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UNIVERSIDAD DE GRANADA

PROGRAMA DE DOCTORADO EN MEDICINA CLÍNICA Y SALUD PÚBLICA

Diseño y Evaluación de Membranas Nanoestructuradas para Regeneración Ósea

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Normativa Universidad de Granada

La presente Memoria sigue la estructura que dicta la normativa del Programa de Doctorado de Medicina y Salud Pública de la Universidad de Granada (https://doctorados.ugr.es/medicinaysalud/pages/organizacion_programa/doctorandos) para las Tesis presentadas como agrupación de trabajos de investigación publicados, que dice textualmente:

…'Aunque no existe una única forma de presentar una tesis por agrupación de artículo, en general se presentará una memoria con una introducción e hipótesis común para los 3 o más artículos presentados y unos objetivos generales u específicos que podrían pertenecer a diferentes artículos; los resultados podrían ser los 3 o más artículos presentados, insertándolos en el propio documento de la Tesis, con o sin un resumen general de los mismos; finalmente un apartado de discusión que trate de justificar la unidad temática y que resuma la idea global de la Tesis Doctoral y las conclusiones, donde se debería responder a cada uno de los objetivos previamente planteados.'…

Resumen

Uno de los principales retos en la Odontología actual es la regeneración de defectos y atrofias óseas. Estas deficiencias de hueso pueden estar causadas por patología infecciosa, tumoral o traumática, y dificultan o impiden la colocación de implantes osteointegrados. En determinados casos, será necesario regenerar parte del hueso de manera previa o simultánea a la colocación del implante. Para este propósito, la técnica más estudiada y con mayor respaldo científico es la Regeneración Ósea Guiada (ROG). Esta técnica requiere la utilización de una membrana, con el fin de producir una compartimentalización del defecto óseo, impidiendo así la colonización del defecto por estirpes celulares no deseadas, permitiendo, por tanto, la proliferación de osteoblastos y la regeneración del tejido óseo.

En el presente trabajo hemos realizado una revisión sistemática de la literatura con metaanálisis para evaluar la eficacia clínica de las membranas existentes cuando se realiza ROG. Se ha determinado que las membranas que con más frecuencia se utilizan para este propósito, son las reabsorbibles de colágeno. Concluimos que la técnica quirúrgica se puede llevar a cabo de manera predecible, pudiendo reducir un defecto horizontal entre 3 y 4,7mm. También sabemos que existen complicaciones en un 8,4% de los casos, siendo la complicación más frecuente es la exposición e infección de la membrana.

De las membranas existentes, ninguna satisface completamente las propiedades ideales de estos biomateriales y además, encontramos que la forma de evaluación de las mismas, data del año 2004 (ISO 22803:2004). Por ello, proponemos mediante una revisión narrativa de la literatura una estrategia completa y actual de análisis experimental de este tipo de membranas para ROG. Una membrana de ROG debe ser evaluada con técnicas basadas en nanotecnología desde el punto de vista tribológico, morfométrico y mecánico. Su bioactividad debe ser evaluada mediante cultivos de células de estirpe osteoblástica donde se determine su capacidad de proliferación, diferenciación y mineralización. Estudios experimentales en animal y estudios clínicos controlados y randomizados son siempre necesarios para determinar su efectividad clínica.

Con objeto de mejorar este tipo de biomateriales se plantea su funcionalización con doxiciclina y se realiza una revisión sistemática de la literatura donde se revisaron los materiales que con mayor frecuencia se emplean como membranas para ROG, los antibióticos usados para su funcionalización, las distintas técnicas de dopado y su eficacia. Encontramos que existen numerosas combinaciones de funcionalización de antibióticos sobre materiales poliméricos. En todas ellas se consigue efecto antibacteriano, pero solo se han evaluado frente a cultivos de bacterias no específicas y con una única especie, lo que limita su relevancia clínica. Los ensayos en humanos son además limitados y de resultados controvertidos.

Finalmente, se propone el diseño y funcionalización con cinc, doxiciclina y dexametasona de membranas poliméricas sintéticas y naturales nanoestructuradas y se realiza un análisis de su interacción con células osteoblásticas. Se determina que la funcionalización de las membranas se puede realizar de modo eficaz y sencillo. La funcionalización más ventajosa desde el punto de vista de proliferación celular es la que se realiza con doxiciclina. En ambos tipos de membranas se consigue una adecuada proliferación celular y mejora de la diferenciación osteoblástica. Se produce una marcada sobreexpresión de genes osteogénicos como BMP-2, ALP, OPG, TGFβ-1 and TGFβ-R1 y se da lugar a una ratio OPG/RANKL más favorable. Los osteoblastos cultivados en membranas poliméricas nanoestructuradas no reabsorbibles y funcionalizadas con doxiciclina presentan mayor expresión de antígenos CD54, CD80, CD86, and HLA-DR, lo que es índice de su posible capacidad inmunomoduladora.

Índice

11.4. Toledano-Osorio M, Manzano-Moreno FJ, Toledano M, Medina-Castillo AL, Costela-Ruiz VJ, Ruiz C, Osorio R. Doxycycline-Doped Polymeric Membranes Induced Growth, Differentiation and Expression of Antigenic Phenotype Markers of Osteoblasts. Polymers (Basel). 2021;13(7):1063. doi: 10.3390/polym13071063.

11.5. Toledano-Osorio M, Manzano-Moreno FJ, Toledano M, Osorio R, Medina-Castillo AL, Costela-Ruiz VJ, Ruiz C. Doxycycline-doped membranes induced osteogenic gene expression on osteoblastic cells. J Dent. 2021;109:103676. doi: 10.1016/j.jdent.2021.103676.

11.6. Toledano-Osorio M, de Luna-Bertos E, Toledano M, Manzano-Moreno FJ, García-Recio E, Ruiz C, Osorio R, Sanz M. Doxycycline-doped collagen membranes accelerate in vitro osteoblast proliferation and differentiation. J Periodont Res. 2022;En prensa. doi: 10.1111/jre.13091.

1. Introducción

1.1. Concepto de regeneración ósea guiada (ROG)

Los defectos óseos alveolares, verticales u horizontales, son la principal condición que dificulta la colocación de implantes dentales osteointegrados mediante una técnica convencional. Estos defectos están, en su mayoría, causados por patología infecciosa, tumoral o traumática (1). La pérdida dental, *per se*, también conlleva una atrofia del hueso alveolar que lo sustenta (2). Esto hace que, en ciertos casos, sea necesario regenerar parte del hueso perdido de manera previa o de forma simultánea a la colocación del implante (2). Para este propósito, la técnica más estudiada y con mayor respaldo científico es la Regeneración Ósea Guiada (ROG). Esta técnica fue descrita por Dahlin y cols. (3), que aplicaron a la regeneración ósea los conceptos previamente descritos para la Regeneración Tisular Guiada (RTG) en defectos periodontales (4,5). Estas técnicas estaban basadas en la utilización de una membrana, que, a modo de barrera física, produjese una compartimentalización del defecto, impidiendo la colonización de éste por estirpes celulares no deseadas. El tipo de célula que primeramente colonizase el defecto, determinaría el tejido que se formaría en él.

El desarrollo y la mejora de estas técnicas se ha basado, fundamentalmente, en la optimización de membranas que, no solamente actúen como meras barreras físicas, sino que toman un papel activo en la regeneración ósea.

1.2. Características ideales de las membranas para ROG

Sanz y cols. (6), como resultado del decimoquinto Workshop Europeo de Periodoncia sobre regeneración ósea, propugnaron la necesidad de desarrollar membranas que, además de su capacidad básica de aislamiento, mostraran biocompatibilidad, actividad biológica, correcta porosidad y oclusividad, buenas propiedades mecánicas, adecuada integración con los tejidos circundantes, tolerancia a la exposición y una ratio de biodegradabilidad adecuada.

Dado que ninguna membrana de las que disponemos en la actualidad cumple todos los requisitos de forma completamente satisfactoria, se hace imperativo el diseño y desarrollo de nuevas membranas de regeneración ósea, con el fin de optimizar y aumentar la predictibilidad de estas intervenciones quirúrgicas.

La introducción de requisitos más exigentes y nuevas membranas de ROG, crea la necesidad de diseñar una batería de pruebas específicas, tanto *in vitro* como *in vivo*, que sean capaces de evaluar las propiedades de las membranas ya mencionados, antes de plantear su uso en un escenario clínico. Debemos resaltar que la normativa ISO que regula la evaluación de estos biomateriales data del año 2004 (ISO 22803:2004) (7).

1.3. Tipos de membranas para ROG

Las membranas para regeneración ósea y tisular se pueden clasificar en dos grandes grupos: reabsorbibles y no reabsorbibles. Las primeras destacan por tener un menor número de complicaciones postoperatorias (6), aunque una capacidad de regeneración más limitada, debido principalmente a su ratio de degradación variable. Por el contrario, las no reabsorbibles tienen una mayor efectividad, pero presentan más complicaciones postoperatorias, destacando la mayor incidencia de exposición y sobreinfección (8,9). Otra de las desventajas de las membranas no reabsorbibles es la necesidad de someter al paciente a una segunda cirugía para su retirada.

En relación con las reabsorbibles, las más representativas son las membranas de colágeno. Este puede ser natural o sintético; y, además, puede estar modificado física o químicamente para producir entrecruzamiento de las fibras de colágeno y aumentar el tiempo de reabsorción, proceso que se conoce como *cross-linking* del colágeno. Además del colágeno, también podemos encontrar otros materiales tales como quitosano o alginato (6). En el caso de las no-reabsorbibles, el politetrafluoruro de etileno (PTFE) es el máximo y, prácticamente, único representante.

El avance de la ciencia de los biomateriales ha causado un aumento de las posibles propuestas de prototipos de membranas con aplicación en el área de la medicina regenerativa. Sin embargo, todavía no se aplican a la clínica.

1.4. Nanoestructura en las membranas para ROG

Una de las propiedades más importantes de las membranas para ROG es que tengan capacidad de estimular la adhesión, proliferación y diferenciación osteoblástica. Desde hace algunos años sabemos que las células poseen receptores que les permiten detectar propiedades de los materiales y de los tejidos a un nivel nanométrico (10). Es interesante, como se ha demostrado, que células madre pueden diferenciarse hacia una u otra estirpe celular dependiendo de las propiedades nanomecánicas del material o tejido sobre el que proliferan (10). Y que, además, su forma y crecimiento dependen de la nanomorfología o nanoestructura del tejido en el que asientan (10). Por estos motivos, proponemos una membrana con una nanoestructura y unas nanopropiedades similares a las del hueso trabecular (11).

El prototipo de membrana que se propone y evalúa a lo largo de esta Tesis Doctoral, se trata, por tanto, de una membrana con textura nanoescalada. Se compone de nanofibras muy similares en dimensiones y morfología a las del colágeno del hueso trabecular, entrecruzadas y con disposición aleatoria, dejando expuestos micro y nanoporos que facilitarán el crecimiento celular (11).

1.5. Propuesta de nueva membrana para ROG

En la presente tesis, se propone la utilización de un polímero no reabsorbible, derivado del polimetilmetacrilato para la síntesis de membranas destinadas a ROG. Se trata de una mezcla polimérica susceptible de *electrospinning* o electrohilado, que posibilita la formación de nanofibras poliméricas de distribución aleatoria. Es una mezcla polimérica que se compone de los co-polímeros: $(MMA)_1$-co-$(HEMA)_1$/$(MA)_3$-co-$(HEA)_2$ [50/50 wt]. En el proceso de fabricación se incorporan a la mezcla polimérica un 5% de nanopartículas de óxido de sílice (SiO_2-NPs), lo que facilita la proliferación celular y la mejora las nano-propiedades mecánicas de la membrana, haciéndolas similares a las del hueso trabecular (11).

Estas membranas han demostrado ser totalmente biocompatibles tanto frente a células de estirpe fibroblástica como a las de origen óseo (11,12). Las membranas son además bioactivas, es decir, que una vez están en contacto con fluidos biológicos, se

produce sobre ellas precipitación de hidroxiapatita. Esto implica que tienen capacidad de biomineralización y una posible integración en el tejido óseo (11,13) 11X. Además, en el caso de las membranas objeto de estudio, se ha demostrado que la incorporación de sílice, promueve y mejora la precipitación biomimética de depósitos de calcio fosfato (11).

La estructura química de las membranas se caracteriza por la exposición de grupos funcionales carboxilo (COO^-), sobre toda su superficie. Esto hace posible el dopado de las mismas con cationes y una gran cantidad de sustancias. Aprovechando esta característica, hemos diseñado y fabricado membranas que funcionan como transportadoras de elementos que pueden promover y favorecer la reparación ósea.

2. Hipótesis

La hipótesis del presente trabajo es que la funcionalización de membranas nanoestructuradas para regeneración ósea guiada con doxiciclina, cinc o dexametasona mejora la proliferación y diferenciación de células osteoblásticas.

3. Objetivos

3.1. Objetivo general

Diseñar y desarrollar una membrana de material polimérico nanoestructurado, funcionalizado con cinc, doxiciclina o dexametasona que permita el crecimiento y diferenciación celular para estimular la regeneración ósea.

3.2. Objetivos específicos

- Objetivo específico 1: Evaluar la eficacia de regeneración ósea de membranas comercializadas y sus posibles complicaciones.

- Objetivo específico 2: Diseñar un protocolo de evaluación de nuevas membranas nanoestructuradas para ser utilizadas en ROG.

- Objetivo específico 3: Formular y fabricar un tejido polimérico nanoestructurado para su uso en ROG, funcionalizado con cinc, doxiciclina o dexametasona.

- Objetivo específico 4: Examinar la capacidad de los nuevos materiales poliméricos funcionalizados para inducir proliferación y diferenciación de osteoblastos.

- Objetivo específico 5: Analizar el efecto de funcionalización de membranas poliméricas de colágeno con doxiciclina y dexametasona en su capacidad para inducir proliferación y diferenciación de osteoblastos.

4. Metodología

Para alcanzar los objetivos propuestos, la metodología de la Memoria de Tesis Doctoral ha sido la siguiente:

- Actividad 1: Realización de una revisión sistemática de la literatura con metaanálisis para evaluar la eficacia clínica de las membranas existentes cuando se realiza ROG.
- Actividad 2: Revisión narrativa de la literatura para diseñar una estrategia completa y actual de evaluación de membranas para ROG.
- Actividad 3: Revisión de la literatura donde se analizaron los materiales existentes de las membranas para ROG, los antibióticos usados para su funcionalización, técnicas de funcionalización y eficacia.
- Actividad 4: Diseño y funcionalización de membranas poliméricas sintéticas nanoestructuradas y evaluación de su interacción con células osteoblásticas.
- Actividad 5: Funcionalización de membranas poliméricas de colágeno y evaluación de su interacción con células osteoblásticas.

Los métodos concretos de cada una de las actividades se describen, en detalle, en cada uno de los artículos publicados que forman parte de la presente Memoria de Tesis. Se presentan *in extenso* en el Anexo al final del documento.

5. Resultados

Siguiendo la normativa del Programa de Doctorado de Medicina y Salud Pública de la Universidad de Granada para las Tesis presentadas como agrupación de trabajos de investigación publicados, se incluyen en el apartado de resultados los seis artículos publicados:

- Toledano-Osorio M, Toledano M, Manzano-Moreno FJ, Vallecillo C, Vallecillo-Rivas M, Rodriguez-Archilla A, Osorio R. Alveolar Bone Ridge Augmentation Using Polymeric Membranes: A Systematic Review and Meta-Analysis. Polymers (Basel). 2021;13(7):1172. doi: 10.3390/polym13071172.

- Toledano-Osorio M, Manzano-Moreno FJ, Ruiz C, Toledano M, Osorio R. Testing active membranes for bone regeneration: A review. J Dent. 2021;105:103580. doi: 10.1016/j.jdent.2021.103580.

- Toledano-Osorio M, Vallecillo C, Vallecillo-Rivas M, Manzano-Moreno FJ, Osorio R. Antibiotic-Loaded Polymeric Barrier Membranes for Guided Bone/Tissue Regeneration: A Mini-Review. Polymers (Basel). 2022;14(4):840. doi: 10.3390/polym14040840.

- Toledano-Osorio M, Manzano-Moreno FJ, Toledano M, Medina-Castillo AL, Costela-Ruiz VJ, Ruiz C, Osorio R. Doxycycline-Doped Polymeric Membranes Induced Growth, Differentiation and Expression of Antigenic Phenotype Markers of Osteoblasts. Polymers (Basel). 2021;13(7):1063. doi: 10.3390/polym13071063.

- Toledano-Osorio M, Manzano-Moreno FJ, Toledano M, Osorio R, Medina-Castillo AL, Costela-Ruiz VJ, Ruiz C. Doxycycline-doped membranes induced osteogenic gene expression on osteoblastic cells. J Dent. 2021;109:103676. doi: 10.1016/j.jdent.2021.103676.

- Toledano-Osorio M, de Luna-Bertos E, Toledano M, Manzano-Moreno FJ, García-Recio E, Ruiz C, Osorio R, Sanz M. Doxycycline-doped collagen membranes accelerate in vitro osteoblast proliferation and differentiation. J Periodont Res. 2022;00:1-12.

Los artículos completos se pueden consultar en el Anexo, adjunto al final de la Memoria.

6. Discusión

Para dar respuesta a los objetivos previamente planteados, se estableció una estrategia de investigación que empezó por una revisión sistemática de la literatura. Mediante este estudio secundario, se evaluó la eficacia clínica de las membranas existentes y utilizadas en clínica, así como las complicaciones más frecuentemente asociadas al uso de estos biomateriales. Además, se pudo realizar un análisis cuantitativo de los datos de los artículos encontrados mediante un metaanálisis (14). Una vez acometida esta tarea, se procedió a realizar una revisión no sistemática de la literatura. En ella, se buscaron las pruebas que se pueden realizar a una membrana para ROG, a la luz de los nuevos requerimientos que se le exigen a una membrana óptima y que fueron propuestos por Sanz y cols. (6). Debemos tener en cuenta que la última revisión de la norma ISO para evaluación de membranas para ROG data del año 2004. Con la información obtenida, se planteó y se propuso una batería completa de pruebas que posibilitan la valoración de la membrana, desde la elección del polímero y la técnica de síntesis de la misma, hasta su utilización en clínica mediante estudios clínicos randomizados (7).

Tras la fase de exploración, se procedió a analizar la interacción de las membranas poliméricas nanoestructuradas con las células osteoblásticas. El estudio celular se comenzó evaluando la proliferación de las células sobre las matrices, utilizando el ensayo de MTT, un test colorimétrico que determina la actividad mitocondrial de las células. También se realizaron estudios de diferenciación celular, midiendo fundamentalmente la actividad de fosfatasa alcalina, el estudio del fenotipo antigénico de las células y técnicas de microscopía electrónica de barrido por emisión de campo (15). Al ver los resultados obtenidos, se decidió ampliar la caracterización de las células y se ejecutó un estudio avanzado de genómica mediante reacción en cadena de la polimerasa cuantitativa (RT-qPCR), para evaluar la expresión de los principales genes relacionados con la proliferación y diferenciación celular: factor de crecimiento transformante beta (TGF-β, por su nombre en inglés) y los tres receptores de esta proteína (TGF-βR1, TGF-βR2 y TGF-βR3), la fosfatasa alcalina (ALP), la osteocalcina (OSC), factor de transcripción relacionado con *Runt* (Runx-2), las proteínas morfogenéticas 2 y 7 (BMP-2, BMP-7), la

osteoprogeterina (OPG) o el ligando de receptor activador para el factor nuclear κβ (RANKL) (16).

Una vez realizado el análisis de los resultados, se planteó introducir el dopado con sustancias como doxiciclina, cinc o dexametasona a membranas disponibles en el mercado y utilizadas en clínica. Se llevó a cabo, entonces, una revisión de la literatura para estudiar los antecedentes de esta técnica, analizando las membranas más utilizadas, las sustancias más frecuentemente empleadas para su funcionalización, la cinética de liberación y el efecto antibacteriano de las mismas (17). Posteriormente, se realizaron los estudios celulares previamente descritos pero, esta vez, utilizando membranas de colágeno disponibles en el mercado dopadas con doxiciclina y dexametasona (18).

Tras ejecutar el metaanálisis, en el que se incluyeron 16 estudios y 292 pacientes, se concluyó que las membranas más utilizadas eran las poliméricas reabsorbibles de colágeno (Tabla 1 del Artículo 1), y con las técnicas de regeneración ósea actuales se conseguía una ganancia horizontal de 3,95mm ($p<0,001$; IC 95% [3,19-4,79]). Dado que existe una heterogeneidad alta, se usó el modelo de efectos aleatorios, obteniendo una elevada significación estadística ($p<0,001$) (Figura 3 del Artículo 1). Como resultado secundario se estudió la aparición de complicaciones postoperatorias. La frecuencia de aparición de complicaciones fue del 8,4%, que se encuentra dentro de la tasa reportada por estudios previos, que la situaron entre 7,95% y 22,7% (2,19). Las complicaciones más frecuentes fueron exposición de la membrana y aparición de dehiscencias del tejido blando. Estas complicaciones podrían reducirse con la utilización de una nueva membrana con mejor adhesión celular y capacidad de integración ósea.

En la revisión narrativa de la literatura (7), se propuso una batería de pruebas ideal, adaptada a la descripción y a los nuevos requerimientos de las membranas con fines regenerativos propuestos por el segundo grupo de trabajo del 15º Workshop Europeo de Periodoncia en Regeneración Ósea (6). Las pruebas más básicas están basadas en la caracterización de superficie de las membranas. Especial hincapié se puso en las propiedades nanomecánicas de las matrices poliméricas, ya que se ha demostrado cómo, por ejemplo, la elasticidad del sustrato puede hacer que células mesenquimales se diferencien *in vitro* hacia una estirpe celular u otra (20). Otro aspecto fundamental de un prototipo de membrana es su citocompatibilidad. Es de suma importancia que el material

del que se componga la membrana resulte prácticamente inocuo para el entorno celular, ya que si no fuera así produciría una respuesta inflamatoria aguda y una reacción de cuerpo extraño. Para ello, se realizan *tests* de viabilidad, exponiendo células fibroblásticas u osteoblásticas al material objeto de estudio y comparando su crecimiento con un control positivo. Uno de los ensayos que más frecuentemente se utilizan para este propósito son los test de viabilidad *LIVE/DEAD*. En este test se usan tinciones y microscopía de fluorescencia para identificar y comparar el porcentaje de células vivas (teñidas de verde) con el porcentaje de células no viables o metabólicamente comprometidas (teñidas de rojo) (7). Para comprobar dicha citocompatibilidad, las membranas objeto de estudio, con distintas concentraciones de sustancias dopadas, fueron sometidas a un cultivo de fibroblastos de mucosa oral. La viabilidad de las células fue analizada mediante liberación de ADN, liberación de LDH y usando el test *LIVE/DEAD*. Los resultados de los tres ensayos demostraron la buena citocompatibilidad de nuestros materiales (12).

La capacidad de las membranas de poseer un efecto antimicrobiano también es un aspecto importante, ya que la colonización bacteriana y posterior infección es una de las causas que pueden impedir la regeneración. Esta capacidad se debe medir, idealmente, mediante la utilización de un modelo de *biofilm*, ya que es la forma en la que las bacterias se agrupan y actúan en el entorno biológico. A este respecto, los prototipos de membrana que se proponen dopados con doxiciclina, demostraron en estudios previos tener una importante actividad antibacteriana *in vitro*, frente a un modelo de *biofilm* subgingival periodontal donde se usaron técnicas de reacción en cadena de la polimerasa cuantitativa (qPCR) (21).

Dado que una de las principales células que va a influir en la regeneración ósea es el osteoblasto, propusimos el estudio de la interacción de esta estirpe celular con nuestras membranas. Para ello, la influencia de las membranas sobre la proliferación y diferenciación de los osteoblastos debió ser evaluada. En relación a los osteoblastos, existen en la literatura muchos modelos celulares para llevar a cabo estos experimentos. Uno de los más utilizados, por su gran relevancia clínica, son los osteoblastos primarios humanos. En cambio, no siempre es el modelo de elección debido fundamentalmente a los largos tiempos de cultivo necesarios para su crecimiento y a su accesibilidad limitada (7,22). Una alternativa muy válida, es la utilización de osteoblastos tumorales, como las células osteoblásticas MG-63. Esta estirpe soluciona los problemas que presentan las

células primarias, ya que tienen un crecimiento mucho más rápido, una disponibilidad ilimitada y apenas tienen variaciones interespecie con los osteoblastos primarios (22). Para evaluar la proliferación se usa principalmente el test que analiza la reducción metabólica del Bromuro de 3-(4,5- dimetiltiazol-2-ilo)-2,5-difeniltetrazol (test MTT) o el ensayo *Alamar Blue*, que mide la actividad mitocondrial celular. Los resultados del MTT de nuestro estudio, demostraron que la proliferación de los osteoblastos siempre fue mejor en los prototipos de membranas con sílice en su formulación. Además, la máxima proliferación se obtuvo en el grupo con sílice y dopado con doxiciclina (Figura 1 del Artículo 3) (15). Ya se ha demostrado, previamente, que las tetraciclinas a nivel local pueden mejorar la proliferación osteoblástica (23). Este hecho podría deberse a dos razones: i) la doxiciclina posee un potente efecto antioxidante (24,25), y sabemos que la proliferación de los osteoblastos se ve favorecida por la presencia de antioxidantes, ya que las especies reactivas de oxígeno disminuyen la reproducción de estas células (26); ii) la doxiciclina posee la capacidad de quelar calcio (11), produciendo ello un aumento de calcio extracelular, hecho que ha demostrado dar lugar a osteoconducción y neoformación ósea (27).

Para la evaluación de la diferenciación celular, hay numerosos ensayos que se pueden llevar a cabo. Los más destacables serían: i) la actividad de fosfatasa alcalina, que mide la cantidad de esta proteína que secretan los osteoblastos; ii) la tinción de alizarina, que evalúa de manera directa la cantidad de mineral depositada por las células; iii) inmunofluorescencia para el estudio de la expresión de proteínas relacionadas con el depósito mineral; o iv) microscopía electrónica de barrido para evaluar la morfología celular, las interacciones intercelulares y las relaciones de las células con el sustrato. En nuestro primer estudio, se llevaron a cabo dos de las técnicas previamente mencionadas; una técnica cuantitativa, la actividad de fosfatasa alcalina; y una técnica cualitativa, la microscopía electrónica de barrido. Los resultados de ambas estuvieron en línea y de acuerdo con las de las pruebas previamente descritas. La actividad de fosfatasa alcalina demostró un aumento de la diferenciación de los osteoblastos cultivados en las membranas con sílice en su formulación, presentando una máxima diferenciación los prototipos con doxiciclina (Figura 4 del Artículo 3) (15). Esto fue también corroborado por las imágenes de microscopía, donde en las membranas con doxiciclina se pudo observar unos osteoblastos con morfologías más elongadas y una más evidente presencia de prolongaciones citoplasmáticas e interconexiones celulares (Figuras 2 y 3 del Artículo

3). Estudios previos han demostrado cómo hay una correlación entre la morfología y el estado metabólico de los osteoblastos, relacionándose las formas celulares más alargadas, fusiformes y expandidas con estados proliferativos y de diferenciación más avanzados (7,28,29).

Uno de los procedimientos más específicos que nos permite valorar la actividad metabólica de las células es la cuantificación de la expresión génica de proteínas relacionadas con la proliferación y diferenciación de los osteoblastos, mediante reacción en cadena de la polimerasa cuantitativa (qPCR). Esta técnica provoca la lisis de las células y evalua el ARN mensajero para la codificación de ciertas proteínas capitales en la actividad metabólica de este tipo celular. En el caso de la proliferación, las proteínas más frecuentemente estudiadas son el factor de crecimiento transformante beta (TGF-β, por su nombre en inglés) y los tres receptores de esta proteína (TGF-βR1, TGF-βR2 y TGF-βR3). En cuanto a la diferenciación, se puede destacar la fosfatasa alcalina (ALP), la osteocalcina (OSC), factor de transcripción relacionado con *Runt* (Runx-2), las proteínas morfogenéticas 2 y 7 (BMP-2, BMP-7), la osteoprogeterina (OPG) o el ligando de receptor activador para el factor nuclear κβ (RANKL). Los resultados del análisis génico se mostraron en línea con los de las pruebas previamente descritas. En líneas generales, las membranas dopadas con doxiciclina produjeron una sobre-expresión de los principales genes relacionados con la proliferación y la diferenciación de los osteoblastos. En el caso del cinc, los resultados fueron algo más controvertidos, ya que en ocasiones se demostró de igual manera un aumento de la expresión de estos genes mientras que otras veces se mostraron valores iguales al grupo con sílice, por lo que dicha sobre-expresión podría ser atribuible a la sílice presente en las membranas y no al cinc. En cuanto a la proliferación, los resultados obtenidos por qPCR están en línea con los obtenidos mediante MTT, quedando esto patente, fundamentalmente, en el aumento significativo en la expresión de TGF-β y TGF-βR1 (Figura 1 del Artículo 4). En relación con los genes relacionados con la diferenciación, encontramos algunos resultados algo contradictorios. La expresión de genes como las BMPs 2 y 7, ALP, RANKL u OPG, están totalmente de acuerdo con los *tests* previamente utilizados. En cambio, hay algunas incoherencias en el gen codificando la Runx-2. Esto puede deberse fundamentalmente a la utilización de células tumorales, que no debemos olvidar que en tienen un crecimiento en ocasiones anómalo y pueden diferir de los patrones que atribuiríamos a células primarias (22).

También se calculó la ratio OPG/RANKL (Figura 2 del Artículo 4). Teniendo en cuenta el significado biológico de la expresión de cada uno de dichos genes, cuanto mayor sea la ratio, mayor potencial osteogénico podría ser atribuido a un biomaterial. En el caso de las membranas dopadas con doxiciclina, se pudo ver cómo esta ratio se aumentó en 28 veces en comparación con las membranas sin dopar (16). Las membranas dopadas con Zn también produjeron una ratio OPG/RANKL significativamente mayor que las membranas sin dopar, siendo 14,2 veces superior (16).

A la luz de los resultados obtenidos en los estudios celulares al dopar las membranas experimentales con sustancias activas, especialmente con la doxiciclina, se planteó el diseño de una revisión sistemática para evaluar el estado del arte en cuanto a la funcionalización con antibióticos locales de membranas de regeneración ósea (17). En ella se vio cómo este enfoque ya había sido propuesto tanto en membranas reabsorbibles como no reabsorbibles. El grupo de antibióticos más frecuentemente utilizados fueron las tetraciclinas, seguidas por el metronidazol. Otros agentes también empleados, aunque en menor medida, son la amoxicilina, eritromicina o vancomicina (Tabla 1 del Artículo 5). La mayoría de estos trabajos, se centraron en la medición de los efectos antibacterianos de dichas membranas, pero pocos de ellos realizaron estudios en relación al efecto que esta modificación podría provocar sobre los osteoblastos. Por ello, se planteó el empleo del tipo de membranas más comúnmente utilizadas en el entorno clínico, las membranas de colágeno (14), para su dopado con doxiciclina. En este estudio, además de la doxiciclina, se utilizó un glucocorticoide buscando el efecto osteoinmunomodulador de estos fármacos, analizando su efecto sobre los osteoblastos. Se ha descrito como los osteoblastos también juegan un papel muy importante en la regulación del sistema inmune, tienen capacidad para liberar citoquinas, y están implicados en la presentación de antígenos, pudiendo llevar a cabo procesos fagocíticos (30). Esta no es una relación meramente unidireccional, sino que se ha visto cómo determinadas células inmunológicas tienen un papel fundamental en la regulación del metabolismo óseo (7,31). Además, se ha demostrado previamente que la dexametasona tiene la capacidad de promover la proliferación y diferenciación, tanto de osteoblastos, como de células madre humanas derivadas de médula ósea (32–34).

Las membranas de colágeno dopadas con doxiciclina o dexametasona mostraron un aumento significativo de la proliferación osteoblástica en el test MTT (Figura 2 del

Artículo 6). Este aumento de la proliferación fue más acentuado en el caso de las membranas dopadas con doxiciclina cuando se analizó la expresión génica. En este grupo se vieron aumentados los genes que codifican la proteína TGF-β y los tres receptores de esta proteína (TGF-βR1, TGF-βR2 y TGF-βR3). La diferenciación osteoblástica se estudió mediante actividad de fosfatasa alcalina, capacidad de mineralización (tinción con alizarina roja) y análisis de genes relacionados con la diferenciación. Los resultados de dichas pruebas presentan resultados acordes (Figuras 3, 4 y 5, respectivamente, del Artículo 6), atribuyendo una efectividad limitada a las membranas modificadas con dexametasona, y una más rápida diferenciación a los osteoblastos que se cultivaron en las membranas a las que se le añadió doxiciclina.

Como se ha mencionado previamente, se ha demostrado cómo las células del sistema inmune tienen un papel primordial en la regulación del metabolismo óseo. Esto hace importante estudiar, de igual manera, el efecto que producen las membranas sobre estirpes celulares inmunológicas. De entre todas las células inmunológicas, los macrófagos juegan un papel fundamental en los procesos de regeneración. En un primer momento, deben facilitar el establecimiento de un ambiente inflamatorio que producirá la eliminación de detritus, células u otros componentes no útiles para la regeneración ósea. En ese momento, se deberá producir un cambio del fenotipo de los macrófagos M1 (pro-inflamatorios) hacia un fenotipo M2 (anti-inflamatorio y pro-regenerativo). Para llevar a cabo este estudio celular, se podrá investigar la polarización de los macrófagos mediante la expresión génica de ciertas proteínas, utilizando qPCR o mediante inmunohistoquímica, marcando con anticuerpos algunos de los antígenos de membrana que diferencian ambos fenotipos celulares (7).

Además de todos los estudios celulares, estas membranas deben ser evaluadas mediante investigación *in vivo*. En primer lugar, los experimentos han de conducirse en diseños preclínicos, siendo de elección en este caso el modelo de animal grande como el conejo neozelandés o el perro *Beagle*. Las pruebas que más comúnmente se ejecutan para evaluar la regeneración son la microtomografía computerizada y los estudios histomorfométricos, usando tinciones de hematoxilina y eosina, *Von Kossa,* o incluso técnicas de fluorescencia.

Nuestro prototipo de membranas ha sido ya evaluado en estudios realizados en calota de conejo (35). Y el último paso necesario de investigación para estas membranas sería el diseño de un ensayo clínico controlado y randomizado.

7. Conclusiones

1º. Las membranas que con más frecuencia se utilizan para ROG son las reabsorbibles de colágeno. La técnica quirúrgica es predecible, pudiendo reducir un defecto horizontal entre 3 y 4,7mm. Existen complicaciones en un 8,4% de los casos y la complicación más frecuente es la exposición e infección de la membrana.

2º. Una membrana de ROG debe ser evaluada con técnicas basadas en nanotecnología desde el punto de vista tribológico, morfométrico y mecánico. Su bioactividad debe ser analizada mediante cultivos de células de estirpe osteoblástica donde se determine su capacidad de proliferación, diferenciación y mineralización. Estudios preclínicos *in vivo* y estudios clínicos controlados y randomizados son siempre necesarios para determinar su efectividad clínica.

3º. Existen numerosas combinaciones de funcionalización de antibióticos sobre materiales poliméricos que han sido analizados en membranas para ROG. En todas ellas se consigue efecto antibacteriano. Solo se han evaluado frente a cultivos de bacterias no específicas y con una única especie, lo que limita su relevancia clínica. Los ensayos en humanos son escasos y de resultados controvertidos.

4º. El dopado de membranas nanoestructuradas poliméricas no reabsorbibles con partículas de sílice mejora la expresión de marcadores osteogénicos. La funcionalización adicional con doxiciclina produce además la sobreexpresión de genes como BMP-2, ALP, OPG, TGFβ-1 and TGFβ-R1, y da lugar a una ratio OPG/RANKL más favorable.

5º. La funcionalización de membranas poliméricas de colágeno con doxiciclina es eficaz para mejorar la proliferación y diferenciación de osteoblastos.

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9. Indicios de calidad objetivos de la Tesis Doctoral

9.1. Trabajos presentados a Congresos

9.1.1. Comunicación Oral presentada en el congreso *CED-IADR/NOF Oral Health Research Congress: Bone-forming Genes Expression of Osteoblasts Cultured on Polymeric Nanostructured Membranes*. Bruselas, 16-19/09/2021.
Indicios de calidad: *Robert Frank Award* a la mejor Comunicación Oral del congreso. Selección del trabajo para representar a la división continental europea de la IADR en su congreso mundial.

9.1.2. Comunicación Oral presentada en el congreso SEPA Sevilla 2021: Expresión génica de osteoblastos cultivados sobre membranas poliméricas nanoestructuradas dopadas con sílice y zinc. Sevilla, 25-27/11/2021.
Indicios de calidad: Mención especial en el Premio a mejor comunicación oral de investigación básica del congreso.

9.1.3. Comunicación Oral presentada en el congreso *2022 IADR/APR General Session: Enhancement of Proliferation and Differentiation Genes Expression of Osteoblasts Cultured on Polymeric Nanostructured Matrices*. *On line* (China), 20-25/06/2022.

9.1.4. Comunicación Oral presentada en el congreso *2022 PER-IADR Oral Health Research Congress: Osteogenic potential of doxycycline doped collagen membranas.* Marsella, 15-19/09/2022.

9.2. Publicaciones Científicas

9.2.1. Toledano-Osorio M, Toledano M, Manzano-Moreno FJ, Vallecillo C, Vallecillo-Rivas M, Rodriguez-Archilla A, Osorio R. Alveolar Bone Ridge Augmentation Using Polymeric Membranes: A Systematic Review and Meta-Analysis. Polymers (Basel). 2021;13(7):1172. doi: 10.3390/polym13071172.

Índice de Impacto: 4,967 Posición: 16/90 Q1 Citaciones recibidas: 8

9.2.2. Toledano-Osorio M, Manzano-Moreno FJ, Ruiz C, Toledano M, Osorio R. Testing active membranes for bone regeneration: A review. J Dent. 2021;105:103580. doi: 10.1016/j.jdent.2021.103580.

Índice de Impacto: 4,991 Posición: 11/92 Q1 Citaciones recibidas: 19

9.2.3. Toledano-Osorio M, Manzano-Moreno FJ, Toledano M, Medina-Castillo AL, Costela-Ruiz VJ, Ruiz C, Osorio R. Doxycycline-Doped Polymeric Membranes Induced Growth, Differentiation and Expression of Antigenic Phenotype Markers of Osteoblasts. Polymers (Basel). 2021;13(7):1063. doi: 10.3390/polym13071063.

Índice de Impacto: 4,967 Posición: 16/90 Q1 Citaciones recibidas: 10

9.2.4. Toledano-Osorio M, Manzano-Moreno FJ, Toledano M, Osorio R, Medina-Castillo AL, Costela-Ruiz VJ, Ruiz C. Doxycycline-doped membranes induced osteogenic gene expression on osteoblastic cells. J Dent. 2021;109:103676. doi: 10.1016/j.jdent.2021.103676.

Índice de Impacto: 4,991 Posición: 11/92 Q1 Citaciones recibidas: 9

9.2.5. Toledano-Osorio M, Vallecillo C, Vallecillo-Rivas M, Manzano-Moreno FJ, Osorio R. Antibiotic-Loaded Polymeric Barrier Membranes for Guided Bone/Tissue Regeneration: A Mini-Review. Polymers (Basel). 2022;14(4):840. doi: 10.3390/polym14040840.

Índice de Impacto: 4,967 Posición: 16/90 Q1 Citaciones recibidas: 6

9.2.6. Toledano-Osorio M, de Luna-Bertos E, Toledano M, Manzano-Moreno FJ, García-Recio E, Ruiz C, Osorio R, Sanz M. Doxycycline-doped collagen membranes accelerate in vitro osteoblast proliferation and differentiation. J Periodont Res. 2022;00:1–12.

Índice de Impacto: 3,946 Posición: 20/92 Q1 Citaciones recibidas: 0

Índices de impacto y posición de la revista según listado *Journal Citation Report*. Citaciones recibidas tomadas de *Scopus*.

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11. ANEXO
Artículos publicados

polymers

MDPI

Systematic Review

Alveolar Bone Ridge Augmentation Using Polymeric Membranes: A Systematic Review and Meta-Analysis

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Abstract: Alveolar bone ridge resorption occurred after natural teeth loss and it can restrict the possibility of dental implants placement. The use of bone regenerative procedures is frequently required. The existing evidence regarding the efficacy of horizontal bone ridge augmentation trough guided bone regeneration (GBR) using polymeric membranes was stated. A systematic review and meta-analysis were performed. Electronic and manual literature searches were conducted. Screening process was done using the National Library of Medicine (MEDLINE by PubMed), Embase, and the Cochrane Oral Health. Included articles were randomized controlled trials and observational studies. Weighted means were calculated. Heterogeneity was determined using Higgins (*I*2). If I2 > 50% a random-effects model was applied. It was found that the mean of horizontal bone gain was 3.95 mm, ranging from 3.19 to 4.70 mm (confidence interval 95%). Heterogeneity is I2 = 99% (confidence interval 95%) and significance of the random-effects model was $p < 0.001$. The complications rate was 8.4% and membrane exposure was the most frequent. Through this study, we were able to conclude that the existing scientific evidence suggests that GBR using polymeric membranes is a predictable technique for achieving horizontal bone augmentation, thus, permitting a proper further implant placement.

Keywords: bone regeneration; polymeric membrane; bone substitutes; ridge augmentation

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1. Introduction

Dental implants have become a predictable treatment option therapy after teeth loss. To perform an optimal implant placement and to improve their long-term prognosis, it is frequently required alveolar ridge augmentation to increase bone volume [1]. Alveolar ridge resorption occurred after teeth loss, trauma, or infections and it can severely restrict dental implant placement [2]. The use of bone regenerative procedures is frequently required before implant placement. Alveolar ridge defects may be classified according to the main resorbed region as horizontal, vertical, or combined defects [2]. The loss of horizontal ridge width occurs more frequently and to a greater extent compared with the loss of vertical ridge height [3].

Different approaches may be used to regenerate atrophic alveolar ridges, but guided bone regeneration (GBR) is the most frequently used technique [2,4]. For GBR various biomaterials are applied: (1) autogenous, allogenic, xenogeneic, and synthetic bioceramics of polymers can be use as bone substitute materials and can be particulated or as a block unit [5] and (2) resorbable or non-resorbable polymeric membranes which will act as barriers, playing an important role by isolating soft tissue and allowing bone to grow. Several combinations of materials may be employed but the use of polymeric barrier membranes is highly encouraged as it prevented significant bone resorption during the healing

period and, thus, greater mean ridge width gain is obtained [3,6]. The barrier polymeric membrane excluded undesirable cells, for example, epithelial and connective tissue cells from populating the wound site, therefore allowing cells with regenerative potential, for example, osteoblasts to colonize the defect and form bone [7]. Non-resorbable membranes, with polytetrafluroethyelene (PTFE) membranes as the maximum representative, have a superior space-making capability but there is a high frequency of wound dehiscence and the subsequent risk of bacterial contamination and infection that require retreatment [5,8,9]. Trying to avoid this problem, antibiotics and metal such as silver, zinc, or copper have been incorporated into the GBR membranes to improve periodontal healing [10–13]. Another drawback of these membranes is the need of a second surgery in order to retrieve the membrane, besides, the difficulty of this process due to their soft tissue integration [5]. Meanwhile, resorbable membranes, which are mainly represented by collagen membranes, are mainly limited by their lack of rigidity, reducing the space-making potential [14]. Another downside of this membranes is their fast degradation rate, which may not meet the necessary period for an optimal tissue regeneration [5]. This problem has been partially solved by the chemical cross-linking of the collagen matrices. This enhances the collagen stability, but it has also been associated with severe inflammation at the surgical site due to the release of chemical residues [5].

The main objectives of this study were to gather all of the scientific evidence about the effectiveness of GBR achieved with polymeric membranes and to quantify the expectable amount of horizontal bone gain than can be obtained.

2. Materials and Methods

2.1. Development of the Protocol

The study protocol was designed according to the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) statement. The developed protocol was registered in the PROSPERO - International Prospective Register of Systematic Reviews database hosted by the National Institute for Health Research, University of York, Center for Reviews and Dissemination (ID: CRD42021232447). Details regarding the PICO question (population, intervention, comparison, and outcome) are the following:

P—Healthy patients, older than 18 years, with a deficient alveolar ridge that needs to be horizontally augmented prior to implant placement.

I—Guided Bone Regeneration with bone graft materials (resorbable or non-resorbable membranes alone or with the addition of bone graft substitutes as autografts, xenografts, alloplasts, or allografts.

C—Defects pre-treatment and post-treatment (between 3 and 7 months of follow-up) or other surgical approaches.

O—Bone gain after treatment, measured with cone beam computed tomography (CBCT). Secondary outcomes are clinical benefits and/or biological complications of GBR.

Articles considered eligible for inclusion were interventional (randomized controlled trials (RCTs)) and observational studies (cohort, case-control studies, and case series). A minimum sample size of 5 patients was required.

Exclusion criteria included: animal studies, in vitro studies, reviews and articles published in a language other than English, studies assessing lateral or vertical bone augmentation or horizontal when in conjunction with immediate implant placement.

2.2. Search Strategy, Data Extraction, and Studies Quality Assessment

Electronic and manual literature searches were conducted by 2 independent reviewers (M.T-O. and R.O.). The following search strategy was followed: (“collagen membrane” OR “extracellular membrane” OR “porcine collagen membrane” OR “porcine derived collagen membrane” OR “cytoplast” OR “PTFE membrane” OR “Bio-guide”) AND (“guide bone regeneration” OR “Bone augmentation” OR “GBR” OR “ridge augmentation”) NOT (“sinus lift” OR “sinus elevation” OR “ridge preservation” OR “socket preservation” OR “animal” OR “dog” OR “pig” OR “rabbit”). A time frame restriction of 15 years was

applied. Screening process was performed at the following information sources: the National Library of Medicine (MEDLINE by PubMed), Embase and the Cochrane Oral Health. Databases were searched for studies published up to including January 2021. Reference lists of the previous reviews and included studies were analyzed trying to search for relevant manuscripts that were missing after the electronic screening.

Data extraction and risk-of bias were assessed by two investigators (M.T-O. and C.V.) in duplicate and thereafter discussed to find agreement. In the case of disagreement, the judgment of a third reviewer (R.O.) was decisive. The following data were extracted: (1) authors and year of publication, (2) study design, (3) participants and number of interventions, (4) bone substitute, (5) membrane, (4) follow-up time, (5) bone gain, and (6) clinical complications.

The study quality and designs were evaluated according to: (i) An adapted version of the Newcastle–Ottawa scale [15] for interventional and observational researches. Studies were considered as having a high, medium, or low methodological quality and (ii) The Joanna Briggs Institute Critical Appraisal tool for case series. Studies were considered as having a high, medium, or low risk of bias [15].

2.3. Data Analyses

Descriptive statistics were used to present the primary outcome—efficacy of GBR in terms of bone gain (mm). Weighted means (CI 95%) were calculated, including total sample size, inverse variance, and standard error of the treatment effect. Heterogeneity was determined using Higgins (I2). If I2 > 50% a random-effects model was applied. Statistical significance was set at 0.05. Data were analyzed with RevMan 5.4 (The Cochrane Collaboration, Oxford, UK). Funnel plot was produced by MedCalc 18.2.1 (MedCalc Software Ltd., Ostend, Belgium) to represent systematic heterogeneity.

3. Results

3.1. Search Results

The electronic search was performed in January 2021, resulting in 523 articles. After duplicate removal and the reading of titles and/or abstracts, 41 articles were selected. A manual search identified eight more manuscripts. Then the full-text of all the selected articles was reviewed for the inclusion criteria. Then, 25 articles were excluded after full reading, and 16 articles were then included in the final selection. A flowchart of the selection and inclusion process, based on PRISMA recommendations is presented in Figure 1. The extracted data for each reviewed article are shown in Table 1.

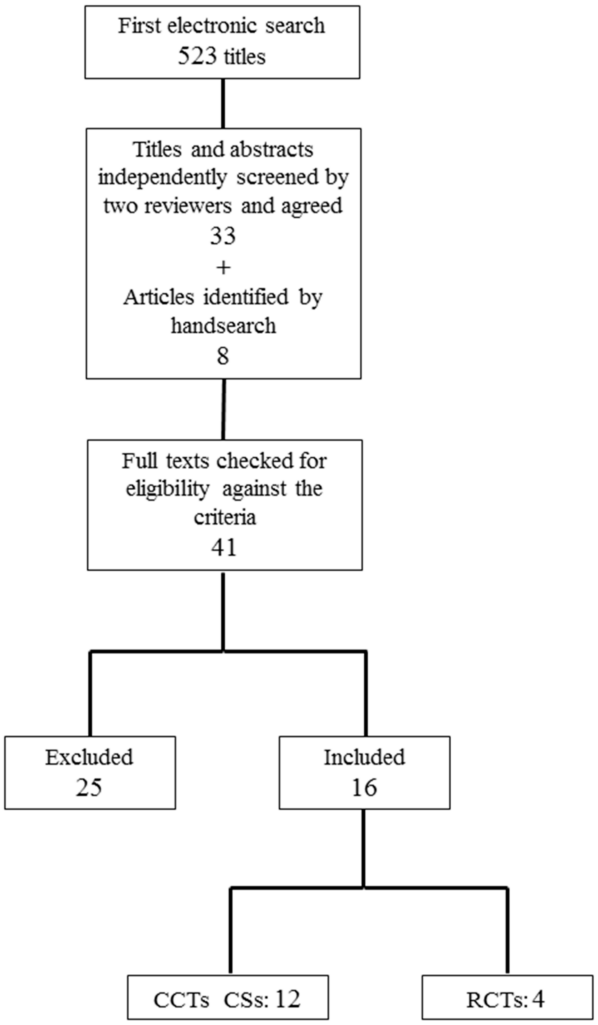

Figure 1. Flow chart of the search results and respective selection process. RCTs: randomized controlled trials, CCTs: cohort and case-control studies, CSs: case series.

Table 1. General overview of the included studies, investigating the outcomes of bone defects treated by guided bone regeneration (GBR) with bone graft materials (polymeric membranes and bone graft substitutes as autografts, xenografts, alloplasts, or allografts).

Author	Study Design	Patients GBRs	Bone Substitute	Membrane	Follow-Up Time	BG (mm) Mean (SD)	Complications
Pushparajan et al., 2013 [16]	CCT	10 patients 10 GBRs	DBBM particles Autogenous	Collagen	6 months	1.44 (0.09)	Not reported
Shalash et al., 2013 [17]	CCT	10 patients 10 GBRs	β-TCP particles	d-PTFE	6 months	1.22 (0.35)	2 membrane exposures
		10 patients 10 GBRs	β-TCP particles DBBM particles	d-PTFE		1.37 (0.35)	
Mordenfeld et al., 2014 [18]	RCT	13 patients 13 GBRs	DBBM particles (90) Autogenous (10)	Collagen	7.5 months	2.9 (1.3)	7 dehiscences
		13 patients 13 GBRs	DBBM particles (60) Autogenous (40)	Collagen		3.5 (1.3)	

Table 1. *Cont.*

Monje et al., 2015 [19]	CS	6 patients 9 GBRs	Illiac crest block DBBM particles	Collagen	5 months	4.93 (0.65)	Not reported
		8 patients 10 GBRs	Mandib ramus block DBBM particles	Collagen		3.23 (0.76)	
Barbu et al., 2016 [1]	RCT	11 patients 11 GBRs	Mandib ramus block DBBM particles + Autogenous	Pericardium	4 months	5.10 (0. 91)	3 patients with pain in donor site
Gultekin et al., 2016 [20]	CCT	12 patients 15 GBRs	DBBM particles Autogenous	Collagen	4–7 months	5.42 (0.76)	1 dehiscence
		12 patients 13 GBRs	Mandib ramus block DBBM particles	Collagen		4.54 (0.59)	
Meloni et al., 2017 [21]	CCT	18 patients 22 GBRs	DBBM particles Autogenous	Collagen	7 months	5.03 (2.15)	3 membrane exposures
Cortellini et al., 2018 [22]	CCT	10 patients 15 GBRs	L-PRF + DBBM particles	Collagen	5–8 months	4.6 (2.3)	1 dehiscence
Mendoza-Azpur et al., 2019 [23]	RCT	20 patients 20 GBRs	DBBM particles Autogenous	Collagen	6 months	5.6 (0.89)	6 membrane exposures 1 infection
		19 patients 19 GBRs	Mandib ramus block DBBM particles	Collagen		5.10 (0.77)	
Lucaciu et al., 2019 [24]	CS	13 patients 20 GBRs	ABBM particles Autogenous	Collagen	4 months	1.96 (1.64)	Not reported
Amaral Valladão et al., 2020 [25]	CS	18 patients 29 GBRs	L-PRF DBBM particles Autogenous	Collagen	7.5–8.5 months	5.9 (2.4)	Not reported
Atef et al., 2020 [26]	RCT	10 patients 10 GBRs	ABBM particles Autogenous	Collagen	6 months	3.65 (1.04)	1 membrane exposure 1 infection
Batas et al., 2020 [27]	CS	6 patients 6 GBRs	Allogenic bone DBBM particles	Collagen	5 months	4.7 (1.22)	Not reported
Hashemipoor et al., 2020 [28]	RCT	21 patients 21 GBRs	FDBA	Collagen	6 months	3.05 (0.98)	1 membrane exposure
		19 patients 19 GBRs	FDBA Autogenous	Collagen		3.10 (1.4)	
Neto et al., 2020 [29]	CCT	18 patients 22 GBRs	DBBM particles	Collagen	6–8 months	2.5 (2.02)	1 membrane exposure
Windisch et al., 2020 [30]	CS	15 patients 18 GBRs	DBBM particles Autogenous	d-PTFE	9 months	8.5 (2.4)	1 membrane exposure

* GBRs: Guided Bone Regeneration procedures; BG: Bone gain; CCT: Cohort and Case-Control Trial, RCT: Randomized Clinical Trial; CS: Case series; DBBM: Demineralized Bovine Bone Matrix; β-TCP: β-tricalcium phosphate; ABBM: Anorganic Bovine Bone Matrix; L-PRF: Leukocyte and Platelet Rich Fibrin; FDBA: Freeze Dried Bone Allograft.

3.2. Studies Quality Assessment and Risk of Bias

The quality assessment and the risk of bias of the selected papers are summarized in Figure 2. Most of the selected studies are classified as high quality or low risk of bias.

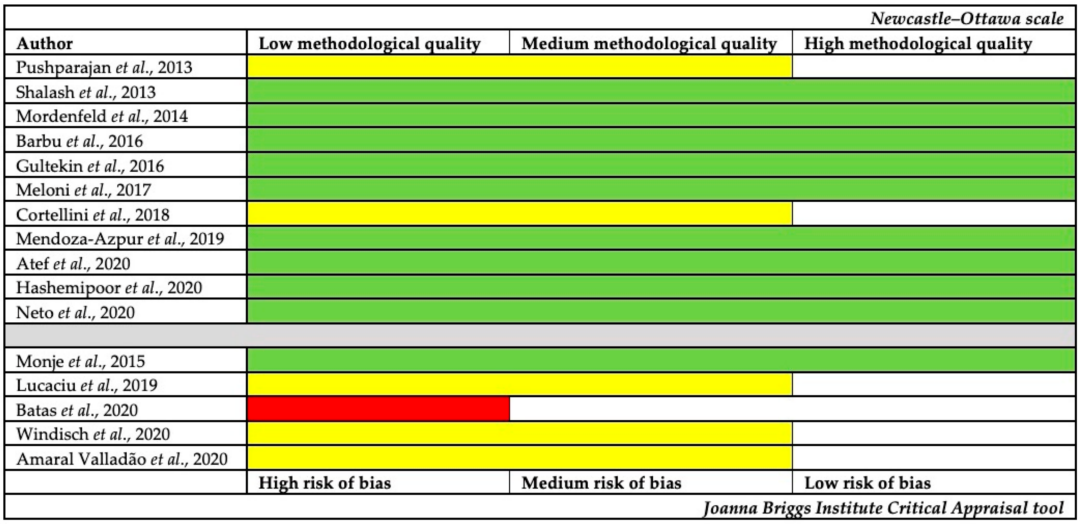

Figure 2. Studies' quality assessment and risk of bias following: Newcastle–Ottawa scale for interventional and observational assays. Studies were considered as having a high (green), medium (yellow), or low (red) methodological quality, and the Joanna Briggs Institute Critical Appraisal tool for case series. Studies were considered as having high (red), moderate (yellow), or low (green) risk of bias.

3.3. Primary and Secondary Outcomes: Horizontal Bone Gain and Complications

Sixteen studies (292 patients and 381 defects) analyzed the regenerative efficacy measured as horizontal bone gain. Main study characteristics are displayed in Table 1.

The mean of horizontal bone gain was 3.95 mm, ranging from 3.19 to 4.70 mm (CI 95%). Heterogeneity is I2 = 99% (CI 95%) and significance of the random-effects model was P < 0.001. Bone gain forest plot graph is displayed in Figure 3. Systematic heterogeneity is displayed at the funnel plot graph (Figure 4).

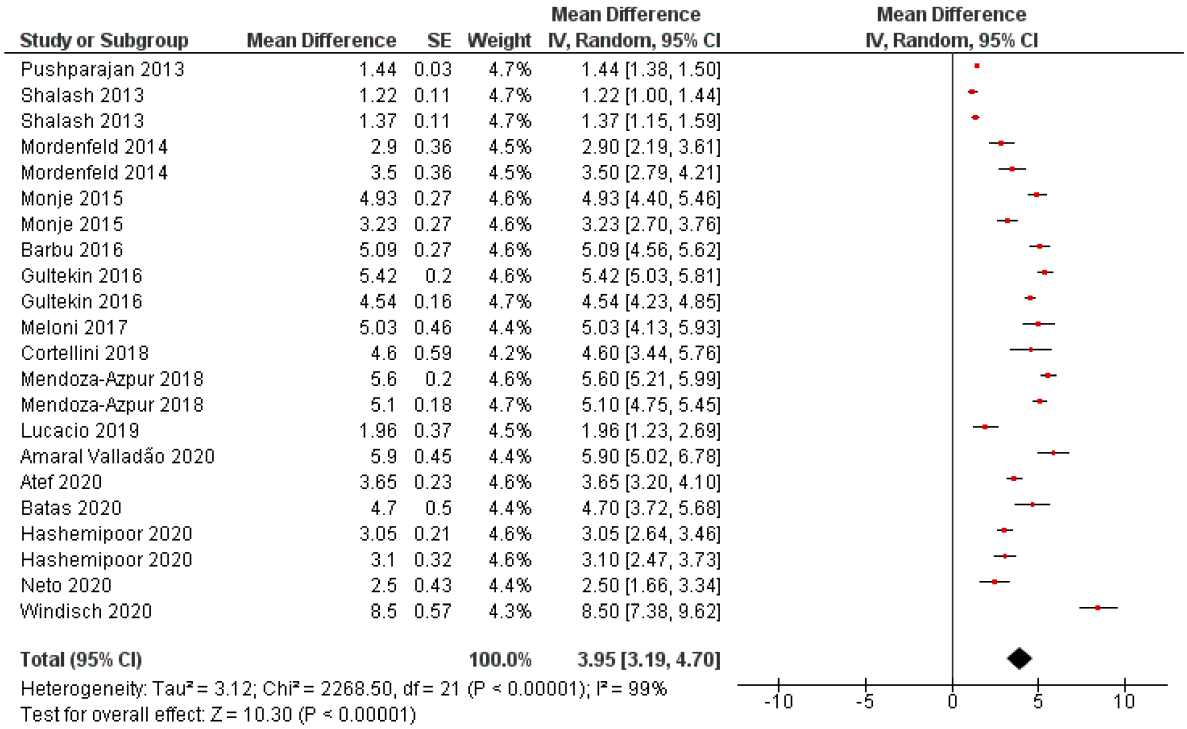

Figure 3. Horizontal bone gain forest plot. Weighted mean is presented at CI 95%. Heterogeneity was determined using Higgins (I2). A random-effects model was applied. Statistical significance was set at 0.05.

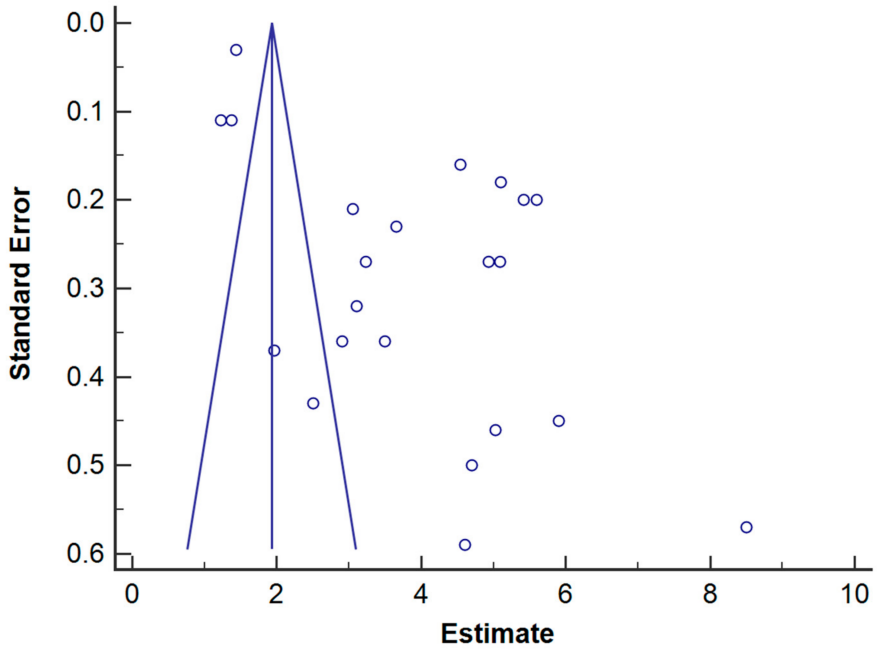

Figure 4. Horizontal bone gain funnel plot. Estimate of bone gain measurement is on the horizontal axis and study precision (standard error) appears on the vertical axis.

The complications rate was 8.4%, while five studies did not report any type [16,19,24,25,27]. The 11 remaining studies demonstrate some clinical complications, including: membrane exposure, the most frequent (15 membranes exposures in seven studies [17,21,23,26,28–30]); nine dehiscences were reported in three studies [18,20,22]; two manuscripts referred to one infection each [23,26] (Table 1).

4. Discussion

The aim of this systematic review and meta-analysis was to obtain the most reliable scientific information regarding the efficacy of bone augmentation procedures in terms of bone gain in cases of horizontal and/or vertical ridge bone deficiencies, when using polymeric membranes for GBR. A great variability of results, measured as bone gain, does exist. Therefore, in this study it is intended to reduce heterogeneity of primary outcomes. For this purpose, only studies that counted with CBCT measurements were included in the review. It has been previously reported that there was variability between measurements performed at CBCT images and direct clinical measuring [31]. The follow-up of the patients included in the present review was set as between 3 and 7 months, and always before implant placement, in order to ensure that GBR processes were not influenced by the implants' outcome.

The first CBCT device (NewTom-9000; Quantitative Radiology, Verona, Italy) was described in 1998. Since then, a number of CBCT machines have been introduced into the market. The cost-effective technology of CBCT led to a speedy ingress into the field of dentistry with demand for commitment of dental professionals and dental educators to explore the applications of CBCT technology [32]. Nevertheless, CBCT is not employed in postsurgical assessments of bone grafts' and implants' position planning until 2006 [33–35]. It is the reason why, although a time frame restriction of 15 years was applied, the first published clinical trial using CBCT for bone augmentation evaluation was not found until 2013, which is the earliest study included in this review.

This systematic review was not limited to clinical trials to achieve more data about the use of polymeric membranes in GBR procedures. Sixteen studies were included, from which only four were randomized clinical trials. Case series, prospective, and retrospective designs were also included to achieve more data about the GBRs. In total, 381 GBRs have been analyzed, involving the mandible and the maxilla. Fourteen studies evaluated just

horizontal bone gain after the augmentation surgery whereas two of them studied horizontal and vertical bone gain. Absorbable membranes were the most used (14 studies). Only three out of the 16 included studies tested non-resorbable membranes; two of them used a titanium reinforced dense polytetrafluroethyelene (Ti-d-PTFE) membrane and one with no reinforcing (d-PTFE). When using absorbable membranes, collagen membranes were the most frequently placed (93% of the studies using absorbable membranes), whereas only one used a commercialized pericardium membrane. The polymeric resorbable collagen membrane is the most employed for GBR procedures, having the higher number of published clinical studies [36]. Main advantages include easy manipulation, weak immunogenicity, a direct effect on bone formation and chemotaxis of gingival and periodontal ligament fibroblasts [37,38]. However, their rapid biodegradation by the enzymatic activity of macrophages and polymorphonuclear leucocytes or bacterial collagenases is their major drawback [39]. Then, the potential of losing space maintenance ability in physiological conditions is high and clinical results may be sometimes unpredictable [36].

Following the main results of the present research, GBR techniques using polymeric membranes may facilitate a horizontal bone gain from 3.19 to 4.70 mm at the alveolar ridge. It is a clinically relevant amount of bone, if we consider that placed implant may usually be from 3.5 to 4.5 mm in diameter, and a minimum of 1.5 mm of remnant bone is required around the placed implants [2]. Therefore, it may be speculated that the achieved horizontal volume after tested GBR techniques, should allow for implant placement with success in most of the clinical cases.

A bone gain of 8.5 $\pm$ 2.4 mm was described in the study that reported the highest horizontal bone gain [26]. It is a case series study in which a Ti-d-PTFE and bovine-derived xenograft in combination with autogenous bone chips. There are not many other studies in which non-resorbable membranes are used to treat horizontal bone defects. It may be that non-resorbable membranes requiring a second surgery and with complications derived from membranes exposition and contamination are preferred when treating vertical defects, in which procedures and healing time is longer and mechanical properties of membranes are a crucial prerequisite [40].

Ten of the selected studies used autologous bone + xenogenic as bone substitute [16,18–21,23–26,30], from which, 70% used particulate autologous bone + xenograft [16,18,21,24–26,30], 20% used autologous bone block [19,23], and one study compared both techniques [20], concluding that mean horizontal bone gain and width after healing were significantly greater in the group of autologous particulate bone compared to bone block.

High bone gain results (5.9 $\pm$ 2.4 mm [25], 5.42 $\pm$ 0.76 mm [20], and 5.03 $\pm$ 2.15 mm [21]) were obtained in three studies which employed a common protocol. They used a collagen membrane with bovine-derived xenograft in combination with autogenous bone chips as bone filler. The highest clinical success when applying this bone combination was previously reported in one study published in 2019 [2]. It may be explained by the fact that an inorganic xenogenous graft could slow down the resorption of autogenous bone and also increase the volume to the grafted area [2].

The encountered rate of complications, 8.4%, is within the rate of other previous meta-analysis about bone regeneration procedures, ranging from 7.95% [2] to 22.7% [6]. Membrane exposure and dehiscences are also the most frequently reported complications in previous studies [2,6].

The attained total heterogeneity data between published studies is very high, 99% (95% CI) (Figure 3); it is also observable in the funnel plot graph (Figure 4). It may be explained by differences in implemented surgical techniques, employed biomaterials and operators. The surgical technique and execution are crucial for the success of bone augmentation procedures. Factors as achieving primary wound closure, adequate angiogenesis, space creation and maintenance, wound stability, membrane exposure, or microorganism colonization may influence the amount of bone regeneration that can occur [3]. The encountered systematic difference between studies may also be due to the small sample size of included studies (namely: 'small-studies effect') [41]. It should be considered that

the experiments' sample size ranges from 6 to 21 patients and from 6 to 29 surgical interventions. A mean bone gain of 5.9 mm (SD: 2.4 mm) was achieved in the study with the greatest sample size [25], a case series in which a combination of demineralized bovine bone matrix particles with autogenous bone, adding leukocytes and platelet rich fibrin was used. However, it should be remarkable that a high statistical significance was obtained at the random-model effects ($p < 0.001$).

Another meta-analysis has been previously published about bone regeneration at the alveolar ridge [6]. Differing from the present study, this study just considered GBRs performed simultaneously with dental implant placement. This surgical strategy is beneficial in terms of reducing the number of interventions. However, it usually negatively affects the total bone gain and increases the complications ratio. Membrane exposure was found in about 23% of the performed GBRs [6]. In this case, the most often used type of intervention was also a xenogeneic particulated grafting material and a resorbable collagen membrane. A mean bone gain of 4.44 mm (ranging from 0.11 to 7.72 mm) was obtained in a systematic review [2], which also studied horizontal bone ridge augmentation procedures but only applying xenogenous graft. It is a value slightly higher than the one obtained in the present research, but with a high standard deviation. Therefore, the present study was the only one considering several bone graft types, which is not reported in any other study. It is considered highly valuable for clinicians and researchers.

This systematic review and meta-analysis possess some strengths which differentiates it from previously published reviews. The registration of the research design in PROSPERO, prior to the beginning of the search, warrants that it has been shown to be associated with increased review quality [42]. Strict inclusion criteria, such as the need for a previous and follow-up CBCT and the exclusion of all the studies with clinical conditions that could alter the results such as immediate implant placement, simultaneal sinus lift, or socket preservation make the results more reliable. In addition, a thorough and rigorous analysis of the risk of bias and methodological quality of the studies included was carried out. As a result of all these methodological premises, a high level of significance ($p < 0.001$) was obtained, even when the random-effects model was applied.

However, the study does not lack of certain limitations. The narrow focus of the question of systematic reviews is a crucial drawback of systematic reviews in general, since they do not allow for complex literature coverage. Apart from this, some of the differences encountered between the clinical trials could have been caused by the small-studies effect, due to their small sample sizes [41].

Additionally, as future perspectives, clinical researchers should try to perform more protocolized and randomized clinical trials in this area, since there is an enormous methodological and clinical heterogeneity in the identified studies.

5. Conclusions

Through this systematic review and meta-analysis, we have been able to conclude that the existing scientific evidence suggests that GBR surgical procedure using polymeric membranes is a predictable technique, in order to achieve horizontal bone augmentation and, usually, the postoperative elapses with no complications. Clinicians can expect to reduce the horizontal bony defect from 3.19 to 4.70 mm, thus, permitting, in most of the cases, proper further implant placement.

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Testing active membranes for bone regeneration: A review

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ABSTRACT

Objectives: Maxillofacial bone defects are the main hindering conditions for traditional dental implant strategies. Guided Bone Regeneration (GBR) is used to handle this situation. The principle of GBR is to use a membrane to prevent the colonization of soft tissue cells of the bone defect and favors the migration of osteogenic linages. Current membranes do not completely fulfill the requirements that an optimal membrane should have, sometimes resulting in non-predictable results. Thus, the need to develop an ideal membrane to perform this duty is clear. Recent developments in bio-manufacturing are driving innovations in membranes technology permitting the active participation of the membrane in the healing and regenerative process trough native tissue mimicking, drug-delivery and cells interaction, away from being a passive barrier. New membranes features need specific evaluation techniques, beyond the International Standard for membrane materials (last reviewed in 2004), being this the rationale for the present review. Nanotechnology application has completely shifted the way of analyzing structural characterization. New progresses on osteoimmmunomodulation have also switched the understanding of cells-membranes interaction.
Data and Sources: To propose an updated protocol for GBR membranes evaluation, critical reading of the relevant published literature was carried out after a MEDLINE/PubMed database search.
Conclusions: The main findings are that a potential active membrane should be assessed in its nanostructure, physicochemical and nanomechanical properties, bioactivity and antibacterial, osteoblasts proliferation, differentiation and mineralization. Immunomodulation testing for macrophages recruitment and M2 phenotype promotion in osteoblasts co-culture has to be achieved to completely analyze membranes/tissue interactions.

1. Introduction

Alveolar and maxilar bone defects are the major hindering conditions for traditional implant strategies. These defects are mainly caused by trauma, tumor or infection [1].

An efficient tool available to palliate this handicap is Guided Bone Regeneration (GBR). GBR is one of the most effective techniques to obtain osteogenesis. It is based on the necessity to isolate the bone defect from soft tissue, in order to prevent that epithelial and connective components migrate and colonize the hard tissue defect. For this reason, to achieve GBR it is indispensable the presence of a membrane, that will act as a barrier. According to Sanz et al. [2], and as one of the consensuses of the 15th European Workshop on Periodontology on Bone Regeneration, besides its occluding and isolation capacity, a membrane for GBR should meet some basic requirements: biocompatibility, biological activity, porosity and occlusive properties, mechanical properties, integration with tissues, exposure tolerance and biodegradability. Currently, there is not a commercialized membrane that meets the optimal characteristics. Recent developments in biomanufacturing are driving innovations in membranes technology to respond to this challenge. The major efforts in recent developments in membranes design are: i) the creation of nanostructured membranes mimicking the native tissue [3], ii) the active participation of the membrane in the healing and regenerative process trough drug-delivery and cells interaction, away

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from being a passive barrier [4,5]. These two new membranes features need specific evaluation techniques. Moreover, it should be considered that the International Standard -ISO- which gives the requirements for a technical evaluation of membrane materials was last reviewed in 2004 [6]. For these reasons, there is a need to develop a membrane for GBR to enable clinicians to accomplish more predictable regenerative surgeries [7], and to define how to examine new membranes characteristics trying to fulfill desired prerequisites of a potential membrane for GBR [2].

The main purpose of this review was to propose a specific and contrasted protocol for GBR membranes evaluation, from *in vitro* to *in vivo* testing and focusing on the most recent membranes evolution.

2. Surface characterization

2.1. Morphological analysis

Nanotechnology application in membranes manufacturing has completely shifted the way of analyzing structural characterization. Nanofibrous scaffolds are preferred as they possess unique properties: high surface area to volume ratio, porosity with interconnected pores, enhanced protein absorption, activation of specific gene expression and intracellular signaling, and promoted cellular reactions [3]. With larger surface to absorb proteins, nanoscaled scaffolds present more binding sites to cell receptors [3,8].

The use of Atomic Force Microscopy (AFM) would give the opportunity to observe membranes nanostructure at an atomic level, being able to access to the very scaffolding of matter, even at the chemical bond scale [9]. AFM can be used to assess nanoroughness, which is an important parameter that will promote protein non-specific adhesion and cellular attachment to the proposed matrices [10,11]. Fiber sizes, fiber to fiber distance and pore sizes can also be measured with Scanning Electron Microscopy (SEM) and AFM [12]. When using SEM for membranes structural characterization, the sample needs to be processed (specially, when analyzing natural polymers) whereas AFM measures accurately on the nanoscale, produces high-resolution images, requires little or no sample preparation and is able to work in humid conditions [13]. Much work has been done on the effect of pore dimension on osteoblast proliferation and differentiation. Scaffolds with interconnected pores usually enhance more bone growth compared with those with closed or non-existent pores [14]. This is because the delivery of osteoprogenitors to the scaffold is improved if the ingrowth of vasculature is facilitated [14,15]. It has been reported that nanometric porosity ranging from 50 to 500 nm selectively enhances protein adsorption (including fibronectin and vitronectin), contributing to cell attachment [11]. Cells growing on membranes containing pores between 5 and 8 μm showed increased osteogenic differentiation [16]. Mimicking collagen nanofiber diameters has been shown to enhance cell attachment on tissues about 1.7-fold [11]. Scaffold architecture greatly influences cell attachment and migration [16], so it is indisputably a fundamental part of tissue analysis. In addition, and to add importance to the fact of working at nanoscale, it has recently been described that nanofibrous materials provide high area-to-volume ratios, mimicking the extracellular matrix of native bone tissue, enhancing cellular adhesion and growth [17]. This fact has determined that novel artificial fibrillar membranes manufactured trough electrospinning are being developed (Fig. 1) [3,7].

2.2. Nanomechanical properties

Measuring nanomechanical properties has been demonstrated to be of great importance, since it has been proved that substrate stiffness can modify cell behavior and cells may probe and respond to mechanics in fibrillar matrices [18]. It has been described that native mesenchymal stem cells have extreme sensitivity to matrix-level elasticity, conditioning their differentiation to specific lineages, including osteogenic phenotypes [19]. However, measuring properties of individual nanofibers or even at a micrometric level is not completely reliable as it does not relate to the clinical use of these materials. These measurements performed on an individual fiber do not take into account the force dissipation due to molecular interactions within the fibers of the network and the force dissipation via interstitial spaces and flows [13]. This is why dynamic nanomechanical analysis is highly recommended instead of the classical static tests.

Polymers of long chains (*i.e.* cross-linked collagen) have unique viscoelastic properties, combining the characteristics of elastic solids and Newtonian fluids [20]. For this reason, specific viscoelastic parameters should be studied. Complex modulus (G*) reflects the contribution of both elastic and viscous components to the material's stiffness, the storage modulus (G') measures the stored energy and represents the elastic portion of the material, the loss modulus (G'') measures the energy dissipated as heat and the tan delta (δ) provides a measure of damping in the material, and it is the coefficient of loss and storage moduli (G'/G'').

To achieve biomimicking, the storage modulus and tan delta values

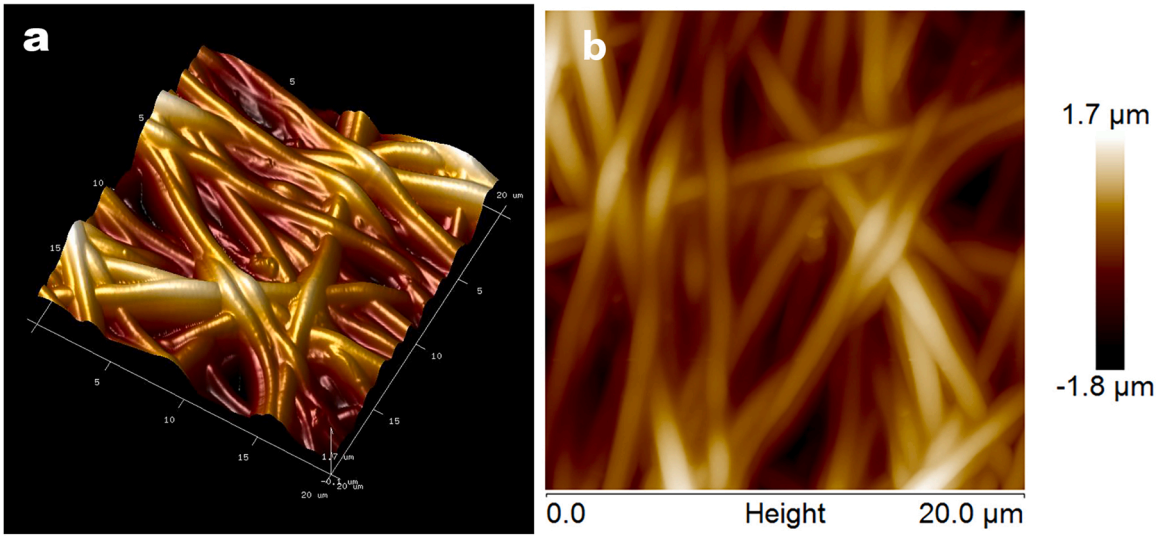

Fig. 1. AFM image of an electrospun nanostructured membrane surface manufactured by NanomyP® (Granada, Spain) using a novel polymer blend: $(MMA)_1$-co-$(HEMA)_1$/$(MA)_3$-co-$(HEA)_2$ doped with 5 % wt of SiO_2-NPs. Overlapped and randomly distributed nanofibers, mimicking collagen fibers, may be observed.

of the matrix should be similar to the calcified trabecular bone, which are around 15 GPa and 0,6; respectively [7,21].

2.3. Wettability

Several studies have used the Water Contact Angle (WCA) method, in order to stablish its hydrophobicity or hydrophilicity [22,23]. Normally, a high value of WCA indicates hydrophobicity, whereas a low value shows that a material is more hydrophilic. It is well known that improved surface hydrophilicity is necessary for cell adherence and growth [24]. Most synthetic biodegradable polymers are hydrophobic; thereby, extensive efforts have thus been devoted towards increasing the hydrophilicity of biomaterials. One convenient measure is to produce chemical modification of the membranes' surfaces introducing polarized groups such as hydroxyl, carboxyl, amino or sulfate groups on polymer surfaces using different techniques as may be plasma treatment [25].

2.4. Bioactivity

Aiming bone regeneration, the ability of a material to chelate calcium phosphate is of essential importance, since it would mean a step forward to obtain primary mineralization. Bioactivity and mineralization can be studied *in vitro*, following the International Standard ISO 23,317 [26], in which it is specified the method for detecting apatite formed on a surface of a material after immersion in simulated body fluid (SBF). SBF is a solution that mimics the blood serum in terms of ionic composition and pH [27]. This method is applicable for surfaces which are intended to be in direct contact with bone. After performing the previous mentioned procedure, the membranes could be analyzed with SEM and Elemental Diffraction Analysis (EDX) trying to look for calcium and phosphate deposits or X-Ray Diffraction Analysis (XRD) to directly detect crystals formation and its main components [8].

2.5. Biodegradation

This section may only be applied to the resorbable membranes, and it is intended to find the average time that the biomaterial would remain with structural integrity. It has been previously described that in order to achieve a predictable GBR process, the membrane should remain physically and mechanically intact for at least an average time of 4–6 weeks [28–31]. This time period may vary depending on individual patients' conditions that negatively influence bone repair rates such as age, systemic and metabolic conditions or big defect size. These factors should be taken into account when selecting a membrane for GBR, opting for a delayed resorption or even a non-resorbable membrane. The main assay employed is a hydrolytic degradation test, achieved by immersing the membrane in a Phosphate Buffered Solution (PBS) at different time points [28,32]. Currently, it is known that the membrane contamination by periodontal pathogens such as *Porphyromonas gingivalis* and *Treponema denticola* (which are able to produce collagenases) and/or membrane exposure to the oral cavity during or after surgery is sometimes unavoidable. It has been assessed that collagen membranes' degradation is up to 80 % faster, when they are immersed in bacterial collagenase if compared to PBS [28]. This may explain the unpredictable clinical results, which is sometimes, attained by resorbable membranes. Moreover, it has been described that pores larger than 100 μm appeared during the degradation processes of membranes [28], which will jeopardize the soft tissue cells barrier effect required for a successful GBR therapy. Knowing the importance of biomaterial stability and maintaining the space in bone regenerations, these results would provide really important information about the membrane that is being tested and the possible clinical situations in which it could potentially be used.

3. Cytocompatibility

Cytocompatibility is defined as the property of a material or substance of not been toxic or harmful to a cell. It is normally tested by the use of cell viability assays. Cell viability is the quantification of the number of live cells and is usually expressed as a percentage of a control material [33]. The two tests which are used the most are: Cell Counting Kit-8 (CCK-8, Dojindo, Japan) and Live/Dead staining. In most of the studies both tests are used together in order to contrast ones results with the other. It has been recently reported [34] that Live/Dead dyes may not be used as an exact quantitative measurement of cell death. Red cells, stained with propidium iodide, have commonly been identified as dead cells, whereas they really represent cells that are injured, dead or starving viable cells. Therefore, red cells percentages should be taken with caution [35]. This may be an explanation for the need to contrast the results by two different methods.

4. Antibacterial effect of the material

After the surgical technique of GBR, the regenerative outcome of the surgery is sometimes compromised by bacterial colonization and infection [36]. This may occur in a more frequent way when the membrane is exposed to the oral cavity due to soft tissue collapse. This situation could be overcome if the membrane shows some bactericidal capacity. This effect can be achieved by modifying the membrane's structure or by adding components able to exert this function. Some of the components that have been described are metal, ions like zinc, copper or silver [1,17,37], or antibiotics, like metronidazole or doxycycline [38–40]. In order to assay the membrane's antibacterial effect, the protocol described by Bueno et al. [39], perfectly meets the required tests. The membranes need to be exposed to a periodontal multispecies biofilm in anaerobic conditions to simulate, as much as possible, the real conditions to which the membrane would be subjected subsequent to bacterial colonization. After culturing the bacteria, quantitative Polymerase Chain Reaction (qPCR) and Live/Dead assay should be conducted to quantify the surviving bacteria and to analyze the dynamics of the biofilm in the presence of the membrane. SEM would also be useful to observe the primary interactions between the initial colonizers and the biomaterial.

5. Other specific assays on membranes to cells interactions

5.1. Testing osteoblasts interactions with membranes

The osteoblast is a complex cell which actively participates in bone metabolism. Its main duties include being responsible for bone formation and regeneration and for the regulation of osteoclast activity. It also possesses immunologic functions, that include: the synthesis of cytokines, expression of antigens implicated in antigen presentation, allogenic stimulation, and phagocytic [41]. Several cell models have been used in *in vitro* studies, including primary human osteoblast cells, primary mouse osteoblast cells, primary bovine osteoblast cells, MG-63, MC3T3-E1 and SaOs-2. Primary human osteoblasts and MG-63 are the ones used the most. Primary human osteoblasts, are obtained from bone tissue of donors, and are the most relevant for clinical studies, but need long isolation procedures, limited accessibility and the cell phenotypes are sensitive to donor-related factors. On the other hand, in the case of MG-63 osteoblast-like cells, there are no interspecies differences with primary human osteoblasts, have a shorter isolation time and there is unlimited accessibly [42]. However, the results need to be extrapolated with caution, taking into account that a tumor line may have an alternative pattern of differentiation from primary human osteoblasts [41]. For this reason, it may be advisable to use at least, two different cell models in order to correctly understand the efficacy of the membrane. Several tests are proposed:

5.1.1. Osteoblasts proliferation

proliferation is defined as the cellular growth rate or as the quantified value for the daughter cell population [33]. Regarding cells

proliferation, the ability of the cells to replicate is the parameter being tested. For this reason, and differing from viability assays, the time points in which the tests are performed are longer (*i.e,* 1, 3, 10, 15 and 21 days); whereas in viability assays it is difficult to find an established time point longer than 48 h. Considering this, it is vital to provide the cells with nutrients (fresh media) during the assay, in order to avoid the entrance into an early stationary phase.

There are several methods to investigate osteoblasts proliferation. The most widely used is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, in which the tetrazolium salt is metabolized by active mitochondria to form insoluble formazan crystals, detected by measuring the absorbance using a microplate reader. It has to be kept in mind that a false return of cell number would be encountered if the biomaterial affected the mitochondrial activity and cells survived via glycolysis. This is one of the reasons why it is recommended that proliferation is studied by different tests, in order to validate the results. Another commonly used method is the Alamar Blue assay. It is also a test which measures the metabolic action of the cells by the mitochondrial activity, in this case by oxidation of resazurin dye by means of a REDOX reaction. An absorbance microplate reader is also needed [7]. In addition, the calcein viability assay can also be used. It is a simple and extremely sensitive quantitative assay to measure the cell viability of adherent and suspension cells. It can detect as low as 50 viable cells in less than 30 min. Calcein is a non-fluorescent, hydrophobic compound that easily penetrates intact and live cells. During the Calcein assay, hydrolysis of Calcein AM by intracellular esterases produces a hydrophilic, strongly fluorescent compound that is retained in the cell cytoplasm and can be measured at Ex/Em = 485/530 nm. The measured fluorescence intensity is proportional to the number of viable cells [43]. Another used assay for cytotoxicity and cell viability with adherent cell cultures is Crystal Violet. The Crystal Violet assay is based on staining cells that are attached to cell culture plates. It relies on the detachment of adherent cells from cell culture plates during cell death. During the assay, dead detached cells are washed away. The remaining attached live cells are stained with Crystal Violet. After a wash step, the Crystal Violet dye is solubilized and measured by absorbance at 595 nm. The amount of Crystal Violet staining in the assay is directly proportional to the cell biomass that is attached to the plate [44].

5.1.2. Osteoblasts differentiation

After testing cells proliferation, the differentiation potential of osteoblasts needs to be ascertained, since it is the stage in which they begin to play their role in matrix production and mineralization. Several methods to study osteoblasts differentiation can be found in literature:

a) Alkaline Phosphatase (ALP) activity. The differentiation and maturation of osteoblasts involves the synthesis of specific bone proteins which contribute to the synthesis of the extracellular matrix and its subsequent mineralization [41]. One of these mentioned proteins is ALP. ALP is one of the firsts functional proteins expressed in the process of calcification [45]. ALP activity is normally quantified using a colorimetric assay which determines early osteoblastic differentiation (*i.e.* 7 days of culturing) [46]. ALP activity is commonly expressed as a relative percentage, considering enzymatic activity of 100 % in a control group without biomaterial.

b) Sirius Red Staining. This colorimetric assay is used to quantitatively measure the amount of collagen (mainly types I and III) produced by osteoblasts. The results may provide a reliable imagen of osteoblasts proliferation, since 85–90 % of the organic extracellular matrix is composed of collagen. Sirius Red Staining, which is an anionic dye that binds to collagen, is diluted in saturated aqueous picric acid solution and added to the membrane. The cells are then washed with HCl to remove all the excess of dye and dissolved in a NaOH solution. Afterwards, the absorbance is measured at 550 nm [47].

c) Alizarin Red staining. Whilst ALP activity is able to measure mineralization indirectly, Alizarin red-S is the optimal assay to measure matrix mineralization in a direct way. This test is normally performed at different time points to evaluate the evolution of the mineralization nodules (*i.e.* 7, 15 and 21 days) [48]. At the different time points, Alizarin Red solution is added to the membrane and washed several times with deionized water in order to reduce non-specific staining. Calcium deposits present in the extracellular collagen matrix will be colored in red, revealing mineralization nodules [46]. These calcium deposits can be measured with a spectrophotometer after dissolving them with cetylpyridiniumchloride.

d) Quantitative Real Time Polymerase Chain Reaction (RT-qPCR). RT-qPCR is used to investigate the expression of genes encoding osteoblast differentiation markers. Before performing the RT-qPCR, the RNA needs to be extracted from the cells. The RNA obtained from the cells is measured by spectrophotometry. Then, the RNA must be transformed into complementary DNA (cDNA) by means of the reverse transcription, in order to avoid working with RNA, a molecule quite instable [49]. The chains of cDNA will then be amplified by PCR following the manufacturer's instructions of the commercialized kit used.

Primers need to be designed using NCBI-nucleotide library and Primer3-design in order to detect messenger RNA (mRNA) of the targeted genes. In Table 1 a list of the most useful primer sequences for the amplification of cDNA by real-time PCR is presented [50,51]. RT-qPCR can now be performed with the cDNA extracted from the cells and the designed primers. It has to be taken into account that each gene needs a specific annealing temperature, ranging from 60 to 65 °C [50]. Ubiquitin C (UBC), peptidylprolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) are commonly used as stable housekeeping genes in order to normalize final results [52,53].

e) Immunofluorescence staining. It has been used for a double purpose. Firstly, and more commonly exerted, to observe cytoskeleton organization. Cells are initially incubated with a Rhodamine-phalloidin dye; and afterwards, 4′,6-diamidino-2-phenylindole (DAPI) dye solution needs to be apply. These two dyes stain specifically actin filaments from the cytoskeleton and DNA from the nuclei of the cells, respectively [54, 55]. Analyzing the cells would give as an image of how well the cells spread over the biomaterial, the emission of filopodia and the shape they acquire when they differentiate. Tsai et al. [56], went a step further and, in addition to studying cytoskeleton and DNA, stained the membranes with osteoblast-specific marker proteins to study differentiation. They used osteocalcin (OCN) and bone sialoprotein (BSP) and primary antibody against osteoblast-specific marker protein OCN and BSP. This gave them the capacity of studying and quantifying the presence of these proteins under a Laser Scanning Confocal Microscope. BSP is a protein which is normally found in the bone matrix. It participates in the hydroxyapatite nucleation, so it has been proposed to be one of the initiators of mineralization of the extracellular matrix [57]. On the other hand, OCN is a specific protein produced by osteoblasts during the mineralization phase [58]. Therefore, BSP and OCN were used as markers of middle and mature stages of osteoblasts' differentiation, respectively [56].

f) Antigenic Phenotype. Osteoblasts maturation and differentiation may also be assessed by analyzing their antigenic phenotype, which is modified along the process of maturation of the cells and may be influenced by growth factors, cytokines, and hormones in the bone tissue, like CD54, CD80, CD86 and HLA-DR [59]. Osteoblasts should be exposed to the biomaterial that is being tested and after that, stained with anti-monoclonal antibodies (anti-Mabs), depending on the antibodies that need to be tested (*i.e*, to detect CD54, osteoblasts should be stained with anti-CD54 monoclonal antibody). After been incubated for approximately 30 min, aliquots are analyzed in a flow cytometer with diode laser at a wavelength of 488 nm to determine the percentage of fluorescent cells. Untreated cells need to be used as controls [46].

g) SEM and Energy Dispersive X-ray spectroscopy (EDX). Using SEM to detect cell differentiation may be useful. It has been described that osteoblasts' morphology is highly influenced by its differentiation stage. Spread morphology has been associated with the expression of differentiation markers and higher metabolic activity, whereas circularity has

Table 1
Primer sequences for the amplification of osteoblasts' cDNA by real-time PCR.

Gene	Sense Primer	Antisense Primer
TGF- β1	5´-TGAACCGGCCTTTCCTGCTTCTCATG-3´	5´-GCGGAAGTCAATGTACAGCTGCCGC-3´
TGF- β R1	5´-ACTGGCAGCTGTCATTGCTGGACCAG-3´	5´-CTGAGCCAGAACCTGACGTTGTCATATCA-3´
TGF- β R2	5´-GGCTCAACCACCAGGGCATCCAGAT-3´	5´-CTCCCCGAGAGCCTGTCCAGATGCT-3´
TGF- β R3	5´-ACCGTGATGGGCATTGCGTTTGCA-3´	5´-GTGCTCTGCGTGCTGCCGA TGCTGT-3´
RUNX-2	5´-TGGTTAATCTCCGCAGGTCAC-3´	5´-ACTGTGCTGAAGAGGCTGTTTG-3´
VEGF	5´-CCTTGCTGCTCTACCTCCAC-3´	5´-CACACAGGATGGCTTGAAGA-3´
OSX	5´-TGCCTAGAAGCCCTGAGAAA-3´	5´-TTTAACTTGGGGCCTTGAGA-3´
BMP-2	5´-TCGAAATTCCCCGTGACCAG-3´	5´-CCACTTCCACCACGAATCCA-3´
BMP-7	5´-CTGGTCTTTGTCTGCAGTGG-3´	5´-GTACCCCTCAACAAGGCTTC-3´
ALP	5´-CCAACGTGGCTAAGAATGTCATC-3´	5´-TGGGCATTGGTGTTGTACGTC-3´
COL-I	5´-AGAACTGGTACATCAGCAAG-3´	5´-GAGTTTACAGGAAGCAGACA-3´
OSC	5´-CCATGAGAGCCCTCACACTCC-3´	5´-GGTCAGCCAACTCGTCACAGTC-3´
OPG	5´-ATGCAACACAGCACAACATA-3´	5´-GTTGCCGTTTT A TCCTCTCT-3´
RANKL	5´-ATACCCTGATGAAAGGAGGA-3´	5´-GGGGCTCAATCTATATCTCG-3´
UBC	5´-TGGGATGCAAATCTTCGTGAAGACCCTGAC-3´	5´-ACCAAGTGCAGAGTGGACTCTTTCTGGATG-3´
PPIA	5´-CCATGGCAAATGCTGGACCCAACACAAATG-3´	5´-TCCTGAGCTACAGAAGGAATGATCTGGTGG-3´
RPS13	5´-GGTGTTGCACAAGTACGTTTTGTGACAGGC-3´	5´-TCATATTTCCAATTGGGAGGGAGGACTCGC-3´

been associated with lower DNA concentrations [60]. In addition, attachment with the neighboring cells by means of extensions or filopodia may indicate cells differentiation [61]. Sometimes, rounded structures can be observed on the surface of osteoblasts which may correspond with mineral deposits, fact that can be confirmed by EDX analysis [61] (Fig. 2).

5.2. Testing macrophages interactions with membranes

Macrophages are cells of the innate immunity that are found nearly in all tissues. They derive from circulant monocites, which in turn, have their origin in hematopoietic stem cells (HSCs). Its main functions include phagocytosis of invading microorganisms, amplifying the inflammatory reaction and recruiting additional immune cells [62]. However, and although all mechanism are still not completely known nor understood, it has been shown that macrophages determine bone regeneration [63]. Macrophages can polarize into a pro-inflammatory phenotype M1 or pro-regenerative M2 phenotype in a context-dependent manner. After any situation which involves bone destruction (*i.e*, pathological fractures, implant placement, etc.), the M1

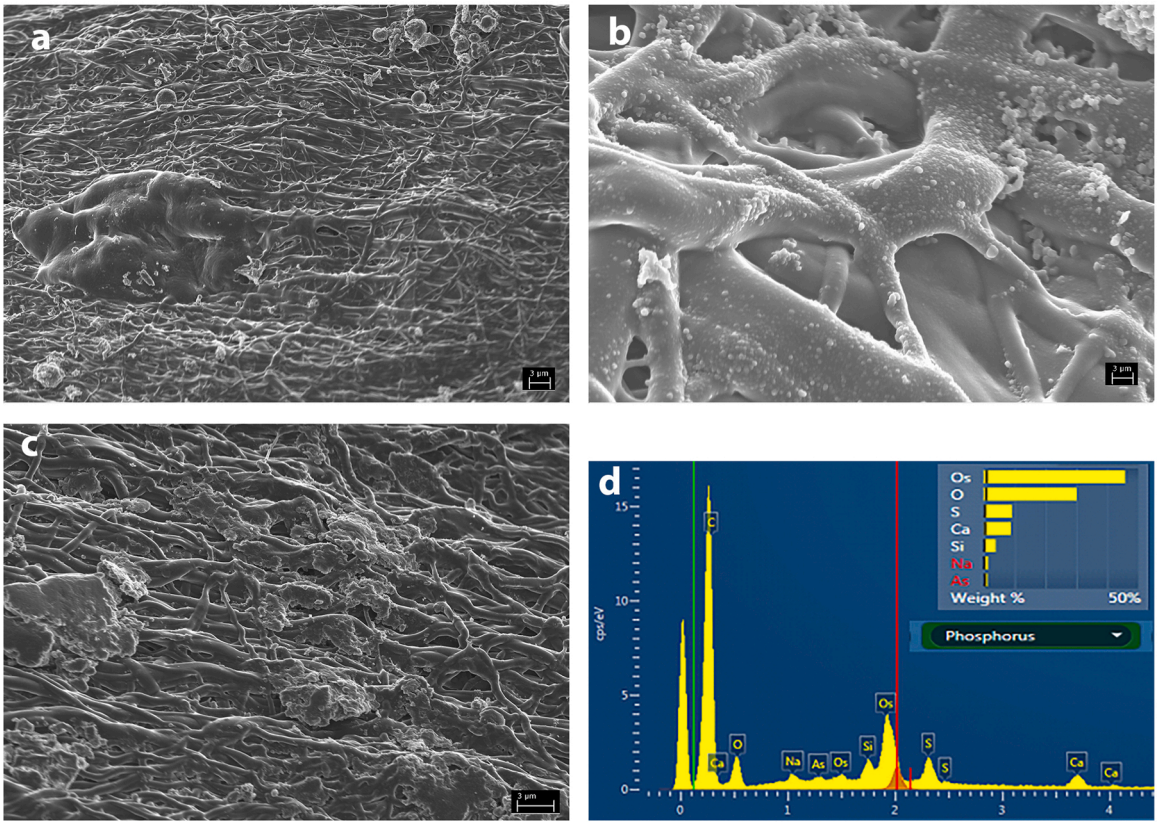

Fig. 2. Surface FESEM images of a non-resorbable polymeric electrospun membrane seeded with osteoblasts cells. a) A flat and elongated osteoblast cell is noticed on the membrane. Long osteoblasts' filapodia may be observed crossing over the membranes' surfaces. b) At higher magnification filapodia are intermingled with membranes fibers and covered by extracellular substance. c) Numerous filapodia are detected on the surface and are difficult to distinguish from nanofibres of the electrospun membrane, mineral deposits are also observed. **d)** Calcium and phosphorous were identified after EDX analysis. Phosphorous is in orange overlapped on the osmium spectrum peak. Silicon is also present at the EDX spectra.

phenotype is needed in order to begin the immune response by producing and releasing pro-inflammatory cytokines and chemokines (IL-1, IL-6, TNF-α, inter alia) that recruit other immune cells. A prolonged time in M1 phase, would lead to fibrous encapsulation and failure of bone regeneration or implant osseointegration [64]. In contrast, if a switch from M1 to M2 phenotype is achieved, it would result in bone regeneration and anti-inflammatory environment. It has been recently ascertained that nanostructured surfaces, hydrophilicity, several chemical radicals as hydroxyl or carboxyl groups or the presence of certain cations (Zn^{2+}, Ca^{2+}, Si^{2+}) at biomaterials may facilitate M2 macrophage polarization [65,66] (Fig. 3). Hence, biomaterials which are capable of controlling and modifying the M1/M2 polarization at tissue-biomaterial interaction locations, will be highly promising for bone regeneration strategies [67].

In addition to this, the role of macrophages in bone regeneration is crucial since it has been recently discovered that there is a cross-talk between macrophages and bone forming cells [63], participating in the process known as osteoimmunomodulation. Luo et al. [68], demonstrated, *in vitro,* that the inclusion of macrophages in an osteoblast culture enhanced osteoblasts differentiation and mineralization, measured by ALP, Alizarin Red Staining and RT-qPCR (through expression of the genes RUNX2, ALP, OCN and BMP2). They even showed that all these parameters where improved just by adding media derived from a 24 h cultured macrophage colony to their osteoblasts cultures [68]. It is speculated that this improvement in mineralization and differentiation could be, partially, due to the ability of the macrophages to diminish Reactive Oxygen Species (ROS) from the osteoblasts [68].

In the light of this information, it would be of utmost importance to investigate the behavior of macrophages when put in direct contact with the biomaterial in order to be able to design a membrane which enables their polarization into M2 phenotype. Studying the impact of macrophages on osteoblasts' activity would help to develop a material with osteoimmunomodulation ability. The analysis of this interaction osteoblast-macrophage-biomaterial could be best achieved by means of a co-culture of macrophages and osteoblasts on the studied membrane [68].

5.2.1. *Macrophages polarization testing*

Firstly, in order to obtain macrophages (M0), THP-1 cells need to be stimulated with the presence of phorbol-12-myristate-13-acetate (PMA) [67]. After this process, M0 macrophages can be cultured on the membranes to observe their polarization pattern when exposed to the biomaterial. After 24−48 h of culture there are different techniques that may be undertaken. RT-qPCR can be used in order to detect differences in the expressions of M1 and M2 markers. For M1 profile; IL-1, IL-6 and TNF-α are normally explored; whereas for M2 markers IL-10, Arg1, CD206 and TGF-β are the selected genes [51,67]. Recommended genes' sequences are presented in Table 2 [51,67]. Immunofluorescence staining can also be used to quantify the M1/M2 ratio. It can be done by

Table 2
Primer sequences for the amplification of macrophages' cDNA by real-time PCR.

Gene	Sense Primer	Antisense Primer
IL-1	5′-GGTTGAGTTTAAGCCAATCCA-3′	5′-TGCTGACCTAGGCTTGATGA-3′
IL-6	5′-GAAAGGAGACATGTAACAAGAGT-3′	5′-GATTTTCACCAGGCAAGTCT-3′
TNF-α	5′-CAGCCTCTTCTCCTTCCTGAT-3′	5′-GCCAGAGGGCTGATTAGAGA-3′
IL-10	5′-GAGGCTACGGCGCTGTCA-3′	5′-TCCACGGCCTTGCTCTTG-3′
Arg1	5′-ACGGAAGAATCAGCCTGGTG-3′	5′-GTCCACGTCTCTCAAGCCAA-3′
CD206	5′-GGGTTGCTATCACTCTCTATGC-3′	5′-TTTCTTGTCTGTTGCCGTAGTT-3′
TGF-β	5′-ACTACTACGCCAAGGAGGTCA-3′	5′-TGCTTGAACTTGTCATAGATTTCG-3′

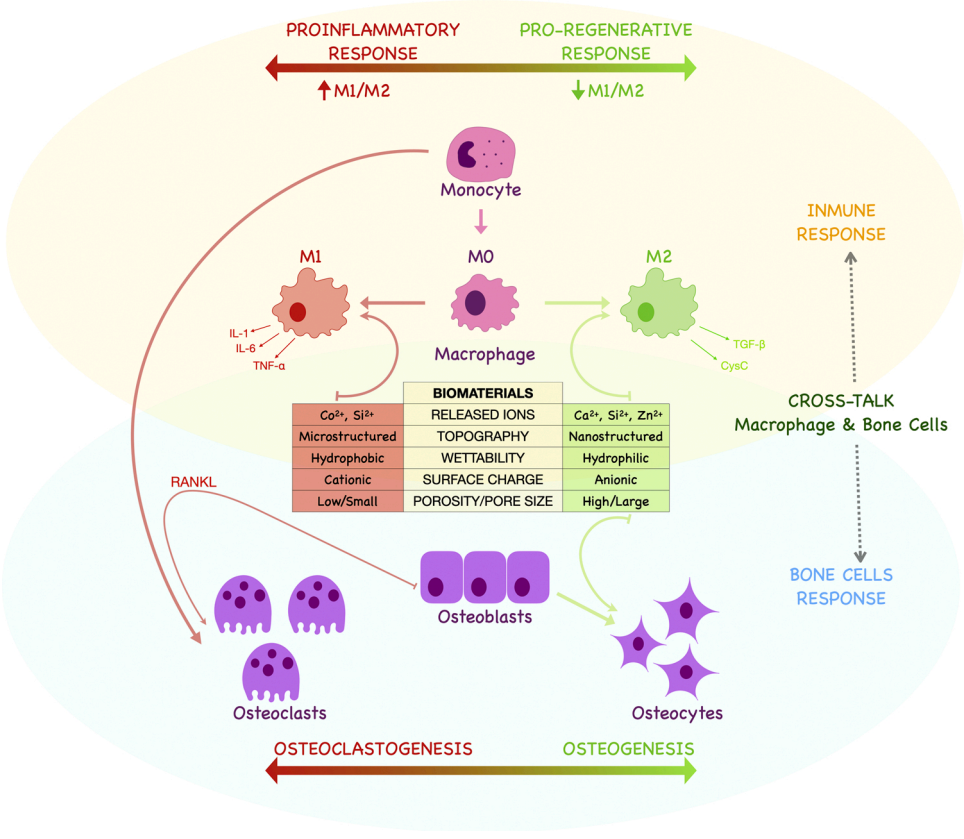

Fig. 3. Biomaterials properties modulate the immune response and the osteoblasts osteogenic potential.

staining the macrophages, in a first step, with specific primary antibodies for M1 and M2, which could be rabbit anti-iNOS and rabbit anti-CD163, respectively; and then a common secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit. Before being analyzed with confocal fluorescence microscope, they should be washed with PBS and the macrophages' nuclei should be dyed with DAPI. The ratio of positive cells (in each case of M1 and M2) needs to be compared with the total cells using a software with an available tool for this purpose [67]. These tests would provide the researchers with enough information to be able to know which profile of macrophages is potentiated with the proposed biomaterial, favoring inflammatory or anti-inflammatory cellular reactions.

5.3. *Macrophages and osteoblasts co-cultures*

The second approach regarding macrophages is focused on investigating how the potential of the osteoblasts is modified, when they are cultured in presence of macrophages. A control group of monoculture of osteoblasts without macrophages is needed in order to compare the results. There are mainly three models of co-culture [68]: a) Conditioned media: after culturing macrophages for 24 h, the media is collected, centrifuged and the supernatant is added to a separate osteoblasts culture; b) indirect co-culture: macrophages are cultured on a specific hanging cells, which are inside the main osteoblasts culture, and c) direct co-culture: osteoblasts and macrophages are cultured together in the same flasks. The first type of co-culture is the one which differs the most from *in vivo* conditions but would be a good way to approach co-culture since the results are more easily interpreted. On the other hand, in direct co-culture, more factors can influence the results, but cell-to-cell contact is achieved leading to more clinically relevant results.

After the co-culture, osteoblasts proliferation and differentiation should be analyzed with some of the techniques described in previous sections, in order to encounter the effects that macrophages may exert on osteoblasts activity.

6. *In-vivo* bone regeneration analysis

The ultimate and most clinically relevant challenge before using a membrane in humans is the *in vivo* testing in an animal model. Animal experimentation should be carried out following the local directives. In Europe, experiments need to be developed following the US National Institute of Health (NIH for Care and Use of Laboratory Animals) and the European Directive 86/609/EEC guidelines concerning animals care and use for experimentation. They should also fulfill the European Directive 2010/63/EU about the animals' protection for scientific purposes and be in accordance with all local laws and regulations [66]. It should be taken into account that, for ethical reasons, the minimum number of animals should be utilized [69]. Therefore, the importance of previously select the correct experimental membrane prototype trough the above described tests is clear. For the present purpose, the most used animal model is the New Zealand calvaria rabbits [8,70–75]. In this model, several calvaria defects are surgically created and occluded with the tested material, leaving always a defect without treatment which would act as negative control. The main difference among studies is the diameter of the defect. The defects can be classified as critical size defects (CSD) or non-critical size defects. CSD were defined by Schmitz and Hollinger in 1986 [76] as "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal". The size of the CSD defect varies depending on the animal that is chosen for the experiment. In New Zealand rabbits is considered as CSD when the diameter of the defect is above 7 mm [66,70]. After the stablished time, the animals are euthanized. The most common ways of analyzing the effect of the applied biomaterials are Micro-CT and different histological techniques. It should be noted that this animal model is not the only one described in the literature. When GBR need to be tested, especially in the case of studying GBR together with osseointegration of dental implants or periodontal regeneration, Beagle dogs [77–79] or Minipigs [80] models are normally used, completely mimicking membranes clinical applications.

It has to be noted that for a first approach of a biomaterial to an *in vivo* testing, the calvarial model might be the most predictable, since primary closing of the incision is always achieved, reducing the risk of infection and influences from saliva or bacterial biofilms prevailing in intraoral models [81]. In contrast, in Beagle dogs and minipigs models, the surgeries are performed on the jaw, increasing the clinical relevance of the results. However, in these animal models there are more factors that can negatively influence the regeneration. These intraoral animal models should be implemented at a second stage, in order to study the behavior of the membranes in a jaw model, when the innate osteogenic properties of the biomaterial have already been stablished.

Regardless of the employed animal model, after the selected healing periods and animals' euthanasia, the following techniques are the most employed to analyze bone regeneration around tested membranes:

6.1. *Micro-CT*

Micro Computer Tomography (Micro-CT) makes possible to analyze the defects' average bone density in the so-called Hounsfield Units (HU). It is interesting to divide the defects in spherical volumes of interest (VOIs) in a rosette arrangement, presenting a central VOI and peripheral VOIs in contact with bone edges (Fig. 4). Using this distribution, the analysis can be carried out from two different approaches, assessing the whole defect or just the central VOI. The central VOI is the most critical part of the defect since it is not in contact with the edges of the defect. Consequently, analyzing this area makes it easier to discriminate between different osteoinductive potential of the tested biomaterials [66].

6.2. *Histomorphometric analyses*

The first step should be to retrieve the blocks from the regenerated bone defect using an oscillating autopsy saw. The obtained bone specimens need to be fixed and dehydrated before they can be included in blocks of acrylic resin and prepared for ground sectioning [66].

Afterwards, the subsequent processing of the samples depends on the staining that is going to be carried out. The most frequent ones include Haematoxylin and eosin (HE) and Masson's trichrome staining [55,82,83] but it needs to be kept in mind that there are some more specific techniques to be performed, allowing the researcher to directly study several specific bone regeneration parameters:

a) Von Kossa (VK) silver nitrate can be applied in order to visualize the mineralized bone. From the images obtained with VK staining, the following structural indexes can be calculates and studied: Bone surface (BS), percentage of bone area [BS/total surface (TS)], bone perimeter (BPm), and bone thickness (BTh) [70].

b) Immunofluorescence staining can also be performed in order to detect M2 markers like CD206, pan-macrophage markers like F4/80, or osteoblasts differentiation markers like VEGF, BMP, RUNX2, etc. [84]. In order to be able to perform this assay, the samples should be embedded in paraffin [84].

c) Calcein may be located and quantified trough fluorescence and it may help researchers to detect the calcium deposited in the last 7 days, in order to differentiate osteoid (newly deposited bone matrix) from mineralized tissue [66].

7. Conclusions and trend to the future

The need of using a membrane in GBR in order to exclude soft tissue's cells for colonizing the bone defect is unquestionable and has been incredibly implemented by oral surgeons. However, it is recognized that the membrane able to compile all desired properties is not still in the

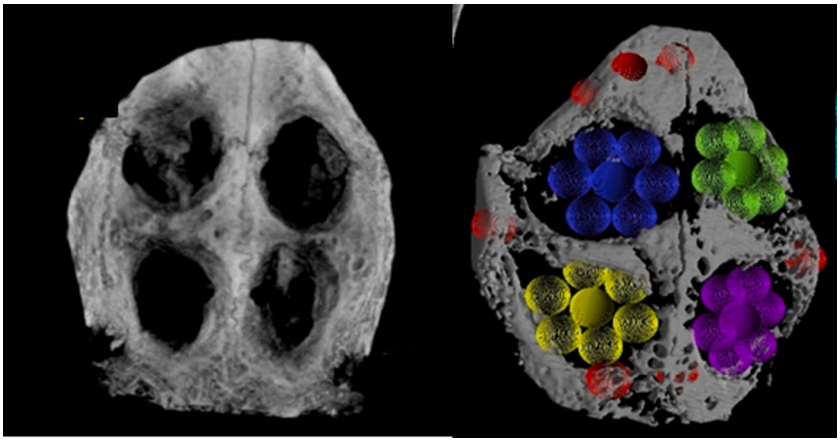

Fig. 4. Micro Computer Tomography (Micro-CT) image of four critical bone defects in a calvarial rabbit (left). At the right image, it is shown that each defect is divided in spherical volumes of interest (VOIs) in a rosette arrangement, with a central VOI and peripheral VOIs in contact with bone edges. VOIs may be analyzed for bone density together (assessing the whole defect) or just the central VOI in each defect.

market, and clinical outcomes are not always predictable. With the actual research and developed technology, the creation of an improved membrane according to the functional and biological requirements is possible. Following the structure of the present review, a potential active membrane should be assessed in its nanostructure. Physicochemical and nanomechanical properties, bioactivity and antibacterial, osteoblasts proliferation, differentiation and mineralization should also be determined. Finally, immunomodulation testing for macrophages recruitment and M2 phenotype promotion in osteoblasts co-culture needs to be achieved in order to completely analyze membranes-host tissue interactions. Membranes which successfully accomplish all these parameters may be prone to create a perfect bone-healing environment and successfully achieve GBR. Hence, appropriately controlled human studies in a clinical scenario are always necessary.

From scientific and clinical perspectives, the challenge of developing an active membrane has been potentiated by new scientific data regarding the mechanisms of GBR, tissue engineering and drug delivery approaches. All this boosts new research questions and may broaden future clinical opportunities for GBR [85].

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Doxycycline-Doped Polymeric Membranes Induced Growth, Differentiation and Expression of Antigenic Phenotype Markers of Osteoblasts

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Abstract: Polymeric membranes are employed in guided bone regeneration (GBR) as physical barriers to facilitate bone in-growth. A bioactive and biomimetic membrane with the ability to participate in the healing and regeneration of the bone is necessary. The aim of the present study was to analyze how novel silicon dioxide composite membranes functionalized with zinc or doxycycline can modulate the osteoblasts' proliferation, differentiation, and expression of selected antigenic markers related to immunomodulation. Nanostructured acrylate-based membranes were developed, blended with silica, and functionalized with zinc or doxycycline. They were subjected to MG63 osteoblast-like cells culturing. Proliferation was assessed by MTT-assay, differentiation by evaluating the alkaline phosphatase activity by a spectrophotometric method and antigenic phenotype was assessed by flow cytometry for selected markers. Mean comparisons were conducted by one-way ANOVA and Tukey tests ($p < 0.05$). The blending of silica nanoparticles in the tested non-resorbable polymeric scaffold improved the proliferation and differentiation of osteoblasts, but doxycycline doped scaffolds attained the best results. Osteoblasts cultured on doxycycline functionalized membranes presented higher expression of CD54, CD80, CD86, and HLA-DR, indicating a beneficial immunomodulation activity. Doxycycline doped membranes may be a potential candidate for use in GBR procedures in several challenging pathologies, including periodontal disease.

Keywords: CD markers; doxycycline; antigenic phenotype; osteoblasts; membrane

1. Introduction

Guided bone regeneration (GBR) is a frequently used surgical technique. GBR techniques are intended to obtain enough alveolar bone volume to accomplish a successful therapy with osseointegrated dental implants. In order to achieve this goal, the use of a membrane, that should not only act as a physical barrier but also as a modulatory barrier, protecting the growth of osteoblastic cells is required [1]. GBR is a tissue engineering therapy that greatly relies on the appropriate selection of the biomaterial. Commercially available membranes can be resorbable or non-resorbable [2]. The shortcoming for resorbable membranes is mainly a lack of space maintenance due to short resorption times.

On the other hand, for non-resorbable membranes, higher rates of wound dehiscences and the need for a second surgery in order to retrieve the membrane are the main drawbacks, as these kinds of membranes do not integrate with the host bone tissue [3].

The search for new osteogenic, bioactive, and biomimetic non-resorbable membranes that integrate with the bone tissue structure is crucial. The electrospinning technique can be used for obtaining scaffolds with attractive features, such as the similarity to the extracellular matrix [4], and may also be successfully used for drug delivery and tissue regeneration [5,6]. Recently, a new membrane barrier based on the electrospun of a polymer mixture of $(MMA)_1$-co-$(HEMA)_1$ and $(MA)_3$-co-$(HEA)_2$ has been developed. These nanofibrous scaffolds closely mimic the bone collagen matrix (fibers with diameters ranging from 50 to 500 nm) [7]. The composite membranes were formed with silica nanoparticles and functionalized with zinc or doxycycline. These new composite membranes combine the mechanical properties of polymeric materials, the bioactivity of SiO_2-NPs [7], osteogenicity provided by the doped zinc [8], and antibacterial properties conferred by the doxycycline [9]. Moreover, zinc has been tested in vivo and in vitro studies, concluding that it plays a crucial role in inducing the viability and proliferation of bone cells as well as enhancing new bone formation and mineralization of extracellular matrix [8,10,11]. Similarly, doxycycline appears to enhance maturation and cells' osteogenic capacity [7,12,13].

These novel membranes should also be tested on the cell's environment. It would be desirable that the membranes favored osteoblast proliferation and differentiation. It may be positive that the membranes could contribute to a positive immunomodulation, hindering the establishment of a pro-inflammatory atmosphere and hampering the penetration of pro-inflammatory cells and cytokines, thus improving tissue regeneration [14]. A growing body of evidence has been found about the immunoregulatory potential of biomaterials. It has been already shown that interactions of osteoblasts with biomaterials can regulate the extent of the response initiated by macrophages, mainly by osteoblastic release of interleukin-6, prostaglandin-E2, or granulocyte macrophage colony-stimulating factor [15,16]. The bone and immune systems are closely related through cellular and molecular interactions [17]. Several regulatory molecules are shared by the immune and skeletal systems, which include cytokines, receptors, signaling molecules, and transcription factors [17,18].

The cluster of differentiation (CD) is a protocol used for the identification of cells' surface antigens, providing targets for immunophenotyping of cells. In terms of physiology, CD molecules can act in numerous ways, often acting as important receptors or ligands to the cell. The behavior of the cell is usually modified by CD proteins playing a role in cell signaling or other functions, such as cell adhesion. Assessing CD osteoblasts' antigenic phenotype is possible by means of flow cytometry. It is an extremely robust technique that has been successfully implemented to probe phenotypic cellular activity in living cells. Namely, the in vitro immunomodulatory properties of bone cells may be ascertained by determining the deficiency or overexpression of the related CD antigenic markers of interest at a specific time point of cells' differentiation stages. It may provide information about cells' potential role in immunomodulation [19].

The aim of the present study was to analyze the proliferation, differentiation potential, and immunomodulation ability of osteoblastic cells cultured on silicon dioxide composite membranes functionalized with zinc or doxycycline. The null hypothesis is that zinc or doxycycline addition on membranes does not affect the proliferation, differentiation, and antigenic phenotype expression of the cultured osteoblasts.

2. Materials and Methods

2.1. Preparation of Nanostructured Polymeric Membranes

Nanostructured membranes were produced by electrospinning using a novel polymeric blend: $(MMA)_1$-co-$(HEMA)_1$/$(MA)_3$-co-$(HEA)_2$ 50/50 wt.%, doped with 5 wt.% of SiO_2-NPs (NanomyP®, Granada, Spain). The membranes were incubated for 2 h in a sodium carbonate buffer solution (333 mM; pH = 12.5), so as to activate them with carboxyl groups (HOOC-Si-M). The partial hydrolysis of ester bonds and the disposal of

carboxyl groups on the surface of the artificial tissue made this functionalization possible [20]. The membranes were then rinsed with distilled water and dried using a vacuum oven [21]. Secondly, the membranes were functionalized with zinc or doxycycline (Dox). Zinc was incorporated using the ability of carboxyl groups to complex divalent cations. On the other hand, Dox was immobilized on the membranes by acid–base reactions between amino groups of Dox and carboxyl groups present in the membranes. HOOC-Si-M were immersed under continuous shaking at room temperature and in aqueous solutions (pH = 7) of both 330 mgL^{-1} of $ZnCl_2$ or 800 mgL^{-1} of Dox [7]. Four different membranes were tested: (1) non-functionalized and SiO_2-NPs undoped membrane (HOOC-M), (2) SiO_2-NPs doped membrane (HOOC-Si-M), (3) SiO_2-NPs doped membrane functionalized with Zn (Zn-HOOC-Si-M), and (4) SiO_2-NPs doped membrane functionalized with Dox (Dox-HOOC-Si-M). The membranes were placed at the bottom of a 24-well plate (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) and sterilized using an ultraviolet radiation sterilization desk (J.P. SELECTA, Barcelona, Spain).

2.2. Cell Culture

The human MG63 osteosarcoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). This cell line has been widely used as an osteoblast model since it shares most of the same characteristics with primary human osteoblasts. MG63 osteoblast-like cells have no interspecies differences with primary human osteoblasts, they have a shorter isolation time and there is unlimited accessibility [1]. The MG63 cell line was maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA) with penicillin 100 IU/mL (Lab Roger SA, Barcelona, Spain), gentamicin 50 mg/mL (Braum Medical SA, Jaen, Spain), amphotericin B 2.5 mg/mL (Sigma, St. Louis, MO, USA), 1% glutamine (Sigma), and 2% HEPES (Sigma) supplemented with 10% of fetal bovine serum (FBS; Gibco, Paisley, UK), as described by Díaz Rodríguez et al. [22]. Cultures were kept in a humidified atmosphere at 37 °C of 95% air and 5% CO_2. Cells were detached from the flask using 0.05% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid (EDTA; Sigma) solution. After this process, they were rinsed and resuspended in complete culture medium with 10% FBS as described by Manzano-Moreno et al. [19].

2.3. Cell Proliferation Assay

Osteoblasts were seeded at 1×10^4 cells/mL per well onto the membranes placed inside the 24-well plate. The cells were then cultured in a humid atmosphere of 95% air and 5% CO_2 at 37 C for 48 h. After this time, cell proliferation was assessed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. For this purpose, the media was replaced by phenol red-free DNEM with MTT 0.5 mg/mL (Sigma) and incubated for 4 h. MTT cellular reduction resulted in the formation of insoluble crystal deposits of formazan that were dissolved by adding dimethyl sulfoxide (Merck Biosciences, Darmstadt, Germany). Absorbance was then measured with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland) at 570 nm [23]. The results were expressed as mean absorbance ± standard deviation (SD). At least three experiments were conducted for each type of membrane.

2.4. Field Emission Scanning Electron Microscopy (FESEM)

Osteoblasts were also seeded at 1×10^4 cells/mL per well onto the membranes placed inside the 24-well plate. The cells were then cultured in a humid atmosphere of 95% air and 5% CO_2 at 37 °C for 48 h. After this time, two membranes of each experimental group were submitted to FESEM (GEMINI, Carl Zeiss SMT, Oberkochen, Germany) observation. Samples were first critical point dried and covered with carbon.

2.5. Alkaline Phosphatase Activity

Early osteoblasts differentiation was assessed by evaluating the alkaline phosphatase (ALP) activity, a colorimetric assay (Diagnostic kit 104-LL, Sigma). This method, measures the conversion of the colorless substrate p-nitrophenyl phosphate to the yellow p-nitrophenol, accomplished by the ALP enzyme. The rate of color shift corresponded with the amount of enzyme present in the culture. Standards of p nitrophenol (0–250 µM) were prepared from dilutions of a 1000 µM stock solution and assayed in parallel. The ALP assay was performed as previously described by Manzano-Moreno et al. [23]. In brief, cell cultures, for 72 h on the membranes placed inside the 24-well plate, were trypsinized (0.05% trypsin, 1 mM EDTA, (Invitrogen Gibco)) and lysed in 100 µL 1 M Tris pH 8.00 by ultrasonification for 4 min. Then, the suspension was mixed with a 7.6 mM p-nitrophenyl phosphate solution at a proportion of 1:10 and incubated for 15 min at 37 °C. Substrate solution was prepared by merging an aqueous solution of 4 mg/mL of 4-nitrophenyl phosphate disodium salt (Sigma) with an equal volume of 1.5 M alkaline buffer (Sigma). The reaction was stopped by adding 1 mL 0.05 N NaOH, and the final absorbance was measured with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland) at 405 nm. The total protein content was estimated by the Bradford method using a protein assay kit from Bio-Rad Laboratories (Bio-Rad Laboratories, Nazareth-Eke, Belgium). All samples were conducted in triplicate.

2.6. Antigenic Phenotype

Antigenic phenotype was assessed by flow cytometry at 48 h of culture using 0.05% trypsin (Sigma) and 0.02% EDTA (Sigma) solution 0.4% EDTA solution. They were then washed and suspended in PBS at a concentration of 2×10^4 cells/mL. Osteoblasts were labeled by direct staining with anti-CD54, CD80, CD86, and HLA-DR monoclonal antibodies (MAbs); CD54/IOL1b, CD80, CD86, and OKDR, respectively (Invitrogen Corp). 100 µL of cell suspension were incubated with 10 µL of each MAb for 30 min at 4 °C in the absence of light. Cells were rinsed and re-suspended in PBS 1 mL, and analyzed at a wavelength of 488 nm in a flow cytometer with a diode laser (FACSCanton II, Becton-Dickinson, Palo Alto, CA, USA) to determine the percentage of fluorescent cells [24].

2.7. Phagocytic Activity

Phagocytic activity was also studied by flow cytometry following the method described by Díaz-Rodriguez et al. [25]. In brief, cultured human MG-63 osteosarcoma cells were cultured for 48 h on the tested membranes. After this time, they were detached from the membranes, washed, and then suspended in complete culture medium with 10% FBS at a density of 2×10^4 cells/mL. Cells were treated by direct staining with labeled latex beads; a 100-µL cell suspension was incubated with 5 µL carboxylated FICT-labeled latex beads 2 µL in diameter (Sigma) for 90 min in darkness at 37 °C. Cells were washed, suspended in 1 mL of PBS, and analyzed in a flow cytometer (Facs Vantage; Becton–Dickinson, Palo Alto, CA, USA). Results were expressed as a percentage of cells positive for phagocytosis and mean channel fluorescence, which correlates with the number of particles phagocytosed by the cells. Tests were performed in triplicate and control assays were carried out at 4 °C, to eliminate the background fluorescence. Results were obtained as the percentage of cells positive for phagocytosis.

2.8. Statistical Analysis

Mean comparisons were conducted by one-way ANOVA and Student–Newman–Keuls multiple comparisons. Significance was set at $p < 0.05$. Data were expressed as means ± standard deviation (SD) in all cases. Before ANOVA, normal distribution was probed by Kolmogorv–Smirnov tests ($p > 0.05$).

3. Results

3.1. Cell Proliferation Assay

Mean and standard deviations of osteoblasts cell proliferation determined by the MTT assay are presented in Figure 1. Significant differences were found between all tested groups and attained means follow the trend: COOH-M < HOOC-Si-M < Zn-HOOC-Si-M < Dox-HOOC-Si-M.

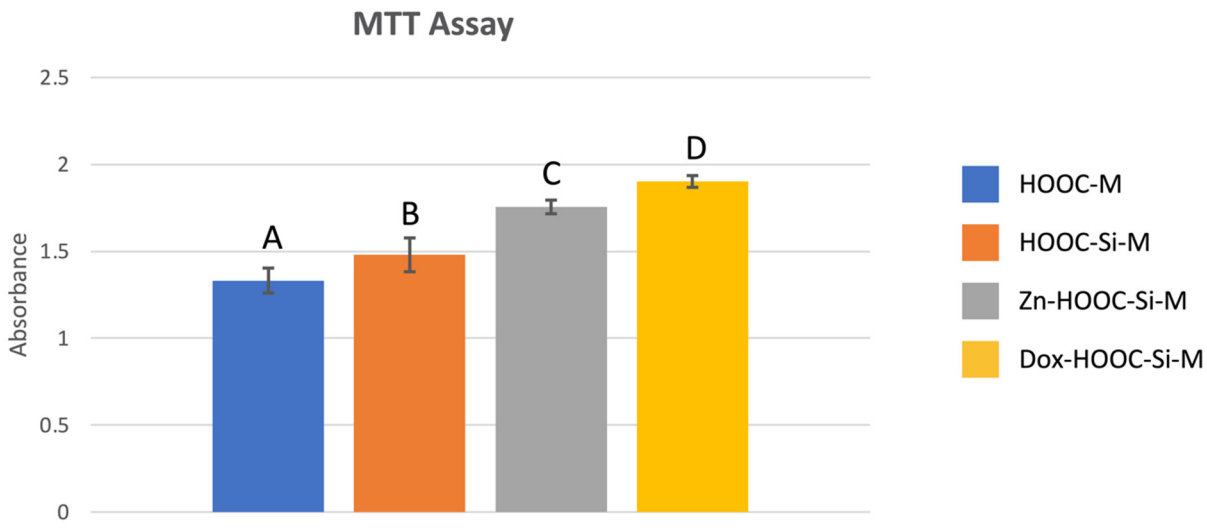

Figure 1. Absorbance mean values and standard deviations obtained after the MTT assay for the different membranes. Distinct letter indicates significant difference between membranes after ANOVA and Student–Newman–Keuls multiple comparisons ($p < 0.05$).

3.2. Field Emission Scanning Electron Microscopy (FESEM)

Selected images from the FESEM analysis are displayed in Figures 2 and 3. Osteoblasts cultured on COOH-Si-M displayed an elongated spindle-shaped morphology and a similar situation was observed for those grown on Zn-COOH-Si-M. By contrast, osteoblasts on COOH-M are round shaped. For osteoblasts on the Dox-COOH-Si-M the elongated morphology was accompanied by larger and more cytoplasmic membrane protrusions compared with those grown on the other membranes. There also exists an alignment of the elongated cells on the tested surfaces, which provided large bio-adhesive areas for the cells. Cell spreading and cell layer thickness is lower at COOH-M than in the other membranes.

3.3. Alkaline Phosphatase (AP) Activity

Mean and standard deviations of alkaline phosphatase expressed as international units (IU) of AP per mg of total proteins are presented in Figure 4. Significant differences were found between all tested groups and attained means follow the trend: COOH-M < HOOC-Si-M < Zn-HOOC-Si-M < Dox-HOOC-Si-M.

3.4. Antigenic Phenotype

The characterization of cultured osteoblasts on the distinct membranes, for the presence of selected surface markers is presented in Figure 5. Osteoblasts cultured on doxycycline functionalized membranes presented higher expression of CD54, CD80, CD86, and HLA-DR ($p < 0.05$), than the rest of the groups. Differences were about (38%, 36%, 53%, and 64%, respectively). Zinc functionalization reduced CD54 expression on osteoblasts about 64% ($p < 0.001$).

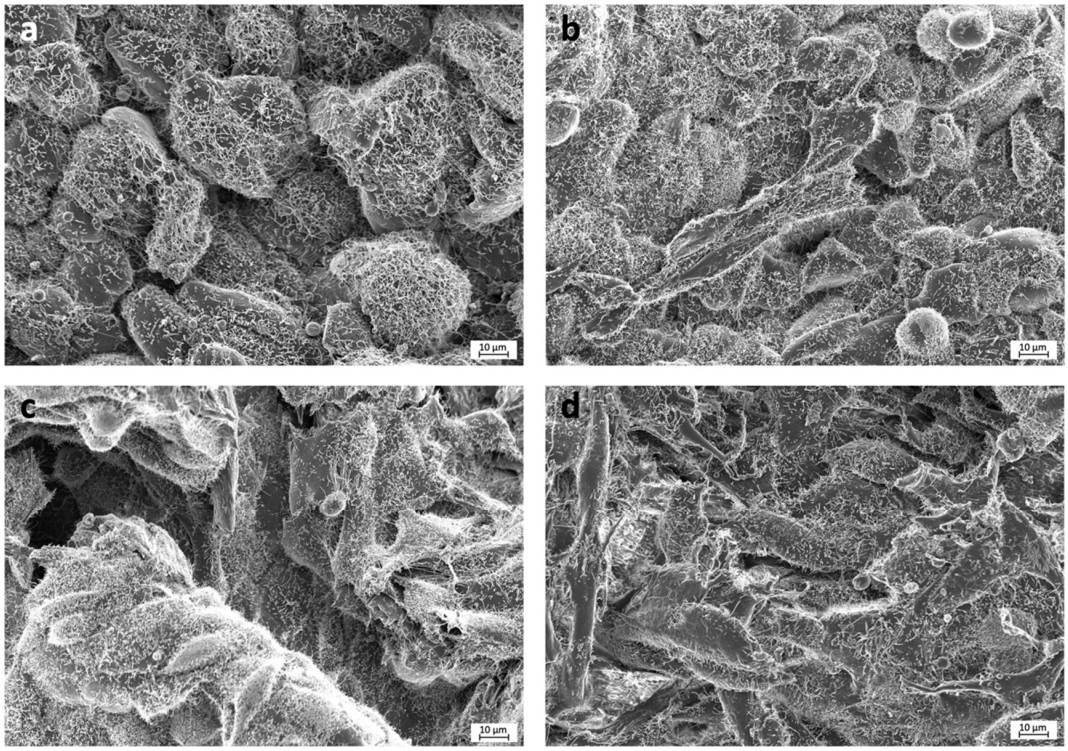

Figure 2. FESEM images of the experimental membranes (**a**) COOH-M, (**b**) COOH-Si-M, (**c**) Zn-COOH-Si, and (**d**) Dox-COOH-Si-M. Cells seeded on COOH-M are round shaped and filapodia are not observed. In the rest of the membranes, elongated cells are observed, some filapodia may also be detected emerging from osteoblasts cytoplasm. Osteoblasts are covered by fibrilar substance and rounded mineral deposits.

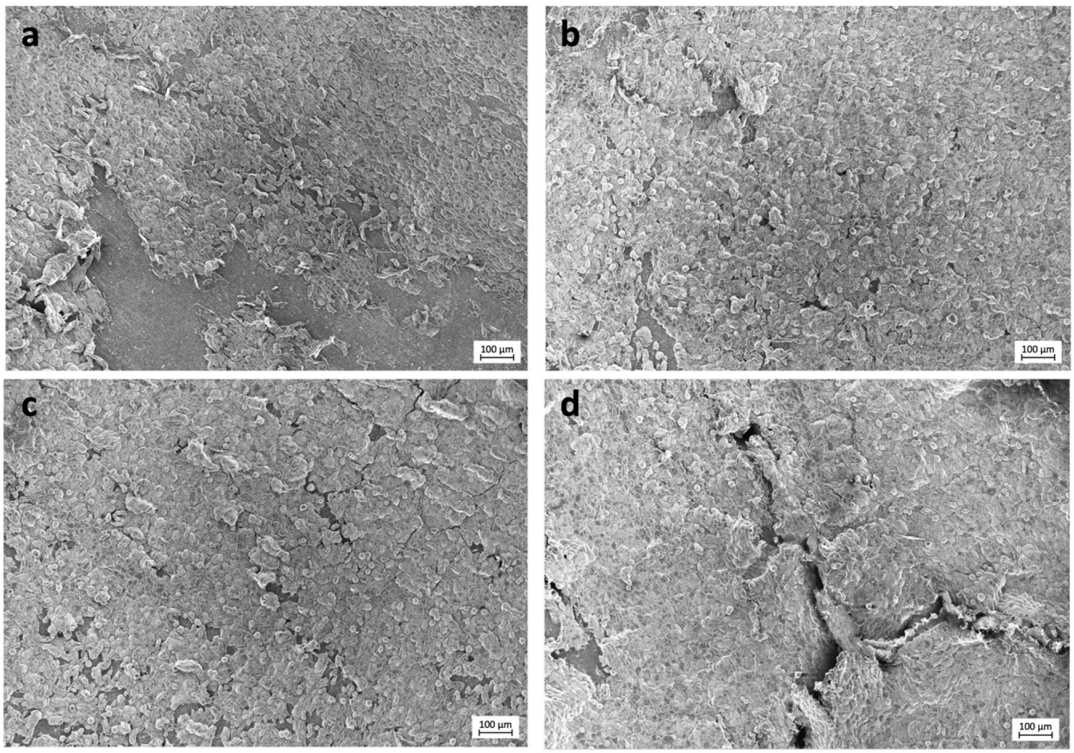

Figure 3. FESEM images of the experimental membranes (**a**) COOH-M, (**b**) COOH-Si-M, (**c**) Zn-COOH-Si-M, and (**d**) Dox-COOH-Si-M. Several aligned osteoblasts connected to each other may be seen. Thicker layers of osteoblasts, constituting a tri-dimensional structure, are evidenced on Zn-COOH-Si-M and Dox-COOH-Si-M.

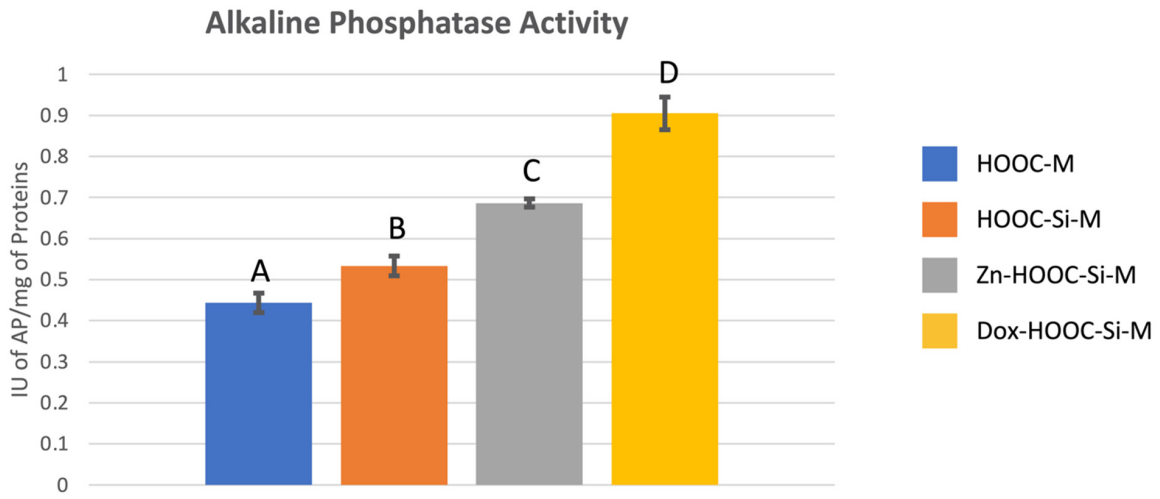

Figure 4. Mean and standard deviation of international units of AP per mg of proteins values obtained with the different membranes. Distinct letter indicates significant difference between membranes after ANOVA and Student–Newman–Keuls multiple comparisons ($p < 0.05$).

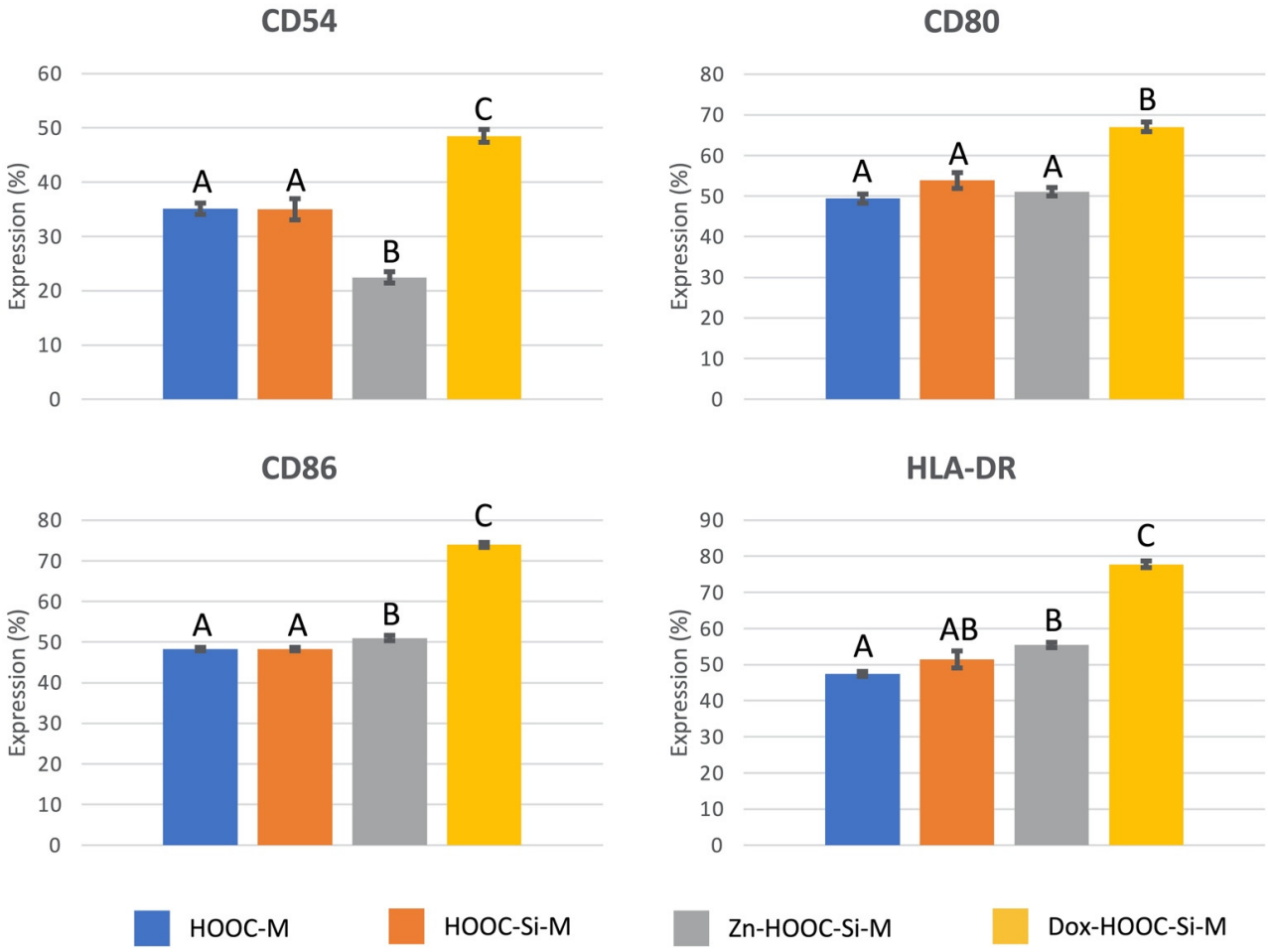

Figure 5. CD-marker antigenic expression on osteoblasts, cultured on the different experimental membranes. Values are presented as percentage of cells expressing the antigen phenotype (CD54, CD80, CD86, and HLA-DR). ANOVA and Student-Newman-Keuls multiple comparisons were performed ($p < 0.05$). Distinct letter indicates significant difference between membranes and always compares each CD-marker independently.

3.5. Phagocytic Activity

Osteoblasts cultured on tested membranes were negative for phagocytosis, except for those osteoblasts grown on the COOH-M where 24.33% of cells were positive, and the standard deviation was 1.52%.

4. Discussion

This research manuscript implies an approach to the study of how osteoblasts' proliferation and differentiation are affected by the different studied nanostructured polymeric membranes. MG-63 osteoblast-like cells were used for this in vitro study. They are one of the cell lines most widely used in literature since there are no interspecies differences with primary human osteoblasts, they have a shorter isolation time and there is unlimited accessibly [1,26]. However, the results need to be extrapolated with caution, taking into account that a tumor line may have an alternative pattern of differentiation from primary human osteoblasts [27,28]. The MTT assay and FESEM were used in order to quantitively measure osteoblasts' proliferation while their differentiation potential was evaluated by means of alkaline phosphatase activity, antigenic phenotype expression, phagocytic activity, and FESEM.

The previous surface characterization of the present membranes demonstrated that the mean fiber diameter was of around 765 nm [7]. Taking into account that mineralized collagen fibrils are about 800 nm in human trabecular bone, these membranes imitate the osseous structure. This collagen bone mimicking has been shown to increase cell attachment on membranes by about 1.7 fold [29]. The best strategy when designing an ideal scaffold is trying to replicate the native tissue and the fibrillar structure, enhancing cellular attachment, proliferation, and differentiation [1,7].

For topical drug application, the in vitro drug release assay does not correlate with the expected drug activity. In these cases, product performance tests in similar conditions to the clinical scenario have been employed. The present methodology involves the determination of the drug activity on osteoblastic cells, considering the liberated drug and the amount of drug permeated into the cells. The effect on cells of the quantity of drug which is not liberated but remained doing its action on the functionalized membrane is also considered. Moreover, it should also be taken into account that membranes are non-resorbable and do not swell or dissolve. Therefore, doxycycline and zinc release is controlled by diffusion of the drug/ions through the liquid permeating the membrane, but as membranes are bioactive [7], an apatite layer can be formed on the surface, hindering the diffusion of the drug/ions to the surrounding tissue [30].

Osteoblasts' proliferation increased on silica-doped membranes, but the best results were obtained on doxycycline functionalized membranes (Figure 1). It has been previously reported that tetracyclines promote the proliferation of osteoblasts [4]; but, the underlying mechanism is not completely understood. Two different events may explain this fact: (i) osteoblasts' proliferation is usually enhanced in the presence of antioxidants, as reactive oxidant species diminish osteoblasts' reproduction [31], and doxycycline presents a potent antioxidant effect [32,33]. (ii) Doxycycline possesses calcium chelating ability that may facilitate calcium deposit on membranes [7]. Increases in extracellular calcium concentration have been shown to stimulate osteoblastic lineage cells, leading to bone formation and osteoconductivity [34].

Osteoblasts with an elongated morphology were observed on the doxycycline doped membranes, with filapodia extensions from the cells to the substrate, producing an interlocked cell structure (Figure 2). This suggests that interplay between the cell and the membrane surface allows for enhanced dynamic propagation and an overall increase in osteoblast activation, as indicated by the filopodia. Correlations between cell morphology and both proliferation and metabolic activity have been previously stated [1,35,36]. Hence, osteoblast spreading apparently favored the metabolic activity and cell elongation favored proliferation, while cellular roundness decreased mitotic activity [35,36]. The same correlation was found in the present study, obtaining the highest osteoblasts proliferation for

the Dox-COOH-Si-M. Rounded and less spread cell shape was found on the COOH-M (Figure 2), coinciding with a lower cell proliferation. The described correlation of cell spreading and proliferation has already been reported, not only for osteoblasts but for other cell types [35]. The cellular morphology seems to be also affected by the presence of silica, inducing a three-dimensional growth (Figure 3). This three-dimensional cellular network is the basis for the known in vitro earlier differentiation of osteoblasts and better osseointegration in vivo [37].

Regarding osteoblasts differentiation, it may be ascertained from phosphatase activity assays that the silica blending and doxycycline doping of the membranes extremely induces osteoblasts' differentiation since it increased the phosphatase activity in about 100% respect COOM-M ($p < 0.001$). The AP activity was also enhanced by 50% when osteoblasts were seeded on Zn-COOH-Si-M when compared with COOH-M ($p < 0.001$). It could be also ascertained that regarding AP activity, Dox and Zn could be said to favor osteoblasts' differentiation, as doping COOH-Si-M with Dox and Zn, raises the AP activity by 70 and 30%, respectively ($p < 0.001$ in both cases). In previous investigations, the modulation of genes related to the osteogenic functional capacity of osteoblastic cells exerted by the studied membranes was evaluated. One of these genes was the one encoding the alkaline phosphatase, and the same tendency that the observed in the present study was registered [28].

It was found that osteoblasts expressed major histocompatibility complex molecules as HLA-DR, and adhesion molecules as CD54, as well as some signaling or co-stimulatory molecules as CD80 and CD86 [38]. The expression of these molecules is up-regulated by the tested doxycycline-doped membranes (Figure 5). Expression of HLA-DR, which has also been described on osteoblasts obtained after mandibular surgery [39], is related to osteoblasts' osteocalcin production when stimulated with 1,25-di-hydroxyvitamin D3 [40]. The co-stimulatory molecules CD80 and CD86 are withal expressed by antigen presenting cells and play pivotal roles in inducing T-cell immunity or tolerance [41,42]. CD80 may primarily inhibit the immune response, working on T-lymphocytes [43].

The co-stimulatory molecule CD86 is a marker expressed in different cell populations such as dendritic cells. To understand the meaning of its over-expression it should be taken into account that the differentiation of osteoclasts is tightly regulated by bone-forming osteoblasts [44]. Osteoblasts express cytokines essential for osteoclastic differentiation, like the receptor activator of NF-kB ligand (RANKL) [45,46], in response to osteotropic hormones and factors [47]. Doxycycline and minocycline have been previously shown to induce the production of dendritic cell markers, like CD86, in the presence of RANKL [48]. Osteoclast's precursors express RANK (RANKL receptors) and differentiate into osteoclasts in response to RANKL emitted by osteoblasts [46,47]. But tetracyclines convert the differentiation pathway, resulting in dendritic-like cells rather than osteoclasts, in the presence of RANKL in vitro and in vivo [48]. As a result, tetracyclines prevent bone loss [48], but the mechanism involved is only partially known. Further research is required in this field. Doxycycline and minocycline inhibited RANKL, which induced osteoclastogenesis, however, no other clear effect on cell growth and phagocytic activity of osteoclasts has been described.

Zinc functionalization reduced CD54 expression on osteoblasts and doxycycline produced its over-expression (Figure 5). CD54 is an adhesion molecule and its presence on osteoblasts is well documented [39,49]. CD54 upregulation on osteoblastic cells has been shown to facilitate osteoclastogenesis [50,51]. CD54 is also known as intercellular adhesion molecule 1, a highly glycosylated immunoglobulin superfamily member that binds the leukocyte integrins. CD54 is constitutively expressed on leukocytes, epithelial, endothelial, and other cells, and it is up-regulated in response to inflammatory mediators [38,52]. CD54 is the mediating protein involved in the contact between mesenchymal stromal cells and macrophages. It has an essential role in regulating immunosuppressive properties of mesenchymal stromal cells [53]. When CD54 is over-expressed by cells, they become more immunosuppressive in inflammatory environments [54]. There is an unconventional

but functional CD54-mediated interaction between pro-inflammatory macrophages (M1) and mesenquimal cells. This crosstalk modulates the immunosuppressive functions of mesenquimal cells and opens important perspectives in therapies for autoimmune and inflammatory diseases [53].

Doxycycline has previously been described as a potent anti-inflammatory and antioxidant substance [33]. These properties are related to beneficial doxycycline actions stated on alveolar tissue inflammatory infiltrates [13]. Oral diseases producing bone loss, such as periodontitis or periimplantitis, are characterized by inflammation and produce the activation of immunological cells leading to the release of pro-inflammatory cytokines and the recruitment of phagocytes and lymphocytes [55].

Phagocytosis is a biological cellular activity through which cells can protect themselves from infectious and non-infectious environmental particles. The process of phagocytosis has been previously identified in osteoblasts [25,56] and requires the recognition of ligands by specific receptors expressed by the phagocyte. Receptor engagement triggers intracellular signaling pathways that initiate appropriate innate immune and pro-inflammatory responses [57]. Osteoblasts grown on tested functionalized membranes possess a diminished phagocytic activity, as an additional sign of anti-inflammatory response to tested doped biomaterials. Just the COOH-M presented about 25% of osteoblasts positive to phagocytosis activity.

Biomaterial-mediated inflammatory response is crucial for bone regeneration. Excessive inflammation may lead to the formation of fibrous tissue, preventing bone cells from integrating with the membranes, resulting in the failure of bone regeneration. However, moderate inflammatory responses may enhance the recruitment and differentiation of osteoblasts, improving osteogenesis [58]. Further research with macrophages in co-culture is required determining immunomodulation and osteogenic differentiation.

Despite recent advancements, the therapeutic capability of biomaterials to regulate the osteoblastic cells-host immune system crosstalk, and the mechanism underlying this immunoregulation is poorly understood [14]. The most important limitation of the present study is the lack of mechanistic assays. When the application of detailed mechanistic assays is being intended [31], it may help to gain insight into a particular mechanism of action, but it also hinders the multi-regulatory processes that are necessary in the biological complexity. However, the efficient use of methods, enabling the identification of cells antigenic phenotype, has an enhanced probability of success in translation of new biomaterials through to the clinic. It presents a different perspective on signaling focused on integrated or holistic responses rather than the resolution of individual events. However, it is recognized that further screening efforts are necessary in order to discover an array that really allows the study and probing of detailed mechanistic studies, for example how these receptors are activated/inactivated and then to ascertain the dissection of cellular pathways. Rather than simplifying, the increasing number of tools should contribute to refine models and to cover further levels of biological complexity. Analogous to a puzzle built from multiple individual pieces, the full representation of cellular activity may transcend following the assembly of different functional outputs.

Since membrane exposure to the oral cavity and contamination of the surgical site are common problems, the linking of antimicrobial [9], anti-inflammatory, and bone regeneration property of doxycycline functionalized membrane makes it a favorable alternative therapeutic tool for GBR procedures prior to implant placement.

5. Conclusions

In the present study, it has been demonstrated that silica loading may offer beneficial effects to experimental membranes in terms of osteogenicity (osteoblasts proliferation and differentiation). Adding zinc to the membranes' formulation showed an enhancement in the proliferation capacity of osteoblast. Furthermore, even better results were obtained when the scaffolds were functionalized with doxycycline. An up-regulation of several antigenic markers with immune-modulatory potential has also been demonstrated for

these Dox-COOH-Si-Ms, which may be potential candidates for use in GBR procedures in several challenging pathologies, including periodontal disease.

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Doxycycline-doped membranes induced osteogenic gene expression on osteoblastic cells

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ABSTRACT

Objectives: To analyze how novel developed silicon dioxide composite membranes, functionalized with zinc or doxycycline, can modulate the expression of genes related to the osteogenic functional capacity of osteoblastic cells.

Methods: The composite nanofibers membranes were manufactured by using a novel polymeric blend and 20 nm silicon dioxide nanoparticles (SiO_2-NPs). To manufacture the membranes, 20 nm SiO_2-NPs were added to the polymer solution and the resulting suspension was processed by electrospinning. In a second step, the membranes were functionalized with zinc or doxycycline. Then, they were subjected to MG63 osteoblast-like cells culturing for 48 h. After this time, real-time quantitative polymerase chain reaction (RT-qPCR) was carried out to study the expression of Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-I, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF- βR2, and TGF-βR3. Mean comparisons were conducted by One-way ANOVA and Tukey tests ($p < 0.05$).

Results: In general, the blending of SiO_2-NPs in the tested non-resorbable polymeric scaffold improves the expression of osteogenic genes over the control membranes. Doxycycline doping of experimental scaffolds attained the best results, encountering up-regulation of BMP-2, ALP, OPG, TGFβ-1 and TGFβ-R1. Membranes with zinc induced a significant increase in the expression of Col-I, ALP and TGF β1. Both, zinc and doxycycline functionalized membranes enormously down-regulated the expression of RANKL.

Conclusions: Zinc and doxycycline doped membranes are bioactive inducing overexpression of several osteogenic gene markers.

Clinical significance: Doxycycline doped membranes may be a potential candidate for use in GBR procedures in several challenging pathologies, including periodontal diseases.

1. Introduction

The basic concepts of Guided Bone Regeneration (GBR) were firstly described by Nyman and Karring in the early 1980s [1]. They found that the cells that first populated a wound area, determined the linage of tissue that would ultimately occupy the space [2]. They also introduced the need to use a barrier membrane, preventing the undesired cells from entering the wound and permitting cells with the capacity to form the desired hard tissue to access the wound space [2].

Since 1980, the techniques and biomaterials used in GBR have been upgraded, enlarging the clinical scenarios in which GBR can be performed and allowing broadening the indications of dental implants to regions with an anatomy that is unfavorable for implant placing. Currently, the employed membranes can be resorbable or non-resorbable. Non-resorbable membranes are made of polytetrafluoroethylene (PTFE) and represent the gold-standard for clinicians, due to its

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higher predictability [3]. However, PTFE is associated with low adhesiveness for cells, total absence of ability to connect to bone tissue and osseointegration, and frequent infections due to the membrane exposition to the oral cavity and lack of antibacterial properties [4]. Therefore, higher rates of wound dehiscences and the necessity of a second surgery to retrieve the membrane after the healing period are associated to the use of these membranes [4,5]. For resorbable membranes, a lack of space maintenance is the drawback, mainly due to low resorption times [4,6,7].

That is why there is a need to develop a membrane for GBR which enable clinicians to accomplish, in a more predictable way, bone regenerative surgeries [8]. Recent developments in biomanufacturing are driving innovations in the technology for membranes designing. The major efforts in membranes design are being put into: i) the creation of nanostructured membranes mimicking the native tissue [9] and ii) the active participation of the membrane in the healing and regenerative process trough drug-delivery and cells interaction, away from being a passive barrier [10–12].

Recently, it has been developed a new biomimetic membrane barrier based on the electrospun of a polymer mixture of $(MMA)_1$-co-$(HEMA)_1$ and $(MA)_3$-co-$(HEA)_2$. Nanofibrous scaffolds mimic closely the scale and morphology of the bone collagen matrix (fibers with diameters ranging from 50 to 500 nm) [8]. The composite membranes were formed with silica nanoparticles and functionalized with zinc or doxycycline. These membranes have previously been subjected to osteoblasts' culture. HOS TE85 human osteosarcoma cells were grown on the membranes for different timepoints; in which the membranes showed a clear enhancement of osteoblasts' viability and proliferation [8]. In different studies, this membranes have also produced an increase the rate of new bone formation in rabbit's skull models [13] and an inhibition of the biofilm formation *in vitro* [14] by virtue of zinc ions and doxycycline, respectively. The new composite membranes should combine both the mechanical properties of polymeric materials and the bioactivity of SiO_2-NPs [8].

The active participation of the novel membranes in the bone healing process and their ability to interact with osteoblasts trying to accelerate bone repair, away from acting as the mere physical barrier described by Nyman and Karring in the 1980s, needs to be further evaluated. For this reason, it is necessary to focus on specific factors influencing the signaling pathway of bone regeneration and osteoinduction, which can be studied using RT-qPCR. The deficiency or overexpression of the related target genes encoding osteogenic markers of interest at a specific time point of cells differentiation stages is crucial. It may provide information about cells differentiation potential and their role in matrix production and mineralization [12].

The objective of the present study was to analyze the modulation of the gene expression related to the osteogenic functional capacity of osteoblastic cells cultured on silicon dioxide composite membranes functionalized with zinc or doxycycline. The null hypothesis is that SiO_2-NPs and zinc or doxycycline addition on membranes does not affect the gene regulation of the cultured osteoblasts.

2. Material and methods

2.1. Preparation of nanostructured polymeric membranes

Nanostructured membranes were manufactured by NanomyP® (Granada, Spain) using a novel polymeric blend: $(MMA)_1$-co-$(HEMA)_1$/$(MA)_3$-co-$(HEA)_2$ 50/50 wt, doped with 5 % wt of SiO_2-NPs. The membranes were incubated during 2 h in a sodium carbonate buffer solution (333 mM; pH = 12.5) in order to activated them with carboxyl groups (HOOC-Si-M). This functionalization is possible due to the partial hydrolysis of ester bonds and the disposal of carboxyl groups on the surface of the artificial tissue [15]. Then, membranes were rinsed with distilled water and dried in a vacuum oven [16]. In a second step, the membranes were functionalized with zinc using the ability of carboxyl groups to complex divalent cations. Doxycycline (Dox), was immobilized on the membranes by acid-base interactions between amino groups of Dox and carboxyl groups present in the membranes. In order to achieve this, HOOC-Si-M were immersed under continuous shaking at room temperature and in aqueous solutions (pH = 7) of both 330 mg L^{-1} of $ZnCl_2$ and 800 mg L^{-1} of Dox [8]. Four different membranes were designed: 1) Non functionalized and SiO_2-NPs undoped membrane (HOOC-M), 2) SiO_2-NPs doped membrane (HOOC-Si-M), 3) SiO_2-NPs doped membrane functionalized with Zn (Zn−HOOC-Si-M) and 4) SiO_2-NPs doped membrane functionalized with Dox (Dox−HOOC-Si-M). The control group in our experiment (HOOC-M), acts as negative control, since it is the group where the independent variable being tested (zinc and doxycycline doping) cannot influence the results. This isolates the independent variable's effects on the experiment and rules out alternative explanations of the experimental results. The membranes were, then, placed at the bottom of 24-well plate (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) and sterilized using an ultraviolet radiation sterilization desk (J.P. SELECTA, Barcelona, Spain).

2.2. Cell culture

The human MG63 osteosarcoma cell line was purchased from the American Type Culture Collection (Manassas, VA). This cell line is commonly used as an osteoblast model because it shares the same characteristics with primary human osteoblasts. MG63 osteoblast-like cells have no interspecies differences with primary human osteoblasts, have a shorter isolation time and there is unlimited accessibility [12]. The MG63 cell line was maintained as described by Díaz Rodríguez et al. [17], in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with penicillin 100 IU/mL (Lab Roger SA, Barcelona, Spain), gentamicin 50 mg/mL (Braum Medical SA, Jaen, Spain), amphotericin B 2.5 mg/mL (Sigma), 1% glutamine (Sigma), and 2% HEPES (Sigma) supplemented with 10 % of fetal bovine serum (FBS; Gibco, Paisley, UK). Cultures were kept in a humidified atmosphere at 37 °C of 95 % air and 5% CO2. Cells were detached from the flask using 0.05 % trypsin (Sigma) and 0.02 % ethylenediaminetetraacetic acid (EDTA; Sigma) solution. After it, they were rinsed and resuspended in complete culture medium with 10 % FBS as described by Manzano-Moreno et al. [18]. Osteoblasts were seeded at 2 × 10^4 cells/mL per membrane, which had previously been placed into a 24-well plate. They were then cultured at 37 °C in a humidified atmosphere of 95 % air and 5% CO_2. Before seeding, the cell density had been adjusted by means of a Neubauer chamber in order to achieve the previously mentioned cell density [19,20].

2.3. RNA extraction and cDNA synthesis (reverse transcription)

After 48 h of culturing osteoblasts with the experimental membranes, the cells were detached using 0,05 %trypsin-EDTA solution (Sigma). A silicate gel technique was used to extract the mRNA, provided by Qiagen RNeasy extraction kit (Qiagen Inc., Hilden, Germany). UV spectrophotometry at 260 nm (Eppendorf AG, Hamburg, Germany) was used to calculate the amount of extracted mRNA from the cells and to determine the contamination with proteins using the 260/280 ratio. After this, 1 μg of mRNA of the osteoblasts cultured on each type of membrane was brought to 40 μL of total volume, reverse-transcribed to cDNA and amplified by PCR with iScriptTM cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA) according to the manufacturer's instructions [21].

2.4. Real-time polymerase chain reaction (RT-PCR)

Primers were design using NCBI- nucleotide library and Primer3-design to spot mRNA of Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-I, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF- βR2, and TGF-βR3 (Table 1).

Table 1
Primer sequences for the amplification of osteoblasts' cDNA by real-time PCR.

Gene	Sense Primer (5′-3′)	Antisense Primer
TGFβ1	TGAACCGGCCTTTCCTGCTTCTCATG	GCGGAAGTCAATGTACAGCTGCCGC
TGFβ-R1	ACTGGCAGCTGTCATTGCTGGACCAG	CTGAGCCAGAACCTGACGTTGTCATATCA
TGFβ-R2	GGCTCAACCACCAGGGCATCCAGAT	CTCCCCGAGAGCCTGTCCAGATGCT
TGFβ-R3	ACCGTGATGGGCATTGCGTTTGCA	GTGCTCTGCGTGCTGCCGA TGCTGT
Runx-2	TGGTTAATCTCCGCAGGTCAC	ACTGTGCTGAAGAGGCTGTTTG
VEGF	CCTTGCTGCTCTACCTCCAC	CACACAGGATGGCTTGAAGA
OSX	TGCCTAGAAGCCCTGAGAAA	TTTAACTTGGGGCCTTGAGA
BMP-2	TCGAAATTCCCCGTGACCAG	CCACTTCCACCACGAATCCA
BMP-7	CTGGTCTTTGTCTGCAGTGG	GTACCCCTCAACAAGGCTTC
ALP	CCAACGTGGCTAAGAATGTCATC	TGGGCATTGGTGTTGTACGTC
Col-1	AGAACTGGTACATCAGCAAG	GAGTTTACAGGAAGCAGACA
OSC	CCATGAGAGCCCTCACACTCC	GGTCAGCCAACTCGTCACAGTC
OPG	ATGCAACACAGCACAACATA	GTTGCCGTTTTATCCTCTCT
RANKL	ATACCCTGATGAAAGGAGGA	GGGGCTCAATCTATATCTCG
UBC	TGGGATGCAAATCTTCGTGAAGACCCTGAC	ACCAAGTGCAGAGTGGACTCTTTCTGGATG
PPIA	CCATGGCAAATGCTGGACCCAACACAAATG	TCCTGAGCTACAGAAGGAATGATCTGGTGG
RPS13	GGTGTTGCACAAGTACGTTTTGTGACAGGC	TCATATTTCCAATTGGGAGGGAGGACTCGC

Final results were normalized using ubiquitin C (UBC), peptidyl-prolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) as stable housekeeping genes [22,23].

The SsoFast™ EvaGreen® Supermix Kit (Bio-Rad laboratories) was used following the manufacturer's recommendations in order to conduct the quantitative RT-PCR (q-RT-PCR). The cDNA samples were placed in 96-well microplates and an IQ5-Cycler (Bio-Rad laboratories) was used to amplify the genetic information. Over 40 cycles were performed, with a specific annealing temperature ranging from 60 to 65 °C and an elongation temperature of 72 °C. PCR reactions were performed in a total volume of 20 μL, of which 5 μL were obtained from the cDNA samples and 2 μL from the specific primer. Standard curves for each targeted gene were constructed by plotting Ct values against log cDNA dilution. Afterwards, nonspecific PCR products and primer dimers were ruled out creating a melting profile and carrying out an agarose gel electrophoresis. The values of mRNA concentration for each gene was expressed as the proportion of ng of mRNA per average ng of housekeeping mRNA [24]. This process was performed in triplicate. Cells were seeded in three independent membranes for each of the four experimental groups (12 samples), and 5 replicates were obtained from each well for PCR analysis (n = 60).

2.5. Statistical analysis

Mean and standard deviations are expressed in ng mRNA/ng HK. Mean comparisons were conducted by One-way ANOVA and Tukey tests. Significance was set at $p < 0.05$. Five independent experiments were performed. Data were expressed as means ± standard deviation (SD).

3. Results

Results from qPCR analysis are displayed in Fig. 1. In general, the results shown that the blending of silica NPs in the tested non-resorbable polymeric scaffold improves the expression of osteogenic genes involved in bone formation and regeneration over the control HOOC-M group. The up-regulation of osteogenic markers in osteoblasts seeded at silica blended nanofibers was observed when analyzing the expression of BMP-2, BMP-7 and OSC ($P < 0.05$, $P < 0.05$ and $P < 0.001$) (3.1, 1.7, and 4-fold change increases, respectively). The expression of genes encoding TGFβ-1, TGFβ-R1 and VEGF in cultured osteoblasts on Si−HOOC-M were found to exhibit a significant overexpression when compared to the control group ($P < 0.008$, $P < 0.001$, $P < 0.009$) (1.2, 2 and 2.2, fold increases, respectively). We did not find changes in the expression of TGFβ-R2 or TGFβ-R3 when silica blending of nanofibers was achieved ($P > 0.6$, $P > 0.29$), neither when analyzing the osteogenic markers OSX, Col 1, ALP or OPG ($P > 0.41$, $P = 1$, $P > 0.98$, $P > 0.97$). RANKL encoding osteoclastogenic factors was down-regulated (1.6 times lower) in osteoblasts seeded on HOOC-Si-M if compared to the control HOOC-M ($P < 0.05$). Runx2 also exhibited down-regulation (1.5-fold change decrease) in the membranes blended with silica NPs if compared to control group ($P < 0.02$).

After, doxycycline doping of experimental scaffolds, several osteogenic markers were up-regulated, as BMP-2, ALP and OPG respect to the HOOC-Si-M ($P < 0.001$); if these values are compared to the control group the fold change increases were 7, 3.5 and 3.2, respectively ($P < 0.001$, $P < 0.001$, $P < 0.001$). TGFβ-1 and TGFβ-R1 were also overexpressed respect to HOOC-Si-M ($P < 0.001$), and respect to the control HOOC-M values were 1.8 and 3.3 times higher, respectively ($P < 0.001$). RANKL was down-regulated about 8.7 times respect to HOOC-Si-M ($P < 0.007$) and represents a 13.9-fold change decrease, if compared to HOOC-M ($P < 0.001$). Col-1 expression was down-regulated, it was about 900 times lower, in the presence of doxycycline ($P < 0.05$) if compared to HOOC-M. No influence of doxycycline was encountered when considering the expression of TGFβ-R2, TGFβ-R3, VEGF, OSC, or BMP7 if compared to HOOC-Si-M ($P > 0.99$, $P > 0.26$, $P > 0.99$, $P > 0.93$, $P > 0.73$, respectively).

When osteoblasts were cultured on zinc-doped membranes, an over-representation of genes encoding Col 1 (2.4-fold increase) was identified if compared to control or HOOC-Si-M groups ($P < 0.02$). ALP was also increased by Zn−HOOC-Si-M if compared with HOOC-Si-M (1.8-fold increase and $P < 0.05$). TGFβ1 values were 1.4 times higher than control ones ($P < 0.001$) and 1.2 greater than values attained with HOOC-Si-M ($P < 0.02$). Down-regulation of VEGF, OSC and RANKL gene expression was found; mean values were about 4.3, 4.6 and 5.2 times lower than those values from the control group, respectively ($P < 0.001$, $P < 0.001$, $P < 0.02$).

The resultant OPG/RANKL ratios were expressed in Fig. 2. They indicate that the highest bone-building osteoblasts activity was achieved when cells were cultured on Dox−HOOC-Si-M; if compared to the control or HOOC-Si-M groups, a 28 and 14.5-fold changes increase was evidenced, respectively ($P < 0.001$). When analyzing the HOOC-Si-*Zn*-M group, the ratio was also different from HOOC-Si-M and HOOC-M; the OPG/RANKL ratio was 7.3 and 14.2 times higher, respectively ($P < 0.05$).

The genes TGFβ-R2 and OSX were not differentially regulated in osteoblasts seeded on silica blended scaffolds, neither in those cultured on zinc nor doxycycline doped membranes (ANOVA P values were 0.44 and 0.23, respectively).

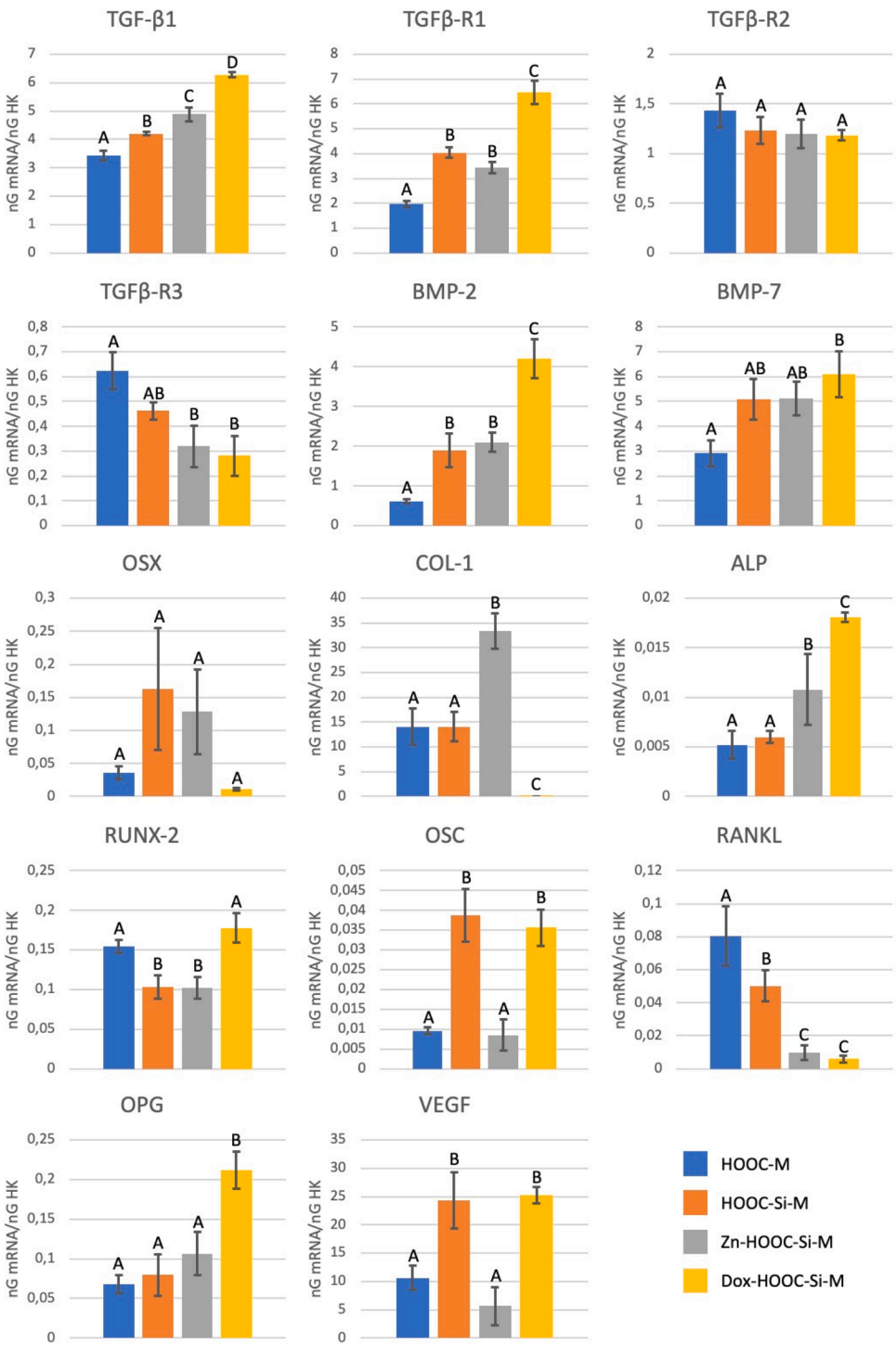

Fig. 1. Quantitative real-time PCR gene expression analysis of TGFβ-1, TGFβ-R1, TGFβ-R2, TGFβ-R3, VEGF, BMP2, BMP7, OSC, RANKL, OPG, OSX, Col-I, ALP, OPG, Runx-2 established for cultured osteoblasts seeded on the several experimental membranes, after 48 h. Results were expressed as mean and standard deviation and presented in ng mRNA/ng HK. Different letters indicate significant difference after One-way ANOVA and Tukey multiple comparisons ($p \leq 0.05$). HOOC-M: Non-functionalized membrane. HOOC-Si-M: SiO_2 nanoparticles-doped membrane. Zn−HOOC-Si-M: SiO_2 nanoparticles-doped membrane functionalized with Zn. Dox−HOOC-Si-M: SiO_2 nanoparticles-doped membrane functionalized with Doxycycline.

4. Discussion

A new approach to addressing difficult tissue regeneration problems in the oral cavity is to engineer new tissue by using selective molecules doped on polymer scaffolds. The objective is to develop membranes with suitable properties which would mimic the natural extracellular matrix and able to induce the proliferation and differentiation of osteoblastic cells. Previous research has demonstrated that osteoblast-specific factors are important at several stages of bone regeneration. Osteogenic markers may be determined by RT-qPCR and are a valuable and sensitive tool to examine osteogenic *in vitro* differentiation [24]. RT- qPCR is considered as a powerful technique capable of accurately quantitating mRNA expression levels over a large dynamic range. This makes RT-qPCR the most widely used method for studying quantitative gene expression [25]. In the present study, it has been tested the capacity that the novel nanostructured polymeric membranes loaded with silica NPs possess to modulate the expression of different osteoblast's genes, which are related to the functional capacity of this population and, therefore, with the bone regeneration process.

Two of the more greatly influenced genes by Dox−HOOC-Si and Zn−HOOC-Si membranes, were RANKL and OPG (Fig. 1). RANKL is produced by osteoblasts and stimulates osteoclasts via its receptor RANK, which is a membrane-bound protein present in osteoclasts and their precursors [24]. This interaction between RANKL and RANK can be

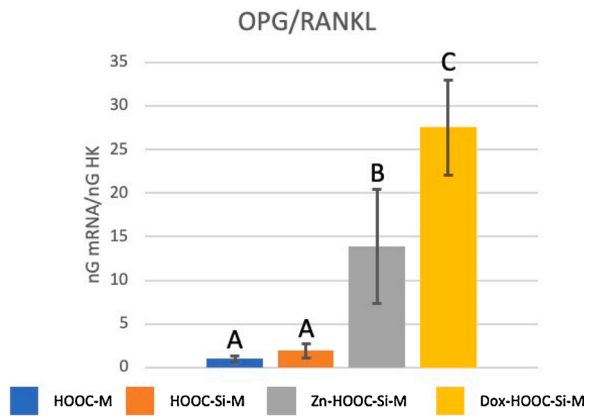

Fig. 2. OPG/RANKL ratio. Different letters indicate significant difference after One-way ANOVA and Tukey multiple comparisons ($p \leq 0.05$). HOOC-M: Non-functionalized membrane. HOOC-Si-M: SiO_2 nanoparticles-doped membrane. Zn−HOOC-Si-M: SiO_2 nanoparticles-doped membrane functionalized with Zn. Dox−HOOC-Si-M: SiO_2 nanoparticles-doped membrane functionalized with Doxycycline.

inhibited by OPG, a soluble protein also produced by osteoblasts which is associated to osteoblasts bone forming activity [26,27]. These underlying cellular mechanisms that mediate the coordinated activity between actions of bone-building osteoblasts and bone-resorbing osteoclasts, are critical for bone remodeling [26]. In our study, RANKL was substantially down-regulated in the presence of Dox and Zn, while OPG was up-regulated in these same groups, leading to a quite positive OPG/RANKL ratio [26]. Due to these results, osteoclasts would presumably be inhibited via RANKL down-regulating and OPG up-regulating. These cellular pathways would produce a positive balance in bone formation [28]. In the present study, attained OPG/RANKL ratios indicate that the highest bone-building osteoblasts activity may be attained by cells cultured on Dox−HOOC-Si membranes; a 28-fold increase was evidenced if compared to HOOC-M (Fig. 2). Similar results were previously found, demonstrating the ability of doxycycline to activate WNT-1b and neutralize Dkk-1. It results in an increase in osteoblast numbers and a decrease in osteoclast cells, also evidenced by a high OPG/RANKL ratio [29,30]. Tetracycline has been shown to significantly repress the RANKL-induced mRNA expression of the MMP-9 target genes in a dose-dependent manner, and produces inhibitory regulation of osteoclast-specific genes. It has been shown to inhibit osteoclast differentiation by modulating MMP-9 mediated proteolysis [31].

Other genes associated with functional mechanisms relevant to bone healing, as TGFβ-1and TGFβ-R1, were also differentially overexpressed (Fig. 1). TGF-β1 is involved in the control of proliferation, migration, differentiation and survival of different kinds of cells. It plays a crucial role on angiogenesis and inflammation. TGF-β1 is able to regulate the development and maintenance of bone [32]. TGF-β superfamily members play a main role in bone tissue repair and remodeling [33]. It is also known to upgrade matrix production and enhance osteoblast differentiation while reducing the ability of osteoblasts to secrete RANKL. Therefore, TGF-β1 could indirectly limits further osteoclast formation [24]. The addition of silica NPs as well as incorporating Zn and Dox to the membrane's formulation, have also shown to increase the expression of TGF-β1; thus, improving the bone formation potential of these novel membranes.

BMP-2 is also an important gene in osteogenic induction [34]. It plays a major role in bone formation/remodeling, development and in osteoblast differentiation [35]. It should be considered that the expression of BMP-2 in osteoblasts seeded on HOOC-Si-M and Dox−HOOC-Si membranes groups was significantly higher than that in the other scaffolds (Fig. 1). It has been previously described that BMP-2 may induce the expression of ALP and other osteoblastic markers [24,36,37]. This finding is also reflected in the results of our study, in which the overexpression of BMP-2 in the HOOC-Si-M and Dox−HOOC-Si membranes can also be observed in the expression of ALP, being Dox−HOOC-Si-M the one with the highest expression of both genes.

The overexpression of TGFβ and BMPs has been associated with an increase in bone formation. This cellular mechanism can be explained in part by virtue of the activation of Smad or MAPKs cascade, which are common pathways for both TGFβ and BMPs [38].

ALP is an enzyme involved in bone tissue mineralization. It increases when mineralization is well progressed during osteoblastic differentiation [39]. ALP is usually classified as an early marker of osteogenic cell differentiation [40]. ALP was significantly upregulated in both Zn−HOOC-Si-M and Dox−HOOC-Si-M (Fig. 1).

It is important to stress that Col-I expression was down-regulated (about 900 times) in the presence of doxycycline (Fig. 1). Collagen type-I is the predominant component of the bone extracellular matrix during osteoblast maturation. Collagen production has an important role in biomineralization. However, Col-I is expressed in high levels at the end of the proliferative osteoblasts state and during the period of matrix deposition [41]. In accordance with this, Col-I is expressed during the proliferative phase being, like ALP, an initial marker of osteoblast differentiation [40]. Both Col-I and ALP are observed in the first stages of osteoblast differentiation and persist in early and mature osteoblasts [24,42]. Then, it may be that Col-I overexpression may only be detected at a certain time of osteoblasts proliferation. At the present study, it was also encountered an upregulation of Col-I at osteoblasts cultures seeded on the Zn−HOOC-Si-M, indicating major activity on un-mineralized matrix deposition. It has previously been shown this effect on osteoblasts proliferating on poly-ethilen-glycol electrospun fibrous composites loaded with Zn_2SiO_4 bioceramic nanoparticles [43].

Runx-2 is as a key transcription factor associated with osteogenic differentiation [44], but exhibited down-regulation (1.5-fold change decrease) in the membranes blended with silica NPs; the same effect was found for silica NPs in bioengineered scaffolds [26]. The osteogenic markers Runx-2, Col-I and ALP and osteonectin, are major bone-related genes during osteogenic differentiation of stem cells. In osteoblasts cultures, it is established that Runx-2 (formerly called Cbfa1), a member of the runt homology domain transcription factor family, plays a crucial role in osteoblast development [43]. These genes are early osteogenic markers, indicating differentiation into mature osteoblasts; attained decreases could be due to the temporal changes in its mRNA expression during osteogenesis [40,45,46].

Present research was performed with MG63 osteosarcome cell line. It may be considered as a study limitation, as per the review by Czekanska et al., 2013 [47], cell lines such as the MG63 demonstrate some distinct similarities with primary human osteoblasts, and should not be used to replace primary cell studies. Therefore, future studies with primary human osteoblasts would be carried out in order to further permit comparisons between both cell types' behavior, when in contact with the tested membranes. Research including alkaline phosphatase activity, antigenic phenotype, phagocytic activity, immunofluorescence staining and Alizarin Red staining should also be performed as they would allow understanding about cell function and behavior under the influence of newly developed membranes.

Our results show that these novel membranes may have crucial implications for tissue engineering strategies, as it has been demonstrated that their modification with silica nanoparticles and doxycycline generated bioactive signaling. HOOC-Si-Dox membranes can effectively direct gene expression and differentiation of osteoblasts and probably of other progenitor cell populations, facilitating bone regeneration. HOOC-Si-Dox membranes do also posse a demonstrated antibacterial effect on periodontal pathogenic biofilms [14]. Therefore, they may be potential candidates for use in bone repair, at those pathologies having an infectious etiologic component (i.e. periodontal disease or peri-implantitis). Doxycycline has also been probed to produce

decreases in inflammatory infiltrates in bone [29], which may also be advantageous in these reparative process.

The main limitation of the present study is the lack of mechanistic assays. However, when these assays are attempted [48], although they contribute to understand a particular mechanism of action, they hamper the multi-regulatory processes present in these complex biological processes of genes expression regulation, and results have not clear application. However, by identifying the over or down-regulation of the targeted genes, the results may be successfully translated to the behavior that the new biomaterials would have in the clinic. The obtained results, pose an insight of integrated or holistic response rather than focusing on individual events. Nevertheless, challenging new experiments for discovering unknown interactions of doxycycline with target genes and molecular pathways descriptions are pendant for future research. Efforts to understand the predictability and translation of these assays to humans will also be performed in the near future.

Zinc and doxycycline have been previously shown to produce beneficial effects in some specific osteoblasts bone-related genes expression [29,30,43]. However, in the present study, a big step-forward is produced, as it has been shown for the first time that appropriately doped GBR membranes may act as carrier of these proliferation and differentiation factors. Novel developed membranes will potentially produce bone repair by the principle of osteoinduction, being active biomaterials in the regenerative process.

5. Conclusions

In the present study, it has been demonstrated that silica loading may offer beneficial effects to experimental membranes; an upregulation of several osteogenic markers and a highly favorable OPG/RANKL ratio were encountered. Moreover, additional doxycycline doping facilitates overexpression of BMP-2, ALP, OPG, TGFβ-1and TGFβ-R1 target genes. Dox-HOOC-Si membranes may be a potential candidate for use in GBR procedures in several challenging pathologies, including periodontal diseases and peri-implantitis.

CRediT authorship contribution statement

Manuel Toledano-Osorio: Investigation, Methodology, Conceptualization, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Francisco Javier Manzano-Moreno:** Investigation, Methodology, Conceptualization, Formal analysis, Data curation, Writing - review & editing. **Manuel Toledano:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - review & editing. **Raquel Osorio:** Investigation, Methodology, Conceptualization, Formal analysis, Data curation, Funding acquisition, Supervision, Writing - original draft, Writing - review & editing. **Antonio L. Medina-Castillo:** Methodology, Visualization, Writing - review & editing. **Víctor J. Costela-Ruiz:** Methodology, Visualization, Writing - review & editing. **Concepción Ruiz:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

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polymers

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Review

Antibiotic-Loaded Polymeric Barrier Membranes for Guided Bone/Tissue Regeneration: A Mini-Review

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Abstract: Polymeric membranes are frequently used for bone regeneration in oral and periodontal surgery. Polymers provide adequate mechanical properties (i.e., Young's modulus) to support oral function and also pose some porosity with interconnectivity to permit for cell proliferation and migration. Bacterial contamination of the membrane is an event that may lead to infection at the bone site, hindering the clinical outcomes of the regeneration procedure. Therefore, polymeric membranes have been proposed as carriers for local antibiotic therapy. A literature search was performed for papers, including peer-reviewed publications. Among the different membranes, collagen is the most employed biomaterial. Collagen membranes and expanded polytetrafluoroethylene loaded with tetracyclines, and polycaprolactone with metronidazole are the combinations that have been assayed the most. Antibiotic liberation is produced in two phases. A first burst release is sometimes followed by a sustained liberation lasting from 7 to 28 days. All tested combinations of membranes and antibiotics provoke an antibacterial effect, but most of the time, they were measured against single bacteria cultures and usually non-specific pathogenic bacteria were employed, limiting the clinical relevance of the attained results. The majority of the studies on animal models state a beneficial effect of these antibiotic functionalized membranes, but human clinical assays are scarce and controversial.

Keywords: barrier membrane; polymer; collagen; antibiotic; bone regeneration

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1. Introduction

In 1982, Nyman et al. [1] proposed the possibility of producing periodontal tissue regeneration in humans by using a barrier membrane. This barrier membrane should avoid soft tissue cell invasion of the regenerating area, maintaining the space and facilitating the periodontal ligament derived cells or bone cells to grow into the defective area. These principles have also been employed to promote guide bone regeneration at those sites where an intraoral bone defect or insufficient bone exists, mainly caused by teeth loss, trauma, tumoral pathology or infections [2]. Currently, these guided tissue regeneration and guided bone regeneration techniques are widely accepted and are often used for clinical applications [3,4].

These occlusive membranes must fulfill several criteria, including space maintaining capacity, mechanical properties, osteoconductivity/osteoinductivity, and biocompatibility [5,6]. Currently, it seems that natural and artificial polymers are the best candidate materials to comply with most of these prerequisites [6]. However, it should be taken into account that in many cases, periodontal guided tissue and bone regeneration are hindered due to contamination and infection of the healing site. It seems that the placement of barrier

membranes at the oral cavity creates a favorable ecological niche that facilitates the growth of some periodontal pathogens [3,4].

In order to improve the barrier membrane function, the incorporation of antibacterials has been suggested to try to inhibit bacterial contamination at the surgical intervention or during the healing period if membrane exposure to the oral cavity occurs (Figure 1) [4,7]. If bacterial colonization and subsequent infection is produced in the early stages of wound healing, the clinical outcomes of the complete procedure will be jeopardized [7,8]. Controlling the membrane's colonization of bacteria and reducing the possibility of infection in the early healing stage increases the predictability of the clinical outcomes [9]. It should be taken into account that some issues discourage the use of systemic antibiotic therapy due to risk of toxicity, acquired bacterial resistance, difficulty in penetrating some areas, and insufficient concentration levels at the infected site to efficiently inhibit the target microorganisms, among others [10]. Therefore, the use of local drug administration is recommended to potentially reduce the drug resistance of the bacteria by lowering the dosage of used antibiotics. The combination of polymeric barrier membranes and antibacterials are preferred in order to facilitate, accelerate, and enhance the effect of guided tissue and guided bone regeneration procedures [4,7].

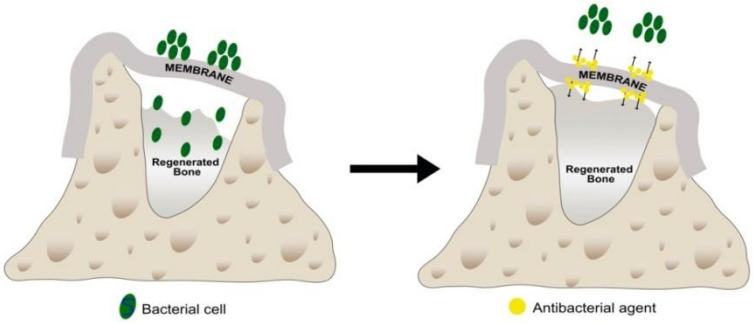

Figure 1. A barrier membrane employed to avoid soft tissue cell invasion, enhancing space maintenance of the regenerating area. The incorporation of antibacterials in the membrane has been suggested to inhibit bacterial contamination during the surgical intervention or the healing period if membrane exposure to the oral cavity occurs, improving the performance of the bone regeneration procedure.

The estimated healing period in bone regeneration is more than 6 months, and for periodontal regeneration, 4 to 6 weeks are necessary [11]. Antibacterials have shorter lifespans and rapid local clearances at bone healing sites. To overcome these points, a polymeric carrier system may play a key role in determining antibacterial activity. In recent years, there has been a strong increase in research focused on appropriate antibacterials and carrying materials for controlled and optimal release. Polymeric-based membranes have been proposed as key biomaterials capable of securing sustained release of antibacterials over a period of time and of affording acceptable release kinetics [4].

The purpose of this study was to review the existing literature on the main findings on antibiotic-loaded polymeric barrier membranes, covering design, manufacturing, loading and release kinetic, antibacterial efficacy, and usefulness for guided bone and tissue regeneration.

2. Methods

Using the National Library of Medicine (MEDLINE by PubMed), The Cochrane Oral Health Group Trials Register, EMBASE, and Web of Science (WOS) a literature search was performed for papers, including peer-reviewed publications from 1963 up to January 2022.

Combinations of several search terms were applied to create a search strategy including the following word combinations: ("Guided Tissue Regeneration" OR "GTR" OR "Guided Bone Regeneration" OR "GBR" OR "Bone Regeneration" OR "Periodontal Regeneration" OR "Bone Tissue Regeneration" OR "Bone formation" OR "Osteogenesis"

OR "Osteogenic regeneration") AND ("Barrier Membrane" OR "Membrane" OR "Barrier" OR "Collagen Membrane" OR "Chitosan-Collagen Membrane" OR "Natural Membrane" OR "Bovine Membrane" OR "Porcine Membrane" OR "Pericardium Membrane" OR "Dermis Membrane") AND ((ions[MeSH Terms]) OR antibiotics[MeSH Terms] OR (antibacterial agents[MeSH Terms]) OR (agents, antimicrobial[MeSH Terms]) OR tetracycline OR doxycycline OR metronidazole OR minocycline OR roxithromycin OR moxifloxacin OR ciprofloxacin)). Bibliographies of eligible articles were also manually searched for missing papers after the electronic searching.

3. Results and Discussion

3.1. Polymeric Materials for Antibacterial-Loaded Membranes

Several polymers have been used as antibacterial carriers for bone regeneration barrier membranes. They can roughly be classified as natural or synthetic, resorbable or non-resorbable [3,6]. The previously-employed polymers for the mentioned medical application are presented in Table 1.

Among all the polymeric biomaterials, the natural collagen membrane is the most widely used as an antibacterial carrier in bone regeneration [5,12–21]. Several factors may explain this finding: (1) among the degradable membranes, collagen-based ones are the most commonly employed in dentistry because of their bioactivity, biocompatibility, and mechanical properties [3,7,22]; (2) they have been found to have many options for loading [23]. It should be considered that the chemical structure of collagen offers versatility, as it contains carboxyl and amino terminals, permitting not only adsorption, but also covalent binding of a great variety of different chemical groups [24]; (3) collagen degradability permits effective antibacterial liberation even if the antimicrobial substance is covalently linked to collagen [23,24].

Other synthetic polymers that have also been used as antibacterial carriers are: poly(lactic acid) (PLA) [25–28], poly(glycolic acid) (PGA) [16,19,29–32], polycaprolactone (PCL) [33–38], or combinations between them [10,39–43]. All these abovementioned polymers are also resorbable. PCL is a slow resorbing polymer as it degrades via an erosion mechanism, hence avoiding the rapid release of acidic byproducts, which may be detrimental to surrounding tissues. PGA and PLA are aliphatic polyesters with a fast degrading behavior [29].

Among the non-resorbable polymers, two are used as antibacterial carriers. One of them is expanded polytetrafluoroethylene (ePTFE) [8,16,19,44,45] and the second is a novel polymer based on hydroxyethyl methacrylate–methyl methacrylate (HEMA–MMA) copolymers that is still in the experimental phase [46,47].

Table 1. Combinations of previously-employed polymers and antibiotics in the designing of barrier membranes for guided bone/tissue regeneration.

Polymeric Material	Origin	Resorbable	Loaded Antibiotic	References
Expanded Polytetrafluoroethylene-ePTFE-	Synthetic	No	Tetracycline	[8,16,19,44]
			Amoxicillin	[16,19]
(MMA)1-co-(HEMA)1/(MA)3-co-(HEA)2	Synthetic	No	Doxycycline	[47,48]
Collagen	Natural or Synthetic	Yes	Minocycline	[12]
			Doxycycline	[14]
			Tetracycline	[16–19]
			Amoxicillin	[15,16,19]
			Metronidazole	[15,20,21]
			Niridazole	[21]
			Tinidazole	[21]
Chitosan		Yes	Minocycline	[49]
			Doxycycline	[50]
Collagen-Chitosan		Yes	Minocycline	[13]

Table 1. *Cont.*

Polymeric Material	Origin	Resorbable	Loaded Antibiotic	References
Poly(lactic acid) -PLA-	Synthetic	Yes	Metronidazole	[25]
			Doxycycline	[28]
			Tetracycline	[27]
			Azithromycin	[29]
Poly(glycolic acid) -PGA-	Synthetic	Yes	Doxycycline	[30]
			Tetracycline	[16,19,51]
			Amoxicillin	[16,19]
			Ornidazole	[31]
Polycaprolactone-PCL-	Synthetic	Yes	Moxifloxacin	[33]
			Metronidazole	[34,37,38,52]
			Vancomycin	[36]
			Salicylic acid	[35]
PGA-PLA	Synthetic	Yes	Tetracycline	[39,40,43]
			Vancomycin	[41]
			Metronidazole	[10,42]
Polyetheretherketone	Synthetic	No	Gentamicin	[53]
Hyaluronic acid	Synthetic	Yes	Hinokitiol	[54]
			Metronidazole	[55]
Cellulose	Synthetic	Yes	Doxycycline	[56]
			Tetracycline	[57]
Hydroxybutyrate	Synthetic	Yes	Metronidazole	[58]
Silk fibroin	Synthetic	Yes	Tetracycline	[32]
Polyvinylidene difluoride-PVDF-	Synthetic	No	Doxycycline	[59]

3.2. Manufacturing Procedure for Polymeric Antibacterial-Loaded Membranes

The most frequently employed manufacturing technique for synthetic polymeric membranes is electrospinning [5,25,29–31,33–38,41,42,47,48,51,52,60,61]. This production method permits the adjusting of the most relevant characteristics of the manufactured membranes. It enables the creation of membranes with desired mechanical properties such as flexibility or elasticity. Fiber diameter may also be adjusted from micro to nanosized. Pore size, which imparts occlusive properties and an interconnected porous network resembling the bone collagen network, which is favorable for long term tissue infiltration and integration [29,47,48], can be controlled. Processing variables for each electrospinning method are different between the evaluated studies and include different voltages, needle to collector distances, and flow rates. These variables, together with the polymeric solution parameters such as surface tension, viscosity, and electrical conductivity of the solution, control the morphology of the electrospun fiber mats [5].

Antibacterials can be loaded in the electrospun nanofiber through: (1) blending, which is a passive loading of the antibacterial into the nanofibers (adding it in the polymeric solution prior to electrospinning) [25,30,31,33,34,36,37,41,51]; (2) coaxial electrospinning, where the antibacterials are embedded inside the electrospun nanofibers in order to improve some different aspects such as release outline (extending the period of drug delivery), drug safety, or drug-loading efficiency of non-soluble substances [37,38,42,55]; and (3) solvent evaporation or immersion techniques after fiber production, which permit physical absorption and chemical bonding of the antibacterials onto the polymers [37,47,48]. The simple electrospinning technique has gained widespread interest in the area of tissue engineering and drug delivery due to its relative ease of use and versatility [62]. Meanwhile, co-axial electrospinning is less employed, as it is a more difficult technique requiring more than a single nozzle [5]. One of the major advantages of electrospun fiber mats is the inherently high surface-to-volume-ratio of formed scaffolds. Not only does this help to enhance drug loading and to accomplish sustained and controlled local drug delivery, but it also improves cell attachment [62].

Collagen, PLA, PLGA, PCL, and other polymeric resorbable membranes have been fabricated through the casting method, by solvent evaporation, or as dried films [10,12,20,39,43,50,55].

In these cases, they are less porous and do not have a fibrous micro or nanostructure resembling collagen. These membranes were loaded by incorporation of the antibacterial in the polymer blend solution [10,12,13,32,39,49,51,55] or by immersion or solvent evaporation techniques after membrane production [14–20,43,50].

When using non-resorbable synthetic membranes, antibacterials are coated on the outer polymer surface through adsorption [8,16,44,59], direct covalent binding of the drug onto the membrane surface [47,48], or by grafting (using intermediary compounds in order to provoke a crosslinking reaction between the antibacterial and the polymeric membrane) [59].

Other manufacturing techniques as supercritical CO_2-assisted processes, 3D printing, porogen leaching, gas foaming, phase separation, or any possible combination between these may also be employed for polymeric membrane preparation [63,64]. Among these techniques, the phase separation process is easy to execute and does not require sophisticated equipment. It is based on the principle that a homogeneous solution of a polymer dissolved in a good solvent can undergo a phase separation, causing solution saturation that will lead to polymer precipitation, followed by a microcellular foam polymer structuration [65]. It is beneficial since it may offer good control of the scaffold structure, particularly in terms of porosity and interconnectivity [63]. These properties play a significant role in tissue regeneration, affecting several cell processes such as adhesion, migration, proliferation, and differentiation [65].

3.3. Loaded Antibacterials for Bone Regeneration in Dentistry

The most frequently loaded antibiotics in bone regeneration membranes are tetracyclines [8,12–14,16–18,27–30,32,39,40,43,47–51,56,57,59]; which are broad-spectrum antibiotics that have been shown to be useful in fighting against most of the bacteria responsible for periodontitis [66]. The most frequently employed tetracyclines have been minocycline [12,13,49] and doxycycline [14,28,30,47,48,50,56,59]. Tetracyclines work by inhibiting protein synthesis in bacteria [67] and have been shown to have a prolonged lifespan and anticollagenase properties, and are well absorbed by bone due to a calcium quelating effect [59].

The second most frequently employed antibiotics are metronidazole [5,10,15,20,21,25,33,38,42,52,55,58] and other nitroimidazoles such as ornidazole [31], niridazole, and tinidazole [21]. These are antibiotics with antibacterial activity for Gram-negative and anaerobic bacteria [25], and they are specific against most of the subgingival [10] and periodontopathic biofilms [21]. Metronidazole's mechanism of action is based on the alteration of nucleic acid synthesis in bacteria [67].

Other encountered, but less used antibiotics for loading membranes were amoxycillin [15,16,19], vancomycin [41] or azithromycin [29]. Amoxycillin and vancomycin are antibiotics targeting the bacterial cell wall [67]. Azithromycin is a macrolide antibiotic extensively recommended for a wide range of anaerobic infections. It mainly acts by altering protein synthesis. However, its main disadvantage is low bio-availability as a result of its poor water solubility, probably limiting its proposed clinical application [29].

3.4. Antibiotic Release Kinetics

Antibiotic release kinetics is not evaluated in all the reviewed studies. When ascertained, it was usually done in vitro. The supernatants are measured at specific time-points after immersion of the loaded membranes in a solution (normally deionized water or phosphate buffered saline). High performance liquid chromatography [31,33,34,37,38,41], inductively coupled plasma atomic emission spectrometry [53], UV-vis spectrophotometry [10,12,13,21,30,50,58,59], and fluorescence [36] are the most used techniques to determine the released concentration of the loaded antibiotics. Loading efficiency, when determined, was usually high, ranging between 30 and 85% [29,52].

Antibiotic release from polymeric membranes is in all cases characterized by two different phases. The first is an initial burst release, which can be described as the liberation produced be-

tween 7 and 10 h [37,55,68], or between 12 and 48 h [10,13,29,31–34,36,39,42,49,50,52,54,58,59]. This rapid release is followed by a slower and sustained liberation that may last from 35 h to 10 days [10,13,21,32,34,37–39,48,52,57,59,65], or in some cases much longer, up to 28 days [12,25,30,33,36,49,53,58]. These described liberation kinetics indicate that some of the antibiotic is always retained by absorption and is rapidly liberated after immersion. The slow and relatively sustained posterior release probably corresponds to the antibiotic that is ionically or covalently linked to the membranes, or to the antibiotic that is liberated at the same time that the membrane degrades. Therefore, the procedures used to load the antibiotic and the type of membrane may be considered as determinant factors affecting antibiotic release. The loading of antibiotics on polymers through chemical conjugation may have a more controlled kinetic release than those processed through physical adsorption [69]. However, it is also necessary to investigate the antibacterial activity of the released antibiotics, since liberation does not always imply biological activity.

3.5. Antibacterial Efficacy of Antibiotic-Loaded Membranes

Most studies investigated antibiotic loaded membrane efficacy using in vitro antibacterial cell assays [12,15,16,19,29–31,33,34,38,41,42,44,48,49,53,56,58,65] and/or in vivo animal models when surgically treating contaminated bone defects [14,36,41]. The efficacy of the membrane as an antibiotic delivery carrier was always confirmed.

Of the antibacterial cell assays, the most employed was the agar disk diffusion test [12,15,29–31,33,34,38,41,42,49,56,58,65]. Other techniques such as the plate-counting method [53], determination of bacterial penetration through membranes [19], bacterial colonization on membranes [44], scanning electron microscopy evaluation of membranes colonization [16,48,53], or number of cells determination by more precise techniques such as quantitative polymerase chain reaction [48] were rarely executed.

It is also worth mentioning that sometimes non-clinically relevant or unspecific bacteria were used for these studies, as in the case of *Peptostreptococcus anaerobius* [31], *Staphylococcus aureus* [12,30,33,36,41,53,56], *Pseudomonas aeruginosa* [33], *Echerichia coli* [30,53,56], *Helicobacter pilori* [37], or *Streptococcus mutans* [16,19,49]. Of periodontally-relevant bacteria *Aggregatibacter actinomycetemcomitans* [15,16], *Porphyromonas gingivalis* [13–15,49] and *Fusobacterium nucleatum* [13,31,34,38,42,68] have been tested. All these studies were based on single bacterial cultures; therefore, results should not be directly extrapolated to the clinical situation. It has to be taken into account that bacteria grow in biofilms, providing them with specific characteristics that make bacteria more resistant and tolerant to antibiotics than when in a planktonic state [70]. Only one recently published study was performed using a subgingival multispecies biofilm model with six different bacterial species [48].

The incorporation of antibacterial agents in membranes is a promising approach that may promote bone formation, especially for some challenging clinical situations when the characteristics of the defect make the site especially prone to membrane exposure and subsequent bacterial contamination and infection. However, despite the promising results encountered in vitro and in preclinical animal models, the value of incorporating antibacterials has not yet been evidenced clinically [4].

In animal models, when treating previously contaminated bone defects, the efficacy has been probed in terms of bone regeneration of doxycycline-loaded collagen membranes [14], vancomycin-loaded PCL-membranes [36], and vancomycin-loaded PGA membranes [41]. Tetracycline-loaded PGA membranes and minocycline-chitosan membranes also induced major regeneration in periodontal defects in beagle dogs [51] and rats [49], respectively.

None of the antibacterial-loaded membranes have been evaluated for efficacy in reducing microbial adhesion and infection in humans. Conversely, several antibiotic-loaded membranes were tested in humans evaluating clinical efficacy when compared to non-antibiotic-loaded membranes. Gain in periodontal attachment level and increases in bone formation were obtained when using tetracycline-loaded ePTFE membranes [44], doxycycline-loaded collagen membranes [18], and metronidazole-loaded collagen mem-

branes [20]. However, in two clinical studies, doxycycline-loaded collagen membranes [28] and tetracycline-loaded ePTFE membranes [8] failed to enhance the periodontal regeneration outcomes when compared to non-antibiotic-loaded membranes. These controversial results may be due to the small sample size of the studies (around 10 to 25 patients) and to the lack of standardization of the employed antibiotic concentration and liberation, which was sometimes extremely low (i.e., 4 wt% [28]) or not reported [8].

3.6. Other Findings Associated with Antibiotics Loaded on Polymeric Membranes

The cytocompatibility of these membranes was sometimes evaluated using different cells lines as osteoblasts [13,37,42,53,60,61], fibroblasts [13,35,37,39,52], epithelial cells [59], and stem cells [5,25,30,32], always with favorable results.

It is relevant that in addition to their antimicrobial activity, doxycycline and minocycline have been shown to enhance osteoblast and/or stem cell proliferation, differentiation, and osteogenic activity [5,13,30,32,33,53,60,61]. Moreover, these antibiotics were shown to inhibit bone resorption and to promote bone formation when assayed in animals [13,29,33,40,43] and in humans [62].

Immunomodulatory effects have been proven for doxycycline-loaded membranes in both cells [71] and animals [47]. The same effect was also shown for metronidazole-loaded collagen membranes in cells [21] or azithromycin-loaded PGA membranes when tested in an animal model [29].

Tetracycline-loaded collagen membranes have also been reported to have slower degradation [17], which may be beneficial for bone regeneration in challenging bone defects.

4. Conclusions

It can be concluded that, taking into account the fact that infection can lead to the failure of the intended bone regeneration, polymeric membranes could be used as carriers for local antibiotic therapy. Due to antibiotic lifespans and the rapid clearance rate existing at the surgical sites, it is impossible for antibiotics to produce a long-term effect without the aid of a carrier facilitating a controlled liberation. The loading efficacy and the kinetic release will depend on the employed polymeric material. The polymeric carrier should ideally have a constant and slow degradation and should be ideally maintained through the complete healing period. Collagen or ePTFE loaded with tetracyclines, and PCL with metronidazole are the most frequently assayed combinations. Antibiotics present the advantage of possessing a wide therapeutic window, making it easier to obtain a beneficial effect whenever the liberation is effective. In the existing studies, even when antibacterial efficacy is often reported, most of the times it is not measured against specific subgingival pathogenic bacteria and it is usually measured using an agar disc diffusion method, which are two variables limiting the clinical relevance of the previously published results. It should be considered that the present literature review lacks of standardization in method; therefore, results need to be taken with caution.

It should also be taken into account that there is relatively scarce experimental evidence that a local antibacterial strategy could be useful in bone regeneration procedures. Apart from several studies on infected periodontal defects locally treated with antibiotics and polymeric membranes [8,18,20,28,44], no specific antimicrobial strategy has been yet clinically validated [4].

Future studies should be performed focusing on: (i) the standardization of adsorption/release abilities of the different polymeric carriers, (ii) antibacterial activity assays using specific and periodontal clinically-relevant biofilm models, and (iii) randomized clinical trials in order to finally determine the safety and efficacy of these novel and innovative procedures; thereby helping to eliminate the barriers limiting the extension of the experimental results to the clinical situation.

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ORIGINAL ARTICLE

Doxycycline-doped collagen membranes accelerate in vitro osteoblast proliferation and differentiation

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Abstract

Objective: The aim of the study was to evaluate the effect of doxycycline- and dexamethasone-doped collagen membranes on the proliferation and differentiation of osteoblasts.

Background: Collagen barrier membranes are frequently used to promote bone regeneration and to boost this biological activity their functionalization with antibacterial and immunomodulatory substances has been suggested.

Methods: The design included commercially available collagen membranes doped with doxycycline (Dox-Col-M) or dexamethasone (Dex-Col-M), as well as undoped membranes (Col-M) as controls, which were placed in contact with cultured MG63 osteoblast-like cells (ATCC). Cell proliferation was assessed by 3-(4,5-dimethylthiazol -2-yl)-2,5-diphenyltetrazolium (MTT) assay and differentiation by measuring the alkaline phosphatase (ALP) activity using spectrophotometry. Real-time quantitative polymerase chain reaction was used to study the expression of the genes: Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-I, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF-βR2, and TGF-βR3. Scanning electron microscopy was used to study osteoblast morphology. Data were assessed using one-way analysis of variance or Kruskal–Wallis tests, once their distribution normality was assessed by Kolmogorov–Smirnov tests ($p > .05$). Bonferroni for multiple comparisons were carried out ($p < .05$).

Results: Osteoblast proliferation was significantly enhanced in the functionalized membranes as follows: (Col-M < Dex-Col-M < Dox-Col-M). ALP activity was significantly higher on cultured osteoblasts on Dox-Col-M. Runx-2, OSX, ALP, OSC, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF-βR2, and TGF-βR3 were overexpressed, and RANKL was down-regulated in osteoblasts cultured on Dox-Col-M. The osteoblasts cultured in contact with the functionalized membranes demonstrated an elongated spindle-shaped morphology.

Conclusion: The functionalization of collagen membranes with Dox promoted an increase in the proliferation and differentiation of osteoblasts.

KEYWORDS
bone regeneration, dexamethasone, doxycycline, membranes, osteoblasts

Title: Doxycycline-doped collagen membranes accelerate *in vitro* osteoblast proliferation and differentiation.

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Abstract

*Objective. T*o evaluate the effect of doxycycline and dexamethasone-doped collagen membranes on the proliferation and differentiation of osteoblasts.

Background. Collagen barrier membranes are frequently used to promote bone regeneration, and to boost this biological activity their functionalization with antibacterial and immunomodulatory substances has been suggested.

Methods. The design included commercially available collagen membranes doped with doxycycline (Dox-Col-M) or dexamethasone (Dex-Col-M), as well as undoped membranes (Col-M) as controls, which were placed in contact with cultured MG63 osteoblast-like cells (ATCC, Manassas, VA, USA). Cell proliferation was assessed by MTT-assay and differentiation by measuring the alkaline phosphatase activity using spectrophotometry. Real-time quantitative polymerase chain reaction (RT-qPCR) was used to study the expression of the genes: Runx-2, OSX, ALP, OSC, OPG, RANKL, Col-I, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF-βR2, and TGF-βR3. Scanning Electron Microscopy was used to study osteoblast morphology. Data was assessed using one-way ANOVA or Kruskal-Wallis tests, once their distribution normality was assessed by Kolmogorv–Smirnov tests ($p > 0.05$). Bonferroni for multiple comparisons were carried out ($p < 0.05$).

Results. Osteoblasts proliferation was significantly enhanced in the functionalized membranes as follows: (Col-M<Dex-Col-M<Dox-Col-M). Alkaline phosphatase activity was significantly higher on cultured osteoblasts on Dox-Col-M. Runx-2, OSX, ALP, OSC, BMP-2, BMP-7, TGF-β1, VEGF, TGF-βR1, TGF-βR2, and TGF-βR3 were overexpressed and RANKL was down-regulated in osteoblasts cultured on Dox-Col-M. The osteoblasts cultured in contact with the functionalized membranes demonstrated an elongated spindle-shaped morphology.

Conclusion. The functionalization of collagen membranes with Dox promoted an increase in the proliferation and differentiation of osteoblasts.

Keywords: bone regeneration, doxycycline, dexamethasone, membranes, osteoblasts.

1. INTRODUCTION

Regenerative therapies aimed to reconstruct the anatomy and function of oral tissues lost due to trauma or disease, have included different technologies and surgical approaches, although guided tissue regeneration (GTR) and guided bone regeneration (GBR)[1–3] are the ones most frequently used and with more predictable outcomes.[4,5] These interventions have in common the use of a membrane that acts as a physical barrier excluding the colonization of the defect by undesired cells and allowing those with capability to regenerate the lost or damaged tissues.[1] With the current understanding of the biological processes of wound healing and regeneration, membrane technology is evolving from a mere physical activity to a more active role combining the barrier effect with biological activity aiming to boost the regenerative process.[6]

One of the key factors in the biological activity of a barrier membrane for bone regeneration (GBR) is its bio-absorbability, since the membrane must maintain its physical integrity during the post operatory wound healing process to predictably achieve the desired regenerative outcomes. Although there is not an ideal resorption time, it is proven that the longer the membrane maintains its function, the regenerated bone will be more dense and mature.[6] Collagen-based membranes, are the most widely used membrane material due to their biocompatibility, bio-absorbability, good handling properties and its biological ability to attract and activate gingival fibroblast, periodontal ligament cells, and osteoblasts, what may lead to enhanced soft and hard tissue healing.[7,8,9] However, as main disadvantage, natural collagen membranes have faster resorption kinetics due to the released of collagenases and proteases by the host, which may be enhanced in presence of a pro-inflammatory phenotype or in unfavorable mechanical environment.[10] This disadvantage has been partially overcome by the utilization of different physical/chemical cross-linking processes.[11] Although cross-linking has improved collagen stability, toxic residues from this process have been reported to induce severe inflammation at the regeneration site.[11]

Furthermore, barrier membranes may be exposed during the healing period, mainly in presence of large osseous defects or when the soft tissue borders of the flap are not properly sutured or with excessive tension. Once exposed in the oral environment, the membranes will become contaminated, what will promote the proinflammatory environment during wound healing and hence, will hamper the desired regenerative outcomes. To counteract these unwanted effects of classical barrier membranes, a new generation of bioactive membranes has been developed where membrane materials are

functionalized with substances either with antimicrobial activity or with immune-modulatory effects, this promoting a pro-healing rather than a pro-inflammatory phenotype.[12-14]

As immune-modulating substances, dexamethasone has been proposed, both due to the immunomodulation effect as well as its intrinsic capacity to stimulate mesenchymal stem cells proliferation and differentiation to osteogenic lineages[15]. Dexamethasone is also a synthetic glucocorticoid that shares this combined effect, thus potentially enhancing bone regeneration. Different antimicrobial substances have also been used to functionalize barrier membranes to prevent bacterial contamination in case of membrane exposure to the oral environment.[16] Tetracyclines have been extensively used since they are highly biocompatible, they are chemically stable at body temperature, do not interfere with wound healing, have a broad-spectrum bactericidal activity and can easily be produced with slow release pharmaco-dynamics.[17] It has been previously reported how doxycycline may enhance the proliferation and differentiation of osteoblasts, as well as this potential in human bone marrow stem cells.[18,19,20] These functionalized membranes, however, have been scarcely tested both pre-clinically as well as in clinical studies.

It was, therefore, the objective of this preclinical *in vitro* investigation to use doxycycline and dexamethasone as bioactive substances to dope commercialized collagen-based membranes and to study their effect on the ability of osteoblasts to proliferate and differentiate.

2. MATERIALS AND METHODS

2.1. Collagen membranes functionalization

Commercially available natural collagen membranes obtained from bovine purified Achilles tendon type I (Symbios®, Dentsply Sirona GmbH, Konstanz, Germany) were trimmed into 7mm diameter discs and doped with doxycycline or dexamethasone. For this process, aqueous solutions of doxycycline hyclate (Dox) and dexamethasone (Dex) (0.2mg/mL and 0.0125mg/mL, respectively) were prepared and 15µL of each, were added to each membrane disc. Hence, three groups of membrane discs were obtained: 1) Undoped (Col-M), 2) Dox functionalized (Col-Dox-M) and 3) Dex functionalized (Col-Dex-M).

2.2. Doxycycline and dexamethasone liberation

Doxycycline and dexamethasone liberation was evaluated by soaking in PBS (pH 7.4) loaded scaffolds at 37 ºC for 24 h, 48 h, 7 d, 14d and 21 d. Doxycycline and dexamethasone concentration in supernatants were measured by a Waters mass spectrometer with a C18 UPLC column (UPLC Synapt G2 Mass Spectrometer Waters, Waters Corp. Milford, MA, USA). In the case of dexamethasone, values were obtained with an UV-Vis detector at 242-nm wavelength (PDA 200 to 500-nm). Calibration curves were created by plotting attained values against known concentrations. The quantities of released drugs in each well were determined using these calibration curves. The cumulative release rate of doxycycline and dexamethasone (%) were calculated with the following equation: (amount of drug liberated at each time point / total loading amount of drug in scaffolds) x 100%.[21]

2.3. Cell Culture

Culture cells from the human MG63 osteosarcoma cell line (ATCC, Manassas, VA, USA) were obtained in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA). After adding penicillin 100IU/mL (Lab Roger SA, Barcelona, Spain), amphotericin B 2.5mg/mL (Sigma, St. Louis, MO, USA), gentamicin 50mg/mL (Braum Medical SA, Jaen, Spain), 1% glutamine (Sigma), and 2% HEPES (Sigma) supplemented with 10% fetal bovine serum (FBS; Gibco, Paisley, UK), cultures were kept in a humidified atmosphere at 37 ◦C with 95% air and 5% CO_2. Then the cells were detached from the flask using 0.05% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid solution (EDTA; Sigma), and then rinsed and resuspended in culture medium with 10% FBS.[22]

2.4. Cell Proliferation Assay

The obtained osteoblasts were seeded at 1×10^4 cells/mL per well onto the functionalized collagen membranes, within a 24-well plate and cultured in a humid atmosphere of 95% air and 5% CO_2 at 37º C. After 48 h, cell proliferation was assayed by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) test. First, the media was replaced by phenol red-free Dulbecco's Modified Eagle Medium (DMEM) with MTT 0.5mg/mL (Sigma), incubated during 4h and the insoluble crystal deposits of formazan from the MTT cellular reduction were dissolved by adding dimethyl sulfoxide (Merck Biosciences, Darmstadt, Germany), and the resulting absorbance (expressed as

mean absorbance ± standard deviation (SD)) was measured with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland) at 570nm.[23] At least three experiments were conducted for each type of membrane disc.

2.5. Alkaline Phosphatase Activity

Early osteoblast differentiation was indirectly assessed by the alkaline phosphatase (ALP) activity measured with a colorimetric assay (Diagnostic kit 104-LL, Sigma). The colour shift corresponds to the amount of ALP enzyme present in the culture, since the ALP enzyme mediates the conversion of the colourless substrate p-nitrophenyl phosphate to the yellow p-nitrophenol. Standards curves of p nitrophenol (0–250µM) were prepared in parallel using dilutions of a 1000µM stock solution.[18] In brief, cell cultures seeded onto the functionalized membranes within 24-well plate during 72 h, were lysed in 100µL of Triton X-100 and then with 1 M Tris pH 8.00 by ultrasonication for 4 min. Then, the suspension was mixed with a 7.6 mM p-nitrophenylphosphate solution at a proportion of 1:10 and incubated for 15 min at 37ºC. A substrate solution was prepared by merging an aqueous solution of 4mg/mL of 4-nitrophenyl phosphate disodium salt (Sigma) with an equal volume of 1.5 M alkaline buffer (Sigma). The reaction was stopped by adding 1mL 0.05N NaOH, and the final absorbance was measured with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland) at 405nm. The total protein content was estimated by the Bradford method using a protein assay kit from Bio-Rad Laboratories (Bio-Rad Laboratories, Nazareth-Eke, Belgium). All samples were conducted in triplicate.

2.6. Matrix Mineralization Evaluation

Mineralized deposition above the membranes was evaluated using Alizarin Red S method. MG63 cells were seeded at 5×10^4 cells/mL/well in a 24-well plate and cultured in osteogenic medium (DMEM supplemented with 5 mM β-glycerophosphate and 0.05 mM ascorbic acid) on the different membrane prototypes at 37°C in a humified atmosphere (95% air and 5% CO_2). After 15 and 21d of culture, the mineral deposition of the cells was evaluated. Ten percent (w/v) cetylpyridinium chloride was used to stop red calcium deposits for 15 minutes. Then, the absorbance of was measured with a spectrophotometer (BioTek ELx800) at a wavelength of 562nm.[24]

2.7. RNA extraction and real-time polymerase chain reaction (RT-PCR)

Cells messenger RNA (mRNa) was extracted using the Qiagen RNeasy extraction kit (Qiagen Inc., Hilden, Germany) and the mRNA amount measured by UV spectrophotometry at 260nm (Eppendorf AG, Hamburg, Germany). 1μg of mRNA from each group was brought to 40μL of total volume, reverse-transcribed to complementary DNA (cDNA) and amplified with iScriptTM cDNA Synthesis Kit (Bio-Rad laboratories, Hercules, CA, USA) by means of the polymerase chain reaction according to the manufacturer's instructions.[25] Then, the NCBI- nucleotide library and Primer3-design were used to design the primers to detect mRNA of the following genes: runt-related transcription factor 2 (Runx-2), osterix (OSX), alkaline phosphatase (ALP), osteocalcin (OSC), osteoprotegerin (OPG), ligand for RANK (RANKL), type I collagen (Col-I), bone morphogenetic proteins 2 and 7 (BMP-2 and BMP-7), TGF-β1 and TGF-β receptors (TGF-βR1, TGF-βR2, and TGF-βR3) and vascular endothelial growth factor (VEGF). Results were normalized using ubiquitin C (UBC), peptidylprolyl isomerase A (PPIA), and ribosomal protein S13 (RPS13) as housekeeping genes.[26,27] The primer sequences have been included in Table 1.

The RT-qPCR was conducted using the SsoFast^{TM} EvaGreen® Supermix Kit (Bio-Rad laboratories). The obtained cDNA (5 μL per sample) was placed in 96-well microplates and amplified by means of an IQ5-Cycler (Bio-Rad laboratories). The annealing and elongating temperatures were set at 60-65ºC and 72ºC, respectively. Over 40 cycles were performed. The Ct values were plotted against the log cDNa dilution to obtain a standard curve for each of the targeted gene. Then, nonspecific PCR products and primer dimers were ruled out creating a melting profile and carrying out an agarose gel electrophoresis. The results were expressed as the proportion of ng of mRNA per average ng of housekeeping mRNA.[18] The whole process was performed in triplicate.

Gene	**Sense Primer (5'-3')**	**Antisense Primer**
TGFβ1	TGAACCGGCCTTTCCTGCTTCTCATG	GCGGAAGTCAATGTACAGCTGCCGC
TGFβ-R1	ACTGGCAGCTGTCATTGCTGGACCAG	CTGAGCCAGAACCTGACGTTGTCATATCA
TGFβ-R2	GGCTCAACCACCAGGGCATCCAGAT	CTCCCCGAGAGCCTGTCCAGATGCT
TGFβ-R3	ACCGTGATGGGCATTGCGTTTGCA	GTGCTCTGCGTGCTGCCGA TGCTGT
Runx-2	TGGTTAATCTCCGCAGGTCAC	ACTGTGCTGAAGAGGCTGTTTG
VEGF	CCTTGCTGCTCTACCTCCAC	CACACAGGATGGCTTGAAGA
OSX	TGCCTAGAAGCCCTGAGAAA	TTTAACTTGGGGCCTTGAGA
BMP-2	TCGAAATTCCCCGTGACCAG	CCACTTCCACCACGAATCCA
BMP-7	CTGGTCTTTGTCTGCAGTGG	GTACCCCTCAACAAGGCTTC
ALP	CCAACGTGGCTAAGAATGTCATC	TGGGCATTGGTGTTGTACGTC

Col-1	AGAACTGGTACATCAGCAAG	GAGTTTACAGGAAGCAGACA
OSC	CCATGAGAGCCCTCACACTCC	GGTCAGCCAACTCGTCACAGTC
OPG	ATGCAACACAGCACAACATA	GTTGCCGTTTTATCCTCTCT
RANKL	ATACCCTGATGAAAGGAGGA	GGGGCTCAATCTATATCTCG
UBC	TGGGATGCAAATCTTCGTGAAGACCCTGAC	ACCAAGTGCAGAGTGGACTCTTTCTGGATG
PPIA	CCATGGCAAATGCTGGACCCAACACAAATG	TCCTGAGCTACAGAAGGAATGATCTGGTGG
RPS13	GGTGTTGCACAAGTACGTTTTGTGACAGGC	TCATATTTCCAATTGGGAGGGAGGACTCGC

Table 1. Primer sequences for the amplification of osteoblasts′ cDNA by real-time PCR.

2.8. Scanning Electron Microscopy (SEM)

Osteoblasts were seeded at 1×10^4 cells/mL onto the membranes discs and placed in the 24-well plate and then cultured in a humid atmosphere of 95% air and 5% CO_2 at 37ºC for 48 h. Then, 2 membranes of each experimental group were subject to critical point drying and covered with carbon. Cell morphology was evaluated with a scanning electron microscope (SEM) (GEMINI, Carl Zeiss SMT, Oberkochen, Germany).[18]

2.9. Statistical Analysis

Data were expressed as means ± standard deviation (SD) for all measured variables. After testing for the normality of the obtained distribution using Kolmogorov–Smirnov test, comparisons among experimental and control groups were conducted by one-way ANOVA for variables following a normal distribution and Kruskal-Wallis one-way ANOVA on ranks for non-parametric distributions. Then post-hoc Bonferroni test for multiple comparisons was applied. Significance was set at $p<0.05$.

3. RESULTS

3.1. Doxycycline and dexamethasone liberation

Cumulative liberation (%) of dexamethasone and doxycycline are displayed in Figure 1. Dexamethasone was released in two phases: i) a first burst during the initial 24 h (0.13µg/mL), where almost 70% of dexamethasone was released, ii) and a second phase from 48h to 14d with a slow release of dexamethasone. From 7 to 14d the whole amount of dexamethasone was released. For doxycycline, a slow and maintained liberation occurred during the evaluation period. After 24h only a 5% of the loaded amount was

liberated and after 21d, 90% of the loaded doxycycline was still remaining within the membrane.

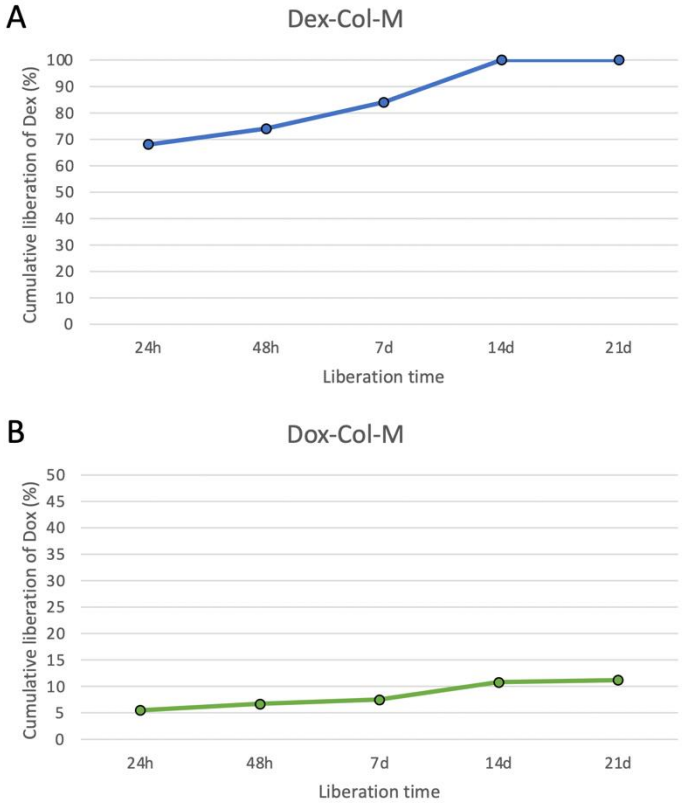

Figure 1. Cumulative liberation (%) of doxycycline and dexamethasone to phosphate buffered saline from experimental loaded collagen membranes measured at different time points.

3.2. Cell Proliferation Assay

The results of the MTT assay are presented in Figure 2. A significantly higher absorbance, indicating higher osteoblastic cell proliferation, was attained in the Dox-Col-M (0.44), when compared with the Dex-Col-M (0.32) and with the control group Col-M (0.23).

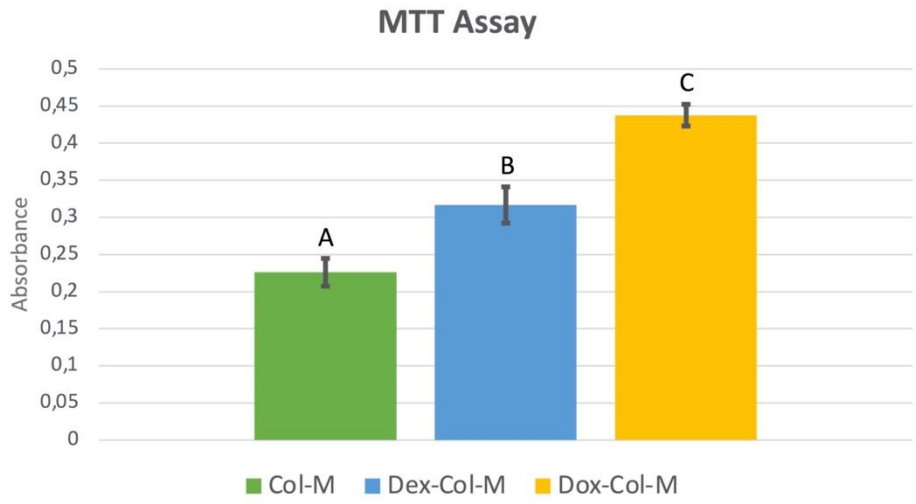

Figure 2. Absorbance mean values and standard deviations obtained after the MTT assay for the different doped membranes. Different letter indicates statistically significant differences between membranes after ANOVA and post-hoc Bonferroni comparisons ($p < 0.05$).

3.3. Alkaline Phosphatase (ALP) Activity

Mean and standard deviations of alkaline phosphatase expressed as international units (IU) of ALP per mg of total proteins are presented in Figure 3. Statistically significant differences were found between Dox-Col-M (0.95IU) and the other two groups (0.11IU for Dex-Col-M and Col-M).

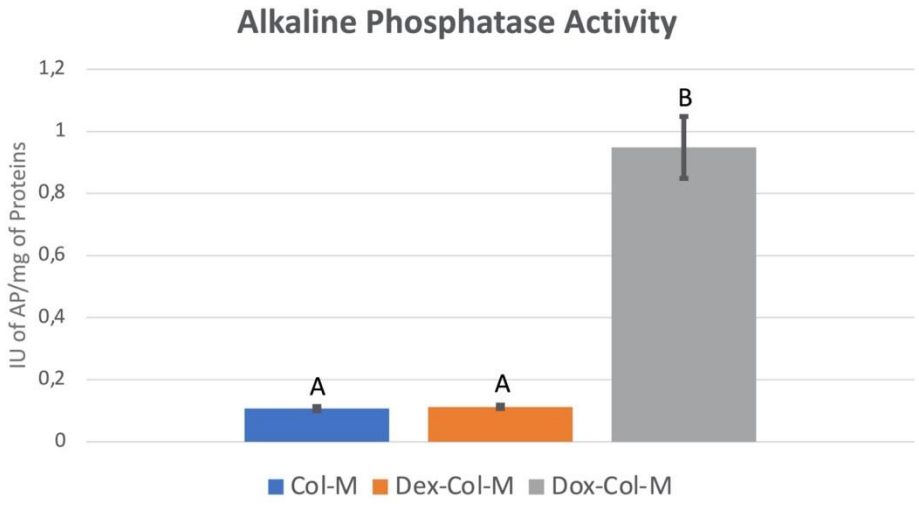

Figure 3. Mean and standard deviation of international units of ALP per mg of proteins values obtained with the different membranes. Distinct letter indicates significant difference between membranes after ANOVA and post-hoc Bonferroni comparisons (p<0.05).

3.4. Mineralization Assay

Means and standard deviations of the attained absorbance for the different membranes at 15 and 21d are displayed in figure 4. After 15d of culture, Dox-Col-M displayed the highest absorbance, when compared to Col-M and Dex-Col-M (p=0.03 and 0.02, respectively), with a mean absorbance of 1.51. These differences were more evident after 21 d of culture, when Dox-Col-M stilled attained the highest mean absorbance (2.76) compared to Col-M (2.06) and Dex-Col-M (1.95). These differences were statistically significant, obtaining p values beneath 0.001 for both comparisons. While after 15 d there were no differences regarding the absorbance of the groups Col-M and Dex-Col-M, after 21 d osteoblasts cultured on Col-M attained a higher mineralization than those cultured on Dex-Col-M (p=0.009).

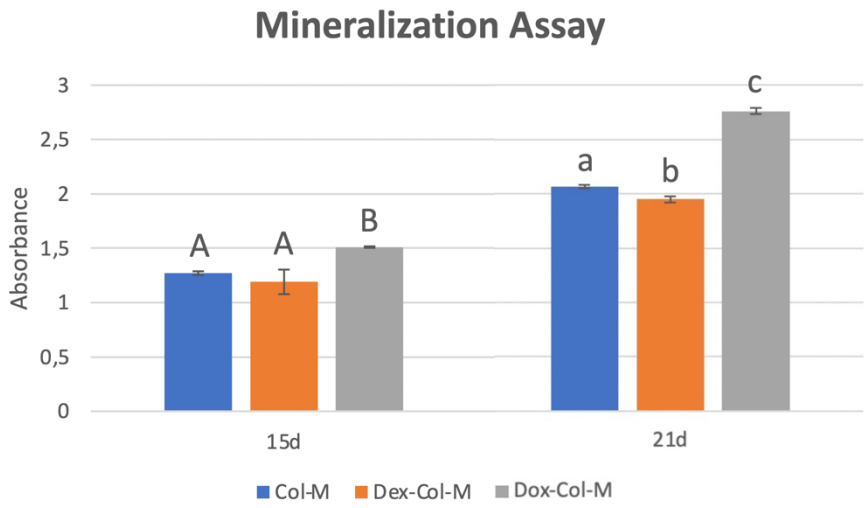

Figure 4. Mean and standard deviation of absorbance obtained with the different membranes using the Alizarin Red S method. ANOVA and post-hoc Bonferroni comparisons (p<0.05) were employed. Distinct capital letter indicates significant difference between membranes in the 15d group and distinct lower case letter indicates significant difference between membranes in the 21d group.

3.5. Real-time Quantitative Polymerase Chain Reaction

Results from the RT-qPCR analysis are displayed in Figure 5. Doxycycline doped membranes caused a significant up-regulation in the expression of osteogenic genes, compared with the other two groups. Specifically, Dox-Col-M significantly up-regulated TGF-β1, TGF-βR1, TGF-βR2, and TGF-βR3 compared to Col-M ($p<0.001$). Dex-Col-M, only demonstrated significant upregulation of TGF-βR3 when compared to Col-M ($p<0.001$).

The expression of ALP and Runx-2 were largely increased by Dox-Col-M (3.3-fold, $p=0.03$ and 3.8-fold increase, $p<0.001$; respectively). Both genes were not altered by Dex-Col-M; in both cases using Col-M as reference. With a similar pattern, OSX and OSC were overexpressed in the presence of Dox-Col-M (2.4-fold increase, $p=0.002$ and 3.3-fold change, $p=0.003$).

In relation to bone morphogenetic proteins, Dox-Col-M produced a positive up-regulation of BMP-2 and BMP-7 (3 and 4.9-fold change, respectively and $p<0.001$ for both comparisons). Both doped membranes, Dox-Col-M and Dex-Col-M showed a marked down-regulation of the expression of RANKL (11.84 and 5.13-fold change respectively, $p<0.001$ in both comparisons).

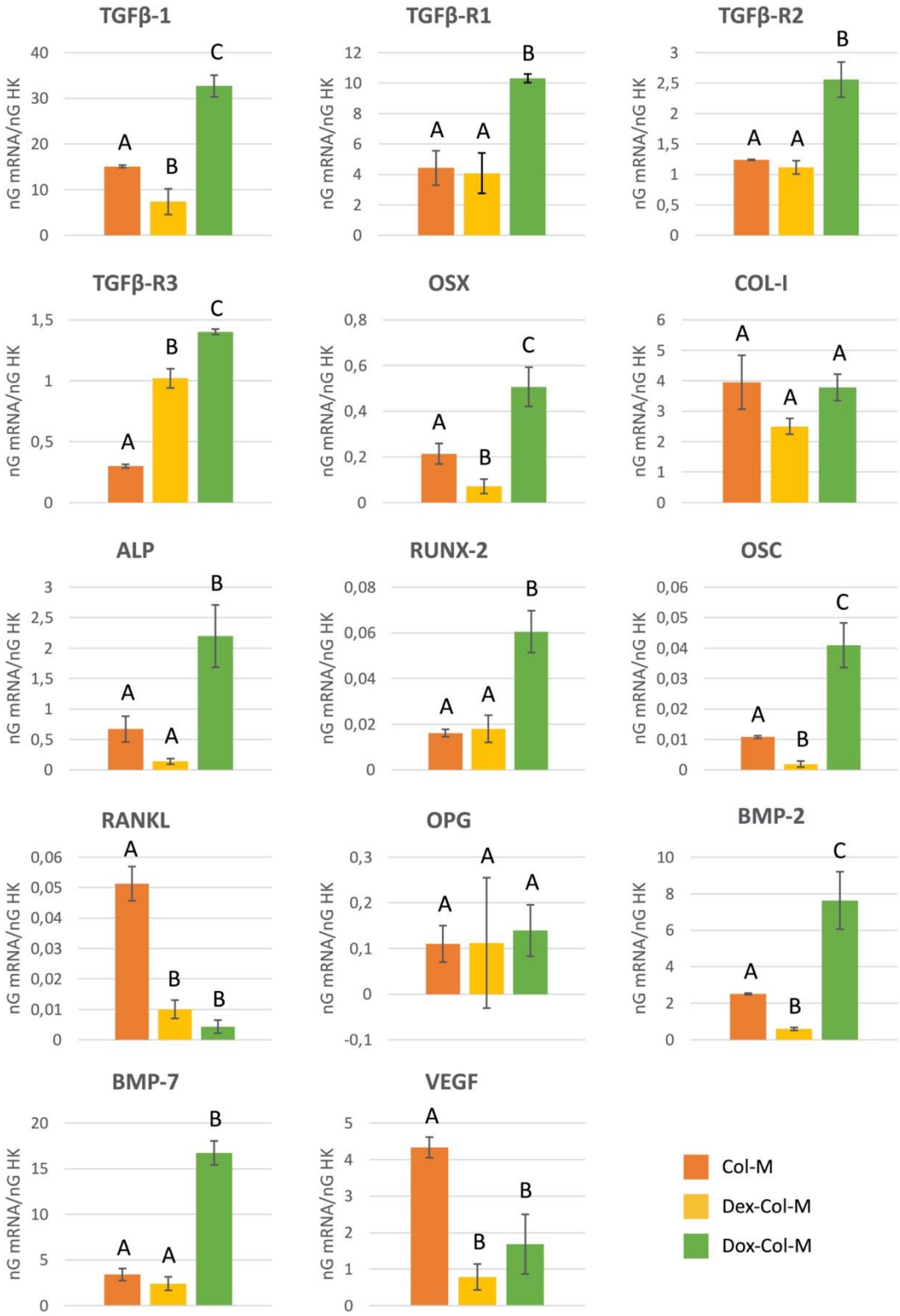

Figure 5. Quantitative real-time PCR gene expression analysis of TGFβ-1, TGFβ-R1, TGFβ-R2, TGFβ-R3, VEGF, BMP2, BMP7, OSC, RANKL, OPG, OSX, Col-I, ALP, Runx-2 established for

cultured osteoblasts seeded on the several experimental membranes, after 48 h. Results were expressed as mean and standard deviation and presented in ng mRNA/ng HK. Different letters indicate significant difference after ANOVA and Bonferroni multiple comparisons ($p \leq 0.05$). Col-M: collagen undoped membranes. Dox-Col-M: doxycycline functionalized collagen membranes. Dex-Col-M: dexamethasone functionalized collagen membranes.

3.6. Scanning Electron Microscopy

Selected SEM images are presented in Figures 6 and 7. In Figure 6, a representative image from each of the three groups is depicted. In the control Col-M group (Figure 6A) a scarce number of cells is present. In the Dex-Col-M group (Figure 6B) a higher number of osteoblasts can be observed on the membranes, while in the Dox-Col-M (Figure 6C), the osteoblasts are even more evident. Figure 7 presents SEM images at higher magnification where rounded osteoblasts emitting cytoplasmatic extensions and forming interconnected clusters of cells can be observed in the control group (Col-M) (Figure 7A, B). In the Dex-Col-M group, the predominant cell morphology is elongated rather than round and cell interconnections and interaction with the substrate can also be encountered (Figure 7C, D). In the Dox-Col-M group, only elongated and spindle-shaped osteoblasts are evidenced. The inter-cellular connections are more apparent and cells grow on different layers, establishing a 3D matrix (Figure 7E, F).

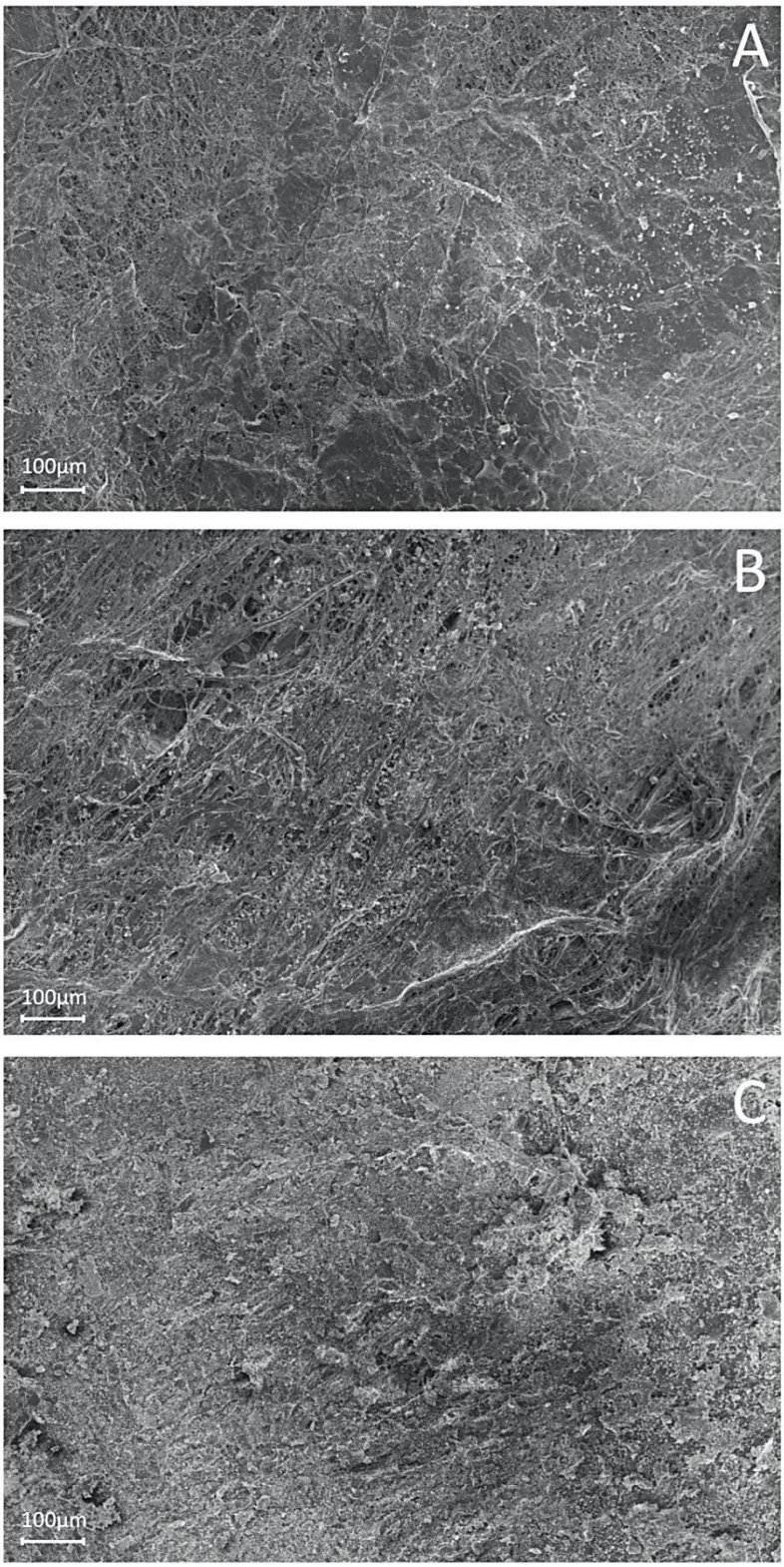

Figure 6. Low magnification (200x) SEM images of the experimental membranes. Osteoblasts are observed on the three images. (A) On Col-M, osteoblasts are flat and no extracellular substance is observable. (B) On Dex-Col-M, osteoblasts are more abundant and some of them are spindle-

shaped, some material secretion is evidenced. (C) On Dox-Col-M, collagen is not even visible as osteoblasts are covering the complete surface. Active extracellular substance can be noticed.

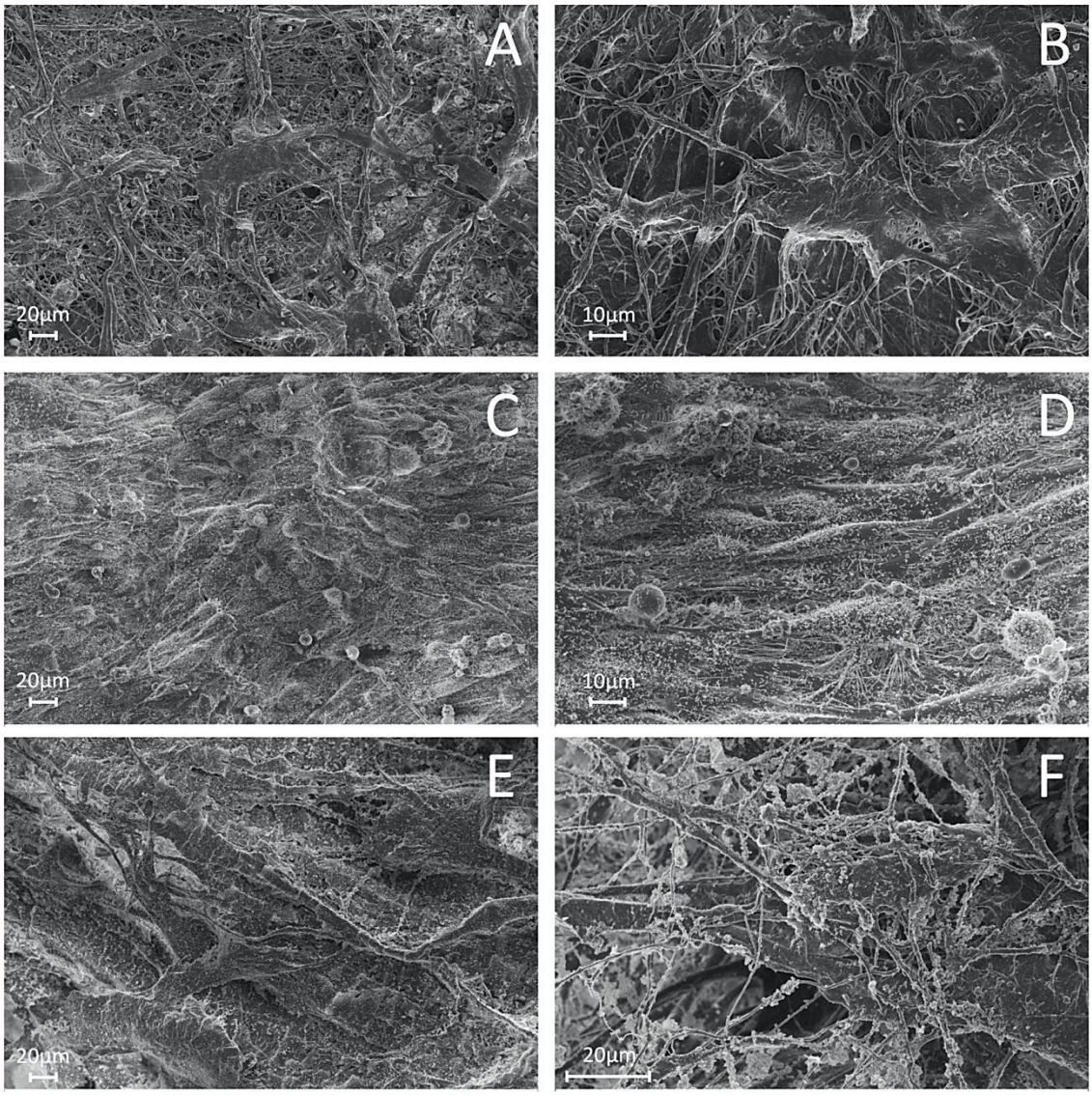

Figure 7. High magnification (600x and 2000x) SEM images of the experimental membranes. (A, B) On Col-M, flat osteoblasts with multiple cytoplasmatic connections are visible. (C, D) On Dex-Col-M osteoblasts are clearly visible, covering the collagen mesh. Many of them are spindle shaped, extracellular substance production is clearly noticed. (E, F) On Dox-Col-M, collagen is not observable. Osteoblasts are fusiform and big in size. They are constituting a three-dimensional layer and have abundant extracellular substance deposits.

4. DISCUSSION

This preclinical *in vitro* investigation aimed to assess the effect of doping natural collagen membranes with doxycycline and dexamethasone on the proliferation and differentiation of cultured osteoblasts. We have used MG-63 osteoblast-like cell model, which together with primary human osteoblasts are the most widely used cell lines to study osteoblast activity.[18,19,28] We selected MG63 osteoblast-like cells, since they share the main characteristics with primary human osteoblasts, but they need shorter isolation time and have unlimited accessibly.[28,29] To study the effect of the different membrane discs on the seeded cells we used different tests to measure their proliferation and differentiation.

Specific drug concentrations need to be determined for each activity and cell type. The dosages used in this investigation were based on previous studies on the in vitro effect of doxycycline and dexamethasone. For doxycycline, a dosage of 1μg/mL (0.2μg of antibiotic added twice a week) was tested for bone marrow cells.[30-31] In the present study, since doxycycline was going to be liberated from collagen in a relatively slow manner, a total amount of 3μg of doxycycline were loaded in each collagen specimen, at the initial stage of the study. In the case of dexamethasone, the loaded amount was 15μL of a $3x10^{-5}$M solution, also based in a previous study where a dexamethasone loaded scaffold was tested using human bone marrow-derived mesenchymal stem cells.[32] It was also considered that concentrations within the range from 1×10^{-7} to 1×10^{-6}M were similar to those of the physiological level of glucocorticoids involved in the regulation of bone remodeling.[33]

It is of interest to highlight the results from the drugs release dynamics, since dexamethasone was released in two phases, with a first burst of release during the initial 24h, what may be due to the dilution of the adsorbed and not trapped molecules onto the collagen fibers.[32] Then, between 48h and 14d, a slow release of the dexamethasone residues within the collagen scaffolds was produced. After 14d, the total amount of loaded dexamethasone was liberated. In contrast, doxycycline maintained a slow-release pattern, what may be speculated it was due to its dependence on collagen degradation.[32]

Proliferation was studied by the MTT assay and by assessing the expression of proliferation-related genes by RT-qPCR (e.g., TGF-β1, TGFβ-R1, TGFβ-R2 or TGFβ-R3). Osteoblasts' differentiation was also quantitatively assayed by ALP activity and by measuring the expression of differentiation-related genes (e.g., ALP, OSC, Runx-2 or

OSX). Finally, we used SEM to indirectly evaluate osteoblasts cell-to-cell interactions and their relation with the substrate, since previous studies have associated changes in osteoblast cell morphology and its proliferation and differentiation rates.[18,28,34,35]

With the MTT assay we measured the ability of the osteoblasts to proliferate by replication (cellular growth rate by quantifying daughter cell population) when cultured on the different disc membranes[28,36] (Figure 2). Since collagen is considered the *gold standard* scaffold for osteoblast proliferation, the present study demonstrated that osteoblasts cultured on the Dox-Col-M and the Dex-Col-M, attained higher mean proliferation values compared to those cultured on the Col-M. In fact, the proliferation of osteoblasts cultured on the Dox-Col-M practically doubled the one in the control group. Previous investigations have also reported the ability of tetracyclines to enhance osteoblast's proliferative capacity.[19,37] This effect was substantiated in the present investigation by the significant up-regulation of the proliferative-related genes demonstrated by RT-qPCR in the Dox-Col-M (Figure 5). Other investigations have also reported the effect of Dox-doped membranes on the expression of TGF-β1 and TGFβ-R1 in cultured osteoblasts.[19] The TGF-β superfamily are a set of proteins that enhance migration, proliferation, and differentiation of different kinds of cells, including osteoblasts[38] and at the same time they enhance matrix production and reduce RANKL synthesis by osteoblasts. It is therefore plausible that the overexpression of these genes indirectly favors bone regeneration by inhibiting osteoclasts activation via RANK[39] band by enhancing osteoblast proliferation and differentiation.[40] However, in the Dex-Col-M, only the TGFβ-R3 genes were up-regulated, while the rest of the osteoblastic proliferation-related genes were under-expressed, compared with the Dox-Col-M group. These results are in agreement with those reported by Walsh *et al.*,[41] who used primary human osteoblasts to evaluate the effect of dexamethasone, although other investigations have also shown up-regulation of osteoblastic proliferation genes when in presence of this glucocorticoid.[42,43] This discrepancy may be due to the use of cells derived from fetal rodents instead of osteoblasts from human origin, which may have marked functional and metabolic differences.[28,41]

We have indirectly assessed the effect of the different membrane discs on osteoblast differentiation by measuring ALP activity. It was demonstrated in this investigation that osteoblasts cultured on the Dox-Col-M had significantly higher ALP production of when compared with the other two groups ($p<0.001$). These results are in agreement with previous studies reporting an increase of ALP production in osteoblasts

in contact with doxycycline.[18,44] Although the underlying pathway remains unclear, it has been suggested that the well-established inhibitory effect of tetracyclines over the matrix metalloproteinases[30,45] may support collagen stabilization and thus osteoblast differentiation. In fact, tetracyclines have shown to increase collagen synthesis.[46] This effect has been corroborated in clinical studies on the effect of low dose doxycycline topical antibiotics, demonstrated significant probing pocket depth reductions when compared to placebo.[47,48]

The effect of Dox-Col-M on the differentiation genes of cultured osteoblasts was also clear demonstrating a significant up-regulation of their expression, compared with the effect of Dex-Col-M and Col-M. Runx-2 (formerly called Cbfa1), a member of the runt homology domain transcription factor family, plays an important role in osteoblast differentiation[49] and together with ALP are the most frequently used markers of early osteoblasts differentiation.[19,49] These results, therefore, corroborate the effect of functionalizing the collagen membranes with doxycycline in the early osteoblastic differentiation stages. The effect of doxycycline on osteoblast differentiation is also supported by the expression patterns of OSC, since OSC is a late marker of differentiation and it is synthesized by mature osteoblasts just before and during matrix mineralization.[50] In the present study OSC was over-expressed in the Dox-Col-M group compared to Dex-Col-M and to Col-M by 21.5 and 3.8 times, respectively ($p<0.001$ in both cases). Since the levels of OSC in the Col-M group and specially, in the Dex-Col-M were very low, it could be argued that the culture time was not long enough to allow for differentiation of mature osteoblasts, since other studies culturing MG-63 osteoblast-like cells for 72h were not able to detect OSC.[50]

The genes encoding for BMP-2 and BMP-7 were also up-regulated by the Dox-Col-M. This is relevant since BMPs plays an important role in osteoblastic differentiation, bone formation/remodeling and overall osteo-induction.[51,52] It has been previously described that BMP-2 may induce the expression of ALP and other osteoblastic markers,[19,53] which is totally in accordance with the results obtained in our study, since the expression of BMPs and ALP followed the same pattern (Figure 5). Since both TGF-β and BMPs genes were up-regulated in the present study, it can be argued that this is due to the activation of Smad or MAPKs cascade, common pathway for both TGF-β and BMPs.[19,54]

One of the main mediators involved in bone homeostasis is RANKL. This marker is a protein synthesized by osteoblasts and its precursors which activates and stimulates

osteoclasts via its membrane-bound protein receptor RANK.[19,39] Thus, the down-regulation of the genes encoding for this protein would result in reduction of osteoclast activation and the subsequent reduction of bone resorption. The down-regulation of this gene was clearly manifested when the osteoblasts were seeded on Dex-Col-M and Dox-Col, compared to Col-M by 5.13 and 11.84 times, respectively (p<0.001 for both comparisons).

It was found that VEGF expression was significantly reduced by Dex-Col-M and Dox-Col-M. It may be due to the inherent anti-inflammatory effect of both dexamethasone and doxycycline,[18,55] leading to a decrease in the production of cytokines and angiogenic factors by the cells.[56]

Using the Alizarin Red Assay, the obtained results by ALP and RT-qPCR were further confirmed. Dox-Col-M, attained higher values of mineralization that Col-M and Dex-Col-M, at both time points. Conversely, Dex-Col-M, after 7d, obtained similar values of mineralization than Col-M and lesser than when the analysis was carried out after 21 d, what could be explained by the dexamethasone release dynamics. The majority of the initially loaded glucocorticoid was liberated between 48h and 7d (Figure 4), thereafter losing its potential effect. However, with doxycycline an 11% of the originally loaded amount was liberated after 21d (Figure 4), thus demonstrating its long-lasting effect on the osteoblastic cells.

The effect of the functionalized membranes on the cultured osteoblasts was also studied morphologically using SEM. Osteoblastic cells were visible on the three groups, although more evident in the Dex-Col-M and even more in the Dox-Col-M group (Figure 6). These results corroborate previous investigations associating cell morphology and the metabolic/differentiation state of osteoblasts.[18,34] Rounded-shape osteoblasts, as the ones observed on Col-M group (Figure 7A, B), have been reduced mitotic activity, thus demonstrating a lower differentiation state and cell activity.[34,35] Conversely, spindle and fusiform-shape cells have been associated with higher proliferation and differentiation states. These morphologies could be clearly identified in the osteoblasts cultured on the membranes doped with Dox and Dex (Figure 7C-F). Furthermore, the osteoblasts grown on the Dox-Col-M group seem to form a three-dimensional cellular network (Figure 7F), which has been associated earlier *in vitro* osteoblast differentiation Schmidt *et al*.,[57] what also corroborates the results from the gene expression analysis, previously reported.

The results from this study, however, should be interpreted with caution due to the preclinical *in vitro* nature of this investigation and the use of MG-63 osteosarcoma

cell line, which despite sharing similar metabolic characteristics with primary osteoblasts and having been widely used in basic research, it is a tumoral cell line that may have alternative patterns of proliferation and differentiation.[28] It should also be remarked that result from cellular in vitro investigation should be extrapolated with cautiousness, since the in vivo wound healing milieu is not present, and this may somehow influence the clinical outcomes. It is however, a relevant investigation since different from previous investigations,[58] we achieved to functionalize commercialized collagen-based membranes with doxycycline and dexamethasone, demonstrating a clear effect on the seeded cultured osteoblasts. The novelty of this investigation resides in the thorough evaluation of the effect of these two substances doped on GBR membranes on osteoblastic cells, including metabolic, differentiation, mineralization and genes expression studies.

Next steps should include *in vitro* antibacterial assays, ideally using a subgingival biofilm model, as the one previously reported by our research group.[59] Furthermore, subsequent preclinical *in vivo* experimental studies are needed before clinical use.[28]

A limitation of the present study is the lack of mechanistic assays. However, although these assays enable the understanding of particular mechanism of action, they are usually unable to depict the complex biological and regulatory processes requiring multiple gene expression and regulation. Nevertheless, new experiments for discovering unknown interactions of doxycycline with target genes and biochemical pathways are needed in future investigations.

In conclusion, this *in vitro* investigation has demonstrated that functionalizing natural collagen GBR membranes with doxycycline significantly enhanced the proliferation and differentiation patterns of cultured osteoblasts, what may open clear possibilities for attaining bioactive GBR membranes, which should be further studied in appropriately designed preclinical *in vivo* and clinical investigations.

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