

Faculdade de Engenharia da Universidade do Porto



FEUP

**Nicotine effects on bone metabolism: in vitro
studies with human osteoclasts and co-cultures of
osteoclasts and osteoblasts in an hydroxyapatite
surface**

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RESUMO

O osso é uma estrutura que, apesar de rígida, é dinâmica e está em constante remodelação. Esta remodelação óssea é importante para regular e manter a massa óssea e envolve a reabsorção do material ósseo por acção dos osteoclastos e a síntese de novo material ósseo mediada pelos osteoblastos. Perturbações neste equilíbrio poderão estar na origem de várias condições patológicas.

O tabagismo está associado a vários efeitos nefastos, tais como perda óssea alveolar associada à doença periodontal, e atraso da regeneração óssea. Entre os componentes do fumo do tabaco, a nicotina parece ser a substância responsável pela maioria destes efeitos negativos ao nível do tecido ósseo.

Numa primeira parte deste estudo, foram avaliados os efeitos celulares e moleculares de uma gama de concentrações de nicotina representativa dos níveis presentes no plasma e saliva de consumidores de tabaco, em precursores osteoclásticos isolados ou em co-cultura dos mesmos com osteoblastos.

Os precursores osteoclásticos (PBMC) foram isolados a partir de sangue periférico humano. As células foram cultivadas isoladamente ou em co-cultura com células osteoblásticas MG63, na presença de nicotina (1.28×10^{-6} - 0.5mg/mL). As culturas de PBMC foram efectuadas na ausência (meio base, BM) ou na presença dos fatores recombinantes M-CSF e RANKL (M+R). Quando indicado, foram também adicionados ao meio de cultura, inibidores das vias de sinalização MEK e NFkB e um inibidor da produção de PGE2.

As culturas foram caracterizadas durante 21 dias, para a actividade da fosfatase ácida resistente ao tartarato (TRAP), presença de células multinucleadas positivas para a TRAP, presença de células com anéis de actina, receptores de vitronectina e de calcitonina, expressão de genes que especificam a linhagem osteoclástica e reabsorção de fosfato de cálcio.

Nas culturas de PBMC, as concentrações baixas de nicotina estimularam a osteoclastogénese. Este efeito foi observado quer em BM, quer em M+R. A presença da nicotina nas culturas celulares efectuadas em BM, realçou a importância da via de

sinalização MEK e da produção de PGE2 na osteoclastogénese. Na presença de M+R, a produção de PGE2 também aparentou ser *upregulated* pela nicotina. Nas co-culturas, a via de sinalização MEK e a produção de PGE2, parecem ser afetadas pela presença da nicotina.

Na segunda parte deste estudo, os precursores osteoclásticos, isolados ou em co-culturas com células osteoblásticas MG63, foram cultivados sobre discos de hidroxiapatite, que é um biomaterial que apresenta uma elevada biocompatibilidade. As culturas celulares foram expostas a uma gama de concentrações de nicotina representativa dos níveis plasmáticos e salivares, e posteriormente caracterizadas.

As PBMC (estimuladas ou não estimuladas), e as co-culturas MG63+PBMC foram cultivadas em discos de HA, e tratadas com nicotina numa gama de concentrações de 1.28×10^{-6} - 0.1mg/mL. As culturas celulares foram caracterizadas, através de marcadores de activação, diferenciação e actividade funcional das células osteoclásticas. Foi também realizado SEM no sentido de avaliar a atividade de reabsorção.

Os resultados obtidos foram semelhantes aos verificados nas placas de cultura de plástico, uma vez que nas culturas de PBMC, as concentrações baixas de nicotina estimularam a osteoclastogénese, enquanto as concentrações altas tiveram um efeito inibitório. Por outro lado, nas co-culturas, a nicotina diminuiu a osteoclastogénese de uma forma dependente da concentração.

Estes resultados sugerem que a nicotina tem a capacidade de actuar directamente nos precursores osteoclásticos, induzindo ou inibindo (concentrações baixas ou altas, respectivamente) a osteoclastogénese. Esta aparenta também afectar o desenvolvimento osteoclástico através de mecanismos indirectos, como por exemplo através das células osteoblásticas. Concluindo, a nicotina parece afetar a osteoclastogénese de diferentes formas, e o resultado destes efeitos pode levar a alterações significativas do metabolismo ósseo.

ABSTRACT

Bone is a rigid and dynamic mineralized tissue that undergoes a constant remodeling. Bone remodeling is important for the regulation and maintenance of skeletal mass, and it involves break down (resorption), mediated by osteoclasts, and build-up (synthesis), mediated by osteoblasts. Imbalances between bone formation and bone resorption may result in several diseases.

Smoking is associated with deleterious effects in bone metabolism, usually related to periodontal bone loss and delay in bone regeneration processes. Among the cigarette smoke components, nicotine appears to be a major responsible for these effects.

In the first part of this work, osteoclastic precursors, isolated (unstimulated and stimulated) or co-cultured with osteoblastic cells, were exposed to a range of salivary and plasmatic nicotine concentrations and the cellular and molecular effects were assessed.

Osteoclastic precursors (PBMC) were isolated from human peripheral blood. Cells were cultured isolated or co-cultured with osteoblast-like MG63 cells in the presence of 1.28×10^{-6} - 0.5mg/mL nicotine. PBMC cultures were performed in the absence (base medium, BM) or presence of recombinant M-CSF and RANKL (M+R). When indicated, MEK and NFkB signaling pathway inhibitors, and a PGE2 production blocker were also added to culture media. Cultures were tested throughout 21 days for tartarate-resistant acid phosphatase activity and staining, presence of cells with actin rings and expressing vitronectin and calcitonin receptors, expression of osteoclast-related genes and ability to resorb bone.

In PBMC cultures, nicotine stimulated osteoclastogenesis at low doses. This effect was observed either in BM or M+R. In cultures performed in BM, the presence of nicotine seemed to turn MEK pathway and the PGE2 production more important for osteoclastogenesis. In addition, in the presence of M+R, PGE2 production appeared to

be upregulated by nicotine. Finally, in the co-culture system, MEK pathway and PGE2 production seemed to be involved in the nicotine-induced osteoclastogenic response. In the second part of this work, osteoclastic precursors, isolated or co-cultured with osteoblastic cells were seeded over hidroxiapatite (HA) disks, which is a high biocompatible biomaterial. Cell cultures were exposed to a range of salivary and plasmatic nicotine concentrations and the cellular response was assessed.

PBMC (unstimulated or stimulated), and MG63+PBMC co-cultures were seeded over HA disks and were treated with 1.28×10^{-6} - 0.1mg/mL nicotine. Cell cultures were assessed for several markers associated with the differentiation, activation and functional activity of osteoclastic cells. Scanning electron microscopy was also performed to examine evidence of resorption.

The results were similar to the ones performed in regular tissue culture plastic wares, once in PBMC cultures nicotine stimulated osteoclastogenesis at low doses, and had a lethal effect at high doses. On the other hand, in co-cultures, nicotine decreased osteoclastogenesis, in a concentration-dependent way.

These results suggest that nicotine has the ability to act directly on osteoclastic precursors, inducing or inhibiting (low or high concentrations, respectively) osteoclastogenesis. Furthermore, it may also affect osteoclast development through indirect mechanisms, that is, *via* osteoblastic cells. Thus, nicotine can affect osteoclastogenesis by different ways and the interplay between these effects can ultimately lead to significant changes in bone metabolism.

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LIST OF ABBREVIATIONS

α -MEM - α -minimal essential medium
ALP - Alkaline phosphatase
ARRF - Activation - resorption - reversal - formation
BM - Base medium
BMP - Bone morphogenetic protein
BMU - Basic multicellular unit
CA2 - Carbonic anhydrase II
CATK - Cathepsin K
Cbfa1 - Core binding factor α 1
CLSM - Confocal laser scanning microscopy
COX - Cyclooxygenase
CTR - Calcitonin receptor
EDTA - Ácido etilenodiamino tetra-acético
ERKs - Extracellularly regulated kinases
GADPH - Glyceraldehyde 3-phosphate dehydrogenase
GH - Growth hormone
GM - CSF - Granulocyte-macrophage colony stimulating factor
HA - Hydroxyapatite
IGF - Insulin-like growth factor
IL - Interleukin
MAP - Mitogen-activated protein
MAPK - Mitogen-activated protein kinase
MEK - MAPK-ERK kinase
M-CSF - Macrophage colony stimulating factor
MMP - Matrix metalloproteinases
M+R - Recombinant M-CSF and RANKL
NF- κ B - Nuclear factor kappa B
OPG - Osteoprotegerin
OSX - Osterix
PBMC - Peripheral blood mononuclear cells
PDTC - Pyrrolidine dithiocarbamate
PG - Prostaglandins
PGE2 - prostaglandin E2
PM - Proliferative monocyte
pNPP - *para*-nitrophenylphosphate
PTH - Parathyroid hormone
RANK - Receptor for activation of NF- κ B
RANKL - RANK ligand
RT-PCR - Reverse transcription polymerase chain reaction

SEM - Scanning electron microscope
TGF- α - - Transforming growth factor - alpha
TGF- β - Transforming growth factor-beta
TNF - Tumor necrosis factor
TRAF - TNF receptor-associated factor
TRAP - Tartrate-resistant acid phosphatase
U0126 - 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene
VEGF - Vascular endothelial growth factor
VN - Vitronectin
VNR - Vitronectin receptor

CHAPTER 1

Chapter1 - State of art

1. Bone Tissue

Bone is a unique material that confers multiple mechanical and metabolic functions to the skeleton and has a ceaseless activity during organisms live. Bone structure is being study in detail since the appearance of light microscope 300 years ago[1]. Innumerous studies until now revealed that bone tissue has a complex hierarchically organized structure that allows them to respond to the different functional requirements for which they are responsible[1, 2].

Thus, bone is a specialized connective tissue, with a biphasic structure composed by an inorganic extracellular phase and an organic phase, which comprises bone cells and organic extracellular matrix, and to accomplish is role as the load-bearing structure of the body, it is formed from a combination of dense compact bone and trabecular bone that is re-inforced at points of stress[2, 3].

1.1- Bone Structure

The two major classes of bone tissue that significantly contribute to the structural strength of the skeletal system are called: cortical or compact bone which is a dense tissue found on the outer surface and cancellous, trabecular or spongy bone which is less compact, has a soft, spongy texture, and can be found in the more intern areas of the bone.

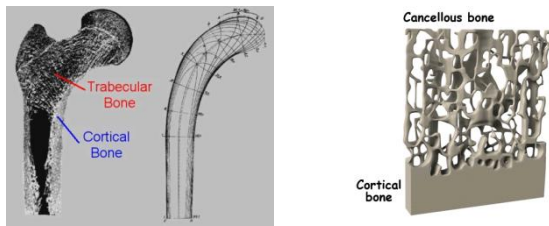


Figure 1 - Trabecular and cortical bone. (http://www.feppd.org/ICB-dent/campus/biomechanics_in_dentistry/ldv_data/img/bone_12.jpg and http://patientsites.com/media/img/722/sacral_insufficiency_anatomy02.jpg).

Cortical bone is mainly found in the shaft of long bones and the surfaces of flat bones and is highly compact due to the circular nature of the osteons or Haversian systems that make up this structure. Within each Haversian system, there is a central canal called Haversian canal, where blood vessels, lymphatics, nerves and connective tissue can be found. Each Haversian canal is surrounded by concentric layer of the matrix material called rings or lamellae. Within the lamella is a network of osteocytes embedded in lacunae and connected by canaliculi.

Osteons are also formed by another important element that is collagen fibers. In cortical bone these are disposed in a concentric form, forming cylindrical structures, and giving to this type of bone a unique feature.

So, the Haversian canal, the lacunae, and the canaliculi within an osteon are all connected to each other, and by lateral passages called Volkmann's canals between the Haversian canals, allowing adjacent osteons to communicate with each other. Consequently the osteocytes with the lacunae are in communication with each other and with a supply of nutrients.

This specific anatomical distribution of cortical bone is therefore optimized to handle compressive and bending forces.

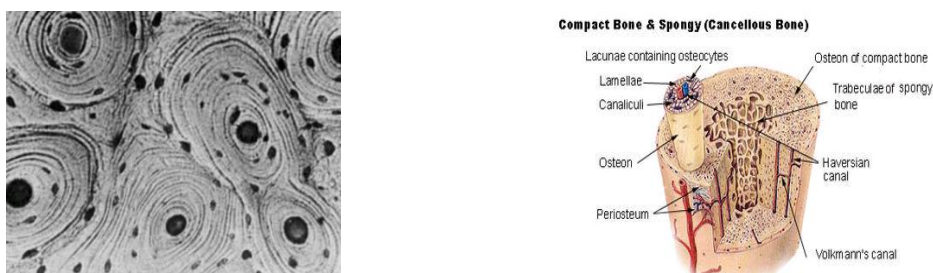


Figure 2 - Lamellar structure of osteons in cortical bone .Cortical bone is the more dense tissue usually found on the surface of bones. It is organized in cylindrical shaped elements called *osteons* composed of concentric lamellae. (http://www.feppd.org/ICB-campus/biomechanics_in_dentistry/ldv_data/img/bone_10.JPG and http://upload.wikimedia.org/Wikipedia/commons/thumb/3/34/Illu_compact_spongy_bone.jpg/500px-Illu_compact_spongy_bone.jpg)

Trabecular or Cancellous bone is another type of bone structure organized in a porous sponge-like pattern [2]. This kind of bone is less numerous and forms the ends of long

bones and the inner parts of flat bones[4]. It consists of a network of hard, interconnected filaments called "trabeculae" interspersed with marrow and a large number of small blood vessels [5]. Trabeculae are aligned along lines of stress; this connectivity adds considerably to its strength[4]. Contrasting with cortical bone, collagen fibers in trabecular bone are parallel to one another.

This spongy mesh-like bone is then ideally suited to withstanding compressive stress and hence is the predominant bone in vertebrae[4].

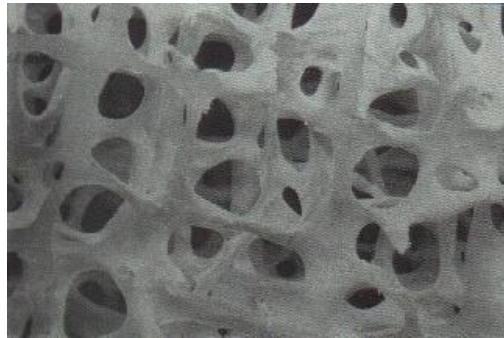


Figure 3 - Trabecular bone. Trabecular bone is quite porous and it is organized in *trabecules* oriented according to the direction of the physiological load. The configuration of the trabecular structures is highly variable and it depends on the anatomical site. (<http://www.medstudents.com.br/endoc/img/endoc6f2.jpg>).

Woven bone usually laid down very quickly, most characteristically in the fetus and in the callus that is produced during fracture repair. This type of bone has a random arrangement of collagen fibres, with non-uniformly sized and distributed osteocytes. In general each bone has an outer layer of cortical bone overlying trabecular bone and the medullary cavity[4, 6].

The cortical bone has an outer membrane called the periosteum. The periosteum has two layers: an outer fibrous layer and an inner one, which has osteogenic potential and lays down new bone allowing the bone to enlarge, a process known as periosteal apposition. The inner surface of the cortex has another lining called the endosteum[4] (Fig 4).

Concluding the specific anatomical distribution of cortical and trabecular bone reflects their respective compressive and tensile strengths. Thus trabecular bone is ideally suited to withstanding compressive stress and hence is the predominant bone in vertebrae.

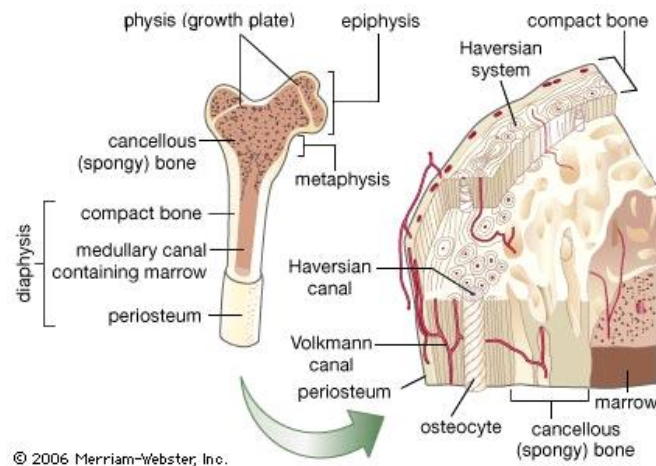


Figure 4 - Adult bone Structure (<http://media-2.web.britannica.com/eb-media/27/72127-035-FE2CB14F.jpg>)

1.2- Inorganic Phase

The inorganic or mineralized phase of the skeleton contributes about two-thirds of its weight and it is mainly composed by calcium phosphates, where hidroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is the most important of them, and this phase is also characterized by the presence of numerous foreign ions [7, 8]. Among the minor mineral constituents of bone is magnesium, sodium, potassium, and a number of trace elements (zinc, manganese, fluoride, and molybdenum) considered to be adsorbed on the bone surface, although it is not known whether these trace elements have a role in skeletal metabolism or merely represent adsorption resulting from their presence in foodstuffs[8].

Due to the characteristics mentioned above the inorganic phase was reported to have two major biological functions. The first function is characterized by the tiny crystals of alkaline mineral hidroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ that enclose the collagen fibrils forming a composite material with the required properties of stiffness, flexibility and strength. With these properties this composite material is able to resist to the external and internal forces to which it is subjected during gait, prehension, etc, and is able to preserve the shape of the organism as a whole and to protect vital organs such as the bone and lungs[3, 9]. The second function of the inorganic phase acts as an ion reservoir, and consequently plays a critical role in maintaining the concentrations of these ions in the extracellular fluid, which in turn is vital for a number of important biochemical reactions and physiological activities[9].

1.3- Organic Phase

The organic phase of bone comprises 25-30% of the total matrix and plays a wide variety of roles, influencing profoundly the structure and also the mechanical and biochemical properties of the tissue[10, 11]. Collagen constitutes approximately 90% of the organic matrix and is classified into several different types, with only Type I collagen being pertinent to the structure of bone[3]. Collagen have a quaternary structure, constituted by three α -chains that are interconnected and twist on each other in a right-handed way. The triple helix motif is composed of three polypeptide chains whose amino acid sequence consists of Gly-X-Y repeats. In about one third of the cases X is a proline and Y is a hydroxyproline, being the presence of hydroxyproline essential to stabilize the triple helix and a unique characteristic of collagen molecules [12].

Collagen in bone undergoes a calcification process and becomes hard, unlike the collagen in our skin and tendons. This calcification process is a result of collagen fibrils interacting with hydroxyapatite crystals in the extracellular matrix of bone. In addition to Type I collagen, other organic constituents of the matrix include various proteins and polysaccharides, which, along with Type I collagen, also function in the bone calcification process[13].

The remaining 10% of the organic matrix is composed a complex assortment of smaller, non-structural proteins, including osteonectin, osteocalcin, phosphoproteins, sialoprotein, growth factors and blood proteins[3].

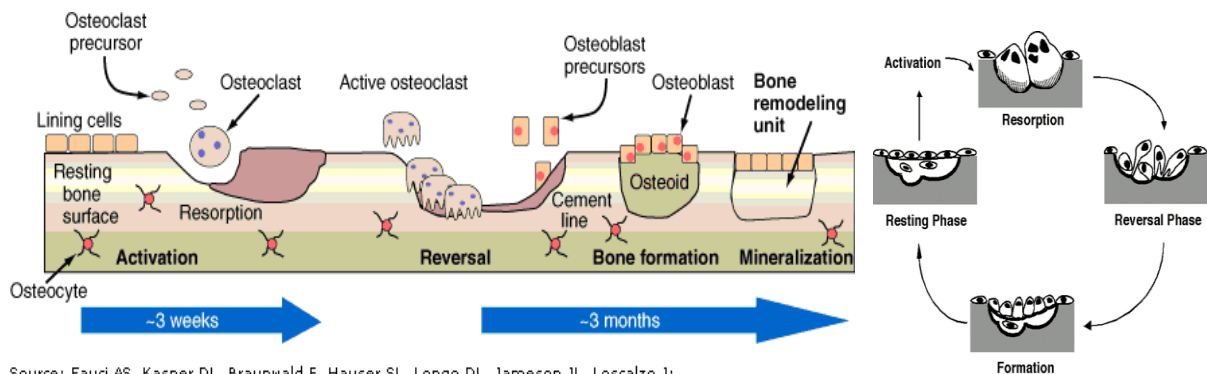
1.3.1- Bone cells and Bone Remodeling

The elaboration, maintenance and resorption of bone tissue results from the interaction of three cell types: osteoblasts, osteocytes and osteoclasts[5, 10, 14, 15]. All of them have defined tasks and are thus essential for the maintenance of a healthy bone tissue, being involved in a process called bone remodeling, which is important for the regulation and maintenance of skeletal mass[10, 16]. The coordinated action of these cells is described as the 'basic multicellular unit' (BMU), and the activity of the unit is regulated by mechanical forces, bone cell turnover, hormones (e.g. parathyroid hormone (PTH), growth hormone (GH)), cytokines and local factors[4]. Thus bone remodeling occurs in BMUs which contain as previously revered, different cell types with different origins and specific functions related to bone metabolism[16]:

- Osteoprogenitor cells, which ultimately differentiates into osteoblasts and osteocytes derived themselves from primitive stem cells;

- Osteoblasts, the cells that produce the bone extracellular mineralized matrix;
- Osteocytes, which are osteoblasts completely embedded in bone matrix and are isolated in lacunae;
- Osteoclasts, the exclusive bone-resorptive cells, line the leading region of the BMU;

Thus bone remodeling (Figure 5) is by far the more active process, reflecting the process of “real-time” tissue replacement during bone turnover and repair, and it involves break down (resorption), mediated by osteoclasts, and build-up (synthesis), mediated by osteoblasts[15-17] . This cyclic sequence is also called the activation-resorption-reversal-formation (ARRF) sequence and takes about 3-6 months to be completed in humans[15]. Activation results in the lining cells of the endosteal surface being retracted and digestion by matrix metalloproteinases of the endosteal collagenous membrane. Osteoclasts are then recruited, followed by fusion of activated osteoclasts to become multinucleated osteoclasts. Activated osteoclasts mediate resorption of the underlying bone. Subsequently, osteoblasts are recruited to the resorption cavity and lay down new osteoid, which eventually becomes calcified. The rate of bone turnover varies according to the type of bone, being highest in sites where trabecular bone predominates such as vertebrae and lowest at sites high in cortical bone such as the hip [4, 14]



Source: Fauci AS, Kasper DL, Braunwald E, Hauser SL, Longo DL, Jameson JL, Loscalzo J: *Harrison's Principles of Internal Medicine*, 17th Edition: <http://www.accessmedicine.com>
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Figure 5 - Schematic representation of bone remodeling process. The cycle of bone remodeling is carried out by the basic multicellular unit (BMU). Bone is then continuously remodeled at these discrete sites in the skeleton in order to maintain the integrity of the tissue. First, osteoclasts are activated, and the resorption phase takes approximately 10 days. Osteoblast precursors are then recruited, proliferate and differentiate into mature osteoblasts. They produce bone mineralized matrix, ending the remodeling process. (<http://www.endotext.org/parathyroid/parathyroid1/figures/figure6.jpg>).

2. Osteoblast

Osteoblasts are the cells responsible for depositing the proteinaceous and calcified extracellular matrix and secreting the growth factors necessary for osteogenesis [18]. These cells differentiate from mesenchymal stem cells.

Upon termination of bone matrix synthesis, osteoblasts can either undergo cell death by apoptosis (programmed cell death) or differentiate into osteocytes or bone lining cells, which are inactive osteoblasts [19]. Osteocytes are the most abundant cell type in bone and are thought to act as mechanosensors, regulating osteoclast and osteoblast activities [20].

Osteoblasts are thought to regulate the local concentrations of calcium and phosphate in such a way as to promote the formation of hydroxyapatite. Osteoblasts express relatively high amounts of alkaline phosphatase. Alkaline phosphatase has been long thought to play a role in bone mineralization [21].

In the process of maturation of osteoblasts, genes like core-binding factor $\alpha 1$ (Cbfa1) and osterix (Osx) play a very important role. Osteoblasts have also a role in the regulation of bone resorption through RANKL expression, that binds to its receptor, RANK, on the surface of pre-osteoclast cells, inducing their differentiation and fusion. This interaction is essential and, together with M-CSF, sufficient for osteoclastogenesis *in vitro* [22]. On the other hand, osteoblasts secrete a disulfide-linked dimeric glycoprotein (osteoprotegerin, OPG) that has a very inhibitory effect on osteoclastogenesis and bone resorption [20, 21].

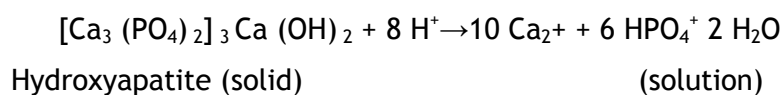
3. Osteoclasts characteristics and function

Osteoclasts play an essential role in bone homeostasis. They are cells that descend from a monocyte/macrophage CD14⁺ lineage and are responsible for the function of resorbing bone matrix. They are multinucleated cells, formed by fusion of their mononuclear precursors derived from the pluripotential haematopoietic stem cells, and subsequent differentiation, at or near the bone surface, in a process named osteoclastogenesis [23-25]. Osteoclast differentiation is primarily controlled by the stromal/osteoblast-derived proteins, macrophage colony stimulating factor (M-CSF), a polypeptide growth factor, and RANKL [22].

Osteoclastogenesis has been shown to be strongly dependent on two growth factors, namely M-CSF (monocyte-colony stimulation factor) and RANKL (receptor activator of nuclear factor- κ B ligand) [4, 22, 23]. M-CSF plays a key role along several osteoclastogenic steps, mainly in proliferation of the osteoclast progenitors. M-CSF leads to the fusion of the osteoclasts progenitor cells, originating multinucleated osteoclasts [4]. RANKL is a

homodimeric glycoprotein, member of the tumor necrosis factor (TNF) family that also has a key role in osteoclastogenesis. RANKL binds to its receptor, RANK, on the surface of osteoclastic precursor cells, and this RANK/RANKL interaction, together with M-CSF, induce the expression of genes that typify the osteoclast lineage, including calcitonin receptor (CTR), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), carbonic anhydrase II (CA2) and B3 class of integrins, leading to development of mature osteoclasts[22]

The mature, multinucleated osteoclast is activated by signals, which leads to initiation of bone remodeling [22]. The osteoclast cell body is polarized, and in response to activation of RANK by its ligand it undergoes internal structural changes that prepare it to resorb bone. To resorb bone effectively, osteoclasts must attach themselves firmly to the bone surface using specialized actin-rich podosomes. By means of these podosomes, they form tight seals with the underlying bone matrix in roughly circular extensions of their cytoplasm[26]. Thus, when osteoclasts are polarized and have formed sealing zone a high number of intracellular acidic vesicles are targeted to plasma membrane inside the sealing zone to form the ruffled border membrane. The fusion of intracellular acidic vesicles to the plasma membrane releases a large quantity of acid into resorption lacuna and initiates mineral dissolution[27]. So resorption lacuna is acidified by the export of hydrogen ions mediated by H⁺-ATPase [28] (Figure 6). The cytoplasm of the osteoclast is very rich in mitochondria, the source of ATP required to drive the H⁺ pumps[29]. The source of the protons for acid secretion is carbonic acid; its slow spontaneous generation from CO₂ produced by oxidative phosphorylation is augmented by expression of CA2 in osteoclasts[30]. Osteoclastic acid transport deposits H⁺ onto the mineralized matrix which leads to the release of calcium into the resorption lacuna, according to the following equation[31].



Resorption continues with the export of lytic enzymes (TRAP and pro-CATK) into the resorption lacuna [32]. Through this process the osteoclast erodes the underlying bone [22]. Degradation products of collagen and other matrix proteins, produced by the action of the protease CATK, are transported by transcytosis to the basolateral membrane and released into the circulation[33]. However, the mechanisms involved in disposal of Ca²⁺

and phosphate, which accumulate within the resorption lacuna, are still unclear [4, 22]. Upon resorption, bone-stored growth factors, such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), and transforming growth factor-betas (TGF- β), which are embedded within the calcified matrix, are released and activated [34]. These local factors, along with the systemic factors parathyroid hormone (PTH), estrogens, and prostaglandins, are constantly released in the bone marrow cavity by osteoclastic bone resorption, and recruit new osteoblasts to fill the gap created by osteoclasts.

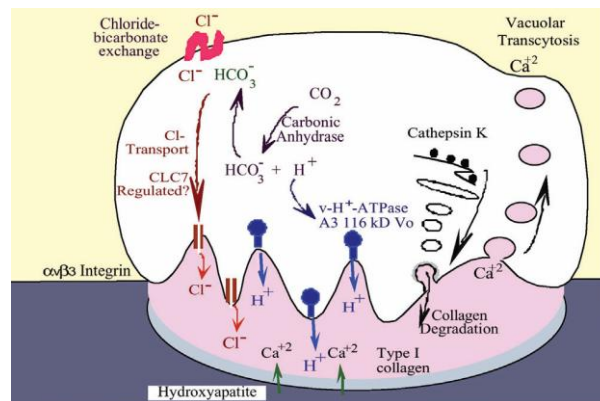


Figure 6 - The acid-transport pathway of the osteoclast. The osteoclast consumes large amounts of energy to drive HCl secretion, which produces calcium, water, and phosphate from the strongly basic calcium salt hydroxyapatite, $[\text{Ca}_3(\text{PO}_4)_2]_3\text{CaOH}_2$, that comprises bone mineral. The source of the energy is oxidative phosphorylation of glucose in mitochondria. Protons are derived from carbonic acid, a process facilitated by high expression of carbonic anhydrase II in osteoclasts (Source: [35]).

3.1- Osteoclasts precursors

Until a few years ago, very little was known about the molecular events that occur during osteoclast differentiation and osteoclastic bone resorption. This lack of knowledge was due to the osteoclasts fragility when isolated from bone, the difficult to isolate them because they are embedded in a calcified matrix and the few number of osteoclast that exist [24, 36]. The development of several techniques over the years allowed some knowledge in the identification of osteoclast precursors, the characterization of their surface phenotype, the identification and characterization of factors that affect osteoclast activity and formation [24, 36].

Fujikawa *et al.* [37] reported that macrophages and osteoclasts are derived from the same population of haematopoietic precursors. Mononuclear precursors of osteoclasts circulate in the monocyte fraction and express a monocyte/macrophage phenotype, acquiring

osteoclast phenotypic features in the process of differentiation into mature functional osteoclasts[27].

It is also known that osteoclast precursors reside in spleen, bone marrow and peripheral blood mononuclear cells (PBMC) [16, 38]. PBMC are heterogeneous populations that are judged by criteria such as surface marker expression, size and function[39, 40]. They comprise lymphocytes (T, B and natural killer cells) and monocytes/macrophages. These cells are found within the circulating blood and are not sequestered within the lymphatic system, spleen, liver or bone marrow[41].

A research conducted by Quinn *et al.*[42] suggested that osteoclasts are derived from monocytes that expressed the macrophage associated antigen CD14 (CD14+) [43]. Based on these results Massey *et al.* [44]conducted a study where he only observed osteoclast formation in the CD14+ population from PBMC, in the presence of both M-CSF and RANKL, confirming that the CD14+ system is a pure population of osteoclast precursors. He also noticed that no expression of osteoclast markers was observed in the absence of RANKL, whereas RANKL dose dependently induced the expression of cathepsin K, tartrate-resistant acid phosphatase (TRAP), and matrix metallo proteinase (MMP)-9.

Human monocytes are commonly considered to be non-proliferating [45]; however several studies have defined a subpopulation of human monocytes which is capable of proliferating *in vitro*[40, 46, 47]. During the phenotyping studies, Clanchy *et al.* [47] noticed that, following culture and sorting by flow cytometry, the proliferative monocytes (PM), could give rise to tartrate-resistant acid phosphatase-positive (TRAP+) multinucleated cells upon culture with M-CSF and RANKL. Based on this preliminary observation, and the likelihood that the PMs represent a relatively less mature monocyte population on account of their ability to proliferate, it was reasoned that they may retain differentiation capability and therefore be osteoclasts precursors [40].Recently, it was found that osteoclasts precursors derive from CD14+CD16- monocyte subpopulation[40, 48, 49].

Although until now much has been done in order to understand the origin of the osteoclast and the factors that affect its differentiation. Many results have been obtained from these studies, but there is still a lot to be discovered. For example in mouse, monocyte subpopulations appeared to have different roles during inflammatory reactions (manifested, for example, by their ability to migrate to sites of inflammation) making this achievements interesting to further study monocyte subpopulations in humans[40, 50].

3.2- Osteoclastogenesis

A significant breakthrough in our understanding of osteoclastogenesis occurred when murine systems using co-cultures of bone marrow or spleen cells and stromal cells yielded

osteoclasts. The close contact between stromal and bone marrow cell types was essential for osteoclastogenesis, and suggested that stromal-derived factors stimulate this process. It is now known that this system allowed for production of two haematopoietic factors that are both necessary and sufficient for osteoclastogenesis, the M-CSF (monocyte-colony stimulation factor) and RANKL (receptor activator of nuclear factor- κ B ligand) [4, 22, 23]. While M-CSF is thought to be critical for the proliferation of the osteoclast progenitors, RANKL is critical for precursor differentiation into mature, multinucleated osteoclasts[4, 51].

M-CSF is a homodimeric glycoprotein produced by several cell types, like, for example, granulocytes, endothelial cells, fibroblasts, osteoblasts, lymphocytes and some tumoral cell lines[23]. M-CSF expression by osteoblastic stromal cells is required for progenitor cells to differentiate into osteoclasts, but M-CSF on its own is unable to complete this process[26]. This requirement for M-CSF was discovered by the observation that the absence of a functional M-CSF in the *op/op* mouse causes not only a macrophage deficiency but also a lack of osteoclasts, resulting in an osteopetrotic phenotype [24, 26, 52-54]. A subsequent study conducted by Sundquist *et al.*[55] , revealed that although injection into *op/op* mice improves the skeletal sclerosis in these animals, the presence of osteoclasts in some skeletal sites in untreated *op* mice, demonstrates that the development of some osteoclasts is independent of M-CSF. Additionally Nilsson *et al.*[56] showed that the osteopetrosis and hematopoietic deficiencies evident in young *op/op* mice, are not permanent but are progressively corrected with age. Furthermore, was found that although M-CSF is necessary for entry of precursors into the early preosteoclasts pathway, it inhibit osteoclastogenesis at high doses [53].

Although M-CSF is a secreted product, osteoclastogenesis requires contact between osteoclast precursors and stromal cells or osteoblasts. Thus, stromal cells and osteoblasts synthesize a surface-residing molecule, essential for osteoclastogenesis, whose identity long remained enigmatic. The unknown factor turned out to be RANK-L, and the receptor on osteoclasts and their precursors was subsequently identified as RANK [16, 24, 57, 58].

RANKL also known as TNF-related activation-inducing cytokine (TRANCE), is a type II transmembrane protein, member of the tumor necrosis factor (TNF) family[22, 23]. RANKL was initially identified as a cytokine produced by T cells and needed for their interaction with dendritic cells and later it was found that this protein mediates the differentiation of T and B lymphocytes. RANKL is produced as a membrane bound protein in osteoblasts, bone marrow stromal cells, activated T lymphocytes and smooth muscle cells and cleaved into a soluble form by a metalloprotease [20]. RANKL expression by osteoblasts coordinates bone remodelling by stimulating bone resorption by local osteoclasts, which in

turn stimulate bone synthesis by closely adjacent osteoblasts by a process called 'coupling'. RANKL has two receptors, one is RANK initially isolated from the dendritic cell cDNA library¹⁰ and the other is the osteoprotegerin (OPG)/ osteoclastogenesis inhibitory factor (OCIF)[58].

RANKL binds to its receptor, RANK, on the surface of osteoclastic precursor cells, and this RANK/RANKL interaction, together with M-CSF, induce the expression of genes that typify the osteoclast lineage, including calcitonin receptor (CTR), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), carbonic anhydrase II (CA2) and $\beta 3$ class of integrins, leading to development of mature osteoclasts[22, 23]. A major breakthrough on understanding of osteoclastogenesis process was achieved with the identification of the other receptor OPG, that is a soluble protein member of the TNF receptor superfamily that blocks osteoclast formation *in vitro* and bone resorption *in vivo*[22, 24]. Thus RANKL activity can be blocked by the decoy receptor OPG, because this is able to bind to its respective ligand, blocking RANK/RANKL interaction and, thus, preventing osteoclast differentiation and activation. In vitro and in vivo osteoclast differentiation from precursor cells is blocked in a dose-dependent manner by recombinant OPG[24]. Indeed, over expression of OPG in mice resulted in osteopetrosis, due to inhibition of osteoclast maturation, whereas OPG-deficient mice exhibit osteoporosis [20, 21]. Expression of RANKL and OPG is therefore coordinated to regulate bone resorption and density positively and negatively by controlling the activation state of RANK on osteoclasts [22].

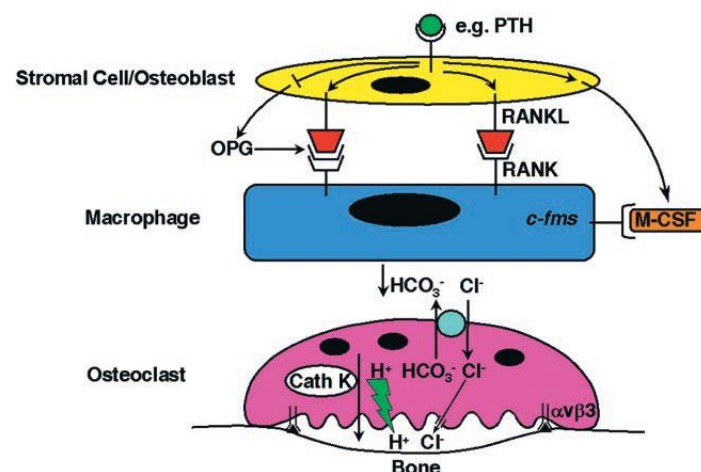


Figure 7 - Mechanisms of osteoclastogenesis and osteoclastic bone resorption. Stromal cells and osteoblasts express RANKL and M-CSF, which are up-regulated by osteoclastogenic molecules, for example PTH. RANKL and M-CSF, interacting with their receptors on monocyte/macrophage cells, induce commitment to the osteoclast phenotype, a process inhibited by OPG.(SOURCE: [57])

Thus RANK/RANKL interaction activates several signaling pathways which are crucial for osteoclastogenesis[23]. This RANK/RANKL intracellular interaction induces recruitment and activation of cytoplasmic tumor necrosis factor receptor-associated factors (TRAFs), leading to the activation of multiple signaling cascades such as MAP, NF- κ B, Src, and Akt.

Among the several TRAF proteins, TRAF-6 is the most important, because is necessary for RANK-induced NF- κ B activation and *in vitro* osteoclastogenesis, and its deletion leads to osteopetrosis[53].

The most notably activated pathway by RANK is NF- κ B. NF κ B is a family of transcription factors composed of five polypeptide subunits and plays a critical role in expression of a variety of cytokines involved in osteoclast differentiation, including IL-1, TNF- α , IL-6, GM-CSF, and other growth factors. It was reported that mice deficient in both the p50 and p52 subunits of NF κ B develop osteopetrosis due to a severe defect in osteoclast differentiation. Thus deletion of NF- κ B subunits, may affect the production of growth factors that are critical for osteoclast differentiation [24, 53, 59]. PDTC (Pyrrolidine dithiocarbamate) has been reported has a potent NF- κ B inhibitor, due to its efficacy and good tolerability presented in numerous *in vivo* studies of inflammation[60]. PDTC acts by stabilizing cytosolic I κ B- α thus preventing the activation and nuclear translocation of the NF- κ B transcription factor. Due to PDTC proprieties Strait *et al.* [59] conducted a study where they tested if the use of PDTC was an effective approach for ameliorating bone loss induced by estrogen deficiency by suppressing osteoclastic bone resorption, concluding that the use of NF- κ B inhibitors may represent novel anticatabolic therapeutic agents for the amelioration of postmenopausal bone loss.

MAPK (mitogen-activated protein kinases) signaling pathways have been also suggested to be critical for osteoclast differentiation [61]. MAPKs play a role in mediating intracellular signal transduction and regulating cytokine production by mononuclear cells in response to a variety of extracellular stimuli. In response to appropriate stimuli, the MAPKs are activated by phosphorylation on both adjacent threonine and tyrosine residues that are separated by a single amino acid. For extracellularly regulated kinases (ERKs), the best studied of MAPK families, this intervening amino acid is glutamate; for the p38 MAPK family, it is a glycine. Whereas ERK has been associated classically with growth- and differentiation-inducing signals, p38 MAPK is involved in inflammatory cytokines and environmental stress inducers [62]. The involvement of MAPKs in osteoclast differentiation have been studied using specific inhibitors of MAPK-ERK kinase (MEK). The ERK-1 kinase is activated by RANK signalling, and seems to be regulated upstream by activation of MEK . The small molecule ERK inhibitor U0126 potentiate RANKL induced osteoclast differentiation, suggesting that the ERK pathway is involved in negative regulation of osteoclastogenesis [22].

Also Prostaglandins (PGs) are likely to play an important role in the physiologic and pathologic responses of skeletal tissue. They are potent agonists that can stimulate and inhibit bone resorption and formation. Cyclooxygenase (COX) is the rate-limiting enzyme in

the conversion of arachidonic acid to PGs. There are two forms of COX, COX-1 and COX-2. COX-2 is an inducible primary-response or immediate early gene. COX-2 expression is induced by many factors through the transcriptional pathway in osteoblasts. Stimulated production of PGs by osteoblasts requires both the induction of COX-2 expression and the availability of arachidonic acid substrate[63, 64]. PGs are complex, potent regulators of bone cell function in vivo, being implicated in bone resorption associated with inflammation and metastatic bone disease, and in bone formation associated with fracture healing and heterotopic ossification [63, 65]. The exogenous prostaglandin E2 seems to stimulate bone resorption and formation. Indomethacin, is a prostaglandin inhibitor, and has been used in several studies in order to clarify the specific pathways of PGs action in bone [66, 67].

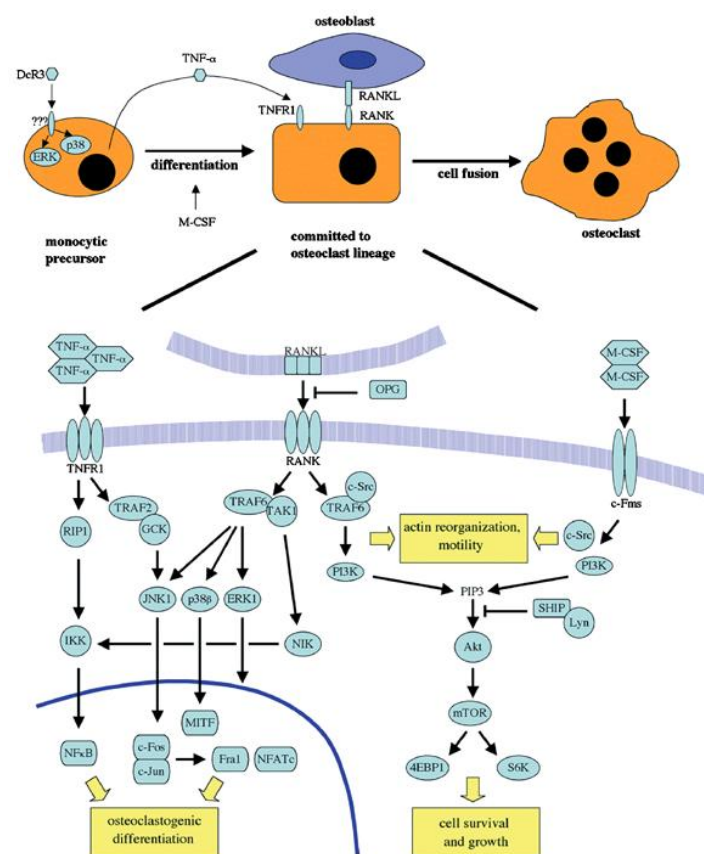


Figure 8 - Signaling pathways involved on osteoclastogenic differentiation. Binding of RANKL to RANK commits monocytic precursor cells to the osteoclastic lineage. Activation of RANK (which is prevented by OPG binding to RANKL) results in the recruitment of adaptor molecules, TRAFs and activation of several downstream signaling pathways.

4. Biomaterials

Biomaterials, traditionally defined as materials (synthetic and natural; solid and sometimes liquid) that are used in medical devices or in contact with biological systems, have been used since antiquity, but recently their degree of sophistication has increased significantly[68].

One of the primary reasons that biomaterials are used is to physically replace hard or soft tissues that have become damaged or destroyed through some pathological process[69]. For example in case of bone diseases such as osteogenesis imperfecta, osteoarthritis, osteomyelitis, and osteoporosis in which bone does not function, the treatment will involve bone repair or replacement. Thus the primary purpose of a tissue-engineered scaffold is to use engineering principles to incite and promote the natural healing process of bone which does not occur in these critical-sized defects[70]. Tissue-engineered biomaterials are biologic-material combinations in which some component of tissue (e.g. cytokines, growth factors, and cells) have been combined with a material to create a device for the restoration or modification of tissue or organ function[71].

Different biomaterials, including bioceramics, biopolymers, metals, and composites have been used in bone tissue engineering, to form bone scaffolds[72]. An ideal synthetic scaffold must be capable of presenting a physiochemical biomimetic environment while biodegrading as native tissue integrates and actively promote or prevent desirable and undesirable physiological responses, respectively. Thus a synthetic bone scaffold must be able to provide temporary mechanical support to the affected area, act as a substrate for osteoid deposition, contain a porous architecture to allow for vascularization and bone in-growth, encourage bone cell migration into the scaffold, support and promote osteogenic differentiation in the non-osseous, synthetic scaffold (osteinduction), enhance cellular activity towards scaffold-host tissue integration (osseointegration), degrade in a controlled manner to facilitate load transfer to developing bone, produce non-toxic degradation products, not incite an active chronic inflammatory response, be capable of sterilization without loss of bioactivity and deliver bioactive molecules or drugs in a controlled manner to accelerate healing and prevent pathology[70].

Although biomaterials are primarily used for medical applications, they are also used to grow cells in culture, to assay for blood proteins in the clinical laboratory, in processing biomolecules in biotechnology, for fertility regulation implants in cattle, among others. The commonality of these applications is the interaction between biological systems and synthetic or modified natural materials.

Thus, biomaterials have a vast scale of applications medical and non-medical (Table 1).

Medical uses	Non-medical uses
Artery graft	Arrays for DNA and diagnostics
Breast implant	Bioremediation materials
Cochlear implant	Biosensors
Ear drainage tube	Bioseparations, chromatography
Dental implant	Biofouling-resistant materials
Feeding tube	Biomimetics for new materials
Glaucoma drainage tube	Cell culture
Hydrocephalous shunt	Controlled release for agriculture
Intraocular lens	Electrophoresis materials
Joints (hip, knee, shoulder)	Fuel cells (biomass)
Keratoprosthesis	MEMS
Left ventricular assist device (LVAD)	Muscles (artificial) and actuators
Mechanical heart valve	Nanofabrication
Nerve guidance tube	NEMS
Ophthalmic drug delivery device	Neural computing/biocomputer
Pacemaker	Smart clothing for biowarfare
Renal dialyzer	Yeast array chip
Stent	
Tissue adhesive	
Urinary catheter	
Valve, heart	
Wound dressing	
X-ray guide	
Zirconium knee joint	

Table 1 - Uses for biomaterials. (Source [73])

4.1 Classification

Biomaterials can be classified based on their chemical composition and biological behavior. Chemical composition divides them into the following categories: metals, polymers, ceramics, and composites[69, 74].

Classification by biological behavior is based in tissue response to the implant surface. In general, there are three terms in which a biomaterial may be described in or classified into representing the tissues responses[74, 75]:

- **Bioinert Biomaterials** - The term bioinert refers to any material that once placed in the human body has minimal interaction with its surrounding tissue, examples of these are stainless steel, titanium, alumina, partially stabilised zirconia, and ultra high molecular weight polyethylene. Generally a fibrous capsule might form around bioinert implants hence its biofunctionality relies on tissue integration through the implant.
- **Bioactive Biomaterials** - refers to a material, which upon being placed within the human body interacts with the surrounding bone and in some cases, even soft tissue. This occurs through a time - dependent kinetic modification of the surface, triggered by their implantation within the living bone. An ion - exchange reaction between the bioactive implant and surrounding body fluids - results in the formation of a biologically active carbonate apatite (CHAp) layer on the implant that is chemically and crystallographically equivalent to the mineral phase in bone. Prime examples of these materials are synthetic hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$], glassceramic A-W and bioglass®
- **Bioresorbable Biomaterial** - refers to a material that upon placement within the human body starts to dissolve (resorbed) and slowly replaced by advancing tissue (such as bone). Common examples of bioresorbable materials are tricalcium phosphate [$\text{Ca}_3(\text{PO}_4)_2$] and polylactic- polyglycolic acid copolymers. Calcium oxide, calcium carbonate and gypsum are other common materials that have been utilized during the last three decades.

Thus bone substitutes can be of synthetic or natural origin. On this last case it can be include three types of graphs that are used nowadays[76, 77]:

- Autografts - fresh, living bone tissue harvested from somewhere else on the patient's body such as the hip. Autografts are nonimmunogenic and contain osteoblasts and osteoprogenitor stem cells, which are capable of proliferating.
- Allografts - living bone tissue donated by other individuals that is sourced from a bone bank. Allograft material is freeze-dried, demineralized, irradiated and treated with ethylene oxide to prevent the transmission of disease.
- Xenografts - made of naturally derived deproteinized cancellous bone from another species (such as bovine or porcine bone).

Thus bone grafts (Table 2) are used to provide structural, stability and linkage, and to stimulate osteogenesis and bone healing in fractures[76, 78]. However, there are some disadvantages associated with each one of these types, like for example autographs, can require a second surgical site and morbidity at the donor site, and allografts and xenografts may have problems of compatibility and rejection. This is why the development of a synthetic scaffold that mimics the complicated physiochemical attributes of bone, is a challenge and a target of innumerous researches[70, 76].

Type	Characteristics
I.	Autograft A. Iliac crest B. Locally harvested C. Vascularized or non-vascularized cortical D. Aspirated and/or enriched bone marrow stromal cells
II.	Allograft A. Mineralized B. Demineralized [demineralized bone matrix (DBM)]
III.	Hydroxyapatite (HA) blocks and granules A. Formed by sintering or precipitation B. Formed by conversion of calcium carbonate
IV.	Soluble calcium-based granules A. Tri-calcium phosphate (TCP) B. Calcium sulfate
V.	Silicon-containing calcium phosphates
VI.	Bone morphogenetic proteins (BMPs) A. BMP-2 B. BMP-7 C. Growth differentiation factor-5 (GDF-5)
VII.	Injectable cements A. Polymethyl methacrylate B. Calcium phosphate cements C. Silica-containing cements

Table 2 - Bone grafts and bone grafts substitutes. (Source [78])

4.2 Osseointegration

The loosening of implants from bone tissues has been a cause of problems in reconstructive surgery and joint replacement. Brånemark introduced the term “osseointegration” to describe the stable fixation of titanium to bone tissue. Thus this first definition defined osseointegration as a direct structural and functional connection between ordered living bone and the surface of a load-carrying implant. Nowadays an implant is regarded as osseointegrated when there is no progressive relative movement between the implant and the bone with which it has direct contact[79, 80]. Histologically osseointegration is defined as the direct anchorage of an implant by the formation of bony tissue around the implant without the growth of fibrous tissue at the bone-implant interface. This phenomenon is not isolated, but instead depends on two essential processes[81]:

- The growth of capillaries, perivascular mesenchymal tissues, and osteoprogenitor cells into the three-dimensional structure of an implant (osteoconduction).

However, in this case bone conduction is not only dependent on conditions for bone repair, but also on the biomaterial used and its reactions. Examples of osteoconductive materials are glass ceramic and materials based on calcium phosphate (HA, biocomposites, tricalcium phosphate), while copper and silver are examples of materials where conduction is not possible.

- The stimulation of osteoprogenitor cells to differentiate into osteoblasts (osteoinduction) that then begin new bone formation (osteogenesis). Osteoinduction is then a process that starts immediately after the injury and is very active during the first week thereafter, even though the action of the newly recruited preosteoblasts is not obvious until several weeks later, in the callus stage. Examples of osteoinductive materials are the demineralized bone matrix and bone growth factors. On the other hand, autologous spongy tissue and autologous cortical bone are examples of osteogenic materials.

Therefore osteoinduction, osteoconduction and osseointegration are interrelated but not identical phenomena, being the two first processes important for the successful of the last one[81]. In conclusion the main goal of osseointegration research is achieving a functional osseointegration after surgery to the long-term performance and remodeling of the bone.

2. Hydroxyapatite

Direct mimicry of biological processes represents an important strategy in modern biomaterials[73]. Thus, being alkaline mineral hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ the major component of the inorganic phase of bone, the use of synthetic hydroxyapatite(HA) that is similar to bone apatite seems to be advantageous for replacing the hard tissue over other synthetic materials[82, 83]. Due to its excellent biocompatibility, it promotes new bone formation along its surface and creates a biological fixation when implanted in bone [72, 82, 83]. In contrast to other materials like for example titanium that is a bioinert material, HA is a bioactive material, allowing that a direct chemical bond (without any so-called intervening fibrous tissue) between natural bone tissue and the implant occur[77]. HA has been used clinically in the form of powder, porous structure, or dense body[82]. However, due to HA low strength, they cannot be used on their own as major load-bearing implants in the human body [70, 75, 82]. For this reason hydroxyapatite has been used as film coatings on for example metallic alloys[75].

There are a several number of techniques for coating HA on metals, including plasma spraying, flame spraying, ion sputtering, chemical vapour deposition and thermal

decomposition [84]. Despite the technique used, Hydroxyapatite (HA) coatings have shown to promote or enhance implant fixation compared with metal substrates alone in experimental and clinical studies [75, 85, 86]. However, until now there is no agreement on the optimal coating composition, thickness, or crystallinity [86].

5. Nicotine

Over the last years, cigarette smoke has been associated with several pathologies like for example osteoporosis and periodontal bone loss as well as with implant failures [87-90]. These conditions share an imbalance between tissue destruction and tissue repair[91]. Although tobacco smoke contains more than a thousand potentially toxic substances, nicotine was suggested to be the major responsible for these effects. As a result nicotine has been implicated in many disease processes and has been shown to be of the highest importance when understanding the negative effects of smoking [88, 92, 93].

Nicotine ($C_{10}H_{14}N_2$) is a tertiary amine composed of a pyridine and a pyrrolidine ring.

Inhaling cigarette smoke, nicotine is rapidly absorbed from lung into the arterial circulation and is rapidly distributed to body tissues[94]. In smokers, plasmatic concentration of nicotine range between 4 - 75 ng/mL, while those found in saliva vary between 96 ng/mL -1, 6 mg/mL [95, 96].

Several researches showed that nicotine is responsible for the decrease of the proliferation of red blood cells, macrophages, and fibroblasts which are important elements of healing [90, 92].

The high rate of implant failure among other reasons has also been attributed to nicotine. This substance leads to bone graft morbidity and impaired osseous healing [92, 93, 97]. These effects of nicotine in bone implant were confirmed and presented recently by Seiichi *et al.*[92], where they demonstrated that nicotine administration had a negative effect on bone wound healing around implants and that nicotine negatively affected the osseointegration of titanium implants in the rat femur. On the other hand previous studies like the one conducted by Dimitra *et al.* [98]obtained different results since nicotine exposure in a short period of time, even in a high dose did not revealed a significant impact on bone healing or implant osseointegration in the femur and tibia of rabbits. However it was argued that these different results may be due to the different concentrations of nicotine used in the different studies[92].

Because the above diseases of bone result from an imbalance between bone formation by osteoblasts and bone resorption by osteoclasts, Pereira *et al.* [88]carry out a research with the aim of characterize the behavior of human bone marrow derived of osteoblastic cells in the presence of nicotine at concentrations representatives of those present in plasma and saliva of tobacco users, and they results raised the possibility of nicotine be involved in the local modulation of the osteoblastic activity at the oral cavity. A posterior research conducted by the same authors, but now with the aim of analyze the behavior of human bone marrow osteoblastic cells cultured on the surface of routinely used plasma-sprayed titanium implants in the presence of plasmatic and salivary nicotine levels, reported that

higher concentrations of nicotine caused dose-dependent inhibitory effects, suggesting the possibility of a direct modulation of the osteoblast activity as a contributing factor to the overall effect of nicotine in the bone microenvironment around dental implants[93].

Due to these recently findings, it is of crucial importance to characterize the role of nicotine in osteoclasts activity, once these cells are responsible for resorbing bone matrix, and play an essential role in bone homeostasis.

CHAPTER 2

Chapter2 - Cellular and molecular effects of nicotine on human osteoclasts and co-cultures of osteoclastic and osteoblastic cells

1. Introduction

Despite its rigid structure, bone is a very dynamic tissue that is constantly being remodeled and shaped by the action of both osteoblastic and osteoclastic cells ([4, 16]. Osteoblasts are the cells responsible for depositing the proteinaceous and calcified extracellular matrix and, thus, synthesizing new bone[18]. In addition, they are also key players in the differentiation of osteoclasts, by secreting several osteoclastogenic modulators, such as the receptor activator of the NF κ B ligand (RANKL), and the macrophage-colony stimulating factor (M-CSF)[20-22, 99]. Osteoclasts are cells that descend from the monocyte/macrophage CD14⁺ lineage and are responsible for resorbing bone matrix. They are formed by fusion of their mononuclear precursors and subsequent differentiation, at or near the bone surface[24, 25]. Bone homeostasis is maintained through a strict regulation of both osteoblastic and osteoclastic activities. Imbalances between those activities can lead to skeletal diseases[58, 100].

Over the last years, cigarette smoke has been associated with several bone disorders like, for example, osteoporosis and periodontal bone loss as well as with implant failures [87-90]. Although tobacco smoke contains more than a thousand of potentially toxic substances, nicotine has been suggested to be the major responsible for these effects. In smokers, plasmatic concentration of nicotine range between 4 - 75 ng/mL, while those found in saliva vary between 96 ng/mL -1, 6 mg/mL [95, 96]. Nicotine has been implicated in many disease processes and has been shown to be of the highest importance toward the understanding of the negative effects of smoking on bone tissue [88, 92, 93].

In that context, previous studies have addressed the effects of nicotine on osteoblastic cells. Generally, it was observed that it depresses osteoblast activity in a dose-dependent way [88, 93, 101]. In terms of osteoblastic cell proliferation, there is no agreement on the results of the several published studies, probably because of the different culture conditions, namely, nicotine concentration ranges, and cell models. Some of these studies showed that low concentrations of nicotine (concentration range of 0.01-0.2 mg/mL) increase cell proliferation and that high concentrations (0.3-1mg/mL) of nicotine inhibit cell proliferation [88, 93, 102]. Other studies showed that nicotine suppresses cellular proliferation at all the range of nicotine concentration used [91, 103]. Finally, some studies showed that it stimulates osteoblastic cells proliferation [104, 105].

Intriguingly, despite the fact that osteoclastic cells are key players on bone metabolism, the effects of nicotine on those cells are very scarcely documented. Moreover, the few published studies about that issue were not conducted with human cells but instead with mouse and porcine cells [106, 107]. It was reported that co-cultures of mouse bone marrow cells and clonal stromal cells from mouse bone marrow, ST2 cells, exposed to nicotine at a concentration range of 10 to 250 µg/mL, displayed an inhibition of osteoclastogenesis [107]. On another study, it was observed that the presence of nicotine stimulated the resorbing activity of porcine osteoclastic cells [106]. The apparent contradiction in the results can be simply due to the different origins of the analysed cells, which reinforces the need to perform such experiments in human osteoclastic cells. In that context, the aim of the present work is to evaluate the role of nicotine in human osteoclastic differentiation and activity, either in unstimulated or stimulated osteoclast precursor cell cultures (maintained in the absence or presence of recombinant M-CSF and RANKL, respectively), or in co-cultures of osteoclastic and osteoblastic cells. For that goal, cell cultures were exposed to a range of salivary and plasmatic nicotine concentrations of 1.28×10^{-6} - 0.5mg/mL. Cell cultures were assessed for several osteoclast-related features. Moreover, the involvement of MEK and NFkB signaling pathways as well as the PGE2 production on the observed cellular response was also addressed.

2. Materials and Methods

2.1 Cell cultures

2.1.1 *Peripheral blood mononuclear cells (PBMC)*

Osteoclast precursor cells were isolated from the blood of healthy donors with 25-35 years old, after informed consent. PBMC were isolated as described previously [108]. Briefly, blood was diluted with PBS (1:1) and applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). After centrifugation at 400g for 30 min, PBMC were recovered and washed twice with PBS. PBMC cells were counted with a cytometer (Celltac MEK-5103). Typically, around 70×10^6 PBMC were obtained for each 100 mL of processed blood. Cells were seeded at 1.5×10^6 PBMC/cm² and maintained in α -MEM supplemented with 30% (V/V) human serum (from the same donor where cells were obtained), 2 mM L-glutamine, 100 IU/mL penicillin (Gibco), 2.5 μ g/mL streptomycin (Gibco), 2.5 μ g/mL amphotericin B (Gibco). PBMC cultures, performed in the absence (base medium, BM) or presence of 25ng/mL M-CSF and 40ng/mL RANKL (M+R) [109], were treated with different concentrations of nicotine (0.000128 - 50×10^{-2} mg/mL). Cell cultures were performed for 21 days at 37°C in a 5% CO₂ humidified atmosphere. The culture medium was replaced once a week, and nicotine in the culture medium was renewed at each medium change.

2.1.2 *Co-cultures of MG63+PBMC cells*

MG63 osteoblast-like cells, were cultured in α -minimal essential medium (α -MEM) (Gibco) supplemented with 10% foetal bovine serum, 100 IU/mL penicillin (Gibco), 2.5 μ g/mL streptomycin (Gibco), 2.5 μ g/mL amphotericin B (Gibco) and 50 μ g/mL ascorbic acid until reaching about 70-80% confluence. Then, cells were enzymatically detached with 0.05% trypsin and 0.5mM EDTA. Cells were seeded at 10^3 cells/cm² and incubated for 24 hours in the same culture medium described above. After that incubation, PBMC (1.5×10^6 cells/cm²) were added and cocultures were maintained in the same culture conditions as those described for PBMC cultures.

2.2 Characterization of the cell cultures

2.2.1 *TRAP activity quantification*

TRAP activity was assayed by *para*-nitrophenylphosphate (*p*NPP) hydrolysis method. Cell cultures were washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100. Cellular extracts were incubated with 12.5 mM *p*NPP in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8) for 1 hour at 37°C. After addition of 5 M NaOH, the absorbance of the samples was measured at 400 nm in an ELISA plate reader (Synergy HT, Biotek). TRAP activity was normalized with total protein content, quantified by Bradford's method [110], and results were expressed as nmol/min.µg_{protein}⁻¹.

2.2.2 *Histochemical staining of TRAP*

Cell cultures were fixed with 3.7% formaldehyde for 10 min and then washed with distilled water. The cells were stained for TRAP with Acid Phosphatase, Leukocyte (TRAP) kit (Sigma), according to manufacturer's instructions. Shortly, after fixation with formaldehyde cells were incubated with naphthol 0.12 mg/mL AS-BI, in the presence of 6.76mM tartrate and 0.14 mg/mL Fast Garnet GBC, for 1 h at 37°C in the dark. Cell layer was washed, stained with haematoxylin and visualised by light microscopy. Multinucleated cells (MC) positive for TRAP were counted.

2.2.3 *RT-PCR analysis*

RNA was extracted from cell cultures with RNeasy®Mini Kit (Qiagen) according to manufacturer's instructions. RNA was quantified by measuring the absorbance of the samples at 260 nm. The expression of GAPDH, and the osteoclast-related genes TRAP, CATK, CA2, c-myc and c-src [53] was performed by RT-PCR. For that, half microgram of total cellular RNA from each sample was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55°C. The primers used are listed on Table 3. The RT-PCR products were separated in a 1% (w/v) agarose gel and analysed with ImageJ 1.41 software. The values obtained were normalized to the corresponding GAPDH value of each experimental condition.

Gene	5' Primer	3' Primer
GAPDH	5'-CAGGACCAGGTTACCAACAAGT-3'	5'-GTGGCAGTGATGGCATGGACTGT-3'
c-myc	5'-TACCCTCTCAACGACAGCAG-3'	5'-TCTTGACATTCTCCTCGGTG-3'
c-src	5'-AAGCTGTTCTGGAGGCTTCAA-3'	5'-TTGGAGTAGTAGGCCACCAG-3'
TRAP	5'-ACCATGACCACCTTGGCAATGTCTC-3'	5'-ATAGTGGGAAGCGCAGATAGCCGTT-3'
CATK	5'-AGGTTCTGCTGCTACCTGTGGTGAG-3'	5'-CTTGCATCAATGGCCACAGAGACAG-3'
CA2	5'-GGACCTGAGCACTGGCATAAGGACT-3'	5'-AAGGAGGCCACGAGGATCGAAGTT-3'

Table 3 - Primers used on RT-PCR analysis of cell cultures.

2.2.4 Visualization of cells with actin rings and expressing, VNR and CTR by confocal laser scanning microscopy (CLSM)

Cell cultures were washed twice with PBS and fixed with 3.7% (v/v) para-formaldehyde for 15 min. After fixation, cells were permeabilized for 5 min with 0.1% (v/v) Triton X-100 and stained for actin with 5 U/mL Alexa Fluor® 647-Phalloidin (Invitrogen), and for VNR and CTR with 50 µg/mL mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems), respectively. Detection of IgGs anti-VNR and IgGs anti-CTR was performed with 2mg/mL Alexa Fluor1 488-Goat anti-mouse IgGs.

2.2.5 Calcium phosphate resorption assay

Cell cultures maintained in the absence or presence of 0.0032×10^{-2} mg/mL nicotine (a concentration that elicited a significant response on both PBMC and MG63+PBMC cultures) were performed on BD BioCoat™ Osteologic™ Bone Cell Culture Plates (BD Biosciences). After 21 days of culture, cells were bleached with 6% NaOCL and 5.2 NaCl, following the manufacturer's protocol and the remaining calcium phosphate layers were visualized by phase contrast light microscopy. Resorption lacunae were identified and total resorbed area was quantified with ImageJ 1.41 software.

2.2.6 Assessment of the intracellular mechanisms involved in the osteoclastogenic response

In order to assess the influence of some osteoclastogenic-related signalling pathways in the observed cellular response, cell cultures performed in the absence or presence of 0.0032×10^{-2} mg/mL nicotine (a concentration that caused a significant effect either in PBMC cultures or in co-cultures of MG63+PBMC) were treated with different specific

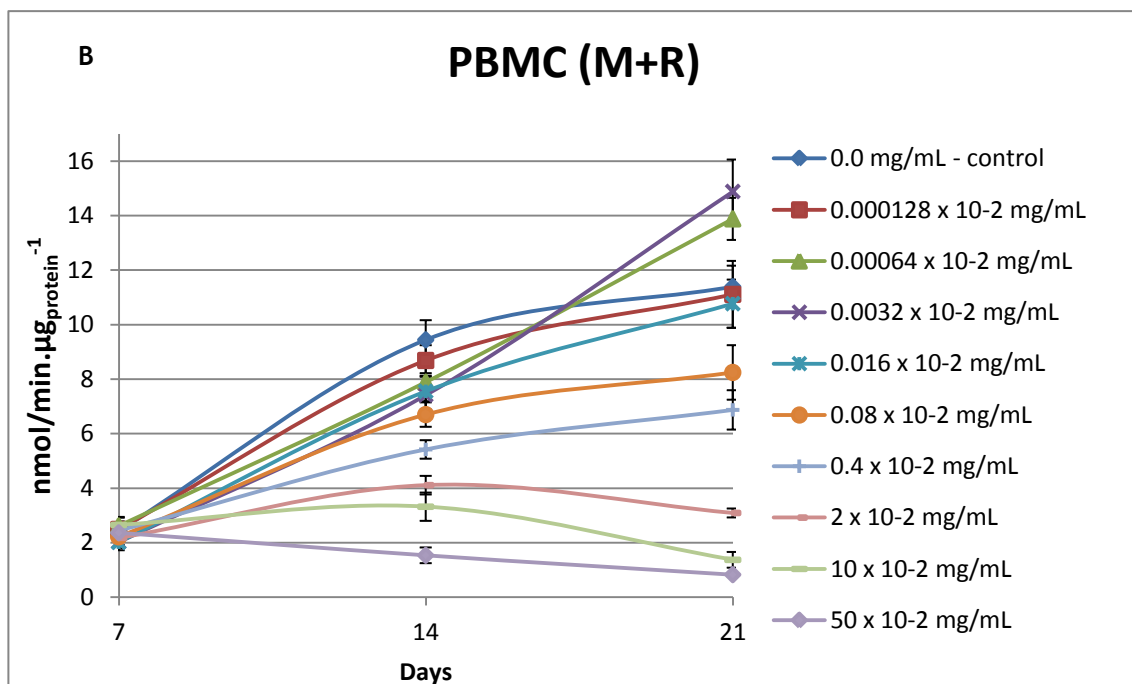
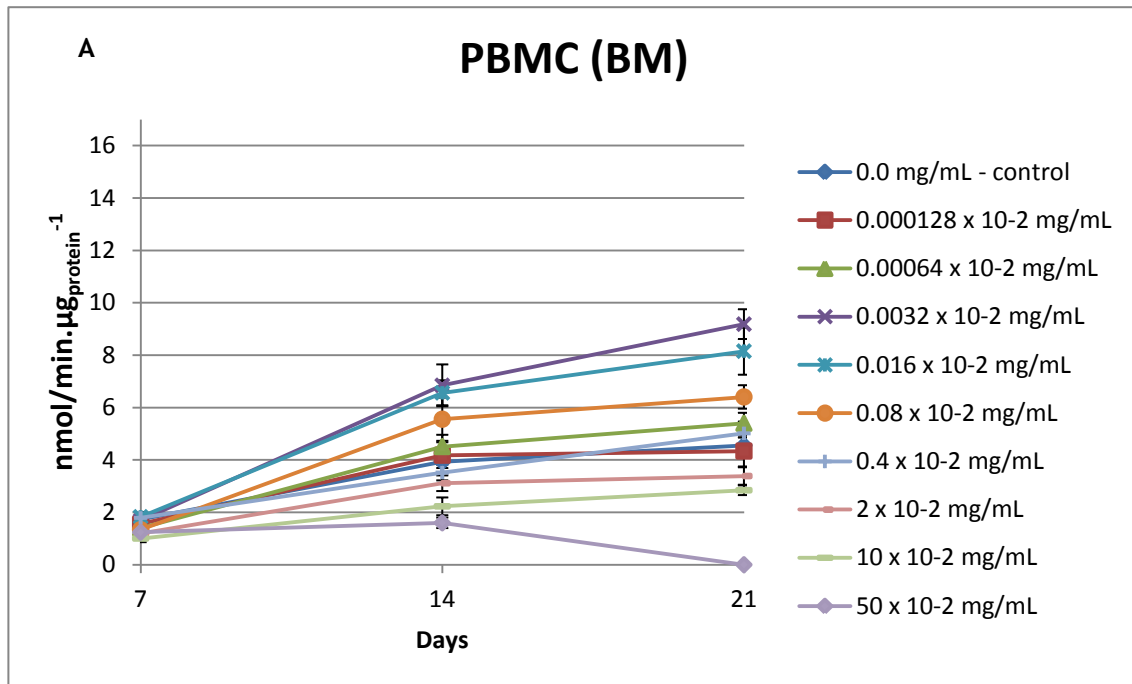
inhibitors, namely: U0126, a MEK signalling pathway inhibitor, that was tested at 1 and 10 μM , because of the contradictory osteoclastogenic effects observed with low concentrations of this molecule[61, 111-113]; PDTC, a NF κ B signalling pathway inhibitor, was used at 10 and 100 μM , because the lower concentration has been previously described as the IC₅₀ on rat osteoclastic differentiation [114]; indomethacin (1 μM) a PGE₂ synthesis blocker, was also included in the study due to the fact that PGE₂ is associated with an increase on osteoclastic development [66, 115].

3.1- Statistical analysis

Data presented in this work are the means of three separate experiments performed with cells from four different blood donors. Statistical differences were assessed using an unpaired Student's t-test with Bonferroni correction for multiple comparisons. All data are expressed as the mean $\pm$ standard deviation of 3 examinations, with similar results obtained in each experiment. For values of $p \leq 0.05$, differences were considered statistically significant

3 Results

3.1 TRAP activity and histochemical staining



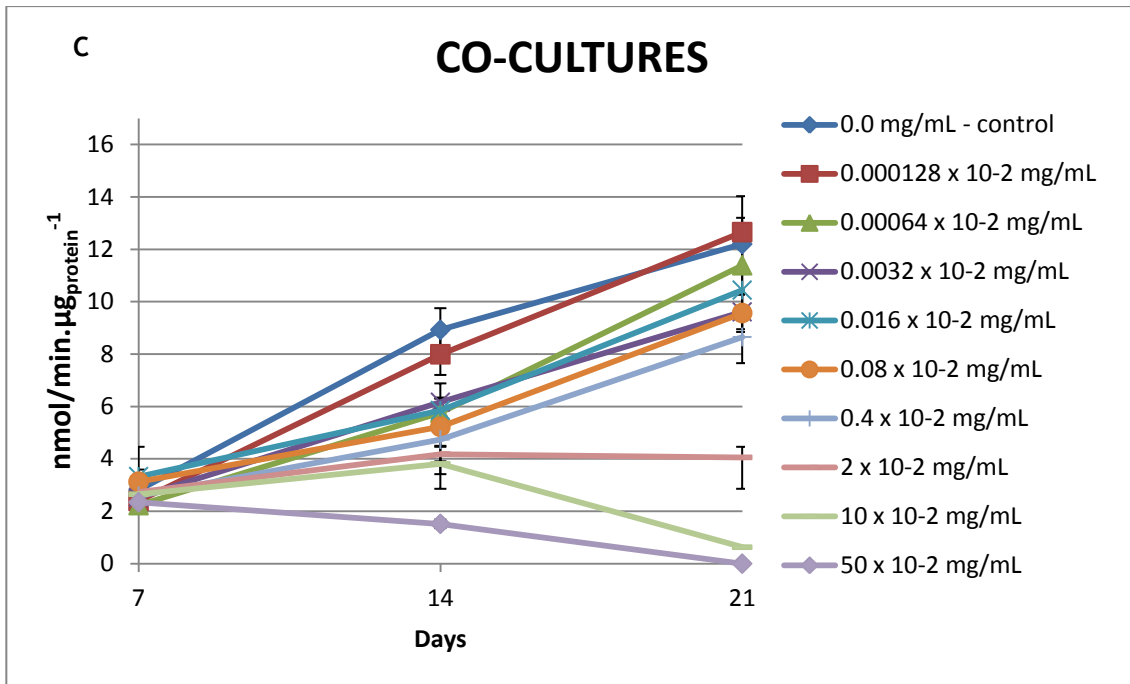
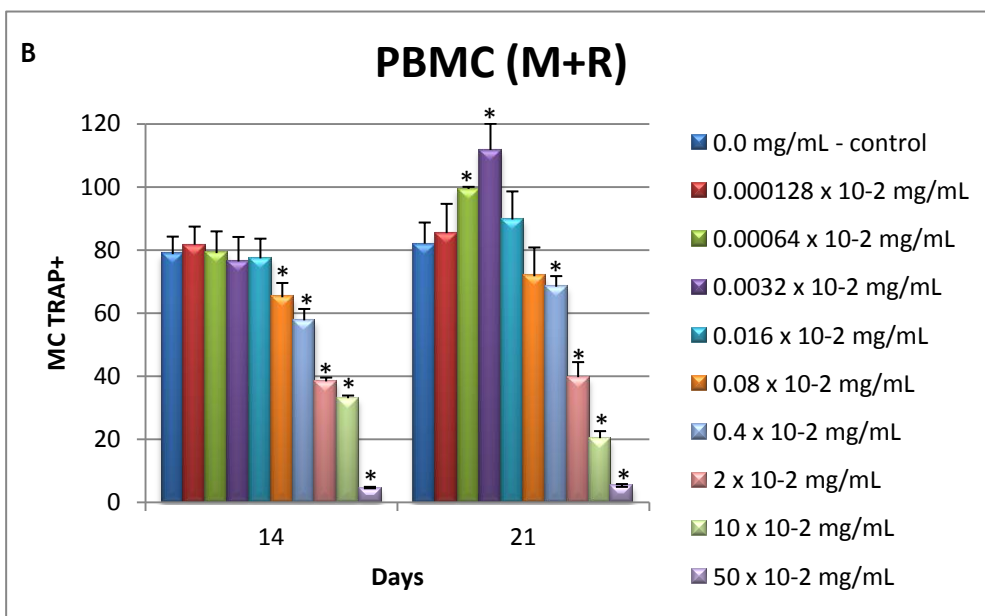
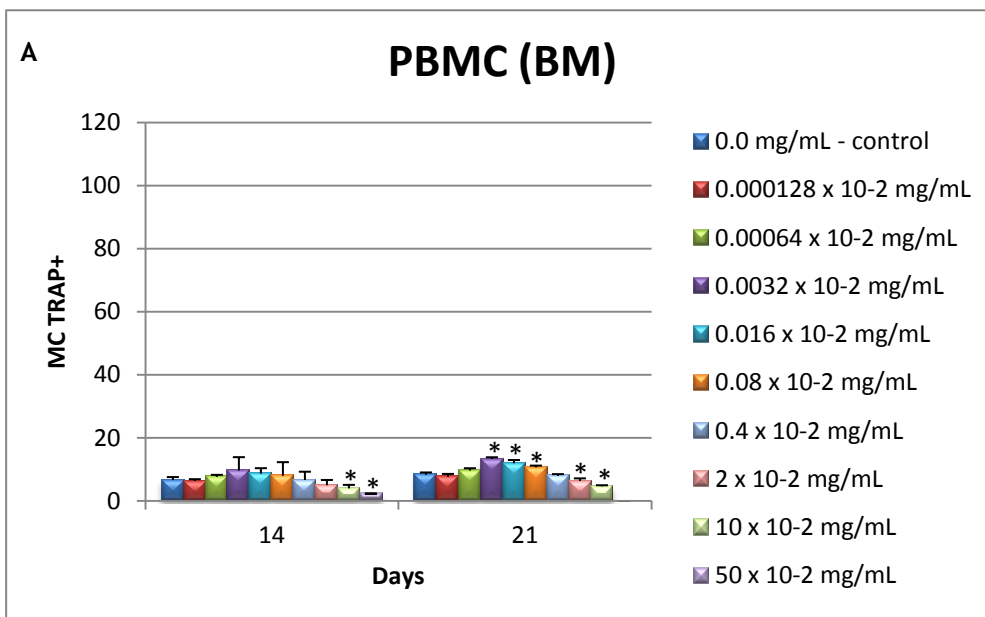


Figure 9- TRAP activity of PBMC cultures, performed in the absence (A) or presence (B) of recombinant M-CSF and RANKL and co-cultures of MG63 + PBMC (C). Cell cultures were maintained in the absence (control) or presence of different concentrations of nicotine. BM - base medium; M+R - supplementation with M-CSF and RANKL.

In base medium (BM), PBMC cultures performed in the absence of nicotine displayed a low TRAP activity (Figure 9A). Although the lowest concentration of nicotine did not affect cellular response, it was observed an increase on TRAP activity with an increase on nicotine concentration that reached the maximum value at a concentration of 0.0032×10^{-2} mg/mL (34% higher than the control). Concentrations higher than that elicited a decrease on the cellular response.

Cultures performed in the presence of M-CSF and RANKL (M+R) displayed significantly higher values of TRAP activity, when compared with cultures performed in BM (Figure 9B). Nevertheless, a similar pattern of cellular response induced by the presence of nicotine was observed. Again, the maximum response was achieved with 0.0032×10^{-2} mg/mL nicotine, being about 29% higher than the one obtained in the absence of that molecule.

Co-cultures of MG63 + PBMC displayed a different behavior, since for nicotine concentrations higher than 0.00128×10^{-2} mg/mL (whose response was similar to the control), TRAP activity exhibited a dose-dependent decrease in response to the presence of that molecule (Figure 9C).



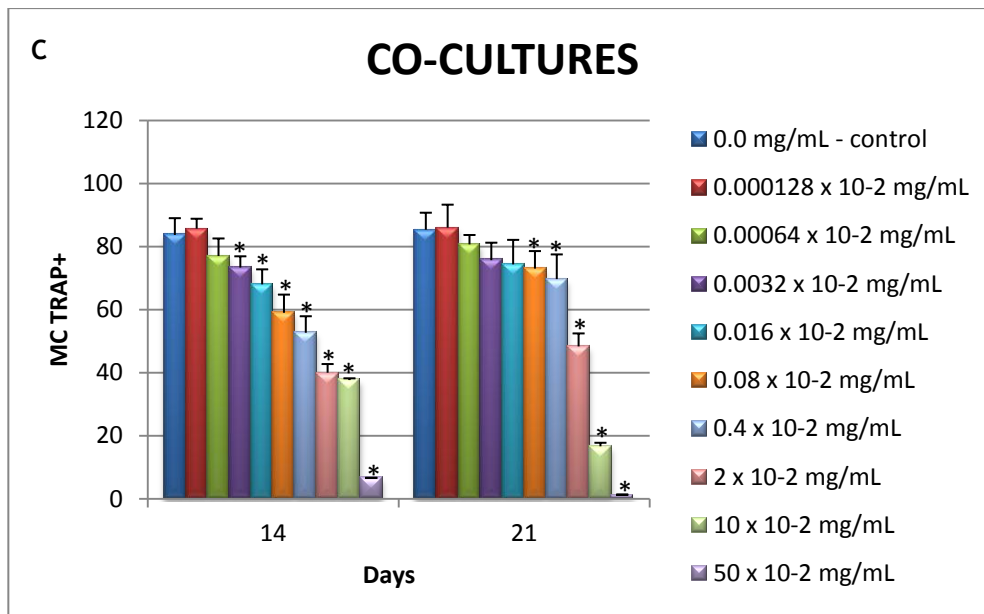
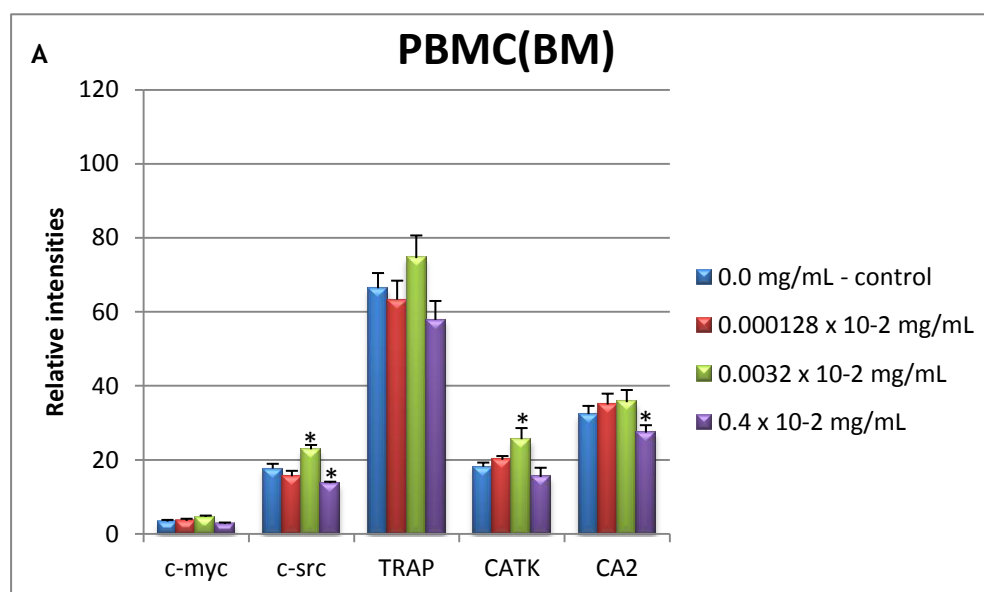


Figure 10 - TRAP staining of PBMC cultures performed in the absence (A) or presence (B) of the osteoclastogenic inducers M-CSF and RANKL, and co-cultures of MG63 + PBMC (C). Cell cultures were maintained in the absence (control) or presence of different concentrations of nicotine. BM - base medium; M+R supplementation with M-CSF and RANKL; MC - multinucleated cells. * Significantly different from the control.

Globally, the pattern of results obtained for TRAP histochemical staining was identical to those observed for TRAP activity in all the three culture conditions tested, that is, PBMC (BM), PBMC (M+R) and MG63 + PBMC co-cultures (Figure 10A-C).

3.2 RT-PCR analysis of osteoclastic cultures



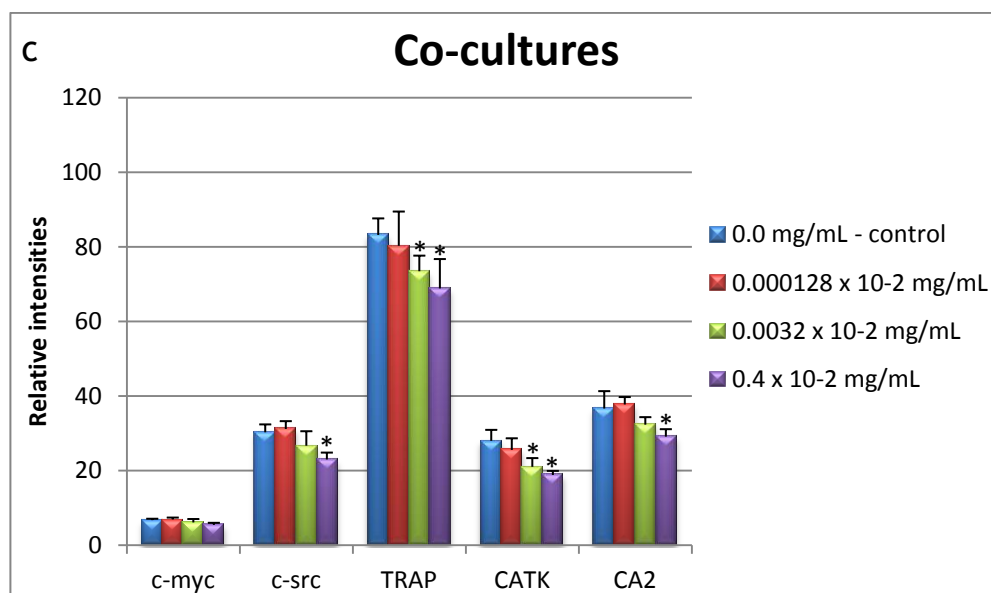
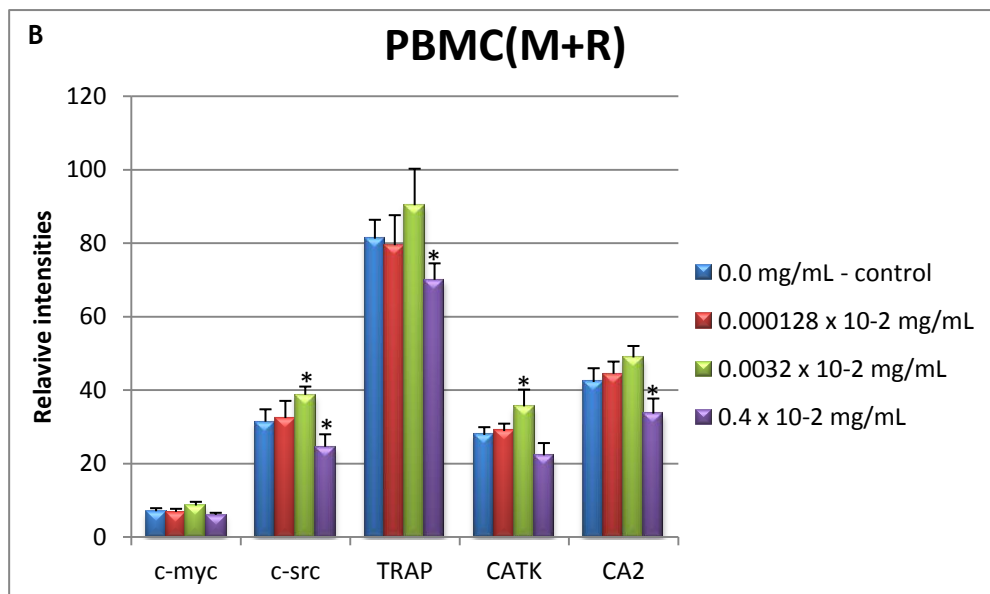


Figure 11 - Densitometric analysis of the RT-PCR products, normalized by GAPDH, of PBMC cultures performed in the absence (A) or presence (B) of recombinant M-CSF and RANKL, and co-cultures of MG63 + PBMC (C). Cell cultures were maintained in the absence (control) or presence of different concentrations of nicotine. BM - base medium; M+R - supplementation with recombinant M-CSF and RANKL. * Significantly different from the control.

PBMC cultures, performed in BM or M+R, and co-cultures of MG63 + PBMC were treated with 0.000128×10^{-2} , 0.0032×10^{-2} and 0.4×10^{-2} mg/mL nicotine and were assessed for the expression of several osteoclast-related genes by RT-PCR (Figure 11).

All the cultures expressed the analyzed genes. PBMC cultures (BM) revealed low expression levels, that were not affected by the presence of 0.000128×10^{-2} mg/mL nicotine (Figure

11A). However, when cell cultures were treated with 0.00032×10^{-2} mg/mL, the expression levels increased (about 5 -17 % higher). On the other hand, cell cultures performed with 0.4×10^{-2} mg/mL nicotine exhibited a lower response than the control (about 7- 13% lower).

PBMC cultures (M+R) performed in the absence or presence of 0.000128×10^{-2} mg/mL nicotine revealed identical expression levels of the osteoclastic genes, that increased (5-12%) following treatment with 0.0032×10^{-2} mg/mL nicotine (Figure 11B). Once again, the presence of the highest nicotine concentration caused a significant decrease (7-13%) in the cellular response.

Co-cultures of MG63 + PBMC cells exhibited a nicotine dose-dependent decrease on the expression levels of the analyzed genes, although the lowest tested concentration did not affect the cellular response (Figure 11C).

3.3 Presence of actin rings and expression of VNR and CTR

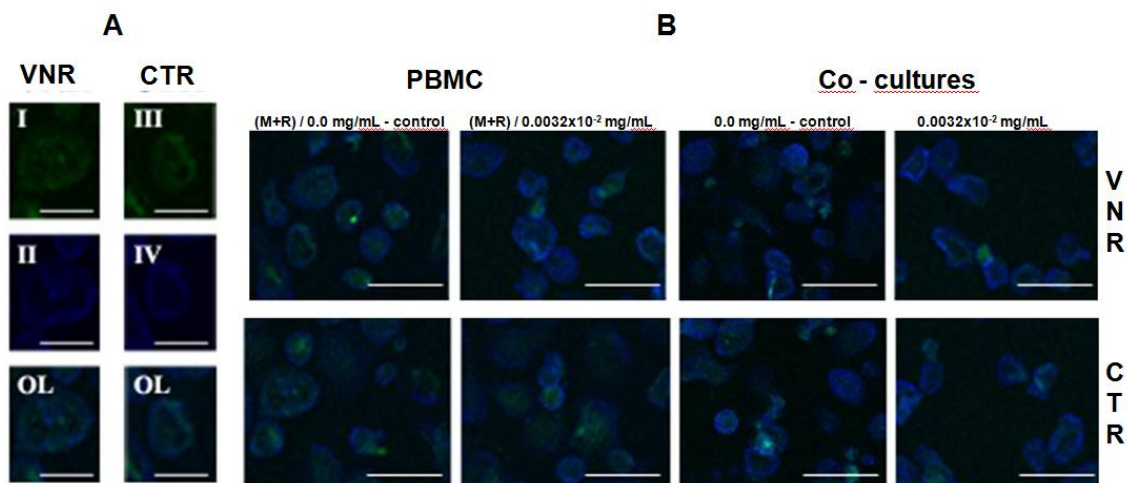


Figure 12 - PBMC cultures and co-cultures of MG63 + PBMC visualized by CLSM after 21 days of culture. A: Representative images: osteoclast stained blue for actin (II and IV), and green for vitronectin receptors, VNR (I) or calcitonin receptors, CTR (III); OL, overlay. B: CLSM visualization of PBMC cultures (M+R) and co-cultures of MG63 + PBMC, performed in the absence or presence of 0.0032×10^{-2} mg/mL of nicotine. White bars represent 60 mm (A) or 100 mm (B).

PBMC cultures and co-cultures of MG63 + PBMC cells were stained for actin and for VNR and CTR and visualized by CLSM (Figure 12A and 12B). It was possible to visualize cells displaying the analyzed osteoclast-features in all tested conditions. However, the amount of osteoclastic cells was somehow in line with the results obtained for TRAP activity and histochemical staining, and RT-PCR, that is, the presence of low doses of nicotine elicited

an increase on that value on PBMC cultures, and a decrease on MG63 + PBMC co-cultures (Figure 12B).

3.4 Calcium Phosphate Resorbing ability

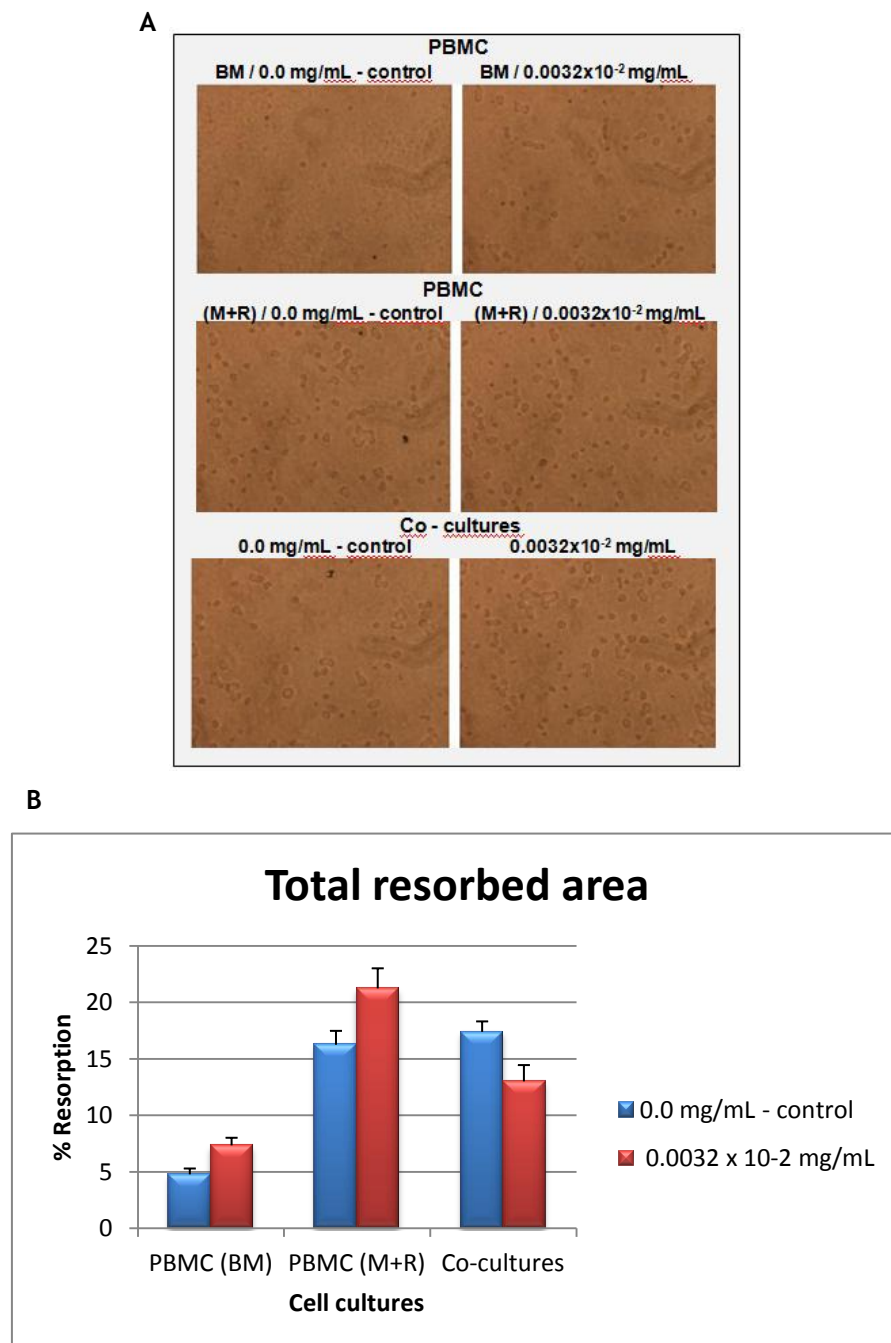
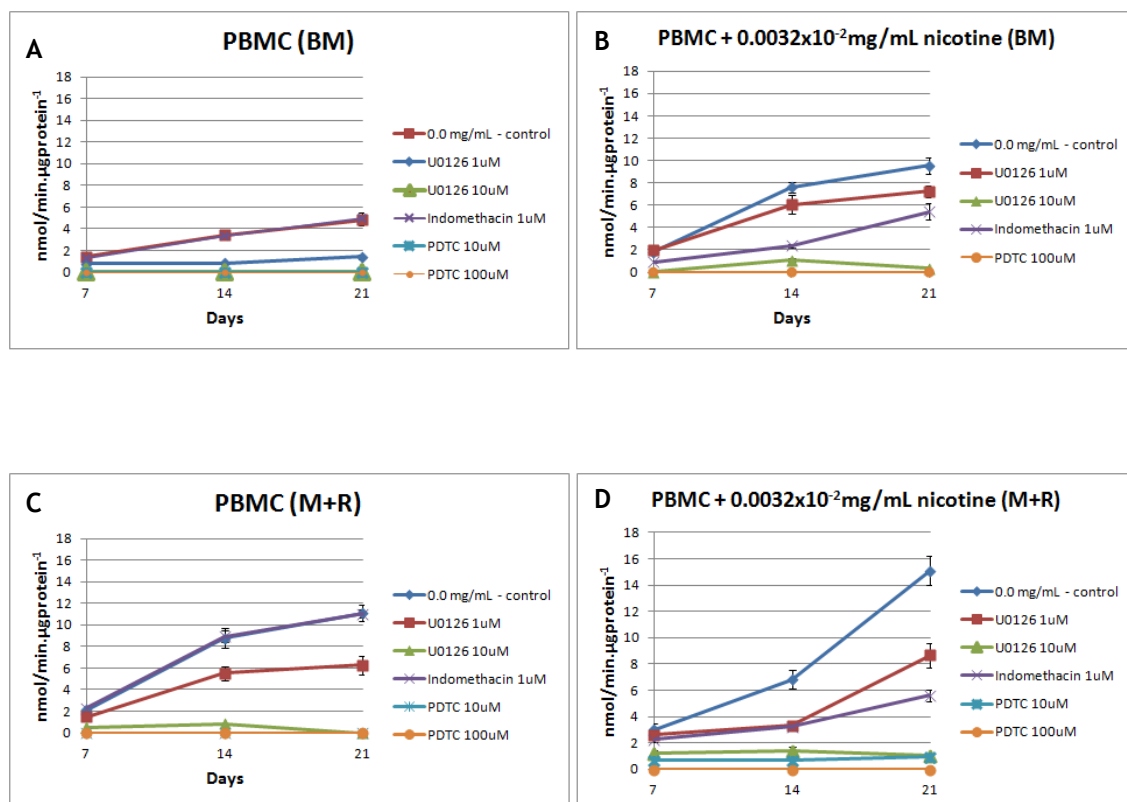


Figure 13 - Calcium phosphate resorbing ability (A) and total resorbed area (B) of PBMC cultures maintained in BM and M+R and MG63 + PBMC co-cultures. Cell cultures were performed in the absence or presence of 0.0032×10^{-2} mg/mL of nicotine. BM - base medium; M+R - supplementation with M-CSF and RANKL. * Significantly different from the control.

Cell cultures maintained in the absence or presence of 0.0032×10^{-2} mg/mL nicotine were cultured on calcium phosphate coated-plates. After 21 days of cultures, cells were removed, the resorption lacunae were identified (Figure 13A) and total resorbed area was quantified (Figure 13B).

It was observed that the presence of nicotine induced a significant increase on the resorbed area on PBMC cultures, performed either in BM or M+R conditions (21 and 13% higher than the corresponding control, respectively). On the other hand, on co-cultures of MG63 + PBMC cells, nicotine elicited a decrease on the resorbing activity (about 14% lower than the control).

3.5 Intracellular mechanisms involved in the osteoclastogenic response



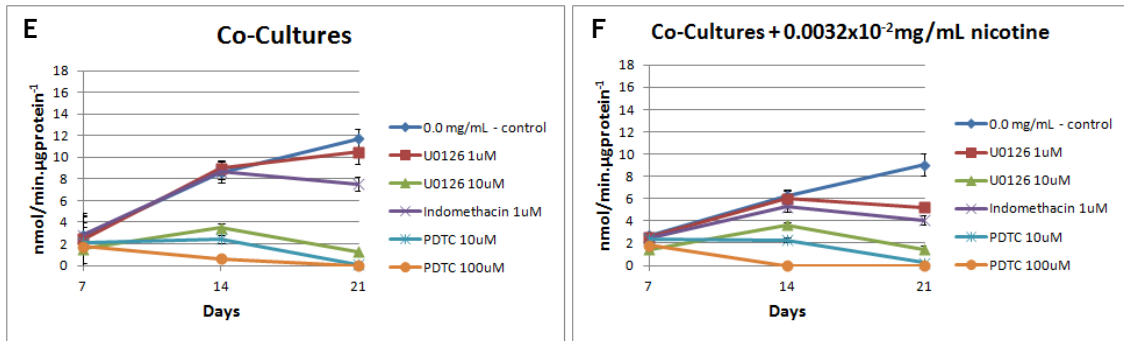


Figure 14 - TRAP activity of PBMC cultures and co-cultures of MG63 + PBMC cells, performed in absence or in the presence of $0,0032 \times 10^{-2}$ mg/mL of nicotine, and treated with the signaling pathways inhibitors. BM - base medium; M+R - supplementation with M-CSF and RANKL.

PBMC cultures performed in BM and maintained in the absence of nicotine displayed a significant decrease on TRAP activity induced by the presence of either U0126 or PDTC (Figure 14A). On the other hand, indomethacin did not affect the cellular response. Compared to the control, in the presence of nicotine (Figure 14B) the inhibition elicited by $1 \mu\text{M}$ U0126 was less pronounced. Nevertheless, treatment with $10 \mu\text{M}$ U0126 or both tested concentrations of PDTC caused a sharp decrease on TRAP activity to almost negligible or even null values. Indomethacin also elicited a significant inhibitory effect on the cellular response.

In M+R, PBMC cultures performed in the absence of nicotine (Figure 14C) revealed a significant inhibition caused by $1 \mu\text{M}$ U0126. That inhibition was total when the concentration of the inhibitor increased to $10 \mu\text{M}$, or in the presence of PDTC (both concentrations). Treatment with indomethacin did not affect TRAP activity. Comparatively, in the presence of nicotine (Figure 6D) the pattern of response was similar for the treatment with U0126 or PDTC. However, the presence of indomethacin elicited a significant decrease on the cellular response.

In MG63 + PBMC co-cultures maintained in the absence of nicotine (Figure 14E), the presence of U0126 only caused a significant decrease on TRAP activity in the higher tested concentration (the lower one did not affect the cellular response). On the other hand, in the presence of either concentrations of PDTC, the values of TRAP activity were almost negligible. Indomethacin also elicited a significant inhibition on the cellular response. In the presence of nicotine (Figure 14F), the lower concentration of U0126 was able to cause a significant inhibition on TRAP activity, while the higher concentration or the presence of PDTC revealed an almost absolute inhibitory behavior. Finally, in that culture condition, the inhibitory effect of indomethacin was potentiated.

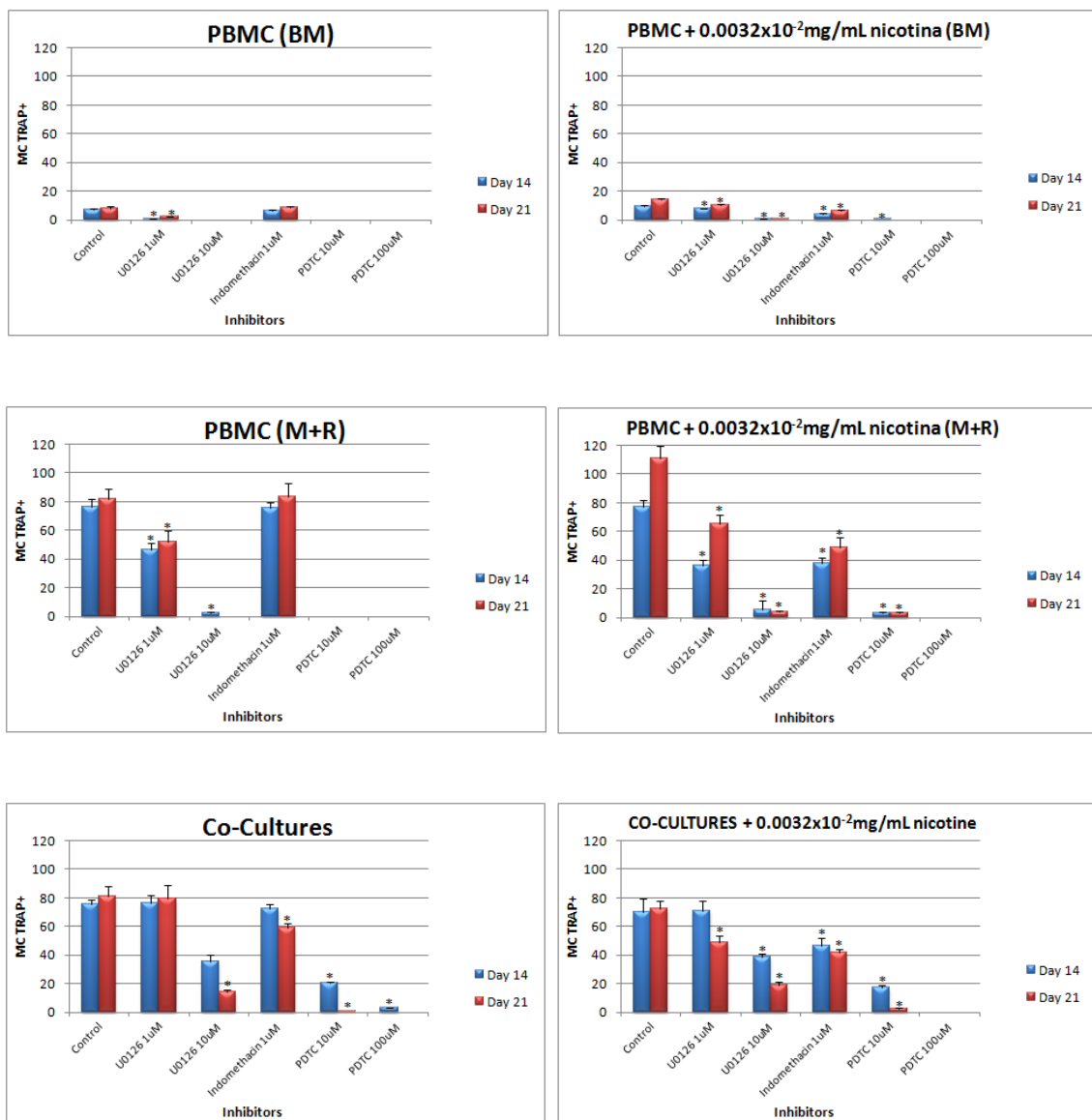


Figure 15 - TRAP staining of PBMC cultures and co-cultures of MG63 + PBMC cells, performed in absence or in the presence of $0,0032 \times 10^{-2}$ mg/mL of nicotine, and treated with the signaling pathways inhibitors. BM - base medium; M+R - supplementation with M-CSF and RANKL; MC - multinucleated cells.

In general, the pattern of the results obtained for the number of multinucleated cells positive for TRAP for each culture condition (Figure 15) was in line with that obtained for TRAP activity.

4 Discussion

Although it is recognized that smoking can lead to skeletal complications [87-90], the underlying involved mechanisms are still unknown. The present study examined the effect of nicotine on human osteoclast development and activity in two distinct culture conditions, that is, osteoclast precursor cells cultured isolated or co-cultured with osteoblastic cells. Furthermore, in the former condition, the effects on spontaneous and induced osteoclastogenesis were addressed.

It was observed that PBMC cultures exposed to nicotine displayed an increase of TRAP activity at low concentrations, reaching a maximum value at a concentration of 0.0032×10^{-2} mg/mL. Concentrations higher than that elicited a decrease on TRAP activity. This behavior was observed independently of the presence of recombinant M-CSF and RANKL, which suggests that nicotine, does not need a previous commitment of the osteoclast precursors to modulate osteoclastogenesis. It also modulates it taking advantage of the ability of PBMC to spontaneously generate some mature osteoclasts, a behavior characterized before [116]. Nevertheless, once the concentration values that caused an increase on osteoclastogenesis were similar in both cases (stimulated and non-stimulated PBMC), and the percentage of increase was somehow identical in both conditions, the present data strongly suggests that nicotine may act by specific mechanisms on osteoclast precursors, independently of the presence of M-CSF and RANKL, directing them towards osteoclast differentiation. Finally, it is important to mention that the presence of low doses of nicotine not also stimulated osteoclast development but also increased its resorbing ability. The lower levels of nicotine (0.000128×10^{-2} - 0.0032×10^{-2} mg/mL) were representative of the nicotine plasma concentrations reported in smokers [95, 117, 118], and the latter value significantly affected the cellular responses. Higher concentrations (0.016×10^{-2} - 50×10^{-2} mg/mL), representative of the nicotine salivary levels [96, 119] elicited a decrease on the cellular response. In contrast, co-cultures of MG63 + PBMC cells exhibited a dose-dependent decrease on osteoclastogenesis, in response to the presence of nicotine. The effects become significant for concentrations higher than 0.000128×10^{-2} mg/mL. This behavior is clearly in contrast with the one observed for PBMC cultures, and strongly suggests that nicotine can also affect the osteoclastogenic process indirectly, *via* osteoblastic cells. In that context, it is known that osteoblasts have the ability to affect osteoclastogenesis through the expression of several modulators [23, 120, 121]. Thus, it is tempting to speculate that nicotine can induce changes in the expression of those molecules. The clarification of this issue and the full identification of those modulators are now underway.

The involvement of MEK and NFκB signaling pathways and the PGE2 production on the nicotine-induced modulation of osteoclastogenesis was also addressed. Globally, and despite the culture condition, NFκB pathway appeared to be crucial for the process. Nevertheless, some important differences were also noted. In PBMC cultures performed in BM, it was observed that osteoclastogenesis promoted by nicotine was less dependent on MEK pathway, while PGE2 production seemed to be important for the process. In the presence of M+R, MEK pathway was not affected by nicotine, but once again PGE2 synthesis was upregulated by that molecule. Finally, in MG63 + PBMC co-cultures, MEK pathway and PGE2 production appeared to be important for nicotine-induced effects on osteoclastogenesis. It was reported previously that nicotine can affect MEK pathway [122-125], which can help to explain the involvement of that signaling pathway in the observed cellular responses. Also, PGE2 production seemed to be affected by nicotine [126, 127]. More precisely, it was observed that it increases the synthesis of PGE2 by platelets and endothelial cells[126, 127], and, more important, by PBMC [128]. Those results are in agreement to the observed involvement of PGE2 in the osteoclastogenic modulation by nicotine.

At the best of our knowledge, only two papers were published before regarding the effects of nicotine on osteoclastic cells. Yuhara *et al.* (1999) used a nicotine concentration range of 10 to 250 µg/mL on osteoclast-like cells formed during co-culture of mouse bone marrow cells and clonal stromal cells from mouse bone marrow, ST2 cells [107]. It was observed that the exposure of these cells to nicotine reduced the formation of TRAP-positive multinucleated cells and the formation of resorption pits on slices of dentine. In another study, Henemyre *et al.* (2003), evaluated the effect of nicotine (at a concentration range of 0.03 to 1.50µg/mL) on porcine mature osteoclasts, by measuring the resorbed surface area of a calcium phosphate substrate[106]. They concluded that the number of osteoclasts increased in a linear relationship with the increase of nicotine concentrations, although they did not find any correlations between osteoclast number and the amount of resorption. Since the range of nicotine concentrations used in both reports are significantly different from those used in the present study, and the previous results were not obtained with human cells, it is not possible to directly compare the literature reports with the present data. Nevertheless, there is an apparent contradiction between the results obtained with rat and porcine cells, which can suggest, *per se*, that osteoclastic cells from different origins might respond differently to nicotine.

In conclusion, our results demonstrate that in PBMC cultures nicotine has the ability to act directly on human osteoclastic precursors, inducing (low concentrations) or inhibiting (high concentrations) the osteoclast differentiation. This effect was observed both for

spontaneous or induced osteoclastogenesis. However, in an osteoclast/osteoblast co-culture system, nicotine revealed an inhibitory effect that was dependent on its concentration, which suggests that that molecule may also act indirectly in the process, *via* the osteoblastic cells. To our knowledge, this is the first report that displays data on human osteoclastic precursors, exposed to nicotine isolated or co-cultured with osteoblastic cells. The full clarification about the effects of nicotine on bone metabolic activities is crucial, in order to understand and prevent the deleterious effects of smoking in bone tissue.

CHAPTER 3

Chapter3 - Nicotine-induced modulation of osteoclastogenesis in a hydroxyapatite surface: *in vitro* studies with human osteoclasts and co-cultures of osteoclasts and osteoblasts

1 Introcuccion

Direct mimicry of biological processes represents an important strategy in modern biomaterial engineering [73]. Thus, being the alkaline mineral hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ the major component of the inorganic phase of bone, the use of synthetic hydroxyapatite (HA) appears to be advantageous for replacing that hard tissue over other synthetic materials. Due to its excellent biocompatibility, it promotes new bone formation along its surface and creates a biological fixation when implanted in bone [72, 82, 83]. Due to that, synthetic HA has been widely and successfully used for bone reconstruction over the years [129].

Cigarette smoke, and particularly nicotine, has been implicated in several deleterious physiological processes and disorders. Among them, some are known to affect directly the bone tissue, such as periodontitis, higher rate of tooth loss and dental implant failure [130-136]. It is known that nicotine negatively affects blood flow to the bone and tissues surrounding, which difficults the healing processes and can also lead to problems with the delivery of nutrients to bone cells [97]. Despite some controversial results regarding the effect of nicotine on osteoblasts, the majority of the studies suggest that nicotine inhibits the differentiation and activation of osteoblastic cells [88, 92, 93, 101, 107]. These inhibitory effects of nicotine on the osteoblast activity suggest that it may have an important role in modulating bone metabolism, and that it can also be a factor that may determine the survival rate of dental implants. Taken together, nicotine can cause bone

graft morbidity and impaired bone healing, and also compromise the bone integration of implants.

Because the extent of bone resorption that occurs at an implant surface seems to be critical for the fate of a prosthetic device, since implant failure may occur due to little or excessive remodeling in the bone that surrounds the implant, and being osteoclasts the cells responsible for bone resorption, during the past years osteoclastic bone resorption on HA have been well documented [137-142]. Generally, it was observed that compared to other biomaterials cells on HA expressed more pronounced actin rings and larger resorption lacunas [139, 140]. Moreover, (HA)-coated titanium implants have been successfully used, since they prove to improve implant fixation compared to uncoated implants, by promoting bone formation around the implant [143-146].

In addition to the very low amount of studies regarding the effects of nicotine on osteoclastic cells, to the present there is no published data about the modulation of the osteoclastic behavior by nicotine over a HA surface. For this reason, the aim of the present study is to evaluate the role of nicotine in osteoclastic differentiation and activity, either in unstimulated and stimulated (maintained in the absence or presence of recombinant M-CSF and RANKL, respectively) osteoclast precursor cells or in co-cultures of osteoclasts and osteoblasts-like MG63 cells performed over HA disks. For that goal, cell cultures were exposed to a range of salivary and plasmatic nicotine concentrations of 1.28×10^{-6} - 0.1 mg/mL. Cultures were assessed for several markers associated to the differentiation, activation and functional activity of osteoclastic cells. Scanning electron microscopy was also performed to examine evidence of resorption.

2 Materials and Methods

2.1 Preparation of hydroxiapatite (HA)

Commercial Hydroxyapatite (HA) (Plasma Biotall Limited) was used to prepare disks with 16 mm of diameter. The disks were obtained using 1 g of dry powder under uniaxial compression stress of 140 Kg/cm² (Mestra snow P3). The sintering temperature used was of 1300°C, with a 15 min plateau and applying a heating rate of 20 ° C/min. The sintering cycle was completed with a cooling process inside the furnace (Termolab/Eurotherm 2408). The mass of the HA discs was assessed by using an analytical balance (Metler AG285). After the sintering process, the disks were polished with a Dual Disc Polishing Machine (Stuers Rotopol-1), with waterproof sandpaper (# 320, # 500 and # 1000).

2.2 Isolation of human peripheral blood mononuclear cells (PBMC)

The cells were isolated from the blood of healthy donors with 25-35 years old, after informed consent. PBMC were isolated as described previously [108]. Briefly, blood was diluted with PBS (1:1), supplemented with 2 mM EDTA and applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). After centrifugation at 400g for 30 min, PBMC were recovered and washed twice with PBS + 2mM EDTA. Typically, around 70 x 10⁶ PBMC were obtained for each 100 ml of processed blood. PBMC cells were counted with a cytometer (Celltac MEK-5103).

2.3 Cell cultures

2.3.1 PBMC

PBMC were seeded on HA disks at 1.5x10⁶ cells/cm² and maintained in α -MEM supplemented with 30% human serum (from the same donor from which PBMC were collected), 100 IU/ml penicillin, 2.5 μ g/ml streptomycin, 2.5 μ g/ml amphotericin B and 2 mM L-glutamine. Cell cultures were performed in the absence (base medium, BM) or presence (M+R) of the osteoclastogenic inducers M-CSF (25 ng/mL) and RANKL (40 ng/mL) [23]. Cells were maintained in the absence (control) or presence of different nicotine concentrations (0.000128-10x10⁻²mg/mL). Cell cultures were incubated in a 5% CO₂ humidified atmosphere at 37°C. Culture medium was replaced once a week and nicotine

was renewed at each medium change. Colonized HA disks were characterized at days 14 and 21.

2.3.2 Co-culture of MG63 cells with PBMC

MG63 cells, established as an osteoblastic cell line from a human osteosarcoma, were cultured in α -minimal essential medium (α -MEM) (Gibco) supplemented with 10% foetal bovine serum, 100 IU/ml penicillin, 2.5 μ g/ml streptomycin, 2.5 μ g/ml amphotericin B and 50 μ g/ml ascorbic acid until reaching about 70-80% confluence. After enzymatic detachment with 0.05% trypsin and 0.5mM EDTA, cells were seeded (10^3 cells/cm²) over HA disks in the same experimental conditions described above. Cultures were maintained at 37°C for 24 hours. Then, 1.5×10^6 cells/cm² of PBMC cells were added and cell cultures were performed in the experimental conditions described for PBMC cultures.

2.4 TRAP activity quantification

TRAP activity was assayed by *para*-nitrophenylphosphate (pNPP) hydrolysis method. Cell cultures were washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100. Cellular extracts were incubated with 12.5 mM pNPP in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8) for 1 hour at 37°C. After addition of 5 M NaOH, the absorbance of the samples was measured at 400 nm in an ELISA plate reader (Synergy HT, Biotek). TRAP activity was normalized with total protein content and results were expressed as nmol/min. μ g_{protein}⁻¹. Cellular protein content was quantified by Bradford's method[110], using bovine serum albumin as a standard. In summary cell cultures were washed twice with PBS and solubilised with 0.1 M NaOH. After addition of Coomassie® Protein Assay Reagent (Fluka), the samples were incubated for 2 minutes at room temperature and the absorbance was quantified at 596nm in an ELISA plate reader (Synergy HT, Biotek).

3.4 RT-PCR analysis

RNA was extracted from cell cultures with RNeasy®Mini Kit (Qiagen) according to manufacturer's instructions. RNA was quantified by measuring the absorbance of the samples at 260 nm. The expression of the housekeeping gene GAPDH, and the osteoclast-related genes TRAP, CATK, CA2, c-myc and c-sr[147] was performed by RT-PCR. For that, half microgram of total cellular RNA from each sample was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55°C. The primers used are listed on Table 4. The RT-PCR products were

separated on a 1% (w/v) agarose gel and the observed bands were subjected to a densitometric analysis with ImageJ 1.41 software. Values were normalized for the corresponding GAPDH value of each experimental condition.

Gene	5' Primer	3' Primer
GAPDH	5'-CAGGACCAGGTTACCAACAAGT-3'	5'-GTGGCAGTGATGGCATGGACTGT-3'
c-myc	5'-TACCCTCTCAACGACAGCAG-3'	5'-TCTTGACATTCTCCTCGGTG-3'
c-src	5'-AAGCTGTTCTGGAGGCTTCAA-3'	5'-TTGGAGTAGTAGGCCACCAG-3'
TRAP	5'-ACCATGACCACCTTGGCAATGTCTC-3'	5'-ATAGTGGGAAGCGCAGATAGCCGTT-3'
CATK	5'-AGGTTCTGCTGCTACCTGTGGTGAG-3'	5'-CTTGCATCAATGGCCACAGAGACAG-3'
CA2	5'-GGACCTGAGCACTGGCATAAGGACT-3'	5'-AAGGAGGCCACGAGGATCGAAGTT-3'

Table 4 - Primers used on RT-PCR analysis of cell cultures

3.4 Visualization of cells with actin rings and expressing vitronectin and calcitonin receptors (VNR and CTR, respectively) by Confocal Laser Scanning Microscopy (CLSM)

Cell cultures were washed twice with PBS and fixed with 3.7% (v/v) para-formaldehyde for 15 min. After fixation, cells were permeabilised for 5 min with 0.1% (v/v) Triton X- 100 and stained for actin with 5 U/mL Alexa Fluor1 647-Phalloidin (Invitrogen), and for VNR and CTR with 50 mg/ml mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems), respectively. Detection of IgGs anti-VNR and IgGs anti-CTR was performed with 2mg/ml Alexa Fluor1 488-Goat anti-mouse IgGs.

3.5 Ca^{2+} quantification on culture medium

Cell cultures maintained in the absence or presence of 0.000128 and 0.0032×10^{-2} mg/mL of nicotine (a concentration that elicited a significant response on both PBMC and MG63+PBMC cultures) were performed on HA disks. After 7 days of culture, the sampling of the cell culture supernatant was performed, following the CalciFluor™ Assay manufacturer's instructions. Samples were collected at day 14 and at day 21 of culture. The fluorescence of the samples was measured in a fluoremeter with plate reader (Synergy HT, Biotek). The concentration of Ca^{2+} in the samples was corrected with the corresponding values obtained in the absence of HA disks and in the absence of cells.

3.6 Scanning Electron Microscopy (SEM)

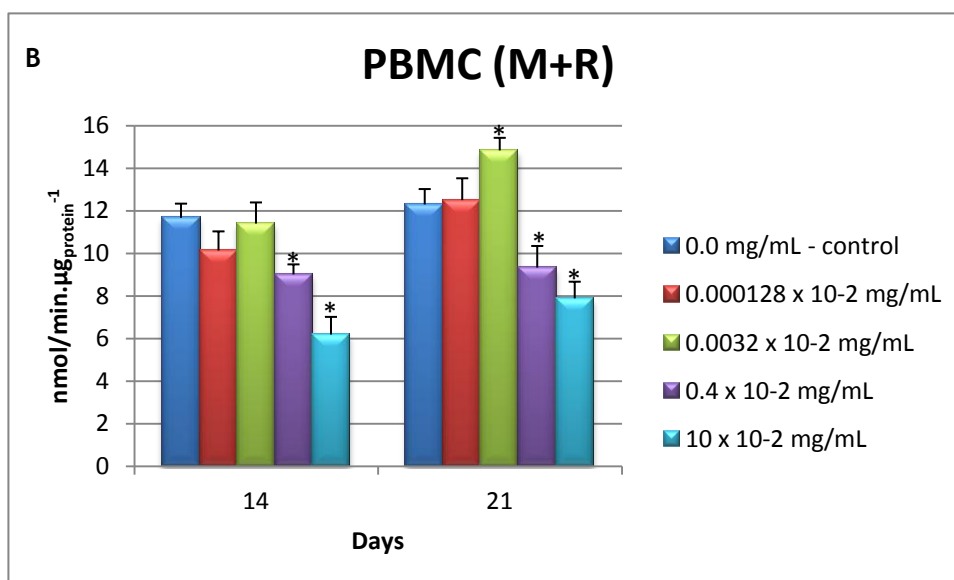
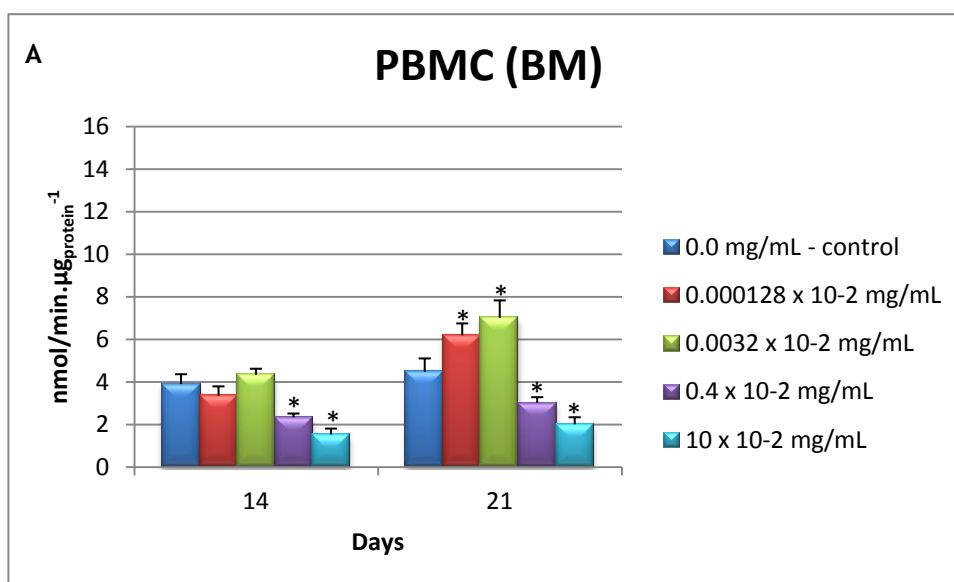
Cells were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate. The samples were dehydrated in graded series of ethanol solutions and critical-point dried. Specimens were mounted onto aluminium supports using Araldite™ and then sputter-coated with palladium- gold and observed in a Joel JSM 35C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

3 Statistical analysis

Data presented in this work are the means of three separate experiments performed with cells from four different blood donors. Statistical differences were assessed using an unpaired Student's t-test with Bonferroni correction for multiple comparisons. All data are expressed as the mean $\pm$ standard deviation of 3 examinations, with similar results obtained in each experiment. For values of $p \leq 0.05$, differences were considered statistically significant.

4 Results

4.1 TRAP activity



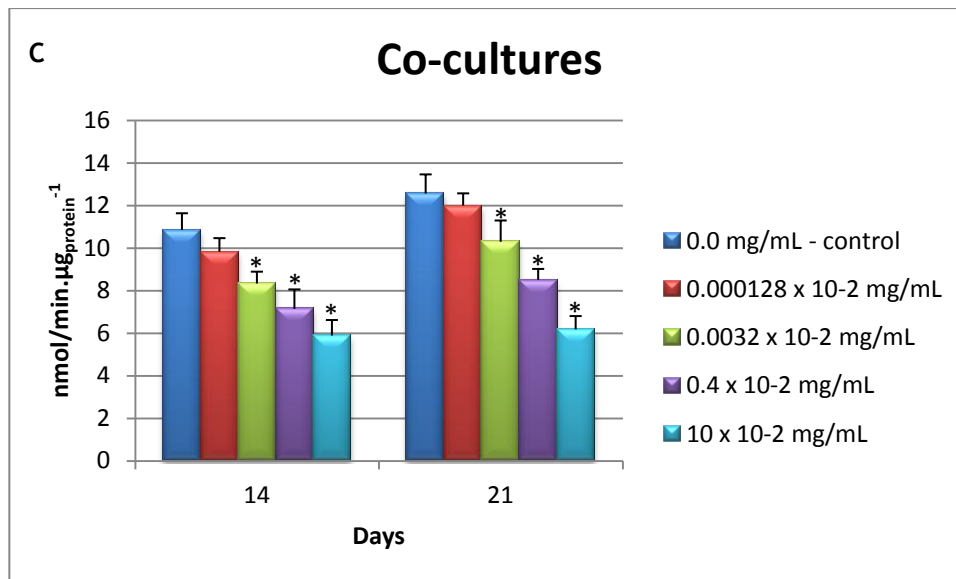
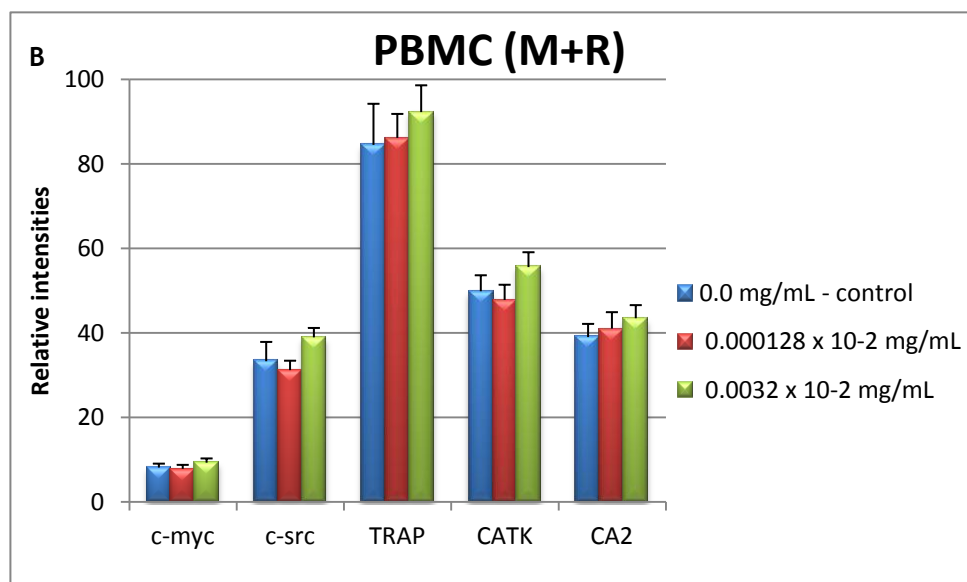
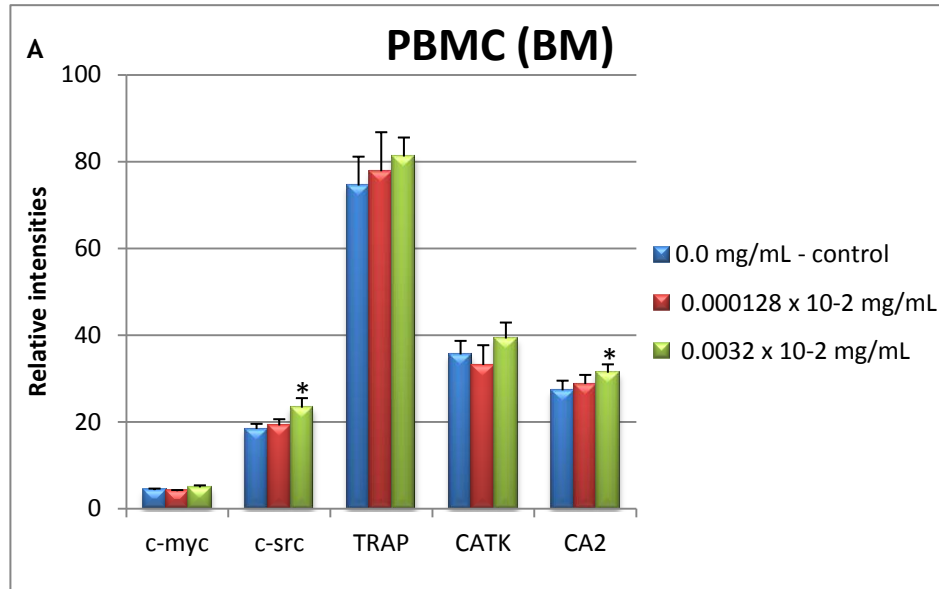


Figure 16 - TRAP activity of PBMC cultures, performed in the absence (A) or presence (B) of recombinant M-CSF and RANKL, and co-cultures of MG63 + PBMC (C), over HA disks. Cell cultures were maintained in the absence (control) or presence of different concentrations of nicotine. BM - base medium; M+R - supplementation with M-CSF and RANKL. * Significantly different from the control.

PBMC cultures performed in BM, in the absence of nicotine or in the presence of the lowest concentration of that molecule, revealed a low and identical TRAP activity, that increased in the presence of 0.0032×10^{-2} mg/mL nicotine (22% higher than the control at day 21) (Figure 16A). Concentrations higher than that elicited a significant decrease on the cellular response. Cultures performed in the presence of M+R displayed significantly higher values of TRAP activity, when compared with those maintained in BM (Figure 16B). Nevertheless, a similar pattern of cellular response induced by the presence of nicotine was observed. Again, the maximum response was achieved following supplementation with 0.0032×10^{-2} mg/mL nicotine, being about 9% higher than the control.

On the other hand, co-cultures of PBMC and MG63 cells displayed a different behavior, since for nicotine concentrations higher than 0.00128×10^{-2} mg/mL (whose response was similar to the control) TRAP activity exhibited a dose-dependent decrease in response to the presence of that molecule (Figure 16C).

4.2 RT-PCR analysis of osteoclastic cultures



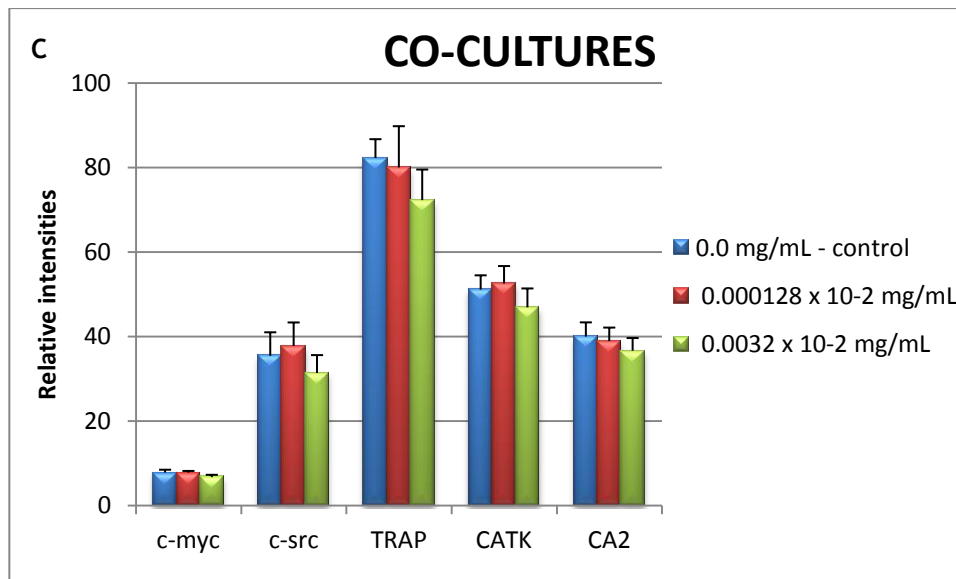


Figure 17 - Densitometric analysis of the RT-PCR products, normalized by GAPDH, of PBMC cultures performed in the absence (A) or presence (B) of recombinant M-CSF and RANKL, and co-cultures of MG63 + PBMC (C), over HA disks. Cell cultures were maintained in the absence (control) or presence of different concentrations of nicotine. BM - base medium; M+R - supplementation with recombinant M-CSF and RANKL. * Significantly different from the control.

PBMC cultures performed in BM or M+R conditions and co-cultures of PBMC + MG63 cells were treated with 0.000128×10^{-2} and 0.0032×10^{-2} mg/mL of nicotine and were assessed for the expression of several osteoclast-related genes by RT-PCR (Figure 17).

All the cultures expressed the analyzed genes. PBMC cultures (BM) revealed low expression levels. Although the presence of 0.000128×10^{-2} mg/mL nicotine did not significantly affect the cellular response, that response increased (about 4 -12 %) in the presence of 0.0032×10^{-2} mg/mL nicotine (Figure 17A).

PBMC cultures (M+R) performed in the absence or presence of 0.000128×10^{-2} mg/mL nicotine revealed identical expression levels of the osteoclastic genes, that increased (4-8%) following treatment with 0.0032×10^{-2} mg/mL nicotine (Figure 17B).

Co-cultures of PBMC and MG63 cells exhibited similar expression levels when performed in the absence of nicotine, or in the presence of the lowest tested concentration of that molecule. Nevertheless, supplementation with 0.0032×10^{-2} mg/mL nicotine caused a decrease (about 4-8%) on the cellular response (Figure 17C).

4.3 Presence of actin rings and expression of VNR and CTR in PBMC cultures

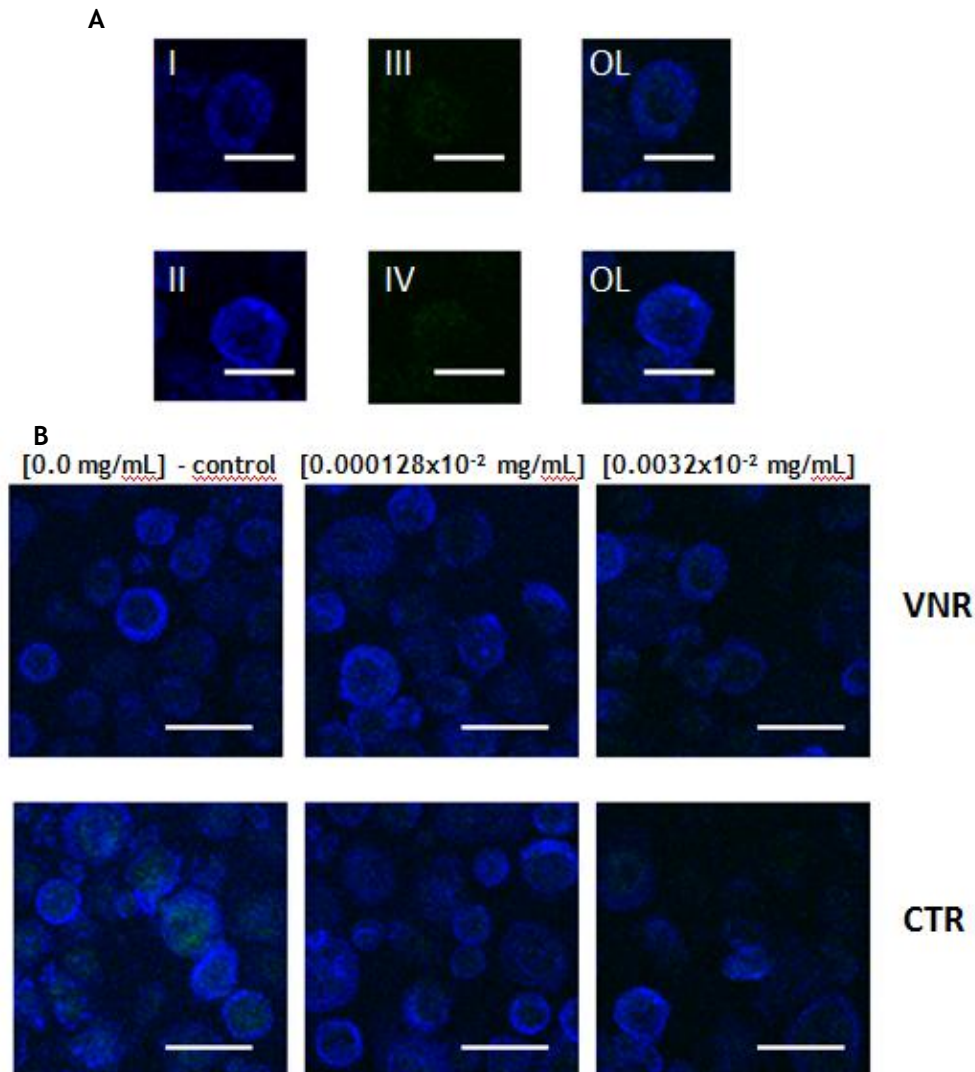
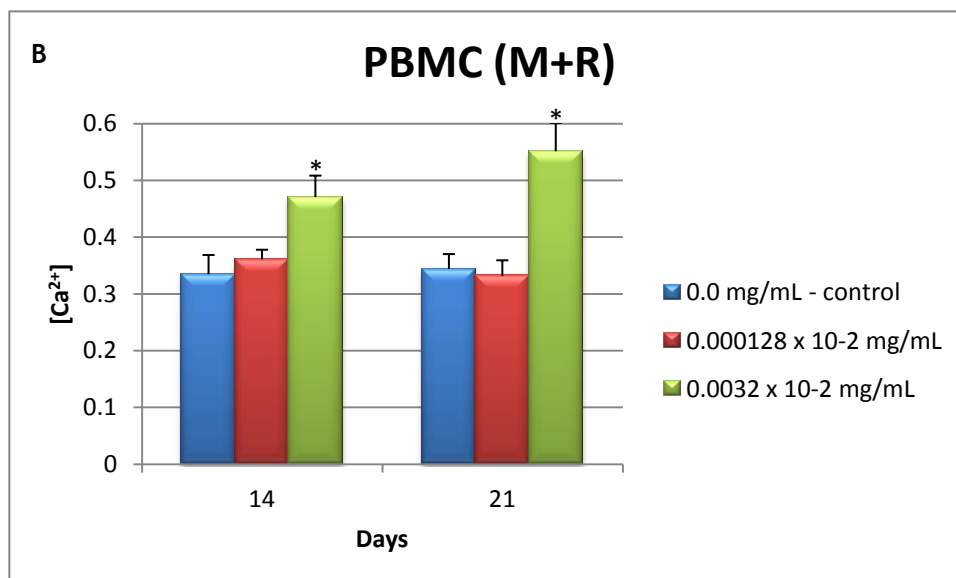
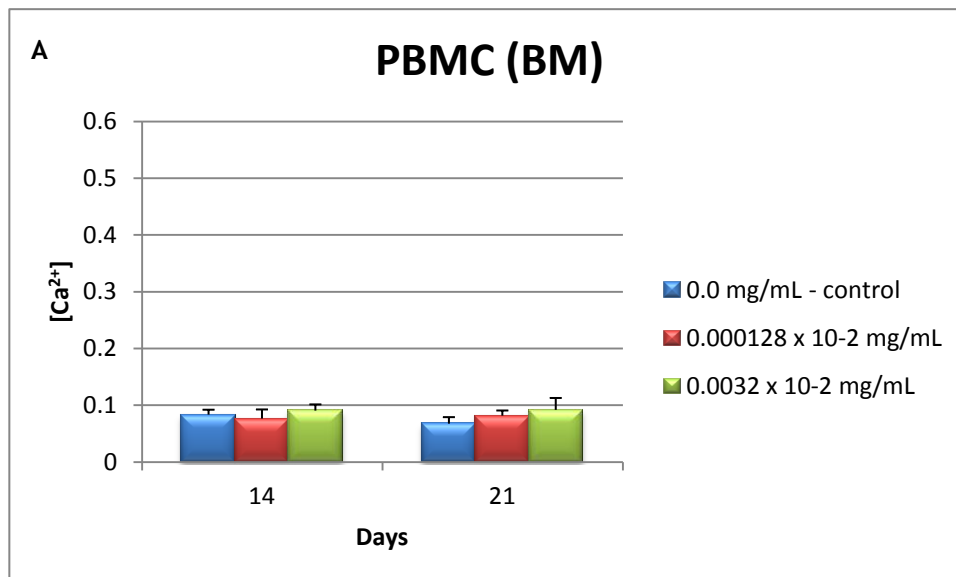


Figure 18 - Co-cultures of MG63 + PBMC on HA visualized by CLSM after 21 days of culture. A: Representative images: osteoclast stained blue for actin (II and IV), and green for vitronectin receptors, VNR (I) or calcitonin receptors, CTR (III); OL, overlay. B: CLSM visualization of MG63+PBMC co-cultures (M+R) performed on the surface of HA, maintained in the absence or presence of 0.000128 x10⁻² mg/mL and 0.0032x10⁻² mg/mL of nicotine. White bars represent 80 mm (A) or 120 mm (B).

Cell cultures maintained in the absence or presence of nicotine were stained for actin and for VNR and CTR and visualized by CLSM (Figure 18). It was possible to visualize cells displaying the analyzed osteoclast-features (Figure 18A) in all tested conditions. However, the amount of osteoclastic cells was somehow in line with the results obtained for TRAP activity and RT-PCR analysis (Figure 18B). Some micrographs obtained for the MG63 and PBMC co-cultures are depicted in Figure 18B.

4.4 Osteoclastic resorbing ability of HA



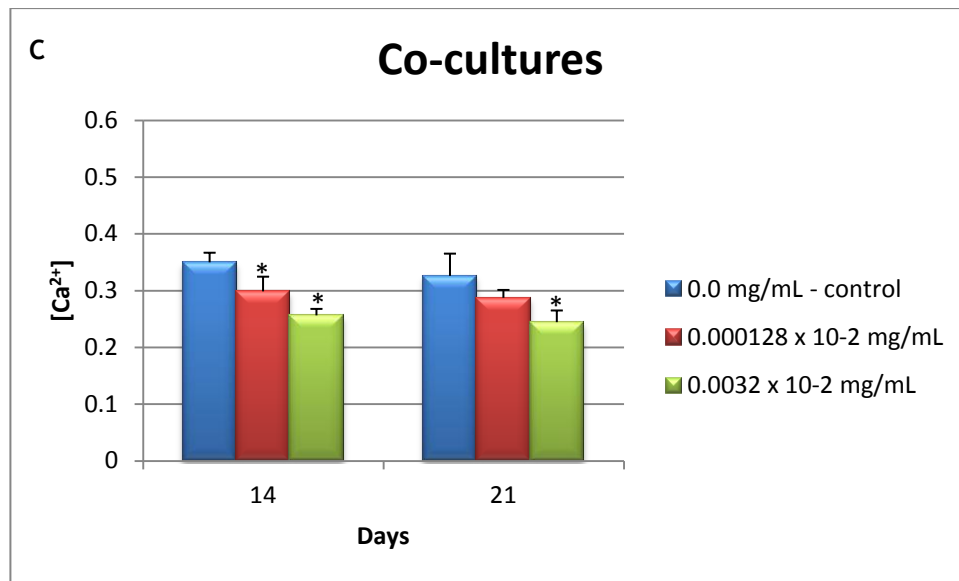


Figure 19 - Osteoclastic resorption activity of PBMC cultures maintained in BM (A) and M+R (B) and MG63 + PBMC co-cultures (C). Cell cultures were performed in the absence or presence of 0.000128×10^{-2} mg/mL and 0.0032×10^{-2} mg/mL of nicotine. BM - base medium; M+R - supplementation with M-CSF and RANKL. * Significantly different from the control.

In all tested conditions it was observed a release of Ca^{2+} to the culture media (Figure 19), and that value was identical at day 14 and at day 21. For cultures performed in BM (Fig. 19A), the amount of calcium present in culture medium was low and was not affected by the presence of the lowest concentration of nicotine. Nevertheless, at day 21, treatment with 0.0032×10^{-2} mg/mL nicotine caused a slight increase on that value (15%). In the presence of M+R (Figure 19B), a significant increase in the extracellular calcium concentration was achieved. Once again, supplementation of PBMC cultures with 0.000128×10^{-2} mg/mL nicotine did not change the cellular behavior, but the presence of 0.0032×10^{-2} elicited a significant increase (23%). In the case of MG63 + PBMC co-cultures (Figure 19C), it was observed a dose-dependent decrease in the release of Ca^{2+} to the culture medium, being about 14% lower than the control when co-cultures were treated with 0.0032×10^{-2} mg/mL nicotine.

4 Scanning Electron Microscopy

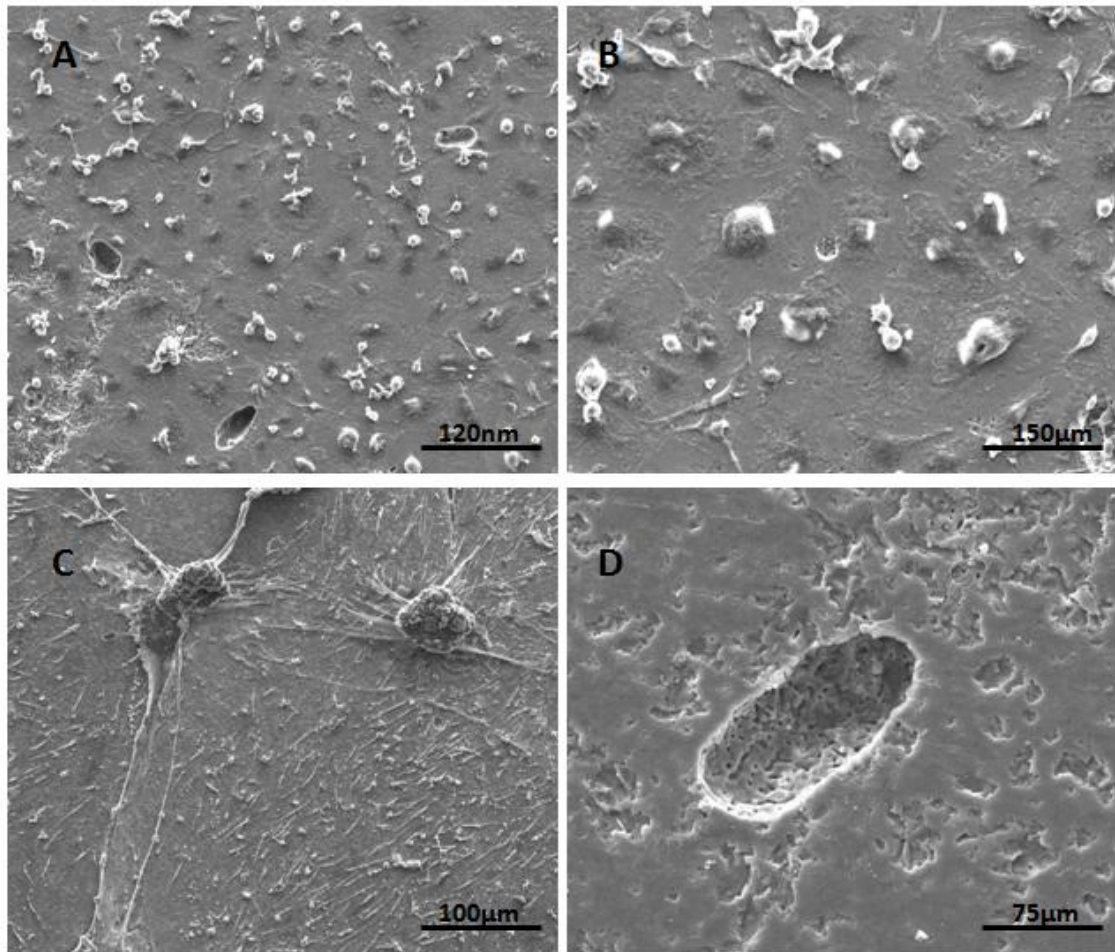


Figure 20 - SEM images of PBMC cultures (A, B and D) and co-cultures of MG63 and PBMC (C) performed over HA samples in the presence of 0.0032×10^{-2} mg/mL nicotine.

Figure 5 shows representative SEM images of PBMC cultures (panels A, B and D) and co-cultures of MG63 and PBMC (panel C) performed over HA samples in the presence of 0.0032×10^{-2} mg/mL nicotine. It was observed a homogeneous distribution of osteoclastic cells, sometimes associated with resorption lacunae (panel B). Also, the presence of lacunae was clearly seen after the removal of the cell layer (panel D).

5 Discussion

Previous studies have proven that due to its excellent biocompatibility, synthetic hydroxyapatite (HA), which is a phosphocalcic ceramic, is a good choice for bone reconstruction strategies. Furthermore these studies also demonstrate that osteoclast-like cells resorb sintered synthetic HA *in vitro* [73, 82, 83, 148, 149]. Thus, being osteoclasts the cells responsible for bone resorption, they partially dictate the fate of an implant, once an incorrect remodeling in the bone that surrounds the implant, can lead to implant failure. Innumerable studies revealed that nicotine is the most toxic chemical compound found in tobacco [150]. Nicotine has also proven to have a negative impact on implant osseointegration, therefore increasing the risk of implant failure [132, 133].

Once nicotine and bone resorption, are key factors to the long-term success of implant strategies, and because little information is available about that issue, the present work intended to look deeply into the role of nicotine in osteoclastic differentiation and activity over HA disks.

It was reported that plasmatic levels of nicotine tend to be in the range of 4 - 75 ng/ml, and that salivary nicotine concentrations can reach values between the range of 96 ng/ml - 1, 6 mg/ml [95, 96]. The concentrations used in the present study (up to 0.1 mg/mL) fall within those ranges and, thus, are considered to be representative of the physiological concentrations of nicotine in smokers.

It was observed that the presence of a low concentration of nicotine (0.0032×10^{-2} mg/mL) in isolated osteoclastic precursors enhances osteoclastogenesis when the precursor cells were cultured over HA disks. The effects were obtained not only for osteoclast differentiation, but also for the ability of the osteoclastic cells to resorb the HA disks. Nicotine concentrations higher than 0.0032×10^{-2} mg/mL caused a dose-dependent decrease on osteoclastogenesis, which might be due to toxicity of that molecule, when present at high concentrations. Globally, the lower levels of nicotine (0.000128×10^{-2} and 0.0032×10^{-2} mg/mL) used in this study are more representative of the nicotine plasma concentrations, while higher concentrations (0.4×10^{-2} - 1×10^{-2} mg/mL) are more close to the nicotine salivary levels achieved by those individuals. Moreover, the observed effects were achieved regardless of the presence of M-CSF and RANKL. Thus, it seems that a previous commitment of osteoclast precursor cells towards osteoclastic differentiation does not appear as a requirement for nicotine to exert its pro-osteoclastogenic effects. In that context, it is important to highlight that the overall relative nicotine-induced stimulation of the process was identical in both tested conditions (BM and M+R), which reinforces the idea that nicotine may act on osteoclast precursor cells by an independent and specific mechanism. The effects of nicotine on osteoclast development were also assessed before,

but not with human cells [106, 107] . Furthermore, those studies were not conducted with any biomaterial. Some controversial results were obtained since in one of them, that was performed with co-culture of mouse bone marrow cells and clonal stromal cells from mouse bone marrow (ST2 cells), nicotine decreased the formation of TRAP-positive multinucleated cells and the formation of resorption pits[107], while in the other study, that was conducted with porcine mature osteoclasts, the number of osteoclasts increased in a linear relationship with the increase of nicotine concentrations[106]. These different results may be due to the use of different cells, ranges of nicotine and/or methodologies. Regarding the co-cultures of human osteoclastic and osteoblastic cells, a different behavior was observed. In this case, the presence of nicotine elicited a dose-dependent inhibition of osteoclastic development and resorbing activity, which is in a clear contrast with the results achieved in PBMC cultures. Combining the two informations, it is tempting to suggest that nicotine affects not only directly the osteoclastic precursor cells, but also the osteoclastogenic properties of osteoblastic cells. Taking into account that in bone tissue the latter cell type is a key player in the modulation of osteoclastogenesis[151] , nicotine may induce changes in the expression of their osteoclastogenic molecules. Osteoblasts are known to produce many different molecules that can affect osteoclast differentiation, such as M-CSF, RANKL, TNF- α , IL-6, IL-17, among others [99, 151]. The identification of the osteoblastic molecules whose expression is affected by nicotine is now underway.

Our results revealed a negative effect of high concentrations of nicotine on osteoclast-like cells seeded on HA, namely in their differentiation and resorbing ability. This can affect the remodeling of the bone that surrounds the implant, increasing the chances of implant failure. Literature also supports the negative influence of nicotine on osteoclast-like cells activity[107]. High concentrations of nicotine was also reported to suppress the proliferation, alkaline phosphatase (ALP) activity and collagen synthesis, of osteoblast-like cells, on plasma-sprayed titanium implants [93, 101]. This apparently non-specific inhibition of cellular activities strongly suggests that nicotine concentrations higher than 0.0032×10^{-2} mg/mL may display a toxic effect on bone cells. Once the highest concentrations used in the present work fall within the range of those observed in the saliva of smokers, it can help to explain the high-rate of dental implant failures in those individuals[96, 119].

In conclusion, our results, suggest that nicotine has the ability to interfere directly with the osteoclastic cells capability of differentiate and resorb synthetic hydroxyapatite, inducing (low concentrations) or inhibiting (high concentrations) osteoclastogenesis. However, in an osteoclast-osteoblast co-culture system, the inductive effects of low doses

of nicotine were not observed. Moreover, a dose-dependent inhibition was displayed by the co-cultures, which is in line with a nicotine-induced osteoblastic mechanism of regulating osteoclastogenesis. Thus, it seems obvious that although nicotine can affect osteoclastogenesis, its effects on the process are complex and depend not only on its concentration but also in the cell types that are present. Also, the others innumerable molecular species contained in cigarette smoke, that can possibly have toxic effects, may affect the process.

Unraveling the detailed mechanisms involved in the effects of smoke components on bone tissue are of crucial importance and can ultimately lead to the improvement of the design of orthopaedic/dental implants, aiming a better biological response and therapeutic use in bone regeneration strategies.

CHAPTER 4

Chapter4 - Final Remarks

During the last decade a significant effort has been done towards understanding the effect of smoking on bone regeneration. However, although it is now known that smoking can lead to several pathologies and also to implant failure, the underlying involved mechanisms are still unknown.

In this study the effect of nicotine in osteoclastic differentiation and activity, either in unstimulated and stimulated (maintained in the absence or presence of recombinant M-CSF and RANKL, respectively) osteoclast precursor cells (PBMC) or in co-cultures of osteoclasts and osteoblasts-like MG63 cells was assessed. It was demonstrated that in PBMC cultures nicotine has the ability to act directly on human osteoclastic precursors, inducing (low concentrations) or inhibiting (high concentrations) the osteoclast differentiation. This effect was observed both for spontaneous or induced osteoclastogenesis. However, in an osteoclast/osteoblast co-culture system, nicotine revealed an inhibitory effect that was dependent on its concentration, which suggests that that molecule may also act indirectly in the process, *via* the osteoblastic cells. In the second part of this work, the role of nicotine in osteoclastic differentiation and activity, either in unstimulated and stimulated (maintained in the absence or presence of recombinant M-CSF and RANKL, respectively) osteoclast precursor cells or in co-cultures of osteoclasts and osteoblasts-like MG63 cells, performed over hydroxyapatite (HA) disks, was also assed. The results were in line with the previous ones, once they also suggest that nicotine has the ability to interfere directly with the osteoclastic cells capability of differentiate and resorb synthetic hydroxyapatite, inducing (low concentrations) or inhibiting (high concentrations) osteoclastogenesis. It was also observed that in an osteoclast-osteoblast co-culture system, the inductive effects of low doses of nicotine were not observed. Moreover, a dose-dependent inhibition was displayed by the co-cultures, which is in line with a nicotine-induced osteoblastic mechanism of regulating osteoclastogenesis.

In summary, this work provides new insights to next in vitro studies that aim to clarify the effects of nicotine on bone metabolic activities, being these crucial to understand and prevent the deleterious effects of smoking in bone tissue and also to the improvement of the design of orthopaedic/dental implants that can somehow minimize the long-term problems observed in smokers.

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