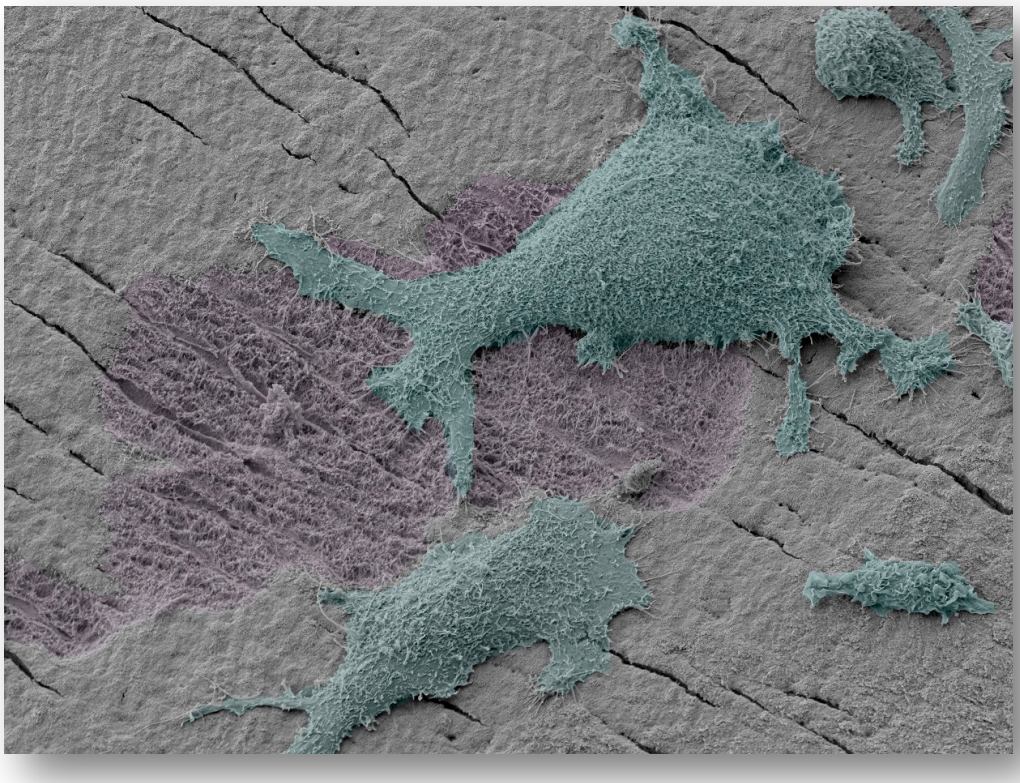
EFECTO DE LOS BISFOSFONATOS SOBRE EL OSTEOBLASTO



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RESUMEN

Los bisfosfonatos (BFs) son una familia de análogos sintéticos del pirofosfato inorgánico en los cuales el oxígeno que une los grupos fosfato es reemplazado por un átomo de carbono. Estos fármacos son ampliamente utilizados para el tratamiento de trastornos óseos, como la osteoporosis, la enfermedad de Paget, hipercalcemia maligna, displasia fibrosa y metástasis óseas producidas por cáncer de mama y próstata. Existen dos tipos principales: BFs no nitrogenados y BFs nitrogenados. Estas dos clases de BFs actúan sobre diferentes dianas intracelulares y tienen diferentes mecanismos de acción a nivel molecular cuya finalidad última es inhibir la reabsorción ósea mediada por osteoclastos.

Los BFs se prescriben frecuentemente por su conocida capacidad para inhibir la formación de osteoclastos. En este sentido su actividad *in vitro* e *in vivo* está bien documentada. Sin embargo, los mecanismos de actuación sobre el hueso y sus efectos directos sobre los osteoblastos no están tan claros.

Aunque son útiles en el tratamiento de algunas patologías óseas, los BFs también han sido asociados con el desarrollo de osteonecrosis mandibular. La fisiopatología de este cuadro clínico no es del todo conocida, aunque se barajan varias teorías, como una disminución en el recambio óseo y la consecuente acumulación de microfracturas, un efecto tóxico sobre los osteoblastos, un efecto antiangiogénico, necrosis avascular, y una reducción en la viabilidad de los fibroblastos y queratinocitos orales.

Los objetivos principales de esta tesis fueron evaluar el efecto de distintas concentraciones de tres BFs nitrogenados (pamidronato, alendronato, e ibandronato) y un BF no nitrogenado (clodronato) sobre el crecimiento, la diferenciación, y el perfil antigénico de los osteoblastos, utilizando para ello la línea celular MG-63.

La revisión de la literatura a cerca del efecto de los BFs sobre el osteoblasto constató que, si bien existe un efecto patente en la estimulación de estas células en estudios *in vitro*,

también existen efectos indirectos sobre la remodelación ósea que no explican el comportamiento de estos fármacos cuando se utilizan *in vivo*.

Cuando estudiamos el efecto de los BFs a bajas concentraciones sobre el osteoblasto encontramos que los nitrogenados aumentaron la proliferación de estas células, modulando la expresión de moléculas co-estimulatorias asociadas a una función inmunológica, y disminuyeron su capacidad de diferenciación. Resultados similares se obtuvieron cuando tratamos estas mismas células con un BF no nitrogenado (clodronato). Sin embargo, cuando tratamos los osteoblastos con altas concentraciones de BFs nitrogenados y no nitrogenados observamos que la proliferación de las células osteoblásticas MG-63 disminuye. Este efecto supresor se mostró, como pudimos comprobar, debido a la detención del ciclo celular y la inducción de apoptosis/necrosis.

Estos hallazgos sugieren que los BFs actúan de distintas formas sobre el osteoblasto, alterando su fisiología y limitando su capacidad reparativa, hecho que podría estar directamente relacionado con el desarrollo de osteonecrosis mandibular por bisfosfonatos (ONB).

ABSTRACT

Bisphosphonates (BPs) are a family of inorganic pyrophosphate synthetic analogues in which the oxygen linking the phosphates has been replaced by carbon. They are widely used to treat bone disorders, including osteoporosis, Paget´s disease, hypercalcemia of malignancy, fibrous dysplasia, and bone metastases of breast and prostate cancer. There are two main types: non-nitrogen-containing BPs and nitrogen-containing BPs. These two types of BPs have different intracellular targets and different action mechanisms with the aim to inhibit osteoclast-mediated bone resorption.

BPs are widely prescribed due to their ability to inhibit osteoclast formation and activity. In this sense, its activity *in vitro* and *in vivo* is well documented. However, the mechanisms underlying their action on bone and their effects on osteoblasts are not fully understood.

Although useful in the treatment of some bone diseases, BPs have also been associated with the development of bisphosphonate-related osteonecrosis of the jaw (BRONJ). The physiopathology of BRONJ in not well known, although various proposals have been made, including: a decrease in bone turnover and subsequent accumulation of microfractures, a toxic effect on osteoblasts, an antiangiogenic effect, producing avascular necrosis, and a reduction in the viability of fibroblasts and oral keratinocytes.

The principal aims of this thesis were to evaluate the effect of different concentrations of three nitrogen-containing BPs (pamidronate, alendronate, and ibandronate) and one non-nitrogen-containing BP (clodronate) on growth, differentiation, and antigenic profile of osteoblasts, using the MG-63 cell line.

The literature review of *in vitro* studies demonstrated a role for BPs in osteoblast stimulation. However, their indirect effects on bone remodeling do not explain the behavior of these drugs *in vivo*.

When we studied the effect of low concentrations of nitrogen-containing bisphosphonates, we observed an increase on MG-63 osteoblast-like cells proliferation, modulating their expression of co-stimulatory molecules associated with immune function, and decreasing their differentiation capacity. Similar results were obtained on osteoblasts treated with a non nitrogen-containing bisphosphonate (clodronate). However, high doses of nitrogen-containing or non-nitrogen-containing BPs reduced the proliferation of MG-63 osteoblast-like cells by arresting cell cycle and inducing apoptosis/necrosis.

These findings suggest that BPs exert their effect on osteoblasts by altering their physiology in different ways, which would explain the disruption of their repair capacity and may be directly related to the development of BRONJ.

I. MARCO TEÓRICO

1. TEJIDO ÓSEO

El tejido óseo es un tejido duro muy complejo con una estructura dinámica que responde ante mediadores fisiológicos, metabolitos, estrés funcional, y ante otros cambios y estímulos medioambientales.[1] Sus funciones principales son proporcionar soporte estructural al organismo y proteger los órganos, a la vez que mantener la homeostasis mineral. Histológicamente, el hueso está formado por un componente celular y una matriz orgánica calcificada, constituida por fibras y por una sustancia fundamental que contiene sales cálcicas, que constituyen la característica principal de este tejido, y le confieren sus diversas funciones.[2]

1.1. Componente celular

1.1.1. Osteoblastos:

El término *osteoblasto* describe una línea de células que cambia considerablemente sus propiedades en los diferentes estadios de desarrollo o diferenciación. Estas células son las responsables de la síntesis y secreción de matriz ósea, o componente orgánico de la matriz extracelular del nuevo hueso, también conocida como matriz osteoide. El linaje osteoblástico también incluye al preosteoblasto, al osteocito, y a la célula *lining* ósea también llamada osteocito de superficie, osteoblasto inactivo, célula lineal endóstica o célula mesenquimal plana.[3,4]

Morfológicamente, estas células muestran un cuerpo en forma de cubo o prisma cuadrangular, del cual parten extensiones citoplasmáticas alargadas. El núcleo es redondo y contiene un nucléolo voluminoso. El citoplasma es rico en ribosomas y mitocondrias, el retículo endoplasmático rugoso es abundante, y el aparato de Golgi se encuentra en posición yuxtanuclear.[5] Estas características son propias de una célula con función eminentemente sintética.

Cuando no se requiere más síntesis de matriz ósea, los osteoblastos pierden esta

capacidad y permanecen como osteoblastos inactivos, células de revestimiento óseo o bien resultan atrapados en la matriz orgánica que posteriormente se calcifica, incluyéndose en el hueso como osteocitos.[2,4] La función de las células de revestimiento es sintetizar y secretar las enzimas que eliminan la capa de osteoide que recubre a la matriz mineralizada. De esta forma, los osteoclastos pueden adherirse al hueso e iniciar la resorción.[6]

1.1.1.1 Origen (ontogenia) de los osteoblastos

Los osteoblastos proceden de células mesenquimales primitivas pluripotenciales, que dan lugar a una progenie con capacidad de diferenciación más limitada, incluso monopotencial. Estas mismas células mesenquimales son capaces de diferenciarse bajo diferentes circunstancias en otras líneas celulares, como los condrocitos o los fibroblastos. Los mediadores que deciden cualquiera de estas vías no son del todo conocidos, aunque está demostrado que los glucocorticoides, la superfamilia del factor de crecimiento transformante beta (TGF-β) y las proteínas morfogenéticas óseas (BMPs) desempeñan una función reguladora en estadios de diferenciación tempranos de algunas líneas celulares.[2]

Algunos investigadores mantienen la hipótesis de que, a partir de un único precursor derivado de células mesenquimales, se originan los osteoblastos, los adipocitos y las células hematopoyéticas secundarias de la médula ósea. Cada una de estas líneas se distingue en base a su morfología y función. En los últimos años este precursor ha sido identificado como células Westen-Baiton, células progenitoras mesenquimales y células fibroblásticas estromales de la médula. [7]

1.1.1.2. Diferenciación

Basándonos en estudios morfológicos e histológicos, las células osteoblásticas muestran una diferenciación secuencial que incluye osteoprogenitores, preosteoblastos, osteoblastos y osteocitos. Los osteoblastos se identifican por su morfología, su localización en el hueso, por su capacidad de sintetizar matriz mineralizada y por su actividad fosfatasa alcalina.

Tanto *in vivo* como *in vitro*, el tejido óseo pasa sucesivamente por tres estadios funcionales: proliferación celular y síntesis de los componentes orgánicos de la matriz ósea; maduración de la matriz ósea (cambios en la composición y organización de la matriz que la hacen competente para ser mineralizada); y depósito mineral.[8,9]

Existen algunos indicadores de los distintos grados de desarrollo y maduración del osteoblasto; así en estudios *in vitro*, se ha comprobado que la síntesis de colágeno tipo I disminuye con la maduración, la actividad fosfatasa alcalina decrece con la mineralización, la osteopontina aparece antes que otras proteínas como la sialoproteína y la osteocalcina.[2]

En otros estudios *in vitro*, parece que las BMPs son potentes agentes inductores de la diferenciación[10,11], así como el fluoruro, la *1-25*(*OH)*2 vitamina D y el ácido retinoico.[12,13]

1.1.1.3. Identificación

1.1.1.3.1. Identificacion morfológica

Las células osteoblásticas, son células grandes que poseen un cuerpo celular cúbico o prismático, con un núcleo redondeado y un nucléolo voluminoso, que con frecuencia se encuentra en el extremo de la célula más distante de la superficie ósea. Su aparato de Golgi está bien desarrollado, y situado entre el núcleo y la base celular. Las mitocondrias son alargadas y bastante numerosas. El citoplasma es azulado, con un retículo endoplásmico rugoso muy desarrollado, característico de las células que llevan a cabo una intensa síntesis de proteínas. En ocasiones se observan gotitas lipídicas y cuerpos densos limitados por membrana que al parecer son lisosomas. Dado que estas células son las responsables de la formación del tejido óseo, se ubican en el frente de avance del hueso que crece o desarrolla; disponiéndose en una capa epiteloide de células cuboides conectadas con otras, a través de expansiones finas y cortas. [14]

1.1.1.3.2. Identificación bioquímica

Los marcadores de recambio óseo son compuestos químicos cuya presencia puede ser detectada en el suero, plasma, u orina, y que idealmente reflejan el recambio óseo, es decir, la resorción, la formación o una combinación de ambas.

Estos compuestos pueden reflejar la presencia de matriz mineralizada (hidroxiapatita, es decir, calcio y fosfato), de matriz no mineralizada (colágeno, osteocalcina [OSC], metaloproteinasas de la matriz, osteopontina, osteonectina, etc.), y de células (osteoclastos, osteoblastos, y osteocitos).

Algunos compuestos pueden tener varios papeles (la OSC es una parte de la matriz no mineralizada además de tener propiedades hormonales, y la fosfatasa alcalina es una enzima que inicializa la mineralización y además un marcador de la función osteoblástica).[15]

La osteocalcina o proteína ósea Gla, es sintetizada específicamente por los osteoblastos[16] y representa alrededor del 15% de las proteínas no colagénicas de la matriz osteoide. Su molécula (49 aminoácidos) contiene tres restos de ácido gamma-carboxiglutámico y un aminoácido que une iones calcio, entre sus dos grupos carboxilo. Como otras proteínas que poseen este aminoácido, su síntesis depende de la vitamina K.[17,18]

En los humanos, el gen de la osteocalcina, está localizado en el cromosoma 1 y está regulado a nivel transcripcional por la 1,25-(OH)2 vitamina D3.[19] Es un marcador específico y un indicador de la fase final de la diferenciación de los osteoblastos. Esta proteína juega un papel importante en el mantenimiento de la calcificación ósea y en la inhibición de la formación del cartílago.[20,21]

La osteopontina, es sintetizada por osteoblastos pero también puede estar presente en otros tejidos conectivos. Es una proteína de 317 aminoácidos y con un 20% de su peso molecular correspondiente al ácido siálico. Tiene la capacidad de combinarse con la hidroxiapatita ($Ca_{10}(PO_4)_6(OH)_2$) y también de facilitar el anclaje de las células óseas a la matriz mineralizada.[22]

El marcador bioquímico más utilizado para el reconocimiento del fenotipo osteoblástico es la actividad fosfatasa alcalina (ALP). Esta enzima libera fosfato inorgánico a partir de ésteres fosfóricos, con pH óptimo de acción de 8,6.[23]

El análisis de esta enzima por técnicas inmunocitoquímicas se utiliza para identificar poblaciones de osteoblastos en el hueso humano en desarrollo, así como los preosteoblastos del tejido conectivo mesenquimal y del estroma medular.[24]

Otra característica que nos permite la identificación bioquímica del osteoblasto es la síntesis y secreción de colágeno. Según algunos estudios realizados en osteoblastos de calvaria fetal de ratas[5,25], el colágeno tipo I, que constituye el 90% de la matriz ósea orgánica, es sintetizado por los osteoblastos durante los últimos periodos de proliferación y los primeros periodos de maduración de la matriz.[26]

1.1.1.3.3. Identificación antigénica

La caracterización del perfil antigénico de los osteoblastos es relativamente reciente. Estas células poseen un perfil antigénico propio, y en parte coincidente con el perfil que expresan otras células que también se originan en la médula ósea.

Nuestro grupo de investigación puede ser considerado pionero en el estudio del perfil antigénico de las células osteoblásticas. Sirvan como ejemplo las numerosas publicaciones sobre este tema.[27–29,29–32]

El antígeno CD10 también es conocido como antígeno de la leucemia linfoblástica aguda (cALLa); es una glicoproteína con actividad endopeptidasa y está presente en la superficie de algunos tipos celulares: linfocitos pre-B, fibroblastos, “stem cells”, células epiteliales renales y células deciduales estromales, entre otras.[33,34] Se ha descrito la expresión del antígeno CD10 en determinadas poblaciones de osteoblastos humanos, tanto en los aislados en primo cultivo a partir de explantes óseos como en los presentes en cortes de tejido óseo, así como en osteoblastos humanos transformados (línea de osteosarcoma humano MG-63).[27,29,31,34] Esto sugiere que la expresión de este antígeno puede ser una

propiedad fenotípica de las poblaciones de osteoblastos y considerarse un nuevo marcador del fenotipo osteoblástico, para utilizarse como dato complementario en la caracterización de esta población celular.

El antígeno CD44 es una molécula de adhesión funcional expresada en osteoblastos.[35] Pertenece a la familia de las glicoproteínas transmembrana codificada por un solo gen, que tienen capacidad de unirse al ácido hialurónico, y por tanto, son consideradas como receptores del mismo. Se encuentra presente en distintos tipos celulares: leucocitos, fibroblastos, células epiteliales y los propios osteoblastos.[36] Se discrepa sobre el estadio de la diferenciación osteoblástica en la que este antígeno es expresado. Unos autores describen su expresión sólo en osteocitos, mientras que otros indican ya su presencia en osteoblastos.[2,28] Su función principal es el mantenimiento de la homeostasis de los tejidos a través de la conexión de distintos componentes de la matriz, como el ácido hialurónico, el condroitín sulfato y la osteopontina, con la superficie celular. También se ha descrito que el CD44 funciona como inhibidor de la pérdida ósea causada por procesos inflamatorios.[37]

Las moléculas CD54, CD80, CD86 y HLA-DR ejercen algunas de las funciones ya descritas para el CD44 y también se detectan en osteoblastos. Están implicadas en los mecanismos de presentación antigénica y en la activación de células T. Para llevar a cabo esta función, las células presentadoras de antígenos desarrollan una primera señal que estimula a las células T, a través del antígeno de clase II HLA-DR, y una segunda señal coestimulante que depende de los antígenos CD80 y CD86. Otras moléculas, como el CD44 y el CD54 complementan esta señal estimuladora. La detección de todos estos antígenos, algunos en la superficie y otros de forma citoplasmática en los osteoblastos han hecho postular a diferentes grupos de investigación que los osteoblastos pueden tener funciones típicamente inmunológicas, como la presentación de antígenos o la fagocitosis en el tejido óseo.[38–40]

Otros antígenos típicamente expresados en células hematopoyéticas han sido

investigados en los osteoblastos humanos en cultivo. Como ejemplo podemos observar la expresión negativa del CD45. Sin embargo, el anticuerpo CD34, que se asocia con un origen hematopoyético y está presente en células endoteliales y células hematopoyéticas inmaduras, es positivo en los osteoblastos humanos en cultivo. En cuanto a la expresión de antígenos correspondientes a las células del linaje B, los osteoblastos expresan CD20 y CD23 y no así CD19; mientras que el antígeno de células T (CD3) no se detecta. CD56, un antígeno propio de células NK, se detecta en una amplia proporción de células. Los antígenos del linaje mielomonocítico: CD11b, CD13, CD16 y CD36 son positivos y no así el CD14, CD15 y CD33. También se expresan antígenos como CD25 y CD38. El CD68 no se detecta en los osteoblastos en cultivo. Finalmente cabe señalar la expresión positiva, en el 100% de las células, de antígenos específicos de las células foliculares dendríticas (FDC y DRC-1).[27,28]

Intracitoplasmáticamente, nuestro grupo también ha descrito la presencia de IL-4, IL-12, IL-15, IL-18 e IFN-γ en osteoblastos en cultivo, y ha comprobado que su expresión puede verse modulada por distintas citoquinas y factores de crecimiento.[32]

1.1.1.3.4. Identificación genética

Hasta hace cierto tiempo, se conocía un sólo gen específico de los osteoblastos, asociado con el proceso de diferenciación de los mismos: el gen ligado a la expresión de la osteocalcina.[16] Sin embargo, estudios más recientes en biología molecular, desarrollo biológico y genética humana y animal han identificado al gen *Cbfa (RUNX2) ,* que se describe como un factor de transcripción específico de osteoblastos, así como una llave reguladora de su diferenciación.[41,42] Se expresa en los osteoblastos diferenciados y modula la expresión de osteocalcina, el último gen que se expresa durante la diferenciación osteoblástica. La expresión de *Cbfa1* es regulada por distintos factores, como las BMPs e incluso el propio gen.[43] También se ha demostrado que la proteína que codifica este gen está implicada en los procesos de interacción epitelio-mesénquima de la morfogénesis dental.[44]

1.1.1.4. Papel funcional del osteoblasto

La función más conocida de los osteoblastos es la síntesis de los componentes de la matriz ósea y el control de la actividad de resorción de los osteoclastos mediante la liberación del ligando del receptor activador nuclear Kappa-B (RANKL) y Osteoprotegerina (OPG).[4,6]

También se ha demostrado que estas células poseen funciones asociadas al sistema inmunológico, como la capacidad fagocítica, la estimulación de los linfocitos T o la síntesis de citoquinas[32,38,40,45], lo que sugiere que durante la diferenciación celular osteoblástica, una subpoblación de las células puede verse involucrada en funciones inmunes, tales como la fagocitosis y la activación de células T. Como propusieron Heinemann y cols. en el 2000[46], la situación *in vitro* tal vez refleje la situación *in vivo*. Es posible que cuando los osteoblastos están en contacto con microorganismos o bien materiales endoprotésicos puedan, en un momento dado, desarrollar funciones inmunológicas para eliminarlos. Esta actividad puede conllevar cambios morfológicos, antigénicos y bioquímicos en dichas células. En algunas situaciones en las que se liberan citoquinas, como los procesos inflamatorios, esta función puede ser esencial, ya que dichas citoquinas son moléculas que pueden modular y acentuar el fenotipo antigénico de los osteoblastos. En este caso el rol de dichas células pasaría a ser puramente inmunológico, y esto podría detener la secuencia de diferenciación y maduración, en detrimento de la formación ósea. Este proceso ha sido propuesto por algunos autores como un mecanismo de emergencia y no como un proceso fisiológico.[40]

1.1.2. Osteocitos

Los osteocitos son las células encargadas de mantener la integridad estructural de la matriz mineralizada. Éstos son el resultado de la diferenciación de los osteoblastos dentro del tejido óseo ya formado, y constituyen más del 90% de las células del hueso humano maduro. Estas células poseen una morfología estrellada, con un núcleo alargado, y un citoplasma con menor número de organelas que los osteoblastos, ya que su actividad metabólica es escasa.[6,47] También se caracterizan por emitir múltiples prolongaciones citoplasmáticas que,

a través de una red de conductos en la matriz mineralizada les permiten conectar con otros osteocitos. Cuando hacen contacto, los osteocitos forman una red viviente dentro del hueso que puede intercambiar nutrientes y residuos. También pueden comunicar cuáles de las partes del hueso están sometidas a estrés y necesitan reforzarse, además de determinar cuáles son las partes que pueden dejar deteriorarse. Este equilibrio metabólico, con los cambios electrolíticos que conlleva les hace ser responsables de mantener la homeostasis del calcio.[6,48]

1.1.3. Osteoclastos

Son las células más voluminosas del tejido óseo. Son células multinucleadas cuya función principal es la de reabsorber el tejido óseo.[49]

A diferencia de los osteoblastos, los osteoclastos se originan en las células precursoras hematopoyéticas. Por eso los factores de transcripción y de crecimiento que los modulan son comunes a estas. Por ejemplo, el RANKL, que es un miembro de la familia de los ligandos y receptores del factor de necrosis tumoral (TNF). Sabemos que el remodelado óseo depende del equilibrio entre la formación y la reabsorción óseas, que siguen un ciclo (*turnover*) contínuo. La reabsorción ósea se desencadena y se regula a través de un sistema consistente en RANK y su ligando (RANKL).

Los osteoclastos se encuentran situados en la superficie ósea en la que comienza el proceso de reabsorción. Para poder llevar a cabo este proceso, los osteoclastos se unen a la superficie del hueso acidificando el medio mediante la secreción de enzimas y protones, rompiendo así, los enlaces existentes entre la hidroxiapatita y el colágeno.[47,50]

De su morfología, se puede destacar que poseen varios nucléolos, y que el citoplasma presenta abundantes microvellosidades, mitocondrias, lisosomas, vesículas y vacuolas.[51]

1.2. Matriz ósea

La matriz ósea, dónde se ubican las células, consta de una matriz orgánica impregnada en

sales minerales. Está formada por colágeno, principalmente tipo I, en un 90%, y por proteínas no colagénicas en un 10%. La dureza y la resistencia a la compresión del tejido óseo se deben a las sales minerales, mientras que la elasticidad y resistencia a la tracción se deben al colágeno.[6]

1.2.1. Matriz orgánica

1.2.1.1. Colágeno

Es una proteína de tres cadenas polipeptídicas, y de unos 1000 aminoácidos que se enrollan entre sí de forma helicoidal, y se unen a través de puentes de hidrógeno. Cada molécula, se alinea paralelamente con la siguiente, y así, forman una fibrilla de colágeno, que posteriormente se agruparán dando lugar a las fibras de colágeno.[52,53]

1.2.1.2. Sustancia fundamental

Las proteínas no colagénicas constituyen entre un 10 y un 15% de las proteínas óseas. Algunas de estas proteínas son de origen exógeno, y otras son sintetizadas por el propio osteoblasto.[54] Estas proteínas son principalmente de cuatro tipos diferentes: proteínas de adhesión celular, proteoglicanos, proteínas gamma-carboxiladas y factores de crecimiento.

Las proteínas de adhesión sintetizadas por las células son la fibronectina, la trombospondina, la osteopontina y la sialoproteína ósea. Los proteoglicanos son macromoléculas que contienen cadenas de polisacáridos ácidos (glicosaminoglicanos) adheridas a una proteína nuclear central. En el hueso encontramos dos tipos de glicosaminoglicanos: el condroitín sulfato y el heparín sulfato.[54,55]

La OSC o proteína ósea Gla, y la MGP son proteínas gamma-carboxiladas. La OSC es una proteína que se ubica en el hueso principalmente, aunque también se encuentra en la dentina, mientras que la MGP se halla en el cartílago y en el hueso.[16,17]

Existen otras proteínas fosforiladas como la osteonectina, o sialoproteínas como la osteopontina.[2]

Algunos factores de crecimiento, como el factor de crecimiento derivado de la insulina (IGF) y el factor de crecimiento transformante beta (TGF-β1), son secretados por los osteoblastos, y pueden estimular el crecimiento de las células osteoblásticas de forma autocrina o paracrina.[56]

1.2.2. Sales minerales

Las sales minerales presentes en el tejido óseo son principalmente carbonato y fosfato cálcico, que se encuentran en forma de fosfato cálcico amorfo y de cristales de hidroxiapatita ($Ca_{10}(PO_4)_6(OH)_2$).

Junto al fosfato cálcico, están presentes numerosos iones, como el magnesio, el potasio y el sodio, entre otros.[6]

1.3. Formación del tejido óseo

El tejido óseo puede formarse de dos maneras diferentes. La primera es la osificación intramembranosa o endoconjuntiva, en la cual las células mesenquimatosas indiferenciadas se transforman en osteoblastos en el seno del tejido conjuntivo. Cuando ya se ha constituido una pequeña zona de tejido óseo, otras células se diferencian en osteoblastos y se disponen alrededor formando una hilera osteoide, y así sucesivamente.

Los huesos de la bóveda craneal, la maxila y la mayor parte de la mandíbula se forman mediante este proceso.[2,6]

El segundo tipo de formación del tejido óseo es la formación endocondral, en la que la formación ósea es similar al proceso anterior, pero algo más complejo, ya que el tejido mesenquimal pasa por un estadio intermedio de cartílago antes de ser reemplazado por tejido óseo.[6]

Independientemente del tipo de formación del tejido óseo, todo se podría resumir en cuatro fases básicas:

- Diferenciación de los osteoblastos a partir de células madre.
- Secreción de la matriz orgánica por parte de los osteoblastos.
- Mineralización de la matriz orgánica.
- Aparición de los osteoclastos para iniciar el proceso de reabsorción.

1.4. Modelado y remodelación ósea

El modelado es una actividad organizada del tejido óseo que permite el crecimiento dimensional del hueso y regula la resistencia de éste, mediante la actividad de los osteoblastos y los osteoclastos situados estratégicamente.[47,51]

La remodelación es un proceso continuo y secuencial de degradación y reparación del hueso, que da lugar a un cambio estructural de la arquitectura ósea. Para que esto funcione correctamente debe haber un equilibrio entre la formación ósea y la resorción. En este proceso participan tanto osteoblastos como osteoclastos.[57,58] Parece ser que la destrucción de la matriz ósea mineralizada se produce por la activación de una bomba de protones que existe en el interior de los osteoclastos, que funciona liberando HCl al exterior y disolviendo la estructura ósea. Posteriormente, y unido a la liberación del Ca^{2+} al medio extracelular se produce una liberación de somatomedinas, TGF-β1 y BMPs que inducen la migración de células mesenquimales para producir osteoblastos y nueva formación ósea.[59]

Este proceso de remodelado sugiere que existe una conexión estrecha entre los osteoblastos y los osteoclastos, aunque la base bioquímica de esta interrelación no está totalmente establecida.

El hueso alveolar es una parte especializada del hueso maxilar y mandibular que constituye la estructura de soporte primario para los dientes. Aunque básicamente es comparable a otros huesos del organismo, y la estructura celular y mineral de este tejido es similar, el hueso alveolar está sujeto a una alta tasa de cambios y remodelado debido a las demandas funcionales de la masticación. Esta elevada tasa de remodelación es esencial para la adaptabilidad del hueso alveolar.[60]

El mecanismo de aposición-reabsorción ósea está regulado por el complejo RANKL-OPG. El RANKL sintetizado por los osteoblastos, se une a un receptor de membrana de los osteoclastos, denominado RANK, produciendo un estímulo de la diferenciación osteoclástica, favoreciendo como consecuencia la resorción ósea. La actividad biológica de este factor, está regulada por la OPG, receptor soluble de la familia TNF. La OPG es sintetizada también por los osteoblastos e inhibe la diferenciación osteoclástica al unirse al RANKL.[61,62]

El hecho de que los osteoblastos sinteticen OPG y RANKL nos sugiere la existencia de un bucle de retroalimentación negativo en el que el osteoblasto podría modular, por sí mismo, la diferenciación de precursores osteoclásticos al presentar moléculas RANKL en su superficie celular, cuya actividad sería regulada, a su vez, por la producción de OPG.[62]

Desajustes en el equilibrio síntesis-reabsorción pueden dar lugar a una disminución de la masa ósea, tal y como ocurre en ciertas enfermedades como la osteoporosis. Los agentes promotores de la resorción actúan incrementando la formación de osteoclastos y activando a los ya existentes. Actualmente se considera que en este proceso están implicados, además de factores hormonales, distintos tipos celulares.[63–65]

Algunos estudios sugieren que los osteoblastos se ven sometidos a procesos de muerte celular o apoptosis en los lugares de remodelación. Hay numerosos signos en los lugares de resorción que son consecuencia de la inhibición de la actividad osteoclástica, como el aumento de los niveles de calcio[66] y de fosfato.[67]

De la misma forma, hay muchos mecanismos implicados en la atracción de precursores de osteoblastos a los lugares de resorción, como son algunos constituyentes de la matriz ósea, entre los que se encuentran fragmentos de colágeno, OSC o factores de crecimiento.[12]

2. BISFOSFONATOS

Los bisfosfonatos (BFs) son potentes inhibidores de la actividad osteoclástica, por lo que son ampliamente utilizados en clínica para prevenir la pérdida ósea asociada a ciertas condiciones como la osteoporosis, la enfermedad de Paget, mieloma múltiple y las enfermedades óseas metastásicas.[68]

Se trata de compuestos sintéticos que tienen una estructura química similar al pirofosfato inorgánico (P-O-P), regulador endógeno del metabolismo óseo. Contienen dos grupos fosfato unidos mediante enlaces fosfo-éster, en los cuales el átomo central de oxígeno presente en el pirofosfato inorgánico es reemplazado por un átomo de carbono (estructura P-C-P), lo que los hace más resistentes a la ruptura por las pirofosfatasas y a la hidrólisis.[69,70] La estructura general de los BFs es bastante fácil de modificar, por lo que las diferentes generaciones de BFs varían mucho dependiendo de sus características biológicas, terapéuticas y toxicológicas.[71]

Hay dos grandes grupos de BFs que se diferencian por su acción a nivel molecular. El primer grupo comprende los BFs no nitrogenados, como el clodronato, el etidronato y el tiludronato. Este tipo de BFs son incorporados, metabolizados y transformados en análogos del ATP no hidrolizables. Esta acumulación de metabolitos da lugar a la inhibición del metabolismo celular y provoca la muerte de los osteoclastos[72], probablemente mediante la inhibición de enzimas ATP-dependientes, como la adenina nucleótido translocasa (ANT), esencial en la función mitocondrial.[73]

El segundo grupo lo componen los BFs nitrogenados o aminobisfosfonatos como el zoledronato, alendronato, pamidronato y risedronato, siendo estos más potentes que los anteriores. Este grupo de BFs reduce la actividad de los osteoclastos inhibiendo a un importante enzima de la vía del mevalonato, la farnesil difosfato sintetasa, y bloqueando la prenilación protéica de pequeñas GTPasas como Ras, Rac, y Cdc42.[74,75]

Los BFs presentan tres propiedades clave: 1), la fuerte unión al hueso debido a la alta afinidad por la hidroxiapatita[68,76], 2), la habilidad para inhibir la función de los osteoclastos[74], e indirectamente, 3), la estimulación de los osteoblastos para producir inhibidores de la formación de los osteoclastos.[77]

Estos fármacos se caracterizan por una baja absorción intestinal, y son excretados por vía renal. Su vida media en el torrente sanguíneo es muy corta y oscila entre 30 minutos y 2 horas[78], pero una vez absorbidos por el tejido óseo, estos fármacos pueden persistir durante más de 10 años en los tejidos esqueléticos, dependiendo del tiempo de recambio óseo.[79–81]

Como se ha descrito anteriormente, la capacidad de los BFs para inhibir la formación de los osteoclastos y su actividad *in vitro* está bien documentada.[82,83] Sin embargo, sus efectos sobre la diferenciación osteoblástica y sobre su función no son del todo conocidos.

Ciertos estudios han indicado que los BFs podrían actuar a través de los osteoblastos para inhibir la reabsorción ósea, estimulando la liberación de ciertos factores inhibidores de la actividad de los osteoclastos como la osteoprotegerina.[84]

Algunos estudios *in vivo* han indicado que el tratamiento con BFs no tiene efectos adversos sobre la diferenciación osteoblástica o sobre la formación ósea. En este sentido, Recker y cols en 2008[85] demostraron que los pacientes que recibían infusiones tempranas de zoledronato mantenían las funciones osteoblásticas normales; así como, la mineralización de la matriz ósea, a pesar de verse reducido el remodelado óseo. Sin embargo, otros autores han mostrado efectos inhibitorios de los BFs sobre la función de los osteoblastos y sobre la aposición mineral *in vivo*.[86,87]

Los datos *in vitro* disponibles son también contradictorios, e incluyen estudios experimentales muy diferentes, utilizando células madres mesenquimales, osteoblastos primarios humanos, líneas celulares de osteosarcoma (MG-63 y SaoS-2) y fibroblastos. Así, concentraciones micromolares de zoledronato han mostrado un descenso en la proliferación,

mineralización, viabilidad y actividad fosfatasa alcalina en células de calvaria de rata MC3T3-E1 y en células de osteosarcoma humano MG-63.[88–90]

Pan y cols. en 2004 identificaron que el tratamiento de células osteoblásticas con altas concentraciones de zoledronato (5-25 micromolar) aumenta el depósito mineral, si bien se acompaña de una reducción del número de células como consecuencia de un efecto citotóxico.[91] Otros estudios concluyen que concentraciones nanomolares de zoledronato pueden promover la proliferación y la diferenciación de células madres mesenquimales.[92] Además, en cultivos murinos de médula ósea, la formación de nódulos mineralizados se ha visto estimulada por concentraciones nanomolares de etidronato y alendronato, pero inhibida por concentraciones micromolares de los mismos fármacos.[93]

3. OSTEONECROSIS MAXILAR POR BISFOSFONATOS

Los BFs han sido asociados con algunas reacciones adversas, como problemas gastrointestinales, fracturas atípicas de huesos largos, fatiga, anemia, disnea, nauseas y problemas renales entre otros, pero sin duda, la patología más grave con la que se asocian es con el desarrollo de osteonecrosis maxilar.[94,95]

En los años 2003 y 2004 se describieron los primeros casos de lesiones óseas necróticas expuestas en el hueso maxilar asociadas al uso de BFs.[96,97] Todos los casos que se reportaron ocurrieron en pacientes que estaban siendo tratados con BFs nitrogenados del tipo zoledronato y pamidronato para el control de la hipercalcemia provocada por mieloma múltiple o por cáncer de mama y próstata. El 78% de los casos se originó tras la realización de una exodoncia dental, y solo el 22% de los casos apareció de forma espontanea. Actualmente se han descrito multitud de casos en relación con otros tipos de BFs, incluso en pacientes tratados con estos para otras patologías como la osteoporosis y la enfermedad de Paget. [18,95,98]

La ONB se describe como un área de hueso expuesto en la región maxilofacial que no cicatriza entre 6 y 8 semanas después de su identificación , que aparece en pacientes que están recibiendo o han estado expuestos a BFs y que no han recibido radioterapia en la región craneofacial.[99,100]

Actualmente, la historia natural y la fisiopatología de la ONB no están claras. En la mayoría de los casos, el cuadro clínico ha sido identificado en pacientes oncológicos que han recibido altas dosis de BFs con quimioterapia concomitante, y que frecuentemente están inmunodeprimidos. La sobre supresión del recambio óseo que da lugar al secuestro de los BFs en la mandíbula, unida a una vascularización comprometida parecen ser los eventos que inician el desarrollo de las lesiones. Además, la inhibición de la cicatrización, el trauma operatorio, la anatomía específica de la cavidad oral, la presencia de una micloflora muy diversa, y la puesta en marcha de procesos inflamatorios han sido propuestos como factores potenciales que pueden contribuir al desarrollo del proceso.[101–107]

Las diferencias en la fisiología ósea de los diferentes lugares del esqueleto pueden estar implicadas en el desarrollo de la ONB. Los huesos craneofaciales provienen de los huesos largos en la línea de desarrollo, que surgen de la cresta neural, y su mecanismo de osificación es intramembranoso. Las diferencias entre la composición de la matriz, la expresión de proteínas morfogenéticas óseas, la degradación osteoclástica y otros factores sugieren diferencias funcionales en el remodelado y en las propiedades mecánicas de los huesos según su localización.[108–111]

Los osteoblastos que se convierten en osteocitos poseen una vida media de 180 días. Estos osteocitos no son meras células quiescentes, sino que actúan como mecanorreceptores de presión/tensión, regulando la reabsorción y la remodelación ósea mediante la liberación de osteoprotegerina. Debido a las altas presiones y tensiones a las que está sometida la parte posterior del hueso mandibular y la lámina dura esta regulación/remodelación ósea debería ser continua y adaptada a las fuerzas de la oclusión. Los BFs inhiben la reabsorción ósea por

parte de los osteoclastos, por tanto, transcurridos los 180 días de vida media de los osteocitos, éstos mueren, y el hueso se vuelve esclerótico, hipermineralizado, acelular y avascular, y termina necrosándose y exponiéndose a la cavidad oral.[94] Esto podría explicar en parte, el por qué las lesiones de ONB aparecen principalmente en el hueso de la mandíbula.

La incidencia de ONB se encuentra en un rango de entre un 1.2%[112] y un 6.7%[113,114] en pacientes con cáncer tratados con BFs intravenosos. El riesgo de desarrollar este proceso en pacientes con osteoporosis tratados con BFs es inferior, y oscila en torno a 1 caso por cada 10000 pacientes por año (0.1%).[115]

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III. JUSTIFICACIÓN

Los BFs son fármacos ampliamente utilizados en el tratamiento de las enfermedades óseas metabólicas y en las metástasis óseas del cáncer de mama y próstata, por su capacidad para inhibir la reabsorción ósea. El papel que juegan los BFs en la inhibición de la formación de los osteoclastos y su actividad *in vitro* está bien documentado. Sin embargo, los efectos que estos fármacos producen en la diferenciación y función osteoblástica no están claros. Los datos *in vitro* disponibles en la literatura son contradictorios, e incluyen estudios con modelos experimentales muy diferentes, utilizando desde células madres mesenquimales hasta cultivos de osteoblastos primarios.

El hecho de que la función de los BFs sobre los osteoblastos no esté clara, unido a un incremento en la incidencia de osteonecrosis mandibular asociada a dichos fármacos nos lleva a plantearnos como odontólogos, profundizar en este campo, con el objeto de conocer mejor la fisiopatología de este cuadro clínico. La valoración de los resultados obtenidos, puede constituir una herramienta de utilidad clínica, ya que ayudaría a la selección de ciertos fármacos cuya acción se centre fundamentalmente en el osteoclasto, sin inhibir o alterar la fisiología de las células formadoras de hueso, lo que podría ayudar a prevenir la aparición de ONB.

IV. HIPÓTESIS

Los BFs utilizados a bajas dosis ejercen un efecto estimulador de la capacidad de crecimiento y de diferenciación, a la vez que modulan otras funciones propias de las células formadoras de hueso, lo que se traduce en un efecto positivo sobre el metabolismo óseo y por tanto sobre la remodelación de este tejido. Sin embargo, a dosis elevadas, tal y como ocurre en el hueso cuando se administran durante un largo periodo de tiempo, debido a su fuerte unión a la hidroxiapatita, estos fármacos poseen un efecto tóxico y negativo sobre el osteoblasto.

V. OBJETIVOS/AIMS

GENERALES

Analizar el efecto de los BFs sobre la fisiología del osteoblasto, con objeto de determinar el papel de estas células en el desarrollo de ONB.

ESPECÍFICOS

1. Identificar la evidencia científica disponible a cerca del efecto de los BFs sobre las células formadoras de tejido óseo a nivel biológico, celular y molecular.

2. Determinar el efecto de bajas concentraciones de tres BFs nitrogenados (pamidronato, alendronato, e ibandronato) sobre el crecimiento, la diferenciación, y el perfil antigénico del osteoblasto.

3. Analizar el papel del osteoblasto en el desarrollo de ONB mediante el estudio del efecto de diferentes concentraciones de clodronato, un BF no nitrogenado, sobre el crecimiento, diferenciación, y perfil antigénico del osteoblasto.

4. Evaluar el efecto de altas concentraciones de tres BFs nitrogenados (pamidronato, alendronato, e ibandronato) y un BF no nitrogenado (clodronato) sobre el crecimiento del osteoblasto.

OVERALLS

To evaluate the effect of BPs on osteoblast physiology, in order to determine the role of these cells in the development of BRONJ.

SPECIFICS

1. To identify the available scientific evidence about the effect of BPs on bone tissue-forming cells at biological, cellular, and molecular levels.

2. To determine the effect of three nitrogen-containing bisphosphonates (pamidronate, alendronate, and ibandronate) at low concentrations on osteoblast growth, differentiation, and antigenic profile.

3. To analyze the role of osteoblasts in the development of BRONJ by studying the effects of different concentrations of clodronate, a non-nitrogen-containing bisphosphonate, on osteoblast growth, differentiation, and antigenic profile.

4. To evaluate the effect on osteoblast growth of high concentrations of three nitrogen-containing bisphosphonates (pamidronate, alendronate, and ibandronate) and one non-nitrogen-containing bisphosphonates, clodronate.

VI. RESULTADOS Y DISCUSIÓN/RESULTS & DISCUSSION

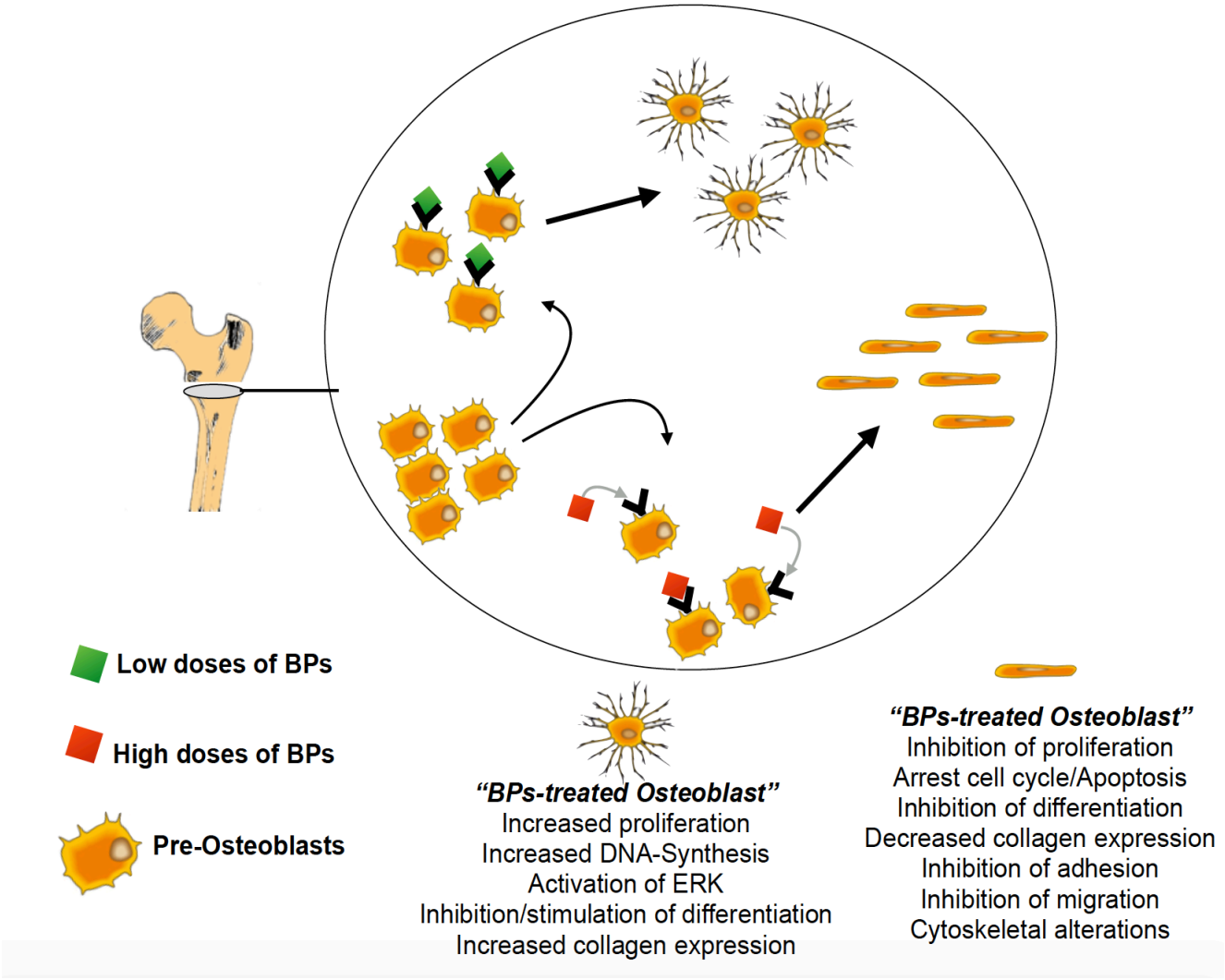
EFFECTS OF BISPHOSPHONATES ON OSTEOBLAST FUNCTION. A LITERATURE REVIEW

Abstract

In recent years, many studies have been performed to analyze the effect of bisphosphonates (BPs) on osteoblasts obtaining a plethora of different, inconsistent results. The aim of this review was to evaluate the action of BPs on bone tissue-forming cells at biological, cellular, and molecular level. A search of the literature was carried out in MEDLINE and EMBASE databases using the search terms bisphosphonates, diphosphonates, BPs, alendronate, pamidronate, etidronate, clodronate, zoledronate, ibandronate, risedronate, osteoblasts, and osteoblast-like cells. We included experimental studies on the effects of BPs on different parameters of osteoblasts *in vitro* (growth, differentiation) and on their relationship with other cells involved in bone metabolism. In vitro studies have demonstrated a role for BPs in osteoblast stimulation. However, their impact on the osteoblast *in vivo* remains unclear, due to their numerous indirect effects on the remodeling cycle mediated by a reduction in bone resorption. These studies of BPs require careful interpretation taking confounding factors into account, including differences in BP concentrations or models, which may explain the inconsistent results obtained. It should also be borne in mind that osteoblasts are exposed *in vivo* to different BP concentrations in the bone microenvironment.

Keywords: Bisphosphonates; osteoblast; proliferation; differentiation.

Graphical Abstract



Introduction

Bisphosphonates (BPs) are synthetic analogues of pyrophosphate that are usually administered to treat bone disorders such as osteoporosis, Paget's disease, fibrous dysplasia, hypercalcemia, and other diseases characterized by increased bone resorption. They avidly bind to bone and are internalized by osteoclasts to inhibit resorption. They are administered both orally and intravenously and are divided into two classes: low-potency non-nitrogen-containing BPs and more potent nitrogen-containing BPs. These two classes have distinct intracellular targets and molecular mechanisms of action that lead to inhibition of osteoclast-mediated bone resorption (Das and Crockett, 2013).

The therapeutic effects of BPs depend on their effect on osteoclasts, but their effect on osteoblasts or bone-forming cells is not well established, despite numerous articles on this topic in the literature (Manzano-Moreno *et al*, 2015a,b). BPs have been shown to specifically bind to osteoblastic cells (Bellido and Plotkin, 2011), and recent reports in humans and animals suggests that BPs may affect bone formation, activating different osteoblastic functions such as differentiation and proliferation (Casado-Díaz *et al*, 2013).

Although useful in the treatment of some bone diseases, BPs have also been associated with the development of BP-related osteonecrosis of the jaw (BRONJ) (Marx, 2003; Ruggiero *et al*, 2004). Despite the significant incidence of BRONJ and the increasing interest in understanding its pathophysiology, the underlying molecular mechanisms are still only partially known. In particular, *in vivo* observations have not elucidated the influence of BPs on bone-forming cells (Saracino *et al*, 2012).

Different mechanisms underlie the complex phenomenon of bone tissue formation, in which bone tissue-forming cells play an essential role. Osteoblasts derived from pluripotent mesenchymal stem cells (MSCs) synthesize and organize the bone matrix (osteoid). Their highly developed endoplasmic reticulum and Golgi apparatus produce large amounts of type I collagen (Col-I) and calcium-binding proteins such as osteocalcin and osteopontin

(Dominguez *et al*, 2011). Osteoblasts express alkaline phosphatase (ALP), leading to matrix mineralization; hence, Col-I and ALP are markers of osteoblast activity (Murshed *et al*, 2005; Dominguez *et al*, 2011). The differentiation of osteoblasts from mesenchymal precursors and their maturation are closely regulated by transcription factors, including run-related transcription factor 2 (Runx2/Cbfa1) and several cytokines and growth factors, such as bone morphogenetic proteins, transforming growth factors, and fibroblast *growth factors* (Ducy, 2000; Reyes-Botella *et al*, 2002; Pérez *et al*, 2006; Ruiz *et al*, 2007).

The aim of this literature review was to evaluate the action of BPs on bone tissue-forming cells at biological, cellular, and molecular levels.

Material and Methods

Literature Search

MEDLINE (using the PubMed search engine) and EMBASE biomedical databases were used for a electronic search of the literature between December 2009 and December 2014. The following search terms were used: (BPs OR diphosphonates OR BPs OR alendronate OR pamidronate OR etidronate OR clodronate OR zoledronate OR ibandronate OR risedronate) AND (osteoblasts OR osteoblast-like cells). We used algorithms and search strategies that can be reproduced by any researcher. The reference lists of articles were reviewed to detect additional relevant articles not found using the search criteria.

Selection criteria

Inclusion criteria were: experimental studies that show the effects of different BPs on different parameters of osteoblasts *in vitro* (growth, differentiation) and/or their association with other cells involved in bone metabolism. Exclusion criteria were clinical studies, human or animal studies, histomorphometric studies and case reports.

Results and discussion

The reviewed literature described different mechanisms of action of BPs on bone-forming cells that were therefore related to distinct action hypotheses. Results were divided into different sections that reproduce bone tissue formation processes, from the differentiation of mesenchymal cells into osteoblasts to the mineralization of newly formed bone tissue, in which osteoblastic cells are also involved. The selected studies centered on the effect of BPs on: osteoblast growth (table 1), differentiation (table 2), extracellular matrix (ECM) formation, migration, relationship of osteoblasts with other cell types, and osteoblast cytoskeleton.

Effect of BPs on growth

There is controversy among authors with respect to proliferation, although the inconsistent results can be attributed to the use of different BP doses. The characteristic structure of BPs contains numerous variations through changes in lateral chains that can lead to extensive modifications in their physicochemical, biological, therapeutic, and toxicological characteristics (Dominguez *et al*, 2011).

Several *in vitro* studies have shown that BPs activate osteoblastic proliferation. In a study by Yoshitani *et al* (2011), an MTS assay was performed using MG-63 and HOS osteosarcoma cell lines. At lower concentrations, the cellular viability of HOS and MG-63 were higher than that of untreated controls. The lowest concentration of alendronate enhanced cellular viability and motility, which correlated with the expression of connexin-43 (Cx-43) at mRNA and protein levels. Thus, Manzano-Moreno *et al* (2015a) observed increased osteoblast (MG-63) cell proliferation at 24 h of culture after treatment with the nitrogen-containing BPs alendronate, pamidronate, and ibandronate at doses ranging between 10 μM and 1 nM. However, the highest stimulating effect was observed at the lowest treatment dose (1 nM). Other authors (Morelli *et al*, 2011) performed binding assays

using [^{3}H]-olpadronate ([^{3}H]OPD) in osteoblasts and found [^{3}H]OPD to be displaced from its binding site by micromolar concentrations of lidadronate, alendronate, and etidronate. Osteoblast exposure to these treatments increased DNA synthesis and the consequent cell proliferation.

Walter *et al* (2010, 2011) analyzed the effect on human osteoblast viability of BPs (ibandronate, pamidronate, zoledronate, and clodronate) at different concentrations (0, 5, 50, 100, 200, 500 μM). At 72 h, the viability of osteogenic cells was reduced by all BPs in a dose-dependent manner. At the lowest concentrations (5–50 μM), the impact of zoledronate on osteoblasts was greater than that of the other BPs, and the lowest effect was observed with clodronate. Koch *et al* (2010) evaluated the effect of BPs (zoledronate, ibandronate, and clodronate) on osteoblast proliferation by cell count and gene expression analysis of cyclin D1 *in vitro*. Cyclin D1 is an important regulator of the cell cycle and a surrogate of cell proliferation. Their study suggested that BPs exerted a limited influence on osteoblast proliferation, with the exception of zoledronate; no significant impact on osteoblast proliferation was observed during the first 6 days, a reduction in Cyclin D1 gene expression was recorded from day 7 with zoledronate treatment.

Kaiser *et al* (2013) studied the influence of the nitrogen-containing BPs zoledronate, ibandronate, and pamidronate on molecular and cellular functions of osteoblasts, focusing on cell proliferation, viability, and apoptosis. Cell viability was analyzed by MTT assay, and cell proliferation was determined with the crystal violet staining technique. Apoptotic nuclear morphology was assessed by staining the cells, visualizing the nuclei with fluorescence. They showed that all three BPs tested reduced osteoblast viability and proliferation at high concentrations. In parallel, zoledronate and pamidronate also induced apoptosis in osteoblasts. Some authors, such as Yoshitani *et al* (2011) and Lezcano *et al* (2012), investigated the role of BPs and their relationship with Cx-43, a molecule required to activate the antiapoptotic effect of BPs. BPs can also exert antiapoptotic effects on

osteoblasts by opening Cx-43 hemichannels, components of gap junction channels that connect adjacent cells, and they can mediate the activation of intracellular signaling cascades.

Other authors completed this information by discovering new osteoblast binding or target sites for BPs. The best known are those of the mevalonate pathway. They postulate for the first time the presence of specific BP-binding sites in one or more of the protein tyrosine phosphatases on the surface of and within the osteoblast. These sites may be blocked by BPs, resulting in a reduction of the tyrosine desphosphorylation of bone signaling proteins. As a consequence, tyrosine phosphorylation levels may rise, leading to stimulation of osteoblast proliferation (Morelli *et al*, 2011).

Evidence has been published suggesting that some of the beneficial effects of N-BPs are due to the prevention of osteoblast and osteocyte apoptosis, mediated by the activation of extracellular signal-regulated kinase (ERK) at low concentrations (Plotkin *et al*, 1999). It has been shown that this anti-apoptotic effect is dependent on the expression of Cx-43, the major gap junction protein expressed in osteocytes. N-BPs cause the opening of Cx-43 hemichannels, followed by Src kinase and ERK activation (Plotkin *et al*, 2002).

The fact that Cx-43 is required for the anti-apoptotic action of BPs has raised the possibility that the interaction of BPs with Cx-43 present in the cell membrane results in hemichannel opening, thereby initiating intracellular survival signaling. However, although Cx-43 is necessary for BP-induced survival of osteoblastic cells, it was not found to be indispensable for BP cell binding (Bellido and Plotkin, 2011).

Although most authors have utilized MTT assays to study osteoblast growth, others have performed cell cycle or binding assays. All BPs have been found to have a dose-dependent effect, which is positive at doses <10 μM but inhibitory at higher doses.

Other studies have shown that BPs may increase or decrease osteoblastogenesis as a function of their concentration, finding a pro-osteoblastogenic effect of BP at concentrations

ranging from 1 nM to 1 μM but an inhibitory effect at concentrations above 10 μM (Kang *et al*, 2012; Manzano-Moreno *et al*, 2015a,b).

The normal morphology of cells was altered after the addition of zoledronate acid (≥5 Mμ), and cell viability was significantly inhibited at concentrations > 0.1 μM for MC3T3; zoledronate induced apoptosis in MC3T3 at a dose of 100 μM (Patntirapong *et al*, 2012).

Effect of BPs on osteoblast differentiation

Most studies in the literature focus on the expression of genes that specifically induce osteoblast differentiation. Thus, Casado-Díaz *et al* (2013) subjected MSCs, which can differentiate into both osteoblasts and adipocytes, to the action of risedronate, finding a rise in osteogenic markers, including those of ECM mineralization (Runx2, ALP, Col-I) and in osteocalcin between treatment days 7 and 21.

Manzano-Moreno *et al* (2015a) treated osteoblast-like (MG-63) cells with 10 μM, 0.1 μM or 1 nM of pamidronate, alendronate, or ibandronate. Cell differentiation was evaluated at 7, 15, and 21 days by spectrophotometric study of nodule formation and ALP activity at 24 h of culture. A decrease in ALP activity was observed after 24 h of treatment and a decrease in calcium deposition after 15 and 21 days ($p<0.05$) at all doses tested.

In contrast, Patntirapong *et al* (2012) showed that zoledronic acid treatment decreased bone nodule formation at all concentrations tested (0.01–100 μM). Furthermore, ALP activity in both cells was markedly reduced when exposed to zoledronic acid (>1 μM for MC3T3 and >5 μM for MSC). Zoledronic acid also downregulated Runx 2 and Col-I mRNA expressions. In this *in vitro* study, zoledronic acid mediated defective bone mineralization by directly disrupting osteoblast/osteoprogenitor cellular activities at several levels, i.e., cell proliferation, osteoblast differentiation, and osteoblast function of both pre-osteoblastic cells and MSCs.

However, according to Dominguez *et al* (2011) the rationale behind the development of the newer compounds (nitrogen-containing-BPs, heterocyclic BPs) is their more potent antiresorptive activity without the elevated mineralization inhibition observed with first-generation compounds, requiring a higher dose to inhibit resorption.

Nonetheless, other authors have demonstrated the involvement of BPs in the induction of osteoblast precursor proliferation and stimulation of the development of mineralized nodules in murine and human bone marrow cultures *in vitro*. In particular, clodronate promoted osteoblast differentiation in cultures of osteoblast-like cells (e.g., ST2 and MC3T3-E1 cells) and in rat organ cultures.

Kang *et al* (2012) investigated the effect of alendronate on the expression of inhibitors of DNA binding/differentiation (Id) genes in osteoblast differentiation. The expression of osteoblast differentiation markers such as ALP, (Col-I), and osteocalcin, and the expression of Id-1 and Id-2 were measured by real-time polymerase chain reaction (RT-PCR). Alendronate treatment not only significantly increased ALP activity but also the expression of ALP, Col-I, Osteocalcin, Id-1 and Id-2 genes.

Ohe *et al* (2012) tested the effects of BPs on the expression of two critical genes for osteoclastogenesis, (macrophage colony stimulating factor) M-CSF and osteoprotegerin (OPG), in the process of osteoblast differentiation from human MSCs for up to 2 and 3 weeks (for RT-PCR) or 3 and 4 weeks (for ELISA). Treatment of differentiating cells with the nitrogen-containing BPs alendronate or pamidronate (10 nM) increased the expression of OPG, which suppresses osteoclastogenesis, whereas it decreased the expression of M-CSF, which promotes pre-osteoclast formation.

Moon *et al* (2011) evaluated the effect on MC3T3-E1 cell differentiation of alendronate fixed on titanium surfaces. Their study showed that alendronate- immobilized titanium substrates significantly enhanced ALP activity and calcium content in MC3T3-E1 cell osteogenic differentiation. RT-PCR results showed that mRNA expressions of

osteocalcin and osteopontin, osteogenesis markers, were upregulated in MC3T3-E1 cells cultured on alendronate-immobilized titanium.

In another study, Fujita *et al* (2011) investigated the effects of the nitrogen-containing BP risedronate on osteoblast differentiation in human and rat MSCs. They found that risedronate dose-dependently suppressed the formation of mineralized nodules (0.3 to 10 μM) and the mRNA expression of differentiation marker genes such as bone sialoprotein and osteocalcin in MSC-derived osteoblasts. Treatment with high-concentration risedronate (300 μM) was also found to induce chromatin condensation, a feature of apoptosis, in MSCs. Risedronate induced chromatin condensation was suppressed by a pan-caspase inhibitor zVAD-FMK and a cell permeable isoprenoid analogue geranylgeraniol. These results indicate that risedronate suppressed osteoblast differentiation and induced caspase and isoprenoid depletion-dependent apoptosis, suggesting that the antiresorptive effect of risedronate is not mediated by a decrease in the RANKL expression in MSC-derived osteoblasts.

Wang and Stern (2011) reported that the effects on osteoblasts vary with modifications in the BP molecule and concentration, which both result in qualitatively different responses. The mechanism underlying the differential effects of high and low BP concentrations on osteoblast activity was examined by comparing the effects of 10 nM and 100 μM of risedronate on gene expression in UMR-106 rat osteoblastic cells. Interestingly, the low but not the high concentration of risedronate transiently and selectively upregulated various genes associated with cell differentiation.

Koch *et al* (2011) compared the osteogenic differentiation potency of nitrogen-containing BPs (zoledronate and ibandronate) with that of non-nitrogen-containing BPs (clodronate) and explored whether their osteogenic effect was dose-dependent. They stimulated human osteoblasts with zoledronate and ibandronate at concentrations of 50, 5 and 0.5 μM for 1, 2, 5, 10, and 14 days and with clodronate at concentrations of 5000, 50,

and 5 μM. At each time point, RT-PCR was used to quantify the gene expression level of the osteoblast-specific differentiation markers of the homeobox transcription factors MSX1 and MSX2, the distal-less homeobox 5 (Dlx5), the Run-related transcription factor 2 (Runx2/CBF1a), and osteocalcin. They observed a significant difference between the nitrogen-containing and non-nitrogen-containing BPs. At 10 days, concentrations of 50 μM zoledronate or ibandronate enhanced the gene expression of all differentiation markers by several hundred fold in comparison to non-stimulated controls after 10 days, whereas clodronate had a lesser influence on gene expression, even at concentrations >5000 μM. However, lower concentrations of zoledronate or ibandronate reduced the gene expression.

Valenti *et al* (2010) evaluated cell proliferation and expression of COX-2 and bone ALP (b-ALP) genes in bone marrow cells and MLO-y4 osteocytes treated with risedronate alone or in co-treatment with the selective COX-2 inhibitor NS-398 or dexamethasone. This *in vitro* study confirmed an increase in the viability and expression of b-ALP gene in bone marrow cells in a dose-dependent manner.

Some authors found that BPs exerted inhibitory effects on the expression of osteoblast differentiation marker genes. Increasing concentrations of zoledronate and pamidronate (5–100 μM) for 72 h produced a sustained regulation of osteogenic marker genes, resulting in a dose-dependent increase in osteocalcin and bone sialoprotein expression and a decrease in ALP and collagen type I a2. In addition, pamidronate induced a substantive enhancement of osteonectin mRNA expression. No significant changes were noted in ibandronate-treated cells (Kaiser *et al,* 2013).

Effect of BPs on formation of the extracellular bone matrix

Sun *et al* (2012) analyzed the effects of alendronate on the expression and activity of matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that cleave multiple components of the ECM. Alendronate at doses of 10 nM to 1 μM did not alter the

expression of MMP-1, MMP-2, MT1-MMP, or TIMP-2 but inhibited MMP-2 activity at 1000 μM and 10000 μM in the zymograms. In conclusion, non-toxic levels of alendronate (10 nM to 1 μM) did not alter MMP expression in MG-63 cells or inhibit MMP-2 activity.

Boanini *et al* (2012) studied the effect of alendronate and zoledronate on co-cultures of osteoblasts and osteoclasts, finding a significant increase in Col-I synthesis between days 3 and 7 of culture.

Koch *et al* (2010) evaluated the effect of nitrogen-containing and non-nitrogen-containing BPs on osteoblast gene expression of the ECM protein Col-I. Collagen gene expression was enhanced by nitrogen-containing BPs but was decreased after day 10. The non-nitrogen-containing BP clodronate did not significantly influence collagen gene expression.

Simon *et al* (2010) used ELISA and RT-PCR to investigate the expression of collagen types I, II, III, and V in human osteoblasts and osteosarcoma cells (SaOS-2 cells) after constant exposure for 28 days to zoledronate and pamidronate. According to the RT-PCR results, zoledronate inhibited gene expression below 16% at all concentrations tested. An amplification of ECM was only possible with pamidronate at concentrations of 1 μM.

In regard to bone matrix formation, authors have analyzed the effects on collagen synthesis. In general, the effects attributed to BPs on this aspect of the bone metabolism were positive. However, some authors demonstrated that long-term exposure to zoledronate and pamidronate can have a negative influence.

Treatment with BPs and its influence on migration in bone forming cells

Koch *et al* (2011) investigated the effect of BPs on migration and cell adhesion by using PCR-RT to study the expression of integrin vb3 and tenascin C genes, finding that zoledronate inhibited human osteoblast migration after 50 h of stimulation. Integrin avb3 and tenascin-C gene expressions were affected by BPs in a cell line-dependent manner, with

a decrease in gene expression levels over time. The non-nitrogen-containing BP clodronate produced a reduction in the gene expression levels.

Walter *et al* (2011) examined the effects of BPs on the migration ability of osteoblasts using a 24-well Boyden Chamber. They quantified cell migration by means of a Microplate Fluorescence Reader with extinction wavelength at 480 nm and emission at 520 nm. Only nitrogen-containing BPs inhibited the migration ability of osteoblasts, with no significant differences among zoledronate, pamidronate, and ibandronate.

Treatment with BPs and its influence on the relationship of bone-forming cells with other cells

Various studies have analyzed the effect of BPs on the osteoblast-osteoclast relationship *via* the RANKL-OPG complex. Giner *et al* (2011) studied the effects of alendronate on OPG production (ELISA) and on OPG and RANKL expression (RT-PCR) in primary cultures of human osteoblasts and reported an increase in the mRNA expression of RANKL. In addition, OPG gene expression was moderately enhanced and RANKL gene expression strongly increased by nitrogen-containing BPs, with maximum concentrations reaching 50 μM, whereas the non-nitrogen-containing BP clodronate had much smaller effects on OPG and RANKL gene expression, even at concentrations of 5000 μM.

Saracino *et al* (2012) investigated the possibility that BRONJ results from changes in osteoblast activity induced by factors released from soft-tissue cells exposed to zoledronic acid. They found reduced osteoblast proliferation and osteogenic properties in osteoblasts grown in medium conditioned by zoledronic acid-exposed NCTC 2544 cells, and an increase in their capacity to induce osteoclast differentiation and inflammatory processes. These observations demonstrated for the first time that the exposure of soft tissues to zoledronic acid significantly increases the production of proinflammatory factors and reduces that of anti-inflammatory factors. This in turn affects the properties of osteoblasts.

The lesser proliferation of osteoblasts grown in zoledronic acid-conditioned culture media translates into a reduction in their regenerating capacity during healing.

Shimizu *et al* (2012) reported that the non-nitrogen-containing BP alendronate indirectly inhibits osteoblast function through the coupling of osteoclasts to osteoblasts by interaction between ephrinB and EphB.

Mansour *et al* (2011) found that zoledronate treatment affected the expression of CXCL12 and IL-7. Although it had no direct effect on B lymphocyte proliferation, it impacted on osteoblast binding/recruitment. B lymphocytes depend on the interaction between B cells and stromal bone cells.

Treatment with BPs and its influence on the cytoskeleton of bone-forming cells

Cytoskeletal elements are critical for cell morphology and signal transduction and are involved in numerous cellular processes, including motility, intracellular transport, and differentiation. The actin cytoskeleton plays a key role in determining cell morphology, and its reorganization is subject to modulation by interactions with the ECM (Small *et al*, 1999).

Gangoiti *et al* (2013) have evaluated the in vitro alterations on osteoblastic morphology by environmental scanning electron microscopy, in actin cytoskeleton and apoptosis induced by AGEs. They reported that high concentrations of alendronate (10 μM, as found in an osteoclastic lacuna) exacerbated osteoblastic morphological and cytoskeletal alterations. However, low doses (10 nM, typical of extracellular fluid levels to which osteoblasts can be exposed for most of their life cycle) had no effect on cell morphology and were able to prevent AGE-induced alterations in osteoblasts and their resulting death.

Kazmers *et al* (2009) explored the effect of nitrogen-containing BPs on RhoA, a small GTP-binding protein (G proteins) of the Ras family, which stabilizes the actin cytoskeleton and promotes the formation of focal adhesions. RhoA is expressed in osteoblasts and is activated by PTH 1–34, a PTH analog that does not increase cAMP. The

authors selected alendronate as Rhoa inhibitor because it reduces intracellular geranylgeranyl pyrophosphate by inhibiting farnesyl pyrophosphate synthase. Alendronate produced a reduction in actin stress fibers, FA density, and FA size but had no effect on edge actin bundle density, cellular area, or circularity.

Conclusions

In conclusion, in vitro studies have demonstrated a role for BPs in osteoblast stimulation. However, their impact on the osteoblast *in vivo* remains unclear, due to their numerous indirect effects on the remodeling cycle mediated by a reduction in bone resorption. *In vitro* studies of BPs require careful interpretation, taking into account confounding factors, including differences in BP concentrations or models, which may explain the inconsistent results obtained. It should also be borne in mind that osteoblasts are exposed *in vivo* to different BP concentrations in the bone microenvironment.

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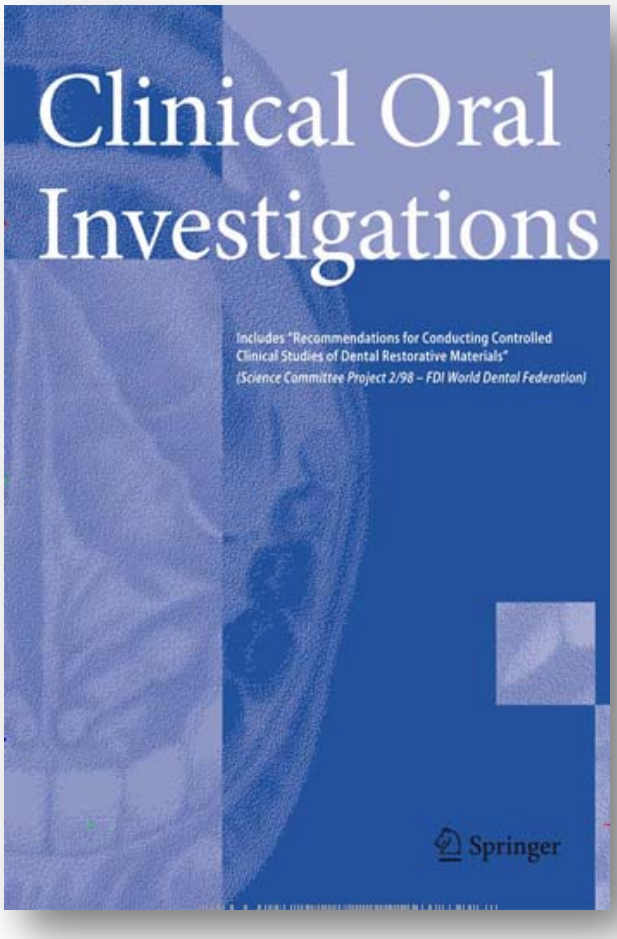
Table 1. Effect of bisphosphonates on osteoblast growth.

Author and year	Cell type	BPs	Doses assayed	Methods	Results
Kaiser et al. 2013	Primary human osteoblasts	Zoledronate, Ibandronate, Pamidronate	From 5 to 100 µM	MTT assay, Cristal violet staining	All BPs tested reduced osteoblast viability and proliferation at high concentrations (50 and 100 µM). Zoledronate and pamidronate also induced apoptosis in osteoblasts
Kang et al. 2012	C2C12	Alendronate	1000 µM to 10 nM	MTT assay	High doses (> 100 µM) of alendronate decreased C2C12 cells viability
Koch et al. 2010	Primary human osteoblasts	Zoledronate, Ibandronate, Clodronate	50 µM	Cell counting , Gene expression of cyclin D1	The BPs tested exerted a limited influence on osteoblast proliferation, with the exception of zoledronate that caused a reduction in Cyclin D1 gene expression
Lezcano et al. 2012	MLO-Y4 ROS17/2.8	Alendronate	1, 10, 100 µM	[^{3}H]-Thymidine incorporation asssay, Trypan blue uptake	10 and 100 µM of alendronate increase osteoblast proliferation µM of alendronate has no effect on cell proliferation
Manzano-Moreno et al. 2015a	MG-63	Alendronate, Ibandronate, Pamidronate	0.001, 0.1, 10 µM	MTT assay	Increase on osteoblast proliferation at 24 h of culture after treatment with the BPs tested at doses ranging between 10 µM and 0.001 µM. However, the highest stimulating effect was observed at the lowest treatment dose (0.001 µM)
Manzano-Moreno et al. 2015b	MG-63	Alendronate, Ibandronate, Pamidronate, Clodronate	10, 50, 100 µM	MTT assay, Annexin-V apoptotic assay	All the BPs assayed produced a significant and dose-dependent reduction in MG-63 proliferation and an increased percentage of cells in apoptosis at the highest doses assayed (50, 100 µM)
Morelli et al. 2011	ROS17/2.8 Primary rat osteoblasts	Olpadronate	1, 10, 100 µM	[^{3}H]-Thymidine incorporation asssay	Osteoblasts exposure to 1, 10, 100 µM of olpadronate increased DNA synthesis and the consequent cell proliferation
Patntirapong et al. 2012	MC3T3-E1	Zoledronate	0'01 to 100 µM	MTT assay	Cell viability was significantly inhibited at concentrations > 0.1 µM and apoptosis was induced at a dose of 100 µM
Walter et al. 2010	Human osteogenic cells	Ibandronate, Pamidronate, Zoledronate Clodronate	0, 5, 50, 100, 200, 500 µM	MTT assay	The viability of osteogenic cels was reduce by all BPs in a dose-dependent manner. Pamidronate and zoledronate showed the greatest negative effect on cell viability
Walter et al. 2011	Human osteogenic cells	Ibandronate, Pamidronate, Zoledronate Clodronate	0, 5, 50, 100, 200, 500 µM	Calcein viability assay, Tunel assay	At lower concentrations (5-50 µM), the impact of zoledronate on osteoblasts was the strongest compared to other BPs. Clodronate decreases osteoblast viability only at 100 µM. Pamidronate and zoledronate showed the greatest effect inducing apoptosis on osteoblasts.
Yoshitani et al. 2011	MG-63 HOS	Alendronate	1, 100 nM, 1, 10, 100 µM	MTS assay	Dose-dependent inhibitory effects on osteoblast viability at concentrations higher tan 10 µM. Concentrations ranging from 1 nM to 10 µM.increased cell viability compared to the controls.

Table 2. Effect of bisphosphonates on osteoblast differentiation.

Author and year	Cell type	BPs	Doses assayed	Methods	Results
Casado-Díaz et al. 2013	Mesenchymal stromal cells (MSCs)-derived osteoblasts	Risedronate	1, 10 nM	ALP activity, RT-PCR, mineralization assay	Risedronate increased the levels of osteogenic marker genes (ALP, RUNX-2, Col-I and osteocalcin), ALP activity and matrix mineralization
Fujita et al. 2011	MSCs-derived osteoblasts	Risedronate	0.3, 1, 3, 10 ¥ M	RT-PCR, Mineralization assay	Risedronate dose-dependently suppressed the formation of mineralized nodules (0.3 to 10 μM) and the mRNA expression of differentiation marker genes such as bone sialoprotein and osteocalcin
Kaiser et al. 2013	Primary human osteoblasts	Zoledronate, Ibandronate, Pamidronate	From 5 to 100 μM	RT-PCR	Increasing concentrations of zoledronate and pamidronate (5–100 μM) for 72 h produced an increase in osteocalcin and bone sialoprotein m RNA expression and a decrease in ALP and Col-I a2 mRNA expression. No effect was observed with ibandronate treatment
Kang et al. 2012	C2C12	Alendronate	1, 0.1 μM and 10 nM	ALP activity, RT-PCR	Alendronate treatment not only significantly increased ALP activity but also the expression of ALP, Col-I, Osteocalcin, Id-1 and Id-2 genes
Koch et al. 2011	Human osteoblasts	Zoledronate, Ibandronate, Clodronate	0.5 to 5000 μM	RT-PCR	Zoledronate and ibandronate at concentrations of 10 μM enhanced the gene expression of all differentiation markers (MSX-1, MSX-2, Dlx5, RUNX-2 and osteocalcin), whereas clodronate had less influence on gene expression
Manzano-Moreno et al. 2015a	MG-63	Alendronate, Ibandronate, Pamidronate	0.001, 0.1, 10 μM	ALP activity, mineralization assay	A decrease in ALP activity was observed after 24 h of treatment and a decrease in calcium deposition after 15 and 21 days at all doses tested
Moon et al. 2011	MC3T3-E1	Alendronate	1, 5 mg/ml	Calcium contents assay, ALP activity, RT-PCR	Alendronate significantly enhanced ALP activity and calcium content in. RT-PCR results showed that mRNA expressions of osteocalcin and osteopontin were upregulated
Ohe et al. 2012	MSCs-derived osteoblasts	Alendronate, Pamidronate	10 nM	RT-PCR	Alendronate and pamidronate increased the expression of OPG, and decreased the expression of M-CSF
Patntirapong et al. 2012	MC3T3-E1	Zoledronate	0.01 to 100 μM	Mineralization assay, ALP activity, RT-PCR	Zoledronate decreased bone nodule formation at all concentrations tested. ALP activity was markedly reduced when exposed to zoledronate (>1 μM). Zoledronate also downregulated Runx 2 and Col I mRNA expressions
Valenti et al. 2010	MLO-y4	Risedronate	0.1 to 10 μM	RT-PCR	Risedronate increased the expression of ALP gene in a dose-dependent manner
Wang et al. 2011	UMR-106	Risedronate	10 nM, 100 μM	RT-PCR	10 nM of risedronate transiently and selectively upregulated various genes associated with cell differentiation



NITROGEN-CONTAINING BISPHOSPHONATES MODULATE THE ANTIGENIC PROFILE AND INHIBIT THE MATURATION AND BIOMINERALIZATION POTENTIAL OF OSTEOBLAST-LIKE CELLS

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ORIGINAL ARTICLE

Nitrogen-containing bisphosphonates modulate the antigenic profile and inhibit the maturation and biomineralization potential of osteoblast-like cells

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Abstract
Objectives The aim was to evaluate the effect of three nitrogen-containing bisphosphonates at different concentrations on osteoblast growth, differentiation, and antigenic profile, using the MG-63 cell line as osteoblast model, in order to determine the role of osteoblasts in bisphosphonate-related osteonecrosis of the jaw (BRONJ).
Materials and methods Osteoblasts were incubated in culture medium with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate. Proliferative capacity of the osteoblasts was determined by spectrophotometry (MTT) at 24 and 48 h of culture. Flow cytometry was used to study antigenic profile (CD54, CD80, CD86, HLA-DR) and phagocytic activity. Cell differentiation was evaluated at 7, 15, and 21 days by the study of nodule formation and alkaline phosphatase activity (ALP) at 24 h by spectrophotometric assay.
Results Pamidronate, alendronate, and ibandronate each exerted a significant stimulatory effect on MG63 proliferation that depended on the dose and treatment duration ($p<0.05$). In general, a significantly decreased expression of CD54, CD80, and HLA-DR membrane antigens was observed after 24 h of treatment with each nitrogen-containing bisphosphonate ($p<0.05$), but there was no significant difference in phagocytic activity versus controls. A decrease in ALP activity was observed after 24 h of treatment and a decrease in calcium deposition after 15 and 21 days ($p<0.05$).
Conclusion Nitrogen-containing bisphosphonates can increase the proliferation of MG-63 osteoblast-like cells, modulate their expression of co-stimulatory molecules associated with immune function, and decrease their differentiation capacity, generally at low doses.
Clinical relevance These findings suggest that low doses of nitrogen-containing bisphosphonates exert their effect on osteoblasts by altering their physiology, which would explain the disruption of their repair capacity and may be directly related to the development of BRONJ.

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Keywords Nitrogen-containing bisphosphonates · Osteoblast · Proliferation · Phenotype · Differentiation · BRONJ

Introduction

Bisphosphonates (BPs) are a family of pyrophosphate synthetic analogues in which the oxygen linking the phosphates has been replaced by carbon. They are commonly used as antiresorptive agents in the treatment of metabolic bone diseases with increased bone resorption (e.g., osteoporosis, Paget's disease, multiple myeloma, malignant hypercalcemia, etc.) and in bone metastases of breast and prostate cancer [1–4]. There are two major types of BPs with different

molecular modes of action, non-nitrogen-containing BPs and nitrogen-containing BPs [5].

BPs are widely prescribed, and their ability to inhibit osteoclast formation and activity in vitro and in vivo is well documented [6, 7]. However, the mechanisms underlying their action on bone and their effects on osteoblasts are not fully understood, although various proposals have been made, including a decrease in bone turnover and subsequent accumulation of microfractures; a toxic effect on osteoblasts; an antiangiogenic effect, producing avascular necrosis; and a reduction in the viability of fibroblasts and oral keratinocytes [8–10]. Although useful in the treatment of some bone diseases, BPs have also been associated with the development of bisphosphonate-related osteonecrosis of the jaw (BRONJ) [11, 12].

Bone formation and bone remodeling processes go beyond osteoblast proliferation and differentiation and are regulated by a large number of paracrine, autocrine, and endocrine factors, including growth factors, hormones, and cytokines [13, 14]. Besides their essential role in bone formation and repair, osteoblasts possess immunological functions, including T lymphocyte stimulation, phagocytic activity, and cytokine synthesis [15–23]. Osteoblasts express CD54, CD80, CD86, and HLA-DR surface antigens according to their degree of differentiation and/or activation, and their antigenic profile is modulated by the presence of different cytokines and growth factors [17, 18, 22].

The objective of the present study was to evaluate the effect of three nitrogen-containing BPs (pamidronate, alendronate, and ibandronate) at different concentrations on osteoblast growth, differentiation, and antigenic profile using the MG63 cell line, in order to determine the role of osteoblasts in BRONJ. MG63 cells are widely used as osteoblast model because they share the same characteristics.

Materials and methods

The nitrogen-containing BPs pamidronate (Sigma-Aldrich, St. Louis, MO), alendronate (Sigma), and ibandronate (Sigma) were selected for the study. The doses in all assays were 10^{-5}, 10^{-7}, or 10^{-9} M, which are within the therapeutic dose range [24].

Cell culture

Human MG63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA). MG63 cell line was maintained as described by Diaz-Rodríguez et al. [22] in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 μg/mL gentamicin (Braum Medical SA, Jaen, Spain), 2.5 μg/mL amphotericin B (Sigma), 1 % glutamine (Sigma), and 2 % HEPES (Sigma), supplemented with 10 % fetal bovine serum (FBS) (Gibco, Paisley, UK). Cultures were kept at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. Cells were detached from the culture flask with a solution of 0.05 % trypsin (Sigma) and 0.02 % ethylenediaminetetraacetic acid (EDTA) (Sigma) and then washed and suspended in complete culture medium with 10 % FBS.

Cell proliferation assay

Osteoblasts were seeded at 1×10^4 cells/mL per well into a 24-well plate (Falcon, Becton Dickinson Labware, NJ) and cultured at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2 for 24 h. Next, the medium was replaced with DMEM containing pamidronate, alendronate, or ibandronate at a dose of 10^{-5}, 10^{-7}, or 10^{-9} M. After 24 or 48 h of culture, the cell proliferation was measured by MTT assay, as described by Manzano-Moreno et al. [25], replacing media with phenol red-free DMEM containing 0.5 mg/mL MTT (Sigma) and incubating for 4 h. Cellular reduction of the MTT tetrazolium ring resulted in the formation of a dark-purple water-insoluble deposit of formazan crystals. After incubation, the medium was aspirated, and dimethyl sulfoxide (DMSO, Merck Biosciences, Darmstadt, Germany) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (Sunrise™, Tecan, Männedorf, Switzerland). Results were reported as mean absorbance (570 nm)±standard deviation (SD). At least three experiments were conducted for each treatment, using the mean value in the analysis.

Antigenic phenotype by flow cytometry

Osteoblasts (MG-63 cell line) were previously treated with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate for 24 h at 37 °C. Cells were then detached from the cultured flask by treatment with 0.4 % (*w/v*) EDTA solution, washed, and suspended in phosphate-buffered saline (PBS) at 2×10^4 cells/mL. Cells were labeled by direct staining with anti-CD54, CD80, CD86, and HLA-DR monoclonal antibodies (MAbs) (CD54/IOL1b, CD80, CD86, and OKDR, respectively, all from Invitrogen Corp, Carlsbad, CA). Aliquots of 100 μL of cell suspension were incubated with 10 μL of the appropriate MAb for 30 min at 4 °C in the dark. Cells were washed, suspended in 1 mL of PBS, and immediately analyzed in a flow cytometer with diode laser (FACSCanton II, Becton Dickinson, Palo Alto, CAL) at a wavelength of 488 nm to determine the percentage of fluorescent cells. Untreated cells were used as controls. The percentage of antibody-positive cells was calculated from counts of 2,000–3,000 cells. At least three experiments were run for each antigen in all cultures.

Phagocytic activity

Phagocytic activity was studied by flow cytometry. Cultured human MG-63 cells were treated with10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate. Untreated cells were used as controls. Cells were detached from the culture flask by treatment with a solution of 0.05 % trypsin and 0.02 % EDTA, washed, and then suspended in complete culture medium with 10 % FBS at 2×10^4 cells/mL. Cells were labeled by direct staining with labeled latex beads. Then, 100 μL of cell suspension was incubated with 200 μL of carboxylated FICT-labeled latex beads with diameter of 2 μm (Aldrich, St Louis, MO) for 30 min at 37 °C in darkness. Cells were washed, suspended in 1 mL of PBS, and immediately analyzed in a flow cytometer (FACSCanton II). Results were expressed as percentage of cells positive for phagocytosis and mean channel fluorescence, which correlates with the number of phagocytosed particles.

Alkaline phosphatase activity

The effect on osteoblast-like cell differentiation was assessed by evaluating the alkaline phosphatase (ALP) activity of MG-63 cells cultured in non-osteogenic medium and treated with BPs. ALP activity was quantified by using a colorimetric assay (Diagnostic kit 104-LL, Sigma) to determine early osteoblastic differentiation. The assay measures the conversion of the colorless substrate *p*-nitrophenylphosphate by the enzyme ALP to the yellow product *p*-nitrophenol, with the rate of color change corresponding to the amount of enzyme present in solution. Standards of *p*-nitrophenol (0–250 μM) were prepared from dilutions of a 1,000 μM stock solution and assayed in parallel. The ALP assay was performed as described by Sandrini et al. [26]. The cells in non-osteogenic medium with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate or without drugs (control group) were seeded at 1×10^4 cells/mL per well into 24-well plates and cultured for 7 days under standard conditions. Then, cells were lysed with 0.1 % (*v*/*v*) Triton X-100 at 37 °C. The samples were centrifuged at 1,500 rpm and the supernatants stored at −70 °C until used. ALP activity was determined with *p*-nitrophenylphosphate as substrate. An aliquot (50 μL) of cell lysate solution was added to 50 μL of ALP substrate (Sigma) and then incubated at 37 °C for 45 min in darkness. The enzymatic reaction was stopped by adding 50 μL of 0.1 M NaOH, and the absorbance was measured at 405 nm with a spectrophotometer (Biotek ELx800). The total protein content was estimated by the Bradford method using a protein assay kit from Bio-Rad Laboratories (Nazareth-Eke, Belgium). All samples were run in triplicate, and the ALP activity was expressed as a percentage, considering enzymatic activity in the absence of BPs as 100 %.

Nodule formation and matrix mineralization

The presence of calcium deposits in the cell matrix was analyzed by the alizarin red S method. MG-63 cells were seeded (5×10^4 cells/mL/well) in a 6-well plate and cultured in osteogenic medium with different concentrations of the BPs at 37 °C in a humidified atmosphere of 95 % air and 5 % CO_2. The medium was replaced after 4 days and then every 3 days. The matrix mineralization of each cell line was measured at 7,

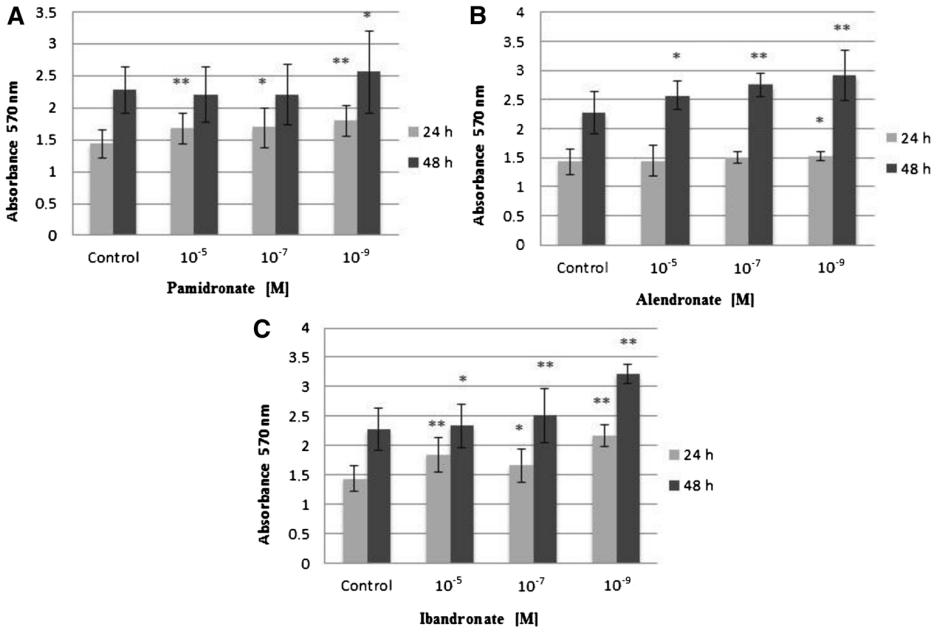

Fig. 1 Effect of **a** pamidronate, **b** alendronate, and **c** ibandronate at different doses (10^{-5}, 10^{-7}, 10^{-9} M) on osteoblast proliferation in MG-63 cell line after 24 and 48 h of incubation. Data are expressed as means+SEM. We compared data between each treatment and control culture by analysis of variance (ANOVA). **P<0.05*; ***P<0.001*

15, and 22 days of culture. Wells were washed with 150 mM sodium chloride, fixed in cold 70 % ethanol for 5 min, and rinsed three times with distilled water. Wells were then incubated for 10 min with 1 mL of 2 % (*u/v*) alizarin red S solution buffered at pH 4 with sodium hydroxide, then rinsed five times with distilled water, and finally washed with PBS to reduce non-specific staining. Calcium deposits present in the extracellular collagen matrix were colored red, revealing mineralization nodules, which were detained for 15 min with 10 % (*w/v*) cetylpyridiniumchloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured with a plate/reader spectrophotometer (Biotek ELx800).

Statistical analysis

SPSS 20.0 (IBM, Chicago, IL) was used for all data analyses. Mean values (±SD) were calculated for each variable. A two-way repeated-measures analysis of variance (ANOVA) was performed to examine the effects on proliferation, nodule formation, and ALP synthesis as a function of the BP type (pamidronate, alendronate, or ibandronate), treatment duration, and concentration. When a significant interaction was identified, the Bonferroni correction was applied for planned pair-wise comparisons. Antigenic profile and phagocytic activity were compared using the Student's *t* test; $p<0.05$ was considered significant. At least three experiments were performed for each assay.

Results

Cell proliferation assay

Each amino-BP studied stimulated osteoblast proliferation capacity as a function of dose and treatment duration (Fig. 1). In comparison to controls, a significant increase in proliferation ($p<0.05$) was observed with ibandronate at doses of 10^{-5}, 10^{-7}, and 10^{-9} M after 24 and 48 h of treatment; with alendronate at all doses after 48-h treatment but at only 10^{-9} M after 24-h treatment; and with pamidronate at all doses after 48-h treatment (10^{-5}, 10^{-7}, and 10^{-9} M), but at only 10^{-9} M after 24-h treatment.

Antigenic phenotype by flow cytometry

The flow cytometry results in Fig. 2 show that, in general, treatment with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate for 24 h significantly decreased the expression of CD54, CD80, and HLA-DR membrane antigens versus non-treated controls MG63 cells after 24 h of culture ($p<0.05$). The CD86 marker showed a variable expression depending on the BP and dose.

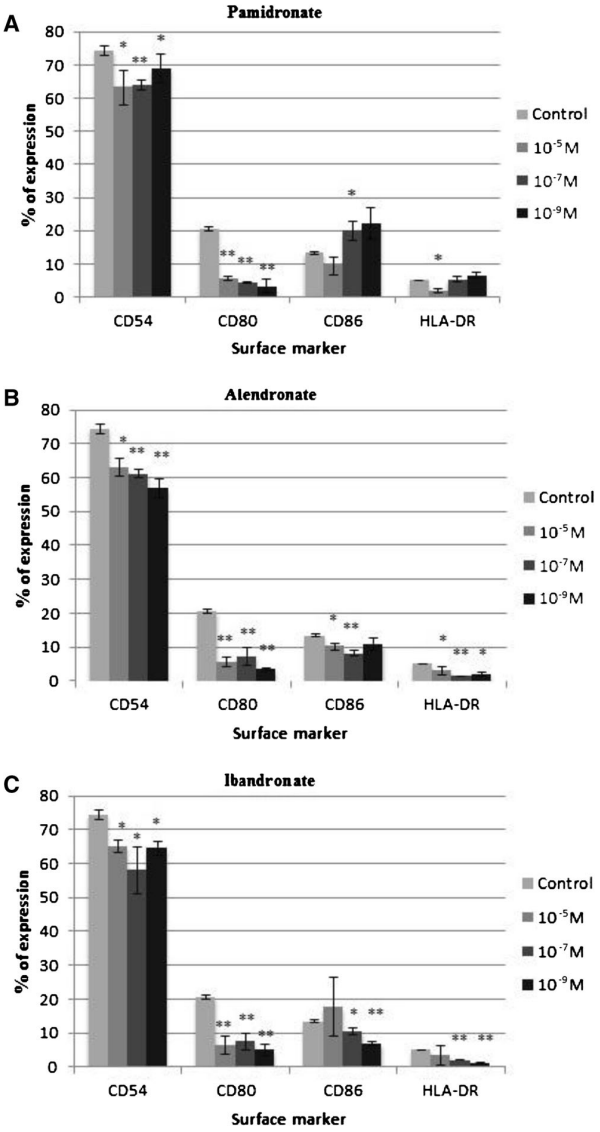

Fig. 2 Percentage expression of osteoblast MG63 cell surface markers after 24-h treatment with pamidronate (**a**), alendronate **(b),** or ibandronate (**c**) at doses of 10^{-5}, 10^{-7}, or 10^{-9} M.*$p<0.05$; **$p<0.001$

Phagocytic activity

Flow cytometry with fluorescent latex beads (Fig. 3) showed no significant differences in phagocytic activity between MG63 cells treated with different doses of BPs and non-treated controls cells after 24 h of culture.

Alkaline phosphatase activity

Results in Fig. 4 show a decrease in the expression of this differentiation marker versus control cells in all 3 drugs after 24 h of treatment; this difference only reached significance at doses of 10^{-7} and 10^{-9} M with pamidronate ($p=0.047$ and

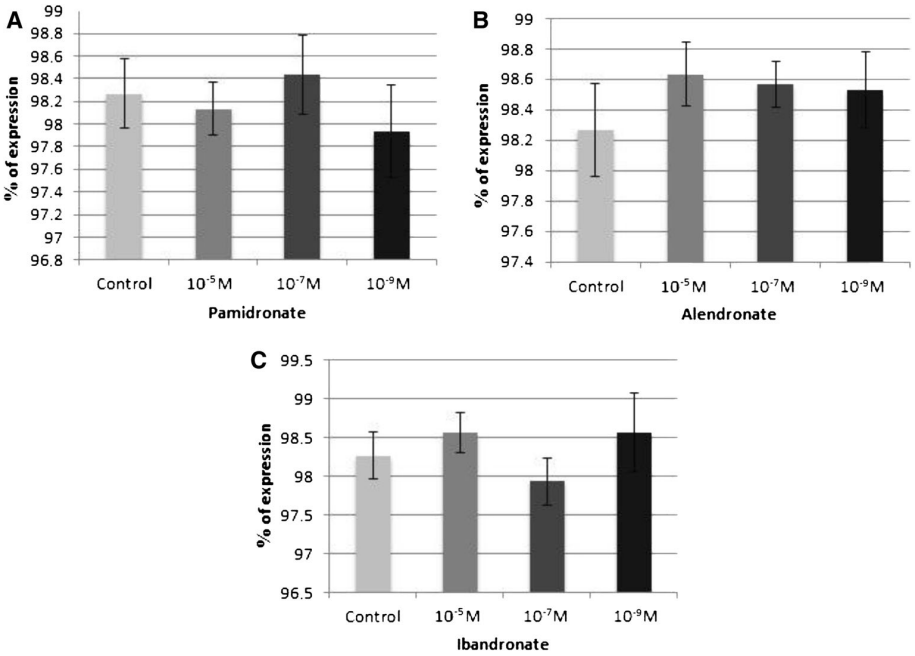

Fig. 3 Percentage of cells with flow cytometry-determined phagocyte capacity after 24-h treatment with pamidronate (**a**), alendronate (**b**), and ibandronate (**c**) at doses of 10^{-5}, 10^{-7}, or 10^{-9} M

p=0.002) or ibandronate (p=0.034 and p=0.028) and at a dose of 10^{-9} M with alendronate (p=0.032).

Nodule formation and matrix mineralization

The effect of the three BPs on nodule mineralization is depicted in Fig. 5. It shows the amount of Alizarin Red staining measured colorimetrically at 7, 15, and 21 days of culture in osteogenic medium with 10^{-5}, 10^{-7}, or 10^{-9} M of pamidronate, alendronate, or ibandronate. There were no significant differences between treated and control (untreated) groups at 7 days of culture. However, in comparison to the control group, the calcium deposition was reduced (p<0.05) after treatment with each BP for 15 or 21 days at all concentrations tested.

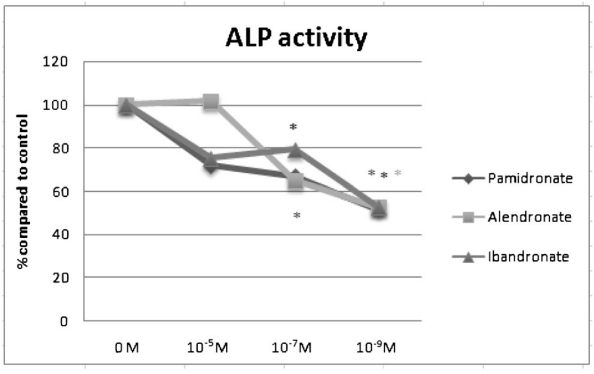

Fig. 4 ALP activity of MG63 line cell after 24-h treatment with pamidronate, alendronate, or ibandronate at doses of 10^{-5}, 10^{-7}, or 10^{-9} M, in non-osteogenic medium, expressed as a percentage, considering the enzymatic activity value in the absence of bisphosphonates as 100 %. *p<0.05

Discussion

The results of this in vitro study demonstrate that therapeutic doses of pamidronate, alendronate, or ibandronate exert an effect on osteoblasts, increasing their growth capacity, inhibiting their differentiation and mineralization, and modulating their antigenic profile. This implies major changes in the complex physiology of the osteoblast, which is regulated by multiple local and systemic factors that may regulate the activity of a specific transcription factor [16, 19, 27]. TGF-β1 is known to play an important role in regulating and stimulating the differentiation of osteoprogenitors during fracture repair [28] and has been described as responsible, among other growth factors, for the biostimulatory effect on osteoblast cells of various treatments [29, 30]. The MG63 human osteosarcoma line was selected as osteoblast model in this study because it is the most widely used cell line in studies on the effects of drug or other treatments on the osteoblast [31–33], although the limitations of studying a tumor line should be borne in mind.

The three BPs investigated in the present study stimulated osteoblast growth as a function of the dose and treatment duration, observing the highest proliferation rates at the lowest concentrations. These results are consistent with previous

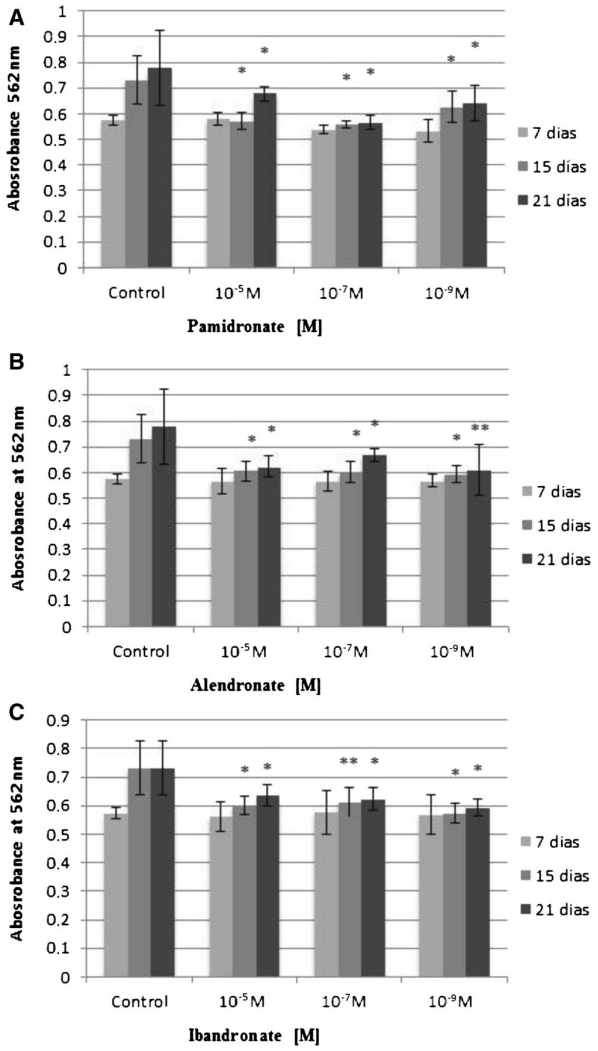

Fig. 5 Quantitative study of the mineralization of MG63 cells (nodule formation) as a function of treatment duration after culture in osteogenic medium supplemented with pamidronate (**a**), alendronate (**b**), or ibandronate (**c**) at doses of 10^{-5}, 10^{-7}, or 10^{-9} M.*$p<0.05$;**$p<0.001$

in vitro findings that BPs exert a pro-osteoblastogenic effect at lower concentrations (10^{-5} to 10^{-9} M) but an inhibitory effect at higher concentrations [34–37]. In common with previous reports, the present results suggest that BPs exert a beneficial effect on the osteoblast by enhancing its proliferative capacity, although account should be taken of the inverse dose-rate effect observed at the assayed doses.

The differentiation and maturation of osteoblasts involve the synthesis of specific bone proteins (e.g., ALP) that contribute to the synthesis of the extracellular matrix followed by its mineralization. The effects of BPs on this differentiation and maturation process are controversial, with some studies finding that BPs reduce ALP synthesis [38] and others suggesting that low doses of nitrogen-containing BPs increase ALP activity and nodule formation. In the present study, low doses of BPs reduced ALP activity and nodule formation [34, 35, 39], indicating an inhibitory effect on the differentiation and maturation of the osteoblast as bone-forming cell.

In addition, the BP treatment modulated the expression of antigens involved in the immunologic function of the osteoblast. Thus, 24 h of treatment produced a significant decrease in CD54, CD80, and HLA-DR antigens, whose expression is elevated in the MG63 cell line, although its effect on CD86 expression was variable and depended on the BP type and dose. No published data are available on the effects of BPs on the osteoblastic expression of these biomarkers.

CD54 expression on pre-osteoblasts and immature osteoblasts is elevated and depends on the degree of cell differentiation and maturation [20]. However, its expression can be modulated in the presence of various substances, notably cytokines, growth factors, platelet-rich plasma, bacterial lipopolysaccharide (LPS), and certain pharmaceuticals [19, 40–43]. In in vitro studies, human osteoblasts obtained by primary culture from bone samples showed a significantly reduced expression of CD54 and CD86, with no change in their expression of CD80 or HLA-DR after TGFβ1 treatment; and no change in these molecules after treatment with FGFb, PDGF-BB, or IL-2 but a significant increase in their expression after treatment with IL-1β, IFNγ, and LPS [19]. These data, alongside findings on the expression of cytokines (IL-4, IL-12, IL-15, IL-18, and IFNγ) in the osteoblast and their modulation by different factors (FGF, TGFβ1, and PDGF) and cytokines (IL-1 and IFNγ), suggest that the functional capacity of osteoblasts is modified during their differentiation and maturation, with a gain in their bone-forming function at the expense of their immunological function.

Naidu et al. [44] reported that TGF-β1 expression was increased by BPs, and others have found that this growth factor enhances proliferation and osteoblast differentiation in its initial phase but inhibits its differentiation and maturation and suppresses matrix mineralization in later phases [45, 46]. In the present study, the three BPs increased osteoblast proliferation and reduced ALP synthesis and mineralization nodule formation in inverse relation to their dose, which may be explained by the BP-induced increase in TGF-β1 synthesis, which would in turn be consistent with the reduced expression of co-stimulatory molecules in the presence of this growth factor. Nevertheless, under physiologic conditions, this inhibition of osteoblast differentiation would imply an increased expression of these markers (CD54, CD80, CD86, and HLA-DR), but this was not observed in the presence of these BPs. It should be borne in mind that TGF-β1 exerts its action via three receptors (types I, II, and III), and there is a need to investigate the effect of BPs on the expression of these receptors in order to improve our understanding of the response of osteoblasts in their presence.

On the other hand, numerous studies have associated mandibular osteonecrosis with BP treatment [11, 12, 47, 48]. The

underlying mechanism has not been elucidated, but the lack of vascular supply or bone remodeling and/or regeneration has been implicated as possible causes. Bone tissue loses its capacity to remove bone areas that are unhealthy or have completed their useful life, limiting or reducing its functional adaptation capacity [49].

The BPs most frequently related to BRONJ are those that contain nitrogen [11, 12, 50, 51], which were assayed in the present study. These drugs bind strongly to hydroxyapatite crystals. Although all BPs share the same phosphorus-carbon-phosphorus core, distinct BPs differ markedly in their affinity for hydroxyapatite binding, and this may play an important role in determining their duration of action [52]. The lesser or greater binding of the BP to hydroxyapatite as a function of the type of BP may explain the different degrees of adverse effects on bone tissue exerted by these drugs in long-term treatments.

In physiologic conditions, the osteoblast loses its immunologic capacity in favor of its bone-forming capacity. Thus, the differentiation and maturation of the osteoblast imply a reduced expression of markers of T cell activation, cytokine synthesis, and phagocyte capacity with an increase in the synthesis of bone morphogenetic proteins and in the mineralization of the extracellular matrix. However, the osteoblast loses its bone-forming capacity in the presence of BPs through their inhibition of its differentiation and maturation. In parallel, this inhibition of differentiation would lead to an increased expression of co-stimulating molecules, which is related to its functional capacity as an immune cell, although BPs inhibit the expression of these molecules, as evidenced in the present study.

In conclusion, the results of the present study suggest that low doses of amino-BPs exert their effect on osteoblasts by altering their physiology, which would explain the impaired repair capacity of the cell population; however, further studies are necessary to determine the mechanisms by which this effect takes place in order to understand the development of BRONJ. It would also be of interest to study the effects of these BPs on primary osteoblast culture lines or other typed osteoblast lines in order to confirm the effects of these drugs on the osteoblast.

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

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THE EFFECT OF CLODRONATE ON ANTIGENIC PROFILE, GROWTH AND DIFFERENTIATION OF OSTEOBLAST-LIKE CELLS

Abstract

Purpose: To evaluate the role of osteoblasts in bisphosphonate-related osteonecrosis of the jaw (BRONJ) by studying the effects of different concentrations of clodronate, a non-nitrogen-containing bisphosphonate, on osteoblast growth, differentiation, and antigenic profile.

Methods*:* Osteoblasts were incubated in culture medium with different doses of clodronate. Their proliferative capacity was determined with a spectrophotometric technique (MTT). Flow cytometry was used to study the antigenic profile. Cell differentiation was evaluated by the study of nodule formation and alkaline phosphatase (ALP) activity was measured by spectrophotometric assay.

Results: Clodronate had a significant stimulatory effect on osteoblast-like cells (MG-63) proliferation ($p<0.05$). A significant decrease in the expression of CD54, CD80, CD86 and HLA-DR membrane antigens *versus* controls was observed after 24 h of treatment with the different clodronate doses assayed ($p<0.05$). A significant decrease ($p=0.004$) in ALP activity was found after 24h of treatment with the lowest dose (10^{-9} M), and a significant decrease in calcium deposition was found after 15 and 21 days of treatment ($p<0.05$).

Conclusion: Non-nitrogen-containing bisphosphonates such as clodronate increase the proliferation of MG-63 osteoblast-like cells and decrease their differentiation capacity, generally at low doses, and modulate the expression of co-stimulatory molecules associated with immune function. Clodronate exerts its effect on osteoblasts by altering their physiology and impairing their repair capacity, which may underlie the development of Bisphosphonates-related osteonecrosis of the jaw (BRONJ). However, Further research in

this line is warranted to fully elucidate the mechanisms by which bisphosphonates can produce this disease.

Key terms: Clodronate; osteoblast; proliferation; phenotype; differentiation.

1. Introduction

Bisphosphonates (BPs) are a class of pharmacological agents chemically related to inorganic pyrophosphate, in which the oxygen linking the phosphates has been replaced by carbon; they are mainly used in the treatment of bone disorders (e.g., osteoporosis, Paget´s disease, multiple myeloma, malignant hypercalcaemia, etc.) (1). There are two main types of BPs with different molecular action mechanisms: non-nitrogen-containing BPs and nitrogen-containing BPs. Non-nitrogen-containing BPs are used in the treatment of osteoporosis and act by reducing bone resorption and potentially increasing bone mass, through their well-documented ability to inhibit osteoclast formation *in vitro and in vivo* (2, 3). Clodronate, a widely used non-nitrogen-containing bisphosphonate in osteoporosis treatment, suppresses bone resorption by incorporating non-hydrolysable ATP analogues with no releasable energy content into osteoclasts, producing their death. In contrast, nitrogen-containing BPs (e.g., alendronate, pamidronate, or ibandronate) interrupt the mevalonate pathway of cholesterol synthesis by inhibiting the enzyme farnesyl diphosphate synthase and blocking the prenylation of small GTPases, thereby altering the function of osteoclasts (4). However, the action mechanisms of BPs on bone and their effects on osteoblasts are not fully understood. Recent studies suggested that the inhibition of bone resorption and osteoclast recruitment may be due, at least in part, to a direct action of BPs on the osteoblast (5, 6).

Although useful in the treatment of some bone diseases, BPs have also been associated with the development of bisphosphonate-related osteonecrosis of the jaw (BRONJ). Numerous hypotheses have been proposed to explain the physiopathology of this clinical condition, including: a decrease in bone turnover and subsequent accumulation of microfractures; a toxic effect on osteoblasts; an antiangiogenic effect, producing avascular necrosis; and a reduction in the viability of fibroblasts and oral keratinocytes (7, 8). Examination of the effects of BPs on osteoblasts may provide a better understanding of the role of these cells in BRONJ.

The proliferation and differentiation of osteoblasts are complex processes that play a key role in bone physiology and are regulated by a large number of paracrine, autocrine, and endocrine factors, including growth factors, hormones, and cytokines (9, 10). The main function of osteoblasts is to synthesize bone matrix components and control the bone-resorbing activity of osteoclasts, although they were recently proposed to have additional functions related to the immune system, including phagocytic activity, T lymphocyte stimulation, and cytokine synthesis (9). Osteoblasts are also characterized by an unusual antigenic phenotype, with the expression of antigens such as CD54, CD80, CD86, and HLA-DR. Their antigenic profile is modulated by the presence of different cytokines and growth factors (11).

The objective of this study was to explore the role of osteoblasts in BRONJ by studying the effect of different concentrations of a non-nitrogen-containing bisphosphonate (clodronate) used in the treatment of osteoporosis on osteoblast growth, differentiation, and antigenic profile, with the MG-63 cell line as osteoblast model.

2. Materials and Methods

A non-nitrogen-containing bisphosphonate, clodronate (Sigma Chem. Comp., St. Louis, MO), was selected for this study.

2.1. Cell culture

The human MG-63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA). This cell line is commonly used as an osteoblast model because it shares the same characteristics. MG-63 cell line was maintained as described by Diaz-Rodríguez et al. (2009)(11) in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 μg/mL gentamicin (Braum Medical SA, Jaen, Spain), 2.5

μg/mL amphotericin B (Sigma), 1% glutamine (Sigma), and 2% HEPES (Sigma), supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, UK). Cultures were kept at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. Cells were detached from the culture flask with a solution of 0.05% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma) and then washed and suspended in complete culture medium with 10% FBS.

2.2. Cell proliferation assay

Osteoblasts were seeded at 1 x 10^4 cells/mL per well into a 24-well plate (Falcon, Becton Dickinson Labware, NJ) and cultured at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. Next, the medium was replaced with DMEM containing clodronate at a dose of 10^{-5}, 10^{-7}, or 10^{-9} M. After 24 or 48h of culture, cell proliferation was measured by MTT assay, as described by Manzano-Moreno et al. (2013)(12), replacing media with phenol red-free DMEM containing 0.5 mg/mL MTT (Sigma) and incubating for 4 h. Cellular reduction of the MTT tetrazolium ring resulted in the formation of a dark-purple water-insoluble deposit of formazan crystals. After incubation, the medium was aspirated, and dimethyl sulfoxide (DMSO, Merck Biosciences, Darmstadt, Germany) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (Sunrise^{TM}, Tecan, Männedorf, Switzerland). Results were reported as mean absorbance ± standard deviation (SD). At least three experiments were conducted for each treatment, using the mean value in the analysis.

2.3. Antigenic phenotype by flow cytometry

Osteoblasts were previously treated with 10^{-5}, 10^{-7}, or 10^{-9} M clodronate for 24 h at 37ºC. Cells were then detached from the cultured flask by treatment with 0.4% (w/v) EDTA solution, washed, and suspended in phosphate-buffered saline (PBS) at 2 x 10^4 cells/mL. Cells were labeled by direct staining with anti-CD54, CD80, CD86, and HLA-DR

monoclonal antibodies (MAbs) (CD54/IOL1b, CD80, CD86, and OKDR, respectively; all from Invitrogen Corp, Carlsbad, CA). Aliquots of 100 µL of cell suspension were incubated with 10 µL of the appropriate MAb for 30 min at 4ºC in the dark. Cells were washed, suspended in 1 mL of PBS, and immediately analyzed in a flow cytometer with diode laser (FACSCanton II, Becton Dickinson, Palo Alto, CAL) at a wavelength of 488 nm to determine the percentage of fluorescent cells. Untreated cells were used as controls. The percentage of antibody-positive cells was calculated from counts of 2000-3000 cells. At least three experiments were run for each antigen in all cultures.

2.4. Alkaline phosphatase (ALP) activity

The effect on osteoblast-like cell differentiation was determined by evaluating the ALP activity of MG-63 cells cultured in non-osteogenic medium and treated with clodronate. ALP activity was quantified by using a colorimetric assay (Diagnostic kit 104-LL, Sigma) to determine early osteoblastic differentiation. The assay measures the conversion of the colorless substrate *p*-nitrophenylphosphate by the enzyme ALP to the yellow product *p*-nitrophenol, with the rate of color change corresponding to the amount of enzyme present in solution. Standards of *p*-nitrophenol (0-250 µM) were prepared from dilutions of a 1000 µM stock solution and assayed in parallel. Cells in non-osteogenic medium with 10^{-5}, 10^{-7}, or 10^{-9} M of clodronate or without drug (control group) were seeded at 1 x 10^4 cells/mL per well into 24-well plates and cultured for 24 h under standard conditions. Then, cells were lysed with 0.1% (v/v) Triton X-100 at 37ºC. Samples were centrifuged at 1500 rpm and the supernatants stored at -70ºC until used. ALP activity was determined with p-nitrophenylphosphate as substrate. An aliquot (50µL) of cell lysate solution was added to 50µL of ALP substrate (Sigma) and then incubated at 37ºC for 45min in darkness. The enzymatic reaction was stopped by adding 50µL of 0.1M NaOH, and the absorbance was measured at 405 nm with a spectrophotometer (Biotek ELx800). Total protein content was estimated by the Bradford method using a protein assay kit from Bio-Rad Laboratories

(Nazareth-Eke, Belgium). All samples were run in triplicate, and the ALP activity was expressed as a percentage, considering enzymatic activity in the absence of clodronate as 100%.

2.5. Nodule formation and matrix mineralization

The presence of calcium deposits in the cell matrix was analyzed by the Alizarin Red S method. MG-63 cells were seeded (5 x 10^4 cells/mL/well) in a 6-well plate and cultured in osteogenic medium with different concentrations of clodronate at 37ºC in a humidified atmosphere of 95% air and 5% CO_2. The medium was replaced after 4 days and then every 3 days. The matrix mineralization of each cell line was measured at 7, 15, and 22 days of culture. Wells were washed with 150 mM sodium chloride, fixed in cold 70% ethanol for 5 min, and rinsed three times with distilled water. Next, wells were incubated for 10 min with 1 mL of 2% (w/v) Alizarin Red S solution buffered at pH 4 with sodium hydroxide, then rinsed five times with distilled water and finally washed with PBS to reduce non-specific staining. Calcium deposits present in the extracellular collagen matrix were colored red, revealing mineralization nodules, which were detained for 15 min with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured with a plate/reader spectrophotometer (Biotek ELx800).

2.6. Statistical analysis

SPSS 22.0 (IBM, Chicago, IL) was used for all data analyses. Mean values (±SD) were calculated for each variable. A two-way repeated-measures analysis of variance (ANOVA) was performed to examine the effects on proliferation, nodule formation, and ALP synthesis as a function of the treatment duration and concentration. When a significant interaction was identified, the Bonferroni correction was applied for planned pair-wise comparisons. The

antigenic profile was compared by using the Student's t-test. $P< 0.05$ was considered significant. At least three experiments were performed for each assay.

3. Results

3.1. Cell proliferation assay

As shown in figure 1, clodronate stimulated the osteoblast proliferation capacity as a function of the dose and treatment duration. At 24 h of culture, a significant increase in proliferation was only observed at the lowest clodronate dose (10^{-9} M) (p=0.024). At 48 h of culture, however, a significant increase (p<0.001) was found at all assayed doses (10^{-5}, 10^{-7}, and 10^{-9} M); the increase was inversely related to the dose.

3.2. Antigenic phenotype by flow cytometry

Flow cytometry results in figure 2 show that treatment with 10^{-5}, 10^{-7}or 10^{-9} M clodronate significantly decreased the expression of CD54, CD80, CD86 and HLA-DR membrane antigens in comparison to untreated control MG-63 cells at 24 h of culture (p<0.05).

3.3. ALP activity

Results in figure 3 show a significant decrease in the expression of this differentiation marker *versus* control cells at the lowest clodronate assayed dose (10^{-9} M) after 24 h of treatment (p=0.004).

3.4. Nodules formation and matrix mineralization

Figure 4 depicts the effects of clodronate on nodule mineralization, showing the colorimetrically-measured Alizarin Red staining at 7, 15, and 21 days of culture in osteogenic medium with 10^{-5}, 10^{-7}, or 10^{-9} M clodronate. There were no significant differences between treated cells and untreated (control) cells at 7 days of culture. At 15 and 21 days of culture, however, the calcium deposition was significantly reduced (p<0.05) in treated *versus* untreated cells at all concentrations tested.

4. Discussion

The results of this *in vitro* study demonstrate that therapeutic doses of clodronate increase the growth capacity of osteoblasts, inhibit their differentiation and mineralization, and modulate their antigenic profile. This may imply major changes in the complex physiology of the osteoblast, which is regulated by multiple local and systemic factors that can regulate the activity of a specific transcription factor (13).

Clodronate stimulated osteoblast growth as a function of the dose and treatment duration, showing the highest proliferation rates at the lowest concentrations. These results are consistent with previous *in vitro* findings that BPs exert a pro-osteoblastogenic effect at lower concentrations (10^{-5} M to 10^{-9} M) but an inhibitory effect at higher concentrations (14, 15).

The differentiation and maturation of osteoblasts involves the synthesis of specific bone proteins e.g., ALP, which contribute to the synthesis of the extracellular matrix and its subsequent mineralization. The effects of BPs on these differentiation and maturation process are controversial, with some studies finding that BPs reduce ALP synthesis (16), and others suggesting that low doses of these drugs increase ALP activity and nodule formation. In the present study, low doses of clodronate reduced ALP activity and nodule formation (14, 17), indicating an inhibitory effect on the differentiation and maturation of the osteoblast as bone-forming cell. The inhibition of ALP synthesis was accompanied by a higher osteoblast growth rate in the presence of the drug, which may be related to the greater growth capacity of the less-differentiated cells.

In addition, the BP treatment modulated the expression of antigens involved in the immunologic function of the osteoblast. Thus, 24 h of treatment produced a significant decrease in CD54, CD80, CD86 and HLA-DR antigens, whose expression is positive in the MG-63 cell line. This effect also has been observed in treated osteoblasts with nitrogen-containing BPs (15). CD54 expression on pre-osteoblasts and immature osteoblasts is

elevated and depends on the degree of cell differentiation and maturation. However, its expression can be modulated in the presence of various substances, notably cytokines, growth factors, platelet-rich plasma, bacterial lipopolysaccharide (LPS), and certain pharmaceuticals (18, 19). In *in vitro* studies, human osteoblasts obtained by primary culture from bone samples showed a significantly reduced expression of CD54 and CD86 after transforming growth factor- β1 (TGF-β1) treatment. TGF-β1 is an autocrine factor that regulates osteoblast proliferation and differentiation (20) and exerts its action *via* three receptors (types I, II, and III). It increases the proliferation and differentiation of osteoblasts in initial phases of treatment but inhibits their differentiation and maturation and suppresses matrix mineralization in subsequent phases (21); it also reduces the expression of CD54 and CD86 (19). Naidu et al. (2008)(22) reported that TGF-β1 expression was increased by BPs. There is need to investigate the effect of BPs on the expression of these receptors in order to improve understanding of the response of osteoblasts to their presence.

The MG-63 human osteosarcoma line was selected as osteoblast model in this study because it is the most widely used cell line in studies on the effects of drug or other treatments on the osteoblast, although the limitations of studying a tumor line should be borne in mind, as the differentiation pattern of MG-63 may differ from that of real osteoblasts.

The osteoblast is a complex cell characterized by its active participation in bone metabolism, being responsible for bone formation and regeneration and for the regulation of osteoclast activity. It also possesses immunologic functions, including the synthesis of cytokines, expression of antigens involved in antigen presentation, allogenic stimulation, and phagocytic capacity (9, 11, 13, 19). In physiologic conditions, the osteoblast loses its immunologic capacity in favor of its bone-forming capacity. Our results show that the osteoblast loses its bone-forming capacity in the presence of clodronate through their inhibition of its differentiation and maturation. The inhibition of differentiation would lead

to an increased expression of co-stimulating molecules, which is related to its functional capacity as an immune cell, although clodronate decreases the expression of these molecules. This change in the physiology of the osteoblast may be related to the expression of TGF-β1 receptors and the adverse effect of BPs on bone tissue.

Since 2003, numerous studies have associated mandibular osteonecrosis with BP treatment (23, 24). One of the most serious complications of BP therapy is BRONJ, defined by the American Association of Oral and Maxillofacial Surgeons as exposed bone in the maxillofacial bone that persists for > 8 weeks in patients under current or previous BP therapy with no history of head/neck radiation (25). Despite the fast-rising in BRONJ cases, the underlying mechanism has not yet been determined, although a lack of vascular supply and absence of bone remodeling/regeneration have been implicated. Bone tissue loses its capacity to remove bone areas that are unhealthy or have completed their useful life, limiting or reducing its functional adaptation capacity (26). In addition, as we have shown in this paper, clodronate promotes important changes in the physiology of the osteoblast, which may be related with the development of BRONJ.

Although the BPs most frequently related to BRONJ are those that contain nitrogen (23, 24), BRONJ has also been associated with the administration of non-nitrogen-containing BPs such as clodronate (27), which bind strongly to hydroxyapatite crystals. Although all BPs share the same phosphorus-carbon-phosphorus core, they markedly differ in their affinity for hydroxyapatite binding, which may play an important role in determining the duration of their action (28). The lesser or greater binding of the BP to hydroxyapatite as a function of the type of BP may explain the different degrees of adverse effects on bone tissue exerted by these drugs in long-term treatments. Non-nitrogen-containing BPs are less potent and bind more weakly to hydroxyapatite, and their action mechanism on the osteoclast involves different pathways; however, both types of BP can produced BRONJ

after long-term treatments. The present results and published data on nitrogen-containing BPs (29, 30) show that both types affect the physiology of the osteoblast.

In conclusion, the present findings suggest that clodronate exerts its effect on osteoblasts by altering their physiology and impairing their repair capacity, which may underlie the development of BRONJ. Further research in this line is warranted to fully elucidate the mechanisms by which BPs can produce this disease.

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FIGURES

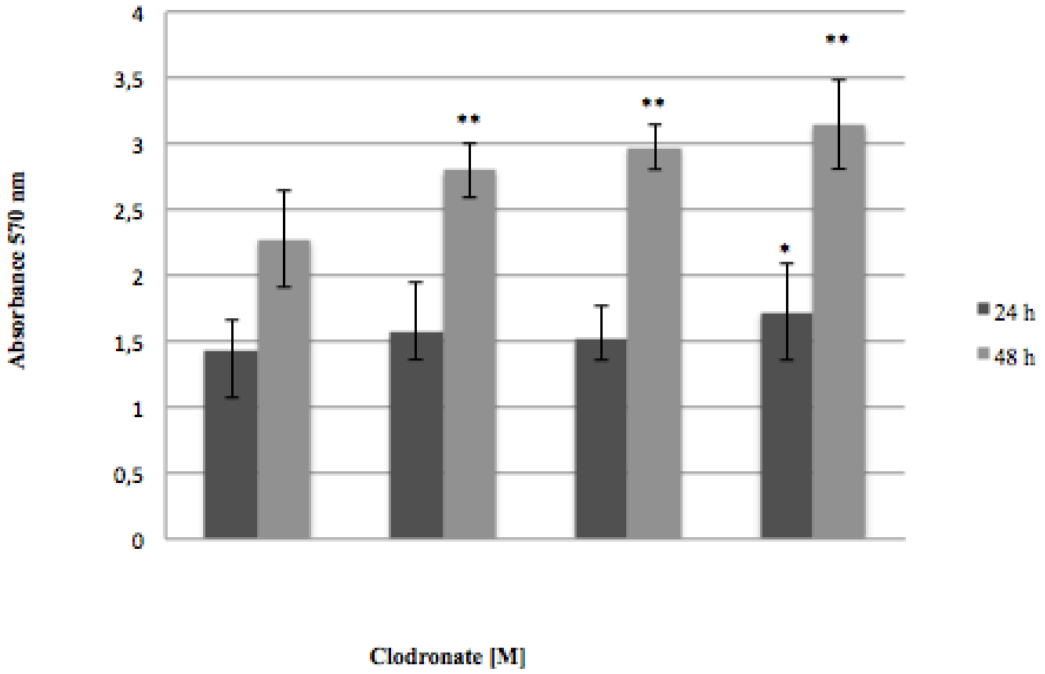

Fig. 1. Effects of clodronate at different doses (10^{-5} M, 10^{-7} M, 10^{-9} M) on osteoblast proliferation (in MG-63 cell line) at 24 h and 48 h of incubation. Data are expressed as means ± SD. We compared data between each treatment and control culture by analysis of variance (ANOVA). *$P<0.05$; ** $P<0.001$

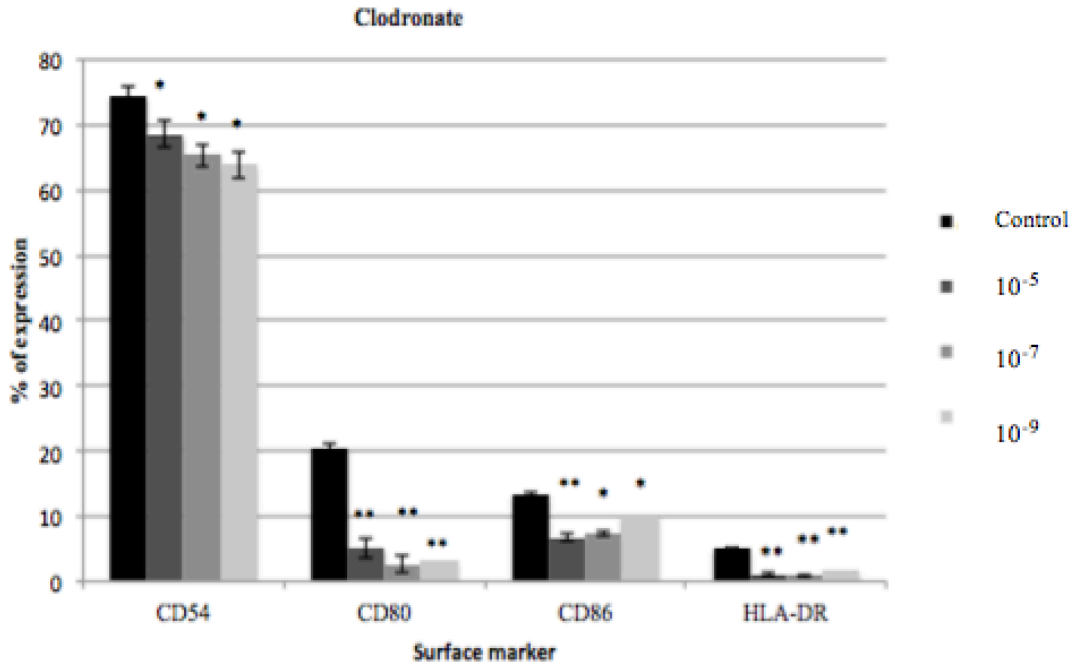

Fig. 2. Percentage expression of different surface markers on osteoblasts (MG-63 cells) treated for 24h with 10^{-5} M, 10^{-7} M, or 10^{-9} M clodronate. *p<0.05; **p<0.001

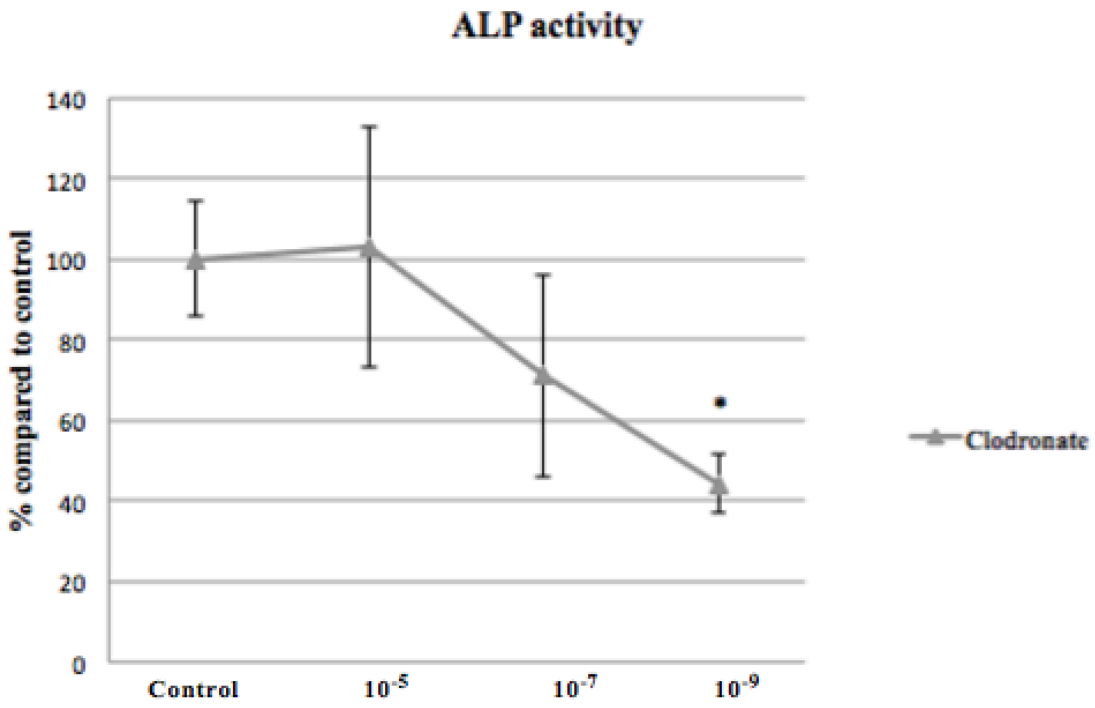

Fig. 3. ALP activity of MG-63 cell line after 24h treatment with clodronate at doses of 10^{-5} M, 10^{-7} M and 10^{-9} M in non-osteogenic medium, expressed as a percentage of enzymatic activity respect to control (in absence of clodronate). *$p< 0.05$

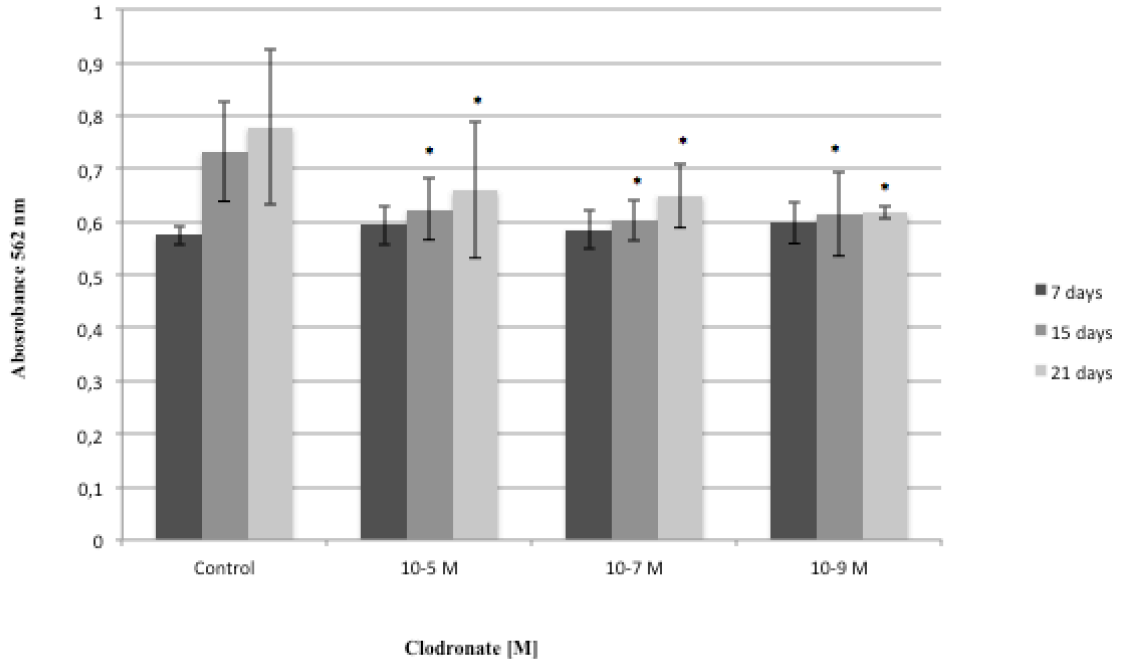

Fig. 4. Quantitative study of mineralization (nodule formation) of MG-63 cells cultured in osteogenic medium supplemented with clodronate (10^{-5} M, 10^{-7} M and, 10^{-9} M) as a function of time.*$p< 0.05$

HIGH DOSES OF BISPHOSPHONATES REDUCE OSTEOBLAST-LIKE CELL PROLIFERATION BY ARRESTING THE CELL CYCLE AND INDUCING APOPTOSIS

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High doses of bisphosphonates reduce osteoblast-like cell proliferation by arresting the cell cycle and inducing apoptosis

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ABSTRACT

Objectives: The study objective was to evaluate the effect on osteoblast growth of high concentrations of three nitrogen-containing bisphosphonates (pamidronate, alendronate, and ibandronate) and one non-nitrogen-containing bisphosphonate (clodronate), using the MG-63 cell line as an osteoblast model, in order to determine the role of osteoblasts in bisphosphonate-related osteonecrosis of the jaw (BRONJ).
Materials and methods: Osteoblasts were incubated in culture medium with different doses of pamidronate, alendronate, ibandronate or clodronate. The proliferative capacity of the osteoblasts was determined by spectrophotometry (MTT-based) at 24 h of culture. Flow cytometry was used to determine the percentage of cells in each cell cycle phase (G0/G1, G2/M, and S) and to discriminate apoptotic cell death from necrotic cell death in the cell cycle at 24 h of treatment.
Results: All the bisphosphonates assayed produced a significant and dose-dependent reduction in MG-63 proliferation at the high doses assayed (10^{-4} and 5×10^{-5} M) in comparison with controls ($p < 0.001$). Cell cycle study revealed that all assayed bisphosphonates significantly arrested the cell cycle in phase G0/G1 at doses of 10^{-4} and 5×10^{-5} M, increasing the percentage of cells in this phase ($p < 0.05$). Apoptosis/necrosis studies showed significant changes compared with control cells, with an increased percentage of cells in apoptosis after treatment with 10^{-4} or 5×10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate ($p < 0.05$).
Conclusions: High doses of nitrogen-containing or non-nitrogen-containing bisphosphonates can reduce the proliferation of MG-63 osteoblast-like cells by arresting the cell cycle and inducing apoptosis/necrosis.

1. Introduction

Bisphosphonates (BPs) are a family of pyrophosphate synthetic analogues in which the oxygen linking the phosphates has been replaced by carbon. They are widely used to treat bone disorders, including osteoporosis, Paget's disease, hypercalcemia of malignancy, fibrous dysplasia, and the bone metastases of breast and prostate cancer (Ralston et al., 1989; Eggelmeijer et al., 1994; Lala et al., 2000; Rodan and Martin, 2000; Lane et al., 2001). BPs can be divided between nitrogen-containing and non-nitrogen-containing forms. Nitrogen-containing BPs, such as pamidronate, alendronate, or ibandronate, interrupt the mevalonate pathway of cholesterol synthesis, inhibiting the enzyme farnesyl diphosphate synthase and blocking prenylation of small GTPases, leading to the impairment of osteoclast function (Lane et al., 2001; Reszka and Rodan, 2004). For their part, non-nitrogen-containing BPs such as clodronate suppress bone resorption by being metabolized into non-hydrolysable ATP analogues that have no releasable energy content, producing osteoclast death (Frith et al., 1997, 2001).

BPs bind to hydroxyapatite crystal but vary in the strength of their binding, which may play an important role in the duration of their action. Differences in the severity of adverse effects on bone

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tissue in long-term BP treatment have been attributed to the lesser or greater binding of the different BPs to hydroxyapatite (Nancollas et al., 2006; Idris et al., 2008). According to some authors, although therapeutic doses are not very high (10^{-5} to 10^{-9} M) (Chen et al., 2002), very high concentrations may reach the bone after prolonged treatment because of the strong binding of these drugs to hydroxyapatite (Marx, 2014).

BPs are widely prescribed, and their ability to inhibit osteoclast formation and activity in vitro and in vivo is well documented (Russell, 2007; Silverman and Maricic, 2007). However, the mechanisms underlying their action on bone and their effects on osteoblasts are not fully understood, although various proposals have been made, including: a decrease in bone turnover and subsequent accumulation of microfractures; a toxic effect on osteoblasts; a decrease in collagen production of osteoblasts (Açil et al., 2012); an anti-angiogenic effect producing avascular necrosis; and a reduction in the viability of fibroblasts and oral keratinocytes (Santini et al., 2002; Mashiba et al., 2005; Landesberg et al., 2008). Although useful in the treatment of some bone diseases, BPs have also been associated with the development of BP-related osteonecrosis of the jaw (BRONJ) (Marx, 2003; Ruggiero et al., 2004). Probably, the infectious conditions that often precede the onset of BRONJ support recent pathogenesis theories stating that local inflammation and associated pH-changes may trigger the release and activation of nitrogen-containing bisphosphonates ultimately resulting in necrosis (Otto et al., 2012).

The objective of this study was to evaluate the effect on osteoblast growth of high concentrations of three nitrogen-containing BPs (pamidronate, alendronate, and ibandronate) and one non-nitrogen-containing BP (clodronate), using the MG-63 cell line, in order to determine the role of osteoblasts in BRONJ. The MG-63 cell line is commonly used as an osteoblast model because it shares the same characteristics.

2. Materials and methods

We studied three nitrogen-containing BPs, pamidronate (Sigma–Aldrich, St. Louis, MO, USA), alendronate (Sigma), and ibandronate (Sigma), and one non-nitrogen-containing BP, clodronate (Sigma).

2.1. Cell culture

Human MG-63 osteosarcoma cell line was purchased from American Type Cultures Collection (ATCC, Manassas, VA, USA) and maintained as described by De Luna-Bertos et al., 2013, in Dulbecco's modified Eagle medium (DMEM; Invitrogen Gibco Cell Culture Products, Carlsbad, CA, USA) with 100 IU/mL penicillin (Lab Roger SA, Barcelona, Spain), 50 μg/mL gentamicin (Braum Medical SA, Jaen, Spain), 2.5 μg/mL amphotericin B (Sigma), 1% glutamine (Sigma), and 2% HEPES (Sigma), supplemented with 10% fetal bovine serum (Gibco, Paisley, UK). Cultures were kept at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were detached from the culture flask with a solution of 0.05% trypsin (Sigma) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma) and were then washed and suspended in complete culture medium with 10% fetal bovine serum.

2.2. Cell proliferation assay

Osteoblasts were seeded at 1×10^4 cells/mL per well into a 24-well plate (Falcon, Becton Dickinson Labware, NJ, USA) and cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 24 h. Next, the medium was replaced with DMEM containing pamidronate, alendronate, ibandronate, or clodronate at a dose of 10^{-4}, 5×10^{-5}, or 10^{-5} M. After 24 h of culture, the cell proliferation was measured by MTT assay, as described by Manzano-Moreno et al. (2013), replacing media with phenol red-free DMEM containing 0.5 mg/mL MTT (Sigma) and incubating for 4 h. Cellular reduction of the MTT tetrazolium ring resulted in the formation of a dark-purple water-insoluble deposit of formazan crystals. After incubation, the medium was aspirated, and dimethyl sulfoxide (DMSO, Merck Biosciences, Darmstadt, Germany) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland.) The results were reported as mean absorbance (570 nm) ± SD. At least three separate experiments were conducted for each treatment, using the mean value in the analysis.

2.3. Cell cycle

Cultured human MG-63 cells treated for 24 h with 10^{-4}, 5×10^{-5}, or 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate, or without BP (control), were detached from the culture flask by treatment with a solution of 0.05% trypsin (Sigma) and 0.02% EDTA (Sigma) and were then washed and suspended in PBS and prepared for study of the cell cycle as reported by García-Martínez et al. (2011). The suspension obtained was placed in 200 μL PBS with 2 mL ice-cold 70% ethanol and 30% distilled H_2O and vigorously mixed. Cells were left for at least 30 min in the cold and then harvested by centrifugation and resuspended in 800 μL PBS. Cells were microscopically examined and, if clumped, passed through a 25-gauge syringe needle. Cells were then incubated at 37 °C for 30 min with 100 μL ribonuclease (RNase) (1 mg/mL) and 100 μL propidium iodine (PI). Finally, samples were analyzed by using an argon-ion laser tuned to 488 nm (Facs Vantage, Becton Dickinson, Palo Alto, CA, USA), measuring forward and orthogonal light scatter and red fluorescence, determining both the area and peak of the fluorescent signal.

2.4. Apoptosis and necrosis analysis

The study of apoptosis and necrosis was performed as described by De Luna-Bertos et al. (2014). Osteoblasts were previously treated with 10^{-4}, 5×10^{-5}, or 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate for 24 h at 37 °C. Next, cells were detached from the culture flask, washed, suspended in 300 μL PBS, and then labeled with annexin V and PI (Immunostep S.L., Salamanca, Spain), incubating 100 μL aliquots of the cell suspension with 5 μL annexin V and 5 μL PI for 30 min at 4 °C in the dark. Cells were then washed, suspended in 1 mL PBS, and immediately analyzed in a flow cytometer with argon laser (Facs Vantage, Becton Dickinson) at a wavelength of 488 nm to determine the percentage of fluorescent cells. We calculated the percentage of annexin-positive (apoptotic) cells and PI-positive (necrotic) cells from counts of 2000–3000 cells.

2.5. Statistical analysis

SPSS 22.0 (IBM, Chicago, IL) was used for all data analyses. Mean values (±SD) were calculated for each variable. A two-way repeated-measures analysis of variance (ANOVA) was performed to examine the effects on proliferation, apoptosis/necrosis induction, and cell cycle as a function of the BP type (pamidronate, alendronate, ibandronate, or clodronate), treatment duration, and concentration. When a significant interaction was identified, the Bonferroni correction was applied for planned pair-wise comparisons. $P < 0.05$ was considered significant. At least three separate experiments were performed for each assay.

3. Results

3.1. Cell proliferation assay

Compared with untreated cells (controls), all the BPs studied reduced osteoblast proliferation capacity at doses of 10^{-4} and 5×10^{-5} M in a dose-dependent fashion at 24 h of treatment ($p < 0.001$) (Fig. 1). At a dose of 10^{-5} M, no significant difference was found between treated and untreated cells for any of these BPs.

3.2. Cell cycle

The percentage of cells in each cell cycle phase (G0/G1, G2/M and S) was determined by flow cytometry. Results are depicted in Table 1. No significant effect on the MG-63 cell cycle ($p > 0.05$) was observed after treatment for 24 h with 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate. The percentage of cells in G0/G1 phase was significantly increased by doses of 10^{-4} and 5×10^{-5} M of each BP tested (p values of 0.049 to <0.001). In parallel, the percentage of cells in G2/M phase was significantly reduced by doses of 10^{-4} M of pamidronate and doses of 10^{-4} and 5×10^{-5} M of ibandronate and clodronate.

3.3. Apoptosis and necrosis analysis

Flow cytometry with annexin V and PI labeling were used to discriminate apoptotic cell death from necrotic cell death in the cell cycle. Fig. 2 shows the percentage of viable cells, necrotic cells, and cells in early and late apoptosis after culture for 24 h in the presence of different doses of pamidronate alendronate, ibandronate, or clodronate.

In comparison with controls, pamidronate treatment significantly increased the percentage of apoptotic cells at doses of 10^{-4} and 5×10^{-5} M ($p = 0.011$ and $p = 0.013$ respectively). Alendronate treatment significantly increased the percentage of apoptotic, late apoptotic, and necrotic cells at a dose of 10^{-4} M ($p < 0.001$, $p = 0.001$ and $p = 0.02$ respectively), whereas treatment with 5×10^{-5} M only increased the percentage of apoptotic cells ($p = 0.007$) compared with controls. Treatment with 10^{-4} and 5×10^{-5} M of ibandronate increased the percentage of apoptotic cells ($p = 0.038$, $p = 0.021$, respectively) compared with controls. Finally, treatment with doses of 10^{-4} and 5×10^{-5} M of clodronate significantly increased the percentage of apoptotic and necrotic cells compared with controls ($p < 0.05$). At a dose of 10^{-5} M, no significant differences were found between untreated cells and those treated with any of the BPs.

4. Discussion

The results of this in vitro study demonstrate that high doses of pamidronate, alendronate, ibandronate, or clodronate have a negative effect on osteoblasts, reducing their proliferative capacity by arresting the cell cycle in the G0/G1 phase and inducing apoptosis. This implies major changes in the complex physiology of the osteoblast, which is regulated by multiple local and systemic factors that may regulate the activity of a specific transcription factor (Pérez et al., 2006; Ruiz et al., 2007; Krischak et al., 2007).

The concentrations of BPs used in this study were considerably higher than the therapeutic dose range of 10^{-5} to 10^{-9} M (Chen et al., 2002) but are comparable with the doses that reach the bone in long-term treatment due to the strong binding of these drugs to hydroxyapatite crystals (Marx, 2014). Nevertheless, various in vitro studies have shown that when nitrogen-containing BPs such as zoledronate bind with calcium phosphate, they lose their adverse effect on different cell populations, including osteoblasts (Schindeler and Little, 2005), dental pulp-derived cells (Cvikl et al., 2011), and human fibroblasts from gingiva and periodontal ligament cells (Agis et al., 2010). However, prolonged or localized acidification may favor the release and activation of the drug

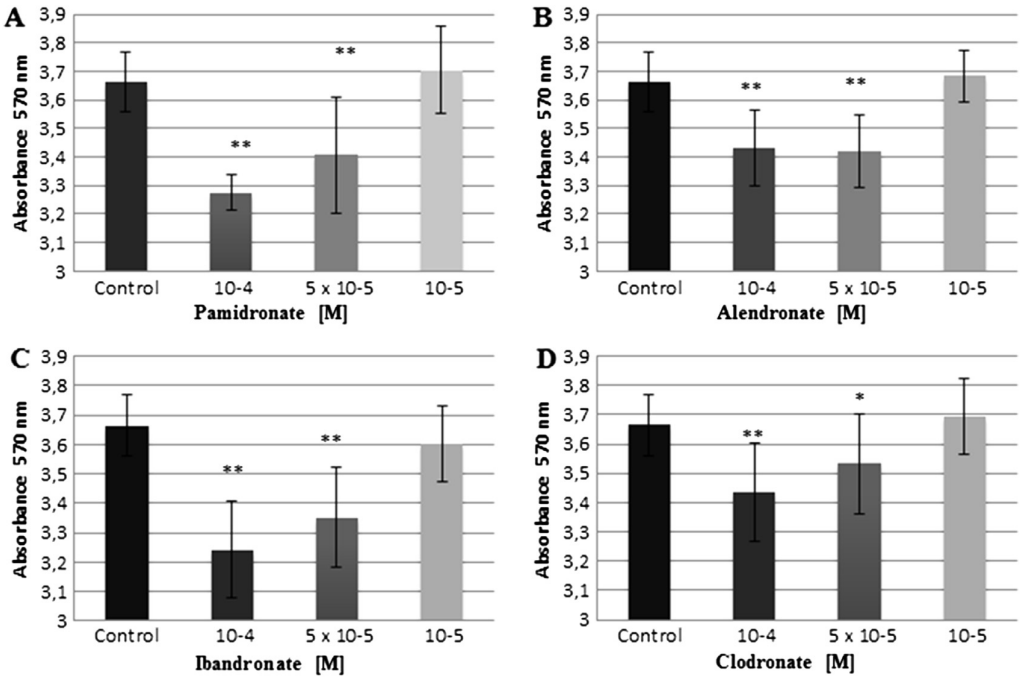

Fig. 1. Effect of four bisphosphonates at different doses (10^{-4}, 5×10^{-5}, 10^{-5} M) on osteoblast proliferation in MG-63 cell line after 24 h of incubation: A) pamidronate; B) alendronate; C) ibandronate; D) clodronate. Data are expressed as means ± SD. *$p < 0.05$; **$p < 0.001$.

Table 1
Percentage of cells in phase G0/G1, G2/M and S of cell cycle MG-63 cells treated for 24 h with 10^{-4}, 5×10^{-5}, or 10^{-5} M of pamidronate, alendronate, ibandronate, or clodronate, or without BP (control).

Treatment	% of cells in phase G0/G1		% of cells in phase G2/M		% of cells in phase S	
	Mean ± SD	*p*-value ≤	Mean ± SD	*p*-value ≤	Mean ± SD	*p*-value ≤
Control	33.07 (0.21)	–	12.46 (0.13)	–	54.45 (0.32)	–
Pamidronate						
10^{-4} M	43.23 (2.53)	**0.001**[a]	6.36 (2.8)	**0.02**[a]	50.41 (15.14)	0.68
5×10^{-5} M	36.78 (0.72)	**0.001**[a]	8.48 (2.68)	0.12	58.73 (2.86)	0.06
10^{-5} M	37.53 (2.48)	0.08	9.83 (2.14)	0.16	52.62 (0.68)	0.089
Alendronate						
10^{-4} M	37.36 (1.27)	**0.03**[a]	10.11 (1.93)	0.16	52.52 (2.13)	0.19
5×10^{-5}M	37.68 (1.74)	**0.04**[a]	12.01 (1.12)	0.56	50.3 (1.2)	**0.005**[a]
10^{-5}M	35.2 (1.25)	0.63	11.68 (0.66)	0.11	53.11 (1.81)	0.32
Ibandronate						
10^{-4} M	38.18 (2.13)	**0.025**[a]	11.09 (0.75)	**0.036**[a]	50.72 (4.96)	0.32
5×10^{-5}M	39.84 (4.24)	**0.049**[a]	9.51 (1.47)	**0.026**[a]	50.64 (5.42)	0.34
10^{-5}M	35.9 (3.7)	0.06	8.46 (3.09)	0.089	48.63 (3.54)	0.1
Clodronate						
10^{-4}M	42.77 (0.9)	<**0.001**[a]	5.54 (1.79)	**0.003**[a]	51.68 (0.89)	**0.007**[a]
5×10^{-5}M	50.91 (2.19)	**0.005**[a]	1.41 (1.67)	<**0.001**[a]	47.67 (2.86)	**0.001**[a]
10^{-5}M	36.28 (0.85)	0.064	8.99 (3.52)	0.053	47.72 (2.91)	**0.023**[a]

[a] Significant differences compared with control.

sequestered in the hydroxyapatite crystals of the bone, thereby activating the cascade of events that contribute to the development of BRONJ. This acidification can be produced by infections, hypoxia, co-medication, or microtraumas, among other causes (Otto et al., 2010a, 2010b).

Our results are in agreement with previous in vitro findings that BPs exert a pro-osteoblastogenic effect at lower concentrations (10^{-5} M to 10^{-9} M) and an inhibitory effect at higher concentrations (Im et al., 2004; Idris et al., 2008; Xiong et al., 2009; Kim et al., 2009) but the mechanisms underlying these effects are poorly understood. We also found that high doses of the studied BPs exerted their inhibitory effect on growth by arresting the cell cycle, inducing apoptosis in the cells, similar to the mechanism reported by Tenta et al. (2006) to underlie the effect on the MG-63 cell line of zoledronate, another nitrogen-containing BP. Furthermore, we observed that both nitrogen-containing and non-nitrogen-containing BPs had a toxic effect on the osteoblast through growth inhibition, although the effect of the non-nitrogen-containing BP was less potent. However, in a previous study by our group, low BP doses produced an increase in osteoblast proliferation in parallel with a reduction in their mineralization capacity (Manzano-Moreno et al., 2014). These findings were

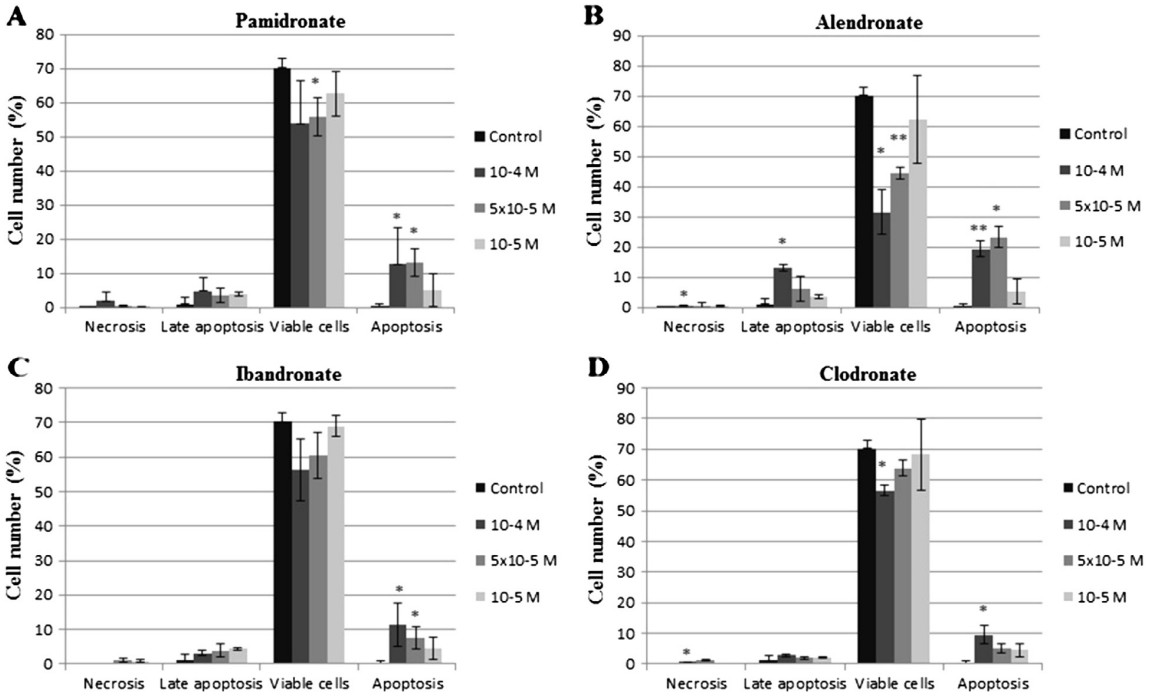

Fig. 2. Percentage of annexin V binding and propidium iodine (PI) uptake of MG-63 cells at 24 h of culture with bisphosphonates at different doses (10^{-4}, 5×10^{-5}, 10^{-5} M): A) pamidronate; B) alendronate; C) ibandronate; D) clodronate. Data are expressed as means ± SD. *p <0.05; **p <0.001.

consistent with the report by Koch et al. (2011) of an increase in osteoblast differentiation after the administration of 10^{-5} M zoledronate or ibandronate, although a reduced expression of differentiation markers was observed at low doses.

Bone is a highly complex tissue in which bone formation and bone remodeling processes go beyond osteoblast proliferation and differentiation and are regulated by a large number of paracrine, autocrine, and endocrine factors, including growth factors, hormones, and cytokines (Eriksen, 2010; Neve et al., 2011). Various authors have reported that BPs can modulate or regulate these factors, thereby affecting the functional capacity of the osteoblast. Thus, Naidu et al. (2008) found that high concentrations of alendronate and zoledronate reduced the viability of osteoblasts but increased TGF-β1 expression. TGF-β1 is known to play an important role in regulating and stimulating the differentiation of osteoprogenitors during fracture repair (Eingartner et al., 1999) and has been described as one of the growth factors responsible for the biostimulatory effect on osteoblast cells of various treatments (Wang et al., 2002; Chen et al., 2004). This growth factor has been found to enhance proliferation and promote osteoblast differentiation in its initial phase but inhibit its differentiation and maturation and suppress matrix mineralization in later phases (Gebken et al., 1999; Balooch et al., 2005). García-Moreno et al. (1998) evaluated the cytotoxic effect of alendronate on primary human osteoblasts and detected no viable cells at 48 h in culture at high concentrations and found a total inhibition of type I collagen synthesis. Pamidronate and zoledronate have also been associated with an acute reaction phase in some patients, who develop fever with an increase in circulating inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α (Kinney et al., 2000; Boskey et al., 2002; Mohammad et al., 2009). It has been established that inflammation can inhibit bone regeneration capacity (Mundy and Bonewald., 1990; Balooch et al., 2005; Alliston, 2006).

The present data and previous findings suggest that high BP concentrations suppress osteoblast growth, inducing apoptosis as a consequence of changes in osteoblast physiology, possibly by preventing their differentiation through inhibition of type I collagen production, a vital component of bone extracellular matrix (Açil et al., 2012). This may result from changes in the expression of one or more of the three main receptors (types I, II, and III) by which TGF-β1 exerts its action on the osteoblast, given that BPs have been reported to increase TGF-β1 synthesis (Naidu et al., 2008; Jia et al., 2013), although the effect of BPs at the level of TGF-β1 receptors is unknown. Moreover, it is possible that the increase in TGF-β1 levels may inhibit osteoblast differentiation and maturation at bone level over the long term.

Numerous studies have associated mandibular osteonecrosis with BP treatment (Marx, 2003; Ruggiero et al., 2004; Boonyapakorn et al., 2008; Walter et al., 2010). The underlying mechanism has not been elucidated, but the lack of vascular supply or bone remodeling and/or regeneration have been implicated as possible causes. Bone tissue loses its capacity to remove bone areas that are unhealthy or have completed their useful life, limiting or reducing its functional adaptation capacity (Yépez-Guillén et al., 2013). In addition, data published by Stockmann et al. (2013) on cancer patients receiving intravenous BPs suggest that MHC class II polymorphisms are genetic risk factors for the development of BRONJ, in line with recent findings on the potential role of inflammation and infection in the pathogenesis of BRONJ.

The BPs most frequently related to BRONJ are those that contain nitrogen (Marx, 2003; Ruggiero et al., 2004; Walter et al., 2007; Marx et al., 2007), although the long-term administration of non-nitrogen-containing BPs such as clodronate can also produce BRONJ and give rise to an elevated accumulation of the drug in bone (Montazeri et al., 2007). The high BP doses reaching the bone in prolonged treatments may have dual adverse effects on the osteoblast: a direct cytotoxic effect, in which the cell dies by apoptosis; and an indirect effect, in which osteoblast differentiation and maturation are inhibited. Thus, the drug accumulated in the bone through its binding to hydroxyapatite can be released and therefore activated, mainly due to local factors such as an infection. High concentrations of the free drug may exert adverse effects on cell populations in the oral cavity, including osteoblasts, hence contributing to the onset of BRONJ (Otto et al., 2010a,b).

One study limitation is that the MG-63 human osteosarcoma cell line was used as the osteoblast model, although this is the most widely used cell line in studies on the effects of drug or other treatments on the osteoblast (Lee et al., 2013; Boanini et al., 2014; Mattioli-Belmonte et al., 2014). It would also be of interest to study the effects of these BPs on primary osteoblast culture lines or other typed osteoblast lines in order to verify the mechanism of action of these drugs on osteoblast growth.

5. Conclusion

In conclusion, the results of this study suggest that high doses of BPs reduce the proliferative capacity of osteoblasts by arresting their cell cycle and inducing apoptosis, explaining the impaired repair capacity of treated cells. Further studies are required to clarify the mechanisms underlying this effect and thereby increase understanding of the development of BRONJ. However, we think that BRONJ is a multifactorial disease in which the type of bisphosphonate and the dose of the drug plays a decisive role.

Conflicts of interest statement

The authors declare that they have no conflict of interest.

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VII. CONCLUSIONES/CONCLUSIONS

CONCLUSIONES

1. La evidencia científica muestra que los BFs son fármacos que ejercen numerosos efectos sobre el ciclo de remodelación ósea. Mientras que su acción sobre los osteoclastos está ampliamente demostrada, su efecto sobre el osteoblasto está aún en discusión.

2. Los BFs nitrogenados (pamidronato, alendronate, e ibandronato) aumentan la proliferación de las células osteoblásticas MG-63, modulan la expresión de moléculas co-estimulatorias asociadas con una función inmune, y disminuyen su capacidad de diferenciación, generalmente a bajas dosis.

3. El clodronato, un BF no nitrogenado con actividad anti-resortiva mediante un mecanismo de actuación diferente al de los BFs nitrogenados, muestra a bajas dosis un efecto sobre el osteoblasto, similar a los BF nitrogenados, aumentando la proliferación de las células osteoblásticas MG-63, disminuyendo su capacidad de diferenciación, y modulando la expresión de moléculas co-estimulatorias asociadas con una función inmune.

4. Altas dosis de BFs nitrogenados y no nitrogenados reducen la proliferación de las células osteoblásticas MG-63 mediante la detención del ciclo celular y la inducción de apoptosis/necrosis.

Conclusión Global

Estos hallazgos indican que los BFs ejercen su efecto sobre el osteoblasto alterando su fisiología, lo cual podría traducirse en una limitación en su capacidad de reparación, hecho que podría estar directamente relacionado con el desarrollo de ONB.

CONCLUSIONS

1. The scientific evidence shows that BPs exert numerous effect on bone remodeling cycle. Although the effect of BPs on osteoclast is well documented, their effects on the osteoblast remains under discussion.

2. Nitrogen-containing BPs (pamidronate, alendronate, and ibandronate) increase the proliferation of MG-63 osteoblast-like cells, modulate their expression of co-stimulatory molecules associated with immune function, and decrease their differentiation capacity, generally at low doses.

3. Clodronate, a non-nitrogen-containing BP with anti-resorptive activity and different action mechanism to that of nitrogen-containing BPs, exerts a similar effect on osteoblast at low doses, increasing the proliferation of MG-63 osteoblast-like cells, decreasing their differentiation capacity, and modulating the expression of co-stimulatory molecules associated with immune function.

4. High doses of nitrogen-containing and non-nitrogen-containing BPs reduce the proliferation of MG-63 osteoblast-like cells by arresting cell cycle and inducing apoptosis/necrosis.

Global Conclusion

These findings suggest that BPs exert their effect on osteoblasts by altering their physiology, which would explain the disruption of their repair capacity and may be directly related to the development of BRONJ.