

FACULDADE DE ENGENHARIA DA UNIVERSIDADE DO PORTO

Reciprocal interaction between human microvascular endothelial cells and mesenchymal stem cells on macroporous granules of nanostructured-hydroxyapatite agglomerates.

Marta de Sousa Laranjeira

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This thesis was supervised by:

Professor Fernando Jorge Monteiro

Faculdade de Engenharia da Universidade do Porto

INEB – Instituto de Engenharia Biomédica

Professora Maria Helena Fernandes

Faculdade de Medicina Dentária da Universidade do Porto

The research described in this thesis was conducted at:

INEB – Instituto de Engenharia Biomédica, Divisão de Biomateriais, Universidade do Porto, Portugal;

FMDUP - Faculdade de Medicina Dentária, Universidade do Porto, Portugal;

FEUP – Faculdade de Engenharia, Universidade do Porto, Portugal

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Aos meus pais

“ Pelo sonho é que vamos,
comovidos e mudos.

(...)

Chegamos? Não chegamos?

Partimos. Vamos. Somos.”

Sebastião da Gama, *Pelo sonho é que vamos*

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Abstract

Hydroxyapatite (HA) possesses exceptional biocompatibility, osteoconductive and bioactive properties due to its chemical composition that is quite similar to that of mineral bone. It has been used in several clinical applications in numerous forms such as powders, granules, and prismatic blocks, depending on the aim of the medical application. However, recent developments in nanotechnology lead to new methodologies to produce nanostructured-HA. Surface properties such as topography, chemistry, wettability and charge can be changed in nanostructured materials when compared to microstructured surfaces. These changes are known to influence surface energy and mediate initial protein interactions and subsequent cellular adhesion, leading to appropriate extracellular matrix development.

Bone regeneration is a complex process influenced by several factors such as, physiological conditions, extracellular matrix, cell-to-cell interaction and vascularisation. The lack of a functional microvasculature is associated with insufficient supply of oxygen, nutrients and inappropriate metabolic waste products removal from porous large size implants inner parts, leading to hypoxia, poor cell survival and death. Furthermore, angiogenesis and osteogenesis are closely associated to important reciprocal interactions between endothelial cells and osteoblasts or their respective precursors.

Porous HA bone graft substitutes can mimic more closely trabecular bone structure, promoting tissue ingrowth and a rapid neovascularisation, leading to successful bone regeneration at the implant site. Furthermore, 3D porous structures can be used as a strategy in bone tissue engineering, by pre-seeding the scaffold with endothelial cells and osteoblasts or its precursors, expecting improved bone regeneration through the complex cross-talk between different types of cells.

The aim of the present work was to develop, optimize and characterize macroporous granules with nanostructured HA aggregates, using polyurethane sponge impregnation and burn-out method and study the reciprocal interaction between human microvascular endothelial cells and mesenchymal stem cells on this nanostructured-HA 3D surfaces.

In the first part of this work, two different types of porous granules were prepared using in one case nanostructured-HA and in the other microstructured-HA, both prepared through a polyurethane sponge burn-out method. Physico-chemical and structural characterization was performed using Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction, Scanning electron microscopy (SEM) and mercury intrusion porosimetry. Results showed that nanostructured-HA granules presented increased surface area and porosity, when compared to microstructured-HA granules. Both presented highly interconnective porosity, with average micro- and macro-porosity dimensions adequate for cell adhesion and bone ingrowth. *In vitro* studies showed that MG-63 human osteoblast-like cells were able to adhere, proliferate and migrate through the macropores of both granulated samples and a higher growth rate was found on nanostructured-HA as compared to microstructured-HA granules. Cells were able to maintain their characteristic morphology and expressed similar levels of osteoblastic-associated gene markers.

In the second part of this work, it was decided to optimize a 2D co-culture system to study in detail the interactions between Human mesenchymal stem cells (HMSC) and Human dermal microvascular endothelial cells (HDMEC), since no consensus exists in the literature regarding the optimal conditions for such type of cell culture. Co-cultured cells were maintained for up to 35 days and HDMEC and HMSC were cultured as monocultures in the same experimental conditions, to be used as controls.

MTT and DNA assay showed that viability/proliferation of HDMEC, HMSC and co-culture increased until day 21. Concerning cell growth patterns in monoculture, both type of cells adopted a characteristic growth pattern for each type of cells. Regarding the co-cultures, HDMEC formed clusters and HMSC organized themselves around them, suggesting that their growth was conditioned by the endothelial cells groups. HMSC and HDMEC differentiation seem to have been enhanced in co-culture conditions, with a very expressive up-regulation and/or earlier expression of osteoblastic and endothelial related genes and ALP activity. Furthermore, HMSC were able to mineralized, as confirmed by alizarin assay and SEM observations.

The next step was to apply this co-culture system in 3D conditions in order to investigate HMSC and HDMEC behaviour, and their interaction/communication in our

macroporous nanostructured-HA granules. The results achieved regarding this final work suggest that these granules provide a good environment for both HMSC and HDMEC cells since they were able to support cells adhesion, survival and proliferation, for 21 days. Over-expression of typical endothelial genes as well as the increased expression levels of genes related with osteogenesis and angiogenesis was also evident. Furthermore, an increased ALP activity and matrix mineralization was also observed in HMSC and co-cultured cells. Taking into account all the results achieved it is believed that the developed granules may be considered a promising class of implants for bone regeneration surgery and also for tissue engineering applications since they conciliate advantages of nano-sized HA and porous granules.

RESUMO

A hidroxiapatite (HA) apresenta uma biocompatibilidade, osteocondutividade e bioatividade excepcionais relacionadas com a sua composição química semelhante à componente mineral do osso. Este cerâmico é usado em diversas aplicações clínicas sob as mais variadas formas tal como pós, grânulos, blocos prismáticos, dependendo do objetivo da aplicação médica em causa. Contudo, avanços recentes em nanotecnologia visam o desenvolvimento de novas metodologias para a produção de HA nanoestruturada. As propriedades da superfícies de um biomaterial tais como a topografia, a química, a molhabilidade ou a carga são diferentes quando falamos de superfícies nanoestruturadas, ou quando comparadas com superfícies microestruturadas. Estas alterações vão influenciar as energias das superfícies e interferir na interação inicial com as proteínas e subsequentemente na adesão celular, levando a um desenvolvimento apropriado da matriz extracelular.

A regeneração óssea é um processo complexo influenciado por vários aspetos, tais como, as condições fisiológicas, a matriz extracelular, a interação entre as células e a vascularização. Falhas no desenvolvimento de uma rede microvascular funcional podem conduzir a um fraco fornecimento de oxigénio, nutrientes e a uma inapropriada remoção dos resíduos resultantes da atividade metabólica das partes internas de implantes de maiores dimensões, causando situações de hipoxia e morte celular. Para além disso, a angiogenese e a osteogenese são processos fortemente associados e implicam uma interação recíproca entre as células endoteliais, os osteoblastos e os seus respetivos precursores.

Os substitutos ósseos de hidroxiapatite porosa mimetizam a estrutura do osso trabecular, promovem o crescimento do tecido ósseo e uma rápida neovascularização para o interior do implante ósseo. Estas estruturas porosas tridimensionais podem ser usadas numa estratégia de engenharia de tecidos, já que estão aptas a ser colonizadas com células endoteliais, osteoblastos ou com os seus precursores, esperando-se que uma posterior implantação destes materiais colonizados com células leve a uma melhor regeneração óssea, conseguida através da complexa interação entre estes dois tipos de células.

O objetivo do presente trabalho foi o de desenvolver, otimizar e caracterizar os grânulos macroporosos tridimensionais com agregados de hidroxiapatite nanoestruturada, usando o método de impregnação de uma esponja de poliuretano e o estudo das interações recíprocas entre células humanas endoteliais da derme e células estaminais mesenquimais, em estruturas tridimensionais de hidroxiapatite nanoestruturada.

Na primeira parte do trabalho, foram preparados dois tipos diferentes de grânulos porosos usando HA nanoestruturada e HA microestruturada, através do método de impregnação de uma esponja de poliuretano. As caracterizações físicoquímica e estrutural foram realizadas através das seguintes técnicas: espectroscopia de infravermelho por transformada de Fourier, difração por raios x, microscopia eletrônica de varrimento e porosometria por intrusão de mercúrio. Os resultados demonstraram que os grânulos de HA nanoestruturada apresentam maior porosidade e área de superfície, quando comparados com os grânulos de HA microestruturada. Ambos os grânulos apresentam uma porosidade interconectada, com uma média de tamanho de macro e microporos adequada à adesão celular e crescimento ósseo. Os estudos *in vitro* realizados mostram que as células da linha celular MG-63 foram capazes de aderir, proliferar e migrar através dos macroporos de ambos os tipos de amostras e foi registrada uma maior percentagem de crescimento celular nos grânulos de HA nanoestruturada, comparativamente aos grânulos de HA microestruturada. As células foram igualmente capazes de manter a sua morfologia característica e expressar níveis similares de genes associados ao fenótipo osteoblástico.

Na segunda parte do trabalho, foi otimizado um sistema de cocultura (2D) para estudar em detalhe as interações entre as células humanas estaminais mesenquimais e as células endoteliais microvasculares da derme, uma vez que não existe consenso na literatura quanto às condições ótimas para o cultivo deste tipo de células. A cocultura foi mantida durante 35 dias e ambos os tipos de células foram igualmente cultivados em monocultura, nas mesmas condições experimentais, para serem usadas como controlo.

Os ensaios de MTT e ADN mostraram que a viabilidade/proliferação celular de ambos os tipos de células em monocultura e cocultura aumentaram até ao 21º dia . As células em monocultura adotaram um padrão de crescimento característico. No que se refere às coculturas, as células endoteliais formaram pequenos agrupamentos, enquanto que as mesenquimais estaminais se organizaram de forma a rodear as células endoteliais, sugerindo que o seu crescimento foi condicionado pelos pequenos grupos de células endoteliais. A diferenciação de ambos os tipos de células parece ter sido estimulada em condições de cocultura, já que foi observado um aumento ou expressão precoce de alguns genes relacionados com o fenótipo osteoblástico e endotelial. A atividade da fosfatase alcalina foi igualmente superior nas coculturas, quando comparada com as monoculturas. Além disso, as células estaminais mesenquimais foram capazes de mineralizar, o que foi confirmado pelo ensaio de alizarina e observações realizadas no MEV.

O passo seguinte foi cultivar as células em cocultura num sistema tridimensional, para estudar o comportamento e interações/comunicações de ambos os tipos de células nos grânulos macroporosos de HA nanoestruturada. Os resultados observados parecem sugerir que os grânulos desenvolvidos promovem um ambiente adequado para ambos os tipos de células, já que permitem a adesão, sobrevivência e proliferação, durante os 21 dias de cultura. Uma sobre-expressão dos níveis de genes relacionada com a osteogénese e angiogénese é igualmente evidente. Além disso, foi observado um aumento da atividade da fosfatase alcalina e a formação de uma matriz mineralizada nas células mesenquimais estaminais em monocultura e cocultura.

Tomando em consideração todos os resultados alcançados, prevê-se que os grânulos desenvolvidos neste trabalho possam vir a ser considerados uma classe de implantes promissora para cirurgias relacionadas com a regeneração óssea, bem como aplicações de engenharia de tecidos, já que conciliam as vantagens da HA nanoestruturada com as dos grânulos porosos.

Résumé

L'Hidroxiapatite (HA) possède une biocompatibilité ainsi que des propriétés ostéoconductive et bioactives exceptionnelles dues à leur composition chimique qui est très proche de celle de la partie minérale de l'os. L'hydroxiapatite a été utilisée dans plusieurs applications cliniques sous nombreuses formes, telles que des poudres, des granulés et de blocs prismatiques, et ceci en fonction de l'objectif de l'application médicale. Toutefois, les développements récents dans le domaine des nanotechnologies ont permis d'utiliser des nouvelles méthodes pour produire des nanostructures-HA. Les propriétés de surface telles que la topographie, la chimie, la mouillabilité et la charge peuvent être changées dans les matériaux nanostructurés par rapport aux surfaces microstructurées. Ces changements sont connus par leur influence sur les énergies de surface, mais aussi dans la médiation des interactions initiales entre les protéines et l'adhésion cellulaire qui s'en suit, conduisant au développement de matrices extracellulaires appropriées.

La régénération osseuse est un processus complexe influencé par plusieurs facteurs tels que, les conditions physiologiques, la matrice extracellulaire, l'interaction cellulaire et la vascularisation. L'absence d'une microvascularisation fonctionnelle est associée à une alimentation insuffisante en oxygène, de nutriments, et une élimination inappropriée des produits de déchets métaboliques de l'intérieur des grands implants poreux, ce qui entraînerait une hypoxie, une faible survie et la mort cellulaire. En outre, l'angiogenèse et l'ostéogenèse sont fortement liées à une interaction importante et réciproque entre les cellules endothéliales et les ostéoblastes ou leurs respectifs précurseurs.

Les substituts de greffes osseuses à base de HA peuvent imiter de très près la structure osseuse trabéculaire, favorisant ainsi la croissance tissulaire et une néovascularisation rapide, qui entraîneraient une régénération osseuse réussie au site de l'implant. En outre, des structures poreuses en 3D peuvent être utilisées comme une stratégie en ingénierie du tissu osseux, en ensemençant l'échafaudage avec les cellules endothéliales et les ostéoblastes ou de leurs précurseurs, et s'attendre à une régénération osseuse améliorée à travers l'interférence complexe entre les différents types cellulaires.

L'objectif de cette étude est de développer, d'optimiser et de caractériser des granulés macroporeux avec des agrégats de nanostructures HA en 3D, par l'imprégnation de la mousse de polyuréthane, et par traitement thermique. Cela a pour objectif d'étudier l'interaction réciproque entre les cellules endothéliales microvasculaires humaines et les cellules souches mésenchymateuses sur ces surfaces nanostructurées-HA en 3D.

Dans la première partie de cette étude, deux types différents de granulés poreux ont été préparés en utilisant d'un côté des nanostructures-HA et de l'autre des microstructures-HA, par le procédé d'imprégnation d'une éponge de polyuréthane. La caractérisation physico-chimique et structurale a été réalisée en utilisant la Spectroscopie Infrarouge à Transformée de Fourier (ou FTIR), la diffraction des rayons X, la microscopie électronique à balayage (MEB) et la porosimétrie au mercure. Les résultats montrent que les granules nanostructurés-HA présentent une surface et une porosité plus élevée par rapport aux granules microstructurés-HA. Les deux types de granulés présentent une porosité très interconnectée, avec des tailles moyennes de micro- et macroporosité suffisantes pour l'adhésion cellulaire et la croissance tissulaire de l'os. Des études *in vitro* ont montré que la lignée ostéoblastique humaine, MG-63, étaient en mesure d'adhérer, de proliférer et de migrer à travers les macropores des deux types d'échantillons granulés et qu'un taux de croissance plus élevé a été enregistré sur les granules à nanostructures-HA par rapport aux microstructures. Par ailleurs, les cellules ont pu maintenir leur morphologie caractéristique et ont exprimé des niveaux similaires de gènes de marqueurs ostéoblastiques.

Dans la deuxième partie de ce travail, il a été décidé d'optimiser un système de co-culture en 2D dans le but d'étudier en détail les interactions entre les cellules souches mésenchymateuses humaines et les cellules endothéliales microvasculaires dermiques humaines, puisque il n'existe aucun consensus dans la littérature concernant les conditions optimales pour ce type de culture cellulaire, la co-culture des cellules a été maintenue pour un maximum de 35 jours. Des cellules souches mésenchymateuses humaines et endothéliales microvasculaires dermiques humaines ont été cultivées comme des monocultures dans les mêmes conditions expérimentales pour être utilisées comme contrôles.

Les essais MTT et ADN ont montré que la viabilité/prolifération des cellules endothéliales microvasculaires dermiques humaines, cellules souches mésenchymateuses humaines et de la co-culture augmente jusqu'au 21^{ème} jour. En ce qui concerne les modèles de croissance des cellules dans la monoculture, les deux lignées cellulaires ont adopté un modèle de croissance caractéristique de leur type cellulaire respectif. Quant aux co-cultures, les cellules endothéliales microvasculaires dermiques humaines ont formé des grappes et les cellules souches mésenchymateuses humaines se sont organisées autour de celles-ci, ce qui laisse à suggérer que leur croissance est conditionnée par les groupes de cellules endothéliales. La différenciation des cellules souches mésenchymateuses humaines et endothéliales microvasculaires dermiques humaines semble s'améliorer dans les conditions de co-culture, avec une surexpression significative et/ou une expression antérieure des gènes ostéoblastiques et endothéliales et les gènes liés à l'activité ALP. En outre, HMSC ont pu se minéraliser, ce qui fut confirmé ultérieurement par le rouge d'alizarine et les observations au MEB.

L'étape suivante consistait à appliquer ce système de co-culture dans des conditions de 3D afin d'examiner le comportement des cellules souches mésenchymateuses humaines et endothéliales microvasculaires dermiques humaines, et leur interaction/communication dans nos granules macroporeux à nanostructure-HA. Les résultats obtenus suggèrent que ces granules fournissent un bon environnement pour les deux types cellulaires, HMSC et HDMEC, puisque celles-ci étaient en mesure de soutenir leur adhésion, survie et prolifération, pendant 21 jours. La surexpression de gènes typiques endothéliaux ainsi que les niveaux d'expression accrue de gènes liés à l'ostéogenèse et de l'angiogenèse sont également évidents. En outre, on a observé une activité ALP accrue et une minéralisation de la matrice dans les cellules souches mésenchymateuses humaines et les co-cultures. Tenant compte de tous les résultats obtenus, il est estimé que les granules développés peuvent être considérés comme une classe prometteuse d'implants pour la chirurgie régénératrice osseuse mais également pour les applications d'ingénierie tissulaire, car ils peuvent associer les avantages de la taille nanométrique des cristaux de l'HA et ceux des granules macroporeux.

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CHAPTER I
GENERAL INTRODUCTION

CHAPTER 1

1. Bone Physiology

1.1. Bone Tissue

Bone is a dynamic and highly vascularised specialized connective tissue that forms the main element of the skeleton and continues to remodel throughout the lifetime of an individual [1]. The skeleton provides support, protection to the internal organs and bone marrow as well as allows muscles and ligament attachment, facilitating the locomotion process [2, 3]. In addition to these structural functions it is considered as the main reservoir of mineral ions such as calcium, phosphate and other inorganic ions intimately involved in homeostasis by regulating the concentration of key electrolytes in the blood. It is a dynamic and complex pool of inorganic and organic molecules [1, 3, 4].

1.1.1. Composition

Bone is an organic–inorganic nanocomposite constituted by bone cells and an extracellular matrix, which has a mineral and an organic part. Cortical bone is composed of 35 % (dry weight) organic part and 65 % (dry weight) inorganic part [3, 5].

The extracellular matrix is a physical support for cells, where they can adhere and interact [3]. The organic part of this matrix consists of collagen fibres, predominantly collagen type I, the other components are various non-collagenous proteins for support of cellular functions such as: *osteonectin*, *osteocalcin*, *osteopontin*, *bone sialoprotein*, *proteoglycans* (decorin, biglycan), *glycoproteins* (thrombospondin, fibronectin, fibrillin), *enzymes* (alkaline phosphate, collagenase), and *cytokines* [6, 7]. Type I collagen provides a backbone for the deposition of mineral bone. Bone mineral crystals are aligned with their long axis parallel to the collagen axis [5]. Collagen fibres are responsible for bone elasticity, flexibility and for the matrix organization [8].

Calcium and phosphate are the main components of the mineral part. Carbonate, citrate, sodium, magnesium, fluoride, hydroxyl, potassium and other ions can be found but in smaller amounts. The major mineral phase of bone is

hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) (HA) [4, 9]. This apatite mineral is similar in composition and structure to minerals within the apatite group, which form naturally in the Earth's crust[9, 10]. Collagen and HA are associated and assembled into a microfibrillar composite[11].

1.1.2. Structure

The adult long bones, anatomically, present a tubular shaft named as *diaphysis*. In the centre of this section, is localized the *medullary cavity*, filled in with marrow (Figure 1). *Epiphysis* is found at each end of the bone. Separating these main parts is the *metaphysis* [7]. A fibrous membrane, *periosteum*, covers bone external surface (Figure 1). It is composed of dense connective tissue, presenting blood vessels. The medullary cavity, Havers and Volkmann channels, and the cavities of cancellous bone are lined with a thin membrane, *endosteum* [12].

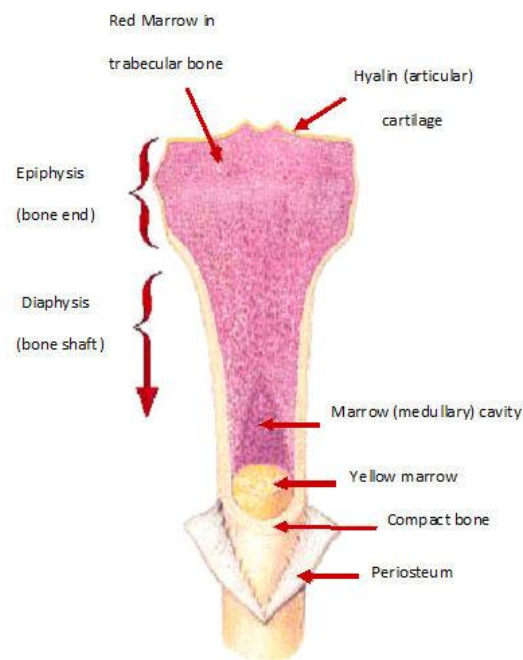


Figure 1 – Long bone anatomy [13].

Bone marrow is divided in yellow and red marrow. The first one is constituted, mainly, by adipose tissue. Red bone marrow fills the cancellous epiphysis of long bones. Large amounts of red bone marrow can also be found in flat bones like those of the ribs, iliac and skull [3, 14, 15].

In bone marrow takes place the hematopoiesis. So, post-natal bone marrow is composed of a hematopoietic tissue and a stromal system [16, 17]. Bone marrow stroma contains several non-hematopoietic cells that support hematopoiesis, including the mesenchymal stem cells (Figure 2) [17].

Mesenchymal stem cells have the capacity of self-renewal and differentiation into several connective tissue lineages. They can give rise to osteoblasts, chondrocytes, adipocytes, tenocytes and nonmesenchymal cells, such as neural cells [17]. They are recruited to repair bone defects, undergo differentiation [3].

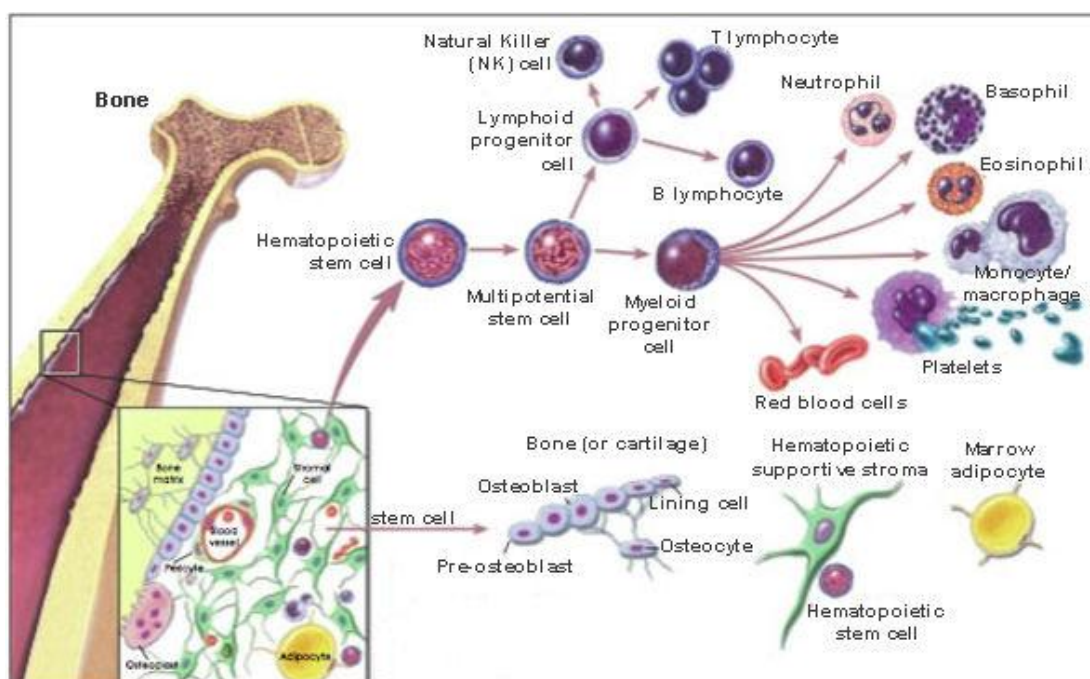


Figure 2 – Hematopoietic and stromal stem cell differentiation [18].

Two different mature bone structures can be identified (morphologically or histologically) in different parts of the bone: compact (dense) and cancellous (trabecular) bone (Figure 3). Both exist in different proportion in several locations of the skeleton. Cortical or compact bone is highly dense and is located on the exterior of the bone immediately underneath the *periosteum*. It constitutes the outer part of all skeletal structures. Its major function is to provide mechanical strength and protection, but it can participate in metabolic responses, particularly when there is severe or prolonged mineral deficit.

This type of bone is organized in cylindrical units, *Haversian systems* or *osteons*, and forms the *diaphysis* of long bones.

Concentric layers called *lamellae*, composed by mineralized collagen fibres, constitute osteons. *Lamellae* are arranged in concentric rings around the *Haversian canal*, which has blood vessels, nerves and lymphatic vessels. Bone tissue has intercommunicating pores systems constituted by *canaliculi* (small canals), *lacunae* (spaces) and *Volkman's* canals which connect with *Haversian canals*. This bone is not remodelled so often as trabecular bone [3, 7, 15]. Trabecular or cancellous bone is porous and is found inside the long bones, throughout the bodies of the vertebrae, and in the inner portions of the pelvis and other large flat bones. It is also more metabolically active than cortical bone. It is present in the *epiphysis* and has a *lacunar-canalicular* network to transport metabolic substances, as the compact bone. Nevertheless, this bone is organized in the form of thin interconnecting spicules and not in a *Haversian* system. Bone turnover occurs with great frequency [3, 7, 8, 14, 15].

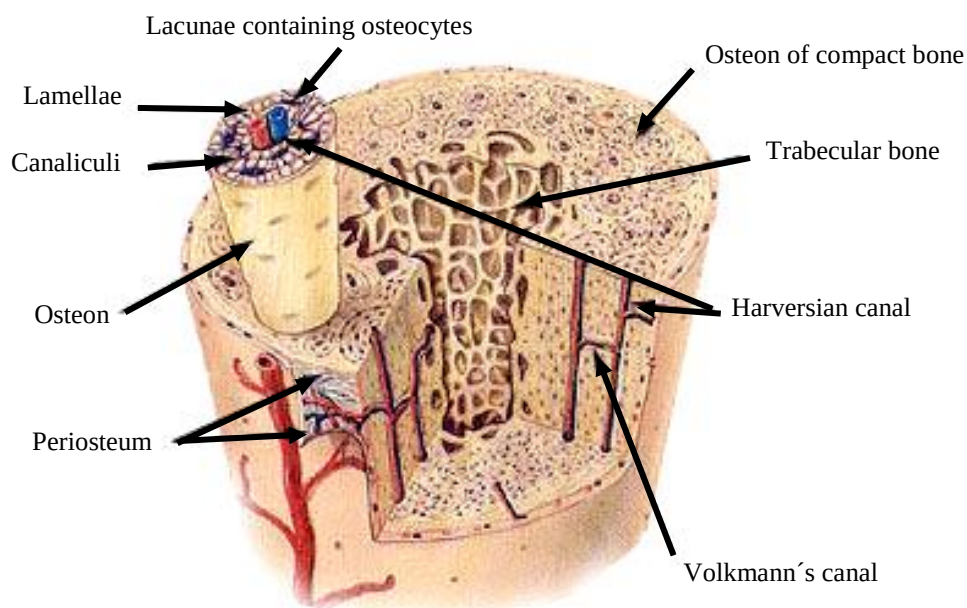


Figure 3 – Constitution of compact and cancellous bone [19].

As with all organs in the body, bone tissue has a hierarchical organization over length scales that span several orders of magnitude from the macro- (centimeter) scale to the nanostructured (extracellular matrix) components (Figure 4).

At the microscopic level, the dense cortical and trabecular bone are composed of mineralized collagen fibrils that are stacked in parallel to form layers (Figure 4). At a nanoscopic scale calcium phosphates are present in the form of needles-like or plates-like nanocrystals with approximately 5-20 nm width and 60 nm length, which are deposited on collagen nano-fibrils (Figure 4) [14, 20]. These nano-crystals of HA give bone adequate biomechanical strength [1, 12].

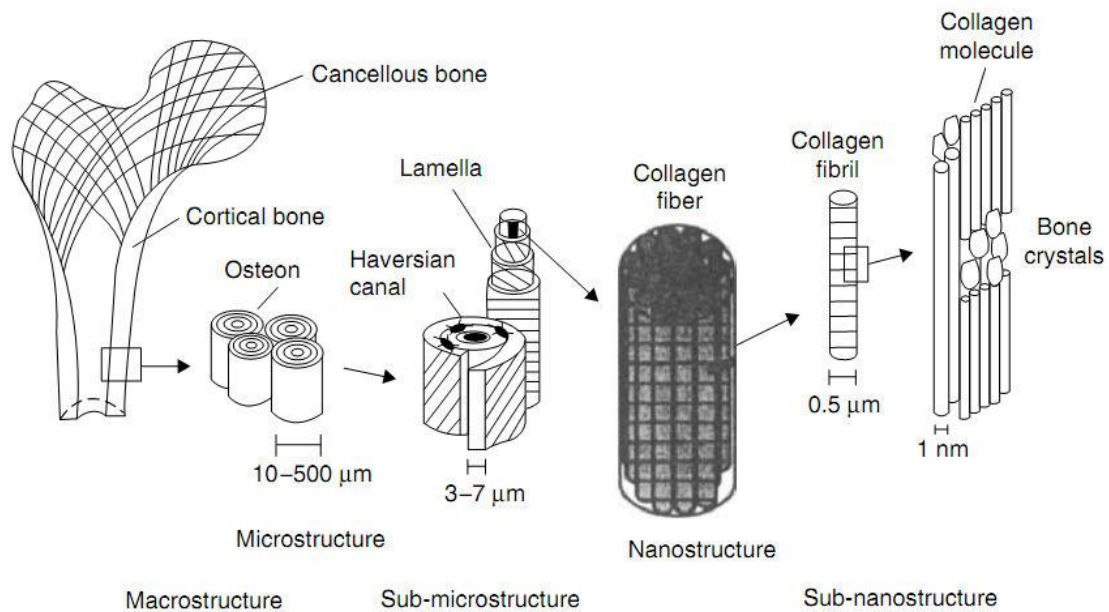


Figure 4 – Bone components from a macroscopic scale to a nanoscopic scale [21].

1.2. Bone Cells

Four main different cells types can be found in bone, namely osteoblasts, osteocytes, osteoclasts and lining cells which are related to bone formation, maintenance and resorption, respectively [6, 7].

1.2.1. Osteoblasts

Mature osteoblasts have their origin in mesenchymal stem cells (MSCs) and their function is to synthesize the organic matrix and participate in mineralization of osteoid (unmineralized ground substance). An osteoblast is a mononuclear cell and has a prominent Golgi apparatus, typical of a protein-producing cell. They are responsible for the secretion of collagen type I and noncollagenous proteins of the matrix [6, 7].

According to morphological studies, osteoblastic cells are categorized in a presumed linear sequence progressing from osteoprogenitors to pre-osteoblasts to osteoblasts and finally to osteocytes and lining cells [6, 22].

In bone marrow, the commitment of MSC to the osteoblastic differentiation is mostly determined by the expression of specific groups of transcription factors such as Runx2 and osterix (OSX) that are the main determinants of MSC osteoblastogenesis [10, 12]. The expression of such transcription factors is promoted through the cross talk between complex signaling pathways including local and systemic growth factors such as bone morphogenic proteins (BMPs), fibroblastic growth factors (FGF), insulin, insulin-like growth factors (IGF) and wingless-type MMTV integration site (Wnt) proteins [22-24].

During the pathway to differentiation they progress through three different developmental stages: proliferation, matrix maturation, and mineralization. Several osteoblastic markers are expressed during these stages and have been identified *in vitro*. RUNX2 is mainly expressed during lineage commitment; histone H4, collagen, TGF β 1 are especially expressed during proliferation; Alkaline phosphate peak occurs during matrix maturation, while osteopontin and osteocalcin peaks occur in the late matrix maturation or early mineralization phases [6, 7, 23].

1.2.2. Osteocytes

During matrix deposition, osteoblasts can be trapped in osteoid and differentiate into osteocytes. These bone cells are embedded throughout the mineralized matrix or osteoid [2, 23]. Osteocytes are in *lacunae* and communicate with each other, and with blood vessels, through minuscule canals, *canaliculi*, filled with cytoplasmic projections. They are responsible for the maintenance of bone matrix (synthesize and resorb matrix to limited extent) and the rapid release of calcium and phosphorous from mineralized bone into the blood [2, 3, 7].

1.2.3. Osteoclasts

Osteoclasts are responsible for bone resorption. They are multinucleated cells, originated from hematopoietic tissue with common differentiation pathways with macrophages. The transcription factor such as PU.1 is responsible for committing

haematopoietic stem cells to the monocyte/macrophage lineage. Afterwards Macrophage colony stimulating factor (M-CSF) signalling is activated to promote macrophage proliferation and the expression of RANK receptor. Additionally, these cells need RANKL that is produced by stromal cells. Under the right stimulus these cells will be differentiated into osteoclasts [25, 26].

Osteoclasts have multiple circumnuclear Golgi stacks, abundant lysosomal vesicles and a high density of mitochondria. Bone degradation begins when these cells attach to a region of the matrix and locally dissolve bone [25].

1.2.4. Bone lining cells

Bone lining cells are present on bone surfaces where bone formation or resorption is not occurring. They are flat, elongated and only a few cytoplasmic organelles can be found. Their function is to cover and protect bone surface. These cells and osteoblasts are originated from osteoprogenitor cells [8].

1.3. Bone Formation, remodelling and healing

The skeleton is metabolically active and constantly remodeling, and both processes are regulated by many local and systemic factors.

The ossification of skeleton occurs by two primary processes: intramembranous and endochondral. Intramembranous ossification occurs, predominantly in the cranial facial bones and parts of mandible and clavicle, where mesenchymal cells condense and differentiate directly into osteoblasts. In most of other bones in the skeleton, endochondral bone ossification occurs, whereby mesenchymal cells condense to form a cartilage matrix [2, 7]. Upon vascular invasion of the cartilage template, the chondrocytes are removed and replaced by osteoblasts and osteoclasts. While the osteoclasts degrade cartilage, osteoblasts produce a bone specific matrix [2, 22].

Bone remodelling is the dynamic physiologic process by which bone mass is maintained constant throughout adult life in vertebrates. Bone remodelling consists of two phases: bone resorption by osteoclasts is followed by bone formation by osteoblasts, and it occurs continually and simultaneously at multiple locations in the skeleton [22].

Bone resorption begins when osteoclasts attach to a site on the bone surface and remove bone by acidification and proteolytic digestion [8], creating a resorption pit. Sequentially, osteoblasts move in and fill the pit with organic matrix, which will be mineralized later. This process allows mineral ion homeostasis and it is regulated by various hormones, like parathyroid hormone, calcitonin, vitamin D, and estrogen [8]. Bone remodelling occurs due to the successive cycles of removal and replacement [6, 22].

Bone fracture or the implant placement result in the loss of continuity of bone tissue at the site of injury and are associated to damage of the surrounding tissues. In both cases, the healing is a complex process that involves multiple cellular and extracellular events [24, 27].

Bone healing can be influenced by several factors like bone type (cortical or trabecular), location and severity of trauma, species and age. Nevertheless, the outcome of successful healing is the reconstruction of tissue continuity [24].

In each case (fracture or implant) ligaments, muscles or blood vessel are damaged. The last one results in haemorrhage. Haemorrhage caused by the fracture or implant procedure results in the activation of the coagulation cascade and the final formation of a blood clot or hematoma [24, 27]. Inflammatory cells (macrophages, monocytes, lymphocytes, and polymorphonuclear cells), fibroblasts and endothelial cells infiltrate the bone. Necrotic tissue and foreign bodies are removed by leukocytes. Next steps are the deposition and formation of granulation tissue, angiogenesis, and migration of osteogenic population (Figure 5) [24, 27, 28]. These cells are going to proliferate and differentiate, invading the bone chamber, or spread over the surface of bone (or implant)[24, 27]. At a final stage, bone is restored to its original shape, structure, and mechanical strength. Much of the normal healing process is driven by growth factors and cytokines [15, 27, 28].

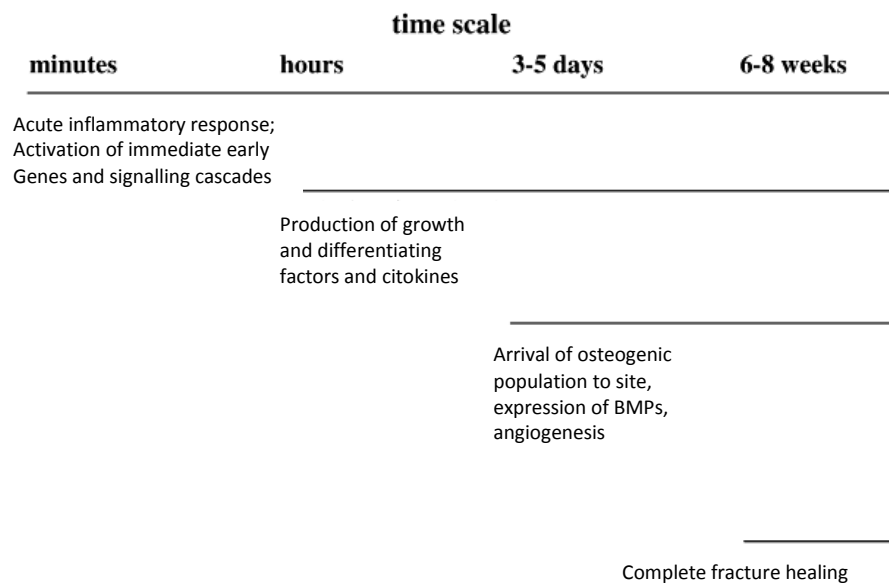


Figure 5 - Biochemical response to bone fracture [27].

1.4. Angiogenesis and Bone repair

Repairing large bone defects remains a major clinical orthopaedic challenge. Bone is a highly vascularised tissue relying on the close spatial and temporal connection between blood vessels and bone cells to maintain skeletal integrity [29].

Blood vessels are essential since cells require: 1. gas exchange for respiration; 2. nutrients for metabolism; 3. metabolic waste removal. If these three conditions are not met, cell viability will be compromised as well as bone repair [30].

They are composed of a single layer of endothelial cells in tube formation and allow for the distribution of blood and its constituents throughout the tissues. Endothelial cells form the lining of all blood vessels and connect with adjacent endothelial cells via vascular endothelial-cadherin (VE-cadherin) and connexions.

The formation of new blood capillaries from the pre-existing network is known as angiogenesis. It is active during embryonic development. At the adult state, angiogenesis is re-activated during wound repair and under several pathological conditions (such as tumour growth, metastasis and cardiovascular disorders).

Angiogenesis starts with endothelial cells migration from basement line into extracellular space and self-organizes into a tubular form. Afterwards a maturation /

stabilization phase occurs. A balance of pro- and anti-angiogenic factors regulates angiogenesis. Pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [31] and basic fibroblast growth factor (FGF) stimulate proliferation and migration of endothelial cells[32]. On the other hand, angiopoietins (Ang) also play an primordial role in the whole process, but their effects are more complex and they are involved in two different phases: Ang1 [33], through its receptors Tie2, is implicated in vascular quiescence while Ang2, by antagonizing Ang1 binding to Tie2, is determinant in the angiogenesis activation phase [34]. Other works described in the literature indicate that Ang-2 is able to activate Tie-2 and promote many of the same biological outcomes as Ang-1. It is believed that Ang-2 behave as an agonist/antagonist depending on the cell and tissue type examined [35].

Platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF β) have also been shown to be also involved in the maturation / stabilization phase. The first one mediates the vascular recruitment of pericytes and smooth muscle cells, and the second ones participate in the extracellular matrix accumulation as well as in the regulation of endothelial cell functions and smooth muscle differentiation [32]. One of the sub-groups of TGF β family are the well known bone morphogenetic proteins (BMPs) which are osteogenic factors such as BMP-2 [36, 37] that can stimulate osteoblast VEGF production and hence modulate vascularisation (Figure 6) [29].

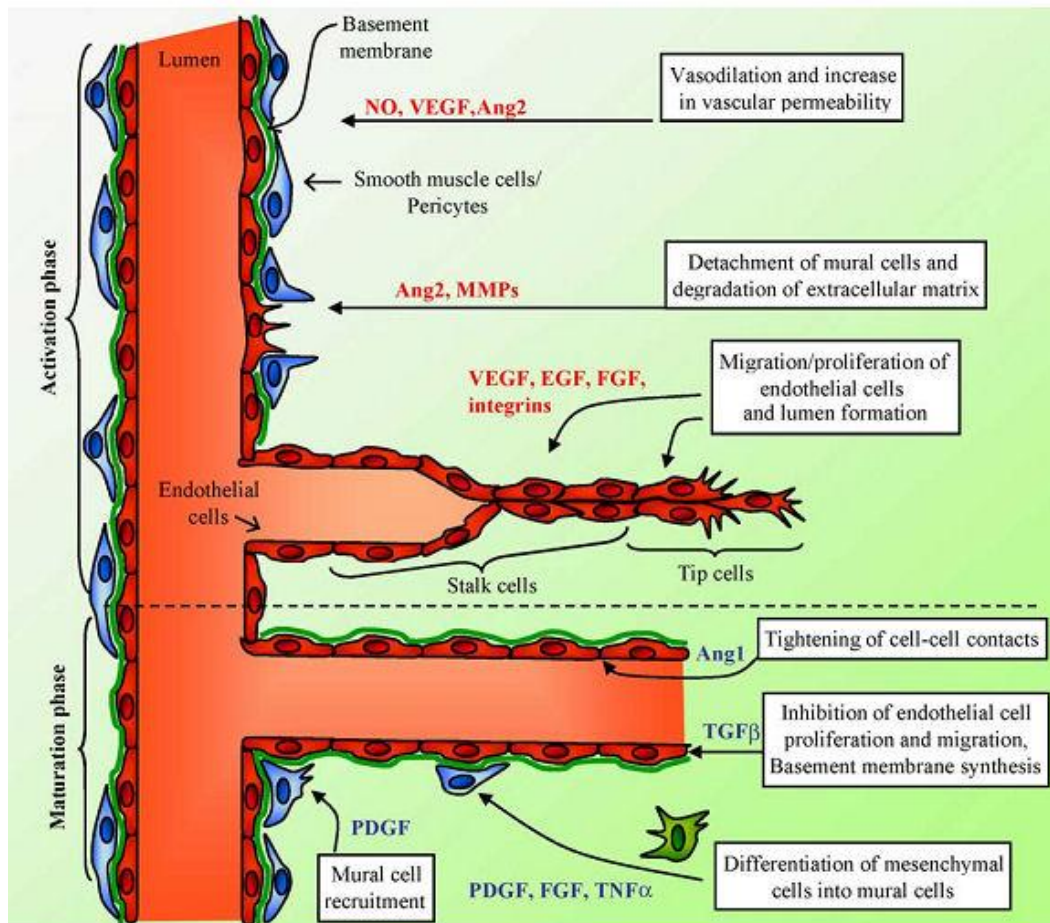


Figure 6- Activation and maturation phase of angiogenic process. Some factors with an active role in the angiogenesis process : Nitric oxide (NO); Vascular endothelial growth factor (VEGF); Angiopoietin 2 (Ang2); Angiopoietin 1 (Ang1), Matrix metalloproteinases (MMPs), Epidermal growth factor (EGF), Fibroblast growth factor (FGF), Transforming growth factor beta (TGFβ), Platelet-derived growth factor (PDGF) and Tumour necrosis factor alpha (TNFα) [29].

VEGF appeared to have a crucial role in angiogenic/osteogenic processes since it has various activities in bone development, such as regulating the endothelial, chondrogenic and osteogenic cells activity, and promoting vascularisation during endochondral bone formation. VEGF can induce haematopoietic stem cell recruitment from the bone marrow, monocyte chemoattraction, being critical for early bone development [29].

Furthermore, several studies have shown that cell-to-cell communication between endothelial cells and osteoblasts are crucial to the coordinated cell behavior

necessary for the development and remodelling of bone. Histological studies indicate that osteoblasts and osteoprogenitors are always located adjacent to endothelial cells in blood vessels at the site of new bone formation. So, it is important to discuss the mode of crosstalk between the endothelial and osteoblastic lineages, giving rise to the hypothesis that bone vascular endothelial cells contribute to the intricate communication pathways that occur in bone and that link different cell types via diffusible signaling molecules, as well as by cell contact-mediated mechanisms. As a result, a detailed knowledge of interaction between bone-forming cells and blood vessel-forming cells appears fundamental to improve bone tissue grafting[38].

2. Bone grafts

As it was mentioned above, bone has a highly regenerative capacity, especially in young population, meaning that most fractures should heal without a major intervention being required. However, large bone defects, as observed after bone tumour resections and severe non-union fractures related with bone trauma, diseases or ageing, require surgical intervention and the presence of a template needed for faster and successful regeneration. Currently, the gold standard treatment to solve these problems is the use of autografts, consisting of tissue transplanted from one site, called donor site, to another called recipient site, in the same patient [39]. Typically, donor site is bone from non-load-bearing sites in the patient (usually an easily accessible site like the iliac crest). The use of autologous bone is the gold standard treatment since cancellous and cortical bone, are usually implanted fresh, providing a source of osteoprogenitor cells and being osteoinductive, osteoconductive, osteogenic as well as avoiding the immune rejection and disease transmission problems related to allogeneic bone (i.e. bone from another human donor) or xenogeneic bone (i.e. bone from an animal source). Nevertheless, these bone implantations show several limitations such as limited availability [39-41], high post-operative pain and morbidity at the implant site [41].

Allografts are tissue transplanted from another member of the same species [39] But they also have disadvantages such as the risk of viral transmission (HIV,

Hepatitis), limited donor bone supply and requirement for immunosuppressant drugs [39-41].

Xenografts are tissue transplanted from another donor species. They can give rise to unfavourable immune response and viral or prion contamination. Due to these aspects, synthetic biomaterials have been developed [6, 15, 40, 41]. In summary, bone-derived grafts still have several limitations, and new synthetic materials have been developed and applied in Medicine. These materials can be an alternative to the use of autografts, allografts or xenografts for tissue reconstruction and regeneration. The search for new bone regeneration strategies is therefore priority due to the debilitating pain associated with bone damage, and the increasing medical and socioeconomic needs of our elderly population.

3. Biomaterials for bone regeneration

Biomaterials have been developed to interface living host tissue. These materials can be used as bone grafts, in alternative to bone-derived grafts, avoiding problems related to autografts, allografts and xenografts. Biomaterials have the advantages of availability, reproducibility and reliability [40].

A wide variety of materials have been used to construct bone implants and replacements, such as polymers, ceramics, metals, and composites that combine together properties of two or more materials from different classes [4]. Each of these categories contains many subdivisions. The metallic material includes pure metals and alloys, ceramics include crystalline ceramics, glasses, glass–ceramics and carbon based materials and polymers include synthetic and natural polymers [42].

Bone grafts for use in bone regeneration must have several characteristics/requirements such as compatibility with human body (must not elicit an unresolved inflammatory response, nor demonstrate extreme immunogenicity or cytotoxicity). This requirement is applied both to intact material and to its degradation products [43]. Furthermore, bone graft materials must present adequate surface properties like chemistry, topography and surface energy [44] in order to improve cell migration, attachment, proliferation and differentiation [44-46].

3.1. Bioceramics

Ceramics are defined as inorganic, non-metallic materials which consist of metallic and non-metallic elements bonded together primarily by ionic and/or covalent bonds [47]. This type of materials has been used by humans for centuries. Although, only more recently, they have been used to improve our life quality, helping in the repair and reconstruction of damage parts of the body (implants, prostheses, prosthetic devices) [15].

All bioceramics when implanted in the body elicit a response of the host tissue, and can undergo both physical and/ chemical modification [28]. So, bioceramics can be divided in three different types based on different attachments and interactions between the implant and the tissue.

Bioinert (or nearly inert) are bioceramics that are stable, almost biologically inactive [48, 49]. These kinds of implants give rise to the formation of a non-adherent fibrous capsule at their interface. For example, Al_2O_3 or ZrO_2 fit in this class of bioceramics, interfacial movements can occur and the implants may become loose quickly [15, 48].

Biodegradable or resorbable materials degrade gradually with time and are replaced by natural tissues. These are the ideal bioceramics, because they stay inside the body just during the time required for tissue regeneration [48, 50]. However, the resorption rates must be similar to the repair rates of body tissues. In addition, they have to give mechanical support (strength and stability) in the early stages of bone healing. Later, the load will be transferred to the new replacement tissue [43, 48]. The degradation can be due to: solubility of the material and local pH (physicochemical dissolution); fragmentation into small particles and biological factors (biological dissolution) [15, 48, 50].

Some bioceramics present *bioactivity* which may be defined as the “*ability to elicit a specific biological response that results in the formation of bond between the tissues and material*” [4]. They are called *bioactive ceramics* and include HA, some composites such as polyethylene-HA, collagen-HA, nano-HA/chitosan, polymer carbon-nanotubes HA, Poly- ϵ -caprolactone-HA, other calcium phosphates, some glasses and glass-ceramics. All the bioactive implants form a hydroxyl-carbonate apatite layer on their surface in simulated physiological solutions. This layer is similar in composition

and structure to the mineral phase of bone. The interfaces between bone and tendons and ligaments are quite similar to the interface between bioactive implant and bone[15, 48, 49].

3.1.1. Hydroxyapatite and other Calcium Phosphates Ceramics

Calcium phosphate ceramics have been applied to repair damage parts of bone tissue. Calcium phosphates may be classified according to their Ca/P ratios. In general, different Ca/P from 2.0 to 0.5 may be synthesized mixing calcium and phosphate ion solution under acid or alkaline conditions (Table 1) [4]. Different phases of calcium phosphate can be used for medical applications as described below:

Table 1- Calcium Phosphates ceramics with their calcium phosphate ratios [48].

Ca:P	Mineral Name	Formula	Chemical name
1.0	Monetite	CaHPO_4	Dicalcium phosphate (DCP)
1.0	Brushite	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	Dicalcium phosphate Dihydrate(DCPD)
1.33	_____	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	Octacalcium phosphate (OCP)
1.5	Whitlockite	$\text{Ca}_3(\text{PO}_4)_2$	Tricalcium phosphate (TCP)
1.67	Hydroxyapatite	$\text{Ca}_{10}(\text{PO}_4)_6 (\text{OH})_2$	
2.0	_____	$\text{Ca}_4\text{P}_2\text{O}_9$	Tetracalcium phosphate

Dicalcium phosphate anhydrite (DCPA, CaHPO_4 ; the mineral monetite) DCPA crystals are triclinic. It is stable at high temperatures and low pH levels (pH 4–5). DCPA undergoes conversion from the unstable states at pH levels greater than 6–7 to a more stable HA phase [51]. DCPA does occur neither in normal nor in pathological

calcifications. In clinical applications, it is used for calcium phosphate cements. It can be also used as source of calcium and phosphate in nutritional supplements, tableting aids, and toothpaste components [52]. Current studies have shown that human osteoblasts were able to adhere, spread and differentiate on three-dimensional (3-D) monetite scaffolds for 21 days in culture. Accordingly, clinical studies in humans with DCPA granules have shown to increase bone regeneration, indicating its biocompatibility and osteoconductivity [53].

Dicalcium phosphate dihydrate (DCPD) ($\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$) has the mineral name of Brushite. Crystals are monoclinic and exhibit a great solubility compared to other calcium phosphate phases mentioned in table 1. It is often found in pathological calcifications [53] such as dental caries, dental calculus, kidney stones and chondrocalcinosis [53, 54]. In surgery, DCPD is used in calcium phosphate cements. In dentistry, for protection against caries it is used in toothpaste together with fluoride-containing compounds [52, 53]. It is used in other applications such as fertilizers, glass production, calcium and mineral supplements in food [52]. It is a precursor to hydroxyapatite (HA) phase, the more stable calcium phosphate phase, since in environments with a pH greater than 6–7, it becomes unstable and transforms into HA. For this reason, several studies have focused on the use of DCPD coatings as an initial step to obtain HA. DCPD is also often used as the preliminary component of bone cements in clinical applications. Its biocompatibility has been proved with several cell lines, including pre-osteoblastic cells and fibroblastic cells[53].

Octacalcium phosphate (OCP) ($\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$) is a precursor of bone and tooth apatite crystals. Spectroscopic analysis suggests that OCP is involved in initial intramembranous bone formation [55]. It is an intermediate phase during the precipitation of a more stable calcium phosphate phase (such as, HA, calcium-deficient HA) [52]. In addition *in vivo* when applied as a bone implant material OCP changes into HA. OCP occurs as one of the components of human dental and urinary calculi [52].

Tricalcium phosphate (TCP) ($\text{Ca}_3(\text{PO}_4)_2$) known as whitlockite, exists in two allotropic forms: α -TCP and β -TCP [3,47]. The extent of dissolution compared to HA decreases following the order: α -TCP > β -TCP > HA [4]. β -TCP is the low temperature polymorph that can be transformed into α -TCP when heat-treated at high temperature. It can be obtained by heat-treating calcium deficient hydroxyapatite (when the Ca/P molar ratio is lower than the value of 1.67 required for HA) above 700 °C [52, 56]. Whitlockite is found in dental calculi and urinary stones, dental caries, arthritic cartilage, as well as in some soft-tissue. In medicine, β -TCP is used in bone cements and in combination with HA, β -TCP is as a bone-substitution ceramic. A good cellular response and biomineralization of human osteoblast cells and bone marrow stromal cells has been achieved in the presence of TCP-containing 3D structures and TCP coatings. Previous works have successfully used antibiotic-loaded TCP to prevent bone infections in ovine cancellous bone defects. Clinically, TCP/HA coatings applied to titanium hip implants have showed a better biocompatibility and decreased bone loss when compared to non-coated implants[56].

Hydroxyapatite (HA) ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) Calcium phosphate ceramics have been applied to repair damage parts of bone tissue. Different phases of calcium phosphate can be used for medical application, but hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) (HA) (Figure 7), has been widely used due to its bioactivity and to its crystallographic similarity to bone and dental tissues [8, 57, 58]. It belongs to the apatite group of minerals and its Ca/P ratio is 1.67. HA crystallizes into hexagonal system and has the unit-cell dimensions $a = b = 9.418\text{\AA}$ and $c = 6.884\text{\AA}$ [10, 15]. Biological apatites (human enamel, dentin, bone and some pathological calcification) are different from pure HA in some characteristics, such as stoichiometry, composition, crystallinity and other physical and mechanical properties. These apatites frequently are calcium-deficient and always carbonate substituted. In a general way, carbonate CO_3^{2-} groups can substitute phosphate group PO_4^{3-} and Ca^{2+} is substituted for Na^+ to balance charges. Small contents of other ions such as K^+ , Mg^{2+} , F^- , Cl^- can also be found in this kind of apatites [8, 15, 48, 49, 59]. The biocompatibility of HA has been demonstrated in *in vitro* cell culture studies with mesenchymal stem cells, primary osteoblasts and several other types of cells.

Osteoblastic cells showed an improved adhesion, proliferation, differentiation and increased alkaline phosphatase activity in the presence of HA. *In vivo* investigations showed an improved bone formation at the implant site in the presence of HA. Furthermore, it was not observed any form of inflammatory reaction, since no inflammatory cells were detected at the implantation site[52].

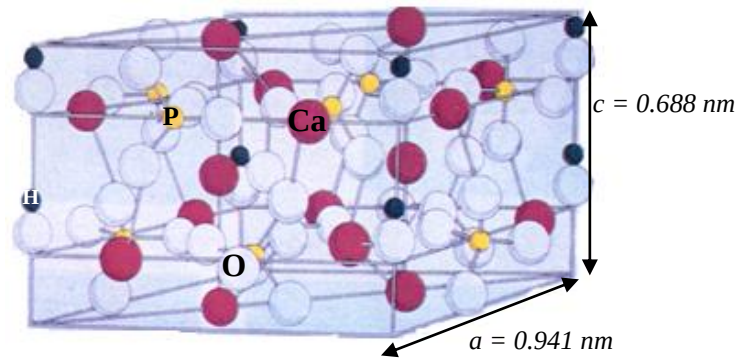


Figure 7 – HA structure [10].

Tetracalcium phosphate (TTCP) ($\text{Ca}_4(\text{PO}_4)_2\text{O}$) cannot be precipitated from aqueous solutions, since it is not very stable in it. It can only be prepared by a solid-state reaction above 1300 °C. TTCP cannot be founded in biological calcifications. It is used in medical applications for the preparation of calcium phosphate cements [52].

3.1.2. Stem-cells - porous bioceramics applications

3D porous ceramics scaffolds are often used in bone tissue engineering. An interconnected porosity is mandatory to allow cell migration, tissue ingrowth, vascularization, flow transport of nutrients and metabolic waste [43, 60]. One of the most promising approaches involves seeding mesenchymal stem cells (MSC) in porous matrices in order to induce bone growth into defected area [60]. They are multipotent adult cells that have the ability to self-renew and the capability of differentiation into a wide range of cell type (such as osteogenic, adipogenic and chondrogenic lineages) and can be modulate by the signals of the local environment that affect their differentiation [61, 62]. These MSC can be isolated from different sources like bone marrow, umbilical cord, adipose tissue and others sources. However, bone marrow is still the most used source, presenting cells with high differentiation potential. As the

number of MSC isolated is very low, they need to be expanded and differentiated to a particular lineage [63, 64].

Cells have to attach to the scaffold, expand, differentiate and organize themselves into bone tissue. Since these cells are autologous, the immune rejection and disease transmittance can be avoided. So, bioceramics with adequate properties can be used for MSC therapy in bone tissue. Furthermore, other types of cells can be seed in co-culture conditions on these 3D structures [65]. Angiogenesis is closely associated to osteogenesis where the reciprocal interactions between endothelial and osteoblast cells take a primordial role in the success of bone regeneration [66-68]. For these reasons, several different co-culture systems have been developed in order to study the interactions between Human mesenchymal stem cells (HMSC) and endothelial cells, as described in detail in chapter 2. The presence of both types of cells help each other to survive and can lead to a faster bone regeneration when implanted in patient.

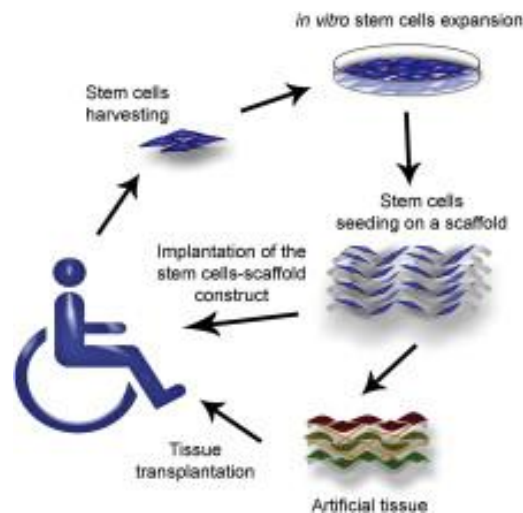


Figure 8- Stem cell-tissue engineering strategies[65].

4. Nanotechnology and Nanomaterials

Nanotechnology comes from the ability to control material properties by assembling such materials at the nanoscale, and it has achieved tremendous progress in the past few years [69]. Recently, nanomaterials, which are materials with basic

structural units, grains, particles, fibers or other constituent components at a nanoscale dimension, have evoked a great amount of attention as they may be used to improve disease prevention, diagnosis, and treatment. These materials at nanoscale can be made of metals, ceramics, polymers, organic materials and composites thereof just like conventional materials [69-71].

Nanomaterials have been pointed out as promising candidate for improving traditional tissue engineering materials, since nanotechnology embraces systems whose materials are in the range of nanometers (10^{-9} m) that can mimic surface properties (topography, energy, etc) of natural tissues, particularly bone [5, 69]. So, bone cells are naturally used to interact with surfaces presenting nanometer roughness, very different from the surfaces provided by traditional orthopedic implants (Figure 9) [5, 72]. These cells directly interact with (and create) nanostructured extra-cellular matrices (ECM) and as a result the biomimetic features and excellent physiochemical properties of nanomaterials play a key role in stimulating cell growth as well as guide tissue regeneration [69, 70].

Furthermore, several studies have shown that nanomaterials exhibit superior cytocompatible, mechanical, electrical, optical, catalytic and magnetic properties compared with micron structured similar materials [5, 69, 72-74]. After decreasing material size into the nanoscale, dramatically increased surface area, surface roughness and surface area to volume ratios can be created to lead to superior physiochemical properties (i.e., mechanical, electrical, optical, catalytic, magnetic properties, etc) [69, 73, 74].

As described, it is expected that the use of nanostructured HA might offer several advantages, being an attractive platform for tissue engineering bone in vivo or ex vivo.

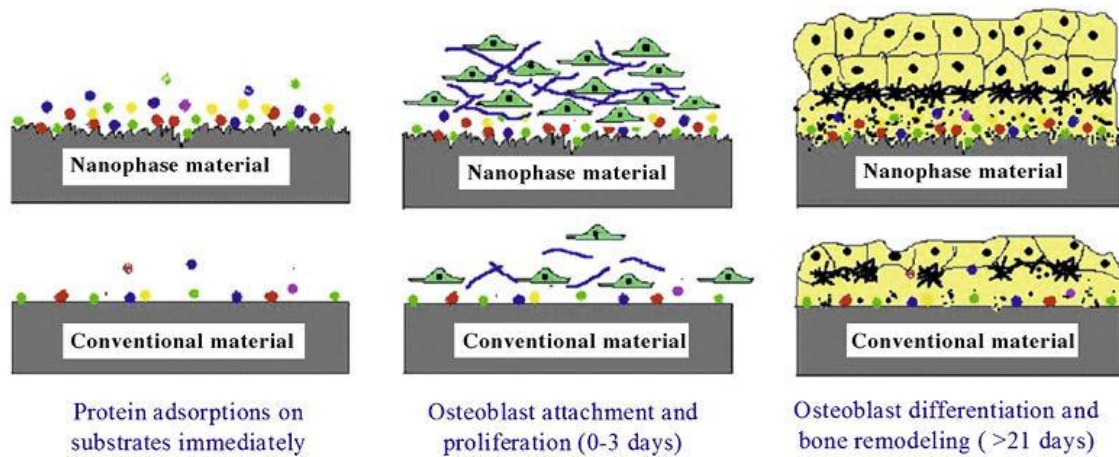


Figure 9- Nanostructured bioactive surfaces promote greater amounts of protein adsorption and consequently should stimulate bone growth comparing with their counterstained microstructured materials [69].

Recent works suggest other nanophase ceramics (such as alumina; titania) [70], polymers (poly(lactic-co-glycolic acid); polyurethane) [71, 75], metals (Ti, Ti6Al4V; CoCrMo) [72, 76] and composites [72], present an enhanced bioactivity and an unique enhanced bone-regeneration, showing the wide range of potentially exciting nanostructured materials for bone applications [69].

5. Aims and outline of the thesis

Aims

The main aim of the present work was to develop 3D macroporous granules with nanostructured HA aggregates and study the reciprocal interaction between human microvascular endothelial cells and mesenchymal stem cells seeded on these granules.

Nanostructured ceramics present improved surface properties when compared to microstructured ones, influencing cell adhesion, proliferation and functionality. Taking this into account, the first aim was to develop, optimize and characterize 3D macroporous granules with nanostructured HA aggregates, through a polyurethane sponge impregnation and burn-out method. The new granules were then compared with similar microstructured-HA granules, regarding the physicochemical characterization and the *in vitro* biological performance, using MG-63 human osteoblasts-like cells.

Bone regeneration requires fast and stable vascularization after applying a bone graft implant. Co-culture systems of osteoblasts or their progenitors and endothelial cells have been used to investigate osteogenesis and angiogenesis, both playing major roles in bone healing. However, in the literature, several works report very different co-culture systems with no consensus regarding the best conditions to do it. So, the second aim of this work was to develop a co-culture system using HMSC and HDMEC to study their long-term interaction and furthermore to apply it on granules.

The potential colonization of biomaterials with endothelial cells and osteoblasts or their progenitor cells, ensuring the proliferation and phenotype differentiation of both cell types, can lead to significant developments for tissue engineering and bone regeneration surgery. As a result, the last aim of the present work was to evaluate the behaviour of HDMEC and HMSC both alone or in co-culture, on macroporous granules composed of nanostructured HA aggregates.

Outline of this Thesis

The treatment of bone defects caused by diseases or trauma requires appropriate bone graft substitutes to generate the ideal conditions for fast bone regeneration. Repairing critical-sized bone defects remains a challenge, since in this cases bone cannot self-regenerate. Several limitations are associated with bone grafts that are currently used nowadays, such as autografts, allografts and xenografts. Autografts limitations are donor site morbidity and limited amount of graft or, in case of allograft and xenografts, the possibility of immune rejection and pathogenic transmission. New bone tissue engineering techniques proposed alternative grafts for the treatment of large bone defects.

Bone is a nanocomposite with a hierarchical structure composed mainly by collagen fibres and calcium phosphates present as needle-like or plate-like nanocrystals with approximately 5-20 nm width and 60 nm length. This unique matrix influences bone cells behaviour, suggesting that this surface where calcium phosphates can be found in the nanometer range size, are critical in all the cell behaviours that are involved in bone regeneration process. Furthermore, previous studies, showed that nanostructured-materials (ceramics, metals, polymers) presented improved surface proprieties such as topography, chemistry, wettability and charge when compared to microstrutured-materials. All these properties are going to influence protein adsorption (type, bioactivity) and subsequent cell adhesion and functionality.

Porous bioceramics in general, promote the in-growth of tissues into pores, providing a large interfacial area between tissue and implant, due to their surface porosity [41, 58, 59]. Porous bioceramics for biomedical applications may be prepared following several pathways, like conversion from natural structures and completely synthetic techniques [59, 61-64]. The first ones start with a natural porous structure, like a coral or trabecular bone, which is converted to the final composition, maintaining the original structure. Other conventional methods, completely synthetic, had been used for creating porous structures, such as foaming techniques (foams and sponges) and introduction of porogenous substances (organic additives, salts). Pores must be open, interconnected and have diameters over 100 μm , to allow for cell

migration and neovascularisation. In CHAPTER II, two different types of highly porous granules were prepared using nanostructured-HA aggregates and microstructured-HA, through a polyurethane sponge impregnation and burn-out method. This technique allows for controllable pore size and a reproducible macroporosity since the polyurethane sponge is used as a template. A physicochemical/structural characterization was done and the MG-63 cell behaviour was also evaluated, with an increased performance of these type of cells in the nanostructured-HA granules.

Much of the available literature describes works done with biomaterials using monocultures systems with different types of cells. However, co-cultures systems have shown an increasing interest since they simulate more closely the *in vivo* environment and reciprocal interactions between different types of cells that are determinant for the biological or pathological process that is being studied. The types of cells chosen to develop a co-culture system are determined in our case by the two main processes involved in bone regeneration. Osteogenesis and angiogenesis are very important steps for successful bone regeneration, since vascular endothelial cells participate in a complex communication network with osteoblasts cells or their precursors. Taken these issues into account, it was decided to develop a co-culture system with human dermal microvascular endothelial cells (HDMEC) and mesenchymal stem cells from human bone marrow (HMSC) since bone marrow stromal cells are regarded as the main source bone stem cells, being capable to differentiate into different type of cells. Since there is no consensus in the literature regarding the optimal conditions for a co-culture system to be set-up (very different data sets for cell ratios and culture media are presented), it was decided to develop a co-culture system between HDMEC and HMSC as described in CHAPTER III to further apply these conditions to our 3D macroporous granules.

Several strategies have been proposed in bone tissue engineering to achieve successful bone regeneration after bone graft implantation, such as, new porous biomaterials, growth factors delivery systems and pre-seeded biomaterials/scaffolds with different types of autologous cells. In addition, a few studies done with nanostructured-HA reported an increased *in vitro* osteoblasts adhesion, proliferation and differentiation with an enhanced ALP activity and calcium phosphate deposition.

CHAPTER IV describes in detail the cross-talk between HDMEC and HMSC macroporous granules with nanostructured HA aggregates on the macroporous nanostructured-HA.

CHAPTER V highlights the general discussion of the work hereby described.

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CHAPTER II

INNOVATIVE MACROPOROUS GRANULES OF NANOSTRUCTURED HYDROXYAPATITE AGGLOMERATES: BIOACTIVITY AND OSTEOBLAST-LIKE CELL BEHAVIOUR

**Innovative macroporous granules of nanostructured hydroxyapatite
agglomerates: bioactivity and osteoblast-like cell behaviour**

M.S. Laranjeira ^{a, b}, M.H. Fernandes ^c, F.J. Monteiro ^{a, b}

^a INEB- Instituto de Engenharia Biomédica, Divisão de Biomateriais, Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal

^bUniversidade do Porto, Faculdade de Engenharia (FEUP), Departamento de Engenharia Metalúrgica e de Materiais, Rua Roberto Frias, s/n, 4200-465 Porto, Portugal

^cLaboratório de Farmacologia e Biocompatibilidade Celular, Faculdade de Medicina Dentária da Universidade do Porto (FMDUP), Rua Dr. Manuel Pereira da Silva, 4200-393 Porto, Portugal

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ABSTRACT

To modulate the biological response of implantable granules, two types of bioactive porous granules composed of nanostructured hydroxyapatite (HA) agglomerates and microstructured hydroxyapatite, respectively, were prepared using a polyurethane sponge impregnation and burn-out method. The resulting granules presented a highly porous structure with interconnected porosity. Both types of granules were characterized using FTIR, X-ray diffraction, SEM and mercury intrusion porosimetry. Results showed that nanostructured-HA granules presented higher surface area and porosity, than microstructured-HA granules. In vitro testing using MG-63 human osteoblast-like cells showed that on both types of surfaces cells were able to adhere, proliferate and migrate through the macropores and a higher growth rate was achieved on nanostructured-HA granules than on microstructured-HA granules (76% and 40% respectively). In addition, these cells maintained similar expression levels of osteoblastic-associated proteins namely COL1, ALP, BMP2, M-CSF and OPG. These innovative nanostructured hydroxyapatite granules may be considered as promising bioceramic alternative matrixes for bone regeneration and drug release application.

KEY WORDS: Biomaterials, Nanomaterials; Hydroxyapatite; Bioceramics; Macroporosity; Granules; Osteoblast response

INTRODUCTION

Hydroxyapatite (HA) is regarded as an exceptionally osteoconductive and bioactive substance, since it forms a strong chemical bond with host bone tissue [1-4], creating suitable local conditions for bone formation when implanted in an osseous environment [5-7]. Nowadays healthcare industry recognizes it as a good bone graft synthetic material [8], owing to its chemical and structural similarity with natural mineral bone [2].

Presently, many production methodologies for obtaining microscale HA have been reported [2], and it has been widely used in several clinical applications in numerous forms (powders, granules, dense, porous blocks, thin and thick films) [8-12].

However with the advent of nanotechnology, nano-sized HA has been prepared and studied in order to improve the biological properties of HA. It is well known that natural bone exhibits hierarchical structures of nanometer dimensions within bone matrix, mainly constituted of collagen (20 wt.%) and calcium phosphates (69 wt.%) [2, 13]. Calcium phosphates are present in the form of needles-like or plates-like nanocrystals with approximately 5-20 nm width and 60 nm length, which are deposited on collagen fibrils [1, 8]. In fact, most nanostructured materials recently developed for bone implant, such as ceramics, polymers and metals [14] have a more dynamic response when compared to counterpart materials with larger particle size [15]. Nanoscaled materials show improved performances due to their large surface to volume ratio and particularly high surface reactivity (unusual chemical/electronic synergistic effects) [2, 8].

HA bioceramics can be applied in dense or porous forms. However, porous structures have a much higher surface area which enables good fixation and allows for bone ingrowth [16, 17]. Pores must be open and interconnected, to promote cell migration, neovascularisation, water circulation, nutrients, gases and metabolic waste removal [7]. Comparing to pre-shaped HA prismatic blocks, macroporous structured granules can easily fill bone defects/voids of irregular and patient-specific shapes, resulting from orthopaedic or periodontal surgeries [12, 18].

Porous bioceramics can be prepared by conversion from natural structures starting with a natural porous structure, like a coral, algae or trabecular bone [19, 20], which is converted to the final composition, maintaining the original structure. Joschek S. *et al* studied the properties of a porous hydroxyapatite ceramic produced by sintering of bovine bone. Macroporous granules have an irregular shape with a macropore average diameter of 300µm and micropores with average of 1.3 µm, showing a total porosity of more than 57% [19].

Also, several distinct methods to create calcium phosphate bioceramics, using synthetic techniques are described in the literature, such as foaming techniques (foams, sponges and gas foaming) [20-22] and introduction of porogenous substances (organic additives, salts) [18, 23]. More recently, solid free form fabrication [24] and freeze casting [25] are also described.

Tas A.C. *et al* used a CaP cement powder as starting material to create spherical granules, using NaCl as a porogen varying in size between 420 μm and 1 mm. The formed granules presented sizes between 2 and 4 mm and about 50% of total porosity [18]. Organic additives are also often used as porogens and high temperatures are then required to burn-out the polymer. E. Chevalier *et al* used pre-gelated starch for granulation, as a pore former in order to prepare milimeter sized spherical granules. The granules presented a pore size between 3 and 10 nm [23]. Pataquiva Mateus A. Y. *et al* prepared hydroxyapatite nanoparticles that were dispersed in a sodium alginate solution. Spherical particles with a uniform size and interconnected microporosity were obtained by droplet extrusion. The final granules presented sizes between 0.5 and 0.8 μm and a theoretical porosity of 45% [26]. As described above, the porous granular structures found in the literature may be macroporous or/and microporous and since nanoceramics represent a promising class of orthopaedic and dental implant formulations with improved biological properties, the main aim of this work was to develop and characterize macro and microporous granules of nanostructured HA aggregates, in order to conciliate advantages of nano-sized HA and porous bioceramic granules. The porous granules reported in this study are believed to be the first macroporous granules produced with a nanostructured HA grain.

For this study, two different types of highly porous granules were prepared using in one case nanostructured-HA aggregates and in the other microstructured-HA, through a polyurethane sponge impregnation and burn-out method. Physicochemical and structural characterizations were performed using different techniques and *in vitro* biological performance was evaluated using MG-63 human osteoblast-like cells.

MATERIALS AND METHODS

Preparation of the porous granules

The porous granules were prepared using the polyurethane sponge impregnation method. Two different type of HA slurries were prepared using nano-sized HA aggregates, kindly provided by Fluidinova S.A. (Maia – Portugal) and a commercial HA (Plasma Biotol – P218). The nano-HA slurry was prepared using 50 (wt%) of water and nano-HA. Commercial HA slurry was prepared as described elsewhere [21]; briefly, a ceramic slurry was prepared using the ratio of 6:4:0,2,

respectively, HA (g), Water (ml) and tensoactive agent (ml). The polyurethane sponges were firstly immersed in the respective slurries and compressed repeatedly, until most of air bubbles were eliminated. Slurry excess was removed by squeezing the polyurethane sponges after being sunk in the slurry. The infiltrated sponges with nanostructured-HA were heat-treated according to several different heat cycles, the best cycle being the following: heating rate of 1°C/min with 1h dwelling time at 600°C followed by a heating rate of 4°C/min with 1h dwelling time at 1050°C and then samples were cooled inside the furnace. After drying, the sponges infiltrated with commercial HA were heat-treated according to the following sintering cycle: heating rate of 1°C/min with 1h dwelling time at 600°C for the polyurethane sponge to burn-out, followed by a heating rate of 4°C/min with 1h dwelling time at 1300°C with samples being cooled inside the furnace. After heat treatment, samples were crushed and sieved in order to obtained granules with sizes between 315 and 500 µm.

Physicochemical characterization

XRD and FTIR

After heat-treatment, sample of both types were ground to fine powders and were analysed by a Rigaku Dmax-III-VC X-ray diffractometer, using CU-K α radiation ($K\alpha = 1.54056 \text{ \AA}$). Data were acquired for 2θ values between 4 and 80°, with steps of 0.02°/s.

Infrared spectra analysis was performed with a FTIR system 2000 from Perkin-Elmer, with 4cm^{-1} resolution and 100 scans. The “as received” nano-sized HA aggregates, nanostructured-HA granules and microstructured-HA granules powders (after heat-treatment) were mixed with potassium bromide (KBr) and thin discs were prepared using a steel die under uniaxial pressing.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) observations were performed with a JOEL JSM-6310F scanning electron microscope. The nanostructured-HA granules and microstrutured-HA granules were attached with AralditeTM in an aluminium support. Finally, they were sputter coated with palladium-gold alloy for about 13 min. SEM

images were used to determinate macropore size and distribution, using “Image J” image analysis software.

Mercury porosimetry method

Mercury porosimetry method (Quantachrome Poremaster model No. 60) was used to evaluate apparent density, total surface area and pores volume percentage on both types of granules (nanostructured-HA and microstructured-HA) (Table 1). The referred equipment allowed for the detection of open porosity in the range of 0.0035 μm to 10.6379 μm . Helium Picnometry was also used to determine real density. About 0.7g of each sample were used in those measurements and reported data were obtained using Quantachrome Poremaster for Windows, version 3.0.

In vitro Biological studies

Cell culture

Mg-63 cells were cultured in minimum essential medium Eagle, alpha modification (α -MEM) containing 10% foetal bovine serum (FBS), 100 $\mu\text{g}/\text{ml}$ penicillin, 10IU/ml streptomycin, 2.5 $\mu\text{g}/\text{ml}$ fungizone and 50 $\mu\text{g}/\text{ml}$ ascorbic acid. Incubation was carried out in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After sub-culturing, cells were washed with phosphate-buffered saline (PBS, Gibco, UK), detached with trypsin solution at 37°C for 5 min, counted using a hemocytometer. Then, cells were seeded on the samples (128 mg/cm^2) and on cell culture plate (control) at a density of 10^5 cells/ cm^2 . Samples of both types were pre-incubated with this medium for 1 hour at 37°C, in a humidified atmosphere of 95% air and 5% CO_2 . Culture medium was changed twice a week. Cultured material samples were observed by CLSM (Confocal laser scanning microscope) and SEM at days 1, 3 and 6. In vitro osteoblast proliferation, alkaline phosphate activity and total protein content were also evaluated at days 3 and 6, for both type of granules and control. RT-PCR amplification was done for cells cultured for 6 days.

Confocal laser scanning microscope (CLSM)

Cells were fixed with 4% formaldehyde (methanol free), permeabilized with 0.1% triton and incubated in 10 mg/ml bovine serum albumin (BSA) with 100 $\mu\text{g}/\text{ml}$

RNAse. F-actin filaments were stained with Alexafluor-conjugated Phalloidin and nuclei were counterstained with 10 µg/ml propidium iodide. Samples were washed with PBS and covered with Vectashield. Images were acquired on a Leica TCP SP2 AOBS.

Scanning electron microscopy (SEM)

Cells were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate. The samples were dehydrated in graded series of ethanol solutions and critical-point dried. Specimens were mounted onto aluminium supports using AralditeTM and then sputter-coated with palladium-gold and observed in a Joel JSM 35C Scanning Electron Microscope equipped with an X-ray energy dispersive spectroscopy voyager XRMA System, Noran Instruments.

Alkaline phosphate activity and total protein content

Cell cultured granules were washed with PBS and with a cell lysis buffer containing 0.1% Triton X-100. The samples were frozen at -20°C and later thawed at 37 °C for 5 min in order to measure the ALP activity. The enzyme activity was assayed by the substrate hydrolysis (p-nitrophenyl phosphate) in alkaline buffer solution (2-amino-2-methyl-1-propanol), at pH 10.5. After 1h incubation at 37°C, NaOH (1M) was added to stop the reaction and hydrolysis product (p-nitrophenol) was measured by absorbance measurements at 405nm using an ELISA reader. The ALP activity results were normalized to total protein content and were expressed in nanomoles of p-nitrophenol produced per min per µg of protein ($\text{nmol min}^{-1} \mu\text{g protein}^{-1}$). Total protein content was measured by Lowry's method with bovine serum albumin used as a standard.

MTT

Mitochondrial dehydrogenase activity of the MG63 cell was determined by using the substrate 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma), which was reduced to formazan that accumulated in the cytoplasm of viable cells. For this propose, 10 µl of MTT solution (5 mg/ml) were added to culture dishes containing 100 µl of medium and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO₂ for 3 h. Formazan salts were dissolved with 200 µL

of dimethylsulphoxide and the absorbance was measured at 600 nm on an ELISA reader.

Resazurin

Cell vitality was assessed with the non-toxic Alamar Blue dye (Resazurin) test simultaneously with MTT assay. Resazurin is reduced by intracellular enzyme activity to resofin, a fluorescent form of Alamar Blue. Fifty microliters of 10% (v/v) fluorescent dye were added to 100 μ l of medium and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 4 h. Then the solution was transferred into a black 96-well plate and fluorescence intensity was measured by a fluorometer (Twinkle™ LB 970, Berthold Technologies) at 530 nm and 590 nm for excitation and emission, respectively.

Reverse-Transcription Polymerase Chain Reaction(PCR)

Total RNA was extracted from cell culture on material using the RiboPure™ Kit according to the manufacturer's instructions and subjected to RT-PCR amplification (Titan One Tube RT-PCR system; Roche, Branchburg, NJ) for 30 cycles. RT reaction mixtures consisted of RNA extracted, Titan RT-PCR buffer, dithiothreitol (DTT), deoxynucleoside triphosphate (dNTP), primers for each gene tested, avian myeloblastosis virus RT (AMV-RT) and water, in a total volume of 25 μ l. Total RNA was reverse transcribed with AMV reverse transcriptase, at 50 °C for 30min, followed by a 2 min denaturation at 94°C. The cDNAs were then amplified with recombinant Taq-DNA polymerase under the following conditions: 30 cycles of denaturation (94 °C/30 s), annealing (50 °C/30 s), elongation (68 °C/45s), and followed by a prolonged elongation of 7 min at 68 °C. Table 1 shows the primer sequences used for PCR amplification. To obtain a semi-quantitative assessment of gene expression, data were expressed as normalized ratios by comparing the integrated density values for all genes tested with those for GAPDH. The PCR products were separated by 2% agarose gel electrophoresis, visualized by Ethidium Bromide staining. The images of the gel were captured with a camera and analyzed with Image J software.

Table 1 – Primers for PCR amplification

Gene	Primer sequence (forward)	Primer sequence (reverse)
GAPDH	5'-CAGGACCAGGTTACCAACAAGT-3'	5'-GTGGCAGTGATGGCATGGACTGT-3'
COL1	5'-TCCGGCTCCTGCTCCTCTTA-3'	5'-ACCAGCAGGACCAGCATCTC-3'
ALP	5'-ACGTGGCTAAGAATGTCATC-3'	5'-CTGGTAGGCGATGTCCTTA-3'
BMP2	5'-GCAATGGCCTTATCTGTGAC-3'	5'-GCAATGGCCTTATCTGTGAC-3'
RANKL	5'-GAGCGCAGATGGATCCTAAT-3'	5'-TCCTCTCCAGACCGTAACTT-3'
M-CSF	5'-CCTGCTGTTGTTGGTCTGTC-3'	5'-GGTACAGGCAGTTGCAATCA-3'
OPG	5'-AAGGAGCTGCAGTACGTCAA-3'	5'-CTGCTCGAAGGTGAGGTTAG-3'

Statistical analysis

Triplicate experiments were performed. The results were expressed as the arithmetic mean $\pm$ standard deviation (SD). Analysis of results was carried out using the Student's t-test, with a significance level of $p < 0.05$.

RESULTS

Physical, chemical and morphological characterization of the macroporous granules

X-ray diffraction technique was used to analyze phase composition and crystallinity of non-heat treated powders of nanostructured-HA aggregates, heat-treated granules of nanostructured-HA aggregates and heat-treated granules of microstructured-HA. The obtained XRD patterns of different powders are shown in Figure 1. X-ray diffraction data do not show any unexpected phases in nano-sized HA powders before and after heat-treatment, since peak positions are in good agreement with HA ICDD standard data (PDF file #9-432). However, XRD analysis of sintered microstructured-HA granules revealed the presence of tricalcium phosphate phases (β -TCP and α -TCP) in residual amounts (Figure 1).

The presence of PO_4^{3-} , OH^- and H_2O groups was confirmed by the FTIR spectrum in all the different powders. FTIR spectra revealed phosphate peaks at 473 cm^{-1} , 560 cm^{-1} , 602 cm^{-1} , 962 cm^{-1} , 1037 cm^{-1} , 1089 cm^{-1} and hydroxyl (OH^-) peaks were detected at 3570 cm^{-1} and 630 cm^{-1} (Figure 2). The broad band observed at $3200\text{--}3600\text{ cm}^{-1}$ indicated adsorbed water on the materials. In "as prepared"

nanostructured-HA powders, peaks at 1456 cm^{-1} and 1416 cm^{-1} are due to carbonate group. Regarding microstructured-HA granules, the peaks presented in the broad band between 2500 cm^{-1} to 2000 cm^{-1} may correspond to the HPO_4^{2-} groups [27]. XRD and FTIR results proved that heat-treated nanostructured-HA was phase pure.

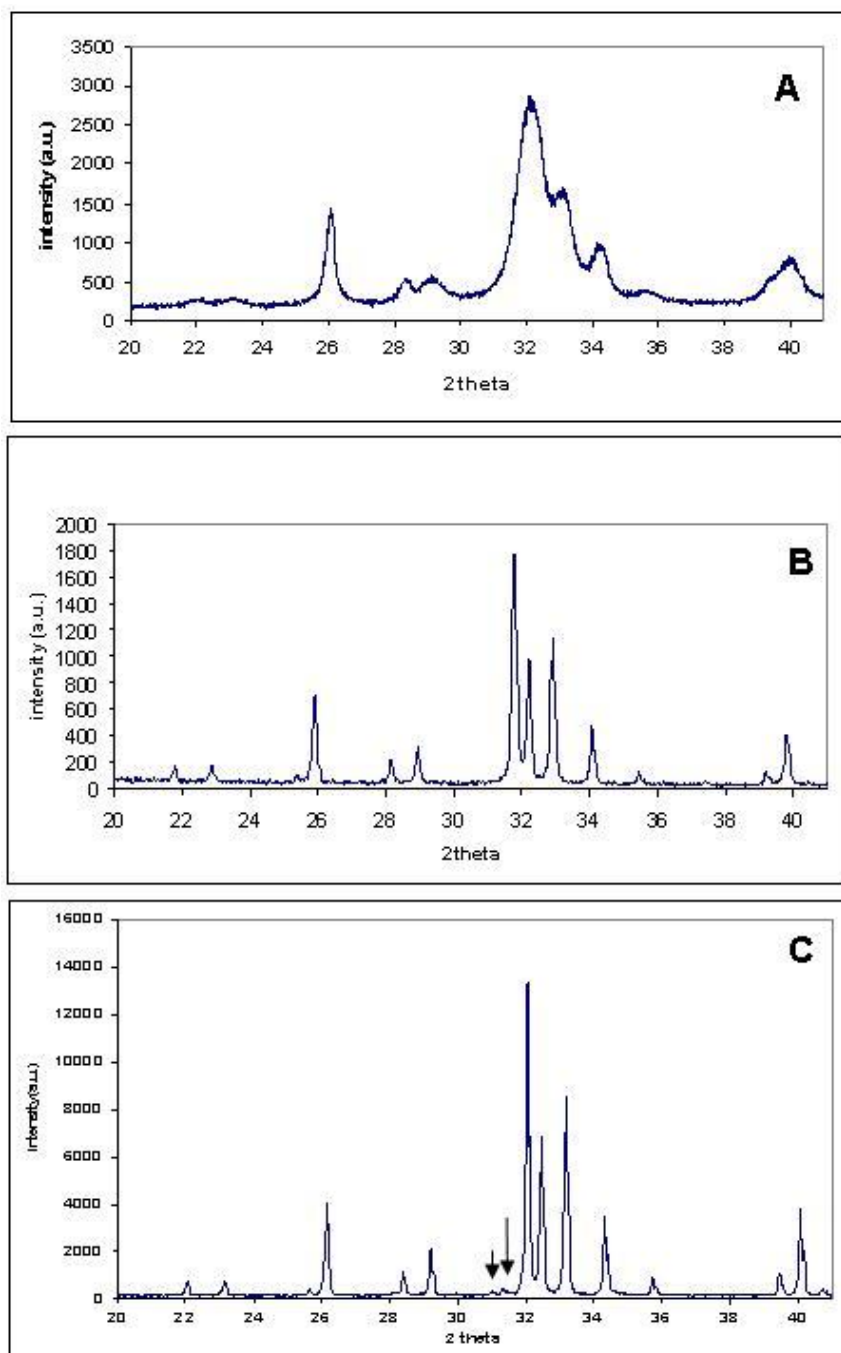


Figure 1 - XRD traces of (A) nanostructured-HA aggregates before heat-treatment, (B) nanostructured-HA granules and (C) microstructured-HA granules (from left to right the arrows show α -TCP and β -TCP, respectively).

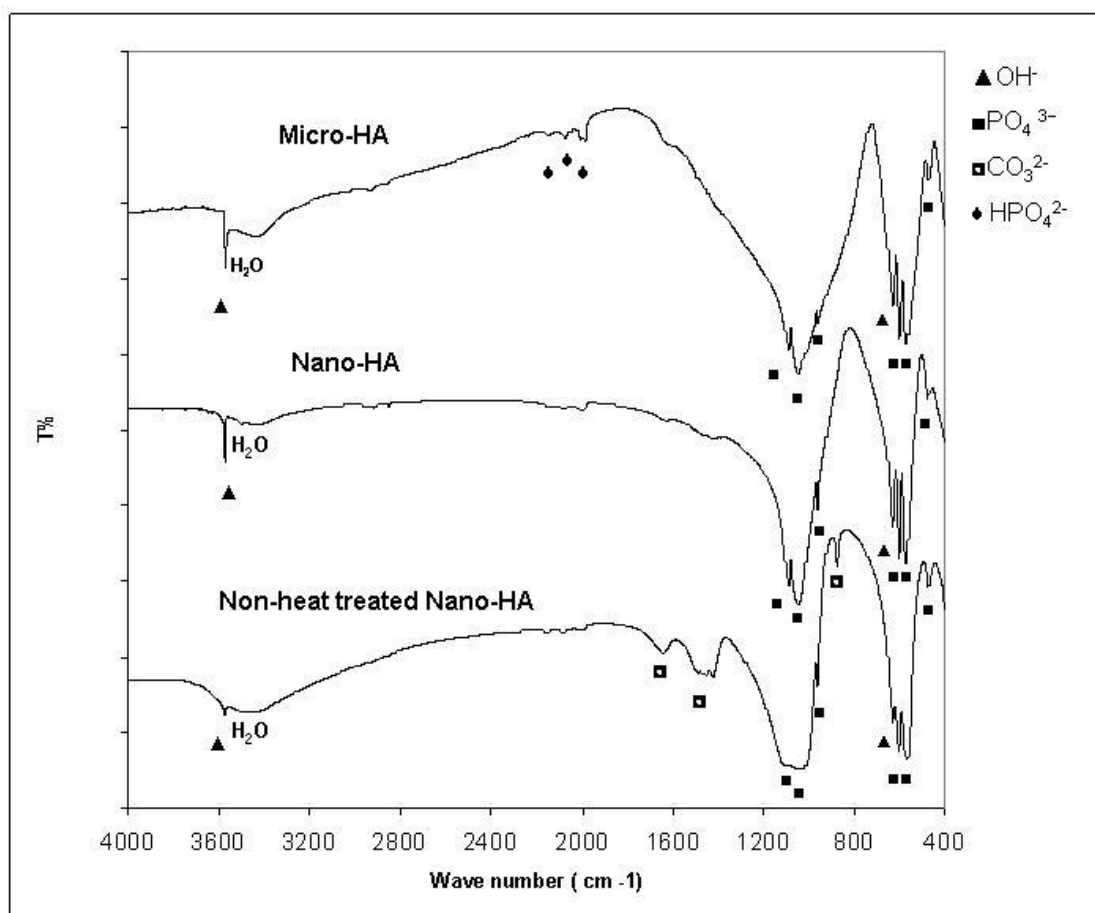


Figure 2 - FTIR spectra of HA samples nanostructured-HA granules; microstructured-HA granules and “as prepared” nanostructured-HA powder.

Samples morphology, microstructure and macroporosity were analysed by scanning electron microscopy (SEM). The starting “as prepared” HA was characterized as spherical aggregates of nanostructured-HA powders which have a wide range of diameter sizes. At higher magnification it was noticed that HA particles were within nano-scale (Figure 3A and B). The heat-treated nanostructured-HA granules presented a highly macroporous and fully interconnected structure (Figure 3C). In SEM analysis it was also possible to observe that nanostructured-HA granules maintained the initial morphology of spherical agglomerates. These nanostructured-HA agglomerates did not collapse during heat-treatment and generated only small contact areas among them (Figure 3D). At higher magnification it was verified that HA grain were maintained in nano-sized range (figure 3E). Regarding the microstructured-HA granules it was possible to observe the micro-sized grains at higher magnification (Figure 3H) with a fully interconnected macroporosity (Figure 3F).

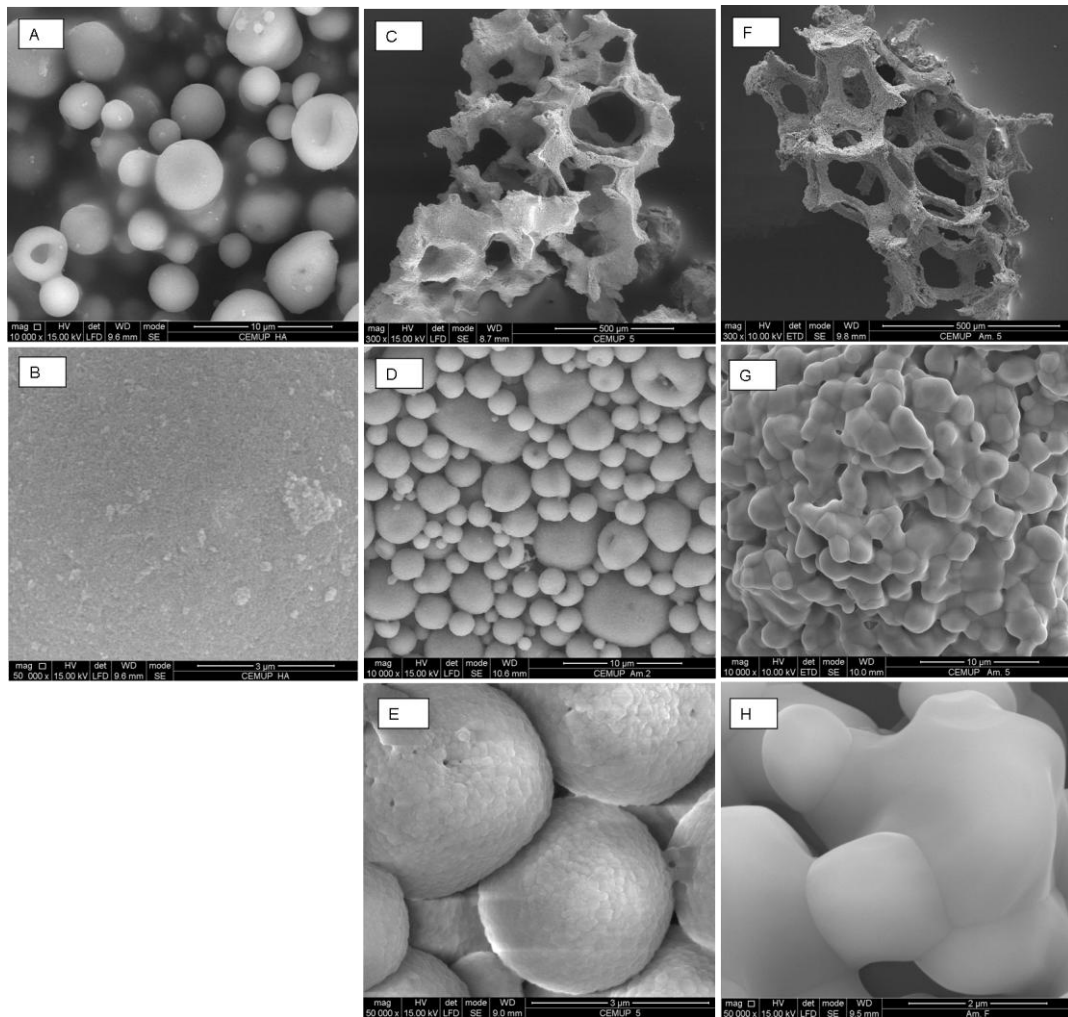


Figure 3 - SEM images of “as prepared” nanostructured-HA aggregates (A,B), nanostructured-HA granules (C,D,E) and microstructured-HA granules (F,G,H).

SEM studies yielded valuable qualitative information but also allowed for the macroporosity assessment using an image analysis Software - Image J (Java). Macropore sizes ranging between 10 and 350 μm were measured by this technique, with an average pore size of 141 μm and 133 μm for nanostructured-HA and microstructured-HA granules, respectively. Pore size distributions are shown in Figure 4 by histogram obtained from the results of image analysis of SEM images. As it may be seen, nanostructured-HA scaffold macroporosity is characterized by a small amount of pores in the range over 300 μm and a large amount of pore ranging from 100 to 200 μm . The same distribution was determined for microstructured-HA scaffolds.

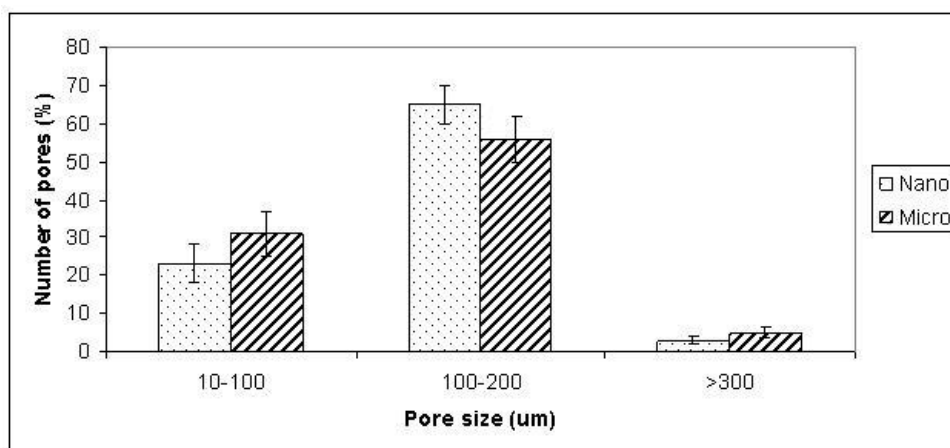


Figure 4 – Histogram with macropore size distribution in nanostructured-HA granules and microstructured-HA granules.

Mercury intrusion allowed for a quantitative study of the actual surface areas. The results showed that nanostructured-HA granules presented a much higher surface area, as compared to microstructured-HA granules. Total porosity volume was also much higher for nanostructured-HA granules than for microstructured-HA granules (Table 2). The micropore mean size calculated for nanostructured-HA and microstructured-HA granules were respectively of 1 μm and 1,3 μm . As the mercury intrusion accounts only for pore diameters in the range of 0,0035 μm to 10,6379 μm , macropores diameter were determined by measuring them through SEM images, as above described.

Table 1 – Results from mercury porosimetry

	Nanostructured granules	Microstructured granules
Total surface area	0,7058 m ² /g	0,3525 m ² /g
Apparent density	1,8253	2,3069
Theorical porosity	41 %	30 %

In vitro biological studies

The biological performance of nanostructured-HA and microstructured-HA granules was evaluated through MG63 osteoblast-like cell culture. SEM results indicated that both types of scaffolds allowed for the attachment and spreading of the cells, while keeping a normal cellular morphology. As it can be seen in Figure 5A, at day

1 of culture, MG63 osteoblast-like cells were already well attached and spread in both materials, presenting a round shape configuration with extended cytoplasm in all directions, adhering to the surface materials.

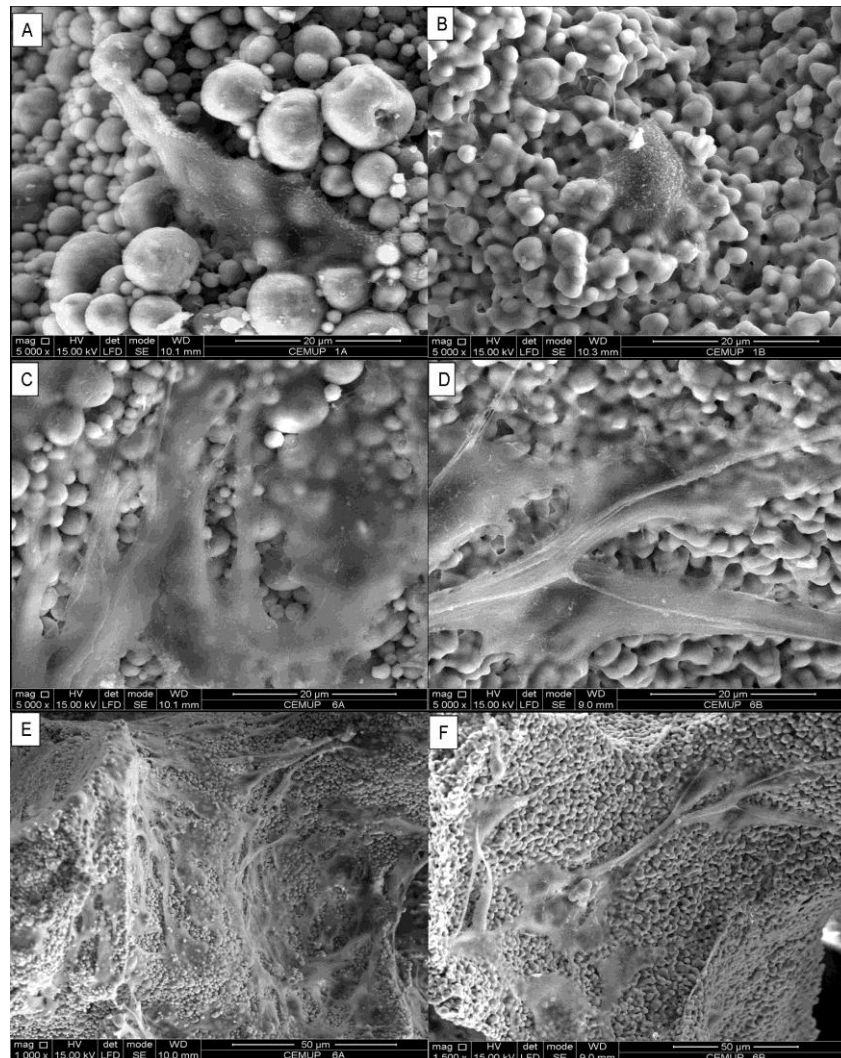


Figure 5 - SEM images of cells cultured on porous nanostructure-HA samples at day 1 (A) and day 6 samples (C, E) at different magnifications, showing macropores and sample's surface covered by cell layers. Cells cultured on porous microstructure-HA samples for day 1 (B) and day 6 samples (D) (F) in different magnifications, showing macropore inner regions and sample's surface.

After six days of culture cells showed a typical morphology presenting a flat configuration with more lamellopodia connecting to neighbouring cells, starting to

form a continuous cell layer. CLSM (Figure 6) observations of seeded granules showed that cells were well spread and almost fully covered the surface after 6 days of culture, on both type of granules. Also, cells were able to migrate into all macropore and covered the entire granules surface. Both SEM and CLSM showed that microstructured HA granules seem to have less cells as compared to nanostructured HA granules.

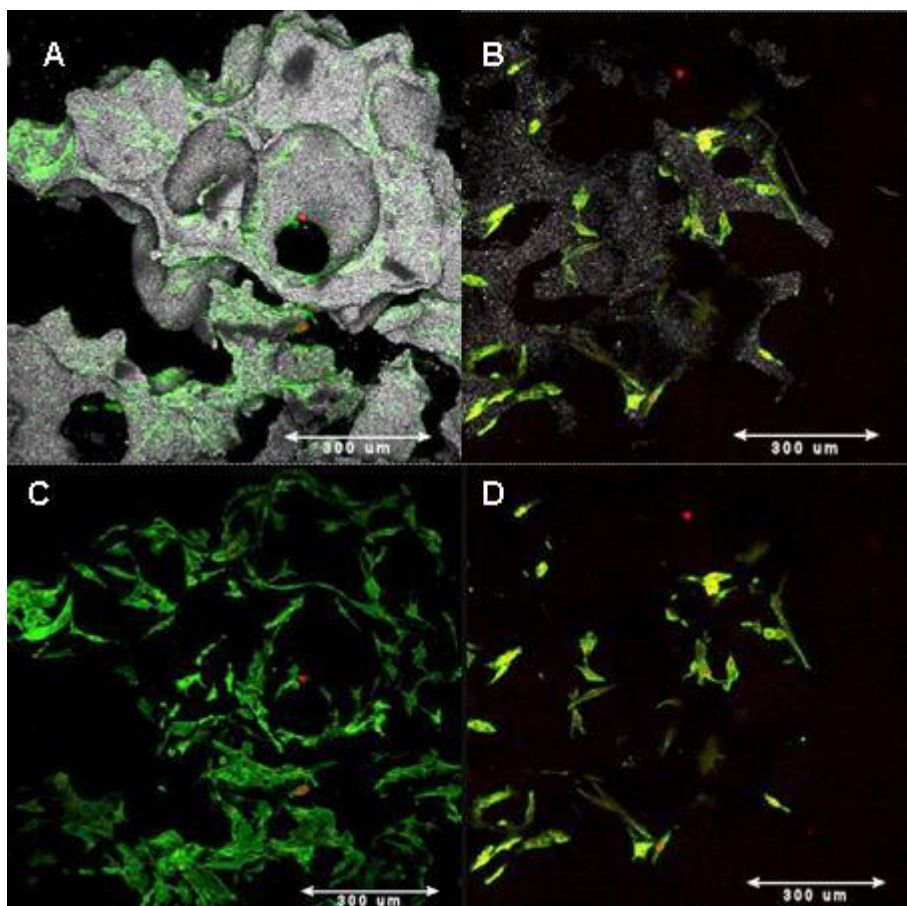


Figure 6 - CLSM images of cells cultured for 6 days on porous nanostructured granules surface and inner regions in the samples (A, C). CLSM images of cells at days 6 on porous microstructured granules surface and inner regions of the sample (B, D).

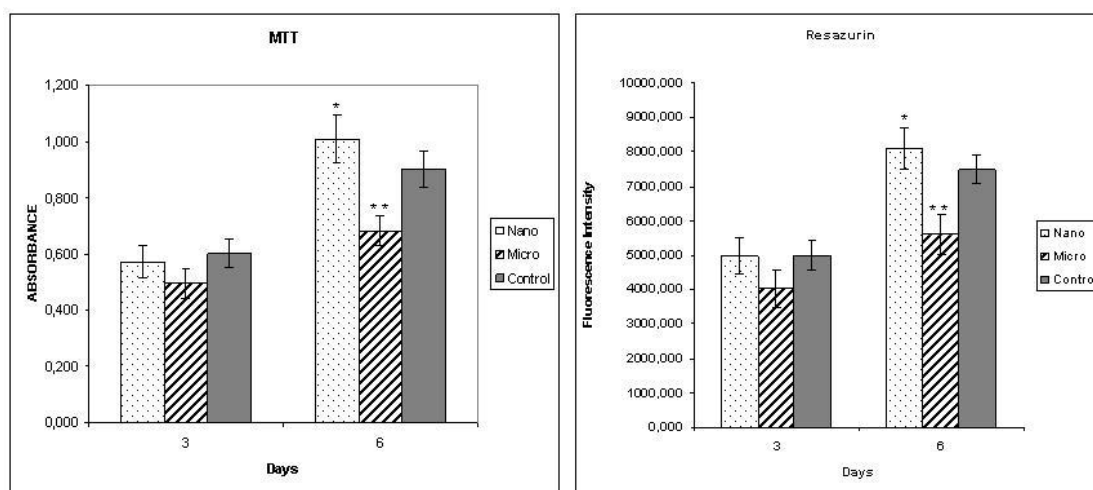


Figure 7 - Cell proliferation of MG63 cells cultured on nanostructured and microstructured granules for 3 and 6 days, estimated by MTT and Resazurin assays (*, significantly different from control cultures and microsized-HA granules / **, different from control cultures and nanostructured-HA granules grown cultures ($p < 0.05$; $n = 3$)).

MTT and Resazurin assays were used to evaluate cell viability and proliferation. The MTT and Resazurin values of cells cultured on nanostructured-HA granules were markedly higher than on microstructured-HA and on control after 3 to 6 days of culture (Figure 7). The differences were statistically significant between both scaffolds and control, at day six. The results of MTT and Resazurin assays indicated a cell proliferation increase between 65% and 76% in the nano-HA scaffolds between day 3 and day 6, when compared to micro-HA scaffolds, which revealed an increase between 37% and 40%. Figure 8 shows that ALP increased with culture time in both materials and control without statistically significant differences.

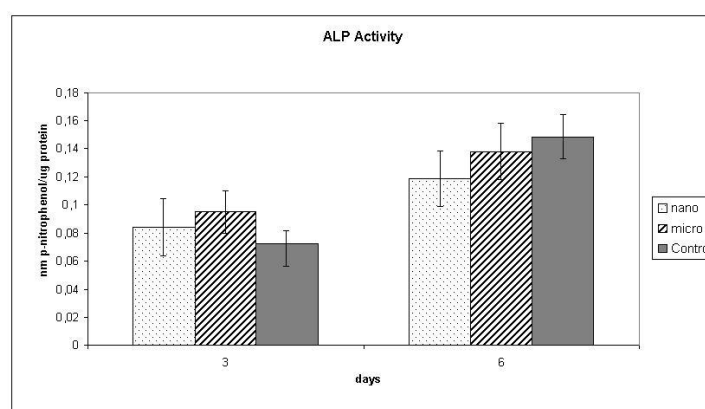


Figure 8 - Results from ALP activity after 3 days and 6 days of culture.

Regarding the PCR results the expression of COL1 genes was higher in nanostructured-HA granules and ALP, BMP2, OPG and M-CSF genes showed a higher gene expression in microstructured-HA granules (Figure 9). In all cases the differences between nanostructured-HA, microstructured-HA granules and the control was considered as not statistically significant.

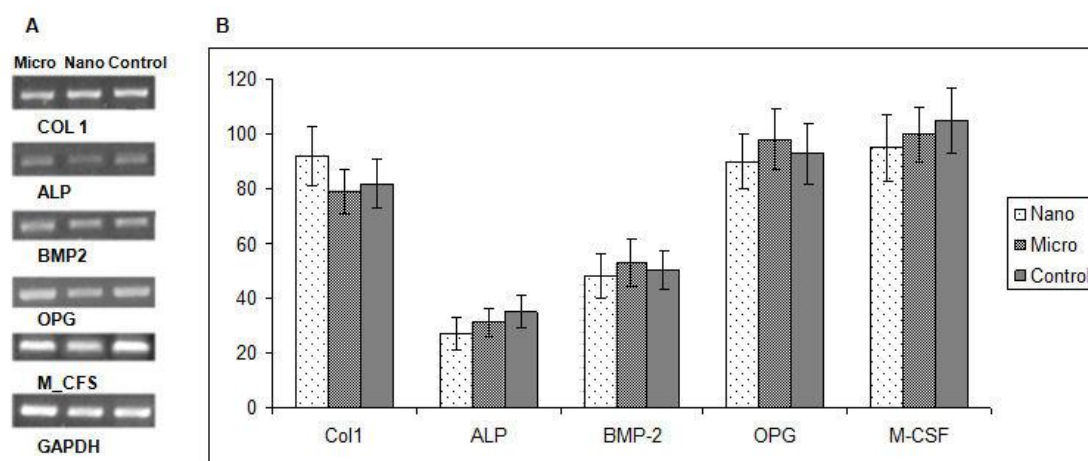


Figure 9 - RT-PCR analysis of the osteoblastic-associated markers: collagen type I (COL1), alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP2), osteoprotegerin (OPG) and macrophage colony-stimulating factor (M-CSF) after 6 days of cell culture on control, nanostructured-HA and microstructured-HA granules. A- Representative agarose gel of the PCR products; B- densitometric analyses of the PCR products, normalized with the corresponding GAPDH value.

DISCUSSION

This work proposed a new bone repairing porous granular material prepared using polyurethane sponge impregnation and burn out method. Two different types of granules were prepared, nanostructured-HA and microstructured-HA granules, and the subsequent morphological and physical characterization was performed.

The two types of granules tested in this work were not heat-treated in similar conditions. While for conventional HA powders, in the micron scale, the temperature of the plateau was 1300°C, for the nanosized HA it was only 1050°C. The reason behind this difference lies on the fact that conventional HA sinters only at 1300 °C, and therefore if heat-treated at lower temperature it will not acquire the necessary

mechanical strength. However, nanosized HA powders show a completely different behaviour, sintering at much lower temperatures. If however these were heat-treated at 1300°C there would occur a complete re-crystallization and considerable crystal growth, thus making it lose all the qualities associated to the nanosized structure. Therefore, although risking comparing the materials introducing an extra variable, there was a clear choice of two different heat cycles, to allow for the adequate comparison between significantly different structures to take place.

The starting material (nanostructured-HA agglomerates) were prepared through a wet chemical route which is the most extensively used method to synthesize HA [26].

The XRD results suggested that wet chemical reaction used for nano-HA synthesis has produced phase pure and homogeneous HA. They also revealed that “as prepared” nanostructured-HA seems to exhibit considerable amount of amorphous phase, even if the apatite phase is clearly presented and HA crystalline phase increased with heat-treatment temperature, as expected. After heat-treatment, XRD results confirmed that no exogenous phases were presented in the nanostructured-HA granules. Both nanostructured-HA and microstructured-HA granules revealed the presence of a crystalline HA phase. Regarding FTIR analysis, “as prepared” nanostructured-HA powders showed the peaks at 1456 cm^{-1} and 1416 cm^{-1} due to carbonate group, which might be incorporated from the atmosphere to these samples during the preparation of nanostructured-HA. The results (Figure 3), showed that CO_3^{2-} groups disappeared after sintering so, these groups might come from the atmospheric carbon dioxide during HA processing and they might be only adsorbed to the surface or weakly bonded in the HA lattice [26]. FTIR analysis indicated characteristic spectra bands for all the studied materials [28-30].

The spaces between these nanoparticles agglomerates may contribute to a very high microporosity in nanostructured-HA granules. Also, it was observed that a highly macroporous interconnected structure was obtained for both type of granules. Nanostructured-HA granules showed a larger surface area, in comparison with microstructured-HA granules and a higher porosity due to the higher microporosity. The total porosity determined by the mercury intrusion porosimetry method reached the maximum value of 41% for nanostructured-HA granules and 30% for

microstructured-HA granules. However, this method accounted pores with less than 10 μ m, so it can be assumed that the porosity is much higher, since macropores of nanostructured-HA and microstructured-HA granules showed an average macropore size of 141 μ m and 133 μ m and reached values up to 350 μ m. So, total porosity should be in agreement with values reported in studies with macroporous granules from Joschek S. *et al* and Tas A.C. *et al* that achieved a total porosity of ~ 50% [18, 19, 31].

It is also reported that to ensure cell viability and function, scaffolds ideally need to exhibit both macro and micro porosity spanning. Pore sizes below 10 μ m are required (microporosity) for cell adhesion. The minimum macropore size required for *in vivo* bone ingrowth into material [32, 33] and to regenerate mineralized bone is considered to be 100 μ m [34]. The pore size in interconnective structures affects cell migration (due to cell size), nutrient delivery and waste exchange [33-35]. In previous studies, it was shown that pore size is also critical for vascular ingrowth into a porous scaffold, assuming that pores above 140 μ m diameter are more prone to achieve a good vascularisation [36]. The nanostructured-HA granules developed in this work fill these requirements, taking in account that larger pores could compromise mechanical stability. Furthermore, the method used to produce the macroporous granules allowed for the production of highly interconnected porosity with controllable pore size and a reproducible macroporosity since a homogeneous polyurethane sponge is used as a template.

The colonization of the porous nanostructured-HA and microstructured-HA with MG-63 suggests a much higher cell proliferation in nanostructured-HA granules, considering MTT and Resazurin results, SEM and CLSM observations.

The expression of COL1, ALP, BMP2, OPG and M-CSF genes, which are typical osteoblastic phenotypes [37-39], were clearly observed after six days of cell cultured on both materials.

The results presented here show an increase of cell proliferation in nano-HA granules in comparison to micro-HA granules and identical gene expression has occurred in both materials.

In the literature, a previous studies by Guo X. *et al* also described significantly higher values on compacts of nano-HA compared to micro-HA after 7 days of cell culture, regarding the MTT assay [40]. Webster T.J. *et al* reported an increase in

osteoblast adhesion onto hydroxyapatite dense samples as well as enhanced proliferation and alkaline phosphatase activity when crystal size decreases to nanoscale. The increased osteoblast adhesion could be due to the greater surface area exhibited by nano-HA and this surface might promote interaction such as adsorption, configuration and bioactivity and selected serum protein(s)[41]. Another study showed that nanostructured surface enhanced the gene expression representative of osteoblast differentiation using human mesenchymal stem cells [42]. The differences between the present work and that work may be related to cell culture time and the culture model used.

In the present work the microstructured-HA macroporous granules produced through polyurethane sponge and burn-out method are not described in the literature, showing also relevant results regarding the highly porous structure and cell culture where cells were able to adhere, proliferate and express bone related genes.

The methodology used combined with nanostructured-HA agglomerates for granules preparation allowed a very high porosity well suited for cell adhesion and proliferation, generating a higher surface area. Previous studies showed that high porosity allows for the incorporation into the granules of large amounts of drugs which may be beneficial if the use of the ceramic as a drug carrier is intended [36]. This could also be an important application for these innovative granules.

CONCLUSION

In this work it was possible to obtain highly porous granules of nanostructured-HA agglomerates, with interconnected porosity. Using SEM and CLSM it was possible to observe cell distribution all over both porous granular structures, meaning that an adequate pore size and distribution were achieved. These innovative granules of nanostructure-HA agglomerates presented a higher surface area and porosity when compared with conventional microstructured-HA. A higher cell proliferation was achieved in nanostructure granules comparing to microstructured granules, with the same gene expression of osteoblastic associated markers. Results showed that porous granules of nanostructured-HA agglomerates provide a more adequate environment for cell adhesion and migration, improving cell response.

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CHAPTER III

RECIPROCAL INDUCTION OF HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS AND HUMAN MESENCHYMAL STEM CELLS: TIME-DEPENDENT PROFILE IN A CO- CULTURE SYSTEM

Reciprocal induction of human dermal microvascular endothelial cells and human mesenchymal stem cells: time-dependent profile in a co-culture system

M.S. Laranjeira^{1, 2*}, M.H. Fernandes³, F.J. Monteiro^{1, 2},

¹ INEB- Instituto de Engenharia Biomédica, Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal

² Universidade do Porto, Faculdade de Engenharia (FEUP), Departamento de Engenharia Metalúrgica e de Materiais, Rua Roberto Frias, s/n, 4200-465 Porto, Portugal

³ Laboratório de Farmacologia e Biocompatibilidade Celular, Faculdade de Medicina Dentária, Universidade do Porto (FMDUP), Rua Dr. Manuel Pereira da Silva, 4200-393 Porto, Portugal

ABSTRACT

Angiogenesis is closely associated with osteogenesis where the reciprocal interactions between endothelial and osteoblast cells play an important role bone regeneration. For these reasons, the aim of this work was to develop a co-culture system to study in detail the time-dependent interactions between Human mesenchymal stem cells (HMSC) and Human dermal microvascular endothelial cells (HDMEC) co-cultured in a 2D system, for 35 days. HMSC and HDMEC were co-cultured in the ratio 1:4, respectively. Single cell cultures were used as control. Cell viability/proliferation was assessed with MTT, DNA quantification and Calcein-AM assays. Cell morphology was monitored by Confocal microscopy and real time PCR was performed. Alkaline phosphatase activity and histochemical staining were evaluated. Matrix mineralization assays were also performed. Cells were able to grow in characteristic patterns maintaining their viability and phenotypic expression through the culture time, compared to HMSC and HDMEC monocultures. HMSC differentiation seems to be enhanced in co-culture conditions, with an up-regulation genes osteogenesis-related genes, and ALP activity. Furthermore, the presence of calcium phosphate deposits was also confirmed. This work reports in detail the interactions between HMSC and HDMEC in a long-term co-culture 2D system. Endothelial and mesenchymal stem cells cultured in the present co-culture conditions ensured the proliferation and phenotype differentiation of cell types, stimulating osteogenesis and the over-expression of angiogenesis-related genes on the same culture system. It is believed that the present work can lead to significant developments for bone tissue regeneration and cell biology studies.

KEYWORDS: Human mesenchymal stem cells, Human dermal microvascular endothelial cells, long term co-culture, reciprocal interaction, bone regeneration, Angiogenesis.

INTRODUCTION

Different approaches as been proposed and developed as strategies to achieve successful bone regeneration, such as growth factors delivery systems and pre-seeded co-cultured cells in biomaterials/scaffolds [1-8]. These strategies must allow a rapid neo-vascularization and bone cell growth with a higher cell proliferation [1, 9]. The lack of a functional microvasculature is associated with insufficient supply of oxygen, nutrients and inappropriate metabolic waste products removal from the porous large size implants inner parts, leading to hypoxia, poor cell survival and death [4, 5, 10-12]. Moreover, an adequate vascular network allows continuous availability of precursor cells and biological mediators involved in cell-to-cell communication [4, 11, 13, 14]. Furthermore, it is well known that angiogenesis and osteogenesis are intimately associated, where the formation of a functional vascular network precedes osteogenesis, being this new vasculature the “point-of-reference” for Harversian bone formation [13-15]. Consequently, for bone regeneration strategies, it is important to understand the interactions between endothelial cells/osteoblasts and mesenchymal stem cells [4]. MSC differ from osteoblasts, because they are multipotent adult cells that have the ability to self-renew and the capability of differentiation into a wide range of cell type (such as osteogenic, adipogenic and chondrogenic lineages) and can be modulate by the signals of the local environment that affect their differentiation [16, 17]. MSCs can be isolated from different sources such as bone marrow, umbilical cord, adipose tissue, skin and amniotic fluid. In the present work, bone marrow-derived mesenchymal stem cells were used, since good results have been achieved in clinic using this cell type for bone, cartilage and spinal cord regeneration [18, 19].

Several co-culture systems have been developed expecting to recreate more closely the *in vivo* environment as compared to single-cell cultures.

Co-culture studies involve mostly short-time protocols and have been assessed with osteoblastic lineages [20-22], human primary osteoblastic cells [7, 23, 24] or osteoprogenitors [25-27] co-cultured with endothelial cells isolated from macro-vascularization [28, 29]. Macrovascular endothelial cells are easier to isolate and maintain in culture compared to microvascular endothelial cells that need a more intensive and time-consuming labour [20]. However, it is worth to notice that inflammatory and angiogenic response following biomaterial applications involves

predominantly microvascular endothelial cells [29-31]. Regarding this, *Lang et al* showed evidences that support phenotypic and physiologic heterogeneity between microvascular and macrovascular endothelial cells [20]. Furthermore, only a few studies were done with cells not committed to osteoblastic phenotype, like human mesenchymal stem cells. In addition, co-culture studies have mainly addressed the influence of endothelial cells on osteoblast proliferation and differentiation [5, 12, 22, 31]. However, the reciprocal situation, i.e. the effect of the osteoblast presence in endothelial cell behaviour, has received less consideration.

In this context, the aim of the present study was to develop *in vitro* culture conditions in which human dermal microvascular endothelial cells (HDMEC) and human mesenchymal stem cells (HMSC) viability, morphology and phenotype gene expression were ensured. For this, the time-dependent interactions between HDMEC and HMSC in a long-term 2D co-culture system are reported in detail, to further apply this strategy for bone regeneration.

MATERIALS AND METHODS

Cell culture

Human dermal microvascular endothelial cells. HDMEC were purchased to Sciencell and were cultured in cell culture plates with endothelial (EC) culture basal medium (Sciencell) containing 5% of foetal bovine serum (FBS; Sciencell), 10 units/mL of Penicillin, 10 µg/mL of Streptomycin (P/S solution; Sciencell) and endothelial cell growth supplement (ECGS; Sciencell). Incubation was carried out in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Human mesenchymal stem cells. Human bone marrow was obtained from orthopaedic surgery procedures, with patient informed consent. Cell suspension was cultured in cell culture plates with minimum essential medium Eagle, alpha modification (α-MEM; Sigma) containing 10% foetal bovine serum (FBS;Sigma), 100µg/mL penicillin (Sigma) and 10 units/mL streptomycin (Sigma). Incubation was carried out in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cells from passage 3.were used. HMSC characterized by flow cytometry were positive for the markers CD105, CD73 and CD90 and did not expressed the markers CD45, CD34,CD14, CD19 and CD31.

Co-culture of HDMEC and HMSC. HDMEC and HMSC, arising from the third subculture, were co-cultured at a cell density of 8×10^3 cells/cm² HDMEC and 2×10^3 cells/cm² HMSC, for a final concentration of 10^4 cells/cm². The medium used was a mixture (50:50) of EC culture medium and HMSC culture medium. Cultures were maintained for 35 days in cell culture plates. Monocultures of HDMEC and HMSC, maintained in the same experimental conditions, were used as control. HDMEC and HMSC monocultures were seeded at 10^4 cells/cm², in order to have the same final cell concentration as that used in the co-culture. Monocultures and co-cultures were characterized throughout the incubation time, as follows.

Cell viability/proliferation.

MTT assay

Mitochondrial dehydrogenase activity of cells was determined by using the substrate 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma), which was reduced to a formazan reaction product that was accumulated in the cytoplasm of viable cells. Ten μ L of MTT solution (5 mg/mL) were added to 96-well culture dishes containing 100 μ L of medium and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂ for 3 h. Formazan salts were dissolved with 200 μ L of dimethylsulphoxide and the absorbance was measured at 550 nm on ELISA reader (Synergy HT, Bio tek) [11]. MTT assay was performed at days 7, 14, 21 28 and 35.

DNA assay

Cell proliferation was evaluated using the PicoGreen DNA quantification assay (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes Inc., Eugene), at 7, 14, 21, 28 and 35 days. Cells were treated with Triton X-100 (0.1%) (Sigma). After adding the PicoGreen reagent to samples, fluorescence was then measured with a plate reader (Synergy HT, Bio tek) at excitation and emission wavelengths of 480 and 520 nm, respectively, and corrected for fluorescence of reagent blanks. The amount of DNA was calculated by extrapolating a standard curve obtained by running the assay with the given DNA standard.

Calcein-AM assay

Cells were incubated with 0.1 μ M calcein-acetoxymethylester (Calcein-AM; Molecular Probes) for 30 min at 37°C. Calcein-AM was converted by viable cells intracellular esterases into membrane-impermeable fluorescent calcein that spread throughout the cell cytoplasm. Fluorescence was visualized by Confocal Laser Scanning Microscopy (CLSM; Leica TCP SP2 AOBS) [11]. Calcein-AM assay was conducted at days 7, 14, 21 28 and 35.

Immunostaining of F-actin cytoskeleton, CD31 and nucleus

Cells were fixed with 4% formaldehyde (methanol free, Sigma) for 15 min, permeabilized with 0.1% triton for 5 min and then incubated in 1% bovine serum albumin (BSA)/PBS for 1h.

Monocultures of HMSC were stained for F-actin, with Alexa-Fluor-conjugated phalloidin (Alexa Fluor® 488 Phalloidin: Molecular Probes) diluted 1:100 in 1% BSA/PBS for 60 min. Nuclei were stained with 10 μ g/mL propidium iodide (Sigma) diluted in PBS for 10 min.

HDMEC and co-cultures were incubated with primary CD31 antibody (PECAM-1 (P2B1) sc-20071; Santa Cruz Biotechnology) diluted 1:100 in 1% BSA/PBS for 45 min and then labelled with the secondary antibody anti-mouse Alexa-Fluor (Alexa Fluor 488 goat anti-mouse IgG1 (Y1); Molecular Probes) diluted 1:1000 in 1% BSA/PBS for 45 min and after that counterstained with 10 μ g/mL propidium iodide (Sigma) for 10 min. Samples were washed with PBS and covered with Vectashield (Vector Laboratories) [11]. CLSM images were acquired on a Leica TCP SP2 AOBS, at days 7, 14 and 21.

Alkaline phosphatase activity and histochemical staining

Cell cultures were washed with PBS and with a cell lysis buffer containing 0.1% Triton X-100 (Sigma). The samples were frozen at -20°C and later thawed at 37°C for 5 min in order to measure alkaline phosphatase (ALP) activity. The enzyme activity, measured at days 7, 14 and 21, was assayed by the substrate hydrolysis (p-nitrophenyl phosphate) (Sigma) in alkaline buffer solution (2-amino-2-methyl-1-propanol) (Sigma), at pH 10.5. After 1h incubation at 37°C, NaOH (1M) was added to stop the reaction

and hydrolysis product (p-nitrophenol) was measured at 405nm using an ELISA reader (Synergy HT, Bio tek) [32].

For histochemical staining, fixed cultures (1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, 10 min) were incubated in the dark for 1h at 37°C in Na-l-naphtyl phosphatase/Fast blue salt (Sigma). After being rinsed with deionized water and air-dried, samples were observed under a stereo microscope (model SZX10, Olympus) [33].

Histochemical staining of calcium deposition

For calcium staining, the fixed cultures (1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, 10 min) were covered with a 1 % 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)[33]. Calcium deposits stained red. The assay was performed at days 7, 14 and 21. Samples were observed under a stereo microscope (model SZX10, Olympus).

Scanning electron microscopy

For SEM observation, fixed cultures were dehydrated in graded series of alcohols and critical point dried. Samples were attached to carbon tape in an aluminium support, coated with gold about 13 min, and observed in a FEI Quanta 400FEG ESEM / EDAX Genesis X4M scanning electron microscope. Samples were collected for days 7, 14 and 21.

Flow cytometry - Cell sorting

Fluorescence-activated cell sorting (FACS) is a specialized method type of flow cytometry that allows the separation of different cell types. It is applied for automatic sorting of heterogeneous cell mixtures depending on the fluorescent characteristics of individual cells, what is usually achieved via the staining of different cell populations with fluorescently labelled antibodies against specific surface protein, in this case FITC-conjugated anti-human CD31 antibody [34]. HDMEC, HMSC and co-cultured cells were detached with trypsin at different time-points, days 7, 14 and 21. Single cell were suspended at a density of 10^5 – 10^7 cells/mL and stained with FITC-conjugated antibody

Anti-Human CD31 (PECAM-1) (BD Biosciences), and then washed and resuspended in a final volume of 250 μ l. Using a BD FACSAria™ II system, CD31-positive (labeled cells, CD31+) and negative (CD31-) populations were gated and sorted into different eppendorfs. HDMEC and HMSC monoculture were used as control, to confirm or not CD31 expression, and to evaluate the auto-fluorescence. The population of HDMEC cells from the co-culture (cHDMEC) was gated in the CD31+ positive cell quadrant, sorted in the exclusion mode, and collected into eppendorfs. The population of HMSC cells from the co-culture (cHMSC) was gated in the CD31- negative cell quadrant, also sorted in the exclusion mode, and collected into different eppendorfs[34]. The number of sorted events was 50,000 per sample. Data processing was performed using FlowJo software 8.7.

Real Time Gene expression by Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR amplification was done for HDMEC and HMSC monocultures and, also, for cHDMEC and cHMSC (populations sorted by FACS from the co-culture) at 7, 14 and 21 days. The following genes were assessed: COL 1, ALP, BMP2, ANGPT2, ANGPT1, VEGF-165, RUNX2, vWF and VE-cadherin. Total RNA was extracted using the NucleoSpin® RNA II Kit (Macherey-Nagel) according to the manufacturer's instructions. The isolated RNA was dissolved in RNase-free water, and the final concentration of RNA was determined by a NanoDrop 1000 instrument (NanoDrop Technologies. NanoDrop 3.0.1 software, Coleman Technologies, Inc.).

The isolated RNA was reverse transcribed with SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen) into cDNA. The protocol was provided by the supplier. cDNA (1 μ l) was loaded in a 96-well plate and the components of SYBR-Green Supermix (iQTM, BioRad, USA) were added according to the manufacturer's instructions. The adopted primers conditions are summarized in Table I. Data was analyzed with the iCycler IQTM software and gene expression was quantified by calculating $2^{-\Delta Ct}$ values and $\Delta Ct = (Ct, \text{reference gene} - Ct, \text{target gene})$ [35].

Table I - Primers for PCR Amplification

Gene	GeneBank	Primers sequences	TM (°C)
GAPDH	NM_00246	Forward 5'-TAACTCTGGTAAAGTGGATATTG-3' Reverse 5'-GAAGATGGTGATGGGATTTC-3'	58
COL1	NM_000088	Forward 5'-GGAATGAGGAGACTCGCAACC-3' Reverse 5'-TCAGCACCACCGATGTCCAAA-3'	58
BMP2	NM_001200	Forward 5'-ATGAAGAATCTTTGGAAGAACTAC-3' Reverse 5'-GGTGATGGAAACTGCTATTG-3'	58
vWF	NM_000552	Forward 5'-AAGAAAATAACACAGGTGAA-3' Reverse 5'-TACTCTCCTCTCTCATTGAC-3'	58
ALP	NM_001177530	Forward 5'-AGCCCTTCACTGCCATCCTGT-3' Reverse 5'-ATTCTCTCGTTCACCGCCAC-3'	58
RUNX2	NM_001024630	Forward 5'-GGGTAACGATGAAAATTATTCT-3' Reverse 5'-TTAATTGCTCTGTGATAGGTA-3'	58
ANGPT1	NM_001199859	Forward 5'-ACCGAGCCTATTCACAGTAT-3' Reverse 5'-ACAGTTGTCATTATCAGCATCTT-3'	58
ANGPT2	NM_001118888	Forward 5'-GTGATTAGACAGAACACCTATGC-3' Reverse 5'-AACAGTGCTCAGAAGAATGC-3'	58
VE-cadherin	NM_001795	Forward 5'-GCAATAGACAAGGACATAACA-3' Reverse 5'-TAGGAAGTGGACCTTGGTAT-3'	58
VEGF-165	AB021221	Forward 5'-TATGCGGATCAAACCTCACCA-3' Reverse 5'-CACAGGGATTTTCTTGTCTTGCT-3'	58

Matrigel tube-like formation assay

Matrigel (Sigma) was diluted with culture cell medium, in the proportion 1:1 to cover 24-well plates bottom. After 1h at 37°C, cells were seeded at a final concentration of 1×10^4 cells/cm² (HDMEC). Phase contrast images were taken under an inverted microscope (Axiovert 200M, Zeiss) after 3 days of culture.

Statistical analysis

Triplicate experiments were performed. The results were expressed as the arithmetic mean $\pm$ standard deviation (SD). Analysis of results was carried out using IBM SPSS Statistics 19 and statistical analysis was assessed using the one way ANOVA, with a significance level of $p < 0.05$.

RESULTS

Cell viability/proliferation and pattern of cell growth*MTT assay and DNA quantification*

Data from the MTT assay (Figure 1A) showed that cell viability of HDMEC and HMSC cultures increased until day 21, decreasing slightly afterwards. HDMEC presented lower values compared to HMSC. Co-cultures showed a similar time-dependent pattern and values were similar to those found in HMSC, but at day 21 values were significantly higher.

Total DNA quantification assay, Figure 1B, provided similar information. HDMEC, HMSC and co-cultured cells proliferated during the first three weeks of culture, reaching a maximum peak at day 21, decreasing afterwards to day 28. HDMEC showed the lowest proliferation values, since less DNA was detected in all time points compared to HMSC monoculture and co-culture. Co-cultures presented similar values compared to HMSC, but a higher proliferation at day 21. It seems HMSC and co-culture cells presented a higher proliferation rate.

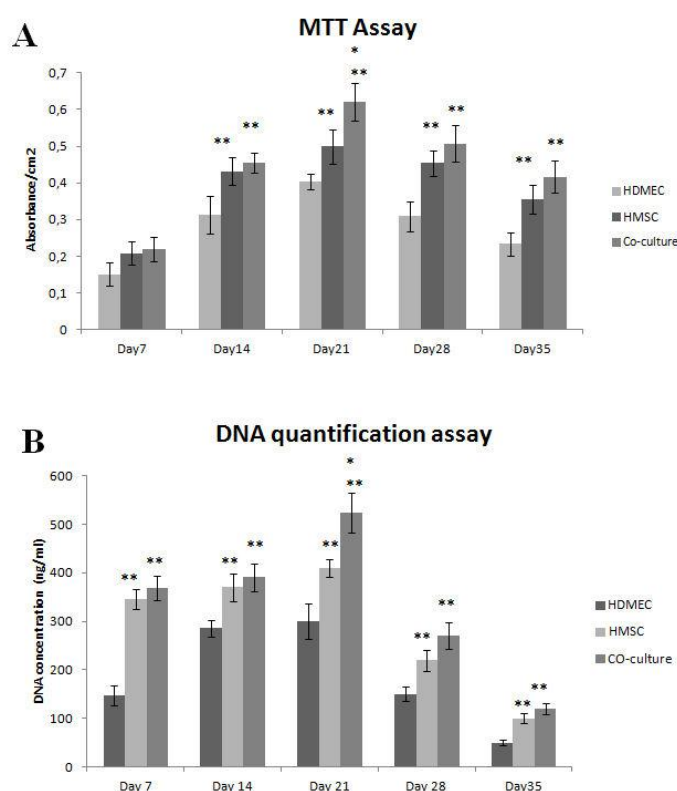


Figure 1 – Cell viability and proliferation of monocultured and co-cultured HMSC and HDMEC estimated by: A) MTT assay and B) DNA quantification. Assays were performed

after 7, 14, 21, 28 and 35 days of culture. Values reported are the mean ($\pm$ SD) [* , significantly different from HMSC, for the same culture time; ** significantly different from HDMEC, for the same culture time ($p < 0.05$; $n=3$)]

Calcein-AM staining

Calcein-AM staining (Figure 2) indicated that HMSC and HDMEC maintained their viability throughout the culture time, both in monocultures and co-culture. In addition, the two cell types exhibited their normal morphology, i.e. an elongated appearance for HMSC and a smaller and rounded shape for HDMEC, and presented extensive cell-to-cell contact. Cells were able to spread and progressively covered the well surface showing a typical cell growth pattern, i.e. the formation of a parallel orientation in HMSC cultures and a tendency for a circular orientation in HDMEC cultures. In the co-cultures, the two cell types were easily identified based on their size and morphology. HDMEC maintained the typical circular orientation and HMSC appeared mainly located around the HDMEC clusters. At day 28, the arrangements were getting bigger, and the pictures showed just the frontiers between the two cell types. At day 35, a decrease in the cell viability was noticed, which is in agreement with the results of the MTT assay.

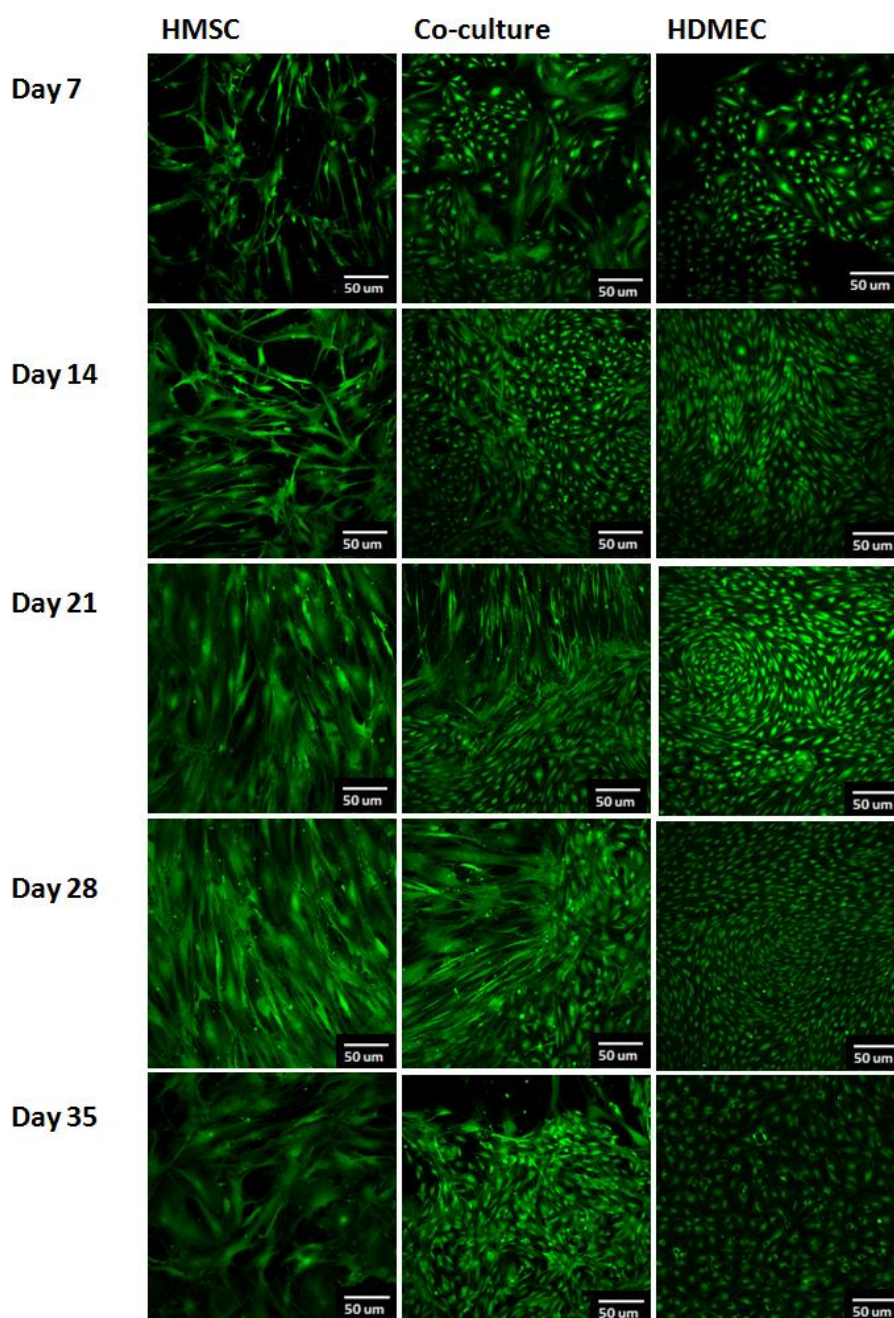


Figure 2 - Confocal laser scanning microscopy (CLSM) images of monocultured and co-cultured HMSC and HDMEC showing cell viability. Cells were incubated with Calcein AM and viable cells present a green fluorescence. Images were collected after 7, 14, 21, 28 and 35 days of culture.

Immunostaining of F-actin, CD31 and nucleus

HMSC cultures were stained for F-actin cytoskeleton and nucleus. At day 7, cells showed an elongated morphology with well defined nuclei and cell-to-cell contacts. Cells adopted an apparently random growth pattern converted to a fibroblast-like

morphology growth pattern at later time points. HDMEC cultures, stained intensively for CD31 and nucleus, showed an evident organization in cell clusters that grew with the culture time. Similarly to what had been suggested by the Calcein-AM staining, a circular growth pattern was identified within these cell clusters. Also, co-cultures, stained for CD31 and nucleus, showed that HDMEC grew in tight clusters being surrounded by HMSC. Figure 3 shows representative CLSM images.

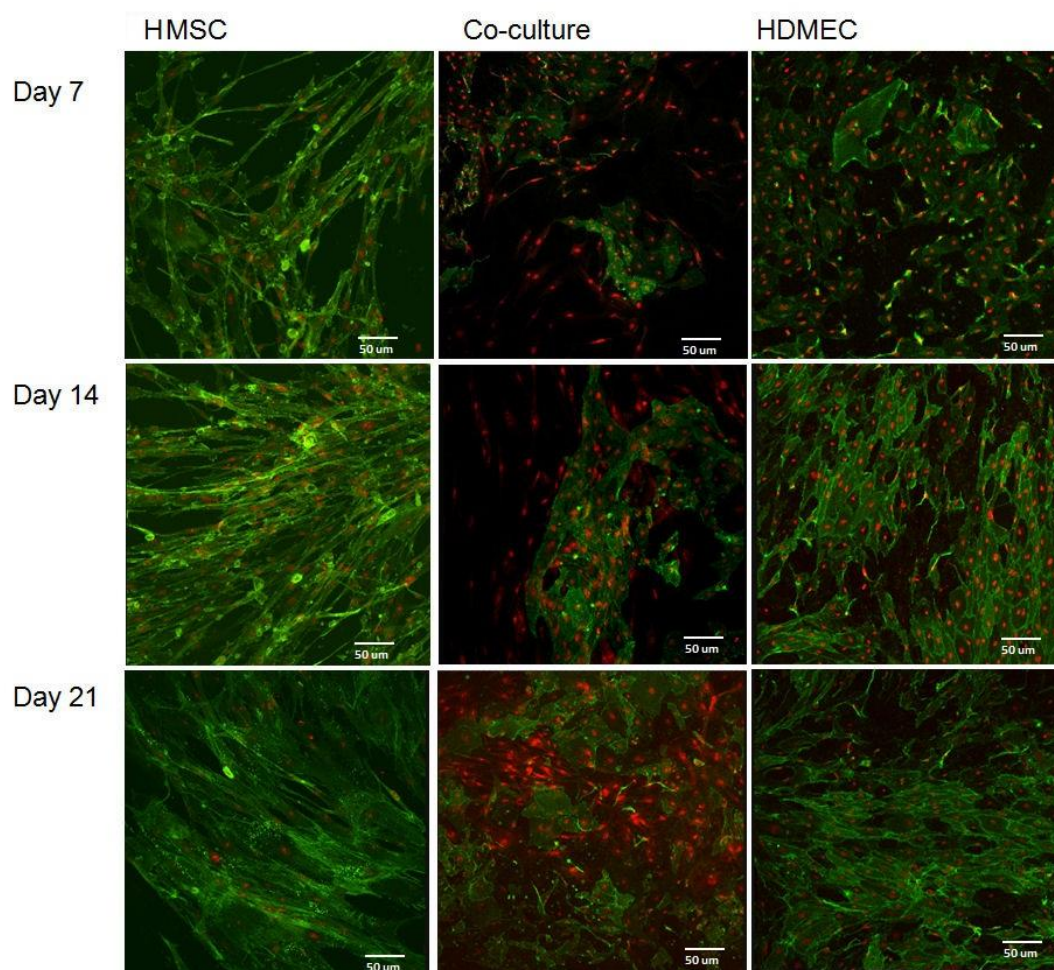


Figure 3 - Confocal laser scanning microscopy (CLSM) images of monocultured and co-cultured HMSC and HDMEC showing cell growth patterns and CD31 expression in cHDMEC and HDMEC. Monocultures of HMSC were stained for F-actin [36] and nuclei with propidium iodide (red). HDMEC and co-cultures were stained for CD31 [36] and nuclei with propidium iodide (red). Images were collected after 7, 14 and 21 days of culture.

ALP activity

Figure 4A refers to ALP activity, an established osteoblastic parameter, measured in isolated and co-cultured HDMEC and HMSC cultures. Enzymatic activity was assessed for 21 days, as MTT and Calcein-AM assays showed some deterioration of the cultures during the last week. ALP was detected in HDMEC, although in very low values. In HMSC cultures, ALP was synthesized during the culture time, but increased significantly between days 14 and 21. Comparatively, co-cultures presented higher ALP activity at days 14 and 21. Histochemical staining (Figure 4B) revealed the presence of ALP positive HDMEC and HMSC in monoculture and co-culture, for all time points of culture (days 7, 14 and 21). HDMEC monocultures presented a weak positive reaction. Regarding HMSC monoculture, more positive cells were observed with culture time. However a higher amount of positive cells was detected in co-cultured cells compared to HMSC monoculture, especially at day 21. These results are in agreement with those described previously for ALP activity.

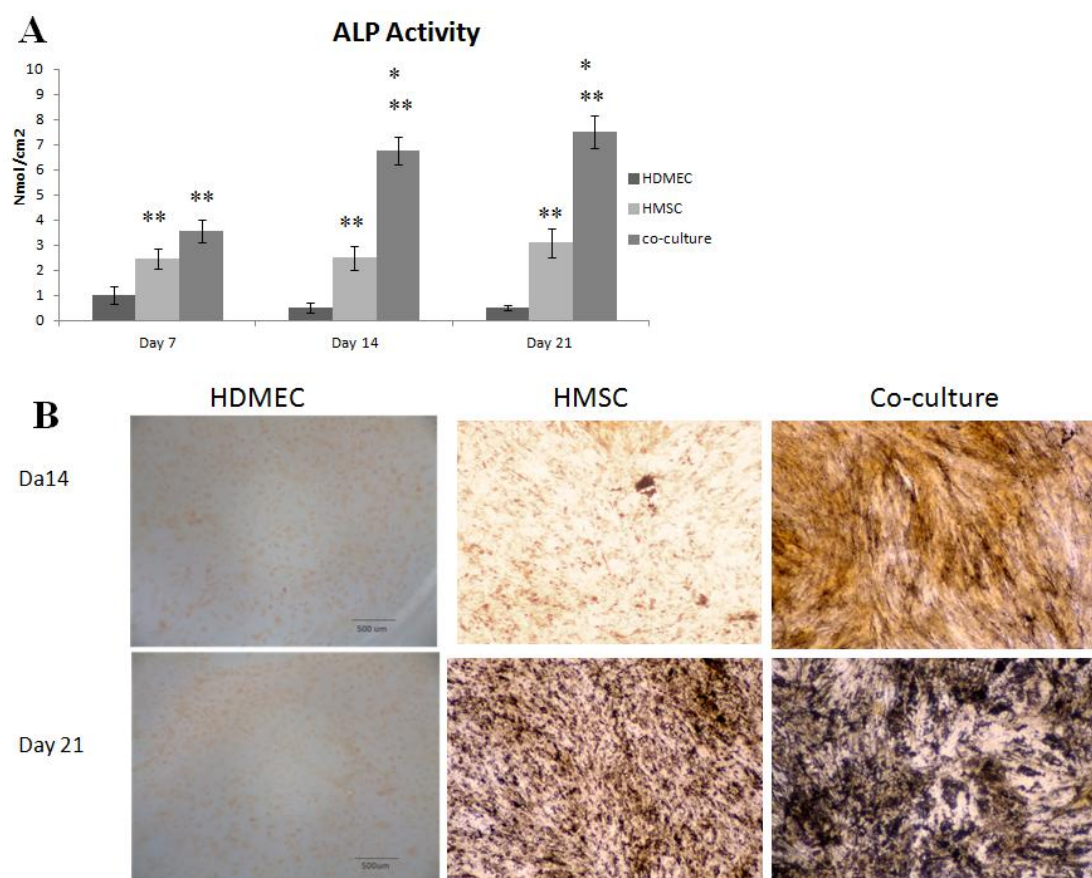


Figure 4 – Alkaline phosphatase assays: A) ALP activity, B) ALP histochemical staining of monocultured and co-cultured HMSC and HDMEC. Values reported are the mean ($\pm$ SD) and assays were performed after 7, 14 and 21 days of culture [ALP activity - *, significantly different from HMSC, for the same culture time; ** significantly different from HDMEC, for the same culture time; ($p < 0.05$; $n = 3$)].

Matrix mineralization

Alizarin red staining for the presence of calcium deposits was positive for co-cultured cells. A very intense colour was detected in the later days of culture Figure 5A. SEM observation of 21-day co-culture revealed an abundant cell layer and the presence of closely associated mineralized structures, showing the presence of Ca and P peaks on X-ray analysis, Figure 5B.

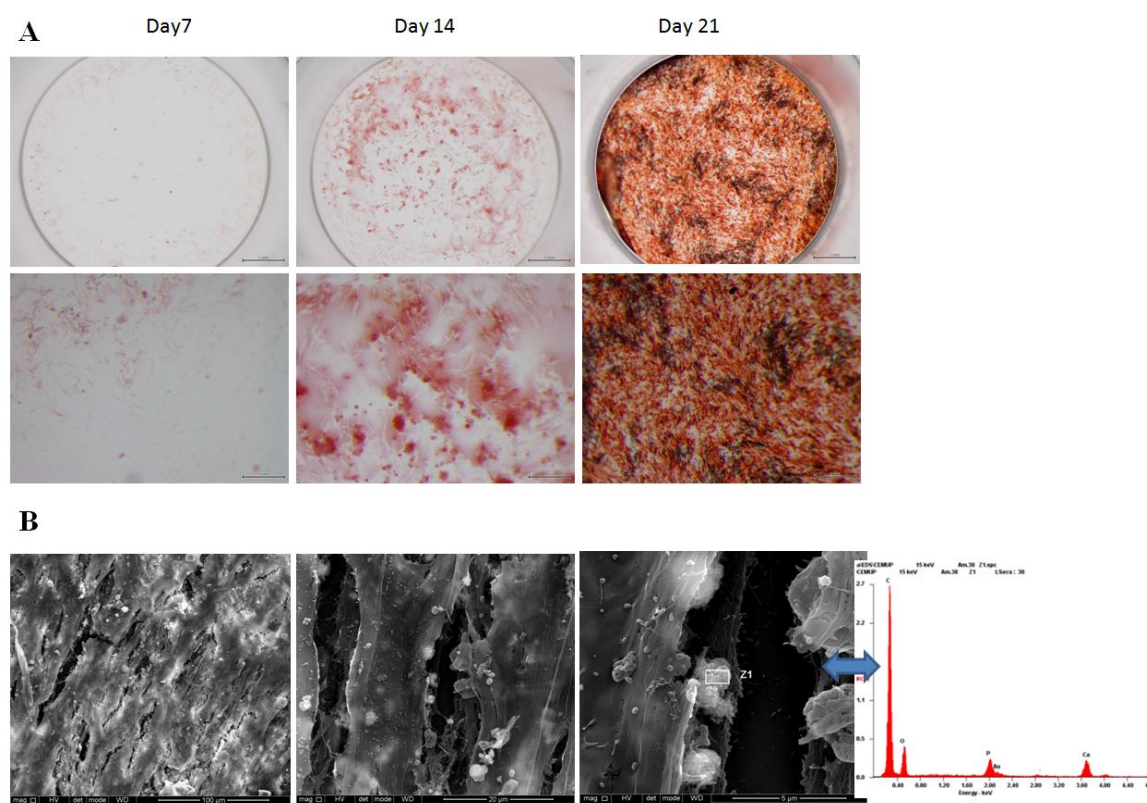


Figure 5 – Matrix mineralization. A) Alizarin red histochemical staining performed in co-cultured cells, at 7, 14 and 21 days. B) SEM images of HMSC and HDMEC co-cultured after 21days. Mineralized globular structures were identified in close association with cell layers, and EDS spectrum showed the presence of Ca and P peaks.

Osteoblasts and endothelial gene expression

Figure 6 shows the results regarding the RT-PCR analyses performed in HMSC and HDMEC monocultures and cHMSC and cHDMEC (the populations sorted from the co-cultures) for the time-dependent expression of phenotype markers. Gene expression was evaluated throughout 21 days. The MTT assay and DNA quantification showed the higher values at day 21, reflecting a high viability of cell cultures, also confirmed by the calcein-AM staining. Cultures were assessed for COL 1, ALP and RUNX2, as genes typically related with osteoblastic differentiation, and ANGPT1 and ANGPT2, angiogenesis factors that modulate endothelial cell differentiation, survival and stability. Two important growth factors, BMP2 and VEGF-165, were also assessed, as well as vWF and VE-cadherin that are important endothelial markers.

HMSC and cHMSC expressed COL 1, RUNX2, ALP, BMP2, ANGPT1 and VEGF-165 in a time-dependent manner. In HMSC, expression of COL 1 and ANGPT1 increased

until day 14 and decreased afterwards, whereas expression of ALP also increased until day 14 but was approximately constant afterwards. Expression of RUNX2 was similar at days 7 and 14 and decreased at day 21. The higher level of expression for BMP2 was observed at day 7, decreasing afterwards and reaching similar levels at days 14 and 21. Maximum levels of VEGF-165 were also attained at day 7, decreasing significantly between days 7 and 14, and increasing slightly afterwards. ANGPT2 was also expressed by these cells, but in very low levels. Comparatively, cHMSC showed significantly higher levels in the gene expression profile of COL 1 (at all time-points), RUNX2 (day 14), ALP (especially, day 21), BMP2 (days 7 and 14), ANGPT1 (all time-points) and VEGF-165 (days 14 and 21).

HDMEC and cHDMEC expressed the endothelial genes markers such as vWF and VE-cadherin with a distinct time-dependent profile. In HDMEC, a high level expression of vWF was observed throughout the culture time, increasing slightly after day 7 and with similar values at days 14 and 21. VE-cadherin expression was low at day 7, but increased until day 14, being approximately constant afterwards. In addition, HDMEC cultures also expressed ANGPT2 and BMP2. ANGPT2 expression levels were similar at days 7 and 14 and higher at day 21. Expression level of BMP2 was higher at day 7 and decreased slightly afterwards. However, levels were lower than those measured on HMSC. VEGF-165 was barely detected. Comparatively, cHDMEC presented higher expression levels for vWF (days 7 and 14, although without statistical significance), VE-cadherin (day 21) and ANGPT2 (days 7 and 14, and maximum levels were attained earlier).

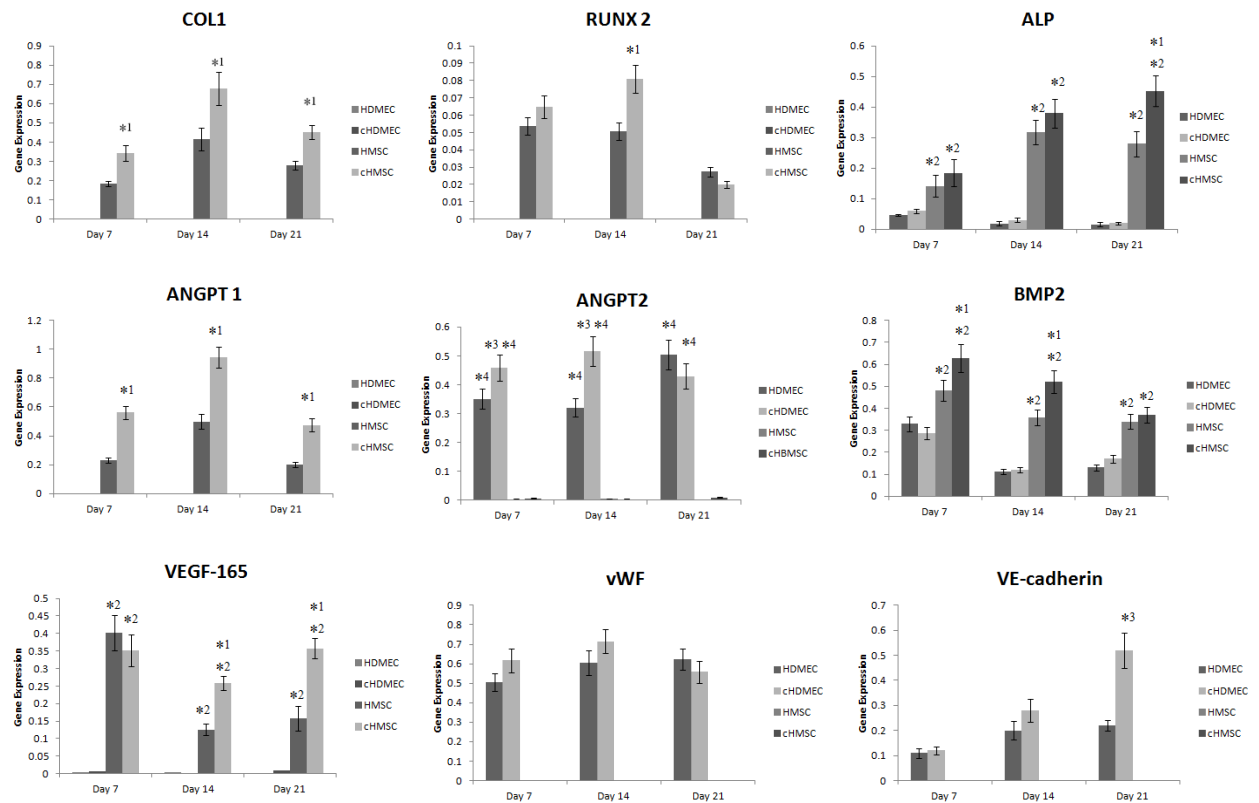


Figure 6 – Real time PCR analyses performed in HMSC and HDMEC monocultures, cHMSC and cHDMEC (the populations sorted from the co-cultures) for gene expression related with osteoblastic and endothelial markers. Values reported are the mean ($\pm$ SD) and assay was performed after 7, 14 and 21days of culture [$*^1$ significantly different from HMSC, for the same culture time; $*^2$ significantly different from HDMEC and cHDMEC, for the same culture time; $*^3$ significantly different from HDMEC, for the same culture time; $*^4$ significantly different from HMSC and cHMSC, for the same culture time ($p < 0.05$; $n = 3$)].

Matrigel tube-like formation assay

HDMEC were able to form a network of capillary-like tubes just after 3 days of culture (Figure 7).

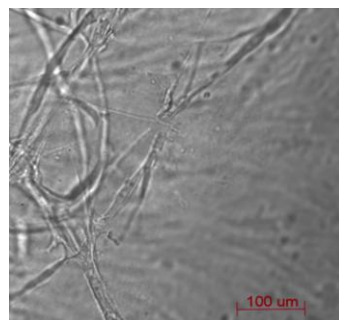


Figure 7 – Capillary-like tubes formed by HDMEC after 3 days of culture in Matrigel.

DISCUSSION

Taking into account the available literature on co-culture systems of endothelial and osteoblast cells, there is no consensus on the optimal conditions for this type of co-culture. Reports described different experimental conditions regarding cell ratio and culture medium. Furthermore, most studies compared co-cultures to monocultures in terms of endothelial or osteoblastic parameters, but none focused on both.

This work proposed an *in vitro* cell culture model system to ensure HDMEC and HMSC co-culture viability, characteristic morphology and phenotype gene expression. This optimized cell culture model system can be used for studying the reciprocal regulation and functional interaction between these two cell types and cell-substrate interactions regarding both osteogenic and angiogenic outcome parameters.

In order to guarantee a continuous cell contact and interaction, the initial ratio of seeded cells (4:1, respectively HDMEC:HMSC) was decided, based on the proliferation rate of each cell type in the present culturing conditions and, also, in flow cytometry studies showing that at this proportion the presence of HDMEC was guaranteed even after 21 days of culture, without affecting the characteristic morphology and phenotype gene expression of each cell type. Cell proportions inferior to 4:1 resulted in overgrowth of HMSC with the disappearance of HDMEC only after 14 days of culture. Furthermore, these results are in accordance with the literature, where *Unger et al* [3] co-cultured HDMEC isolated from juvenile foreskin with MG-63 and human osteoblast cells in endothelial medium, showing that ratios between 5:1 and 10:1, respectively of HDMEC:MG-63 (or primary osteoblasts) resulted in the presence of both cell types after 1 week on cell culture, whereas with other cell ratios no endothelial cells were present after this culture time. Also, *Zang et al* [34] observed that initial rates were not constant during the culture time and changes occurred in the proportions of MG-63 cells and HUVECs cultured together in endothelial medium. Over time, the ratio of MG-63 and HUVECs changed from 1:5 to 1:1 after 5 days of culture. However, the aforementioned studies were done for short time periods, whereas, in the present study, cultures were maintained for 35 days. Furthermore, none of the studies were done with HMSC and HDMEC.

Regarding the choice of cell culture medium, it is difficult to define a common medium for co-cultures, in which all cell types are able to grow. However, in this work, HDMEC were the more sensitive cell type, the ones that required more demands for survival. So, a supplemented endothelial medium should be used to sustain HDMEC viability especially in monoculture, since one of the aims of this work was to compare HDMEC in monoculture with co-cultured HDMECs. The medium used was a mixture (50:50) of EC culture medium (from Sciencell supplemented with ECGS) and HMSC culture medium (α -MEM containing 10% FBS).

Results of MTT assay showed that viability/proliferation of HDMEC and HMSC followed a similar profile, i.e. increased until day 21 and started to decrease at day 28, which was in agreement with DNA quantification and CLSM observations; in addition, HDMSC cells exhibited for all time-points lower values in the MTT and DNA assays, as expected regarding their lower proliferation ratio. Regarding the co-culture, significantly higher values were observed at day 21 compared to monocultures. As the cell plating density was similar in monocultures and co-culture, results suggest that co-cultured cells achieved higher proliferation rates, at least after 3 weeks of culture time. This might be related to cell interactions involving direct cell-to-cell contact or paracrine communication via soluble factors that are known to modulate cell growth [15, 28].

Concerning cell growth patterns, HMSC cultures adopted a fibroblast-like growth and HDMEC cultures showed a circular orientation, as previously described for these cell types [11, 20]. Regarding the co-cultures, HDMEC formed clusters and HMSC organized themselves around these groupments, suggesting that their growth was conditioned by the endothelial cell groups. This organization might possibly reflect or be related with the bone structure where the mineralized collagen fibres are arranged in concentric rings around blood vessels. Furthermore, *in vivo*, the formation of a first microvascular network is required for osteogenesis to occur [20].

HMSC and cHMSC expressed genes typically related to osteoblast differentiation, such as COL 1, ALP and RUNX2. Also ANGPT1, an angiogenic factor that modulate endothelial cell differentiation, was expressed by these cells. BMP2 was also expressed by HMSC and HDMEC, in monoculture and co-culture. HDMEC cHDMEC expressed vWF and VE-cadherin that are important endothelial markers. These results showed that osteoblastic and endothelial phenotypes were achieved and maintained

in monoculture conditions. However, the genetic profile of cHMSC and cHDMEC, was distinct from that observed in the respective control monoculture, concerning the time-dependent expression and, in addition, most of the genes were over-expressed in co-culture conditions.

Collagen type 1 is the most abundant extracellular bone protein, also considered an early bone differentiation marker and an imperative pro-angiogenic substrate being important to drive endothelial migration and proliferation [6]. HMSC cultures, expressed high COL 1 gene levels and, comparatively, in cHMSC, COL 1 gene was significantly over-expressed for all culture time-points. These results suggest that expression of COL 1 was highly stimulated in the presence of HDMEC from early co-culture periods. This agrees to previous studies performed in co-cultures with different type of cells where collagen type 1 gene was up-regulated only after 21 days of co-culture, single time-point analysed [6].

ALP gene expression represents a frequently used early marker for osteogenic differentiation [37]. In the present study, HMSC cultures expressed ALP gene throughout the culture time, and ALP activity showed a similar profile. HDMEC and cHDMEC also express ALP, although at low levels. Comparatively, cHMSC presented higher levels of ALP expression and ALP activity, indicating an enhanced osteogenic differentiation. ALP staining of the co-culture also showed an increased intensity when compared to HMSC monoculture. These observations are consistent with previous studies done with different cell types and cell culture medium [34, 37].

RUNX2 is an earlier marker for osteogenic differentiation, and higher levels were found until day 14, followed by a decrease, suggesting an early commitment of HMSC to the osteoblast phenotype. This gene was significantly over-expressed in cHMSC.

Bone Morphogenetic Proteins (BMPs) induce osteoblast differentiation [38]. In the present work, HMSC monoculture presented high levels of BMP2 expression, especially in the first week. This is in accordance with a previous study done by *Stiehler et al* which showed a decreasing expression level of BMP2 with culture time, under static conditions for 21 days, using an osteogenic medium [39]. In cHMSC, BMP2 expression was greatly induced at days 7 and 14. BMP2 was also detected on HDMEC with a time-dependent pattern similar to that of HMSC, although with significantly

lower levels. Expression of this gene was similar in cHDMEC. Also *Bouletreau et al* showed that BMPs can be expressed by bovine capillary endothelial cells and pointed out that BMP2 expression can be up-regulated under hypoxia or exogenous VEGF conditions [36], playing an angiogenic role at the fracture site. Furthermore, *Deckers et al* demonstrated that BMP2 stimulate angiogenesis through the production of VEGF by osteoblasts [40]. Although just a few works in the literature studied the expression of BMP2 by endothelial cells, the results of the present work showed a significant BMP2 expression by both HMSC and HDMEC monocultures and in co-culture conditions.

Vascular endothelial growth factor (VEGF) is expressed by osteoblasts cells and is involved in the regulation of endothelial differentiation, proliferation, migration, and formation of functional vessels [41]. In the present work, VEGF-165 was expressed by HMSC and over-expressed in cHMSC, suggesting an important role for osteoblasts in the reciprocal interactions of both cell types. A previous study had also shown that VEGF was expressed by cHMSC, although the culture time studied held just a few hours [42]. VEGF expression was also detected in HDMEC and cHDMEC, although in very lower levels.

Several works in the literature studied the complex bidirectional communications between bone marrow stromal cells or mesenchymal stem cells and endothelial cells, to understand the ways they interact and the role of endothelial cells promoting osteogenic differentiation [8, 26-28, 31]. *Greillier et al* [28] proposed three different types of major ways of communication between these cells, such as direct cell-to-cell mechanisms (gap junction communications; adherens and tight junctions communications) and the secretion of diffusible factors that activate specific receptors on the target cells. The gap junctions are composed of aggregations of membrane channels, called connexons, providing direct cytoplasmic connections between adjacent cells [43]. *Villar et al* [37] showed that a functional inhibition of the gap junction channel Cx43 which is expressed by HUVEC and HBMSC decreased the effect of endothelial cells on HBMSC differentiation (ALP and COL 1 expression). Also, *Li et al* [44] showed co-cultured HBMSC with HUVEC (human umbilical vein endothelial cells) expressed higher neural-cadherin (N-cadherin) compared to monocultured cells. Furthermore, a neutralization of N-cadherin led to a down-regulation of ALP and COL 1 gene expression. In normal conditions (without N-cadherin neutralization)

earlier osteoblastic differentiation of HBMSC was achieved when co-cultured with HUVEC. Taking into account these studies, it seems that a direct contact co-culture is important to promote HMSC or HBMSC cells differentiation. Cell-to-cell contacts can be promoted in 2D surface or in a 3D *in vitro* model. 3D scaffolds with [12] and without dynamic conditions [45] and 3D spheroid co-culture systems [17, 46] can be used. Regarding the diffusible factors, several factors have been pointed as determinant in the interaction between these cells, such as BMP2, FGF, PDGF, TGF- β , VEGF and IGF [28]. Saleh *et al* [46] studied the effects of paracrine factors produced by HUVEC on MSC using a serum-free endothelial cell-conditioned medium (CM). The reported results showed that these soluble factors were able to enhance MSC osteogenic differentiation, since the expression levels of genes like ALP, osteonectin and osteopontin were increased when MSC were cultured in CM culture medium. The soluble factors can be secreted by cells or released from the extracellular matrix [47].

As discussed above, in the present study, cHMSC were able to over-expressed gene markers related with osteoblastic differentiation such as ALP, COL 1 and RUNX2. Regarding other osteoblastic markers, as the ability to form a mineralized extracellular matrix, the last event of osteoblast differentiation, alizarin red assay and SEM observation showed the presence of calcium phosphate deposits, with a clear induction in co-culture conditions.

It is believed that BMP2 and VEGF interaction had a primordial role in the HMSC osteoblastic differentiation, observed in the present work. The medium used (described above), had in its composition VEGF that could induced HDMEC to expressed BMP2. A previous study of Bouletreau *et al* [48] also showed that VEGF induced BMP2 mRNA and protein expression in HDMEC. Furthermore, other studies showed that BMP2 is an important growth factor to induce osteoblastic differentiation and mineralization [49, 50]. Jorgensen *et al* [51] supplemented MEM basal culture medium with BMP2 and showed that the presence of BMP2 was able to induce osteogenic differentiation with a mineralized matrix bone marrow stromal cells. Also Kaigler *et al* [52] showed that HDMEC significantly increased BMSC osteogenic differentiation *in vitro* and expressed BMP2. When BMP2 RNA expression was inhibited in HDMEC, BMSC osteogenic differentiation decreased. Several studies also

showed an enhanced bone formation when VEGF and BMP2 were released or expressed simultaneously *in vivo* [53].

Von Willebrand factor (vWF) is a glycoprotein found in endothelial cells, platelets and plasma and it is stored in Weidil-Palade bodies, [54] large rod-shaped organelles that are specific for endothelial cells [55]. This is also an endothelial marker because it is synthesized exclusively by endothelial cells and magakaryocytes [54, 56]. In this study, vWF was expressed by HDMEC in very high levels from the first studied time-point, keeping its high levels for 21 days. A slight increase in the expression of this gene was noticed for cHDMEC at days 7 and 14, evidencing that endothelial phenotype was maintained in both conditions.

It is known that inter-endothelial cell contacts control and regulate permeability of blood vessels wall and angiogenesis. Adherens junctions between endothelial cells are formed by cell adhesion molecules such as VE-cadherin [57, 58]. VE-cadherin is a member of Ca^{2+} dependent adhesion molecules and is also involved in vascular morphogenesis and endothelial survival, being up-regulated during vascular proliferation, essential for angiogenesis [56]. The present work showed that HDMEC cultures expressed VE-cadherin, with increased level expression from days 7 to 21. Over-expression of this adhesion molecule was observed in cHDMEC, with a significantly high increase at day 21.

Angiopoietins (Ang) also play a major role in the whole angiogenesis process. ANGPT1 is related with blood vessel remodelling, maturation and stabilization [59] and ANGPT2 may regulate cell-matrix interactions in the growing vessels to facilitate sprouting [60]. HMSC expressed ANGPT1 achieving its maximum level at day 14, and it is worth to notice the significant increase in the expression of this gene in cHMSC during all culture times. As expected no expression was detected on HDMEC and cHDMEC, since ANGPT1 is expressed by osteoblasts, as reported in the literature [61, 62]. Expression of ANGPT2 was detected in HDMEC and over-expressed in cHDMEC. HMSC and cHMSC also express this gene in very low levels, which was also found in previous studies[60, 63], where ANGPT2 was essentially expressed by endothelial cells. No works were done evaluating the expression of these two genes with sorted cells from co-cultures. Considering the role of these genes in endothelial cells, the expression of ANGPT1 by HMSC and ANGPT2 by HDMEC, and their over-expression in

co-culture conditions, the results strongly suggest the importance of the reciprocal interactions between these cell types.

Different assays are used to study the angiogenic process. The most used models involve a 3D supportive matrix like Matrigel [16, 64], where endothelial cells are able to organize themselves in a network of tubular like blood vessels. Furthermore, *Lozito et al* [16] showed that the extra-cellular matrix components like laminin, collagen and fibronectin alone (without growth factors or the presence of other cell types) were not able to induce vascular differentiation. The same happen when vascular endothelial cell growth factors were used alone. In this work, endothelial cells were seeded on Matrigel to evaluate the capacity of HDMEC to form capillary-like structures, what occurred after 3 days of culture. It seems that endothelial cells require a 3D matrix of extracellular components to organize themselves into capillary-like-structures since many soluble growth factors important for vascular differentiation bind to the extracellular matrix. In summary, it seems that the presence of 3D matrix and angiogenic growth factors is required for capillary-like-structures formation [16]. Furthermore, cell number was quantified by flow cytometry in both monocultures and co-culture. The results obtained showed that endothelial cell number in co-culture decreased with culture time, as previously described in the literature [22, 34]. Possibly, for these reasons, no microvessels like structures were observed in our co-culture system, although the expression of genes associated with angiogenesis was not affected.

Recent studies revealed that bone vascular endothelial cells are members of a complex interactive communication network within bone microenvironment which involves also osteoblasts, osteoclasts, macrophages and stromal cells [65], during bone development and remodelling. The present results provide detailed information regarding the time-dependent gene expression associated with endothelial and osteoblast cells and, also, the effect of the reciprocal interaction of these cell types in their gene expression profile. Results showed that genes typically associated with osteoblast differentiation (COL 1, RUNX2 and ALP) and with endothelial cells (vWF and VE-cadherin) were expressed in monocultures of HMSC and HDMEC, respectively. In addition, results observed in HMSC and HDMEC sorted from co-culture (cHMSC and

cHDMEC) confirm the differentiation of HMSC in co-culture conditions and also a complex reciprocal interaction with an evident induction of both phenotypes.

CONCLUSION

The applied conditions allowed for the continuous interactions between HDMEC and HMSC co-cultured for a considerably long period. Co-cultured cells were able to maintain their viability, morphology, metabolic activity and individual functionality for up to 21 days. The time-detail study of the reciprocal relationships regarding the expression of genes associated with endothelial and osteoblast differentiation seems to indicate that an increase of endothelial markers expression is enhanced by the presence of HMSC. At the same time, HMSC differentiation is induced in co-culture conditions. This co-culture system might be useful for studying the reciprocal regulation and functional interaction between these two cell types and, possibly, be applied as a strategy for bone regeneration.

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CHAPTER IV

RESPONSE OF MONOCULTURED AND CO-CULTURED HUMAN MICROVASCULAR ENDOTHELIAL CELLS AND MESENCHYMAL STEM CELLS TO MACROPOROUS GRANULES OF NANOSTRUCTURED-HYDROXYAPATITE AGGLOMERATES

Response of monocultured and co-cultured human microvascular endothelial cells and mesenchymal stem cells to macroporous granules of nanostructured-hydroxyapatite agglomerates

M.S. Laranjeira^{1, 2*}, M.H. Fernandes³, F.J. Monteiro^{1, 2}

¹ INEB- Instituto de Engenharia Biomédica, Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal

² Universidade do Porto, Faculdade de Engenharia (FEUP), Departamento de Engenharia Metalúrgica e de Materiais, Rua Roberto Frias, s/n, 4200-465 Porto, Portugal

³ Laboratório de Farmacologia e Biocompatibilidade Celular, Faculdade de Medicina Dentária, Universidade do Porto (FMDUP), Rua Dr. Manuel Pereira da Silva, 4200-393 Porto, Portugal

ABSTRACT

Nanostructured materials recently developed for bone implant, such as ceramics, polymers and metals have a more dynamic response when compared to counterpart materials with larger particle size. Recent studies have shown that hydroxyapatite (HA) nanocrystalline have better functional properties that are important to create suitable local conditions for bone formation, when implanted in an osseous environment. Bone formation depends on several complex processes, including a tight communication between endothelial cells and osteoblasts and mesenchymal stem cells. This study examined the interaction between human dermal microvascular endothelial cells (HDMEC) and human mesenchymal stem cells (HMSC), in monoculture and co-culture on macroporous granules of nanostructured-hydroxyapatite agglomerates. The work clearly demonstrated that these granules were able to support cell type's survival, proliferation and individual functionality in a monoculture system, for 21 days. Furthermore, these 3D structures were suitable to maintain a co-culture of HDMEC + HMSC expressing their characteristic phenotype markers and cellular interactions. The osteoblastic phenotype was clearly induced, as evidenced by the over-expression of osteoblastic genes, increased ALP activity and matrix mineralization. Over-expression of typical endothelial genes was also observed. Increased expression levels of genes related with osteogenesis and angiogenesis was also evident. The present work, is believed to be the first described in the literature to study the interactions between HMSC and HDMEC when co-cultured on nanostructured-HA 3D surfaces. Furthermore, the results achieved suggest that nanostructured HA granules may be considered a promising type of implants for bone regeneration and tissue engineering application, in which the granules can be pre-seeded with these two types of autologous cells, before bone graft implant.

KEYWORDS: Human mesenchymal stem cells, Human dermal microvascular endothelial cells, Biomaterials, Nanotechnology, Hydroxyapatite, Osteogenesis, Angiogenesis

INTRODUCTION

Nanomaterials have been pointed as promising candidates to achieve a therapeutic success in tissue engineering and regenerative medicine, since nanotechnology embraces systems whose materials are in the range of nanometers (10^{-9} m) and can mimic surface properties (topography, energy, etc) of natural tissues, particularly bone [1, 2]. So, after a bone biomaterial implant, a wound healing around the graft surface involves a series of events related to proteins adsorption and cell-material interactions such as cell recruitment, migration and adhesion [3]. The nanostructured materials will affect several different types of cells such as mesenchymal stem cells (MSC), osteoblasts, osteoclasts, fibroblasts, endothelial cells and cell-to-cell interaction between the same and other cell types [4].

It is well known that the establishment of a rapid and stable microvasculature after implantation is required to guarantee the metabolic demand of cells recruited to start bone regeneration and, afterwards, to the newly formed tissue around bone material [5-7]. Therefore, several studies have shown that there is a reciprocal interaction between osteoblasts and endothelial cells, during osteogenesis [5, 8-10]. *In vitro* cell culture works have been done as an approach to study the mechanism of molecular and cellular response to microstructured calcium phosphate surfaces properties [7, 11, 12]. Cell adhesion, migration, proliferation and differentiation have been studied on hydroxyapatite (HA) materials, in different single cell culture and co-culture systems, using endothelial and osteoblasts cells [2, 11]. Although, since bone is an organic-inorganic nanocomposite consisting of nano-HA crystals, bone cells are naturally used to interact with nanometer roughness surfaces, very different from the surfaces provided by traditional orthopedic implant. A variety of studies have shown that nano-HA exhibited enhanced osteoblast adhesion and cell proliferation to optimize biological functionality, due to the higher surface area [13-15]. However, there are no studies in the literature reporting the long-term behaviour of a co-culture system with endothelial and mesenchymal stem cells performed on nano-HA surfaces.

Several studies have shown that cells cultured in 2D and 3D conditions can show different cell behaviour in monoculture and in co-culture conditions. The majority of the reports with endothelial and osteoblast/MSC cells co-cultured on conventional 2D surfaces versus 3D structures, showed that 3D culture presented an environment more adequate for cell survival, functionality and cell-to-cell interaction, with an increase of their osteogenic and angiogenic potentials. However, detailed knowledge about the interactions between cells in the context of an artificial 3D scaffold (biomaterial) remains limited [8, 10, 16, 17].

Recently, our work group developed and characterized nanostructured-HA macroporous granules, with a highly interconnected porosity and a mean size of micro and macroporous, appropriate for cell adhesion and bone ingrowth. *In vitro* studies with MG63 osteoblast-like cells showed a higher cell proliferation in nanostructured-HA granules compared to microstructured-HA granules [15]. In this context, the present study investigated for the first time, if human dermal microvascular cells (HDMEC) and human mesenchymal stem cells (HMSC) alone or in co-culture were able to proliferate and migrate into macroporous granules of nanostructured-HA agglomerates, prepared as previously described [15], maintaining their morphology and expression of osteoblastic and endothelial gene markers, without interfering with cell-to-cell communication.

MATERIALS AND METHODS

Granules Preparation

The porous granules were prepared using a polyurethane sponge technique, described elsewhere [15]. Briefly, HA slurry was prepared using nano-sized HA aggregates, kindly provided by Fluidinova S.A. (Maia – Portugal). The nano-HA slurry was prepared using 50 (wt%) of water and nano-HA powders. Slurry excess was removed by squeezing the polyurethane sponges. The infiltrated sponges with nanostructured-HA powders slurry was submitted to a heat treatment of 1050°C. After heat treatment, samples were crushed and sieved, in order to obtain granules.

Cell culture

Human dermal microvascular endothelial cells culture. Commercial available HDMEC (Sciencell) were cultured in cell culture plates with endothelial (EC) basal medium (Sciencell) containing 5% of foetal bovine serum (FBS; Sciencell), 10 units/mL of Penicillin, 10 µg/mL of Streptomycin (P/S solution; Sciencell) and endothelial cell growth supplement (ECGS; Sciencell). Incubation was carried out under standard cell culture conditions at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The culture medium was refreshed every 2 days and cells were trypsinized after reaching 80% of confluence.

Human mesenchymal stem cells culture. Cell suspension from human bone marrow was obtained from orthopaedic surgery procedures, with patient informed consent and was expanded on cell culture plates with minimum essential medium Eagle, alpha modification (α-MEM; Sigma) containing 10% foetal bovine serum (FBS; Sigma), 100µg/mL penicillin (Sigma) and 10 units/mL streptomycin (Sigma). Incubation was carried out under the same standard cell culture conditions described above. HMSC characterized by flow cytometry were positive for the markers CD105, CD73 and CD90 and did not expressed the markers CD45, CD34, CD14, CD19 and CD31.

Cell cultured on the material. Granules were pre-incubated for 1 hour in cell culture medium, at 37°C, in a humidified atmosphere of 95% air and 5% CO₂. For co-culture, HDMEC and HMSC from passage 3, were seeded on granules (128 mg/cm²) at a cell density of 8×10⁴ cells/cm² HDMEC and 2×10⁴ cells/cm² HMSC, for a final concentration 10⁵ cells/cm², and cultured in a mixture (50:50) of EC culture medium and HMSC culture medium. HDMEC and HMSC monocultures were used as control; they were cultured on the granules at a cell density 10⁵ cells/cm², and maintained in the same experimental conditions. Monocultures and co-cultures were characterized throughout the incubation time (7, 14 and 21 days), as follows.

Cell viability/proliferation

MTT assay

Metabolic activity of HDMEC and HMSC monocultures and co-cultures was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. In summary, ten microliters of MTT solution (5 mg/mL in PBS) were added to 100 µL of cell medium and incubated 3h at 37°C. The colonized granules were transferred to a new plate and formazan salts were dissolved with dimethylsulphoxide (200 µL). The resulting solution was transferred to a 96-well plate, and the absorbance was read at 550 nm on an ELISA reader (Synergy HT, Bio tek). MTT assay was performed at days 7, 14 and 21. The absorbance of the samples was normalized for DNA content.

DNA assay

DNA content was quantitatively analyzed over time (7, 14 and 21 days) by the PicoGreen DNA quantification assay (Quant-iT™ PicoGreen® dsDNA Assay Kit, Molecular Probes Inc., Eugene), according manufacturer's instructions. The colonized granules were transferred to a new plate and were treated with Triton X-100 (0.1%) (Sigma) and fluorescence was measured with a plate reader (Synergy HT, Bio tek) at wavelengths of 480 and 520 nm, excitation and emission respectively, and corrected for fluorescence of reagent blanks. The amount of DNA was calculated by extrapolating a standard curve obtained by running the assay with the given DNA standard [18].

Immunostaining of F-actin cytoskeleton, CD31 and nucleus

Cells growing in the granules were fixed (4% formaldehyde, Sigma; 15 min), permeabilized with 0.1% Triton X-100 for 5 min and then incubated in 1% bovine serum albumin (BSA)/PBS for 1h. After been washed with PBS, monocultures of HMSC were stained for F-actin with Alexa-Fluor-conjugated phalloidin (1:100 in 1% BSA/PBS, 1h; Alexa Fluor® 488 Phalloidin: Molecular Probes). Nuclei were stained with 10 µg/mL propidium iodide (Sigma) diluted in PBS for 10 min.

HDMEC and co-cultures growing on the granules were incubated with primary CD31 antibody (1:100 in 1% BSA/PBS, 45 min; PECAM-1 (P2B1) sc-20071; Santa Cruz Biotechnology). Samples were washed four times with PBS and incubated with secondary antibody anti-mouse Alexa-Fluor (1:1000 in 1% BSA/PBS, 45 min; Alexa Fluor 488 goat anti-mouse IgG1 (Y1); Molecular Probes). After that, nuclei were counterstained with 10 µg/mL propidium iodide (Sigma) for 10 min. Samples were washed with PBS and covered with Vectashield (Vector Laboratories). CLSM images were acquired on a Leica TCP SP2 AOBS, at days 7, 14 and 21.

ALP activity

For ALP activity assay, colonized granules were washed with PBS and a cell lyses buffer containing 0.1% Triton X-100 (Sigma). Cell lysates were incubated with p-nitrophenyl phosphate (Sigma) in alkaline buffer solution (2-amino-2-methyl-1-propanol) (Sigma), at pH 10.5, for 1h at 37°C. The absorbance was measured at 405nm using an ELISA reader (Synergy HT, Bio tek) [15]. ALP activity was normalized for DNA content.

Matrix mineralization

Histochemical staining of calcium deposition

Alizarin red S staining is a common method for visualizing nodular and calcium deposition in osteoblast cell cultures. At days 14 and 21, cells cultured in the granules were fixed (1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, 10 min) and were covered with a 1 % 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)[19]. The same proceeding was done in the granules alone (without cells) as a control, since hydroxyapatite is a calcium containing material. Samples were observed under a stereo microscope (model SZX10, Olympus).

Scanning electron microscopy (SEM)

Cells cultured on the materials, were fixed and dehydrated in graded series of alcohols and critical point dried. Samples were coated with gold and observed in a FEI Quanta

400FEG ESEM / EDAX Genesis X4M scanning electron microscope. Samples were collected at days 7, 14 and 21.

Flow cytometry - Cell sorting

HDMEC, HMSC and cocultured cells were extracted from the ceramic with a solution of collagenase and trypsin at different time-points, days 7, 14 and 21. Co-cultured cells were sorted through Fluorescence-activated cell sorting (FACS). Single cell cultures were suspended at a density of 10^5 – 10^7 cells/mL and stained with FITC-conjugated Anti-Human CD31 (PECAM-1) FITC (BD Biosciences), and then washed and resuspended in a final volume of 250 μ l. Using a BD FACSAria™ II system, CD31-positive (labeled cells, CD31+) and negative (CD31-) populations were gated and sorted into different eppendorfs. HDMEC and HMSC monoculture were used as control, to confirm or not CD31 expression, and to evaluate the auto-fluorescence. The population of HDMEC cells from the co-culture (cHDMEC) was gated in the CD31+ positive cell quadrant, sorted and collected into eppendorfs. The population of HMSC cells from the co-culture (cHMSC) was gated in the CD31- negative cell quadrant, also sorted and collected into different eppendorfs. The number of sorted events was 50,000 per sample. HDMEC and HMSC monoculture were used as control, to confirm or not CD31 expression. Data processing was performed using FlowJo software 8.7.

Real Time Gene expression by Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

At days 7, 14 and 21, mRNA was extracted from HDMEC and HMSC monocultures and from cHDMEC and cHMSC (the populations sorted by FACS from the co-culture), using the NucleoSpin® RNA II Kit (Macherey-Nagel) according to the manufacturer's instructions. The final concentration of RNA was determined by a NanoDrop 1000 instrument (NanoDrop Technologies, NanoDrop 3.0.1 software, Coleman Technologies, Inc.). Reverse transcription of the samples was performed with SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen) into cDNA. The protocol was provided by the supplier. cDNA (1 μ l) was loaded in a 96-well plate and the components of SYBR-

Green Supermix (iQTM, BioRad, USA) were added according to the manufacturer's instructions. The genes COL1, ALP, BMP2, ANGPT2, ANGPT1, VEGF-165, RUNX2, vWF and VE-cadherin were assessed for Real time RT-PCR amplification. The adopted primers conditions are summarized in Table I. Data were analyzed with the iCycler IQTM software and gene expression were quantified by calculating $2^{-\Delta Ct}$ values and $\Delta Ct = (Ct, \text{reference gene} - Ct, \text{target gene})$.

Table I - Primers for PCR Amplification

Gene	GeneBank	Primers sequences	TM (°C)
GAPDH	NM_00246	Forward 5'-TAACTCTGGTAAAGTGGATATTG-3'	58
		Reverse 5'-GAAGATGGTGATGGGATTTC-3'	
COL1	NM_000088	Forward 5'-GGAATGAGGAGACTCGCAACC-3'	58
		Reverse 5'-TCAGCACCACCGATGTCCAAA-3'	
BMP2	NM_001200	Forward 5'-ATGAAGAATCTTTGGAAGAACTAC-3'	58
		Reverse 5'-GGTGATGGAACTGCTATTG-3'	
vWF	NM_000552	Forward 5'-AAGAAAATAACACAGGTGAA-3'	58
		Reverse 5'-TACTCTCCTCTCTCATTGAC-3'	
ALP	NM_001177530	Forward 5'-AGCCCTTCACTGCCATCCTGT-3'	58
		Reverse 5'-ATTCTCTCGTTCACCGCCAC-3'	
RUNX2	NM_001024630	Forward 5'-GGGTAACGATGAAAATTATTCT-3'	58
		Reverse 5'-TTAATTGCTCTGTGATAGGTA-3'	
ANGPT1	NM_001199859	Forward 5'-ACCGAGCCTATTCACAGTAT-3'	58
		Reverse 5'-ACAGTTGTCATTATCAGCATCTT-3'	
ANGPT2	NM_001118888	Forward 5'-GTGATTAGACAGAACACCTATGC-3'	58
		Reverse 5'-AACAGTGCTCAGAAGAATGC-3'	
VE-cadherin	NM_001795	Forward 5'-GCAATAGACAAGGACATAACA-3'	58
		Reverse 5'-TAGGAAGTGGACCTTGGTAT-3'	
VEGF-165	AB021221	Forward 5'-TATGCGGATCAAACCTCACCA-3'	58
		Reverse 5'-CACAGGGATTTTCTTGTCTTGCT-3'	

Matrigel tube-like formation assay

Matrigel (Sigma) was diluted in culture cell medium and mixed with the granules, in the proportion 1:1 in 24-well plates. After 1h at 37°C, HDMEC were seeded at a final concentration of 1×10^4 cells/cm². Phase contrast images were taken under a inverted microscope (Axiovert 200M, Zeiss) after 3 days of culture [20].

Statistical analysis

Triplicate experiments were performed. The results were expressed as the arithmetic mean $\pm$ standard deviation (SD). Analysis of results was carried out using IBM SPSS Statistics 19 and statistical analysis was assessed using the one way ANOVA, with a significance level of $p < 0.05$.

RESULTS

Cell viability/proliferation and pattern of cell growth

MTT assay and DNA quantification

HDMEC and HMSC monocultures and co-culture were analysed regarding cells metabolic activity/viability through the MTT assay (Figure 1A). Results showed that cell viability/proliferation increased during culture time on monocultures and co-culture. However, HDMEC presented lower values. HMSC and co-cultures showed a similar time-dependent pattern, but, at day 21, values were significantly higher for co-cultured cells.

DNA levels quantification, Figure 1B, showed that HDMEC, HMSC and co-cultured cells were able to proliferate in granules during the 21 days of culture. HDMEC showed the lowest proliferation values, although with similar time-dependent pattern compared to other two types of culture, being statistically different from HMSC at days 14 and 21. At day 21, co-cultures presented a higher DNA content when compared to HMSC and HDMEC.

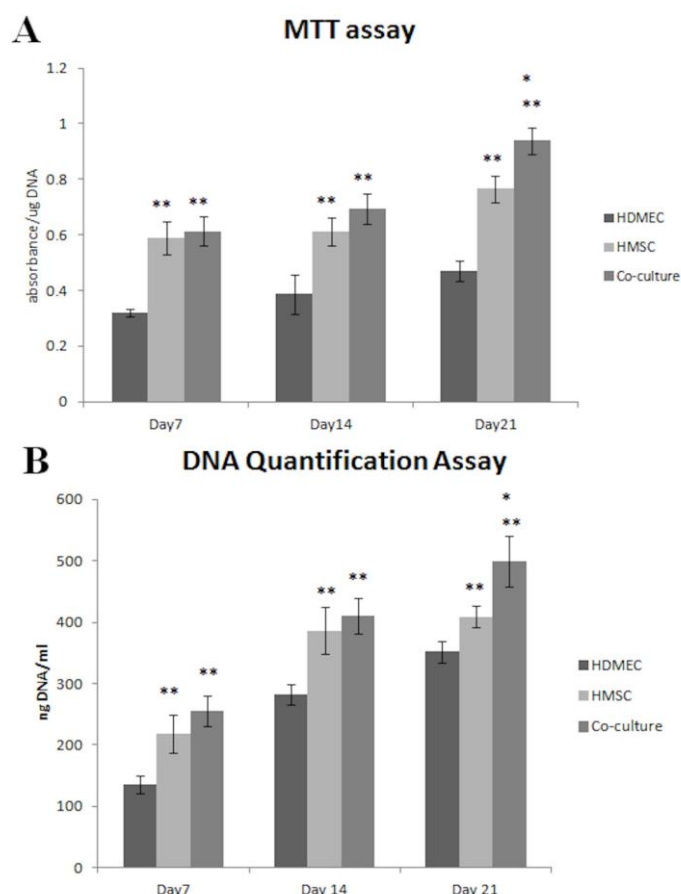


Figure 1 – Cell viability and proliferation of monocultured and co-cultured HDMEC and HMSC estimated by: A) MTT assay and B) DNA quantification. Assays were performed after 7, 14 and 21 days of culture. Values reported are the mean ($\pm$ SD) [* , significantly different from HMSC, for the same culture time; ** significantly different from HDMEC, for the same culture time ($p < 0.05$; $n = 3$)]

Immunostaining of F-actin, CD31 and nucleus

The growth patterns and distribution throughout the macroporous granules surface were observed by CLSM, and representative images of 21-day cultures are shown in Figure 2. HDMEC cultures stained intensively for CD31 and nucleus, attached well to biomaterial and by day 21 granules were completely covered by these cells, which adopted characteristic cobblestone morphology. HMSC cultures showed the typical elongated morphology growth pattern (fibroblastic-like morphology). After 21 days, cells covered the entire surface and dense cell layers could be observed in between granules, establishing bridges across different granules and linking them strongly. In

co-cultured granules, both types of cells were identified, even after three weeks of culture, as shown in Figure 2.

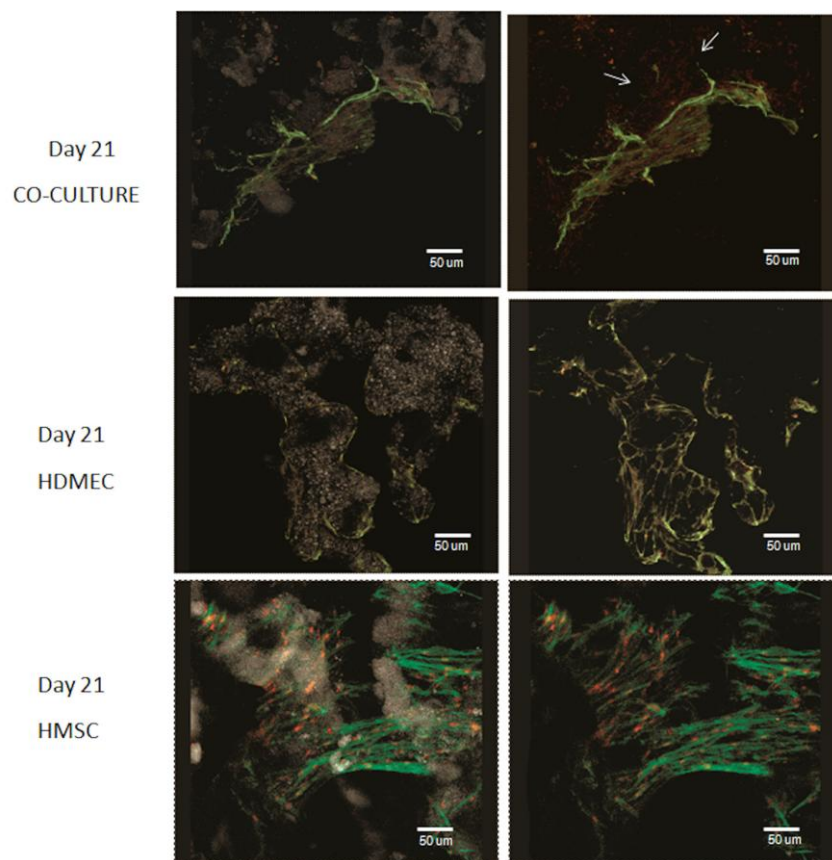


Figure 2 - Confocal laser scanning microscopy (CLSM) images of monocultured and co-cultured HDMEC and HMSC on the granules. Monocultures of HMSC were stained for F-actin (green) and nuclei with propidium iodide (red). HDMEC and co-cultures were stained for CD31 (green) and nuclei with propidium iodide (red). In the co-culture, HDMEC stained green and HMSC stained red and are pointed by white arrows. Images were collected after 21 days of culture.

ALP activity

ALP activity was measured at days 7, 14 and 21 in isolated and co-cultured HDMEC and HMSC seeded in the granules, Figure 3. ALP activity in HDMEC was lower compared to HMSC cultures. Comparatively, co-cultures presented significantly higher ALP activity at days 14 and 21, respectively 84% and 160%.

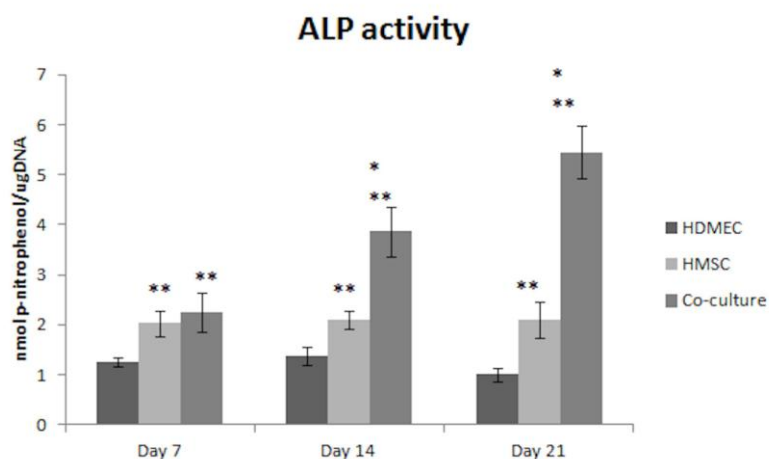


Figure 3 – Alkaline phosphatase (ALP) activity assay. Values reported are the mean ($\pm$ SD) and assays were performed after 7, 14 and 21 days of culture [ALP activity - *, significantly different from HMSC, for the same culture time; ** significantly different from HDMEC, for the same culture time ($p < 0.05$; $n = 3$)].

Matrix mineralization

Alizarin red staining assay was performed to identify the presence of calcium deposits. HMSC monocultures and co-cultures stained positive, with an evident higher intensity in the co-cultures, Figure 4. SEM observation showed that HDMEC and HMSC were able to progressively colonize the macroporous granules, and, by day 21, a thick cell layer, successfully adapted to the underlying surface, Figure 5. Also, granules seeded with co-cultured HDMEC and HMSC exhibited an increased cell proliferation through the culture time and, at day 21, several cell layers covered the material surface. In addition, the presence of calcium phosphate mineralized deposits, closely associated with the cell layer, was identified, Figure 5.

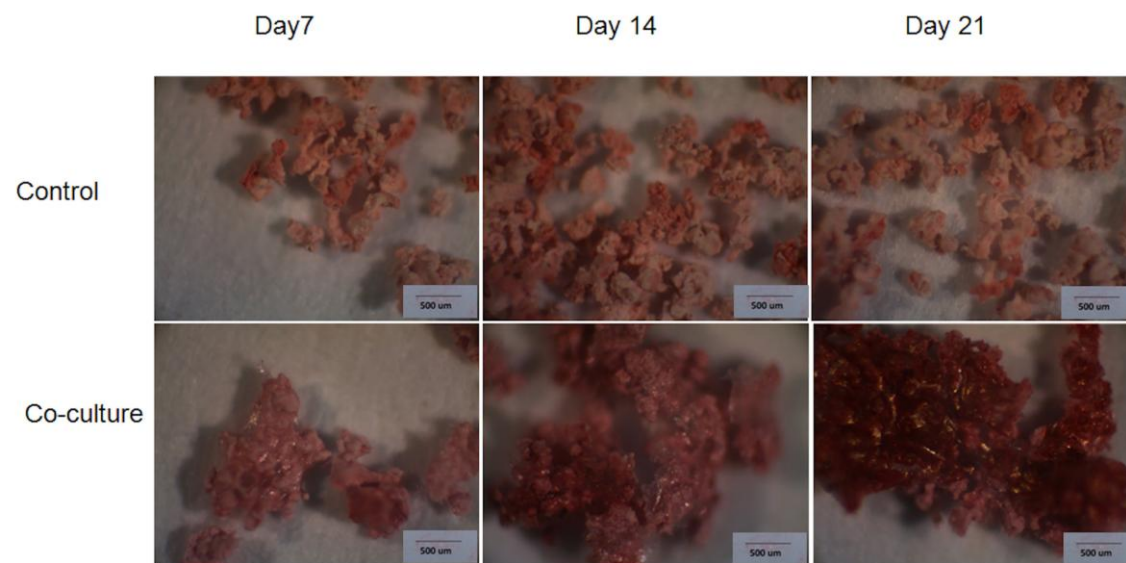


Figure 4 – Alizarin red histochemical staining performed in granules without cells and co-cultured granules, at days 7, 14 and 21.

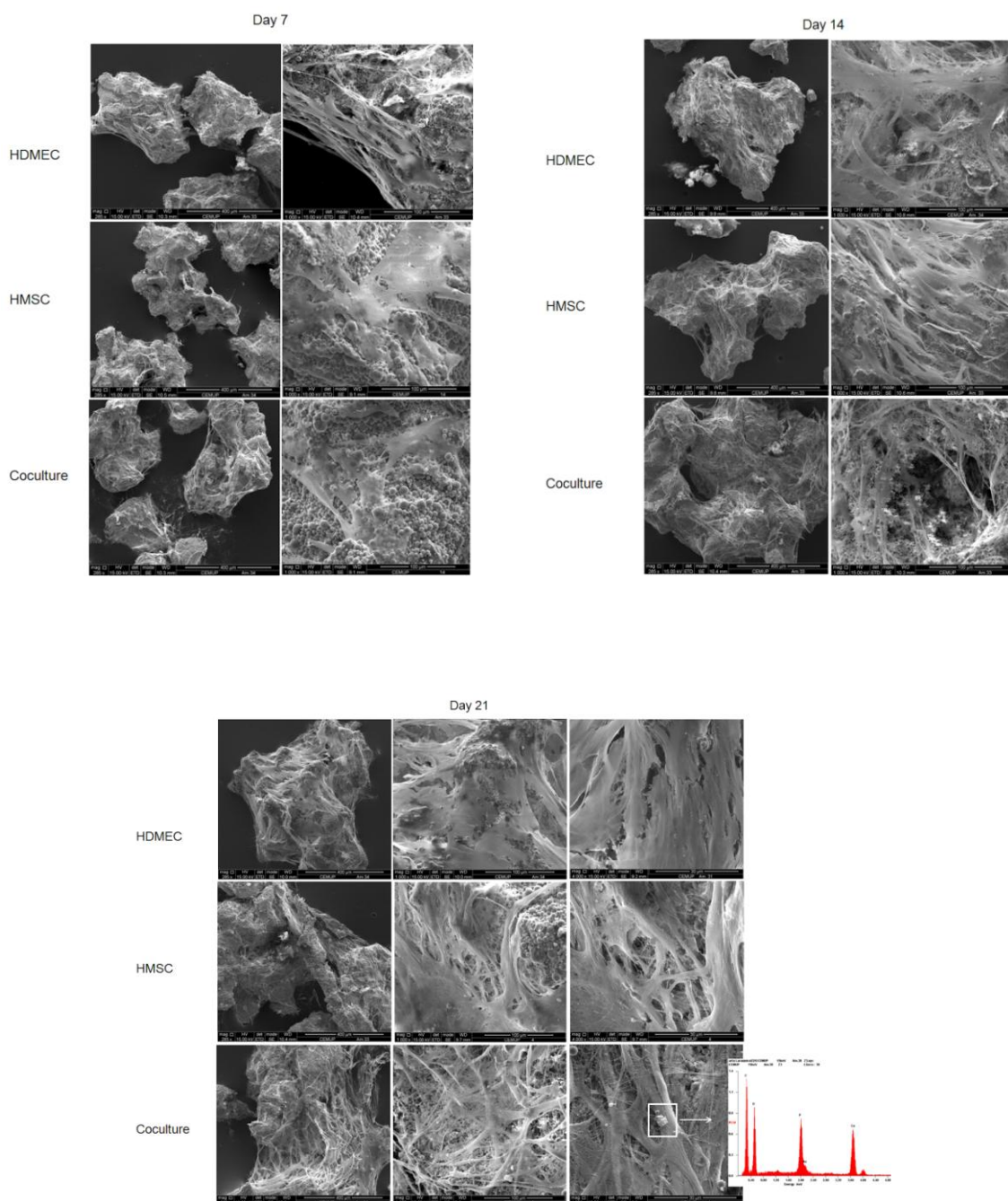


Figure 5 –SEM images of HDMEC and HMSC monocultured and co-cultured after 7, 14 and 21 days on granules. Mineralized globular structures were identified in close association with cell layers, and EDS spectrum showed the presence of Ca and P peaks.

Osteoblast and endothelial gene expression

Real time RT-PCR were performed in HMSC and HDMEC monocultures and cHMSC and cHDMEC (the populations sorted from the co-cultures) to analyse cells ability to

express markers of osteoblastic and endothelial phenotype in cultured nano-HA granules, Figure 6.

HMSC and cHMSC expressed COL1, RUNX2, ALP, BMP2, ANGPT1 and VEGF-165. In HMSC, expression of COL1, RUNX2 and ALP increased until day 14 and decreased afterwards. Expression of BMP2 was high at day 7 and decreased slightly afterwards. Expression of ANGPT1 increased with culture time, whereas VEGF-165 expression decreased with culture time. ANGPT2 was also detected, but in very low levels. Comparatively, cHMSC showed significantly higher levels in the gene expression of COL1 (days 7 and 14, particularly day 7), RUNX2 (days 7 and 14), ALP (all time points), BMP2 (days 7 and 14), ANGPT1 (days 14 and 21) and VEGF-165 (day 21). Regarding, HDMEC and cHDMEC, both expressed endothelial gene markers such as vWF and VE-cadherin with a distinct time-dependent profile. In HDMEC, vWF was expressed during 21 days, decreasing from day 14 to day 21. VE-cadherin expression was low at day 7, and increased afterwards. HDMEC cultures also expressed ANGPT2; levels were similar at days 7 and 14 and higher at day 21. BMP2 expression was also noticed, but levels were significantly lower compared to those expressed by HMSC. VEGF-165 was barely detected. Comparatively, cHDMEC presented higher expression levels for vWF (day 7, although without statistical significance), VE-cadherin (days 7 and 21) and ANGPT2 (day 14).

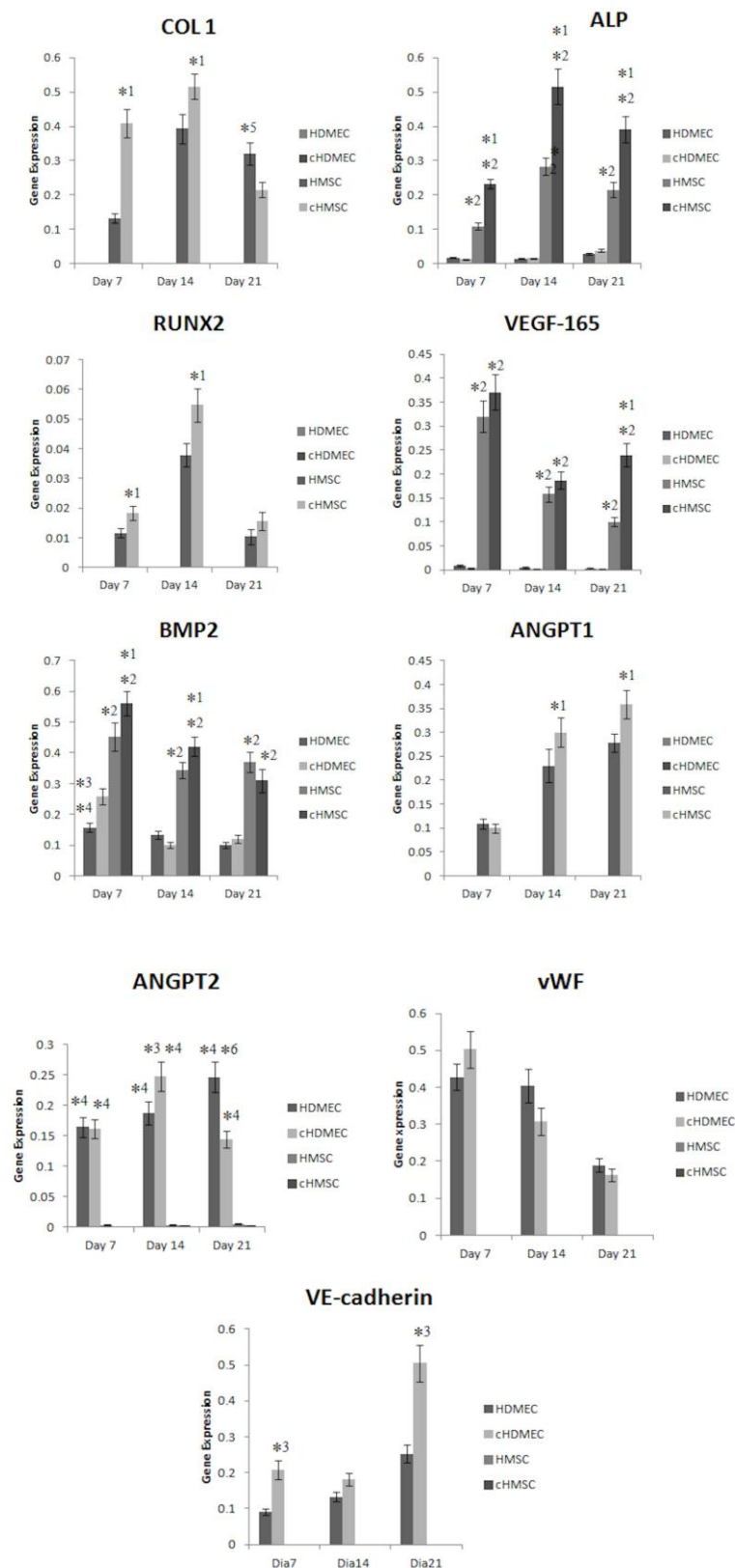


Figure 6 – Real time PCR analyses performed in HMSC and HDMEC monocultures, cHMSC and cHDMEC (the populations sorted from the co-cultures) for gene expression

related with osteoblastic and endothelial markers. Values reported are the mean ($\pm$ SD) and assay was performed after 7, 14 and 21 days of culture [^{*1}significantly different from HMSC, for the same culture time; ^{*2}significantly different from HDMEC and cHDMEC, for the same culture time; ^{*3}significantly different from HDMEC, for the same culture time; ^{*4}significantly different from HMSC and cHMSC, for the same culture time; ^{*5}significantly different from cHMSC; ^{*6} significantly different from cHDMEC; ($p < 0.05$; $n = 3$)].

Matrigel tube-like formation assay

HDMEC were able to form a network of capillary-like tubes just after 3 days of culture in the presence of the granules (Figure 7).

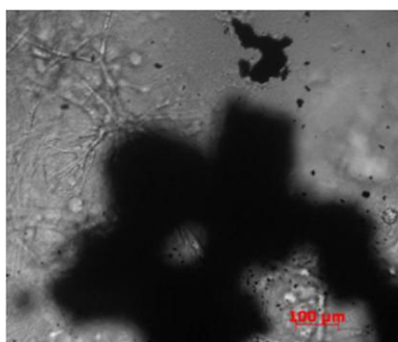


Figure 7 – Capillary-like tubes formed by HDMEC after 3 days of culture in Matrigel with the granules.

DISCUSSION

It is well known that endothelial cells are participant in the construction of a vascular network, necessary to adequate bone regeneration. Bone forming cells recruitment and growth are also essential for bone formation, repair and remodelling. As a result, co-culture endothelial and osteoblasts or their precursors can more close mimic the natural bone regeneration compared to single cell cultures, since bone is composed by multiple cells with essential cell interactions going on. Furthermore, taking into account the available literature, co-cultured endothelial and osteoblasts or mesenchymal stem cells seem to be a potential tool in bone regeneration, since their incorporation into the biomaterial before implantation is expected to speed up the

regeneration process [21, 22]. Also, the behaviour of co-cultured cells can be explored in 3D conditions with specific types of biomaterials, in order to know how the tested materials can affect the cellular interactions. Ideally, biomaterials should be porous, particularly to fill large bone defects.

Nanostructured materials recently developed for bone implants, such as ceramics, polymers and metals, have a more dynamic response when compared to counterpart materials with larger particle size. Nanoscaled materials showed improved performances due to their large surface area and particularly surface reactivity. Laranjeira *et al* [15] have done a previous study, comparing the MG63 cells behaviour cultured in macroporous granules of microstructured-HA and nanostructured-HA. MG63 osteoblast-like cells were able to adhere, proliferate and migrate into macropores in both types of granules. However, a higher growth rate was achieved on nanostructured-HA granules compared to microstructured-HA. Furthermore, cells maintained similar expression levels of osteoblastic-related proteins such as COL1, ALP, BMP2, M-CSF and OPG, during the 6 days culture time.

Taking these aspects into account, the phenotype behaviour of monocultured and co-cultured HDMEC and HMSC was addressed for the first time in macroporous granules of nano-hydroxyapatite developed previous by our work group.

Results regarding MTT assay and DNA quantification showed that HDMEC and HMSC seeded onto HA-granules exhibited a high viability and proliferation that increased with culture time, both in monoculture and co-culture conditions, following a similar time-dependent profile. HDMEC cells showed lower values, but they were able to proliferate in 3D nanostructured-HA surfaces, contrarily to previous studies reporting that HDMEC were not able to attach and proliferate in micro-sized hydroxyapatite porous structures [11]. Regarding the co-culture, significantly higher values were observed at day 21, compared to monocultures. Since the initial plating density was the same in both situations (10^5 cell/cm²), this observation might be related with the over-expression of several important genes involved in the modulation of cell proliferation under co-culture conditions, such as BMP2 and VEGF-165 [10, 23].

Confocal and SEM observations confirmed that cells were able to attach, grow in the macroporous granules and completely covered the available surface with dense cell layers, in monocultures and co-culture. HDMEC monoculture adopted cobblestone morphology and expressed CD31, whereas monocultured HMSC displayed the expected fibroblast-like morphology.

The genetic profile of HDMEC and HMSC in monocultures and co-cultures, after the cell sorting of the two populations (cHDMEC and cHMSC), was also addressed. Genes were chosen regarding their relevance in the two phenotypes. COL1, ALP and RUNX2 play an important role in osteoblast differentiation; BMP2 and VEGF-165 are described as being important growth factors for osteoblasts and endothelial cells, respectively; vWF and VE-cadherin are relevant endothelial markers, and angiopoietins (Ang) play a major role in the angiogenesis process. In general, the results showed that osteoblastic and endothelial phenotypes were achieved and maintained in monocultures and co-culture. Nevertheless, the genetic profile of co-cultured HMSC and HDMEC, after being sorted, was distinct from that observed in the respective control monoculture, regarding the level of expression and time-dependent profile.

Collagen type 1 is the most abundant extracellular bone protein, also considered an early bone differentiation marker and an imperative pro-angiogenic substrate, being important to drive endothelial migration and proliferation [24]. This gene was significantly over-expressed by cHMSC comparatively to HMSC, particularly at early culture times (7 days), suggesting that expression of COL1 was highly stimulated in the presence of HDMEC. Previous studies performed in co-cultures with different cell types and biomaterials (2D and 3D) showed that up-regulation of COL1 occurred only after 21 days of co-culture [24], whereas, in the present work, an early over-expression was observed in the macroporous nanostructured granules.

Regarding another early bone differentiation marker, ALP, which is an enzyme involved in the mineralization of bone extracellular matrix [25], the present work showed that all types of cells seeded in nano-HA granules (HDMEC, cHDMEC, HMSC and cHMSC) expressed ALP gene throughout the culture time. Nevertheless, HDMEC and cHDMEC expressed ALP gene at very low levels, which is in agreement with the

ALP activity assay. ALP gene expression and ALP activity were significantly higher in cHMSC compared to HMSC, indicative of an induced osteogenic differentiation of stem cells, due to HDMEC presence. These results are consistent with previous studies on 3D scaffolds of different biomaterials [26].

Another earlier marker of osteogenic differentiation, RUNX2, was significantly over-expressed in cHMSC compared to HMSC. The higher levels in cHMSC were found at days 7 and 14, followed by a decrease, suggesting again an osteoblastic commitment of cHMSC. Previous studies, where HMSC were seeded on polycaprolactone (PCL) 3D scaffolds showed a similar behaviour in the expression of this gene, reporting that the higher expression was reached at day 14, and also that the osteogenic differentiation was clearly achieved in the 3D structure [26].

Also, the Alizarin red assay and SEM observation showed a clear induction in the formation of mineralized calcium phosphate deposits in co-culture conditions.

Bone Morphogenetic Proteins (BMPs) are members of the TGF- β superfamily cytokines that affect bone and cartilage formation, playing a role on osteogenesis [27]. The present results showed that HMSC and cHMSC presented high levels of BMP2 expression during the culture time, but cHMSC exhibited an induced expression at days 7 and 14. On the other hand, the expression levels decreased with culture time, what has been previously described [28]. The gene over-expression in co-culture conditions might also play a role in HSMC differentiation, since BMP2 induces osteoblast differentiation [27].

Vascular endothelial growth factor (VEGF) is an important angiogenic factor involved in endothelial cells survival and formation of functional vessels [29]. VEGF expression was detected in HDMEC and cHDMEC cultured on granules, although in very lower levels, what is in accordance with previous studies, showing that VEGF is expressed mainly by osteoblasts [30, 31]. In the present work, VEGF was expressed by HMSC and cHMSC, being over-expressed in cHMSC. This might be related with the increased expression of BMP2 also observed in co-culture conditions, as previous studies suggest, BMP2 can stimulate VEGF production by osteoblasts [27], thereby promoting osteogenesis and angiogenesis and modulating the coupling between the

two processes [32]. Also, a study done in 3D scaffold of polycaprolactone showed a clear high concentration of VEGF in co-cultures compared to HMSC monocultures [5]. In the same way, co-cultured osteoprogenitors immobilized in alginate microspheres showed a higher gene expression level of VEGF-165 compared to osteoprogenitors single cell culture[33].

Von Willebrand factor (vWF) gene expression was also assessed, since it is a glycoprotein synthesized exclusively by endothelial cells and megakaryocytes [34, 35], consequently an endothelial marker. vWF was only expressed by endothelial cells as expected, HDMEC and cHDMEC showed the same pattern of expression. Levels decreased with culture time, especially in the last week, although, it is important to notice that endothelial phenotype was maintained during the 21 days culture time, both in monoculture and co-culture conditions. Regarding previous similar studies, Fuchs *et al* co-cultured outgrowth endothelial cells with primary osteoblasts on 3D silk fibroin scaffolds and observed that vWF expression also decreased after 4 weeks of co-culture [36].

VE-cadherin, another endothelial marker, that has an important role in angiogenesis and endothelial cell survival [32, 37] was also expressed by HDMEC, with increasing levels during the culture time. VE-cadherin was over-expressed in cHDMEC at the last time point of co-culture, compared to HDMEC. Also, Aguirre *et al* showed an up-regulation of CD31 and VE-cadherin expression in co-cultured endothelial progenitors and mesenchymal stem cells [20]. Studies in 3D structures also demonstrated a significant expression of this gene in HUVEC cells, with improved endothelialization [38]

The expression of angiogenesis related genes such as ANGPT1 and ANGPT2 was also examined. ANGPT1 regulates endothelial cell survival, maintaining their normal quiescent phenotype and it is also related with blood-vessel maturation, protecting it against inflammation [39-41]. ANGPT2 is expressed by endothelial cells and it is related with vascular remodelling, acting as an angiogenic promotor in the presence of angiogenic stimulators, like VEGF, promoting vessels growth and sprouting [42, 43]. In this work, ANGPT1 gene expression was not detected on HDMEC and cHDMEC, since

this gene is mainly expressed by osteoblasts [44]. This is in accordance with literature, where it is described that ANGPT1 is mainly expressed by endothelial surrounding cells (such as perivascular cells) and act in a paracrine manner on endothelial cells [44, 45]. HMSC expressed ANGPT1 achieving its maximum level at day 21, and it was over-expressed by cHMSC at the same day. Expression of ANGPT2 was detected in HDMEC and cHDMEC. However, maximum level expression was observed earlier in cHDMEC (day 14) compared to that on HDMEC (day 21). Very low levels of ANGPT2 gene were expressed by HMSC and cHMSC, which was also found in previous studies reporting that this gene is essentially expressed by endothelial cells [42, 46]. No works were done evaluating the expression of these two genes with sorted cells from co-cultures of endothelial and osteoblastic or mesenchymal stem cells, in 3D conditions.

This study demonstrated that the co-culture of HDMEC and HMSC in nanostructured-HA 3D granules was successful, recreating an adequate microenvironment for cell survival, proliferation and differentiation of the two phenotypes. Furthermore, to our knowledge, this work is the first to study the proliferation of endothelial cells on bioactive nanostructured-HA 3D porous granules system. It is important to notice that these granules did not affect the endothelial cell response and, this cell type was able to adhere and proliferate through all the available surface, maintaining their phenotype and creating a network of tubular like blood vessels formation, using Matrigel (angiogenic assay) [47, 48]. In addition, the co-cultivation of HDMEC and HMSC on the nanostructured-HA macroporous granules clearly induced important parameters related to the angiogenesis process and HMSC commitment to osteoblastic lineage.

CONCLUSION

This work showed that nanostructured-HA granules presented excellent cytocompatibility properties, since they provided an adequate environment for both HMSC and HDMEC adhesion, migration and proliferation, maintaining the characteristic gene expression markers for each cell type, during the 21 days of culture. In addition, these HA granules appear not to affect the interactions between HMSC and HDMEC since, in co-culture conditions, evident positive effects for both

cell types were demonstrated. The current study highlights the good performance of these granules in supporting a co-culture system of endothelial and osteoblastic cells with evident positive effects in both angiogenic and osteogenic behaviour.

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CHAPTER V

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

General discussion and future perspectives

Recent studies on bioceramics have been developed using nanocrystalline particles of calcium phosphates, since bone is a natural composite of hydroxyapatite (HA) nano-particles and collagen fibers. These studies have shown that nanocrystalline HA shows better functional properties compared to microcrystalline HA, such as surface reactivity and ultra-fine structure, which are important to create suitable local conditions for bone formation when implanted in an osseous environment [1-4].

The first work done in the present thesis was to develop two different types of granules: nanostructured-HA and microstructured-HA, and the subsequent morphological and physical characterization was performed. The XRD results suggested that heat-treatment used in both type of granules nanostructured-HA and microstructured-HA revealed the presence of HA crystalline phase and no exogenous phases were present in the nanostructured-HA granules. FTIR analysis also showed HA characteristic spectra bands for all the materials studied [5-7]. So, the heat-treatments used allowed us to guarantee the presence of HA crystalline phase in the macroporous granules. Regarding the results related to the porosity, both type of granules presented a macroporous interconnected structure. Nanostructured-HA granules showed a higher microporosity and a larger surface area when compared to microstructured-HA granules. Nanostructured-HA and microstructured-HA granules showed an average macropore size of 141 μm and 133 μm , respectively, reaching sizes of 350 μm . Also, the spaces between nanoparticles agglomerates found in nanostructured-HA, may contribute to a very high microporosity in nanostructured-HA granules. It is reported in the literature that porous grafts should present macro and microporosity to guarantee cell viability, growth and function. Furthermore, other studies have showed that 100 μm is the minimum macropore diameter to ensure *in vivo* bone in-growth and regeneration [8, 9]. Furthermore, the interconnected macroporosity present in the granules should allow cell migration and an adequate vascular network formation and in-growth into the graft porous structure, what is known to be essential in nutrient delivery and waste exchange [9-12]. In addition, the polyurethane sponge method, used to produce the macroporous granules, produced

highly interconnected porosity with controllable pore size and reproducible macroporosity, requirements for adequate and fast bone regeneration. Moreover, the *in vitro* biological studies with MG-63 in both types of granules suggested a much higher cell proliferation taking place in nanostructured-HA granules, taking into account MTT and Resazurin results, SEM and CLSM observations. On the other hand, the expression of characteristic osteoblastic related genes (such as COL1, ALP, BMP2, OPG and M.CSF)[13-15], was clearly observed after six days of cell culture on both materials. An increase in cell proliferation was clearly observed in nano-HA granules in comparison with micro-HA granules, and identical gene expression has occurred on both materials. These results are in accordance with the literature, which reports a higher and increased osteoblasts adhesion, proliferation and differentiation on dense samples composed of nano-sized HA compared to similar samples of micro-sized-HA [16]. The cell behaviour observed in the present work and reported in the literature could be due to the greater surface area exhibited in the case of nano-HA and that might promote interactions such as adsorption, configuration, bioactivity and selective serum protein(s) adsorption[17].

After this first approach to characterize the granules, it was decided to design a suitable biological *in vitro* assay using cells from human tissues, to more closely approach the bone tissue environment. Recent studies revealed that bone vascular endothelial cells are members of the complex interactive communication network within bone microenvironment involving also osteoblasts, osteoclasts, macrophages and stromal cells [18], to maintain skeletal homeostasis in bone development and remodelling. It is well known that endothelial cells participate in the construction of a vascular network, necessary to adequate bone regeneration. Bone forming cells recruitment and growth are also essential for bone formation, repair and remodelling. As a result, co-cultures of endothelial and osteoblasts cells or their precursors can more closely mimic the natural bone regeneration, as compared to single cell cultures, since bone is composed by multiple cells with essential cell interactions going on, as referred above.

Endothelial cells and mesenchymal stem cells were chosen taking into account the biomaterial application and its characteristics. In addition, it is well known that angiogenesis and osteogenesis are essential for adequate bone regeneration, where the interactions between endothelial and osteoblasts or stem cells play a major role. Several works in the literature studied the interactions between these two cell types in co-culture systems, although no consensus on the optimal conditions for co-culture was reached. Different experimental conditions such as several cell ratios and culture media were described and most studies compared co-cultures with monocultures, in terms of endothelial or osteoblastic parameters, but few focused on both [19-26]. So, the second work emerged from the necessity to create an adequate co-culture system to further apply it on the porous granules.

In this second work an *in vitro* culture system was developed, in which both human dermal microvascular endothelial cells (HDMEC) and human mesenchymal stem cells (HMSC) viability, morphology and phenotype gene expression were ensured. For this, the time-dependent interactions between HDMEC and HMSC, in a long-term 2D co-culture system was reported in detail.

MTT and DNA quantification assays suggested that co-cultured cells achieved higher viability/proliferation rates, at least after 3 weeks of culture time. This might be related to cell interactions involving direct cell-to-cell contact or paracrine communication via soluble factors that are known to modulate cell growth [25, 27]. Several works in the literature studied the complex bidirectional communications between mesenchymal stem cells and endothelial cells, to understand how they interact [25, 28-31].

Greillier *et al* [25] proposed three different types of major ways of communication between these cells, such as direct cell-to-cell mechanisms (gap junction communications; adherens and tight junctions communications) and the secretion of diffusible factors that activate specific receptors on the target cells. Regarding the diffusible factors, several factors have been pointed as determinant in the interaction between these cells, such as BMP2 and VEGF [25].

In the present work, HMSC and cHMSC expressed genes typically related to osteoblast differentiation, such as COL1, ALP and RUNX2. Also ANGPT1, an angiogenic factor that modulates endothelial cell differentiation, was expressed by these cells. BMP2 was also expressed by HMSC and HDMEC, in monoculture and co-culture. HDMEC and cHDMEC expressed vWF and VE-cadherin that are important endothelial markers as well as ANGPT2 which plays a major role in the angiogenesis process. These results showed that osteoblastic and endothelial phenotypes were achieved and maintained in monoculture conditions. However, the genetic profile of cHMSC and cHDMEC, was distinct from that observed in the respective control monoculture, concerning the time-dependent expression and, in addition, most of the genes were over-expressed in co-culture conditions. cHMSC were able to over-express gene markers related to osteoblastic differentiation such as ALP, COL 1 and RUNX2. Regarding other osteoblastic markers, as the ability to form a mineralized extracellular matrix, the last event of osteoblast differentiation, alizarin red assay and SEM observation showed the presence of calcium phosphate deposits, with a clear induction in co-culture conditions. Furthermore, it is believed that BMP-2 and VEGF interaction had a primordial role in the HMSC osteoblastic differentiation, observed in the present work. BMP2 and VEGF were also over-expressed in co-culture conditions having a role in HMSC osteogenic differentiation, as described in the literature [32-35].

Different assays are used to study the angiogenic process. The most used models involve a 3D supportive matrix like Matrigel [36, 37], where endothelial cells are able to organize themselves in a network of tubular capillary-like structures. In this work, endothelial cells were seeded on Matrigel to evaluate the capacity of HDMEC to form capillary-like structures, that occurred after 3 days of culture. It seems that endothelial cells require a 3D matrix of extracellular components to organize themselves into these structures. Furthermore, the expression of genes associated with angiogenesis was not affected and these were over-expressed in co-culture conditions for some studied time-points.

In the third part of the work, the co-culture system developed was applied on the granules, to be used as a strategy for bone regeneration. Furthermore, taking into

account the available literature, co-cultured endothelial and osteoblasts or mesenchymal stem cells seem to be a potential tool in bone regeneration, since their incorporation into the biomaterial before implantation is expected to speed up the regeneration process [38, 39]. Also, the behaviour of co-cultured cells can be explored in 3D conditions with specific types of biomaterials, in order to know how the tested materials can affect the cellular interactions. Ideally, biomaterials should be porous, particularly to fill large bone defects as it is the case for our macroporous granules.

Taking these aspects into account, the phenotype behaviour of monocultured and co-cultured HDMEC and HMSC was addressed for the first time in macroporous granules of nano-hydroxyapatite.

Results regarding MTT assay and DNA quantification showed that cells seeded onto HA-granules exhibited a high viability and proliferation that increased with culture time, both in monoculture and co-culture conditions. Regarding the co-culture, significantly higher values were observed at day 21, compared to monocultures. Since the initial plating density was the same in both situations, this observation might be related with the over-expression of several important genes involved in the modulation of cell proliferation under co-culture conditions, such as BMP2 and VEGF-165 [25, 27].

The genetic profile of HDMEC and HMSC in monocultures and co-cultures, after the cell sorting of the two populations (cHDMEC and cHMSC), was also addressed. The genes were chosen regarding their relevance in the two phenotypes. COL1, ALP and RUNX2 play an important role in osteoblastic differentiation; BMP2 and VEGF-165 are described as being important growth factors for osteoblasts and endothelial cells, respectively; vWF and VE-cadherin are relevant endothelial markers, and angiopoietins (Ang) play a major role in the angiogenesis process. In general, the results showed that osteoblastic and endothelial phenotypes were achieved and maintained in monocultures and co-culture. Nevertheless, the genetic profile of co-cultured HMSC and HDMEC, after being sorted, was distinct from that observed in the respective control monoculture, regarding the level of expression and time-dependent profile.

Similarly to the previous work (the second work of the present thesis) COL1, ALP, RUNX2 and BMP2 were over-expressed in co-culture conditions. Also, the alizarin red assay and SEM observation showed a clear induction in the formation of mineralized calcium phosphate deposits in co-culture conditions, showing that HMSC were able to differentiate into osteoblasts phenotype.

This study showed that the co-culture of HDMEC and HMSC in nanostructured-HA 3D granules was successful, re-creating an adequate micro-environment for cell survival, proliferation and differentiation of the two phenotypes. Furthermore, to the best of our knowledge, this work is the first to study the proliferation of endothelial cells on bioactive nanostructured-HA 3D porous granules system. It is important to notice that these granules did not affected the endothelial cell response and, this cell type was able to adhere and proliferate through all the available surface, maintaining their phenotype and creating a network of tubular capillary-like structures, using Matrigel (angiogenic assay) [36, 37]. In addition, the co-cultivation of HDMEC and HMSC on the nanostructured-HA macroporous granules clearly induced important parameters (over-expression of the genes COL1, ALP and RUNX2) related to the angiogenesis process and HMSC commitment to osteoblastic lineage.

Future perspectives

The results achieved in the present work suggest that nano-structured HA granules may be considered a promising type of implants for bone regeneration and tissue engineering application, in which the granules can be pre-seeded with these two types of autologous cells, before bone graft implant. They can be potential candidates for bone fillers in large defects, so the next step should be the *in vivo* studies to demonstrate if nanostructured-HA granules accelerate new bone formation.

Due to its characteristics and high porosity, new applications may be given to these granules regarding the local release of drugs or other molecules. Macroporous HA granules can possibly be loaded with drugs to be released at adequately chosen sites. It is well known that systemic administration of drugs requires high dosages and periodic intakes to maintain efficient levels. Furthermore, bone tissue has a limited

blood circulation, and conventional systemic systems can have reduced effects. Localized delivery of drugs with sustained release can be an interesting approach to reduce side-effects.[40, 41]

Furthermore, its macroporous structure also allows the incorporation of polymers such as collagen type 1 in order to create new composites and possibly improve nanostructured-HA properties.

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