingivalis* actúa como un agente patógeno clave. El lipopolisacárido, un componente principal de la membrana externa de las bacterias gram negativas, es el principal factor virulento en la periodontitis, ya que es un potente estimulador de mediadores inflamatorios [232-234].

La terapia periodontal regenerativa está diseñada para restaurar la estructura y función del periodonto, promoviendo así la formación de nuevo cemento, hueso alveolar, ligamento periodontal y gingiva. Se han utilizado una amplia variedad de técnicas y biomateriales para regenerar tejidos periodontales. Sin embargo, la regeneración periodontal no se ha logrado completamente con los enfoques terapéuticos actuales. El uso autólogo tanto de factores de crecimiento derivados de las plaquetas como de matrices de fibrina, representa un concepto interesante en la evolución de la medicina personalizada. El uso del plasma rico en plaquetas ha surgido en este sentido como terapia para la reparación tisular. Sin embargo, la falta de estándares aceptados para definir dichos productos autólogos ha limitado significativamente la traslación con precisión de sus resultados a la práctica clínica. Y lo que es aún más importante, la incoherencia en los resultados

obtenidos a partir de diferentes PRPs puede contribuir a conclusiones contradictorias con respecto a la eficacia general de estas terapias autólogas [235]. A pesar de que existen numerosos parámetros en los que pueden diferir las diferentes preparaciones de PRPs, la inclusión o no de leucocitos ha sido una de las principales fuentes de debate en la literatura [235,236]. Esta preocupación se debe principalmente a los posibles efectos proinflamatorios de los glóbulos blancos.

En el presente estudio se ha evaluado el efecto biológico de la tecnología PRGF, pionera en el uso de plaquetas autólogas [23], en un modelo experimental de periodontitis, abordando a su vez el efecto de la inclusión de leucocitos en sus propiedades biológicas y mecánicas que son clave para los resultados terapéuticos finales. Para simular la situación patológica inflamatoria de la periodontitis se utilizaron lipopolisacáridos de *Porphyromonas gingivalis*. De acuerdo con los resultados obtenidos, la inclusión de leucocitos afecta negativamente a las propiedades mecánicas de la matriz de fibrina y estimula un entorno más proinflamatorio que está directamente relacionado con un aumento en la condición celular inflamatoria y con una reducida respuesta proliferativa. Estos efectos pueden retrasar o incluso impedir el proceso de regeneración. Tal y como se ha mencionado, la inclusión de leucocitos dentro de los PRPs siempre ha sido un tema de debate y sus efectos nocivos han sido ampliamente descritos [205,237-239]. Los resultados obtenidos en el presente trabajo, confirman que el PRGF (libre de leucocitos) es más predecible puesto que sus propiedades (liberación de factores de crecimiento e integridad de la matriz de fibrina) se mantienen prácticamente inalteradas entre el modelo no inflamatorio y el inflamatorio [240,241]. Bajo condiciones “normales”, sólo en el caso de la citoquina IL-8 se detectaron diferencias estadísticamente significativas entre el PRGF y el PRP con leucocitos (L-PRP), siendo los niveles de esta citoquina más elevados en este último. Sin embargo, en condiciones inflamatorias, esas diferencias fueron detectadas en un mayor número de citoquinas. Los niveles de IL-1β, IL-6, IL-8 y TNF-α liberados por las matrices de fibrina del L-PRP fueron estadísticamente superiores a los niveles liberados por las fibrinas de PRGF, en presencia de LPS. A pesar de que las citoquinas juegan un papel importante en la infección y la inflamación, una expresión excesiva puede conducir a la destrucción del tejido [242,243]. Se ha descrito que los niveles de ciertos mediadores inflamatorios, como IL-1 β, TNF-α y prostaglandina E2, se correlacionan con el grado de

daño periodontal y pueden agravar la respuesta inflamatoria [227]. De hecho, la interleuquina IL-1β es una citoquina proinflamatoria esencial que induce la infiltración de células inflamatorias [244]. Así mismo, y junto con el factor TNF-α, desempeña un papel esencial en la pérdida ósea [245,246]. Por otra parte, la citoquina IL-6 puede activar a los osteoclastos, promoviendo también la pérdida ósea y causando resorción. Al mismo tiempo, esta citoquina estimula a las metaloproteasas (MMPs), aumentando la degradación de la matriz. La citoquina IL-8, a su vez, puede inducir la quimiotaxis y activación de los neutrófilos [247-249].

La actividad enzimática de la metaloproteasa MMP-1 liberada por las matrices de fibrina del L-PRP fue de nuevo estadísticamente más elevada que en el caso de la MMP-1 liberada por la matriz de PRGF, tanto en condiciones normales como en condiciones inflamatorias. Este incremento en la actividad enzimática se vio reflejado en un aumento en la degradación de las fibrinas derivadas del plasma enriquecido con leucocitos en condiciones inflamatorias. Las metaloproteasas de matriz son una familia de enzimas proteolíticas responsables de degradar los principales componentes de la matriz extracelular. La metaloproteasa MMP-1 en concreto, es una colagenasa que está involucrada en la ruptura de la matriz extracelular durante la periodontitis. Varios factores de crecimiento y citoquinas como la IL-1β, TNF-α e IL-6 estimulan la producción de esta enzima [249,250].

En el presente trabajo no sólo se observaron diferencias en la liberación de citoquinas sino también en la liberación de factores de crecimiento. En condiciones normales, la concentración de VEGF liberada por las matrices de fibrina del L-PRP fue significativamente superior a la liberada por las fibrinas de PRGF. Sin embargo, en condiciones inflamatorias, no se detectó VEGF en los medios condicionados por las fibrinas del L-PRP. Este hecho implica determinadas limitaciones ya que el factor VEGF es necesario en la reparación de heridas que causan inflamación promoviendo los eventos más tempranos de la angiogénesis [251,252]. Este hallazgo podría explicarse por una posible captación de VEGF por su receptor soluble (sVEGFR) sintetizado por los leucocitos [253,254]. Resultados similares a los obtenidos en este estudio se describieron en un trabajo previo [255] donde también se detectó una disminución en la concentración de VEGF en el medio condicionado por fibrinas de L-PRP a partir del tercer día de ensayo.

El concepto fundamental que subyace a la ingeniería de tejidos es combinar una matriz con células vivas y moléculas biológicamente activas para promover la regeneración de tejidos. Se espera que la matriz provisional sea biocompatible y que estimule entre otras funciones la colonización celular, la migración, el crecimiento y la diferenciación. Las propiedades fisicoquímicas, la morfología y la cinética de degradación también tienen que estar presentes en el diseño de estas matrices. De hecho, deben proporcionar una rigidez y una resistencia mecánica inicial suficiente como para sustituir la pérdida de la función mecánica del tejido dañado [256]. La fibrina es la matriz biológica y transitoria que primero se forma en los lugares donde se produce la lesión. Las propiedades mecánicas y la biodegradación de estas matrices de fibrina son esenciales para determinar su papel en la regeneración tisular como matriz provisional y sistema de administración de biomoléculas activas [257-260]. Las propiedades viscoelásticas dependen en gran medida de la estructura del coágulo de fibrina. Los resultados obtenidos en este trabajo mostraron que la composición celular del L-PRP alteró la estructura del coágulo y disminuyó el alargamiento máximo de la matriz. Alargamientos más elevados indican una mayor elasticidad que a su vez se traduce en fibrinas más maleables y más fuertes permitiéndoles cumplir con la función requerida como biomaterial para ser utilizado en ingeniería de tejidos [258]. De hecho, la fibrina de PRGF ha sido ampliamente utilizada en diferentes aplicaciones como el tratamiento de úlceras, cierre de heridas, ingeniería de tejidos o incluso combinada con otros materiales [241].

El proceso de regeneración periodontal implica la diferenciación de diferentes tipos celulares y su producción en un número suficiente en la ubicación y entorno correctos [256]. Curiosamente, los lipopolisacáridos estimulan a células inflamatorias, como neutrófilos y macrófagos, pero también a células residentes como fibroblastos y osteoblastos. De hecho, los fibroblastos gingivales, que son los principales constituyentes del tejido conjuntivo gingival, pueden interactuar directamente con *Porphyromonas gingivalis* y sus productos bacterianos, incluido el lipopolisacárido, en las lesiones de periodontitis [231]. Por ello, en el presente estudio también se evaluó la respuesta celular de fibroblastos gingivales y osteoblastos alveolares primarios al tratamiento con PRGF y L-PRP en condiciones inflamatorias. Como ya se ha descrito, las matrices de PRGF y L-PRP fueron estimuladas con 10 μg/ml de LPS de *P. gingivalis* durante 72h. El medio

condicionado obtenido bajo estas condiciones se denominó PRGF+LPS o L-PRP+LPS respectivamente. Para el estudio de la respuesta celular las células fueron previamente pretratadas con LPS de *P. gingivalis* durante 72h y posteriormente cultivadas con el medio de cultivo correspondiente + 10 μg/ml de LPS y suplementado con 70% del medio condicionado obtenido previamente y denominado PRGF+LPS o L-PRP + LPS.

El receptor tipo Toll 4 es el receptor principal en la respuesta celular mediada por LPS. La unión a estos receptores desencadena una serie de eventos que conducen finalmente a la activación de factores de transcripción, incluido el factor nuclear-κB (NF-κB) que a su vez induce la producción de citoquinas proinflamatorias [234,261,262]. Las proteínas NF-κB son una familia de factores de transcripción evolutivamente conservados y expresados de forma ubicua que, en mamíferos, consta de cinco miembros: p65 (RelA), RelB, c-Rel, NF-kB1 (p50 y su precursor 105) y NF-kB2 (p52 y su precursor p100) [263,264]. El heterodímero es una de las formas más abundantes del NF-κB [265]. Todas las proteínas de la familia del NF-κB comparten un dominio de homología Rel en su extremo N-terminal que media su unión al ADN, dimerización y translocación nuclear [265]. El factor NF-κB está en el citoplasma de manera latente y requiere una ruta de señalización para su activación. Estas vías de activación del factor NF-κB se desencadenan por una variedad de estímulos extracelulares. Una vez activado, el factor de transcripción NF-κB migra al núcleo para regular la expresión de múltiples genes diana [265-268]. La degradación del inhibidor del NF-κB (IκB) está mediada por su fosforilación mediante la quinasa IκB (IKK), un complejo trimérico compuesto por dos subunidades catalíticas, IKKα e IKKβ, y una subunidad reguladora, IKKγ (también llamada NEMO) [267,269]. En la vía de activación clásica o canónica del NFκB la fosforilación, ubiquitinización y posterior degradación del inhibidor IκB ocurre a través de la subunidad IKKβ en un proceso dependiente de la subunidad reguladora NEMO. El dímero de NFκB más comúnmente liberado en esta vía es el dímero p50-RelA, que se trasloca posteriormente al núcleo para unirse al ADN y activar la transcripción génica [265,270]. Por lo tanto, el inhibidor IκB tiene un papel antiinflamatorio ya que mantiene al factor NF-κB en un estado inactivado en el citosol [265,266]. Esta vía canónica es activada por productos microbianos y citoquinas proinflamatorias como el factor TNF-α o la interleuquina 1 [263,264]. Además de esta vía de activación clásica bien definida, existen otros dos

mecanismos para mediar en la activación de miembros más específicos del factor NF-κB: la vía de activación alternativa (basada en el procesamiento inducible de p100 en lugar de la degradación de IκBα) y la atípica (independiente de las quinasas IKKs).

Los hallazgos obtenidos en el presente trabajo revelaron que la expresión del inhibidor IκBα se incrementó cuando los fibroblastos gingivales y los osteoblastos alveolares se cultivaron en presencia de PRGF + LPS en condiciones inflamatorias. Estos resultados fueron consistentes con los observados para el ratio pNFκB / NFκB. En este caso, el cultivo con L-PRP + LPS indujo una fosforilación de la subunidad p65 del factor NF-κB significativamente mayor en ambos tipos celulares. La fosforilación de la subunidad p65 del factor NF-κB en el residuo de serina 536 regula la localización nuclear, las interacciones proteína-proteína, la activación de la expresión génica y la actividad transcripcional [266,268]. Estos resultados, concuerdan con los obtenidos por Xu *et al.* [206] que compararon los efectos de un plasma rico en plaquetas (P-PRP) con el de un PRP enriquecido en plaquetas y leucocitos (L-PRP) en la reparación del cartílago. Los autores revelaron que el P-PRP mostró mejores resultados en la regeneración del cartílago debido a que la eliminación de los leucocitos redujo las concentraciones de IL-1β y TNF-α e inhibió la activación de la vía NF-κB. El efecto antiinflamatorio del PRGF ha sido también descrito en otro modelo celular de enfermedad de injerto contra huésped ocular [271]. Tal y como se ha descrito, las citoquinas IL-1β y TNF-α activan la vía canónica del factor NFκB [265,266]. Por lo tanto, el entorno proinflamatorio generado por las matrices de L-PRP puede ser el responsable de este incremento en la activación del factor NFκB después del tratamiento celular con L-PRP + LPS. Este ambiente proinflamatorio y la mayor activación del NFκB tras el tratamiento de las células con L-PRP + LPS tuvo también un efecto negativo sobre la proliferación celular. Tanto los fibroblastos gingivales como los osteoblastos alveolares proliferaron significativamente menos cuando se trataron con L-PRP + LPS que cuando se trataron con PRGF + LPS. Estos resultados están en consonancia con los obtenidos en el estudio de Xu *et al.* [206], donde la proliferación de células madre mesenquimales de médula ósea de conejo cultivadas durante 5 y 7 días con L-PRP también se vio afectada. Según los autores, la disminución en la proliferación reflejaba los efectos nocivos de las citoquinas IL-1β y TNF-α.

Por otro lado, el cultivo con L-PRP + LPS también estimuló la síntesis de citoquinas proinflamatorias (IL-1β, IL-6, IL-8 y TNF-α) en ambos tipos celulares en comparación con el tratamiento con PRGF + LPS. Esta respuesta celular se observó tanto a las 24 h como a las 72 h de tratamiento. Tal y como se ha mencionado, el exceso de esas proteínas proinflamatorias puede conducir a la destrucción del tejido. Estos resultados están en la misma línea de evidencia que los obtenidos por McCarrel *et al.* [205]. En ese estudio, el L-PRP aumentó la expresión de los genes IL-1β y TNF-α por parte de las células de tendón equino flexor digital superficial en comparación con el PRP sin leucocitos que estimuló de manera más baja la expresión celular de genes catabólicos.

En nuestro estudio, este aumento en la síntesis celular de citoquinas proinflamatorias está relacionado con el incremento en la activación del factor de transcripción NFκB tras el tratamiento con L-PRP + LPS. De hecho, la fosforilación del factor NFκB da como resultado la producción de esas citoquinas, que a su vez pueden amplificar e incrementar la respuesta inflamatoria, manteniendo activa la ruta del factor NFκB y estableciendo así una respuesta de retroalimentación positiva. A raíz de esta activación, la liberación excesiva de citoquinas proinflamatorias tras la incubación con L-PRP + LPS puede exacerbar la respuesta inflamatoria al estimular el reclutamiento de nuevas células inflamatorias en el sitio de la lesión. La inflamación es una respuesta protectora del huésped a las infecciones y daños en los tejidos, que es beneficiosa para el huésped si se resuelve de manera oportuna. Sin embargo, las respuestas inflamatorias desreguladas pueden causar daños excesivos o duraderos en los tejidos, lo que contribuye al desarrollo de enfermedades inflamatorias agudas o crónicas [272]. Por tanto, el microambiente generado con el tratamiento L-PRP + LPS puede ser propicio para una inflamación no resolutiva que conduzca a una condición fibrótica del tejido (figura 12).

Los resultados obtenidos en este estudio demuestran el papel antiinflamatorio de la tecnología PRGF y cómo afecta la composición celular en los denominados plasmas ricos en plaquetas, demostrando que la inclusión de leucocitos puede exacerbar la respuesta inflamatoria preexistente en un ambiente inflamatorio. En condiciones inflamatorias el tratamiento con PRGF aporta una menor carga de citoquinas proinflamatorias lo que a su vez conlleva a una menor activación del factor NFκB y por ende a una menor síntesis de dichas citoquinas por parte de las células residentes. En estas condiciones inflamatorias,

obtenidas mediante la estimulación con lipopolisacáridos, las fibrinas de PRGF mostraron unas propiedades mecánicas superiores con ausencia de degradación. En resumen, la inclusión de leucocitos en el PRGF afectó de manera negativa a las propiedades mecánicas de las matrices de fibrina y estimuló la creación de un entorno más proinflamatorio que se relacionó directamente con un aumento en la condición celular inflamatoria y con una reducción en la respuesta proliferativa que en última instancia puede afectar a la regeneración tisular en una patología como la periodontitis.

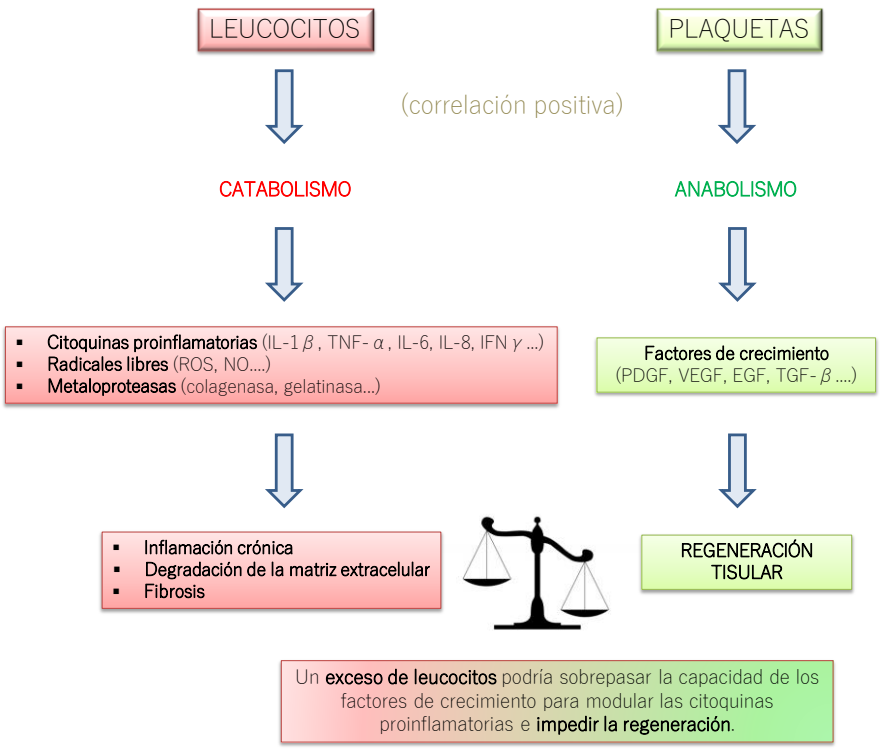

Figura 12. Esquema simplificado del efecto negativo de la inclusión de leucocitos en la capacidad regeneradora del PRP.

Por otra parte, la periodontitis está estrechamente relacionada con la osteonecrosis mandibular inducida por bifosfonatos y ambas patologías tienen una particularidad en común: ambas presentan características proinflamatorias [273]. Teniendo en cuenta por tanto, el papel de la tecnología PRGF como agente antiinflamatorio parece razonable estudiar su uso como tratamiento para la osteonecrosis mandibular inducida por bifosfonatos.

A pesar de que la prevalencia de esta enfermedad no es tan elevada como la de la periodontitis, sus repercusiones son muy significativas tanto para la calidad de vida del paciente como para los recursos médicos demandados [5,6]. Con el envejecimiento de la población y el incremento de la esperanza de vida, es previsible que enfermedades como la osteoporosis y el cáncer aumenten y con ello la necesidad y duración de terapias antirresortivas y antiangiogénicas que finalmente pueden dar lugar a un incremento en la incidencia de patologías como consecuencia de los efectos secundarios derivados del consumo de dichos medicamentos. Éste sería el caso de la osteonecrosis de los maxilares inducida por bifosfonatos.

Esta patología puede tener una morbilidad significativa que varía desde una mínima incomodidad a una pérdida significativa de hueso y función [274]. Existe una gran disparidad en la tasa de incidencia entre los diferentes estudios que puede ser atribuida a variaciones en el régimen de tratamiento, a la propia naturaleza del estudio y a la gran variedad de factores de riesgo que existen [156]. De hecho, algunos autores afirman que es difícil establecer la prevalencia exacta de esta patología ya que se estima que una parte de la enfermedad no está diagnosticada en la población, especialmente en la etapa 0 [275]. Por lo tanto, el número real de afectados podría ser incluso mayor. De hecho, las incidencias publicadas y la prevalencia de BRONJ en pacientes con enfermedades malignas varían entre 1% y 21% [276]. En general, la tasa de incidencia es más alta en pacientes con administración intravenosa de bifosfonatos en comparación con la vía de administración oral [277], siendo el ácido zoledrónico uno de los bifosfonatos intravenosos y nitrogenados más potentes [138,278].

La mandíbula y, en particular, el hueso alveolar, están constantemente expuestos a una fuerte presión masticatoria. Por esta razón, la remodelación del hueso alveolar es más

rápida que la de otros huesos del cuerpo. Debido a la alta tasa de metabolismo óseo, los BPs nitrogenados se acumulan selectivamente en el área del hueso alveolar en altas concentraciones. Además, y debido al gran número de bacterias que residen en la cavidad oral, la infección juega un papel muy relevante en la patología de la osteonecrosis mandibular. Esta infección está estrechamente relacionada con el proceso inflamatorio y con la inmunidad innata, ya que tras una infección las células inmunes son reclutadas produciendo grandes cantidades de citoquinas inflamatorias perpetuando así el estado hiperinflamatorio [279]. De hecho, Muratsu *et al.* [279] demostraron un incremento en la expresión de citoquinas inflamatorias en macrófagos murinos (RAW264.7) tratados con ácido zoledrónico debido a una mayor translocación nuclear del factor NF-κB como consecuencia de la elevada fosforilación del inhibidor IκB-α estimulada por LPS. La acumulación de estos BPs nitrogenados disminuye el metabolismo óseo, lo que evita que tras un trauma inducido o fisiológico se produzca la reparación tisular de manera adecuada, dando lugar a la exposición del hueso necrótico al entorno oral [279], (figura13). Además del efecto inhibitorio sobre los osteoclastos y osteoblastos, los BPs nitrogenados interactúan con las células del tejido blando, como fibroblastos y queratinocitos. Tras la acumulación local de BPs en los maxilares, estos pueden afectar a la mucosa oral circundante, lo que podría conducir a una lesión gingival seguida de una exposición ósea. Esta alteración de la actividad biológica de los tejidos blandos, también podría provocar una cicatrización tardía de la mucosa [278,280,281]. En comparación con otras partes del cuerpo, la gingiva oral es única y muestra características especiales. A diferencia de otros epitelios, la encía oral está en contacto directo con el hueso subyacente. Tras el tratamiento con BPs, existe un efecto citotóxico directo tanto por el suministro sanguíneo como por el propio hueso subyacente enriquecido con BPs. No existe ninguna capa de tejido blando, como grasa o músculo que amortigüe el efecto negativo de los BPs liberados por el hueso subyacente [280].

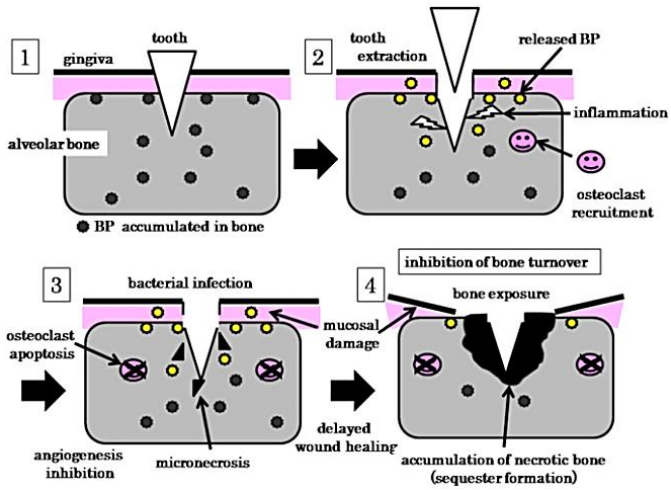

Figura 13. Fisiopatología hipotética de la osteonecrosis mandibular inducida por bifosfonatos. Reproducido con permiso de Ikebe T. [143].

Los resultados presentados en este trabajo muestran el efecto citotóxico dosis-dependiente del ácido zoledrónico sobre la proliferación y supervivencia de fibroblastos gingivales y osteoblastos alveolares primarios humanos. Esta capacidad del ZA para inhibir el crecimiento celular y promover la muerte celular ya ha sido descrita previamente [281-283]. De hecho, se sabe que los BPs nitrogenados inducen apoptosis celular al inhibir la vía del ácido mevalónico [279]. En nuestro estudio, los fibroblastos gingivales fueron más susceptibles a la toxicidad del ácido zoledrónico que los osteoblastos. De hecho, una dosis de 4 y 10 µM fue suficiente para causar un efecto adverso en la proliferación y supervivencia de fibroblastos gingivales, respectivamente. Sin embargo, en el caso de los osteoblastos, las concentraciones de ácido zoledrónico que produjeron efectos citotóxicos similares fueron aproximadamente el doble. Estos resultados son coherentes con el hecho de que los BPs se almacenan en el hueso y se liberan de manera progresiva al microambiente inmediato, afectando así a los fibroblastos gingivales locales [281,284,285]. Es decir, esto implicaría que las concentraciones de ZA a las que estaría expuesto el hueso serían significativamente superiores a las que reciben los fibroblastos gingivales.

En la actualidad no existe un tratamiento definitivo para la osteonecrosis de los maxilares inducida por bifosfonatos, donde la prevención desempeña un papel clave. De hecho, los tratamientos son principalmente paliativos con el objetivo de aliviar los principales síntomas de la enfermedad. Recientemente, se ha propuesto el uso de concentrados autólogos de plaquetas como una interesante alternativa para la prevención y tratamiento de la patología de BRONJ [145,165,286]. Esta terapia autóloga podría representar una herramienta efectiva tanto para acelerar la reparación ósea y de tejidos blandos tras la resección quirúrgica del hueso necrótico como para prevenir la aparición de la enfermedad en pacientes tratados con BPs y sometidos a procedimientos de cirugía oral [165]. Por lo tanto, asumiendo que esta patología se caracteriza por un retraso en la cicatrización oral, el uso de un cóctel de proteínas y factores de crecimiento [287,288] podría mitigar algunos de los efectos negativos del uso de bifosfonatos y promover a su vez, aspectos biológicos clave como la proliferación, migración o viabilidad celular. De hecho, se ha descrito recientemente el uso clínico de la tecnología autóloga PRGF como terapia adyuvante para el tratamiento de pacientes con BRONJ, proporcionándoles una mejora en la vascularización y en la regeneración de los tejidos óseos y epiteliales y en definitiva, mejorando la cicatrización de las heridas [145,165,286,289-292]. Los resultados obtenidos en este estudio *in vitro* podrían explicar en parte los mecanismos que están detrás de estos prometedores resultados clínicos.

Los resultados que se derivan de este trabajo experimental, indican un efecto citoprotector por parte de la tecnología PRGF en las células expuestas al ácido zoledrónico. El uso de estas proteínas autólogas y factores de crecimiento puede limitar los efectos secundarios del tratamiento con BPs tan potentes como el propio ácido zoledrónico, promoviendo la proliferación celular y reduciendo la apoptosis de las células orales tratadas con bifosfonatos. Efectos anti-apoptóticos similares ya fueron descritos previamente en un modelo experimental de enfermedad de Alzheimer tras el tratamiento con PRGF [293,294].

Los bifosfonatos nitrogenados tal y como se ha descrito, pueden dar lugar a un estado inflamatorio y a aumentar el estrés oxidativo local del periodonto. Es decir, estos fármacos están relacionados con una desregulación en la producción de citoquinas proinflamatorias [273,279,295]. Por tanto, la patología de BRONJ está directamente relacionada con el incremento en la producción de marcadores proinflamatorios (TNF-α, IL-1β...) siendo los

macrófagos y la actividad nuclear del factor de transcripción NF κ B necesarios para el origen y desarrollo de esta patología [273]. Como ya se ha descrito, la vía NF- κ B activa la transcripción de muchos mediadores proinflamatorios. La desregulación de esta vía conduce a un aumento persistente en la expresión de varios efectores asociados con la perpetuación de la inflamación [265] que puede provocar que las heridas crónicas no se resuelvan de manera óptima generando cicatrices y fibrosis. De acuerdo con nuestros resultados, el ZA estimuló la activación del factor NF- κ B tanto en fibroblastos gingivales como en osteoblastos alveolares. Los hallazgos obtenidos también revelaron que la adición de PRGF amortiguó el efecto inflamatorio del ácido zoledrónico en las células orales. De hecho, el tratamiento combinado de ácido zoledrónico y PRGF mostró una reducción significativa en la fosforilación de la subunidad p65 del factor de transcripción NF-κB en ambos fenotipos celulares, restaurando los valores obtenidos en ausencia del tratamiento con BPs. Algunos de los factores de crecimiento presentes en el PRGF podrían ser los responsables de estas propiedades antiinflamatorias del plasma. De hecho, el factor de crecimiento HGF tiene la capacidad de suprimir la respuesta inflamatoria al interrumpir la señalización del factor de transcripción NF- κ B [209,296-298]. Existen otros factores de crecimiento como el IGF-I o el PDGF-BB que también pueden actuar como agentes antiinflamatorios mediante la supresión de la señalización del factor NF- κ B inducida por la interleuquina IL-1 β [299].

Por lo tanto, la continua liberación de ácido zoledrónico por parte del tejido óseo y los efectos que este fármaco produce en la proliferación, apoptosis e inflamación tanto del hueso como del tejido blando evitan la resolución espontánea de la reparación tisular. Dentro de las limitaciones de un estudio *in vitro,* este trabajo ha demostrado el papel citoprotector que ejerce la tecnología autóloga PRGF, reduciendo o limitando los efectos negativos derivados del ácido zoledrónico en la células orales y que están detrás de la patología de BRONJ.

En base a las características de la tecnología PRGF, se decidió estudiar su aplicación para terapias avanzadas. La ingeniería tisular y la terapia con células madre han surgido como nuevos enfoques terapéuticos para la reparación de tejidos dañados o perdidos en una amplia variedad de enfermedades. En particular, las células madre mesenquimales son las candidatas más prometedoras entre las células madre adultas debido a su accesibilidad y

a la ausencia de dilemas éticos [12,69,213]. Estas células mesenquimales pueden obtenerse de diferentes órganos y tejidos siendo uno de ellos la pulpa dental por su fácil acceso quirúrgico y los métodos de aislamiento no invasivos. La cantidad de células madre mesenquimales presentes en los tejidos es limitada, por lo que se requiere una expansión *ex vivo* que cumpla los estándares de buenas prácticas de fabricación para producir una dosis adecuada con el fin de lograr resultados terapéuticos óptimos [12,13,300]. Por lo tanto, la identificación de las condiciones óptimas de cultivo es un requisito previo para la aplicación clínica de la terapia con células madre. Los suplementos de crecimiento de origen animal, como el suero bovino fetal, se han usado predominantemente para la expansión de MSCs en protocolos clínicos. Sin embargo, la utilización de productos derivados de animales conlleva limitaciones críticas y serias preocupaciones relativas a la seguridad. Por lo tanto, para evitar complicaciones indeseadas, se están evaluando formulaciones alternativas de medios libres de productos de origen animal. Los esfuerzos recientes se han centrado en el desarrollo de medios de cultivo "humanizados" [300]. De hecho, actualmente, se están explorando muchos productos autólogos o alogénicos derivados de la sangre humana, como suero humano [18,301], plasma rico en plaquetas [19,26], lisado plaquetario [20,302-304] o suero sanguíneo del cordón umbilical [21,305] como posible reemplazo para el FBS. Al igual que éste, los suplementos derivados de componentes de la sangre humana incluyen una variedad de factores esenciales capaces de promover los procesos claves de la reparación tisular. El desarrollo de protocolos de cultivo no xenogénicos y estandarizados que mantengan la viabilidad celular, el fenotipo y el potencial de diferenciación, representa uno de los principales hitos de las terapias celulares para su traslación clínica (figura 14).

En el presente trabajo se ha desarrollado un método de cultivo de hDPSCs totalmente autólogo basado en la tecnología PRGF. Los resultados obtenidos muestran que el suplemento de PRGF puede usarse como sustituto del suero bovino fetal en el medio de cultivo para aislar con éxito hDPSCs. De hecho, no se encontraron diferencias significativas entre suplementos para el porcentaje de explantes a partir de los cuales se obtuvieron células ni para el tiempo que éstas tardaron en salir de los mismos. Por el contrario, el número de células por explante que se recuperó fue significativamente mayor cuando se incluyó PRGF como suplemento del medio de cultivo. Estos resultados

concuerdan con los obtenidos en el ensayo de proliferación celular donde el uso de PRGF como suplemento del medio de cultivo indujo una mayor proliferación frente al FBS tras 72 y 96 horas de tratamiento. Nuestros resultados concuerdan con trabajos previos en los que también se demostró que el PRP puede sustituir al FBS para el mantenimiento a largo plazo de las hDPSCs [306,307]. También se ha descrito la estimulación de la proliferación celular por parte de la tecnología PRGF en células madre obtenidas de médula ósea, ojo o tejido adiposo [26,308,309].

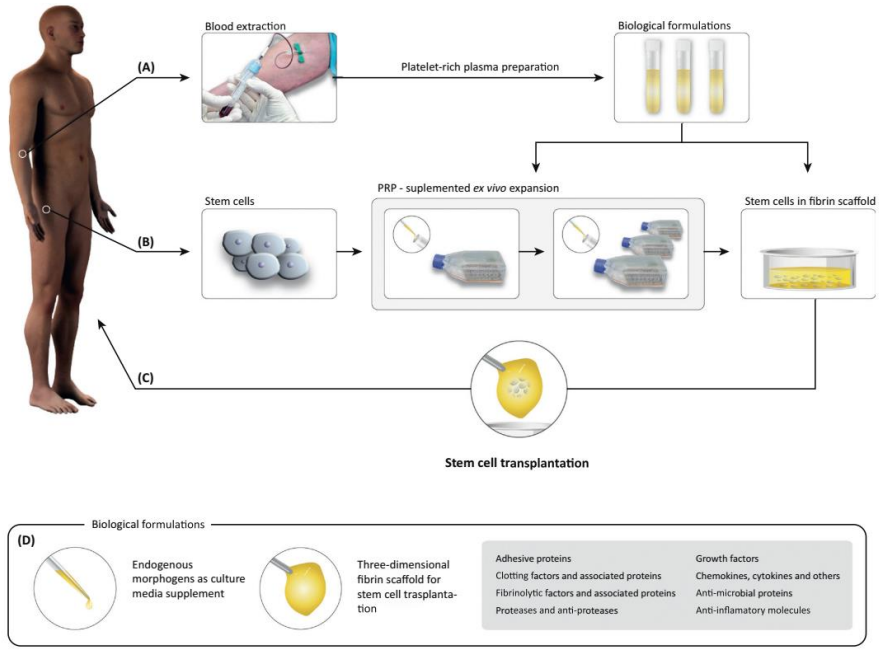

Figura 14. Descripción de un enfoque autólogo para la terapia con células madre. (A) Obtención de las diferentes formulaciones del plasma rico en plaquetas (PRP). (B) Aislamiento de las células madre y su posterior expansión con PRP como suplemento del medio de cultivo. Tras la expansión, libre de productos xenogénicos, las células madre se cultivan en la matriz de fibrina de PRP. (C) Finalmente, las células madre son retransplantadas en un entorno molecular óptimo. (D) Las formulaciones biológicas, tanto la formulación líquida como la matriz de fibrina, contienen morfógenos endógenos que fomentan la expansión segura y eficiente de las células madre y aumentan la supervivencia en el receptor del órgano. Reproducida con permiso de Anitua E *et al.* [213].

La tecnología PRGF ofrece una fuente autóloga de moléculas biológicamente activas que pueden estimular sinérgicamente no sólo la proliferación, sino también la motilidad celular. En este sentido, la presencia de PRGF en el medio de cultivo estimuló significativamente la migración celular (hasta 2'5 veces más) en comparación con el mismo porcentaje de FBS. Varios estudios han demostrado que el PRP aumenta la capacidad de migración celular en células madre mesenquimales [308,310], estos resultados apoyan los obtenidos en este trabajo.

Las células madre mesenquimales representan una interesante alternativa para su uso en procesos de reparación tisular debido a su amplia capacidad proliferativa al tiempo que conservan su potencial de diferenciación multilinaje. Estas MSCs tienen el potencial de diferenciarse hacia varios linajes mesodérmicos, incluidos el linaje osteogénico, adipogénico y condrogénico. Durante la diferenciación de MSCs hacia un tipo de célula específico, existen una multitud de estímulos e inhibidores que juegan un papel importante en el compromiso inicial y en las etapas posteriores de diferenciación. La diferenciación de las MSCs en tipos celulares específicos y maduros está controlada por varias citoquinas, factores de crecimiento, moléculas de la matriz extracelular y factores de transcripción [311]. En este estudio, las hDPSCs demostraron ser capaces de diferenciarse hacia los tres linajes descritos independientemente del suplemento utilizado. Sin embargo, la capacidad de diferenciación osteogénica y adipogénica se vio incrementada en presencia de PRGF. De hecho, la tinción con rojo de alizarina confirmó que la deposición mineral fue más prominente en las células cultivadas con el medio osteogénico suplementado con PRGF y el análisis cuantitativo de triglicéridos también reveló un incremento significativo en las hDPSCs cultivadas con el medio adipogénico suplementado con PRGF con respecto al suplementado con FBS. La diferenciación condrogénica puede lograrse en ausencia de suero [95,312,313], por lo que su sustitución no es necesaria. No obstante, se evaluó si la adición de PRGF al medio de cultivo aumentaba dicha diferenciación. Los resultados mostraron que el potencial de diferenciación condrogénica no se incrementó de manera significativa. Diferentes estudios también han confirmado el potencial de los derivados plaquetarios para mejorar el proceso de diferenciación de las células madre de pulpa dental. Vasanthan *et al.* [215], detectaron la expresión de varios marcadores hepáticos después de sustituir el suero bovino fetal por lisado plaquetario en el medio de

diferenciación de cultivos de células madre de pulpa dental. Del mismo modo, se ha demostrado la eficacia del plasma rico en plaquetas para estimular la diferenciación osteogénica de las DPSCs [20,314,315]. En el caso particular de la tecnología PRGF, también se ha descrito su papel como sustituto del suero bovino fetal para la amplificación y diferenciación de otros tipos de células madre mesenquimales, como las derivadas del tejido adiposo [316], de la médula ósea [309] o del epitelio limbar [26].

Otro posible riesgo a tener en cuenta en relación con las terapias celulares es la considerable expansión celular *in vitro* que se requiere antes de obtener un número suficiente de células para su uso terapéutico. Esta expansión puede conducir a una disminución proliferativa y senescencia celular, acompañadas de un comportamiento celular y potencial regenerador alterados [94]. En este estudio las células madre de pulpa dental de pases tempranos mostraron una morfología típica, en forma de huso, con una elevada tasa de proliferación. Sin embargo, a medida que fueron aumentando los pases en cultivo (hasta pase 11), se detectaron cambios morfológicos, observándose células de mayor tamaño, aplanadas y de apariencia estrellada. Así mismo, se detectó una disminución en la capacidad proliferativa. Estos cambios fueron observados independientemente del suplemento de cultivo utilizado. La tinción de la enzima β-galactosidasa asociada a la senescencia (SA-β-gal) reveló bajos niveles de positividad en el pase cuatro. Sin embargo, hDPSCs de pase 11 mostraron un incremento significativo en el porcentaje de células positivas, independientemente del suplemento utilizado. La actividad de esta enzima ha sido el biomarcador más utilizado para la senescencia debido a la simplicidad del método de ensayo y a su aparente especificidad para las células senescentes [317]. La enzima SA-β-gal muestra una actividad máxima en un rango de pH de entre 4 y 4'5, con una actividad marcadamente inferior a pH 6 (pH subóptimo). De hecho, la actividad de la β-galactosidasa no se detecta a pH 6 en células que estén proliferando, sin embargo, es fácilmente detectable en estas células a pH ácido. La actividad de esta enzima aumenta en las células senescentes debido al aumento en el contenido de lisosomas, que supera un nivel umbral, por lo que es detectable a pH 6 [317-319]. Sin embargo, a pesar de la confianza generalizada en el uso de esta enzima como marcador de senescencia, su papel en este proceso no es del todo concluyente. De hecho, hay varios informes que indican que la actividad de la SA-β-gal también puede

detectarse en estados no senescentes por inactividad derivada de la inhibición por contacto como consecuencia de la incubación prolongada a alta densidad [317,318]. Por ello, es importante combinar diferentes marcadores para aportar resultados fiables. Una de las principales causas de la senescencia replicativa es el acortamiento progresivo de los telómeros, considerándose uno de los mecanismos subyacentes del envejecimiento [320-322]. El proceso de envejecimiento concuerda con una reducción en la capacidad regenerativa de varios tejidos, una disminución en la funcionalidad de las células madre y una caída en la reserva de telómeros. En general, se acepta que la funcionalidad de las células madre limita la homeostasis de los órganos, lo que a su vez limita la longevidad del organismo [323]. Los resultados obtenidos en nuestro estudio demostraron que los valores de telomerasa de las hDPSCs en pase 4 fueron significativamente más elevados que los detectados en dichas células en pase 11. El uso de FBS o PRGF como suplemento del medio de cultivo no supuso diferencias significativas en los niveles de telomerasa detectados en las células en pase 11, siendo en ambos casos inferiores a los de las células de pase 4. Teniendo en cuenta la capacidad del PRGF para estimular la proliferación de las hDPSCs, no sería necesario expandir las células hasta pases muy avanzados para obtener un número apropiado de células, evitando así alcanzar la senescencia replicativa.

Por último, en el caso de que las hDPSCs no se utilicen de inmediato, el proceso de criopreservación sería la alternativa apropiada. Existen trabajos previos en los que se describe el uso del compuesto dimetilsulfóxido como agente crioprotector para la criopreservación de hDPSCs durante diferentes periodos de tiempo [324,325]. Los resultados obtenidos en el presente trabajo demostraron la viabilidad del uso de la tecnología autóloga PRGF para criopreservar hDPSCs junto con el crioprotector dimetilsulfóxido durante al menos un mes. No se encontraron diferencias estadísticamente significativas respecto a la viabilidad celular obtenida tras la descongelación celular. La capacidad de las células madre para adherirse a las superficies de cultivo tras el periodo de congelación tampoco se vio alterada con ninguno de los dos suplementos de cultivo utilizados.

Por lo tanto, la tecnología autóloga PRGF representa una prometedora alternativa al uso de FBS como suplemento del medio de cultivo para aislar, cultivar y criopreservar células

madre de pulpa dental. De hecho, el uso de PRGF estimula de manera significativa los procesos de proliferación, migración y diferenciación de las hDPSCs.

En conclusión, el conjunto de nuestros resultados [326-328] demuestra el papel antiinflamatorio de la tecnología PRGF en un modelo experimental de periodontitis y cómo afecta la inclusión de leucocitos en los denominados plasmas ricos en plaquetas. La presencia de estas células de la serie blanca puede exacerbar la respuesta inflamatoria preexistente en un ambiente inflamatorio y afectar de manera negativa a las propiedades mecánicas de las matrices de fibrina. Además, los resultados derivados de este trabajo experimental, indican un efecto citoprotector del PRGF en las células expuestas al ácido zoledrónico. El uso de estas proteínas autólogas y factores de crecimiento puede limitar los efectos secundarios del tratamiento con BPs promoviendo la proliferación celular, reduciendo la apoptosis y amortiguando la inflamación de las células orales tratadas con ácido zoledrónico, uno de los principales responsables de la osteonecrosis de los maxilares inducida por bifosfonatos. El uso de PRGF como único suplemento del medio de cultivo ha arrojado resultados similares e incluso superiores a los de un medio en cuya composición se incluyen suplementos xenogénicos como el suero bovino fetal, no recomendables para la traslación clínica. De hecho, la capacidad de las hDPSCs para proliferar, migrar y diferenciar se ha visto incrementada tras el cultivo con PRGF.

CONCLUSIONES

CONCLUSIONES

1. Las fibrinas de PRGF preservaron su integridad y mantuvieron una elevada elasticidad, proporcionando propiedades biomecánicas óptimas para la regeneración tisular en un modelo experimental de periodontitis.

2. En estas condiciones inflamatorias el tratamiento con PRGF aportó una menor carga de citoquinas proinflamatorias lo que a su vez conllevó a una menor activación del factor NFκB y por ende a una menor síntesis de dichas citoquinas por parte de las células residentes. Por tanto, la tecnología autóloga PRGF mostró un papel antiinflamatorio en un modelo experimental de periodontitis

3. La inclusión de leucocitos en el PRGF estimuló la creación de un entorno más proinflamatorio que se relacionó directamente con un aumento en la condición celular inflamatoria y con una reducción en la respuesta proliferativa y afectó de manera negativa a las propiedades mecánicas de la matriz de fibrina.

4. La tecnología PRGF exhibió un efecto citoprotector frente al ácido zoledrónico uno de los principales responsables de la osteonecrosis de los maxilares inducida por bifosfonatos. El uso de PRGF estimuló la proliferación, redujo la apoptosis y amortiguó la inflamación de las células orales tratadas con ácido zoledrónico.

5. El uso de PRGF como único suplemento del medio de cultivo arrojó resultados similares e incluso superiores a los del suero bovino fetal, suplemento xenogénico más comúnmente utilizado en los cultivos celulares. De hecho, la capacidad de las hDPSCs para proliferar, migrar y diferenciarse hacia el linaje osteogénico y adipogénico fue significativamente superior tras el cultivo con PRGF.

La relevancia clínica de estos resultados radica en el uso de una tecnología autóloga, de fácil obtención que se postula como alternativa al uso de productos xenogénicos para la terapia celular, representando un nuevo enfoque en el tratamiento eficiente de patologías orales así como en el de otras áreas de la medicina.

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ÍNDICE DE FIGURAS Y TABLAS

Figuras

la formulación líquida como la matriz de fibrina, contienen morfógenos endógenos que fomentan la expansión segura y eficiente de las células madre y aumentan la supervivencia en el receptor del órgano.

Tablas

ANEXO

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Universidad del País Vasco
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