

In Vitro Osteogenic Performance of Bonelike[®] Modulated by Tetracyclines

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.... to Sara

*The ordinary scientific man is strictly a sentimentalist.
He is a sentimentalist in this essential sense, that he is soaked and
swept away by mere associations.*

Gilbert K. Chesterton

Contents

Acknowledgements	v
Publications	vi
Abstract	vii
Resumo	ix
Aim and structure	xi
Foreword	xiii
Chapter 1 – Introduction	1
1. Bone biology	2
2. Bone grafts	10
3. Antimicrobial chemotherapy	13
4. Tetracyclines	14
Chapter 2 - Osteoblastic response to tetracyclines	28
Chapter 3 - Osteoblastic response to tetracyclines in seeded hydroxyapatite and Bonelike®	48
Chapter 4 – General conclusions	66

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Publications

Full papers in SCI journals:

1 – Gomes PS, Fernandes MH. Effect of therapeutic levels of doxycycline and minocycline in the proliferation and differentiation of human bone marrow osteoblastic cells. *Archives of Oral Biology* 2007;52:251-259.

2 – Gomes PS, Santos JD, Fernandes MH. Cell-induced response by tetracyclines on human bone marrow colonized hydroxyapatite and Bonelike®. *Acta Biomaterialia* 2008. doi:10.1016/j.actbio.2007.12.006

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1 - Gomes PS, Fernandes MH. Osteoinductive effect of tetracyclines – in vitro biological evaluation. *Experimental Pathology and Health Sciences* 2007;1:23.

2 - Gomes PS, Santos JS, Fernandes MH. Cell induced response by tetracyclines on human bone marrow colonized Bonelike®. *European Cells and Materials* 2007;14 sup1:64.

Abstract

Nowadays, current bone tissue engineering strategies require the availability of adequate cells, biomaterials and biomodulators in order to achieve a successful regeneration of the bone tissue. Most of the present strategies rely on biomaterials implantation in diseased or damage tissues, although clinical morbidity, associated with inflammatory and infectious processes, arises from the surgical implantation.

Several methodologies aiming to prevent infection establishment and development have been assayed, including the local delivery of pharmacological agents targeting pathogenic microorganisms. Tetracyclines, because of their high affinity to mineralized tissues and their broad-spectrum antibacterial action are suitable candidates to be used in biomaterial-dependent bone regenerative strategies. These drugs also seem to play an important role in the modulation of several immuno-inflammatory bone diseases by mechanisms independent from the direct inhibition of protein synthesis – their principal antibacterial mechanism. Although the current knowledge, tetracyclines' effect over bone physiological metabolism is not fully understood.

In this work, the behavior of human osteoblastic-colonized hydroxyapatite and Bonelike[®] (a glass-modified hydroxyapatite) was assayed, in the presence of representative therapeutic concentrations of doxycycline or minocycline.

Initially, first passage human osteoblastic-induced bone marrow cells were cultured, in conditions known to favour osteoblastic differentiation, in the presence of doxycycline (1 to 25 $\mu\text{g/ml}$) or minocycline (1 to 50 $\mu\text{g/ml}$). Data revealed that low dosage of both tetracyclines (1 $\mu\text{g/ml}$) increased cell proliferation in a significant way, without inducing representative alterations in cell phenotype and functional activity – as verified by high alkaline phosphatase expression and increased mineralization of the extracellular matrix. Higher concentrations of both agents induced dose-dependent detrimental effects which were responsible for the hindrance in cell proliferation and differentiation. Following, cell behaviour was evaluated on seeded hydroxyapatite and Bonelike[®], in the presence of 1 $\mu\text{g/ml}$ doxycycline or minocycline. Both tetracyclines induced cell behavior on cultures established on the surface of the two substrates. Proliferation was enhanced while phenotypic characteristics (alkaline phosphatase expression and mineral deposition over the extracellular matrix) were maintained. In addition, Bonelike[®] presented an improved biological behavior, in comparison to hydroxyapatite.

Overall, results suggest that local delivery of both doxycycline and minocycline may combine an expected local antibacterial activity with a potential anabolic effect regarding osteoblastic proliferation, at the same time that phenotypic expression and functional activity are maintained. Further, the association of a local factor that acts simultaneously as a bone biomodulator and an antimicrobial agent, with a bone regenerative biomaterial, could contribute to a more predictable clinical outcome.

Resumo

Actualmente, as estratégias de engenharia do tecido ósseo assentam na implantação de células, biomateriais e biomoduladores adequados, que permitem uma regeneração favorável do tecido. A maioria das estratégias actuais recorre à implantação de biomateriais nos tecidos lesados, apesar da descrita morbilidade associada aos processos inflamatórios e infecciosos, decorrentes da implantação cirúrgica.

Recentemente têm sido desenvolvidas diversas metodologias que visam prevenir o estabelecimento e desenvolvimento dos processos infecciosos, incluindo a utilização local de fármacos antibacterianos. As tetraciclinas são fármacos potencialmente adequados para este fim, devido à elevada afinidade para os tecidos mineralizados e ao largo espectro antimicrobiano. Estes agentes farmacológicos desempenham, também, um papel importante na modulação de diversas patologias ósseas de base imuno-inflamatória, através de mecanismos independentes da inibição da síntese proteica – o principal mecanismo de acção antibacteriana. Apesar do conhecimento actual, o efeito das tetraciclinas sobre o metabolismo ósseo não está completamente esclarecido.

Este trabalho tem como objectivo avaliar o comportamento de células osteoblásticas humanas cultivadas na superfície de hidroxiapatite e Bonelike[®] (hidroxiapatite modificada por biovidros), na presença de concentrações terapêuticas de doxiciclina ou minociclina.

Inicialmente, as células osteoblásticas humanas, derivadas de medula óssea, foram cultivadas na presença de doxiciclina (1 to 25 µg/ml) ou minociclina (1 to 50 µg/ml), em condições que favorecem o processo de diferenciação osteoblástica. Os resultados demonstraram que concentrações mais baixas de ambas as tetraciclinas (1 µg/ml) aumentaram a proliferação celular de forma significativa, sem, no entanto, induzirem alterações no fenótipo e na actividade funcional das células – verificados pela elevada expressão de fosfatase alcalina e aumento da mineralização da matriz extracelular. Concentrações mais elevadas dos dois fármacos induziram alterações dependentes da dose, responsáveis pela diminuição da proliferação e diferenciação celulares.

Seguidamente, o comportamento celular foi avaliado na superfície da hidroxiapatite e Bonelike[®], na presença de 1 µg/ml de doxiciclina ou minociclina. Ambos os fármacos induziram um efeito positivo no comportamento das culturas osteoblásticas estabelecidas sobre os dois substratos. A proliferação foi induzida enquanto que as características fenotípicas (expressão de fosfatase alcalina e deposição mineral na

matriz extracelular) foram mantidas. Por outro lado, o Bonelike[®] apresentou um comportamento biológico mais adequado que o da hidroxiapatite.

De forma geral, os resultados sugerem que a utilização local de doxiciclina ou minociclina pode combinar uma acção antimicrobiana localizada com um potencial efeito anabólico relativamente à proliferação osteoblástica, ao mesmo tempo que a expressão fenotípica e a actividade funcional são mantidas. Adicionalmente, a associação de um factor local, que actue simultaneamente como um biomodulador do tecido ósseo e um agente antimicrobiano, com um material de regeneração óssea, pode contribuir para uma melhoria da eficácia das estratégias clínicas actuais.

Aim and structure

Tetracyclines comprise a family of broad-spectrum antimicrobial drugs that also have been characterized as modulators of the immuno-inflammatory imbalance, verified in several bone diseases, with experimental and clinical evidence of an overall positive effect on bone. Even so, the effect of these drugs over bone metabolism is not fully understood.

In this work we aim to characterize the *in vitro* response of human osteoblastic-seeded bone regenerative biomaterials to tetracyclines. First, human bone marrow osteoblastic cells were characterized for proliferation and differentiation events in the presence of selected concentrations of doxycycline (1-25 µg/ml) and minocycline (1-50 µg/ml), for the establishment of the biological profile of these antimicrobial agents regarding osteoblastic behaviour. Later, *in vitro* osteoblastic response was evaluated on the surface of hydroxyapatite and over an improved glass-modified hydroxyapatite (Bonelike[®]), in the presence of 1 µg/ml of both doxycycline and minocycline, representative of those attained in biological fluids after oral administration of a therapeutic antimicrobial dose.

This thesis is composed of four chapters:

In chapter 1, a brief review of the literature is conducted and focused on bone biology, bone grafts and tetracyclines.

In chapters 2 and 3, the experimental part of the work is described by reporting published and accepted papers in international referred journals.

Chapter 2 is focused on the evaluation of the behaviour of human bone marrow osteoblastic cells in the presence of doxycycline and minocycline. Cell cultures were evaluated, for 35 days, regarding cell viability and proliferation, and functional events that determine differentiation. This was accomplished by the evaluation of the alkaline phosphatase activity and assessment of matrix mineralization by determination of ionised calcium and phosphorus in the culture medium, histochemical staining for calcium and phosphate deposits, and scanning electron microscopy observation.

In chapter 3, the evaluation of the osteoblastic response to tetracyclines was determined in the surface of hydroxyapatite and a glass-modified hydroxyapatite, marketed as Bonelike[®]. Colonized materials were evaluated for cell

viability/proliferation and differentiation events, by the previously described methodologies.

The last chapter, chapter 4, presents the general conclusions of the described research, summarising the most relevant considerations.

Foreword

Since a long time ago, mankind realized the limited capacity of the human body to self-repair. These events are further impaired by aging and the presence of systemic disease, which lead to the established dream to rejuvenate or replace disabled or diseased body parts. In most of the developed countries worldwide, in terms of economic burden and human cost, the chief challenge of population aging, for health services, comes from chronic illness, disability and tissue morbidity (1). Each year, around ten millions individuals are treated for a variety of conditions ranging from localized tissue loss to end-stage organ failure.

Currently, gold-standard treatment includes the transplantation of healthy biological material from another location (autotransplant), another individual (allotransplant), or even from a donor of another species (xenotransplant). Unfortunately, there has been a chronic shortage of biological material for such treatments that shows no sign of improvement (2).

Nowadays, several new and exciting strategies are being pursued to provide clinicians with adequate technologies and materials to address this need. New products, that were just laboratory curiosities a few years ago are now entering clinics around the world and contribute to the improvement of the quality of life for many thousands of people.

Historically, regarding bone regeneration, the dream started around 1890 when the first total hip replacement was carried out with an ivory prosthesis being glued into place. Since then, a large variety of materials have been developed ranging from early plastic materials to more modern metal, polymeric and ceramic constructs. In the recent past, millions of orthopaedic prostheses made of bioinert materials have been implanted with around 80% survivability of 15 years (3, 4). Although this has enhanced the quality of life for many individuals, the increasing percentage of the aging population requires more than 30 years survivability of devices (5). In order to solve this problem, a conceptual paradigm shift is needed: the established emphasis on the replacement process must evolve towards the regeneration of the biological tissues (1). This conceptual change involves a shift from the material/mechanical perspective to a biological based tissue engineering repair which must be substantiated by the increasing knowledge of tissue molecular and biochemical pathways. The new biological-oriented alternative reaches out to the use of bioactive materials (performing as scaffolds), bioactive modulators (which induce and regulate cellular crosstalk) and

cells (6, 7). Over this new perspective, the modulation of the cell-cell or cell-matrix communication acquires a new preponderance. Biochemical modulators influence intracellular signalling that leads to different events such as the enhancement of cell adhesion, proliferation, migration and differentiation by up- or down-regulating the expression of specific proteins, growth factors and receptors (8). Many molecules ranging from small cytokines to the more complex synthesized therapeutic drugs are known to influence the metabolism of the bone tissue, although, only few of them have been realistically proposed for bone tissue applications (9).

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Chapter 1

Literature overview

1. Bone biology

Nowadays, bone biology is a complex and growing field of research aiming to study the skeleton, as an organ of unappreciated complexities. Several of these complexities affect patterning and cell differentiation during development, physiological and pathological situations. This conjuncture contributes to the maintenance of several life-dependent physiological functions namely: support of soft tissues and lever for muscle action; support of the haematopoiesis; housing for the brain and spinal cord; and regulation of calcium and phosphate homeostasis.

Structure

Bone is a specialized form of connective tissue that functions both as a tissue and an organ system, in higher vertebrates. Its structure includes cells like osteoblasts, osteocytes, lining cells, osteoclasts and a mineralized extracellular matrix which contains organic and inorganic components.

Bone morphological structure can be classified either as cancellous (spongy or trabecular bone) or as cortical (compact or dense bone) (1). Functionally, cancellous bone is closely associated with metabolic processes – it has a higher metabolic rate. Also it appears to respond quicker to changes in mechanical loading and unloading. This may be due to the increased exposure of bone cells to the adjacent marrow and vascular supply, whereas cells within cortical bone tend to be embedded deeper within the mineralized matrix (2). Cancellous bone is formed by a network of calcified trabeculae which are composed of irregular osteon fragments that receive nutritional support from the surrounding marrow. The mineralized trabeculae are not generally penetrated by large blood vessels. The void space between trabeculae is filled with active hematopoietic marrow in continuity with the medullar cavity (1). On the other hand, the cortical bone provides physical and mechanical protection. Interestingly, besides the adequate mechanical strength, its biomechanical properties allow a significant flexibility (3). This bone is denser (around 80% against 20% of the cancellous bone) and, ultra-structurally, several densely packed collagen fibres can be observed, organized as concentric lamellae. The structural units of the cortical bone are designated Haversian systems or osteons. These systems contain central canals with blood vessels, lymph vessels and occasionally nerves, and are surrounded by concentric lamellae. In this arrangement, several canaliculi (in which osteocytes processes travel) connect the canals and disperse lacunae, extending out in a radial manner. Among the lamellae, the lacunae are distributed erratically and several osteocytes are arranged circumferentially around the canal (4). These canaliculi allow

nutritional support and oxygenation of the lacunae while metabolic waste products are also removed by them. The canaliculi connect to the Haversian systems which, in turn, can anastomose with obliquely orientated vascular branches (known as Volkmann's canals) that establish communication between the periosteum and the endosteum (5).

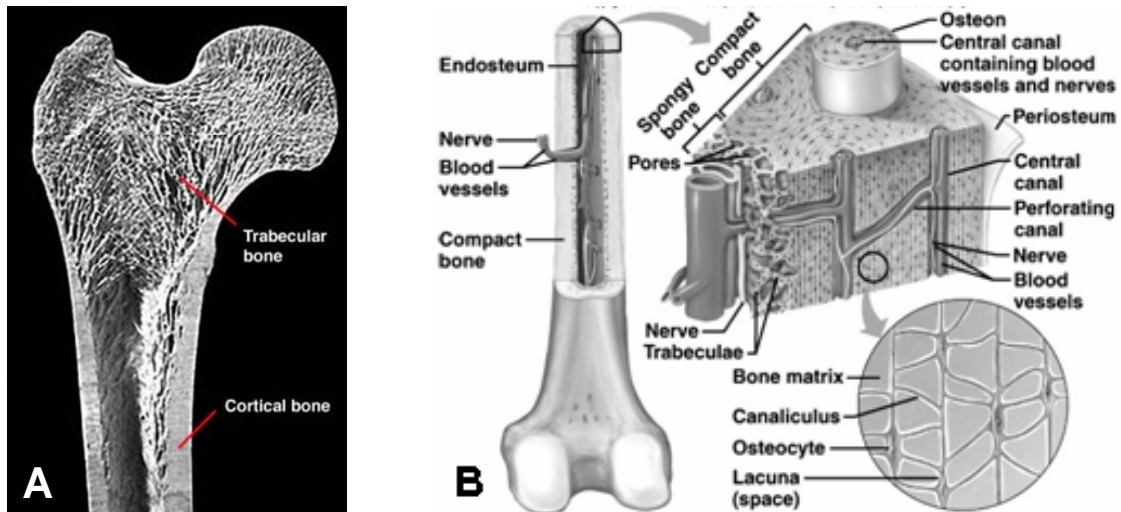


Figure 1 – A) Structure of a longitudinally sliced human femur (6).

B) Morphological structure of bone (7).

Cortical and cancellous bone can be made of either woven or lamellar bone. Woven bone, also named primary bone, can be found during embryonic bone development, which is later resorbed and replaced by lamellar (or secondary) bone. Primary bone can also be found during fracture healing, until the closure of cranial sutures, ear ossicles and epiphysial plates. Comparing to lamellar bone, woven bone has an increased rate of metabolic activity which leads to a quicker turnover during the remodelling process. Structurally, woven bone has a scattered and irregular appearance while lamellar bone is characterized by an orderly arrangement whereas the osteocytes are uniform in size, shape and orientation, in line with other cells and structures of the bone (8).

Cellular composition

There are mainly four different cellular elements in the bone tissue: osteoblasts, osteocytes, bone lining cells and osteoclasts (2). A simpler classification, based on functional activity, may dichotomize these populations into bone forming and bone resorbing cells. Cells can also be classified regarding their embryologic origin and, in this way, osteoblasts, osteocytes and bone lining cells originate from the mesenchyma while osteoclasts are derived from hematopoietic progenitors.

Further, cell location varies along the bone structure: osteoblasts, osteoclasts and bone lining cells are found along the bone surface while osteocytes can only be found in the interior, entrapped by the mineralized extracellular matrix.

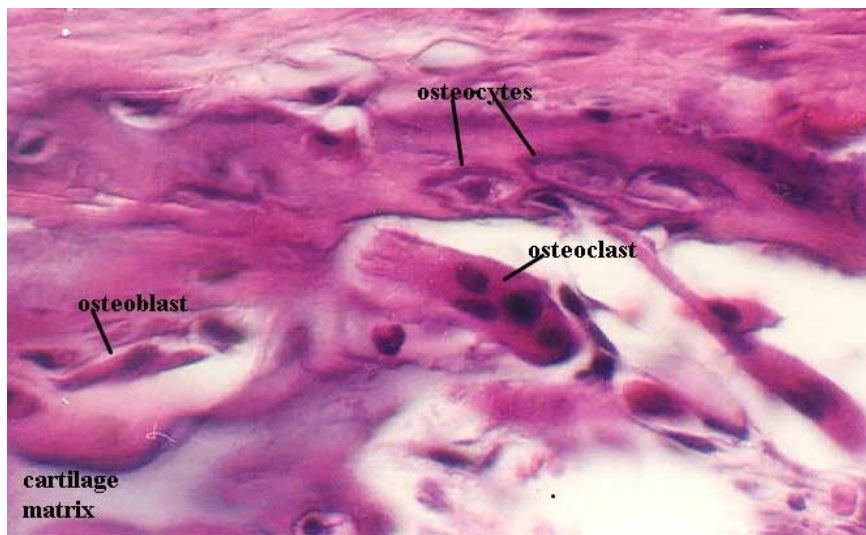


Figure 2 – Location and morphology of the bone cells (9).

Osteoblasts

Osteoblasts are derived from undifferentiated mesenchymal cells which can be found in the marrow, endosteum, periosteum and bone canals (10). These cells may be referred to as "preosteoblasts" and they can migrate from neighbouring tissues or through the vascular system into the target area.

Osteoblasts, found on the bone surfaces undergoing remodeling events, are responsible for the production of the extracellular bone matrix and its subsequent mineralization. They are characterized by several distinct morphological features including: localization of the round nucleus at the base of the cell opposite to bone surface, presence of small amounts of basophilic cytoplasm, presence of a prominent Golgi complex and endoplasmic reticula, both related with the high biosynthetic and secretory activity, among others (11).

The cytoplasmic membrane is rich in alkaline phosphatase, which is important for the initiation of the mineralization process, and is distributed over the entire surface of the cell membrane. The expression of high levels of this enzyme is associated with a shift to a more differentiated state of the osteoblastic differentiation and usually determines the beginning of the mineralization process of an *in vitro* osteoblastic population (12). Eventually, osteoblasts can follow one of three paths: they can (1) remain active cells, (2) become embedded by the extracellular matrix that is subsequently mineralized – turning to osteocytes or, (3) become relatively inactive and, eventually, develop into bone lining cells.

Bone lining cells

These cells are, in contrast to osteoblasts, elongated and they cover most of the bone surface in the mature skeleton, namely the areas that are not being remodeled. They are compactly associated with each other by *tight junctions* or cytoplasmic extensions which also link them to osteocytes. Ultra-structurally, they present fewer organelles than osteoblasts since they are metabolically less active (13). That is why they have been defined as “resting osteoblasts” or “surface osteocytes”.

Their biological role has been a centre for interesting debate in the literature, over the past years. Some authors report that, in the presence of parathyroid hormone, these cells secrete enzymes that remove osteoid, preparing the matrix for osteoclastic activity (2). Others argue that these cells may become activated and enrol osteoblast-like functions, regulate the growth of hydroxyapatite crystals or even establish a barrier between extracellular fluid and bone (8, 10).

Osteocytes

Osteocytes are estimated to comprise more than 90% of the bone cells in adult skeleton. These cells differentiate during the remodelling process in which some osteoblasts become entrapped in the osteoid (non-calcified extracellular matrix). These young osteocytes have similar characteristics to mature osteoblasts.

As these cells mature, and more matrix is laid down and mineralized, osteocytes become located deeper within the tissue and become smaller by cytoplasmic loss. Although fully surrounded by matrix, these cells are located within a lacuna of 1-2 μm wide around the cell. The lacunae have collagen fibrils which support cytoplasmic process that are responsible for intercellular crosstalk, via the established canaliculi (13). Besides allowing cell-mediated exchanges of minerals, this network is also believed to sense mechanical deformation within bone that leads to the activation of bone formation or resorption. During osteoclastic bone resorption these cells are

processed along with the matrix. Osteocytes are non-mitotic cells and their turnover is only achieved during the remodelling process (a combining process of bone resorption and formation).

Osteoclasts

Osteoclasts are giant multinucleated bone resorbing cells. They originate from the fusion of several mononuclear haematopoietic precursor cells. Generally these cells are located on the surface of bone undergoing resorption and present a high mobility which allows them to move along the bone surface, from one site to another (14).

Morphologically, osteoclasts are characterized for having 3 to 20 nuclei that tend to be oval and concentrated mid-cell. There is less endoplasmatic reticulla which is in accordance with the reduced protein production, but an increased number of mitochondria may be observed, as well as several lysosomal vacuoles leading to the common characterization of the cytoplasm as foamy (15). The plasma membrane, facing the bone surface, is characterized as having several folds and finger-like projections, know as “ruffled border”. The deep infolds can wrap around bone prominences or lie along the surface, forming a sealing zone where resorption takes place. The process is initiated by the dissolution of the inorganic phases of the bone by acidification of the microenvironment and the secretion of lytic enzymes (namely TRAP and cathepsin K) (14). The products originating from bone resorption are endocyted by osteoclasts and then transported and released by the cell antiresorptive surface.

Bone matrix

The overall extracellular structure of the bone comprises around 90% of its volume with only the remaining 10% being formed by cells and blood vessels. The extracellular matrix is composed by distinct phases: an organic component (composed essentially by collagens, proteoglycans and several non-collagenous proteins) and an inorganic component rich in calcium salts.

Organic component

Around 80% to 90% of the organic phase is composed by collagenous proteins that are synthesized by osteoblasts, secreted and assembled while in the extracellular environment. The most abundant collagen type is type I but types V, VI, VIII and XII are also present, although in small amounts.

Collagen I molecules form a triple helix of polypeptide chains and are organized into fibrils, extracellularly. These fibrils are highly ordered and establish several crosslinks

that contribute to the formation of a porous structure from which the bone derives its yield strength (16).

Several non-collagenous proteins are also part of bone organic component. Among them, osteopontin, bone sialoprotein, osteocalcin, osteonectin play an important role. Their exact biological role is not yet fulfilled but some functions have already been established. For instance, osteopontin and bone sialoprotein have the RGD sequence that mediates cell attachment and cell signalling pathways. They can also be involved in hydroxyapatite binding but while bone sialoprotein seems to participate in crystal nucleation, osteopontin seems to inhibit mineral growth (17, 18). Osteocalcin may be involved in bone mineralization and calcium homeostasis but it has been stipulated that it can also function as a negative regulator of bone formation (19).

Inorganic component

The inorganic component of the bone is important in strengthening the biological tissue (especially in which relates to the tensile strength) but also plays an important role in ion storing.

This mineral phase of the bone has many similarities to the synthetic hydroxyapatite, chemically described as $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, and with a calcium phosphate ratio of 5:3 (1.67) (20). Nowadays, several differences have been demonstrated, namely in which refers to its composition, crystallinity, stoichiometry, physical and mechanical properties. Bone apatite is chemically characterized by calcium and hydroxyl deficiencies and the range of Ca:P varies between 1.37-1.87. Also, several ionic substitutions within the lattice are reported and seem to alter internal crystal organization (21). The reported substitutions include carbonate, sodium, magnesium, potassium, fluoride, chloride, strontium, lead, barium and more. The exact role of many of these ionic species has not been established yet, although all seem to be important in the physiological biochemical processes of bone remodelling as well as during bone disease or fracture healing (2).

Bone remodelling

Bone is a dynamic and metabolic active specialized connective tissue. In this way, skeletal functions are accomplished by a continuous tissue renewal – remodelling – which occurs throughout life, at approximately two million microscopic sites in the adult skeleton (22). In this process, old bone is removed and new bone is produced in order to replace it.

Cortical bone is remodelled by the removal and refilling of osteons: active osteoclasts resorb the bone on the surface and progress to the interior forming a *cutting cone*.

Soon after the beginning of the resorption process, the reversal phase is started and the whole osteon is resorbed by the osteoclasts. In the following phases, osteoblasts lay down new bone matrix and, during the production of the osteoid, these cells become entrapped and differentiate into osteocytes. This leads to the formation of a new Haversian system.

In the cancellous bone, the remodelling process is different because the lack of mature osteons and large surface area of the trabeculae. In this way, the remodelling process is initiated by the recruitment of osteoclasts to the bone surface which are then activated and start the resorption process. Following, osteoblasts start to lay down extracellular matrix that is subsequently mineralized.

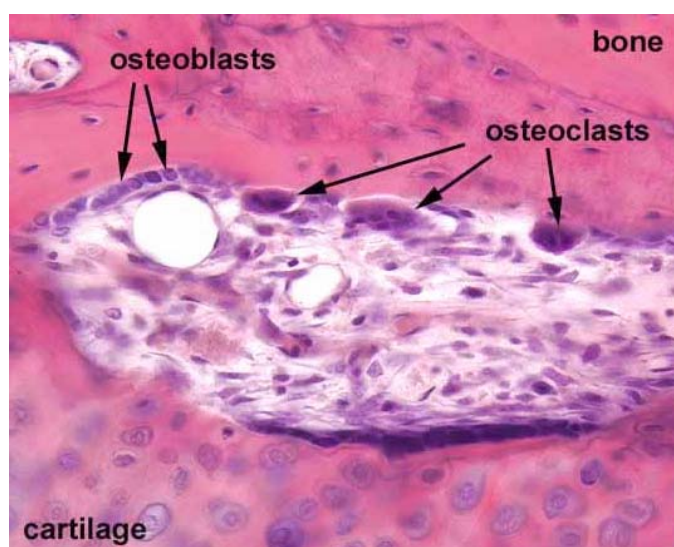


Figure 3 – Location of osteoblasts and osteoclasts during the process of bone remodelling (23).

Regulators of bone metabolism

The overall bone metabolism is under constant and tight regulation by several hormonal and local factors. Parathyroid hormone, vitamin D and calcitonin are three of the calcitropic hormones that seem to play an important role. Parathyroid hormone increases the flow of calcium from the bone into the calcium pool and is responsible for the regulation of calcium extracellular level. It is a strong stimulator of osteoclastic bone resorption (increasing the number of recruited cells and enhancing their activity) and also induces reabsorption of calcium by the kidneys (24). Vitamin D, in its active form of 1,25-dihydroxyvitamin D₃, stimulates intestinal and renal calcium-binding proteins and facilitates active calcium transport. Both inhibitory and inductive actions have been reported over osteoblasts, depending on whether vitamin D is applied during proliferative or differentiation stages (25). Calcitonin is secreted by the thyroid gland in

response to an acute rising of plasma calcium level. Its main role is to inhibit osteoclasts activity by inducing loss of the ruffled borders and dislocate the cells from the underlying bone (26). Other hormones can influence bone cell function and bone metabolism and these include glucocorticoids, thyroid hormone and estrogens.

Besides hormones, several factors can influence bone development through local mechanisms including cell-to-cell and cell-to-matrix interactions. Cytokines, growth factors, prostaglandins and several other proteins can be released from platelets, macrophages, fibroblasts, bone cells and several other cells present in bone microenvironment. Cytokines and growth factors are soluble molecules that act at a local level and mediate cell-to-cell function during growth, development and remodelling processes. On the other hand, prostaglandins are a diverse group of unsaturated fatty acids that regulate several mechanisms during inflammation, blood flow and ion transport (27).

Wound healing

Injury and the subsequent perturbation of homeostatic mechanisms are responsible for the initiation of the healing process that aims to restore the tissue to its original physical and functional properties. This process is influenced by a variety of systemic and local factors that include, among others, the extent of the injury, the loss or maintenance of the basement membrane associated structures, the amount of provisional matrix formation, the extent and degree of cellular necrosis, and the extent of the inflammatory response (28).

Early, following fracture establishment, a haematoma develops in the fracture location – caused by the haemorrhage from the damaged blood vessels – and inflammatory cells (macrophages, monocytes, lymphocytes and polymorphonuclear neutrophils) are recruited along with fibroblasts, pericytes and endothelial cells that are lining capillaries. These cells are surrounded by an extracellular matrix composed by fibronectin, proteoglycans, hyaluronic acid and collagen type III that, gradually, will be substituted by collagen type I (29). This results in the formation of granulation tissue in which the large number of blood vessels (around 60% wt) gives the granular appearance. Angiogenic process is initiated but while the vasculature network is not established, early nutrient and oxygen supply is provided by the exposed cancellous bone and muscular tissue (30). Later, the haematoma is resorbed and replaced by fibrous vascular tissue in which neovascularisation occurs. Meanwhile, recruited osteoblasts (from the bone marrow and blood) initiate osteoid deposition and subsequent mineralization (31). Alternatively, in case of inadequate fixation of the fragments, ossification may not occur and, instead, an unstable fibrous union may develop. The

healing process is completed during the remodelling phase which comprises the restoring of the bone original shape, structure and strength. Adequate strength is usually achieved in 3 to 6 months (31).

2. Bone grafts

Bone grafts are used in several orthopaedic, maxillofacial and dental surgical procedures aiming to restore skeletal integrity and function. Bone grafts have several mechanical and biological functions namely providing the framework for the host bone healing and regeneration, during the repair or replacement in defective and diseased tissues, by trauma, aging, degenerative diseases, etc (32).

Overall and accordingly to their origin, bone grafts can be classified in: 1) autografts, if the grafted tissue is obtained from the same individual; 2) allografts, if the tissue is obtained from a different donor, but from the same specie; 3) xenografts, if the tissue is obtained from a donor from a different specie; and 4) synthetic grafts (alloplastic grafts), which include a wide variety of ceramic, polymeric and composite materials (32).

The biological events associated with the implantation of a bone graft are quite similar to those following bone healing process that occur in fractured long bones, since the introduction of an implant results in the loss of continuity of the bone tissue.

Autografts

For many years, autografts have been considered the gold standard for bone regeneration clinical applications. They consist in the grafting of bone from another site in or on the body of the same individual. This option, besides the established biosafety and prevention of the immunologic rejection, allows for cell osteoconduction, growth factor-dependent osteoinduction and availability of progenitor cell for osteogenesis (33). However some limitations have been associated with autografts, namely: the need of an extra and invasive surgical procedure for bone harvest, with consequent post-operative pain and complications and limited quantity of the bone available for harvesting.

Allografts

The clinical application of allografts aims to solve some of the drawbacks associated with autograft use. In this way, the second surgical intervention is eliminated and the quantity of the material is no longer limited, but some established problems are also associated with allografts (34). These include the risk of disease transmission, and immunological intolerance that leads to graft rejection. In order to limit these potential

risks, allografts are generally treated by freeze-drying techniques, irradiation and demineralization processes (35). These methodologies, in addition to being associated with the increase in cost production and decrease mechanical properties, don't assure complete eradication of the possible disease transmission.

Xenografts

The clinical use of xenografts has become quite popular during the last years. This biological material allows the availability of large amounts of tissue and may be mixed with autografts to augment the graft quantity and control the eventual resorption of the tissue (36). These grafts can be derived from several origins but the most popular are those derived from mammalian bones and coral exoskeletons. Recent techniques of bone deproteination increase host tolerability by reducing graft antigenicity (37). Nowadays, there is quite a concern regarding the possibility of future bovine spongiform encephalopathy development, from the use of bovine derived grafts (38).

Alloplastic materials

Despite the benefits of biological-derived grafts, the continuous concerns regarding biosafety and immunogenicity guided the search for synthetic applications. In this way, three criteria have been established as fundamental for successful grafting: 1) osteogenesis, which assures bone tissue formation; 2) osteoinduction, which induces the formation of new bone by bone-forming cells; and 3) osteoconduction, which provides the structural framework for cell migration, adhesion, proliferation and differentiation (39). Autografts, allografts and some of the mineral bone graft substitutes (namely hydroxyapatite and bioactive glasses) allow for this last property.

In this way, and besides the defined properties, synthetic bone graft substitutes should be biocompatible when implanted in living tissues. Synthetic hydroxyapatite (HA) is a mineral apatite with the chemical composition of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ – similar to the one of natural bone – and has been used as a bone graft material for a long time (40). Although the adequate biocompatibility, its resorbability is very slow and so, it can be combined with other materials (ex: bioactive glasses) to assure a faster graft resorption and adequate substitution by newly formed bone (41).

The bioactive glasses can be produced with the conventional technologies of the glass industry to tailor a great chemical range of properties and of linking speed to the biological tissues. The main advantage of the bioactive glasses is the high superficial speed reaction while the greater disadvantages are the sub-optimal mechanical properties and the meagre break resistance. The out-bending-tensile rigidity of the

greater part of the bioactive glasses varies between 40 and 60 MPa, and they are not therefore useable for loading applications (42).

In fact, monophasic bioactive ceramics, like for example Bioglass[®] or sintered hydroxyapatite, do not show mechanical resistance comparable to that of the cortical human bone. To find a solution to that, several attempts have been made to establish a composite material that reports similar physical and mechanical properties to those of the cortical bone, while maintaining adequate biocompatibility and allowing osteoconduction.

Bonelike[®]

Bonelike[®] is a glass-reinforced HA composite that results from the incorporation of Ca-P₂O₅ based glasses into the matrix of HA. Accordingly, Bonelike[®] displays enhanced bioactivity by reproducing the inorganic phase of HA in bone (which is highly substituted by several ionic species) and improved mechanical properties resulting from the addition of the phosphate based bioactive glasses that, during the sintering process, induces HA decomposition into β -tricalcium phosphate, which in turn, at higher sintering temperatures, is decomposed into α -tricalcium phosphate (43). Mechanical properties are also enhanced by the changes in microstructure that result from the glass-induced reduction in porosity (44). Bonelike[®] appears to induce bone formation through specific activation of osteoblasts with the regulated release of ionic species, namely F⁻, Mg²⁺, Na⁺, among others, into the surrounding medium (45). This composite biomaterial also reports high osteoconduction and bioactiveness, which allowed for bonded bone formation (chemically and mechanically) over its surface (45-53).

Bonelike[®] grafts are currently being used in several successful clinical applications namely in oral and maxillofacial surgeries, implantology and also in orthopaedic procedures (46, 48-53). Regarding oral surgical procedures and implantology, Bonelike[®] has been used for the regeneration of maxillary and mandible bone, after cyst removal and impacted teeth extraction and for *sinus lift* and bone augmentation around implants, respectively (49, 52). In maxillofacial applications, Bonelike[®] has been used for maxilla and mandible reconstruction (53) while in orthopaedics, it has been employed for the regeneration of several bone defects caused by trauma or ageing (46, 48, 51).

Bonelike[®] preparation

Briefly, the preparation of Bonelike[®] was conducted as follows: a 15CaO–65P₂O₅–10CaF₂–10Na₂O (mol%) glass was prepared from reagent grade chemicals that were mixed for 2 minutes and placed in a platinum crucible. Following, the mixed powders were heated for 120 minutes at 1450°C, with a heating rate of 20°C/min. The prepared glass was then crushed in an agate mortar and sieved to granules (size <75 μm). Following, the Bonelike[®] composite was obtained by mixing 2.5% of the attained glass with laboratory-prepared HA, in isopropanol. The resulting mix was dried at 60°C, for 24 hours, sieved to less than 75 μm, and then isostatically pressed at 200 MPa, before sinterization, at 1300°C, for 1 hour. These patented process has been previously reported (54, 55).

3. Antimicrobial chemotherapy

One of the greatest advances of modern medicine was, without a doubt, the development of antimicrobial chemotherapy. The antimicrobial agents are able to interact with targeted bacteria, suppressing their growth that contributes to their destruction. Ideally, these drugs would target pathogenic agents without affecting the cells of the host. However, since the cells and bacteria share many biochemical pathways and macromolecular structures, the antimicrobial agents may induce nonantimicrobial effects that might be undesirable and even unexpected.

In some cases, these effects can be therapeutically useful. For instance, sulfonamides were found to inhibit carbonic anhydrase, which results in the reduction of the intraocular pressure that plays an important role in glaucoma pathogenesis (56); erythromycin, in therapeutic and subtherapeutic antimicrobial doses, induces the gastrointestinal motility (57); clindamycin enhances phagocytic activity against microorganisms (58); fluoroquinolones have been reported to interfere with glucose metabolism, eventually by stimulating pancreatic cells (59); and tetracyclines may act as immunomodulators in inflammatory disorders (60).

Regarding bone associated infections, haematogenous pyogenic bone and joint infections have been proven difficult to cure (61). This complexity is associated with bacterial adherence to necrotic bone and foreign material, drug resistance and limited availability of the antibiotic in the infected bone (62). Further, with biomaterials implantation, the introduction of an implant in a living organism always causes inflammation phenomena and, frequently, infectious processes (63). In the acute

conditions, an effort must be made in order to prevent progression to a chronic stage. This avoids the exacerbation of the infection, facilitates the restoration of the normal anatomy and function and prevents complications such as joint destruction, bone deformity and overgrowth, and amyloidosis (61). These problems can be overridden by the use of local drug delivery strategies in which drug molecules are confined into the scaffold's structure and following, after implantation, the drug is released in a controlled fashion (64). Among several therapeutic options that are available for the pharmacological control of infection, tetracyclines have been proved effective in the treatment of pathogenic bone-associated diseases (65-67).

4. Tetracyclines

Tetracyclines have been used for decades because of their broad spectrum of antimicrobial activity. They were first discovered in 1948 as natural fermentation products of a soil bacterium, *Streptomyces aureofaciens*. The first purified tetracycline was chlortetracycline (68) but, nowadays, three groups of tetracyclines are available: natural products, semisynthetic compounds and chemically modified agents.

Tetracyclines are bacteriostatic agents that inhibit protein synthesis. They inhibit bacterial growth by binding to the 16S part of the 30S ribosomal subunit, preventing the amino acetyl tRNA complexes from binding to the acceptor site on the mRNA-ribosomal complex, blocking the addition of new aminoacids to the peptide chain (69). These antimicrobials are active against a wide range of aerobic, facultative and anaerobic gram-positive and gram-negative bacteria. Tetracyclines are active against *Rickettsia*, *Chlamydia* and *Mycoplasma*, some nontuberculosis strains of mycobacteria, *Legionella* spp., *Ureaplasma*, *Plasmodium* spp. and many spirochetes. They are not active against fungi (69). Intrinsically, tetracyclines are more active against gram-positive than gram-negative microorganisms, although acquired resistance is common. Tetracyclines have been used extensively to treat infectious diseases and as an additive to animal feeds in order to facilitate growth, resulting in the prevalence of resistant strains worldwide. Nonetheless, since therapeutic application was reduced for a long time, clinical use of these drugs has made a come back into being effective against previously resistant bacteria (69).

Upon administration, tetracyclines are widely distributed into the tissues and also attain the cerebrospinal fluid. Also, as they chelate with the calcium ions, they are concentrated at mineralized tissues, namely bone and teeth.

Tetracyclines and related agents are characterized for having a structure that consists of a tetracyclic naphthacene carboxamide ring system. They report a similar spectrum against pathogenic agents but they differ significantly regarding pharmacokinetic profile. Their antibiotic activity is related with the presence of a dimethylamine group at carbon 4 in ring A. Its removal enhances tetracyclines' nonantibiotic actions, which is in the base of the development of chemically modified tetracyclines (CMTs) (70). Further, modifications in the upper peripheral zone of the tetracycline molecule enhances biological targeting – this was accomplished to the synthesis of the semisynthetic compounds such as minocycline and doxycycline (70, 71).

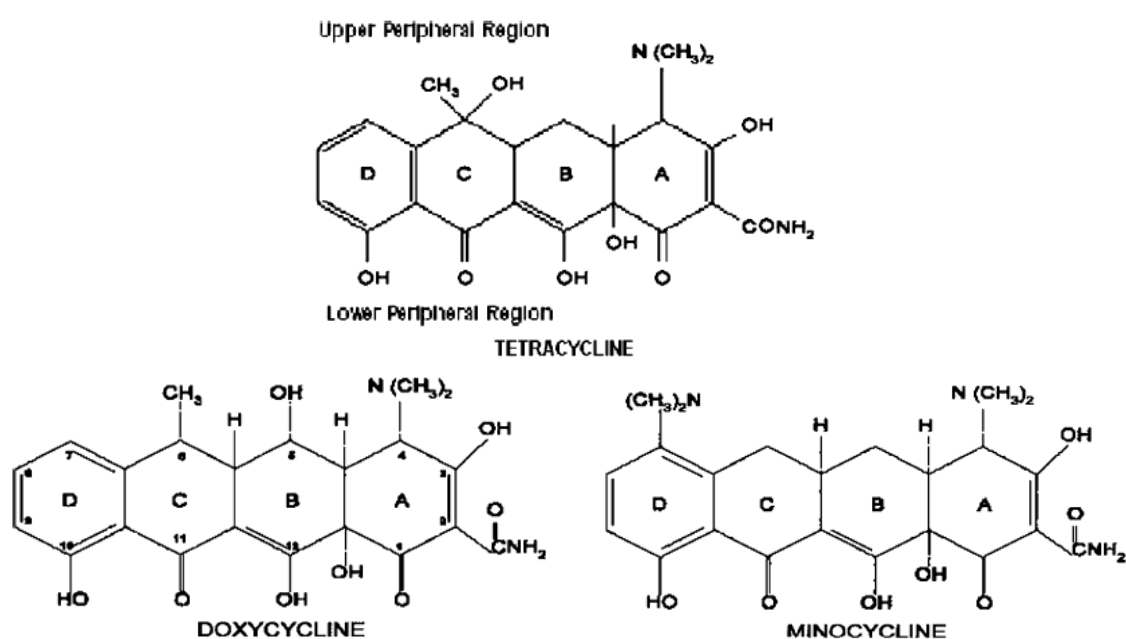


Figure 4 – Chemical structures of tetracycline, doxycycline and minocycline (71).

Many non-antimicrobial properties of tetracyclines may be related with their capacity for divalent cation chelation. They bind primarily Ca^{2+} and Mg^{2+} , mostly along their lower peripheral region (70). Intracellular calcium acts as a second messenger and plays a role in the regulation of several cell processes including secretion, receptor modulation, cell division and diverse metabolic routes. Tetracycline-dependent calcium chelation may interfere with signal transduction events and alter determined cell functions (72). Tetracyclines have been therapeutically used in several hard-tissue related infections. Doxycycline has been engaged with success in the treatment of prosthetic joint infections, osteomyelitis and chronic osteomyelitis associated with orthopaedic hardware (73). In orthopaedic surgery, tetracyclines have been mixed with bone cement for the prevention of infection in bone surgery. Antibiotic-laden bone cement has become the gold standard in the treatment of infected orthopaedic implants and there are confirmatory laboratory and clinical data that support the use of these

materials (74). Further, antibiotic-impregnated cement has proven efficacy in infection prevention and treatment in revision of total hip arthroplasty procedures (75) and infection associated with total hip or knee arthroplasty (76). Tetracyclines have also been used to manage periodontal disease, namely in what refers to the treatment of chronic periodontitis. Several local and sustained-release strategies have been assayed as an adjunct to scaling and root planing procedures with established improvement of the clinical outcome (77). In this context, a low-dose formulation of doxycycline has been introduced by Golub et al. (78) and provided an effective adjunct to mechanical debridement in the management of pathologic collagenolysis in periodontal diseases. These results were later confirmed by placebo-controlled, double-blinded multi centre trials (79, 80) which substantiated the safe application regarding specific side-effects including gastrointestinal disturbances and emergence of tetracycline-resistant pathogenic micro-organisms (81, 82). Also, nonantimicrobial chemically modified tetracyclines have been synthesized and they lack antimicrobial activity while retaining their potential for the inhibition of inflammatory mediators (83, 84).

Non-antimicrobial properties

Tetracyclines and their derivatives are responsible for several actions, which are independent from their antibiotic activity.

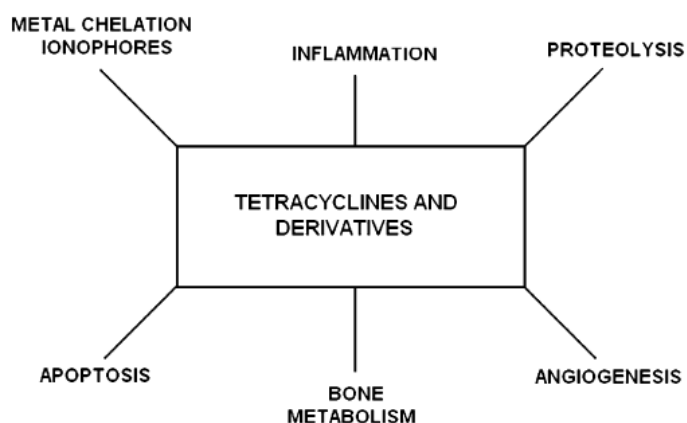


Figure 5 – Non-antimicrobial properties of tetracyclines and associated derivatives (71).

Tetracyclines have proven effective against an array of mediators of the inflammatory cascade. Several direct or indirect mechanisms have been proposed and these include: suppression of neutrophilic migration and chemotaxis (85), inhibition of T cell activation and consequent inhibition of their proliferation (86), inhibition and increased degradation of nitric oxide synthetase (87), pro-inflammatory cytokines inhibition (88,

89), among others. These mediators play an important role on the tissue damage arising from acute or chronic inflammatory processes. The advantage in modifying expression of these mediators makes tetracyclines attractive for therapeutic action over acute and inflammatory disorders in which immuno-inflammatory imbalance is responsible for soft- and hard-tissue damage. These include periodontal disease, rheumatoid arthritis, osteoarthritis, acute respiratory distress syndrome, among others (56).

Further anti-inflammatory action is enhanced by the inhibition of matrix metalloproteinases (MMPs). MMPs are extracellular enzymes that rely on the availability of two cations per molecule (usually Ca^{2+} or Zn^{2+}), to fulfil their enzymatic activity (90). Some MMPs (MMP-1, MMP-8 and MMP-13) are known as collagenases, which is associated with their capacity to break down fibrillar collagens. Tetracycline and its semi-synthetic derivatives (doxycycline and minocycline) have been reported to inhibit collagenolytic activity in humans and several animal models (91-95). Others (MMP-2 and MMP-9) are known as gelatinases and are responsible for the degradation of collagen type IV, which can be found in the basement membrane. Tetracyclines inhibitory action may be established by cation chelating from the active site of the enzymes (60). These enzymes are important for the regulated breakdown of the extracellular matrix of several biological tissues during physiologic turnover processes, namely embryogenesis, bone remodelling, wound healing and biological involution (90). On the other hand, they also play a role in several pathological conditions including rheumatoid arthritis, coronary disease and tumour development, in which they are associated with the invasiveness and metastatical potential of tumour cells (96-98).

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a biological process which is activated in physiological and pathological conditions. In order to facilitate vessel development, matrix degrading enzymes must be recruited and activated, allowing blood vessels to penetrate into the matrix. The MMPs family plays a role in this process. Evidence suggests that minocycline and doxycycline, as well as chemically modified tetracyclines, inhibit tumour-induced angiogenesis (99) and can inhibit MMPs synthesis by endothelial cells (100).

Other important non-antimicrobial property of tetracyclines and related molecules is associated with their eventual antiapoptotic effect. Minocycline was able to prevent neuronal death after mice brain injury, by inhibiting caspase-1 (101). Caspase-1 is a protease that is part of the cysteinyl aspartate-specific proteinases (caspases), which is important in the regulation of the apoptotic events.

Furthermore, the interaction between tetracyclines and the bone system is known for a long time. These agents have been used as diagnostic markers for a long time (102).

Tetracyclines, after uptake, follow the pattern of calcium metabolism which can be visualized by fluorescence microscopy, allowing for qualitative and quantitative determination of the bone remodelling process (103, 104). Further, this technique is being currently used intraoperatively, in which tetracycline fluorescence labelling is used to facilitate the distinction between vital bone that reveals detectable fluorescence after excitation with a black light, in contrast to necrotic bone (105).

Besides contributing to clinical diagnosis regarding bone related pathologies, tetracyclines and derivatives are also expected to modulate, in a direct or indirect way, bone and cartilage metabolism. Early research of bone defect regeneration conducted in dogs reported that defects treated with high concentration of tetracyclines had more regenerative healing and less crestal resorption when compared to control. These results were enhanced when tricalcium phosphate was added to the defect (106). Also, in a dog model, the administration of doxycycline reduced the severity degree of osteoarthritis, with reduced levels of total collagenase activity and inhibition of the proliferation and hypertrophy of chondrocytes (107). Further, a reduction over cartilage collagenase and gelatinase was obtained, after doxycycline administration, in human cartilage extracts (108). These results were confirmed in a recent double-blinded, randomized, placebo-controlled trial which reported a doxycycline-dependent reduction in the rate of joint space narrowing, in knees of obese women with established osteoarthritis (109).

Minocycline also stimulates bone formation and prevents the decrease of mineral density in ovariectomized rats (110). On the other hand, tetracyclines and CMTs also prevent osteopenia in experimental diabetes-induced in rats, by restoring osteoblast structure and function and normalizing collagen metabolism (111-115). Positive results were also verified in pathological induced experimental models of rheumatoid arthritis (116) and osteoporosis (117).

Overall, most of the established investigations, with positive results, were based on the evaluation of bone forming activity by tetracyclines over pathological-induced bone diseases in animal models or clinical trials. Only one recent study was conducted in order to evaluate the effect of tetracycline administration over normal bone remodelling conditions (95). In this situation, increased osteoblastic activity and osteoid formation was attained in the alveolar bone of the squirrel monkeys.

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Chapter 2

Osteoblastic response to tetracyclines

Effect of Therapeutic Levels of Doxycycline and Minocycline in the Proliferation and Differentiation of Human Bone Marrow Osteoblastic Cells

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Abstract

Semi-synthetic tetracyclines (TCs) have been reported to reduce pathological bone resorption through several mechanisms, although their effect over bone physiological metabolism is not yet fully understood. The present study aims at evaluate the behaviour of osteoblastic-induced human bone marrow cells regarding proliferation and functional activity, in the presence of representative therapeutic concentrations of doxycycline and minocycline. First passage human osteoblastic bone marrow cells were cultured for 35 days in conditions known to favour osteoblastic differentiation. Doxycycline (1 to 25 µg/ml) or minocycline (1 to 50 µg/ml) were added continuously, with the culture medium, twice a week with every medium change. Cultures were characterized at several time points for cell proliferation and function.

Present data showed that 1 µg/ml of both tetracyclines, level representative of that attained in plasma and crevicular fluid with the standard therapeutic dosage, increased significantly the proliferation of human bone marrow osteoblastic cells without altering their specific phenotype and functional activity. Long-term exposure to these TCs induced a significant increase in the number of active osteoblastic cells that yielded a proportional amount of a normal mineralized matrix, suggesting a potential application in therapeutic approaches aiming to increase bone formation. The presence of higher levels of these agents led to a dose-dependent deleterious effect over cell culture, delaying cell proliferation and differentiation.

Introduction

An emerging concept on periodontal therapy is the need to block the host response and the bacterial action since both are responsible for tissue damage (1). Bacterial biofilms are known to be associated to the initiation of the inflammatory response as well as periodontal destruction, but the major component of periodontal disease is related to the activation of the host immuno-inflammatory response (2). For instance, most of the soft- and hard-tissue damage (clinically characterized by pocketing, loss of attachment and alveolar bone destruction) occurs when synthesis of endothelial and intercellular adhesion molecules is over-expressed in association with several immune and inflammatory mediators. Inflammatory cells as well as fibroblasts, osteoblasts, epithelial and other structural cells are responsible for the release of several mediators such as prostaglandins, cytokines, eicosanoids, matrix metalloproteinases (MMPs), among others, that are known to mediate the immuno-inflammatory response (3).

Evidence converge to the realization that an imbalance between pro-inflammatory and anti-inflammatory cytokines is linked with the pathogenesis of several diseases associated with bone loss (4, 5). In such diseases, many therapeutic mechanisms have been proposed to block bone resorption and promote bone formation, including gene therapy, administration of bioactive molecules like BMPs, growth factors and MMPs inhibitors (6, 7). Regarding clinical application of MMPs inhibitors, several properties of tetracyclines (TCs) have been reported to contribute to their therapeutic effectiveness in this situation. These include cation-chelation activity, with consequent avidity for mineralized tissues and MMPs inactivation, and long-term clinical safety, all independent from antimicrobial activity (8, 9). Recently, subantimicrobial doses of doxycycline (SDD) have been approved as a host response modifier in the treatment of periodontal disease, and positive effects were observed in clinical trials in patients with chronic periodontitis receiving this therapeutic approach (10-12).

Several reports tend to indicate a bone forming activity by TCs. Most investigations were based on pathological induced bone diseases in animal models, and positive effects were obtained on rheumatoid arthritis (13), osteoporosis condition (14), diabetes induced osteopenia (15-18), cartilage degradation (19-21) and bone loss induced by estrogens deficiency (22). A recent study evaluated the effect of tetracycline upon morphologic characteristics of bone undergoing normal remodeling in squirrel monkeys and also found increased osteoblastic activity and osteoid formation in alveolar bone (23). Several mechanisms have been proposed to explain the beneficial effect including enhancing of bone formation, decreasing of connective tissue

breakdown and diminishing of bone resorption (16-24); the most widely investigated is related to the ability of these agents to inhibit the activity of MMPs. The MMPs family is responsible for most of the degradation process of the constituent macromolecules located in the extracellular matrix of several tissues, including periodontium (24). They are active at physiological pH and their main function is to promote protein breakdown in cell membrane and in the extracellular matrix, which is known to play an essential role in the proliferation and differentiation of cellular populations of the conjunctive tissues (25-29), including osteoblastic cells (25, 26, 29, 30).

To the best of our knowledge, the dose-dependent response of human osteoblastic cells to TCs has not been addressed previously. In this way, to further comprehend the mechanisms associated with the eventual anabolic effect of TCs over bone metabolism, this work evaluated the proliferation and functional activity of osteoblastic-induced human bone marrow cells in the presence of selected concentrations of doxycycline and minocycline – representative of those found in plasma and crevicular fluid after oral administration.

Materials and Methods

Cell Culture

Bone marrow was obtained from patients undergoing orthopaedic surgery procedures. Informed consent to use this biological material, that would be otherwise discarded, was obtained. Bone marrow was cultured in α -minimal essential medium (α -MEM) containing 10% foetal bovine serum, 50 μ g/ml gentamicin, 2.5 μ g/ml fungizone and 50 μ g/ml ascorbic acid. Cultures were incubated in a humidified atmosphere of 5% CO₂ in air at 37 °C, and the medium was changed twice a week. Primary cultures were maintained for 10/15 days till near confluence when adherent cells were enzymatically released with 0.04% trypsin and 0.025% collagenase. The resultant cell suspension was cultured (10⁴ cell/cm²) at the same experimental conditions, but the culture medium was further supplemented with 10 mM β -glycerophosphate and 10 nM dexamethasone. Cell cultures were established for 35 days in absence (control) or presence of doxycycline (1 to 25 μ g/ml) or minocycline (1 to 50 μ g/ml). TCs were renewed in the culture at each medium change, twice a week. Cell cultures were routinely monitored by phase contrast optic microscopy and characterized at days 3, 7, 14, 21, 28 and 35 for cell proliferation and function.

Culture characterization

Cell viability/proliferation and total protein content

Proliferation studies included MTT assay and total protein content. MTT assay was based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product by viable cells. In the last 4 hours of each test time, cells were incubated with 0.5 mg/ml of MTT in the conditions referred above. The medium was then decanted and the stained product dissolved with dimethylsulphoxide before absorbance determination at 600 nm.

Total protein content was determined by Lowry method, after treatment of the cell layer with 0.1 M NaOH for 1 hour. Bovine serum albumin was used as a standard, and absorbance evaluated at 750 nm.

Alkaline phosphatase (ALP) activity

ALP was determined in cell lysates (0.1 % triton) by the hydrolysis (30 minutes at 37° C) of p-nitrophenyl phosphate in an alkaline buffer solution (pH 10.3), followed by colorimetric determination of p-nitrophenol at 405 nm. Results were expressed in nanomoles of p-nitrophenol produced per minute per µg of protein (nmol/min/µg protein).

Histochemical staining for calcium and phosphate deposition

Fixed cultures (1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, 10 min) were stained for the presence of calcium and phosphate deposits in the extracellular matrix. For calcium staining, the fixed cultures were covered with a 1 % S alizarin sodium solution (0.028 % in NH₄OH), pH = 6.4, for 2 min and then rinsed with distilled water and acid ethanol (ethanol, 0.01 % HCL). Calcium deposits stained red.

Phosphate deposits were assessed by the von Kossa assay. Fixed cultures were treated with a 1.0% silver nitrate solution and kept for 1 h under UV light. After rinsing, a 5% sodium thiosulphate solution was added for 2 min and cultures were washed again. Phosphate deposits stained black.

Scanning electron microscopy (SEM)

Fixed cultures were dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analysed in a JeoL JSM 6301F scanning electron microscope equipped with a X-ray energy dispersive spectroscopy (EDX) microanalysis capability (Voyager XRMA System, Noran Instruments).

Ionized calcium (Cai) and phosphorus (Pi) in the culture medium

Culture media from control and TCs-treated cultures were collected every 2-3 days (and cultures reseeded with fresh medium) during the 35 days culture time and analysed for Cai and Pi concentration. Cai and Pi were dosed using Sigma Diagnostics Kits, respectively procedures number 587 and 670. Results were expressed in mmol per litre of medium (mmol/L).

Statistical Analyses

Results presented in this study are from three separate experiments using cell cultures from three different donors (both sexes and aged between 20-50 years old). For each experiment and assay, eight replicas were accomplished. Groups of data were evaluated using a one-way analysis of variance (ANOVA) and no significant differences in the pattern of the cell behaviour were found. Statistical differences found between control and TC-treated cultures were determined by Bonferroni's method with significance set up to $p \leq 0.05$.

Results

Human bone marrow cells (first subculture) were characterized for proliferation and differentiation events during 35 days. Cultures were grown in an osteoblastic-inducing medium in the presence of a concentration range of doxycycline (1 to 25 $\mu\text{g/ml}$) or minocycline (1 to 50 $\mu\text{g/ml}$).

Cell viability/proliferation

Results regarding cell proliferation were measured by the MTT assay in the various experimental conditions and are reported in Fig. 1A.

Cultures grown in control conditions showed a gradual increase in the cell proliferation till day 21, followed by a decrease in the last two weeks. Doxycycline and minocycline caused an initial dose-dependent inhibitory effect (lower MTT reduction at day 3), followed by an increase in the cell proliferation. This initial negative effect ranged from 2 - 3 days in the presence of 1 $\mu\text{g/ml}$, for both TCs, to progressively longer periods at higher levels. The continuous exposure to doxycycline (1 and 5 $\mu\text{g/ml}$) and minocycline (1 to 10 $\mu\text{g/ml}$) caused an evident induction of cell proliferation from the first week onwards. At day 21, the MTT reduction values were around 2 fold higher in the presence of 1 and 5 $\mu\text{g/ml}$, as compared to control cultures. However, cultures treated with levels higher than 1 $\mu\text{g/ml}$ presented a delayed maturation, since maximal

proliferation was achieved later. Doxycycline, at 10 $\mu\text{g/ml}$, caused a significant inhibitory effect in cell growth with a recovery at late incubation time, and treatment with 25 $\mu\text{g/ml}$ resulted in an almost absence of cell growth. At this high concentration range, minocycline presented lower long-term cytotoxic potential, with cell growth being observed in the presence of 25 and 50 $\mu\text{g/ml}$, although after a lag phase of two and three weeks, respectively.

Results regarding total protein content reflected similar information as that obtained from the MTT assay (not shown).

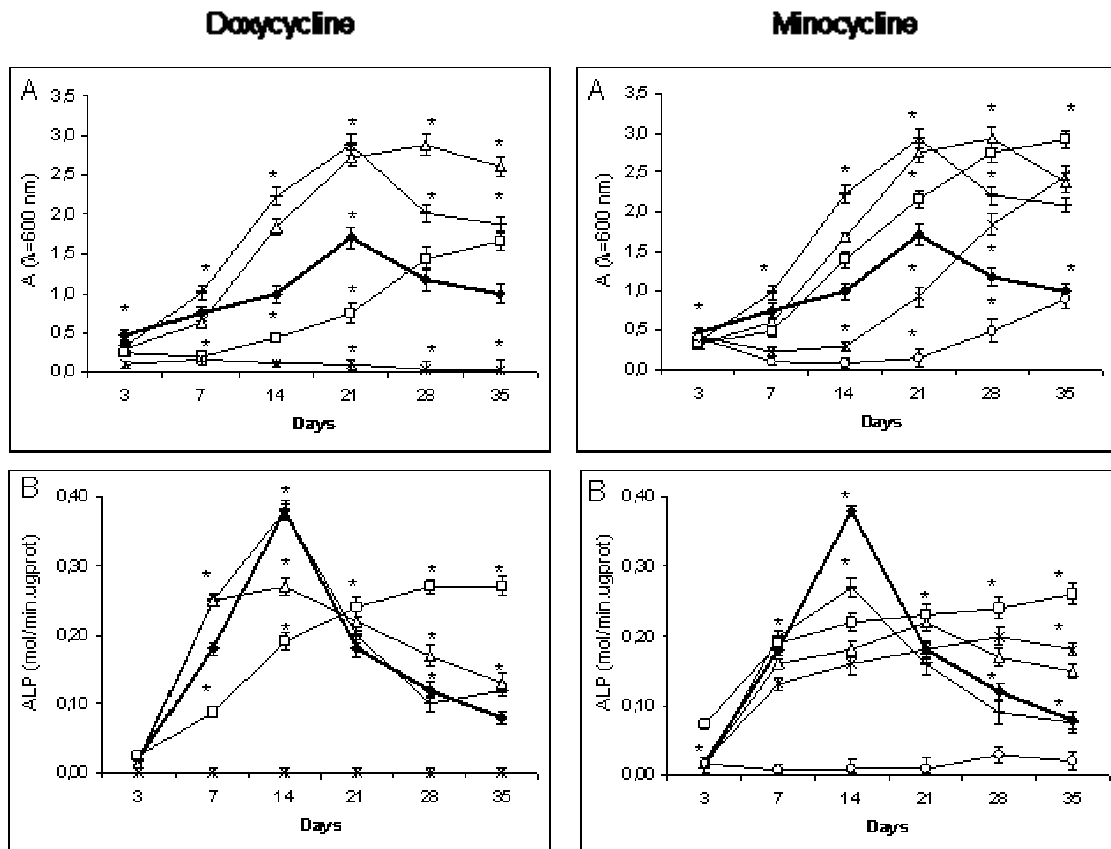


Figure 1. Cell viability/proliferation (A) and alkaline phosphatase activity (B) of human osteoblastic bone marrow cells cultured for 35 days and exposed continuously to doxycycline or minocycline. Control cultures (\blacklozenge , embossed line). Cultures treated with tetracyclines: 1 $\mu\text{g/ml}$ (—), 5 $\mu\text{g/ml}$ (Δ), 10 $\mu\text{g/ml}$ (\square), 25 $\mu\text{g/ml}$ (*) and 50 $\mu\text{g/ml}$ (\circ). * Significantly different from control cultures ($p \leq 0.05$).

Alkaline phosphatase activity

Results concerning ALP activity are shown in Fig. 1B.

Control cultures presented ALP levels that increased with incubation time till day 14, decreasing significantly after that. Doxycycline, at 1 $\mu\text{g/ml}$, did not affect cell behaviour, but treatment with 5 $\mu\text{g/ml}$ caused a slight decrease in maximal levels, although with a

similar pattern of expression. The addition of 10 $\mu\text{g/ml}$ resulted in a significant initial decrease, but with the cells progressively recovering the ability to synthesize this enzyme. In the presence of 25 $\mu\text{g/ml}$, values were very low, reflecting the almost absence of cell growth in this situation. Treatment with minocycline resulted in a higher dose-dependent inhibitory effect in ALP activity, and decreased maximal levels were observed even in the presence of 1 $\mu\text{g/ml}$. Negligible levels were measured in cultures exposed to 50 $\mu\text{g/ml}$.

Matrix mineralization

The presence of calcium phosphate deposits in the extracellular matrix was inferred from the pattern of variation of Cai and Pi in the culture medium during the incubation time (Fig. 2) and visualized by histochemical staining (Table 1, Fig. 3) and SEM observation (Fig. 4).

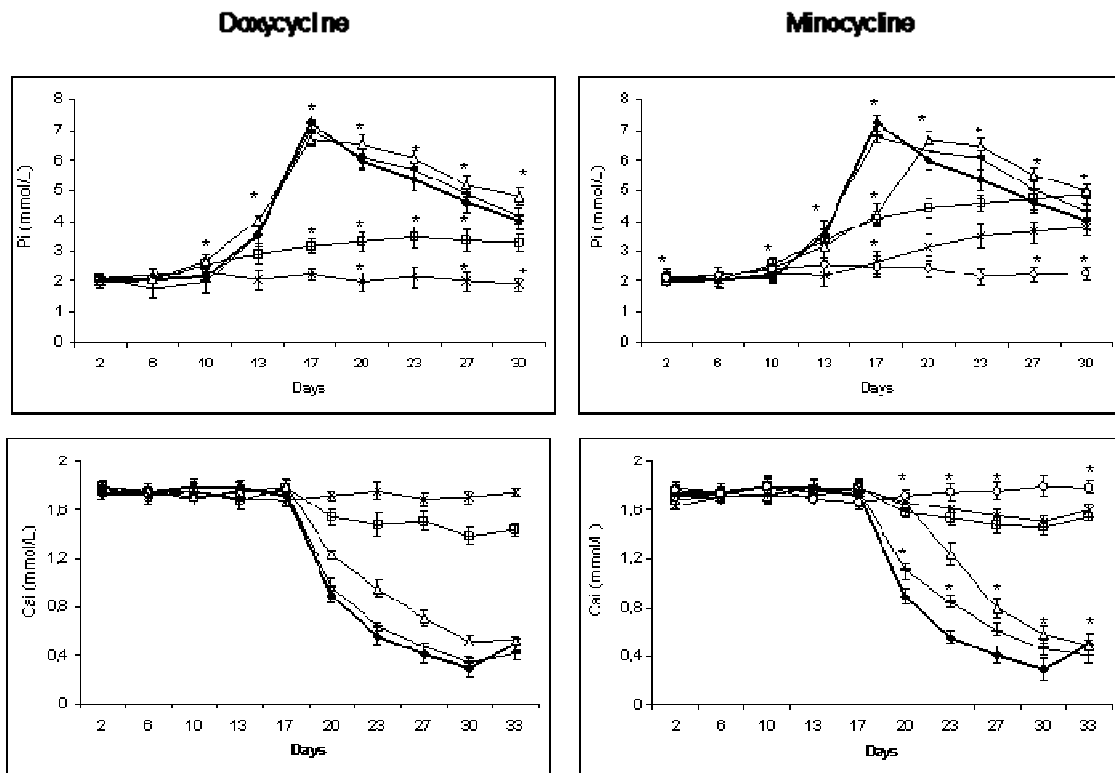


Figure 2. Levels of ionized phosphorus (Pi) and calcium (Cai) in the culture medium from human osteoblastic bone marrow cell cultures, collected at each medium change (twice a week) during the 35-day incubation time. Therefore, levels measured were not cumulative, reflecting changes occurring in intervals of 2-3 days throughout the culture period. Control cultures (\blacklozenge , embossed line). Cultures treated with tetracyclines: 1 $\mu\text{g/ml}$ ($-$), 5 $\mu\text{g/ml}$ (Δ), 10 $\mu\text{g/ml}$ (\square), 25 $\mu\text{g/ml}$ (\circ) and 50 $\mu\text{g/ml}$ (\circ). * Significantly different from control cultures ($p \leq 0.05$).

Pi and Cai levels in the culture medium

Pi and Cai in the culture medium (approximately 2 and 1.8 mmol/L, respectively) originated from calcium and phosphate compounds present in α -MEM and in foetal bovine serum. In addition, β -glycerophosphate added to the culture medium (10 mmol/L) provided a source of Pi, after being hydrolysed by the increasing levels of ALP present in the cultures. Consumption of Pi and Cai from the culture medium reflects the mineralization process, i.e. the formation of calcium phosphate deposits in the extracellular matrix. In the present work, levels of Pi and Cai measured were not cumulative, as culture medium was totally replaced at each medium change. In this way, values reflect changes occurring in intervals of 2-3 days throughout the culture period, providing quantitative information regarding the ongoing mineral deposition in control and TCs-treated cultures.

Medium collected from control cultures presented similar Pi levels for the first 10 days; after that, values increased until day 17 (attaining 7.5 mmol/L), decreasing afterwards. Cai levels were constant until around day 17 and decreased significantly after that (0.3 mmol/L, at day 30). Exposure to doxycycline, 1 and 5 μ g/ml, and minocycline, 1 μ g/ml, resulted in behaviour similar to control, suggesting an identical pattern of calcium phosphate deposition. In the presence of 5 μ g/ml minocycline, mineral deposition began few days later and occurred, apparently, at a lower rate; this observation is suggested by the levels of Pi (maximal values were slightly lower and attained later, around day 20) and Cai (slower rate of decrease, with the onset around day 20). Cultures treated with doxycycline, 10 μ g/ml, or minocycline, 10 and 25 μ g/ml, presented an evident impairment in the production of Pi, reflecting the lower production of ALP in these conditions. Also, only a slight consumption of Cai and Pi from the medium was observed, suggesting a late and faint mineral deposition. Cai and Pi levels remained constant in the medium collected from the cultures treated with 25 μ g/ml doxycycline or 50 μ g/ml minocycline. Results are shown in Fig. 2.

Histochemical staining of calcium deposits. SEM observation

In control cultures, matrix mineralization occurred during the third week. Cultures presented a positive staining for calcium and phosphate deposits from day 21 onwards and the mineral deposition increased with the incubation time. In addition, staining was especially evident in areas of high cell density, with the appearance of nodule-like structures. SEM observation provided similar information. Cultures presented a continuous cell layer with abundant mineral globular deposits closely associated with the fibrous matrix showing the presence of Ca and P peaks on X-ray microanalysis.

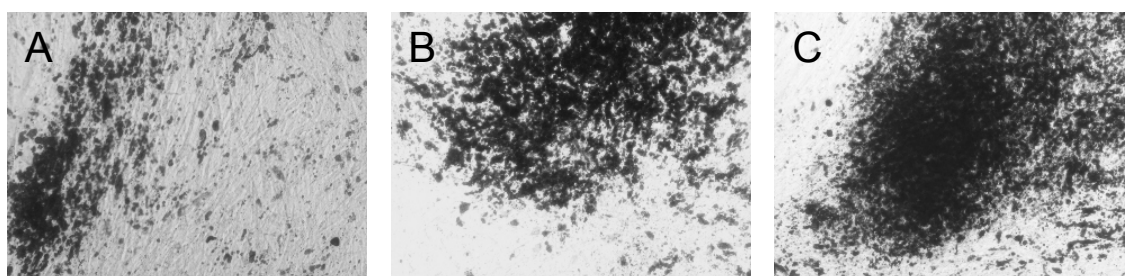


Figure 3. Histochemical assessment of mineral deposition in human osteoblastic bone marrow cell cultures. Representative von Kossa staining at days 21 (A and B) and 35 (C). A – control culture; B and C – cultures treated with doxycycline 1 µg/ml. Magnification 100x.

Cultures treated with the lower concentrations of doxycycline (1 and 5 µg/ml) and minocycline (1 µg/ml) presented similar behaviour to that of control cultures, but a higher abundance of mineral deposition. Comparatively, lower matrix mineralization was observed in the cultures exposed to 5 µg/ml minocycline. In the presence of doxycycline and minocycline, at 10 µg/ml, mineral deposition was observed only at day 35.

Results are summarized in Table 1 regarding the staining intensity resulting from histochemical assessment of calcium and phosphate deposits. Figs. 3 and 4 show the appearance of the cultures, respectively light micrographs of stained cultures and scanning electron micrographs. The areas shown are representative of the corresponding cell layer at the experimental conditions selected.

	Days	14	21	28	35
Control		-	+	+++	+++
Doxycycline	1 µg/mL	-	++	+++	+++
	5 µg/mL	-	++	++	++
	10 µg/mL	-	-	-	+
Minocycline	1 µg/mL	-	++	+++	+++
	5 µg/mL	-	+/-	+	+
	10 µg/mL	-	-	-	+

Table 1. Staining intensity resulting from histochemical assessment of calcium and phosphate deposits (Alizarin red and von Kossa assays) in human osteoblastic bone marrow cell cultures exposed continuously to doxycycline or minocycline, days 14 - 35. Intensity of staining was graded as follows: (-), negative staining; (+/-), fair/absent staining; (+), definite staining but of low intensity; (++) , moderate staining; (+++), intense staining.

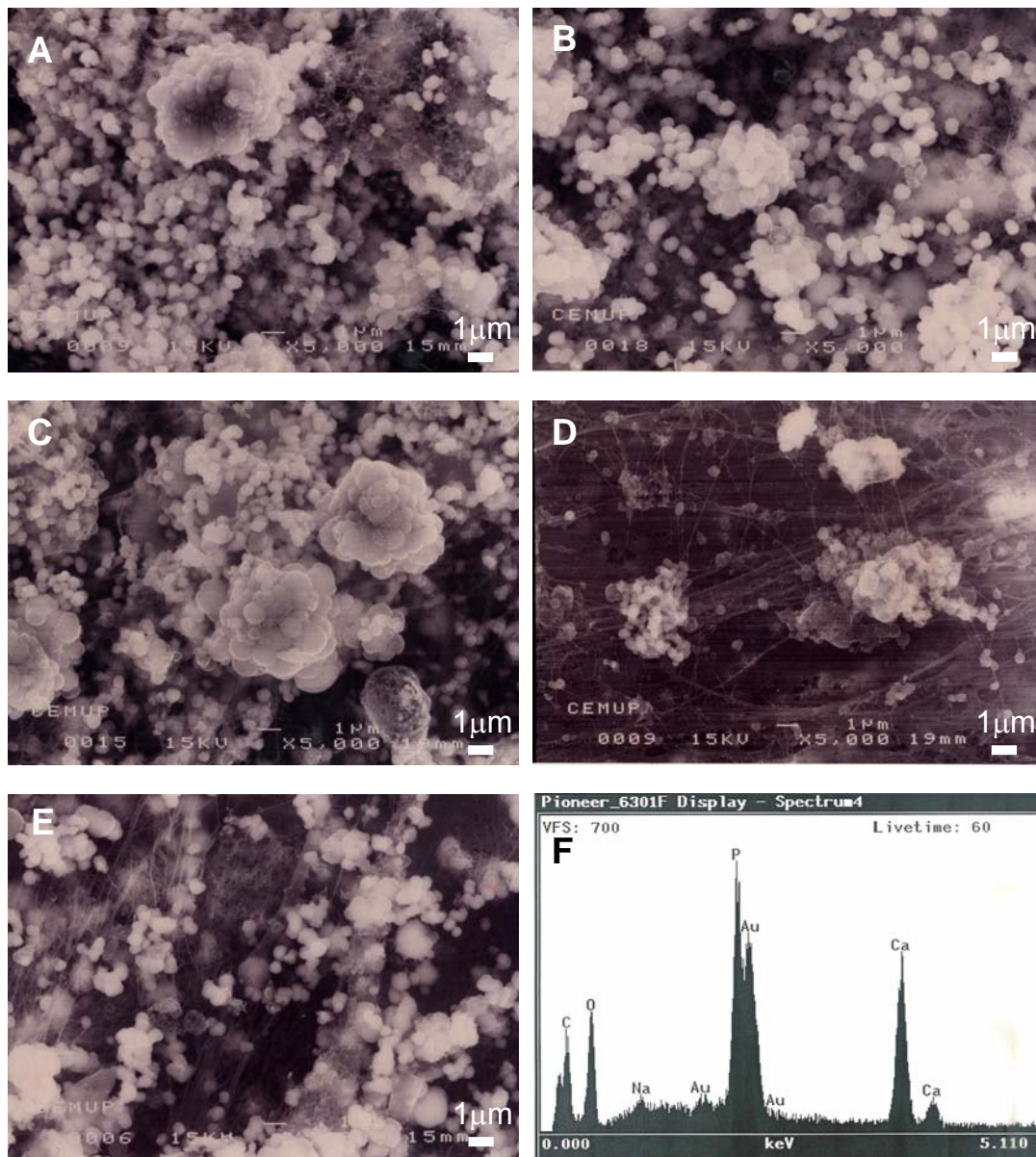


Figure 4. SEM appearance of human osteoblastic bone marrow cell cultures exposed continuously to doxycycline or minocycline, at day 28. (A), 1 µg/ml doxycycline; (B), 5 µg/ml doxycycline; (C), 1 µg/ml minocycline; (D), 5 µg/ml minocycline; (E), control conditions; (F), Representative X-ray spectrum of the mineralized deposits.

Discussion

Human bone marrow cells were cultured in α -MEM with foetal bovine serum, ascorbic acid, β -glycerophosphate and dexamethasone, conditions known to favour the development of osteoblastic phenotype (26). Based on this premise, characterization of the cell behaviour of cultures treated continuously with a selected concentration range of semi-synthetic tetracyclines - doxycycline (1 to 25 $\mu\text{g/ml}$) and minocycline (1 to 50 $\mu\text{g/ml}$) - was assayed.

Bone marrow cells grown in control conditions presented a phase of active proliferation during the first three weeks (Fig. 1A) and synthesis of high levels of ALP (Fig. 1B). Activity of this enzyme increased especially during the second week, suggesting a significant differentiation of the cells in this stage of the culture. ALP appears to play an essential role in the mineralization process providing high levels of phosphate ions to the onset of mineral deposition, being subsequently down-regulated (29, 31). In the present work, hydrolysis of β -glycerophosphate by ALP led to a significant increase in the levels of Pi in the culture medium until day 17, with maximal values being around 8 mmol/L (Fig. 2), suggesting that most of this compound was converted to inorganic phosphate. According to the pattern of Cai and Pi in the medium (Fig. 2), mineral deposition began to occur around day 17 and this was confirmed by the positive staining on Alizarin red and von Kossa assays (Table 1, Fig. 3) and the presence of calcium phosphate deposits intimately associated with the cell layer on SEM observation, from day 21 onwards (Fig. 4). In the last two weeks, the osteoblastic cells became progressively embedded by the mineralising matrix and unable to proliferate, as apparent by the decreased MTT reduction values measured at days 28 and 35 (Fig. 1A). This behaviour is similar to previous results (32) and in line with the established model for the in vitro development of the osteoblastic phenotype (26). Human bone marrow cells in culture presented a continually changing differentiation status, namely a proliferative and poor differentiated cell population, at early time points, and a less proliferative and more differentiated cell population, at later time points, ending with the formation of a cell-mediated mineralized matrix, the event that represents the complete expression of the osteoblastic phenotype (26).

The present data suggest that low doxycycline and minocycline concentrations are able to stimulate the proliferation of osteoblastic-induced bone marrow cells. After an initial inhibitory effect observed during the first 2 - 3 days, treatment with 1 $\mu\text{g/ml}$ caused an increase in the cell growth, maintenance of ALP activity and higher abundance of mineral deposition. Exposure to 5 $\mu\text{g/ml}$ doxycycline resulted in a similar behaviour. Comparatively, minocycline, at this concentration, caused a slight impairment in

osteoblastic function. Higher TCs levels delayed cell proliferation and function. The selected concentration range is of clinical significance as pharmacokinetic studies showed that after oral uptake of 200 mg of doxycycline or minocycline (usual therapeutic dosage), the plasma concentration reached 3 µg/ml at 2 hours and was maintained above 1 µg/ml for 8 to 12 hours (33). Similar and higher levels were found in GCF (33, 34). On the other hand, therapeutic dosage of SDD (20 mg twice daily) resulted in peak serum concentrations around 0.8 µg/ml and steady-state concentrations around 0.48 µg/ml (35).

The effects of TCs over osteoblastic cells were addressed in few previous studies, although in different cell systems and conditions. Williams et al reported that minocycline, at levels up to 3 µg/ml, might increase the efficiency of rat's primary bone marrow stromal cells regarding colony formation capacity (36). Scharz et al reported induction of early differentiation events and cell proliferation improvement with diminished later differentiation events (like osteocalcin production) in osteoblast-like MG63 cells cultured in tetracycline pre-treated dentin (37). In contrast, Homes et al reported that doxycycline (up to 5 µg/ml) addition to the culture of mouse osteoblast-precursors did not result in any anabolic effect in cultures established for 12 days and exposed between 0-5, 5-9 or 9-12 days (38). Differences among studies might be related with the cell system used (human/animal cells, stage of cell differentiation), the pharmacological agent analysed and the type of exposure (continuous/intermittent). This parameter appears to be relevant, as the present results showed that, for a given dose, the effect on cell proliferation is time-dependent, i.e. an initial inhibitory effect followed by an induction on long-term treatment. This probably explains, at least in part, the absence of effect reported by Homes et al that tested short and intermittent doxycycline exposures.

The results of the present in vitro study are in line with those reported by several authors investigating bone metabolism and pathology in animal models. The mouse model was used by Golub et al. to simulate diabetic-induced osteopenia in which it was verified that the pathological situation (characterized by decreased bone formation and not augmented bone resorption) was significantly ameliorated with tetracycline uptake (15). Later, Sasaki et al suggested that minocycline and CMTs would be able to improve osteoblastic structure and function in diabetes-induced osteopenia in rats (16). Li et al reported increased bone formation and mass levels on ovariectomized rats by low dose tetracycline administration. The two concentrations tested reflected 0.24 % - 0.72 % of clinical antibiotic regular dose and while the lower dose increased osteoblastic recruitment, the higher increased osteoblastic activity (39). Recently, tetracycline administration to squirrel monkeys, in a model of normal bone metabolism,

increased the deposition of osteoid in the alveolar process by increasing the number of active osteoblasts (23).

The present data tend to indicate that low concentrations of doxycycline and minocycline - 1 µg/ml - have proanabolic effects over osteoblastic phenotypic cells, regarding proliferation. These TCs, that apparently need long treatment periods (several days) to cause the stimulating effects, may act as promoters of less differentiated stages inducing cell growth, without altering functional activity. This observation is suggested by the significantly increased cell proliferation in the TCs-treated cultures, but similar ALP activity; in this way, the higher abundance of mineral deposition is likely related to the increased number of viable and normal functioning cells in culture. However, in the present work, TCs were present throughout the culture time and, therefore, the effect of these agents was assessed in conditions of a continually changing differentiation status of the culture, making data interpreting difficult. Drug exposure at early and late time points would provide information regarding the responsiveness of osteoblastic cells at different stages of cell differentiation. The mechanism by which these TCs exert their favourable effects over osteoblasts may be speculated. Indirect mechanisms related with their capacity to chelate calcium in the extracellular environment might play a role. Calcium chelation, which is responsible for diminishing free calcium concentration, might inhibit MMPs activity since these enzymes are cation-dependent. Recently, it has also been speculated that the inhibitory action by TCs could be linked to a decreased level of collagenase mRNA, rather than a direct inhibition of the MMPs (19, 40, 41).

Regardless the mechanism involved, it has been widely demonstrated that TCs can inhibit collagenase and/or the breakdown of collagen under a variety of conditions (4, 8, 13, 17). Studies in animal models of bone-deficiency disease showed that TCs were found to increase type I collagen synthesis (42). Moreover, recent studies demonstrated that these agents can also increase collagen synthesis on soft tissues as well (43-45). Evidence that a stable collagenous matrix is important for the progression of osteoblastic differentiation is well known (46, 47) and provided, for instance, by studies showing that inhibition of collagen synthesis or its increased degradation leads to an impairment of osteoblastic cell behaviour (48, 49). In this way, the eventual modulation of the extracellular matrix by TCs might favour osteoblastic cell proliferation. Related to this, it is known that osteoblastic cells sense and respond to calcium ion in a time- and concentration-dependent manner, regarding proliferation and expression of several differentiation markers, including synthesis of collagen type I (50). A variety of studies suggest that the calcium ion, most likely acting through the calcium sensing receptor (CaSR), is a key regulator of osteoblastic cell fate (50, 51). In this way, local

fluctuations in the levels of calcium ion, potentially associated to the calcium quelation properties of TCs, might play a role in modulating osteoblastic cell behaviour.

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Chapter 3

Osteoblastic response to tetracyclines in seeded hydroxyapatite and Bonelike[®]

Cell-induced response by tetracyclines on human bone marrow colonized hydroxyapatite and Bonelike[®]

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Abstract

Semi-synthetic tetracyclines are commonly used antibiotics that also seem to play an important role in the modulation of the immuno-inflammatory imbalance, verified in several bone diseases. The association of a therapeutic agent (that prevents bacterial infection and induces tissue formation) to a biomaterial aiming to repair/regenerate bone defects could contribute to a more predictable clinical outcome.

The present study intends to evaluate the proliferation and functional activity of osteoblastic-induced human bone marrow cells, cultured on the surface of hydroxyapatite (HA) and Bonelike[®], in the presence of therapeutic concentrations of doxycycline and minocycline.

First passage bone marrow cells were cultured for 35 days on the surface of HA and Bonelike[®] discs, in the absence or presence of 1 µg/ml doxycycline and minocycline. Cultures performed in standard tissue culture plates were used as control.

Doxycycline or minocycline induced cell proliferation and increased extent of matrix mineralization in osteoblastic cell cultures established in the three substrates. Also, an improved biological behavior was verified in seeded Bonelike[®] comparing to HA. Results suggest that the local delivery of tetracyclines might associate the antimicrobial activity in implant-related bone infection with an eventual induction of osteoblastic proliferation and maintenance of the characteristic biological activity of these cells.

Introduction

Tetracyclines are commonly used bacteriostatic antibiotics active against a wide range of both aerobic and anaerobic gram-positive bacteria. Their antimicrobial activity is due to the inhibition of bacterial protein synthesis, by binding to the 30S ribosome subunit, blocking the bond to the tRNA, on the mRNA-ribosome complex (1).

In the last years, several observations converge to justify the therapeutic effectiveness of tetracycline (as well as its semi-synthetic derivatives, minocycline and doxycycline) in the modulation of the immuno-inflammatory imbalance verified in several animal and human bone diseases (2-4). Different mechanisms, distinct from the antimicrobial action, have been proposed to justify the pro-anabolic activity of these pharmacological agents regarding bone metabolism. These include enhancing of bone formation, decreasing of connective tissue breakdown and diminishing of bone resorption (5-10). Clinical application of these agents targeting bone might also be favored by their cation chelation activity and consequent avidity for mineralized tissue (11).

Currently, ceramic-based biomaterials have been used in bone tissue repair strategies for their adequate mechanical properties and chemical composition – similar to those of the bone tissue. Among them, HA has generated a great deal of interest in the last years (12). This synthetic bone graft substitute, although lacking osteogenic properties that can only be found on autologous grafts, still offer advantages that include a reduced local tissue morbidity, absence of complications at donor site and unlimited material availability (13). This biomaterial, being similar to the mineral component of natural bone revealed good osteoconductivity and bone bonding ability (14). However, HA presents low load bearing capacity (15) and the introduction of phosphate based glasses as a sintering aid is known to reinforce HA mechanically (16). Glass-reinforced HA with bioactive properties has been applied with success in medical and dental clinical applications aiming to regenerate the bone tissue (17, 18). Bonelike[®] is a registered and patented glass-reinforced HA with improved mechanical properties and enhanced bioactivity that result from the addition of CaO - P₂O₅ based glasses during the liquid phase sintering process of HA, leading to several ionic substitutions in the lattice that are responsible for the reduction of porosity and grain size (19, 20). Recently, it has been successfully applied in regenerative procedures aiming to restore bone structure and function in oral, maxillofacial and orthopedic procedures (21, 22).

Although the wide application of synthetic biomaterials in order to repair/regenerate the bone tissue, several clinical complications are established and prove difficult to remedy. Among them, osteomyelitis, septic arthritis and prosthetic joint infection are specially caused by Gram-positive organisms and known to contribute to a heavy clinical and

economic burden (23). Treatment is often complicated at sites of reduced vascularization, requiring prolonged antimicrobial use, usually associated with surgical drainage or debridement (24). In this way, the selection of the most effective therapeutic approach, based on several parameters that include the specificity of the pathogenic agents, their sensitivity profile, pharmacokinetics of the drug, local vascular supply, presence or absence of a biomaterial and individual factors, is essential in order to minimize tissue and function loss, as well as to reduce discomfort and need of further medical/surgical intervention (24). Also, it is known that local and systemic measures to control the colonization of the surgical wound at the early healing phase, associated with reduction of the infections' spreading, may increase the predictability of the results (25). Tetracyclines have been proven to be effective in several bone and joint related infections (26-28).

Regarding bone regeneration strategies, the association of a biomaterial and a therapeutic agent that might induce bone formation at the same time that prevents bacterial infection could, undoubtedly, contribute to a more predictable clinical outcome. In this way, the objective of this research was to evaluate the proliferation and functional activity of osteoblastic-induced human bone marrow cells, cultured on the surface of HA and Bonelike[®], in the presence of therapeutic concentrations of doxycycline and minocycline.

Materials and Methods

Preparation of samples

Bonelike[®] was prepared with the chemical composition of 65P₂O₅-15CaO-10CaF₂-10Na₂O in mol% from reagent grade chemicals using conventional glass making techniques. The composite was obtained by adding the milled glass to HA powder in 2.5% (wt/wt), using isopropanol as a solvent. The powders were then dried and sieved to less than 75 μm. Disc samples were therefore prepared for *in vitro* testing by uniaxial pressing at 200 MPa, using steel dies to obtain 12 mm diameter discs. The discs were then sintered at 1300°C (using a ramp rate of 4°C/min), with the temperature maintained for 1 hour, followed by natural cooling inside the furnace. Phase identification and quantification was assessed by X-ray diffraction and Rietveld analysis. XRD was performed on Bonelike[®] samples using a Siemens D5000 diffractometer with Cu-K_α radiation ($\lambda=1.5418 \text{ \AA}$). The scans were made in the range of 25–35° (2θ) with a step size of 0.02° and a count time of 2 s/step.

Detailed description of Bonelike[®] preparation has been previously reported (20).

For *in vitro* testing, discs were mechanically polished to the same final topology of 1 μm using silicon carbide paper, ultrasonically degreased, cleaned with ethanol followed by deionised water and finally sterilized, prior to cell culture.

HA samples were also prepared as 12 mm diameter discs in order to compare to the biological behavior of Bonelike[®].

Cell culture

Human bone marrow was obtained from orthopedic surgical procedures conducted in adult patients (aged between 25 and 45 years). Informed consent was obtained to use this biological material that would be otherwise discarded. Bone marrow was cultured in α -MEM culture medium containing 10% foetal bovine serum, 50 $\mu\text{g/ml}$ gentamicin, 2.5 $\mu\text{g/ml}$ fungizone and 50 $\mu\text{g/ml}$ ascorbic acid. Primary cultures were maintained in a humidified atmosphere (5% CO_2 in air at 37 $^\circ\text{C}$) for 10-15 days until sub-confluence condition. At this stage, cells were enzymatically released (0.05% trypsin and 0.025% collagenase) and the resultant suspension was cultured at a density of 10^4 cell/ cm^2 , in the previous described culture medium further supplemented with 10 mM β -glycerophosphate and 10 nM dexamethasone. Cell cultures were established for 35 days and maintained in the surface of the culture plate (control cultures), HA or Bonelike[®] in the absence or presence of doxycycline or minocycline (1 $\mu\text{g/ml}$). Tetracyclines were both renewed at every medium change that occurred twice a week.

Culture characterization

Cell viability/proliferation and total protein content

MTT assay was used to estimate cell viability/proliferation. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product by viable cells. Cultured cells were incubated with 0.5 mg/ml of MTT during the last 4 hours of each test time. The medium was then decanted, the stained product dissolved with dimethylsulphoxide and absorbance determination was conducted at 600 nm in an ELISA reader. Results were expressed as absorbance per square centimeter (A/cm^2).

Total protein content was determined by Lowry method after treatment of the cell layer with 0.1 M NaOH for 1 hour. Bovine serum albumin was used as a standard and absorbance determination was conducted at 750 nm.

Alkaline phosphatase (ALP) activity

Activity of ALP was determined in cell lysates (obtained after treatment of cultured cells with 0.1% triton) by the hydrolysis of p-nitrophenyl phosphate (30 minutes at 37° C) in an alkaline buffer solution (pH 10.3). Colorimetric determination of p-nitrophenol was established at 405 nm. Enzyme activity was normalized by total protein content and results were expressed as nanomoles of p-nitrophenol produced per minute per µg of protein (nmol/min/µg protein).

Ca²⁺ loss from the culture medium

Culture medium from cultures in control and experimental conditions was collected every 2-3 days (and cultures refed with fresh medium) between days 10 and 35 of the culture. Analysis of Ca²⁺ content was conducted using Sigma Diagnostics Kit, procedure number 587. Results were expressed as millimoles per litre of ionized calcium loss from medium (Ca²⁺ mmol/L).

Scanning electron microscopy (SEM)

Glutaraldehyde (3%) fixed cultures were dehydrated in graded alcohols (70, 80, 2 x 90 and 99.8%), critical-point dried, sputter-coated with gold and analyzed in a JeoL JSM 6301F scanning electron microscope equipped with a X-ray energy dispersive spectroscopy (EDX) microanalysis capability (Voyager XRMA System, Noran Instruments).

Statistical Analyses

Results presented in this study are from three separate experiments using cell cultures from different patients. In each experimental situation, three replicas were accomplished. Groups of data were evaluated using a two-way analysis of variance (ANOVA) and no significant differences in the pattern of the cell behaviour were found. Statistical differences found between control and experimental conditions were determined by Bonferroni's method. P-values ≤0.05 were considered significant.

Results

Human bone marrow stromal cells were characterized for proliferation and differentiation events, on the surface of the culture plate, HA and Bonelike[®], in the presence of an osteogenic inducing medium, further supplemented with 1 $\mu\text{g/ml}$ doxycycline or minocycline.

XRD analysis revealed that due to the reaction between the HA matrix and P_2O_5 -based glass during the sintering process, the Bonelike[®] microstructure had a main crystalline phase of HA with β - and α -tricalcium phosphate as secondary phases (Figure 1).

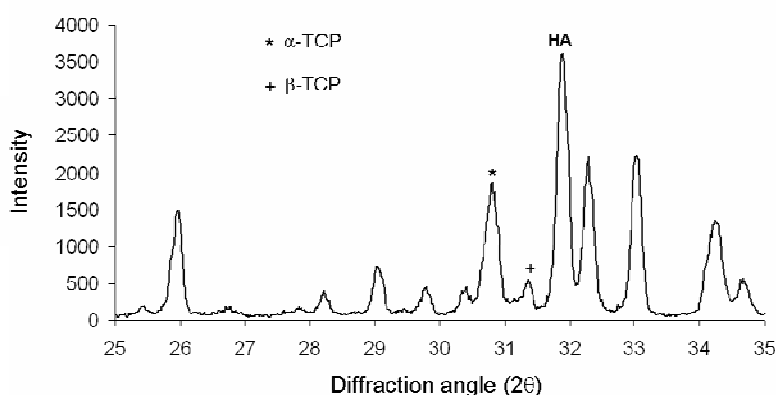


Figure 1. X-ray diffraction pattern of Bonelike[®]. Bonelike[®] is composed of HA and α - and β -TCP phases.

Cell viability/proliferation

The results regarding the evaluation of cell proliferation by the MTT assay are reported on Figure 2A.

Cultures grown on the surface of the culture plate proliferated gradually till day 21, followed by a decrease in the remaining time of culture. Seeded Bonelike[®] presented a similar behavior to control, while cultures established on the surface of HA showed an initial lag phase of approximately two weeks and maximal MTT reduction values were only achieved by day 28. The presence of doxycycline or minocycline (1 $\mu\text{g/ml}$) increased cell proliferation in control cultures and on seeded sample materials. Results were statistically significant ($p \leq 0.05$) at days 21, 28 and 35 for cultures grown on the culture plate, and at maximal MTT reduction values for cultures established on the surface of the materials – day 21 for Bonelike[®] and 28 for HA. The stimulatory effect was evident after an initial lag stage (during approximately the first week) and maximal

MTT values were around 30% and 40% higher in the presence of minocycline and doxycycline, respectively, comparing to those obtained on non-treated cultures.

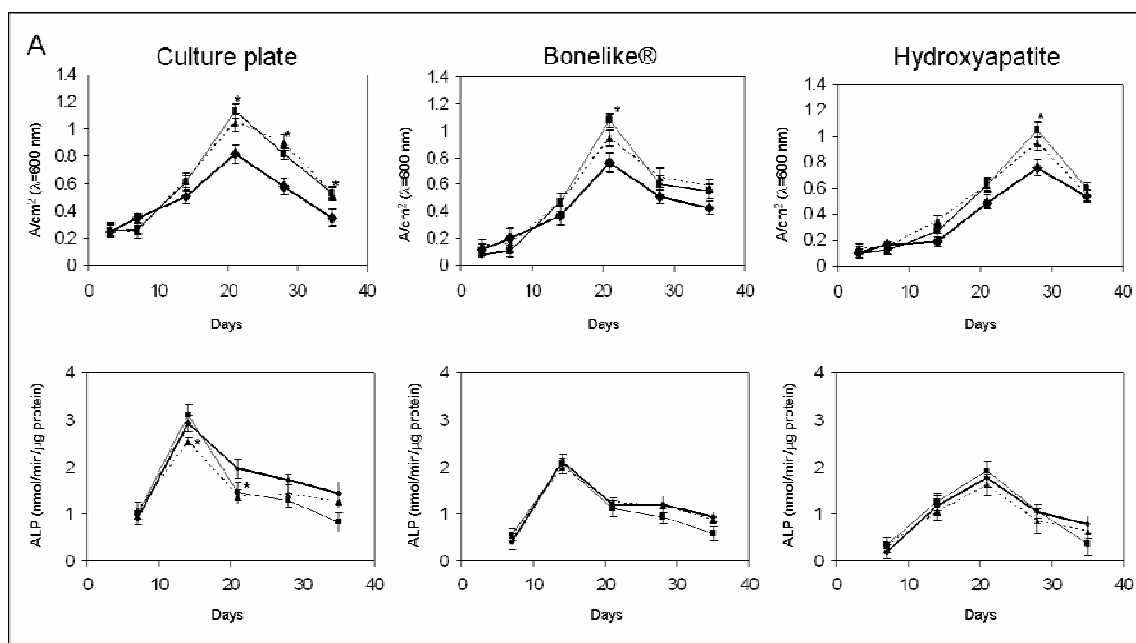


Figure 2. Cell viability/proliferation (A) and alkaline phosphatase activity (B) of human bone marrow osteoblastic cells cultured for 35 days on the surface of culture plate, Bonelike® and HA in the absence (◆) and presence of 1 µg/ml Doxycycline (■) or Minocycline (▲). * Significantly different from control cultures (p≤0.05).

Alkaline phosphatase activity

Results regarding the activity of alkaline phosphatase are presented on Figure 2B.

ALP activity was low during the first week of culture and increased significantly afterwards achieving maximal values by day 14 in control and cultures established on the surface of Bonelike® and, later, by day 21, on seeded HA. Subsequently, ALP activity decreased throughout the remaining culture time. Comparing to control cultures, reduced enzymatic activity was verified in seeded Bonelike® and HA.

No significant differences were found in ALP activity in the presence of doxycycline, although, in minocycline-treated cultures, a tendency for a reduction in the enzyme activity was verified, which was statistically significant (p≤0.05) in the cultures grown on the surface of the culture plate.

Mineralized deposits in the extracellular matrix

The presence of mineralized deposits (calcium phosphate) in the cell layer was evaluated by the analysis of ionized calcium (Cai) loss from the culture medium throughout days 10 to 35 and by SEM observation.

Cai loss from the culture medium

The Cai (in association with Pi) is consumed in the formation of calcium phosphate deposits in the extracellular matrix, reflecting the mineralization process. In the present work, the Cai consumption reflects the changes occurring between every medium change (intervals of 2-3 days), since the medium was totally replaced at every change, outputting values that are not cumulative. Results are presented on Figure 3.

Levels of Cai were determined in the absence of cultured cells and a relatively steady value of 1.7 mmol/L was verified in the absence of materials. A higher variation of Cai levels (between 1.6 and 1.8 mmol/ml) occurred in incubated Bonelike[®] and HA samples. Cultures grown on the surface of the culture plate revealed almost no Cai loss from the culture medium, between day 10 and 17. Since this day onwards, a significant and progressive increase of the Cai loss was verified and, at day 30, values around 1.35 mmol/L were attained. Medium collected from cultures established on the surface of Bonelike[®] revealed a similar behavior. Cai loss regarding cultures grown on the surface of HA, was significant from day 20 onwards, achieving approximately 1.15 mmol/L around day 30.

In the presence of doxycycline and minocycline, levels of Cai followed a similar pattern. However, in cultures established on the surface of culture plate and Bonelike[®], the initial rate of calcium deposition (between days 17 and 23) was higher compared to non-treated cultures, especially in the presence of doxycycline (results statistically significant, $p \leq 0.05$).

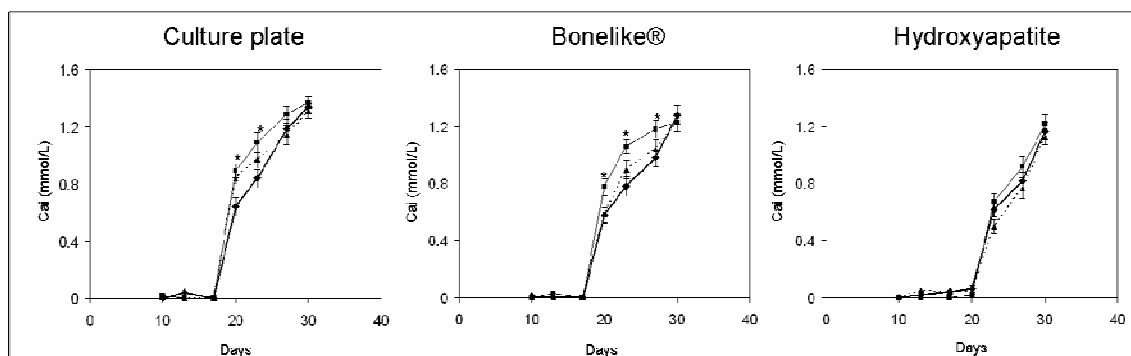


Figure 3. Levels of ionized calcium (Cai) loss from the culture medium regarding human bone marrow osteoblastic cell cultures performed on the surface of the culture plate, Bonelike[®] and HA. Cai loss was not cumulative, as the medium was totally replaced at each medium change (twice a week), and levels measured reflect changes occurring in intervals of 2-3 days throughout the culture period. Cultures were grown in the absence (◆) and presence of 1 µg/ml Doxycycline (■) or Minocycline (▲). * Significantly different from control cultures ($p \leq 0.05$).

SEM

Representative scanning electron micrographs of the cell layer, at day 21, are presented on Figure 4 corresponding to different experimental conditions.

Control cultures and seeded HA and Bonelike[®] revealed a continuous cell layer with distributed globular mineral deposits in close association with the matrix (Figure 4A-C). Treatment with doxycycline (Figure 4D-F) or minocycline (Figure 4G-I) increased mineral deposition. Regarding cultures established on the surface of HA, significantly less mineral deposits were visualized both in the absence (Figure 4C) and presence of doxycycline (Figure 4F) and minocycline (Figure 4I).

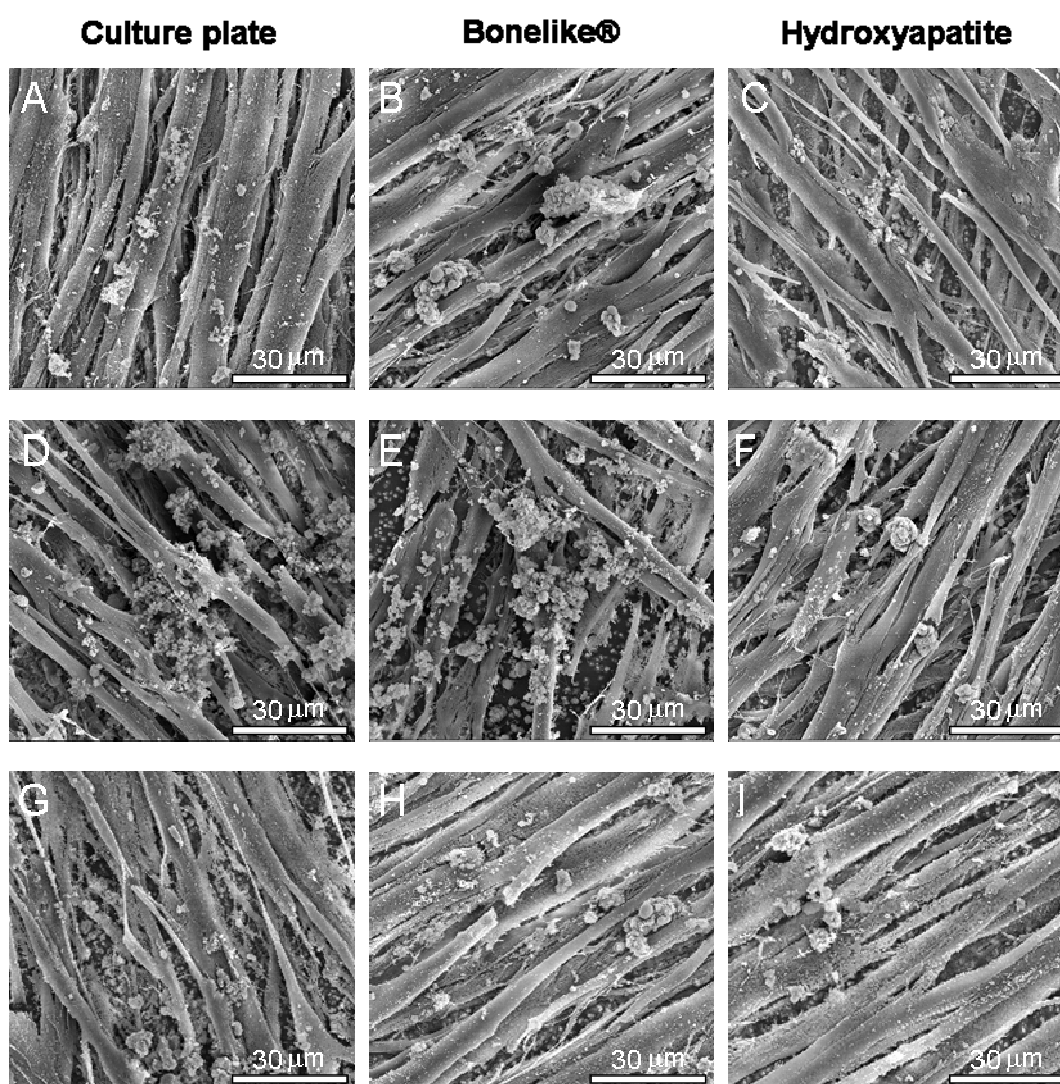


Figure 4. SEM appearance of human bone marrow osteoblastic cell cultures grown in the absence (A-C) or the presence of 1 µg/ml Doxycycline (D-F) and 1 µg/ml Minocycline (G-I), at day 21, in the surface of the culture plate (A, D and G), Bonelike[®] (B, E and H) and HA (C, F and I).

Discussion

Each year, almost two million patients worldwide undergo bone graft surgery in order to repair skeletal lesions resulting from trauma, tumor resection or degenerative diseases (29) and several synthetic graft materials, with osteoconductive and osteoinductive properties have been designed to fulfill this need (30). The implantation of a bone graft causes an inflammatory reaction and, frequently, an infectious process (31). The direct contact of the material surface with blood and/or tissue fluid enhances the formation of an adsorbed protein film. Several of the adsorbed proteins (including fibronectin, vitronectin and fibrinogen) provide anchoring sites for bacteria via the expression of adhesion molecules from the pathogenic agents. When the adhesion process is established, the bacteria proliferate and create an infectious process in close association with the implanted material (32). In order to control the infection, several methodologies have been proposed. Local control offers many advantages over systemic antibiotic chemotherapy (25, 32) and, in addition, the association of antimicrobial activity with a positive modulation of the host response contributes to a more predictable and rapid clinical outcome in bone regeneration therapies (33).

Tetracyclines are broad spectrum antibiotics important in the treatment of bone and joint infections (26-28) which have been associated with a positive effect in bone metabolism (5-10). In previous studies, performed in standard tissue culture plates, we reported that therapeutic levels of doxycycline and minocycline were able to induce proliferation of osteoblastic bone marrow cells while maintaining their specific phenotype (34). In the present study, HA and Bonelike[®], a glass-reinforced HA, were cultured with human bone marrow cells in a medium known to induce osteogenic differentiation (35, 36), and cell proliferation and function were assessed in the presence of 1 µg/ml doxycycline or minocycline, representative of the plasmatic levels attained with standard therapeutic systemic dosages (37).

Bone marrow cells grown on standard polystyrene culture plates, in the absence of tetracyclines, presented active proliferation during the first three weeks associated with expression of high levels of ALP. The maximum levels of ALP, achieved on day 14, indicate the subjacent differentiation process being established at this stage. ALP is associated with the availability of high levels of phosphate ions, essential to the onset of the mineralization process, being subsequently down-regulate (38). Accordingly, the pattern of Cai loss from the culture medium and SEM observation showed that mineral deposition closely associated with the cell layer occurred from day 17 onwards. Over the last two weeks of culture, cell proliferation was greatly reduced by the embedding

of the cells in the mineralized extracellular matrix, as in accordance with the established model for the *in vitro* development of the osteoblastic phenotype (35, 36, 38). Cell response on the surface of Bonelike[®] was similar. HA showed a poor performance during the initial stages of the culture, reflected by a long lag phase, which resulted in delayed maximal values in the MTT assay and ALP activity, compared to Bonelike[®]. Upon cell seeding, surface chemical and topographic changes resulting from the interactions between the material surface and the culture medium are of particular importance to the subsequent cell growth and differentiation events. Appropriate surface features for normal cell behavior seem to take place earlier on Bonelike[®], comparing to HA. Bonelike[®] is composed by an HA matrix with soluble β - and α -tricalcium phosphate phases (Figure 1) and the ongoing release/deposition events of Ca and P ionic species leads to a rapid formation of an apatite layer, according to previous studies (39). This behavior appears to contribute to the improved biological performance of Bonelike[®], both *in vitro* (40, 41) and *in vivo* (22, 42, 43). Comparatively, formation of an apatite layer on the surface of HA occurs slowly (39) with favorable surface conditions for cell growth being observed later. However, the interfacial reactions tend to an equilibrium rendering the surface of the two materials with similar features. Accordingly, after two weeks, cell behavior on HA surface showed a pattern similar to that observed on Bonelike[®] samples. Also, the presence of F in the composition of Bonelike[®] and/or its release may also contribute to the improved initial response, as this ion is reported to stimulate osteoblastic proliferation (44).

Doxycycline or minocycline affected the proliferation of osteoblastic cells in a time-dependent manner. After a small lag phase during the first week, induced cell proliferation was observed in all experimental situations, i.e. control cultures, seeded HA and Bonelike[®]. ALP activity was similar, although somewhat reduced in minocycline-treated cultures performed in standard culture plates. Rate of mineral deposition, as assessed by the pattern of Ca loss from the culture medium, was slightly higher in tetracycline-treated cultures, which is probably related with the presence of increased cell number of functional active cells in these experimental conditions.

Osteoblastic cell response to tetracyclines was addressed in few previous studies, performed in standard tissue culture plates. Minocycline (up to 3 $\mu\text{g/ml}$) was reported to increase the efficiency of rat bone marrow stromal cells regarding colony forming capacity (45). Also, induction of early differentiation events and cell proliferation improvement were observed in osteoblast-like MG63 cells cultured in tetracycline pre-coated dentin (46). In contrast, doxycycline failed to cause any anabolic effect in murine calvarial osteoblasts and MG63 osteoblast-like cells (47). A recent study

assessed the effect of a variety of antibiotics on human bone trabecular bone derived cells and on the cell line MG63, exposed during 48 hours, and found a mean IC₂₀ of 60 µg/ml for tetracycline (48). As mentioned above, we reported previously that long-term exposure of osteogenic-induced human bone marrow cells to 1 µg/ml doxycycline and minocycline resulted in increased cell growth, while higher levels caused dose-dependent deleterious effects (34). Positive effects of tetracyclines over osteoblastic cells were also reported in an in vivo model of normal bone metabolism (10). Tetracycline administration to squirrel monkeys over a period of 17 days increased osteoblastic activity and osteoid formation in alveolar bone (10), consistent with similar effects of other tetracycline compounds in animal models of bone-deficiency diseases (5-8).

The mechanisms underlying the effects of tetracyclines over osteoblastic cells remain unclear. However, changes in the collagenous extracellular matrix, expected from the well established inhibitory effect of tetracyclines over matrix metalloproteinases (MMPs) (4, 49, 50) might play a role. Tetracyclines can inhibit collagenase and/or the breakdown of collagen under a variety of conditions (51-54). Also, additional effects on collagen metabolism may occur, particularly increased collagen synthesis after tetracycline administration verified on pathological models of bone-associated diseases and cell population from soft tissues (53, 54). In addition, it is interesting to refer that progressive consolidation of collagenous connective tissue at the apical portions of periodontal pockets is reported to occur in patients receiving subantimicrobial doses of doxycycline (55, 56). These events might result in improved osteoblastic behavior, since a stable collagenous matrix is known to play a significant role in the osteoblastic proliferation/differentiation sequence (38, 57, 58).

In the present study, treatment with doxycycline or minocycline, 1 µg/ml, induced cell proliferation and increased extent of matrix mineralization in human osteoblastic cell cultures growing on HA or Bonelike[®]. Results suggest that the local delivery of tetracyclines might associate the antimicrobial activity in implant-related bone infection with an eventual induction of osteoblastic proliferation and maintenance of the characteristic biological activity of these cells. In this way, tetracyclines may become suitable candidates to drug confinement and delivery applications regarding the use of ceramic matrices for hard tissue regeneration.

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Chapter 4

General conclusions

Tetracyclines are broad-spectrum antimicrobial agents that are characterized as modulators of several biological effects in cellular mechanisms, unrelated to the inhibition of ribosomal protein synthesis— their principal antibacterial mechanism of action (1).

High chelation activity with metals may account for several non-antimicrobial properties – they may act as carriers or ionophores, capable of delivering calcium to a host of biological targets (2). Tetracyclines are also able to affect calcium release from endoplasmic reticulum and mediate its discharge from mitochondria. In this way, tetracyclines may interfere with several metabolic reactions, cell division, membrane transport, receptor activation, secretion and calcium-mediated motility (1, 3, 4).

In which concerns mineralized tissues, tetracyclines affinity for bone tissue has been known for long and might be explained by the formation of stable chelates with Ca^{2+} , which is present in the extracellular matrix in the form of biological apatite (5). Although this affinity, little is known about the direct or indirect modulation of bone metabolism by tetracyclines.

The aim of this work was to evaluate the proliferation and differentiation events of osteoblastic-induced human bone marrow cells in the presence of therapeutic concentrations of doxycycline or minocycline, grown on the surface of hydroxyapatite and Bonelike[®] (a glass-improved hydroxyapatite).

In order to achieve the established goals, human osteoblastic cells were initially grown, for 35 days, in the presence of doxycycline (1-25 $\mu\text{g/ml}$) or minocycline (1-50 $\mu\text{g/ml}$) – concentrations selected for the establishment of tetracyclines' biological profile regarding human osteoblastic behaviour – on the surface of standard tissue culture plates. Both pharmacological agents, at low concentrations (1 $\mu\text{g/ml}$), reported pro-anabolic effects over osteoblastic cells, in which concerns cell proliferation. After an initial lag phase, an increase in cell growth was verified in association with expression of high levels of alkaline phosphatase and a proportional increased amount of mineralized extracellular matrix. Exposure to 5 $\mu\text{g/ml}$ of doxycycline induced similar results while the same concentration of minocycline impaired osteoblastic function. Higher levels of both tetracyclines impaired greatly cell proliferation and function in a dose-dependent way.

Following, after the established pro-anabolic activity of low-dose doxycycline and minocycline over osteoblastic human bone marrow cells, colonization of hydroxyapatite

and Bonelike[®] was assayed with the same cell type, in the presence of 1 µg/ml doxycycline or minocycline.

During the 35-day culture period, cells grown on Bonelike[®] presented active proliferation during the first 3 weeks, associated with expression of high levels of alkaline phosphatase. In accordance, mineral deposition began around day 17, which was verified by the ionized Ca loss from the culture medium and SEM micrographs. This behaviour was similar to that attained on the surface of the culture plate (control situation). Colonized hydroxyapatite revealed a long initial lag phase – delayed maximal values in the MTT assay and alkaline phosphatase activity. Even so, after 2 weeks, cell behaviour in the surface of the hydroxyapatite was identical to the one verified over colonized Bonelike[®].

The improved biological performance of Bonelike[®] might be explained by the presence of the more soluble phases of α - and β -tricalcium phosphate which results in a more rapid release/deposition events of ionic species leading to an increased apatite layer formation over the biomaterial surface, as described previously (6). In contrast, the formation of the apatite layer over hydroxyapatite occurs slowly but, at later time points, the reaction tends to an equilibrium in which the surface of both materials ends up with similar characteristics (6). Either way, early formation of an apatite layer appears to contribute to the improvement of the initial biological response (7, 8) and this has been verified regarding *in vitro* (9, 10) and *in vivo* (11-13) studies with Bonelike[®].

The addition of doxycycline or minocycline to the culture medium affected cell proliferation on the surface of the culture plate, hydroxyapatite and Bonelike[®]. After an established lag phase, increased proliferation was verified in close association with high levels of expressed alkaline phosphatase. An increased mineralization was verified in tetracycline-treated cultures by the higher C_{ai} loss from the culture medium and SEM micrographs.

Overall, the treatment of human osteoblastic cells with low concentrations of doxycycline or minocycline induced cell proliferation without impairing functional activity, analysed by alkaline phosphatase activity and extent of matrix mineralization. Pro-anabolic actions were also verified on the surface of hydroxyapatite and Bonelike[®], although Bonelike[®] revealed an improved biological response, as in accordance with published literature (9-13).

In this way, these results suggest that the local application of either semi-synthetic tetracyclines (doxycycline or minocycline) might, eventually, combine a targeted antimicrobial activity into the implantation site with the induction of osteoblastic proliferation, without interfering with cell-specific biological activity.

Hydroxyapatite and Bonelike[®] may become suitable candidates to drug confinement and delivery for bone regeneration therapies.

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