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The Influence of Modified Chitosan Substrates on Osteoclastogenesis

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ABSTRACT

Chitosan is a widely used biomaterial, biocompatible and biodegradable, as well as easily modifiable and processed into 2D and 3D substrates. If a biomaterial is to be used as a scaffold for bone regeneration it is important that its degradation takes place within an appropriate time frame. A recent report of osteoclasts cultured on chitosan substrates indicated that chitosan *per se* might inhibit osteoclasts activity, but when modified with collagen, activity was enhanced. Thus, osteoclasts are attractive candidate cells for promoting modified chitosan scaffold degradation, as part of a potential process of bone remodeling *in vivo*. In this context, our main aims were to investigate the capacity of fibrinogen-modified chitosan to induce the formation of osteoclasts, and to evaluate their capacity to degrade this substrate.

To accomplish our goals, it was first necessary to establish a functional human monocyte-derived osteoclasts culture, which was performed on tissue culture polystyrene (TCPS) plates, where different combinations of osteoclast differentiating cytokines, cell densities, and time points of analysis were tested. In order to determine if the cells obtained were functional, Tartrate-Resistant Acid Phosphatase (TRAP) production and the formation of excavation pits on dentine slices were also examined. After optimizing culture conditions, monocytes were induced to differentiate into osteoclasts, on top of chitosan substrates either unmodified or modified by fibrinogen adsorption. Cell maturation, inferred by the formation of multinuclear osteoclasts, was analyzed by actin cytoskeleton and nuclei staining, followed by TRAP staining and quantification of the enzyme activity as measures of cell functionality. In addition, two distinct methods of monocyte isolation were compared: plastic adherence and negative selection. Regarding degradation of chitosan substrates modified with fibrinogen, the polymer was labeled with a fluorescent molecule to allow detection of degradation areas by both Fluorescence Microscopy and Fluorimetry. Finally, osteoclasts behavior in a 3D environment was also studied, on chitosan scaffolds modified or not with fibrinogen. Results were analyzed by confocal and scanning electron microscopy.

Osteoclast differentiation proved to be better in presence of the highest concentrations of Receptor Activator of Nuclear Factor- κ B (RANKL), together with the higher concentrations of Macrophage Colony-Stimulating Factor (M-CSF) tested. So, 50 ng/mL of RANKL and 30 ng/mL of M-CSF were selected to induce cell differentiation,



which resulted in functional osteoclasts according to the TRAP staining and the formation of excavation pits on dentine slices.

On chitosan substrates, the results obtained revealed that upon 7 days of culture already a higher number of differentiating osteoclasts were visible, when compared with TCPS control. Cells were bigger, exhibited larger actin rings and more nuclei per cell. Moreover, chitosan modification by fibrinogen adsorption increased the number of multinucleated cells and the number of nuclei per cell, particularly after 21 days of differentiation. These results were statistically significantly different, in particular when monocytes were isolated by negative selection. Additionally, osteoclasts differentiated on chitosan and modified chitosan substrates were TRAP positive and showed higher enzyme activity in the supernatant than on control conditions.

Quantification of chitosan degradation clearly showed that chitosan substrates were being degraded and indicated that when chitosan is modified with fibrinogen degradation is increased. However, this still requires further confirmation.

Similarly to 2D conditions, osteoclasts cultured on 3D substrates are also bigger and with more nuclei per cell on chitosan modified with fibrinogen. Furthermore, scanning electron microscopy confirmed the distinct morphologies of monocytes differentiated into osteoclasts, when compared with cells cultured without cytokines (macrophages).

Taken together our results show, for the first time, that fibrinogen-modified chitosan substrates potentiate osteoclastogenesis and possibly osteoclast activity, when compared to chitosan alone, supporting the argument that osteoclasts are able to degrade non-mineralized substrates.

RESUMO

O quitosano é um biomaterial amplamente utilizado, biocompatível e biodegradável, sendo facilmente modificado e processado em substratos 2D e 3D. Se um biomaterial for usado como substrato 3D para a regeneração óssea é importante que sua degradação ocorra dentro de um período de tempo adequado. Um estudo recente referente a osteoclastos cultivados em substratos de quitosano indicou que o quitosano, *per se*, pode inibir a actividade dos osteoclastos, mas quando modificado com colagénio, a actividade dos osteoclastos aumentou. Assim, os osteoclastos são candidatos atraentes para promover a degradação de substratos 3D de quitosano modificado, como parte de um potencial processo de remodelação óssea *in vivo*. Neste contexto, os nossos principais objectivos foram investigar a capacidade que o quitosano modificado com fibrinogénio possui, para induzir a formação de osteoclastos, e avaliar a sua capacidade para degradar este substrato.

Para atingir os nossos objectivos, foi inicialmente necessário estabelecer uma cultura funcional de osteoclastos derivados de monócitos humanos, a qual foi realizada em TCPS, e onde foram testadas as diferentes combinações de citocinas necessárias para induzir a diferenciação dos osteoclastos, as densidades celulares e os tempos de análise das culturas. Com o propósito de avaliar se as células obtidas eram funcionais, foi avaliada a produção de TRAP e a formação de lacunas em discos de dentina. Após a optimização das condições de cultura, os monócitos foram induzidos a diferenciarem-se em osteoclastos, em substratos de quitosano modificados, ou não, por adsorção de fibrinogénio. A maturação das células, inferida pela formação de osteoclastos multinucleares, foi analisada através da marcação para a actina do citoesqueleto e núcleos, seguida da coloração para o TRAP e da quantificação da actividade desta enzima, como medidas de funcionalidade celular. Além disso, dois métodos distintos de isolamento de monócitos foram comparados: a adesão em plástico e a selecção negativa. No que concerne à degradação dos substratos de quitosano modificados com fibrinogénio, o polímero foi marcado com uma molécula fluorescente para permitir a detecção de áreas de degradação pelos métodos de Microscopia de Fluorescência e Fluorimetria. Finalmente, foi também estudado o comportamento dos osteoclastos em ambientes 3D, tendo sido usados substratos 3D de quitosano modificados ou não com fibrinogénio. Os resultados foram analisados por Microscopia Confocal de Varrimento a Laser e Microscopia Electrónica de Varrimento.



Verificou-se que a diferenciação dos osteoclastos foi melhor na presença das maiores concentrações de RANKL testadas, juntamente com as concentrações mais elevadas de M-CSF também testadas. Assim, 50 ng/mL de RANKL e 30 ng/ml de M-CSF foram seleccionadas para induzir a diferenciação celular, o que resultou na obtenção de osteoclastos funcionais, de acordo com os resultados obtidos após a coloração de TRAP e a formação de lacunas na superfície dos discos de dentina.

Os resultados obtidos revelaram que, após 7 dias de cultura, era já visível um maior número de osteoclastos em diferenciação nos substratos de quitosano quando comparado com o controlo TCPS. As células eram maiores, exibiam anéis de actina com maior diâmetro e mais núcleos por célula. Além disso, a modificação dos substratos de quitosano por adsorção de fibrinogénio aumentou o número de células multinucleadas e o número de núcleos por célula, particularmente após 21 dias de diferenciação. Estes resultados são estatisticamente diferentes, principalmente quando os monócitos foram isolados pelo método de selecção negativa. Ainda, os osteoclastos diferenciados em substratos de quitosano e quitosano modificado eram TRAP positivos, apresentando uma maior actividade enzimática no sobrenadante do que nas condições de controlo.

A quantificação da degradação do quitosano revelou distintamente que os substratos de quitosano foram degradados ao longo do tempo, e revelou que a modificação de quitosano com fibrinogénio promoveu o aumento da degradação. No entanto, estes resultados requerem posterior confirmação.

De forma semelhante às condições 2D, nos substratos 3D de quitosano modificado com fibrinogénio os osteoclastos eram maiores e com mais núcleos por célula. Além disso, a Microscopia de Electrónica de Varrimento confirmou as morfologias distintas de monócitos diferenciados em osteoclastos, quando comparado com células cultivadas sem citocinas (macrófagos).

Em conjunto os nossos resultados mostram, pela primeira vez, que os substratos de quitosano modificados com fibrinogénio potenciam a osteoclastogénese e, possivelmente, a actividade dos osteoclastos, quando comparado com os substratos de quitosano não modificados, apoiando o argumento de que os osteoclastos são capazes de degradar substratos não mineralizados.



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ACRONYMS

1,25(OH)₂D₃	1,25 dihydroxyvitamin D3 (1,25(OH) ₂ D ₃)
3D	Three Dimensional
AcOH	Acetic Acid
BC	Buffy Coat
BM	Bone Marrow
BMP	Bone Morphogenic Factors
BMU	Basic Multicellular Unit
BSA	Bovine Serum Albumine
Cath K	Cathepsin K
cDNA	Complementary DNA
Ch	Chitosan
CLSM	Confocal Laser Scanning Microscope
CN	Calcitonin
CNR	Calcitonin Receptor
DAPI	4',6-Diamidino-2-Phenylindole
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCL	Chloridric Acid
HFG	Human Fibrinogen
HSC	Hematopoietic Stem Cell
IGF	Insulin Growth Factor
IL	Interleukin
KOH	Potassium Hydroxide
M-CSF	Macrophage-Colony Stimulating Factor
MHC	Major Histocompatibility Complex
min	Minutes
ml	Mililiter
MMP	Matrix Metalloproteinase
MSC	Mesenchymal Stem Cell



NaOH	Sodium Hydroxide
NK	Natural Killer
OB	Osteoblast
OC	Osteoclast
OCIF	Osteoclastogenesis Inhibitory Factor
OCL	Osteoclast-like Cell
OPG	Osteoprotegerin
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffer Saline
PEG	Poly(ethylene glycol)
PFA	Paraformaldehyde
PG	Prostaglandin
PI	Propidium Iodide
PI-3	Phosphatidylinositol-3
PPAR-γ	Peroxisome Proliferative Activated Receptor- γ
PS	Penicillin G-Streptomycin
PTH	Parathyroid Hormone
RA	Rheumatoid Arthritis
RANK	Receptor Activator of Nuclear Factor- $\kappa\beta$
RANKL	Receptor Activator of Nuclear factor- $\kappa\beta$ Ligand
RBC	Red Blood Cells
RFU	Relative Fluorescence Units
RGD	Arginine-Glycine-Aspartic Acid
RNA	Ribonucleic Acid
RPM	Rotations per Minute
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Scanning Electron Microscope
TAC	Tetrameric Antibody Complexes
TBE	Tris/Borate/EDTA
TCPS	Tissue Culture Polystyrene
TNF	Tumor Necrosis Factor
TRAP	Tartrate Resistant Acid Phosphatase
VNR	Vitronectin Receptor
α-MEM	Alpha Minimal Essential Medium



CHAPTER I

INTRODUCTION



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1. BONE SYSTEM

The bone system is composed of bones, cartilage and joints. Bone is formed by cells and extracellular matrix (ECM), which is divided in two components: organic and inorganic. The former is mainly constituted of collagen type I, non-collagenous proteins and proteoglycans, whereas the latter is rich on calcium and phosphorus, appearing as hydroxyapatite crystals, placed into the collagenous matrix. This organization confers rigidity and strength to the bone, allowing the maintenance of some elasticity [1]. The most important bone system functions are support, as the skeleton forms a rigid framework to which are attached the softer tissues and the organs of the body, and movement because bones act as levers when attached muscles contract, causing movement. Bone also acts as mineral and energy storage, as bone matrix is composed primarily of calcium and phosphorus, and by minor amounts of magnesium and sodium, that can be released in small quantities when needed. Protection is another important function ensured by the bone system, which encloses many vital organs, as for example the skull, which protects the brain. Finally, hematopoiesis is a major function assured within the niche created inside the bones that make up the skeletal system – the bone marrow (BM). In an adult it is the BM that produces white and red blood cells and platelets [2].

Osteoblasts (OB), osteocytes and Osteoclasts (OC) are cells of the bone that have different origins [2, 3]. OB are derived from pluripotent mesenchymal stem cells (MSC). During MSC differentiation into OB the expression of phenotypic markers, such as alkaline phosphatases and osteocalcin is increased, whose activation promotes OB differentiation [4]. OB are cellular bone producers and, when forming bone matrix, they get entrapped in it and originate osteocytes [5]. OC on the other hand, derive from pluripotent hematopoietic stem cells (HSC). HSC can give rise to two types of multipotent lineage progenitors: common lymphocyte and common myeloid progenitors, depending on the stimulus they receive [6]. It is the common myeloid progenitor cell that originates megakaryocyte, erythrocyte and granulocyte/macrophage progenitors; and the latter originate OC [7].



1.1. BONE METABOLISM

Bone metabolism includes bone remodeling, where bone resorption is followed by bone formation. These two processes are regulated by local and systemic factors, including Receptor Activator of Nuclear Factor kappa-B ligand (RANKL), Macrophage Colony-Stimulating Factor (M-CSF), Calcitonin (CN), $1,25(\text{OH})_2\text{-D}_3$ (Vitamin D3), among others [8], and requires the co-ordinated actions of OB and OC [9].

1.1.1. BONE REMODELING

Although bone appears to be metabolically inert and static, it is actually a dynamic organ, under the active control of both bone-forming OB and bone-resorbing OC (Figure 1). Bone formation and resorption are functionally coupled such that inhibition of resorption also suppresses bone formation [10, 11]. Replacement of old bone by new bone allows removal of microscopic bone fractures. OC are responsible for degrading bone, removing both organic and mineral parts and forming resorption pits on the bone surface. Previous work shows that these are the only cells that are able to perform this function efficiently [9]. After OC-mediated bone resorption mature OB secrete bone matrix, which becomes mineralized [12].

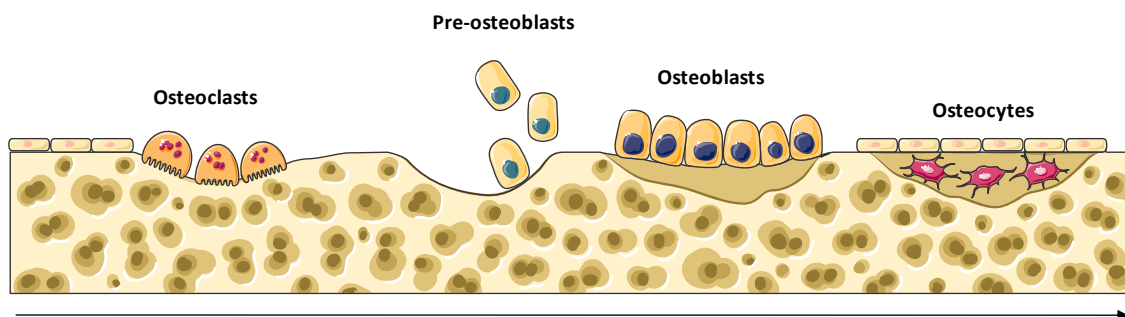


Figure 1 – The bone remodeling process. After bone resorption performed by osteoclasts, osteoblast precursors are recruited and induced to differentiate into mature osteoblasts, which begin to form new bone matrix, giving rise to osteocytes, when inside the matrix. (Adapted from: Servier: Medical Art <http://www.servier.co.uk/medical-art-gallery/>).

By dynamically shifting the equilibrium, bone remodeling regulates extracellular calcium levels and responds to mechanical loading [13, 14]. Numerous factors, systemic and local, are responsible for regulating bone remodeling in healthy adults, among which insulin, insulin-like growth factor-I (IGF-I), bone morphogenetic factors (BMPs) and Wnt proteins are potent bone anabolic factors [15, 16].

Bone remodeling can be divided in three phases: initiation phase, when OC differentiate and resorb bone; transition phase, when resorption is suppressed and bone formation is activated; and termination phase, when bone formation stops [17]. During bone remodeling, a group of OC and OB that cooperates is called a Basic Multicellular Unit (BMU). In cortical bone, a BMU is located at the leading edge of a cylindrical canal. At the tip, OC dig a tunnel, which OB fill, except for the centre channel, forming an osteon. In vertebrates, mature bones consist of dense surface plates of bone, the cortices, within which there is a web of delicate bone struts oriented to oppose loading forces, the trabecular bone. In trabecular bone, a BMU is located on the bone surface, as a hemi-osteon. Both OC and OB precursors are supported by the microvasculature that irrigates the BMU [18].

Physiological bone resorption is known to be controlled by systemic hormones, whose main role is to influence bone stromal cells, especially OB, instead of OC or their progenitors, directly [19]. These osteolytic hormones are responsible for stimulating the production of RANKL by OB, which is essential for OC formation and function [20], and M-CSF, also a critical factor to generate OC both *in vivo* and *in vitro* [21]. Both cytokines are also known to promote active OC survival [22].

To initiate bone resorption, OC adhere to the substrate, by extending their adhesive structures-like podosomes and $\alpha_v\beta_3$ integrin binding. Previous studies have demonstrated that antibodies against this integrin and $\alpha_v\beta_3$ antagonists, such as echistatin and kistrin, are sufficient to interrupt osteoclastic activity *in vivo* and *in vitro* [23, 24]. The area that will be resorbed, is then closed through the sealing zone (constituted by an actin ring), which seals the bone matrix in roughly circular areas. As a result, sealing zones, also designated clear zones, become devoid of cytoplasmic organelles. Next, OC develop ruffled border membranes inside the sealing zones. Ruffled borders are highly convoluted, rich in vesicles containing proton pumps and hydrolases and will be inserted into the bone-apposed surface. In addition, OC contain vacuoles of various kinds, whose functions are linked with secretory and transport processes. A resorptive hemivacuole is formed between cells and bone, to where



hydrochloric acid will be secreted and that will degrade bone mineral (hydroxyapatite). Microelectrode-based pH measurements at the ruffled border have shown pH levels as low as 3 to 4 [25]. The acid secretion of protons is followed by passive transport of chloride ions, which contributes to maintain electroneutrality [26]. Furthermore enzymes like cathepsin K (Cath K) and Tartrate Resistant Acid Phosphatase (TRAP), are secreted by OC, during bone resorption, into the bone to digest collagen fibers and other matrix proteins, under acidic conditions [18]. Cath K has the unique capacity to degrade the triple helix of native collagen, at an acid pH, and compounds that inhibit Cath K strongly suppress bone resorption *in vitro* [18]. After bone degradation, bone debris are transported from the extracellular space under the ruffled border through the cells, leaving the cell at the opposite side of the bone surface, by transcytosis (Figure 2).

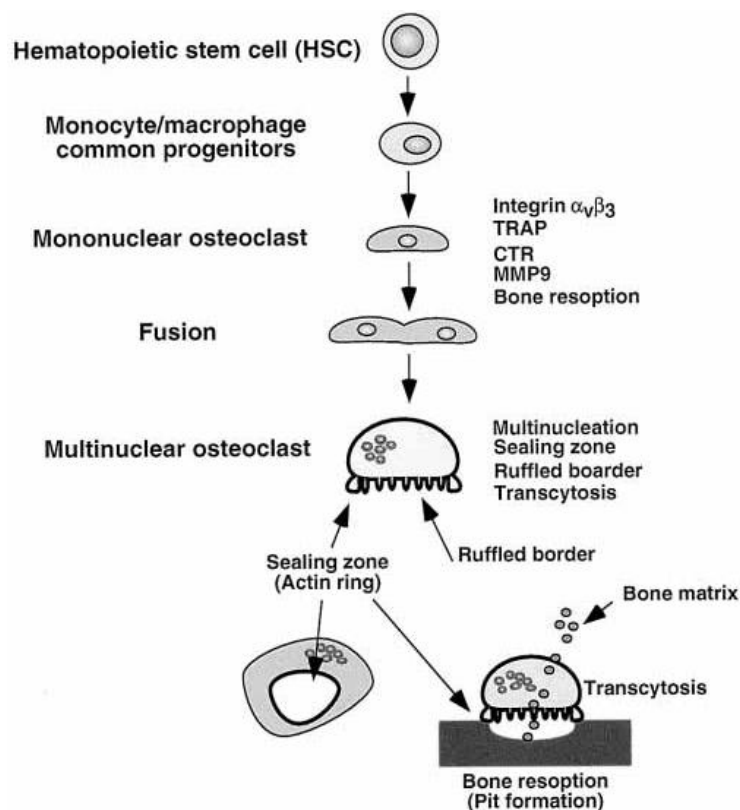


Figure 2 – Osteoclast development from hematopoietic stem cell, multinucleation and bone resorption. Osteoclasts derive from hematopoietic stem cells through osteoclastogenesis. Mononuclear osteoclasts are able to merge and form bigger multinucleated cells, which have the ability to degrade bone to a higher extent. Osteoclasts have two distinct zones; the ruffled border, responsible for secreting hydrochloric acid and hydrolases into the bone, in order to degrade it; and the sealing zone, which is ring shaped, formed by actin, and responsible for preventing the acid leakage from the area that is being resorbed. During bone resorption, some bone debris are formed which are transported across the interior of the osteoclast via transcytosis, in order to form new bone matrix [27]. TRAP: Tartrate Resistant Acid Phosphatase; CTR: Calcitonin Receptor; MMP-9: Matrix Metalloproteinase-9.

While resorbing bone, OC stimulate OB to restock the resorption lacunae with new bone (osteoid), which is then gradually mineralized (Figure 1). OB are responsible for building up bone, synthesizing collagenous fibers that are oriented along lines of stress, and the consequent deposition of hydroxyapatite mineral in the bone matrix helps to resist compression. OB promote mineralization during ossification, being subsequently trapped into their own matrix, and developing into osteocytes, which maintain bone tissue [2, 28]. Osteocytes are also able to communicate with OB and OC by extending dendrites, into small canals, called canaliculi [18]. Communication between OB and OC is quite important, not only when considering bone resorption, but also in bone remodeling. This cellular cross-talk between bone cells and bone surface is mediated by cell-cell and cell-matrix interactions and involves a number of adhesion molecules, such as integrins, selectins and cadherins [3].

1.2. OSTEOCLASTS

OC precursors, which exhibit a monocyte-macrophage phenotype and can be found in the spleen, BM and peripheral blood, are induced to differentiate into OC at or near the bone surface area, by factors of the surrounding environment. Thus, the microenvironment to which common myeloid precursor cells are exposed, may determine if these cells will differentiate into immune or bone-resorbing cells [9].

Activated macrophages¹ and OC share some common cell surface molecules, like integrin receptors and CD68. Moreover, OC express a number of molecular markers some of which shared by macrophages, such as, calcitonin receptor (CNR), vitronectin receptor (VNR), also known as integrin $\alpha_v\beta_3$, RANK (receptor of RANKL), c-fms (receptor of M-CSF), Cath K, among others [29] and they also exhibit Matrix Metalloproteinase (MMP)-9 enzymatic activity (Figure 2). Previous work suggests that MMPs identified in bone may be important in the dissolution of non-mineralized matrix,

¹ Macrophages can be activated when under the influence of some cytokines; they become larger, more motile, stickier, express more Major Histocompatibility Complex (MHC) II proteins on their surface, contain more lysosomal enzymes, and secrete a variety of substances including interleukin-1, tumor necrosis factors. Also, they have an increase in their phagocytic activity.



enabling the formation of a tight seal, between OC and the substrate, for matrix acidification [5].

However, some receptors that mediate macrophage interactions with T cells and the innate immune system, such as immunoglobulin Fc and complement, $\beta 2$ integrins, CD14, [11] are not expressed by OC. Nonetheless, considering that their progenitors express these molecules, perhaps OC formation may be influenced by immune factors that do not affect mature OC [12]. Macrophages and OC are both able to produce the TRAP enzyme [11]. Importantly, a study performed by Janckila and others [30], reported that there are two isoforms of TRAP, TRAP 5a and TRAP 5b. During bone resorption, the OC-specific isoform TRAP 5b, is released by OC into circulation [28]. Conversely, macrophages secrete TRAP 5a but not TRAP 5b *in vitro* [30]. OC can also be distinguished from macrophages morphologically, through the formation of ruffled borders and actin ring structures, in the presence of bone. These cytoskeleton rearrangements are necessary to resorb bone; similarly, OC secretion of H^+ ions and proteolytic enzymes in a restricted area is required for bone resorption, a function that is not performed by macrophages.

Newly differentiated OC present only one nucleus per cell (mononuclear OC), but with progress of their differentiation, they usually merge, originating bigger cells with more than one nucleus (multinucleated OC) (Figure2), in order to cover up a larger area.

During the bone resorption cycle, OC experience dramatic changes to their size and shape, demanding profound cytoskeleton re-organization [31]. An example is the formation of the OC characteristic sealing zone, formed by an actin ring, and which prevents acid and proton leakage from the area that is being resorbed (Figure 2) [27].

Actin is a main component of the cytoskeleton, which plays a role in cell integrity, shape, adhesion and motility [32]. Actin can be filamentous or non-filamentous (unpolymerized); the latter can easily and rapidly suffer transformation into filamentous actin within the cells, in response to specific stimulus [33]. Several proteins have been identified as crucial for the reorganization of the osteoclastic cytoskeleton; these include the tyrosine kinase c-Src [34], as well as the lipid kinase phosphatidylinositol-3 (PI-3) kinase [35]. In fact, PI-3 kinase inhibitors, have been shown to inhibit bone resorption [36].



The identification of cell surface markers expressed on OC, namely RANK and c-fms, made possible to induce OC differentiation *in vitro*, without the need for OC/OB co-cultures. These two membrane receptors interact with RANKL and M-CSF, respectively. Both RANKL and M-CSF combined effects are responsible for inducing OC differentiation, even in the absence of osteoblastic cells [37]. RANKL is a member of the Tumor Necrosis Factor (TNF) family, which was initially characterized as a T-lymphocyte specific protein and later shown to be expressed by almost all cell types in the body, including OB, has a critical cooperation in the regulation and differentiation of OC [38] RANKL is responsible for activating c-jun terminal kinase, sending signals to the Nuclear Factor- κ B [39].

It was been demonstrated by Yasuda and others, that OC formation is induced by osteotropic factors and, the majority of these, act indirectly by binding marrow stromal cells, leading to RANKL expression up-regulation [40]. RANKL will then bind to RANK receptor on OC precursors, inducing OC formation (Figure 3), and stimulating the expression of several osteoclastic molecules, such as TRAP and Cath K [41, 42].

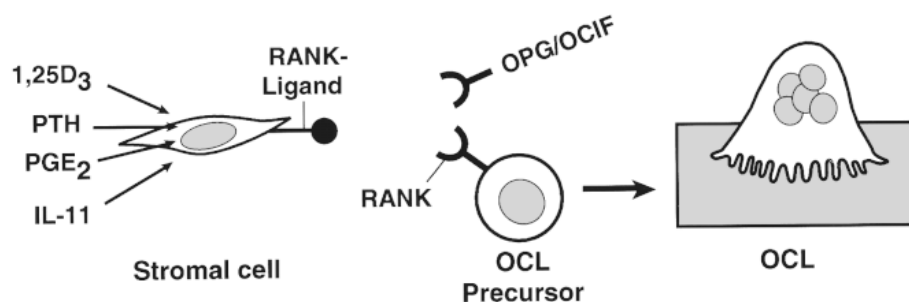


Figure 3 – RANKL role in osteoclastogenesis. RANKL is upregulated by osteotropic factors, such as Vitamin D3 (1,25D3), Parathyroid Hormone (PTH), Prostaglandin E2 (PGE₂) and Interleukin (IL)-11, on marrow stromal cells and osteoblast. Receptor Activator of Nuclear Factor κ B Ligand (RANKL) binds the RANK receptor on osteoclast precursors, inducing osteoclasts formation [39]. OPG: Osteoprotegerin; OCIF: Osteoclastogenesis Inhibitory Factor; OCL: Osteoclast-like cell.

RANKL activity may be blocked by Osteoprotegerin (OPG), as it works as a decoy receptor for RANKL inhibiting its action and blocking OC fusion and differentiation [42]. Expression of RANKL and OPG is regulated by many factors, and both are responsible for controlling osteoclastogenesis *in vivo* and *in vitro*.



Besides OPG, CN, a peptide hormone secreted by the parafollicular cells in the thyroid gland, also largely inhibits bone resorption by OC [44]. Both committed OC precursors and mature OC are known to express CNR, and so they may suffer inhibition by CN [39]. CN stimulates adenylcyclase activity and cyclic adenosine monophosphate (cAMP) accumulation, leading to OC immobilization and contraction, away from the bone surface [43], inhibiting resorption [44]. Importantly, of all cells of the mononuclear phagocyte system, OC are the only ones that express receptors for the anti-osteolytic calcitonin [45].

M-CSF, a homodimeric glycoprotein, is produced by many types of cells, such as granulocytes, endothelial cells, fibroblasts, OB, lymphocytes, among others [46]. M-CSF affects both OC precursors and mature OC, since c-fms, the M-CSF receptor, is also expressed on mature OC, maintaining their survival and chemotactic behavior [47]. M-CSF plays an important role not only in the differentiation, proliferation and survival of mononuclear phagocytic cells [48], but also for OC precursors, as it prevents OC apoptosis, enhances their motility, besides inhibiting bone resorption [49]. In fact, when spleen or bone marrow hematopoietic cells are cultured in the presence of $1,25(\text{OH})_2\text{D}_3$ -stimulated OB or in the presence of M-CSF and RANKL, OC formation takes place [37]. In bone, M-CSF and RANKL expression by OB cells and the presence of osteolytic factors, such as $1,25(\text{OH})_2\text{D}_3$, decrease OPG secretion and increase OB RANKL expression, which enhances osteoclastogenesis [50].

An adherent environment is also essential for OC development, with integrins playing important roles in OC bone resorption function [35]. A previous study has reported that when OC precursors were cultured in the presence of M-CSF and RANKL, but prevented from adhering to the substrate, no multinuclear TRAP-positive OC were formed [51]. The majority of these integrins are able to bind ligands that have in their constitution the arginine-glycine-aspartic acid (RGD) tripeptide, which is present in fibronectin, vitronectin, fibrinogen, osteopontin and thrombospondin, among other proteins that are present in the ECM [52]. OC may be identified by the expression of VNR, which also binds ligands through the RGD sequence.



1.3. OSTEOCLASTS-ASSOCIATED DISEASES

Even though bone has the remarkable capacity to heal without scar formation, disruption of the homeostatic balance of bone removal and replacement can cause bone abnormalities, such as osteopetrosis – due to a deficiency in osteoclastogenesis or lack of functional OC –, osteosclerosis – characterized by the increased bone formation – and osteoporosis – which comes as a consequence of a reduced bone formation, or due to an increased bone resorption [27]. Pathologic bone loss can manifest as osteoporosis, periodontal disease and some inflammatory arthritis; as inappropriate new bone formation is found for example in Ankylosing Spondylitis [4, 42]. Hence, when bone regenerative processes fail, the consequence is patients with large bone lesions or impaired fracture healing, requiring clinical intervention [53].

In rheumatoid arthritis, a disease characterized by local bone loss, osteoclastogenesis occurs at a higher rate than normal and outside the marrow cavity, which is an abnormal situation in healthy adults; this leads to bone and joint degradation, causing ultimately severe pain [54]. Previous studies have demonstrated that in inflammatory arthritis, OC are effector cells, responsible for the degradation of bone and cartilage. So, one way to fight bone degradation in patients with arthritis would be to stop osteoclastogenesis [55]. When OC precursors, generated in the BM, are mobilized through the blood stream to diseases sites, they originate OC, which will play roles both in resorbing bone under abnormal conditions and acting as effector cells, enhancing the inflammatory process [56]. Not only OC, but also T cells are assumed to be responsible for bone loss that occurs as a consequence of pathological conditions, such as infection, inflammation processes [57], and arthritis [58]. In fact, a subset of T cells that produces IL-17, Th-17 cells, has been identified as the exclusive osteoclastogenic T-cell subset [58]. IL-17 induces RANKL on osteoclastogenesis-supporting mesenchymal cells, such as OB. IL-17 also enhances local inflammation and increases the production of inflammatory cytokines, which further promote RANKL expression and activity as it is illustrated in Figure 4. Thus, T cells and OC interactions have a crucial role in the pathogenesis of bone loss, highlighting the importance of modulating the inflammatory response.



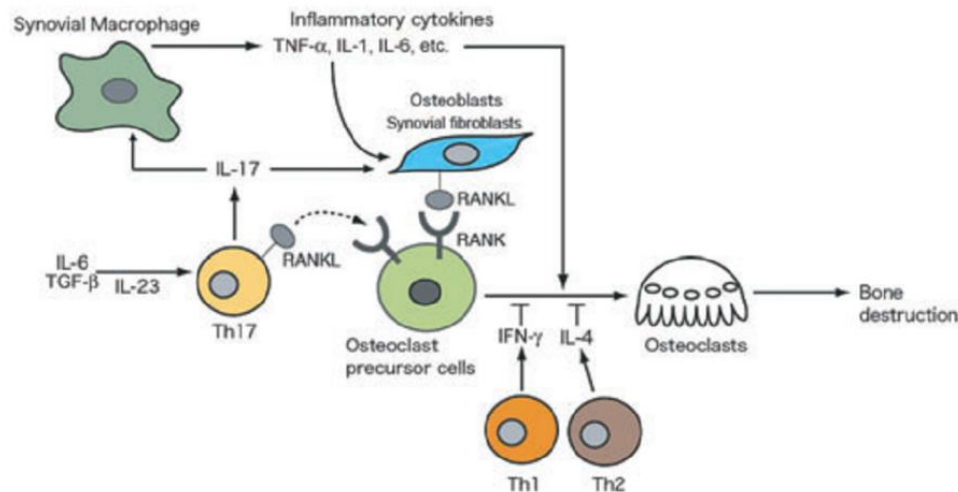


Figure 4 – Regulation of osteoclast differentiation by T cells in inflammation. T helper 17 (Th17) cells have stimulatory effects on osteoclastogenesis, while Th1 and Th2 cells have inhibitory effects through Interferon (IFN)- γ and Interleukin (IL)-4, respectively. IL-17 induces receptor activator of nuclear factor (NF)- κ B ligand (RANKL) on osteoblasts leading to the up-regulation of inflammatory cytokines, such as tumor necrosis factor (TNF)- α , IL-1, and IL-6. These cytokines further increase RANKL on mesenchymal cells and, in addition, act directly on the osteoclast precursor cells to enhance RANKL activity [58]. TGF: Transforming Growth Factor.

2. CHITOSAN INFLUENCE ON OSTEOCLASTOGENESIS

A biomaterial can be any substance (other than a drug), natural or synthetic, which treats, augments or replaces any tissue, organ or body function [59]. It needs to be safe, reliable, economic, and physiologically acceptable. Classical biomaterials, as metals, ceramics and polymer composites, have been successfully implanted, in order to replace mechanical functions of bone tissues, such as teeth, hips, knees, intervertebral disks, among others. However, their limited ability to modulate the repair and regeneration of host tissues, has restricted their use for tissue engineering applications, motivating the development of new materials [60]. Also, long term clinical success is limited by implant loosening, a consequence of the loss of surrounding bone. This loss is associated with fibrous capsule formation and production of inflammatory cytokines [61].

Presently, new materials are being developed, using different strategies, with the aim to control specific cell-binding interactions, release growth factors, degrade at a controlled rate and respond to environmental cues [62]. According to Hench and Polak [63], these “third-generation biomaterials” should support self-healing processes of the bone tissue and combine good resorptivity with osteoconductivity and osteoinductivity [64].

One material currently being used is chitosan, a naturally derived polymer that mimics the nature of the ECM, which has gained popularity due to its good characteristics, namely biocompatibility, ease of processing and ability to encapsulate cells and bioactive molecules [65-68]. Also, chitosan’s biodegradability, high viscosity [69, 70] and the fact that (in a chemical analysis) chitosan is reminiscent of glycosaminoglycans, – ECM components, with great importance in bone formation – it is also able to bind ECM components, growth factors and cytokines [71]. This is an important issue for bone engineering, because chitosan is able to concentrate added factors, near to the growth site. Although chitin-based tissues are not present in the human body the repeating unit of chitin, N-acetylglucosamine, is present in glycosaminoglycans and in glycoproteins [72].

Chitosan degradation *in vivo* is performed by hydrolases, such as lysozymes, which are able to recognize and to break down chitosan [73]. Besides lysozymes, there are other hydrolases also able to degrade chitosan, such as cellulases, amylases, proteases and chitosanases, among others [73]. Chitinases, for instance, are enzymes present in leukocytes and human serum capable of degrading chitosan [72]. Importantly, macrophages are capable of secreting very large amounts of chitinase, under specific circumstances [72]. An example of chitinases is chitotriosidase, which is also expressed in humans and has the ability to recognize chitin and chitosan, generating small diffusible chitin/chitosan fragments, capable of activating human macrophages [74, 75]. Although, products resultant from the degradation of synthetic polymers are known to be inflammatory, potentially damaging the surrounding tissue [76], recent reports revealed that chitosan degradation products support angiogenesis and show anti-inflammatory properties [77].

Biomaterial implantation, if intended for subsequent bone regeneration, should lead to the coordinated regulation of both OC and OB. For this reason, it is important to evaluate OB and OC differentiation and activity in response to biomaterials commonly used for bone regeneration [78]. To allow OC precursors to merge and form



multinuclear, fully differentiated and activated OC, cells must be able to migrate, along the substrate, not only to degrade it, but mainly to fuse with each other [73]. Chitosan substrates can be modified with molecules that contribute to cell migration and subsequent fusion. Recent studies of OC cultured on chitosan substrates modified with collagen, reported that OC activity was enhanced and also the expression of their markers was increased, when compared to OC cultured on unmodified chitosan substrates [73]. Also, some authors argue that it is necessary the presence of a mineralized substrate to induce OC resorptive behavior, as OC resorb bone if the mineral is exposed on the bone surface, but do not resorb bone that is unmineralized or has been demineralized [79, 80]. However, other authors suggest that OC activation occurs when they adhere to a rigid substrate, whether the adhesive ligand is vitronectin, fibronectin or collagen [81]. A previous study indicates that OC activity, and consequently their capacity to present resorptive behavior can be indirectly analysed by other characteristics and the functions that they are able to perform [82]. One of such features is the ability of OC to form actin rings, which are formed on bone and have been shown to correlate with its resorption [83]. Interestingly, OC do not form actin rings on plastic substrates but instead they form podosome belts [83]. A second characteristic is the secretion of TRAP, by OC, which has been shown to take place during bone resorption [84]. Finally, the third one is the formation of ruffled border and sealing zones, that are characteristic of resorbing OC [82]. All of these characteristics have been shown on OC cultured on chitosan substrates [73].

Fibrinogen is a protein present in blood plasma, essential for the coagulation of blood, which is converted into fibrin by the action of thrombin in the presence of ionized calcium. Fibrinogen is reported to be required for normal platelet function and wound healing [85]. Moreover, fibrinogen is effective in promoting cytokine secretion from Peripheral Blood Mononuclear Cells (PBMC), suggesting that this protein may actively modulate the inflammatory process [85]. Importantly, it has been proposed that fibrinogen has an important role in tissue repair, since it has been shown to stabilize wound fields and to support local cell proliferation and migration of inflammatory, endothelial and stromal cells, contributing for tissue repair [86]. Finally, a previous study reports that poly(ethylene glycol) (PEG) conjugated to fibrinogen materials have potential osteogenic properties in a tibial bone defect model [87].



3. AIMS OF THIS STUDY

Although a lot is known about the biology of OC, the ability of these cells to degrade a biomaterial is still comparatively poorly understood. Similarly, the capacity of a specific substrate to induce and support osteoclastogenesis remains largely to be elucidated.

Having this into consideration, the main goal of this work is to evaluate the degradability of Human Fibrinogen (HFG)-modified chitosan substrates by human osteoclasts. In order to accomplish this, three specific objectives were delineated, namely:

- Establish functional human monocyte derived OC;
- Determine if chitosan modified with HFG is capable of inducing the formation of OC-like cells;
- Determine the degradability of HFG-modified chitosan substrates by OC.





CHAPTER II

MATERIALS & METHODS



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1. PREPARATION OF CHITOSAN SUBSTRATES

1.1. CHITOSAN PURIFICATION

High molecular weight (Mw) Chitosan (France Chitine, Orange, France, degree of N-acetylation of 11-12%, Mw $324 \pm 27 \times 10^3$, endotoxin-free) was hydrated using Milli-Q water, and then dissolved in 0.1 M Chloridric Acid (HCl) under stirring and filtered. Chitosan was next precipitated with 0.1 M Potassium Hydroxide (KOH), washed with Milli-Q water, freeze-dried (48 hours) and finally milled (IKA, Germany/Deutschland) until a fine powder was obtained.

1.2. CHITOSAN FILMS

After purification chitosan was dissolved in AcOH 0.2 M to a final concentration of 0.5% and the solution was degassed, filtered through 0.2 μm pore filters (Millipore, MA, USA) and stored at 4°C protected from light. Chitosan films were prepared by adding a single drop of chitosan solution directly into each well of a 96-well (80 μL per well) or 24-well (200 μL per well) tissue culture polystyrene (TCPS) plates (BD, Biosciences, NJ, USA) and allowing it to dry at 37°C for 48h (Drop Method). Films were then neutralized with 0.1 M Sodium Hydroxide (NaOH), washed twice with distilled water and dried overnight at 37°C. Prior to cell culture, films were sterilized with filtered 70% ethanol and washed twice with PBS. Where indicated, chitosan films were further modified with either HFG (Sigma-Aldrich) or collagen type I (Millipore). For that, films were incubated for 2 hours with a HFG solution (100 μg /ml in Phosphate Buffer Saline (PBS)) or with a collagen type I solution (2.5 $\mu\text{g}/\text{cm}^2$ in 30% ethanol). All films were equilibrated with complete culture medium for 1 hour, at 37°C before cell culture.



1.3. FITC-LABELED CHITOSAN FILMS

To prepare fluorescent chitosan, a 5% chitosan modification – 5% of amine groups with fluorescein isothiocyanate (FITC) (Sigma-Aldrich) – was performed. Purified chitosan (500 mg) was dried overnight, in a vacuum oven, and then hydrated (400 mg) in 400 mL of 1% (v/v) Acetic Acid (AcOH) at 4°C until complete dissolution. Then, 44 mg of FITC were dissolved in 400 ml of methanol and mixed with 400 mL of chitosan, to achieve an estimated 5% modification. Both solutions (chitosan and FITC) were mixed, at Room Temperature (RT), under constant stirring, protected from light, for 3h. Following this, FITC-labeled chitosan was precipitated with 1.0 M NaOH and washed with Milli-Q water, until no fluorescence could be seen in the supernatant. FITC-labeled chitosan was finally lyophilized, dried, weighted, and stored in a dessicator protected from light until use. FITC-labeled chitosan was dissolved in AcOH 0.2 M to a 1% final concentration, and the solution was degassed and stored at 4°C, protected from light until use. Fluorescently-labeled chitosan films were prepared by spincoating (SCS Cookson Electronics Spincoater-model G3P-8), for 2 minutes at 9000 RPM to ensure a homogenous distribution of the polymer. Briefly, 80 μ L of the FITC-chitosan solution was deposited onto round glass coverslips (13 mm diameter), previously washed overnight with nitric acid 65%, washed several times with distilled water, and finally calcinated overnight at 220°C. The spinning procedure was repeated four times, in order to produce a four-layer film, so that FITC could be detected by fluorescence microscopy. Films were then neutralized with 0.1 M NaOH, washed twice with distilled water and dried overnight at 37°C. Prior to cell culture, films were sterilized with filtered 70% ethanol, washed twice with PBS and incubated with complete culture medium for 1 hour, at 37°C. Where appropriate, chitosan films were further modified with HFG, by incubating them for 2 hours with a HFG solution, as it was previously described (please see section 1.2, Chapter 2).

1.4. 3D CHITOSAN SCAFFOLDS

Chitosan three-dimensional (3D) porous scaffolds were prepared starting from chitosan acidic solutions via thermally induced phase separation and subsequent sublimation of



the ice crystals [88]. Briefly, dry chitosan was hydrated in Milli-Q water overnight and solubilized in AcOH for 24 hours. The solution was then poured onto 24-well plates (800 μ L/well), frozen at -20°C, and subsequently lyophilized at -80 °C during 48 hours. Next, the top layers of scaffolds were cut in appropriated dimensions and kept protected from light at 4°C until their use. Prior to cell culture, scaffolds were immersed in absolute ethanol, hydrated in a serial dilution of ethanol solutions, and finally equilibrated in culture medium. Where necessary HFG was adsorbed to the scaffold and excess removed by pipetting, before cell culture.

2. MONOCYTE ISOLATION, CULTURE AND DIFFERENTIATION

Monocytes were isolated by two different methods, the plastic adherence method (adherence method) and the negative selection method, using the RosetteSep human monocyte enrichment isolation kit (Stem Cell Technologies SARL, Grenoble, France) (negative selection method).

2.1. ISOLATION OF MONOCYTES FROM BUFFY COATS, BY PLASTIC ADHERENCE

Buffy Coats (BC) (kindly donated by Instituto Português do Sangue) from healthy blood donors, were used to isolate monocytes, by simple cell adherence. BC were directly layered on Histopaque and centrifuged at 800xg, for 30 minutes, at RT, without brake. A gradient of: Red Blood Cells (RBC), Histopaque, PBMC and plasma was obtained, as illustrated in Figure 5.



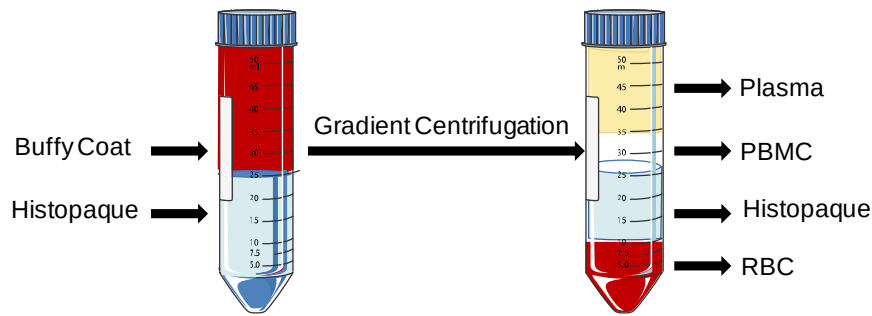


Figure 5 – Gradient resulting from buffy coat centrifugation over Histopaque. Histopaque was placed at the bottom of a 50 mL conical tube and the same volume of buffy coat was overlaid gently to form 2 layers. Centrifugation was performed at 800xg for 30 minutes at room temperature (approximately 23°C), without brake, in order not to disturb the gradient. The resulting gradient shows different layers for: red blood cells (RBC) (bottom) followed by Histopaque, then the Peripheral Blood Mononuclear Cells (PBMC) layer and on top the plasma.

The PBMC layer was collected carefully into a new conical centrifuge tube and cells were washed three times with PBS at 300xg, for 10 minutes. If required, contaminating RBC were removed using RBC Lysis Buffer (4.99×10^{-3} M ammonium chloride and 7.49×10^{-2} M Tris-Base, pH of 7.2) for 8 minutes at 37°C, followed by a PBS wash. Cells were resuspended in α -Minimal Essential Medium (MEM) (Gibco, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Lonza, Basel, Switzerland) and 1% penicillin G-streptomycin (PS) (Invitrogen). Cells were counted in a Neubauer Chamber using a trypan blue dye (Sigma-Aldrich) – exclusion assay, in order to distinguish live from dead cells (dead cells incorporate the blue dye) – and plated directly on the substrates at the indicated density, or on Petri dishes at a density of 50×10^6 cells/10mL. After a two hour incubation period at 37°C, lymphocytes were removed (non-adherent fraction) and monocytes were left in the wells or recovered using PBS-Ethylene-Diamine Tetraacetic Acid (EDTA) (5 mM). In the second approach cells were then counted and plated on TCPS or on the chitosan substrates, modified or not with HFG or collagen type I, at the indicated densities. Monocytes were allowed to differentiate into OC with the indicated concentrations of RANKL and M-CSF for up to 21 days at 37°C, 5%CO₂. Culture medium supplemented with these cytokines was replaced every three to four days.

2.2. ISOLATION OF MONOCYTES, BY NEGATIVE SELECTION, FROM BUFFY COATS

BC (kindly donated by Instituto Português do Sangue) from healthy blood donors, were used to isolate monocytes, by negative selection, using RosetteSep isolation kit and a method adapted from the manufacturer's instructions (Oliveira MI *et al*, submitted). BC were centrifuged at 1200xg, for 20 minutes, without brake, so that a gradient composed of 3 different layers (RBC, PBMC and plasma) was formed (Figure 6).

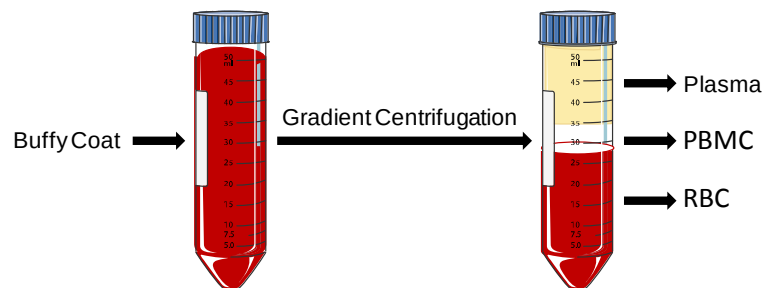


Figure 6 – Gradient resulting from buffy coat centrifugation. The buffy coat was centrifuged at 1200xg for 20 minutes at room temperature (approximately 23°C), without brake, in order not to disturb the gradient. Three layers were visible at the end: Red Blood Cells (RBC) at the bottom with Peripheral Blood Mononuclear Cells (PBMC) immediately after and plasma at the top.

Secondly, the layer containing PBMC was collected into a new conical centrifuge tube, together with some RBC and incubated for 20 minutes with RosetteSep human monocyte enrichment isolation kit, according to the manufacturer's instructions. The mixture was then diluted at a 1:1 ratio with PBS 2% FBS before being layered over Histopaque and centrifuged as previously. A second gradient formed by RBC and unwanted cells entrapped in the immunorosettes at the bottom and then, Histopaque, followed by monocytes and plasma on the top, was obtained (Figure 7). The enriched monocyte layer was carefully collected into a new conical tube, and washed with PBS. The washes were performed to ensure platelet removal. Cells were then resuspended in α -MEM supplemented with 10% FBS and 1% PS, counted and plated on TCPS or on the chitosan substrates, modified or not with HFG or collagen type I, at the indicated densities. Cells were allowed to differentiate into OC with the indicated concentrations of RANKL and M-CSF for up to



21 days at 37°C, 5%CO₂. Culture medium supplemented with these cytokines was replaced every three to four days.

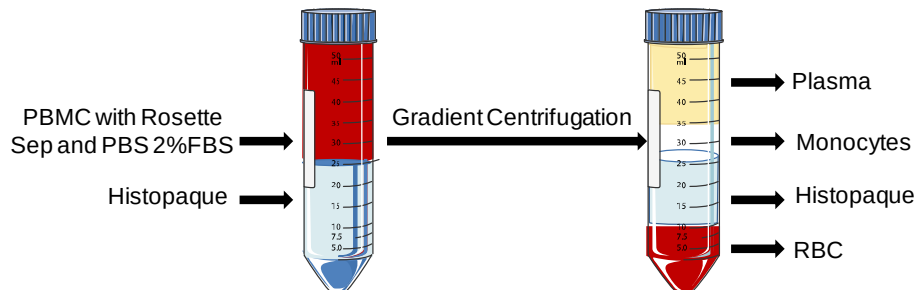


Figure 7 – Monocyte separation. Peripheral Blood Mononuclear Cells (PBMC) population with RosetteSep and PBS 2% FBS is layered over Histopaque and centrifuged. Red blood cells (RBC) along with the other cells that formed the immunorosettes are denser and centrifuge through the Histopaque, while monocytes band over it and can be recovered at the interface with plasma. Centrifugation was performed at 1200xg, for 20 minutes, at room temperature (approximately 23°C), without brake, in order not to disturb the gradient.

3. ANALYSIS OF OSTEOCLAST CULTURES

3.1. HEMATOXYLIN-EOSIN STAINING

OC were stained with the Hemacolor® Kit (Merck), according to the manufacturer's instructions. Cells were washed in PBS, fixed for 2 minutes with Hemacolor Solution 1 (Fixing Solution), rinsed twice with distilled water and then stained with Hemacolor Solution 2 (red) for 2 minutes and Hemacolor Solution 3 (blue) for 1 minute. Cells were finally rinsed once more, dried and observed under an Inverted Fluorescence Microscopy (Zeiss Axio Vert). Images were acquired with AxioCam HRc (Zeiss) and using AxioVision Rel. 4.8 software.

3.2. F-ACTIN AND NUCLEI FLUORESCENT STAINING

3.2.1. 2D SAMPLES

To stain OC cytoskeleton and nuclei, culture medium was removed from the wells and cells were washed three times with PBS, at RT, fixed with Paraformaldehyde (PFA) (Sigma-Aldrich) 4% for 10 minutes and washed again three times with PBS. Cells were then permeabilized for 5 minutes with 0.1% (v/v) Triton X-100 in PBS, washed once more with PBS and finally incubated with 5 μ M Alexa Fluor® 488 (green) or 594 (red) phalloidin (Molecular Probes, Invitrogen) in a 1% (w/v) Bovine Serum Albumine (BSA) (Gibco, Alfacene) / PBS solution, for 60 minutes, in the dark, at RT. At the end of the incubation time, samples were washed three times with 1% (w/v) PBS/BSA solution and mounted using Vectashield with DAPI (Vector). Samples were kept at -20°C protected from light until analysis by Inverted Fluorescence Microscopy (Zeiss Axio Vert Microscope). Images were acquired with AxioCam MRm (Zeiss) using the AxioVision Rel. 4.8 software. Image analysis and cell counting was performed with Cell Note software [89].

3.2.2. 3D SAMPLES

All the incubation steps were performed under gentle shaking (50 rpm) in an orbital shaker. Culture medium was removed from the wells and cells were washed three times with PBS, at RT, fixed with PFA 4% for 30 minutes and washed again three times with PBS. Cells were then permeabilized for 15 minutes with 0.1% (v/v) Triton X-100 in PBS, washed once more with PBS and finally incubated with 5 μ M Alexa Fluor® 488 (green) phalloidin in a 1% (w/v) BSA/PBS solution, for 2 hours, in the dark, at RT. After this incubation period, phalloidin was removed, cells were washed three times with 1% (w/v) PBS/BSA solution and PI (3 μ M) was added to the scaffolds, followed by a 20 minutes incubation. Finally, scaffolds were mounted using Fluoromount® mounting media (Sigma), followed by analysis by Scanning Electron Microscopy (SEM) (Section 3.3, Chapter 2) or by Confocal Laser Scanning Microscope (CLSM, Leica, SP2 AOBS)



with a LCS Software (Leica). Stacks of images were collected and final images were obtained by projecting these stacks using the LCS Software.

3.3. PREPARATION OF SAMPLES FOR SEM ANALYSIS

To prepare biological samples for SEM, OC cultured on dentine slices or on 3D chitosan scaffolds were washed with PBS buffer at RT and 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate solution was added to each well. Fixation was performed at RT, for 30 minutes, under gentle shaking (50 rpm), in an orbital shaker. Samples were then washed three times with cacodylate buffer. Finally, cells were incubated for 10 minutes in each of a serial dilution of ethanol solutions 50, 60, 70, 80, 90 and 99% v/v, in order to dehydrate them, and stored in absolute ethanol, at 4°C, until being critical point dried, mounted onto appropriate supports with araldite glue, sputtered-coated with gold and examined by SEM.

4. OSTEOCLAST FUNCTIONALITY ASSAYS

4.1. TRAP STAINING

The TRAP assay (Sigma-Aldrich) was performed according to the manufacturer's instructions. OC at days 7, 14 and 21 of differentiation were fixed in citrate/acetone solution, at RT for 30 seconds, washed in deionized water and dried for at least 15 minutes. A solution containing water, acetate, naphthol and 1 capsule of fast garnet GBC salt was prepared, mixed, filtered, warmed up and incubated with the cells, for 1 hour, at 37°C, in the dark. At the end of the incubation period, cells were washed in deionized water, stained with acid hematoxylin solution (counter-staining), washed



once more and finally allowed to air dry. Cells were then analyzed with a Stereomicroscope (SZX10, Olympus) coupled to a digital camera (DP21, Olympus) or with the Inverted Fluorescence Microscope (Zeiss Axio Vert), where images were acquired with AxioCam HRc (Zeiss) and using AxioVision Rel. 4.8 software.

4.2. MEASUREMENT OF TRAP RELEASE

The quantification of TRAP release into the supernatant was performed as previously described [82]. At culture day 20, fresh complete culture medium was added to the cells and after incubation for 24 hours supernatants were removed for measurement of enzyme released. Cell lysates, for assessment of enzyme present in the corresponding cells, were prepared by washing cells with PBS, incubating in 100 μ L 0.1% Triton X-100 in water (v/v), for 10 minutes and collecting the lysate into a tube. To quantify the activity of TRAP, the conversion of p-nitrophenyl phosphate (phosphatase substrate) (Sigma-Aldrich) into p-nitrophenol, in the presence of sodium tartrate was measured. 80 μ L of the supernatant or the lysate of each condition was transferred to a 96-well plate, containing 80 μ L of 0.09 M citrate buffer, 20 mM phosphatase substrate and 80 mM tartaric acid and incubated at RT for 40 minutes. In order to stop the reaction, 40 μ L of 0.5 M NaOH was added to each well. Serial dilutions of p-nitrophenol (Sigma-Aldrich) were performed, to create a standard curve, and the absorbance of all samples was analysed on a plate reader (Biotek, Powerwave Microplate Reader), at 405 nm. For each sample assayed, the activity of TRAP was measured in the supernatant and lysate of cells and enzyme activity released into the supernatant was calculated as a percentage of total enzyme activity [82].

4.3. RESORPTION ASSAY WITH DENTINE SLICES

To evaluate OC resorption activity, monocytes cultured on top of dentine slices, at a density of 0.5×10^6 cells per slice (diameter of approximately 0.35 cm), in the presence,



or not, of 50 ng/mL of RANKL and 30 ng/mL of M-CSF. Cell culture medium was changed every three to four days. At the end of the incubation period (culture day 21), discs were stained with hematoxylin-eosin as described before (section 3.1, Chapter 2) and the extent of resorption was assessed by reflected light microscopy, under inverted light microscopy (Zeiss Axio Vert) Images were acquired with AxioCam HRc (Zeiss) using the AxioVision Rel. 4.8 software, or prepared for SEM analysis as described above (Section 3.3, Chapter 2).

5. EXPRESSION OF SPECIFIC OSTEOCLAST MARKERS, AT THE mRNA LEVEL

5.1. RNA EXTRACTION

At day 17 of culture, cells were washed with PBS buffer, lysed in Tripure (Roche) (100 μ L/well of a 24-well plate), collected by gently scrapping and stored at -80°C until used. Then, chloroform (200 μ L/ml of TriPure) was added to the cell lysate and incubated at RT for 15 minutes. The solution was centrifuged at 12 000xg for 15 min at 4°C originating three different phases: a transparent upper phase where RNA is present, a white interphase, formed by DNA and proteins, and a red organic lower phase (mainly chloroform). The upper phase was then collected, RNA precipitated with isopropanol for 15 minutes at RT, and the sample centrifuged at 12 000xg for 10 minutes at 4°C. Finally, RNA was washed with ethanol, centrifuged at 7500xg for 5 minutes at 4°C and then resuspended in RNase-free water.



5.2. RNA QUANTIFICATION

The purity and yield of RNA obtained was determined by measuring the ratio of absorbance at 260 and 280 nm using the Thermo Scientific NanoDrop® 1000 Spectrophotometer. A ratio of 2.0 is generally accepted as highly pure RNA; whereas ratios lower than 1.65, may indicate the presence of protein, phenol or other contaminants [90]. The integrity of total RNA was assessed by running an RNA sample in a 1% agarose gel and stained with gel-red. 2µL of RNA were mixed with bromophenol blue and loaded in a total volume of 6 µl. The gel run at 100V for 30 min in 1x TBE buffer and visualized on the Gel Doc™ XR+ System from BioRad.

5.3. REVERSE TRANSCRIPTASE – POLYMERASE CHAIN REACTION (RT-PCR)

RT-PCR is a technique for amplifying a defined sequence of RNA molecule. The RNA strand is first reverse transcribed into its complementary DNA (cDNA), followed by the amplification of the resulting DNA using polymerase chain reaction. 176.4 ng of RNA were reverse-transcribed using the Superscript II RT enzyme (Invitrogen) and according to standard protocols. The resulting cDNA was amplified as follows: a 25 µl reaction was prepared, containing 2 µl of cDNA, 2 µL of dNTPs, 2 µL of each specific primer (Table 1), 2.5 µl of 10x reaction buffer, 0.2 µL of TaqDNA polymerase and 14.3 µL of ultra pure water.

The amplification protocol used for each gene is indicated in Table 2. The housekeeping gene GAPDH was used as positive control at all conditions tested. Amplification products were loaded into 2% agarose gel stained with ethidium bromide. The gel run at 80V, for approximately 40 minutes and visualized on the Gel Doc™ XR+ System from BioRad.



Table 1 – Primers used for RT_PCR.

Primers	Sequence	PCR Product	T _{annealing}
Trap-F	GATCCTGGGTGCAGACTTCA	212 bp	57°C
Trap-R	GCGCTTGGAGATCTTAGAGT		
Cath K-F	GATACTGGACACCCACTGGGA	464 bp	57°C
Cath K-R	CATTCTCAGACACACAATCCAC		
CNR-F	GACAACTGCTGGCTGAGTG	321 bp	57°C
CNR-R	GAAGCAGTAGATGGTCGCAA		
Rank-F	TGTGGCACTGGATCAATGAG	262 bp	57°C
Rank-R	GTCTTGCTGACCAATGAGAG		
VNR-F	AAGTTGGGAGATTAGACAGAGG	355 bp	57°C
VNR-R	GAAGCTGCTCCCTTTCTTGT		
GAPDH-F	TCAAGGCTGAGAACGGGAAG	200 bp	60°C
GAPDH-R	AGAGGGGGCAGAGATGATGA		

Table 2 – Amplification Protocol used for Cath K, VNR, CNR, RANK and TRAP.

Cath K and VNR

Step	T (°C)	Time	Number of Cycles
1	95	4'	30
2	95	45''	
3	57 (T annealing)	45''	
4	72	1'	
5	72	10'	

CNR and RANK

Step	T (°C)	Time	Number of Cycles
1	95	5'	33
2	94	1'	
3	57 (T annealing)	1'	
4	72	2'	
5	72	10'	

TRAP

Step	T (°C)	Time	Number of Cycles
1	95	5'	20
2	94	1'	
3	57 (T annealing)	1'	
4	72	2'	
5	72	10'	



6. ANALYSIS OF CHITOSAN DEGRADATION

OC were induced to differentiate on top of FITC-labeled chitosan films (Section 1.3, Chapter 2). At days 3, 7, 10, 14 and 17 of culture, fluorescence measurements were performed on a Microplate reader (Biotek Synergy MX) using the Area-scan mode. Measurements were performed from the bottom of the plate, at the wavelength range of 485 to 528 nm.

Cells were also analyzed under the Inverted Fluorescence Microscope (Zeiss Axio Vert) and images acquired with AxioCam MRm (Zeiss) and using AxioVision Rel. 4.8 software. Images were taken at the indicated days, in order to follow up the evolution of the film degradation. These images were then analyzed with ImageJ 1.44 Software. The particle analysis tool was used on black and white images of the FITC-channel of fluorescence. Brightness and contrast were automatically adjusted and images were then thresholded. All back areas with more than 5 pixels were analyzed and percentage of the total area calculated.

7. STATISTICAL ANALYSIS

Statistical analysis was performed using Prism5 software, vs. 5.0a. Results were analyzed for Gaussian distribution using D'Agostino and Pearson normality test and samples were non-parametric. The Mann-Whitney test was used to compare two samples, whereas comparison between more than two samples was performed using the Kruskal-Wallis followed by Dunns comparison test. A value of $p < 0.05$ was considered statistically significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All data are expressed as mean $\pm$ SD.





CHAPTER III

RESULTS & DISCUSSION



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1. CULTURE AND CHARACTERIZATION OF OSTEOCLASTS

1.1. OPTIMIZATION OF OSTEOCLAST CULTURE CONDITIONS

OC share the same precursor as macrophages and are frequently differentiated *in vitro* from peripheral blood monocytes, in presence of M-CSF and RANKL. In the literature a range of different concentrations of M-CSF and RANKL are described to obtain OC [29, 44, 91, 92], so the first step of this work was to determine the combination of concentrations of the two cytokines that would lead to the highest rate of monocyte differentiation into OC. Several combinations were tested, which are summarized in Table 3.

Monocytes were isolated from BC, by the adherence method. PBMC were plated at 1.25×10^6 cells/cm², before the adherence step. As monocytes constitute 8-10% of PBMC, and the adherent fraction is enriched in monocytes, it was estimated that $0.1-0.125 \times 10^6$ monocytes/cm² would remain attached after lymphocytes that are in suspension were washed away. This adherent fraction was then incubated with complete culture medium, supplemented with the different concentrations of RANKL and M-CSF (Table 3). No cytokines, or the highest concentration of each cytokine alone, were used as controls.

Table 3 – Different combinations of RANKL and M-CSF concentrations tested for inducing OC differentiation. Cells cultured without stimulation or with just one cytokine were used as controls.

[Cytokines] (ng/mL)	RANKL			
M-CSF	0	30	40	50
0	X			X
20		X	X	X
30		X	X	X
40	X	X	X	X



At day 7 induction of multinucleated cells was analyzed, and their functionality evaluated at day 21. The times selected to analyze cultures was based in the literature, where 7 days are reported to be necessary for OC precursors to form [44], and 17 or 21 days are required to obtain mature and functional OC [29, 93].

Figure 8A and B illustrate each cytokine combination tested and Figure 8C shows that the majority of cells have, at the most, 2 nuclei. However, it is already evident that cells cultured for 7 days in the presence of 50 ng/mL of RANKL and 30 ng/mL of M-CSF are bigger (Figure 8B, image h), with more than one cell presenting 2 nuclei per cell, than cells cultured in the presence of other cytokine combinations. To quantify the results observed, four images were collected for each condition, cells counted and divided into two categories, according to the number of nuclei per cell: 2 nuclei and more than 2 nuclei per cell. This division was intended to distinguish between binucleated cells and truly multinucleated cells. As shown in Figure 8C more multinucleated cells are found when the highest concentration of RANKL (50 ng/mL) was combined with the two higher concentrations of M-CSF (30 ng/mL and 40 ng/mL), with more cells with 2 nuclei and some truly multinucleated. All control samples showed smaller cells with few cases of multinucleated cells (Figure 8A and C).

A TRAP staining performed at day 21 to evaluate OC-like cells functionality revealed that these are TRAP producers (Figure 9), as seen by the brown staining produced as a result of tartrate substrate hydrolysis by the TRAP enzyme. These results are in agreement with the ones presented above for multinucleated cells at day 7, as higher concentrations of RANKL and M-CSF also lead to stronger TRAP staining (Figure 9, images e, h and i). Our results are also in accordance with previous studies by Sorensen [94] and Kudo [44].

It is possible to see in Figure 9A.b that cells cultured in the presence of only M-CSF are also TRAP producers (visible formation of brown agglomerates). This is due to the fact that macrophages are also capable of producing TRAP, as previously mentioned. Although they produce a different isoform than that produced by OC, this cannot be distinguished by this staining [30].



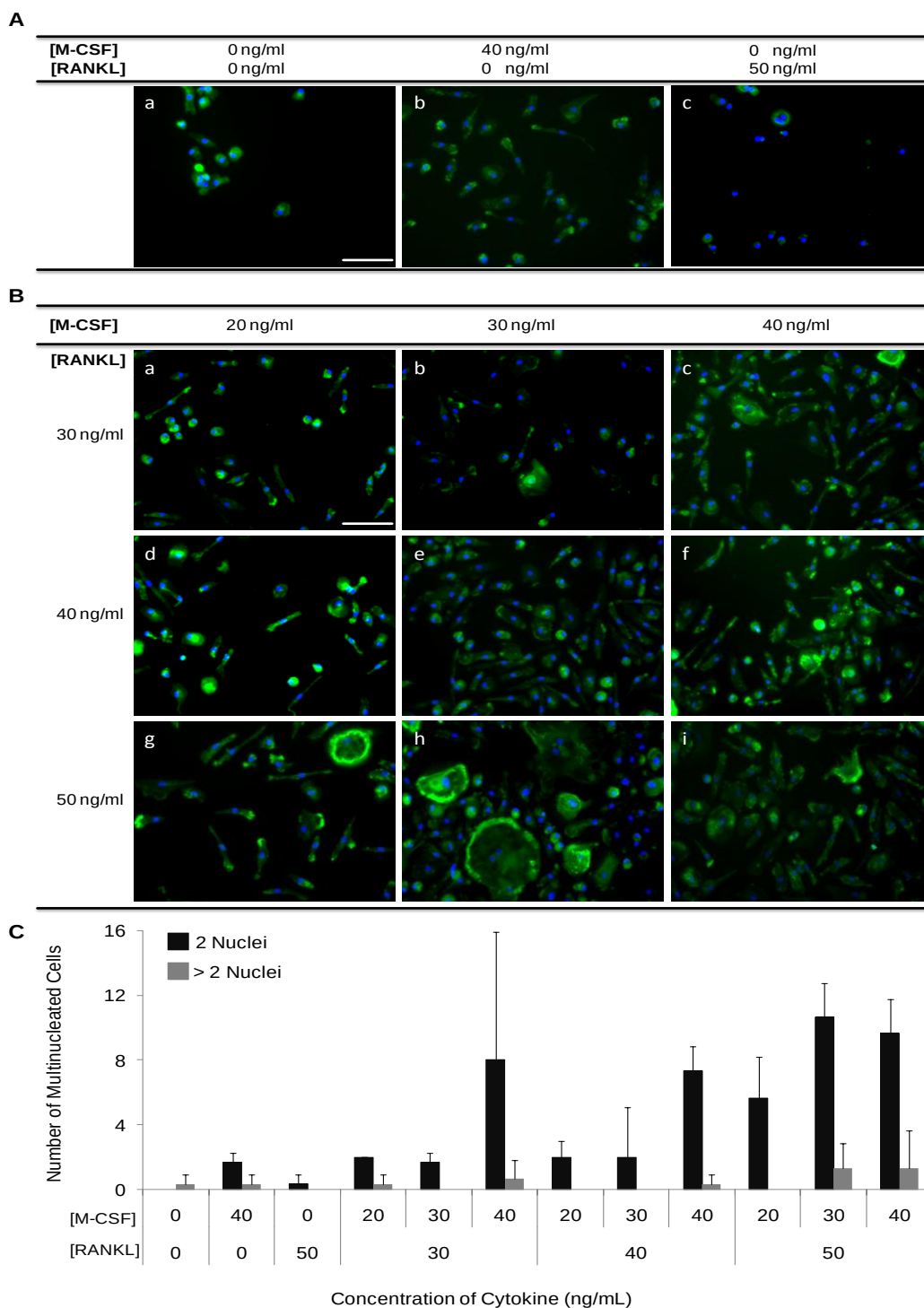


Figure 8 – Osteoclast-like cells differentiated on the presence of higher concentrations of RANKL with higher concentrations of M-CSF have more nuclei per cell. Monocytes cultured for 7 days: **A:** without cytokines or in the presence of M-CSF or RANKL alone or **B:** in presence of the indicated concentrations of osteoclast differentiating cytokines, M-CSF and RANKL. The presence of multinucleated cells (formation of characteristic actin rings, surrounding 2 or more nuclei) was evaluated by actin cytoskeleton staining, using Alexa Fluor® 488 conjugated phalloidin (green), and DAPI for nuclei staining (blue). Scale bar corresponds to 100 μ m. **C:** Quantification of the results illustrated in A and B. Multinucleated cells were divided into 2 categories: 2 nuclei (black bars) and more than 2 nuclei (dark grey bars). The average of 4 experimental replicates obtained for 1 biological replicate is shown. Errors bars represent standard deviation. M-CSF: Macrophage Colony-Stimulating Factor; RANKL: Receptor Activator of Nuclear Factor κ B.



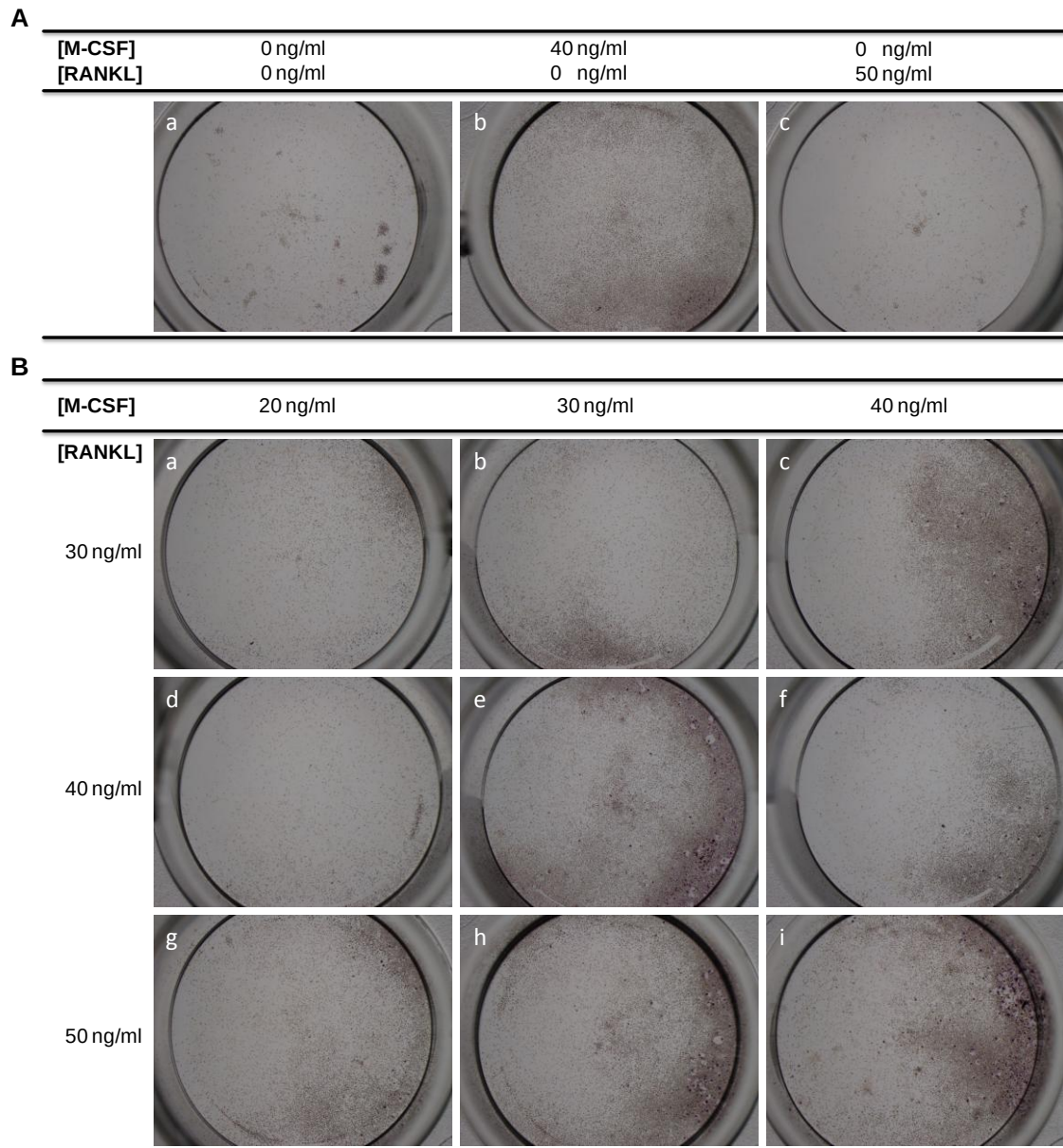


Figure 9 – Osteoclast-like cells differentiated for 21 days, are TRAP producers. Monocytes cultured: **A:** without cytokines or in the presence of M-CSF or RANKL alone or **B:** in presence of the indicated combinations of osteoclast differentiating cytokines. Images were obtained using a Stereomicroscope coupled to a digital camera and the full well of a 24-well plate is shown (diameter = 1.6 cm). M-CSF: Macrophage Colony-Stimulating Factor; RANKL: Receptor Activator of Nuclear Factor κ B.

Taking together the results obtained for the formation of multinucleated OC-like cells and TRAP activity, three combinations of cytokine concentrations were chosen to further optimize of OC culture: 1) 40 ng/mL of RANKL with 30 ng/mL of M-CSF; 2) 50 ng/mL of RANKL with 20 ng/mL of M-CSF; 3) 50 ng/mL of RANKL with 30 ng/mL of M-CSF.

A new experiment, similar to the described above was performed, to confirm the results obtained and further discriminate between the most similar conditions. PBMC were seeded at a density of 1.8×10^6 cells/cm² and cultured for 21 days, in culture medium supplemented with the three different combinations of cytokines. The difference in the initial cell density was an adjustment due to the change of culture plates from 24 to 96-well plates.

In order to follow the formation of OC-like cells along time, cultures were analyzed at days 7, 14 and 21. Figure 10A, B and C illustrates the formation of multinucleated cells along time, showing that at day 7 cells are mostly small, but cell size and the number of nuclei per cell increase by day 14 and even more by day 21, especially in the presence of combined OC differentiating cytokines. This is reflected in the quantification of results, presented in Figure 10D to F. Particularly combinations of 40 or 50 ng/mL of RANKL with 30 ng/mL of M-CSF, exhibit an increasing number of cells with 2 and 3 to 9 nuclei, when we compare days 7 and 21 of culture. Noteworthy, 50 ng/mL of RANKL combined with 30 ng/mL of M-CSF presented at day 21 some cells with more than 10 nuclei, which were quantified separately.

Next, we wanted to evaluate functionality of obtained OC-like cells. Figure 11 shows that the strongest TRAP staining was obtained for 50 ng/mL of RANKL combined with M-CSF (Figure 11B, images e and f).

Taking together the results of the two experiments described above, the combination of 50 ng/mL of RANKL and 30 ng/mL of M-CSF was chosen to differentiate OC, as it was the most consistent at inducing large multinucleated OC-like cells that were TRAP positive. Also, at this point it was decided that cells would be cultured up to day 21 to allow OC to become fully differentiated, and that cultures would be analyzed at weekly intervals: at day 7 for OC precursor's formation, day 14 to evaluate the evolution of multinucleated cells formation and day 21 for analysis of fully mature OC.



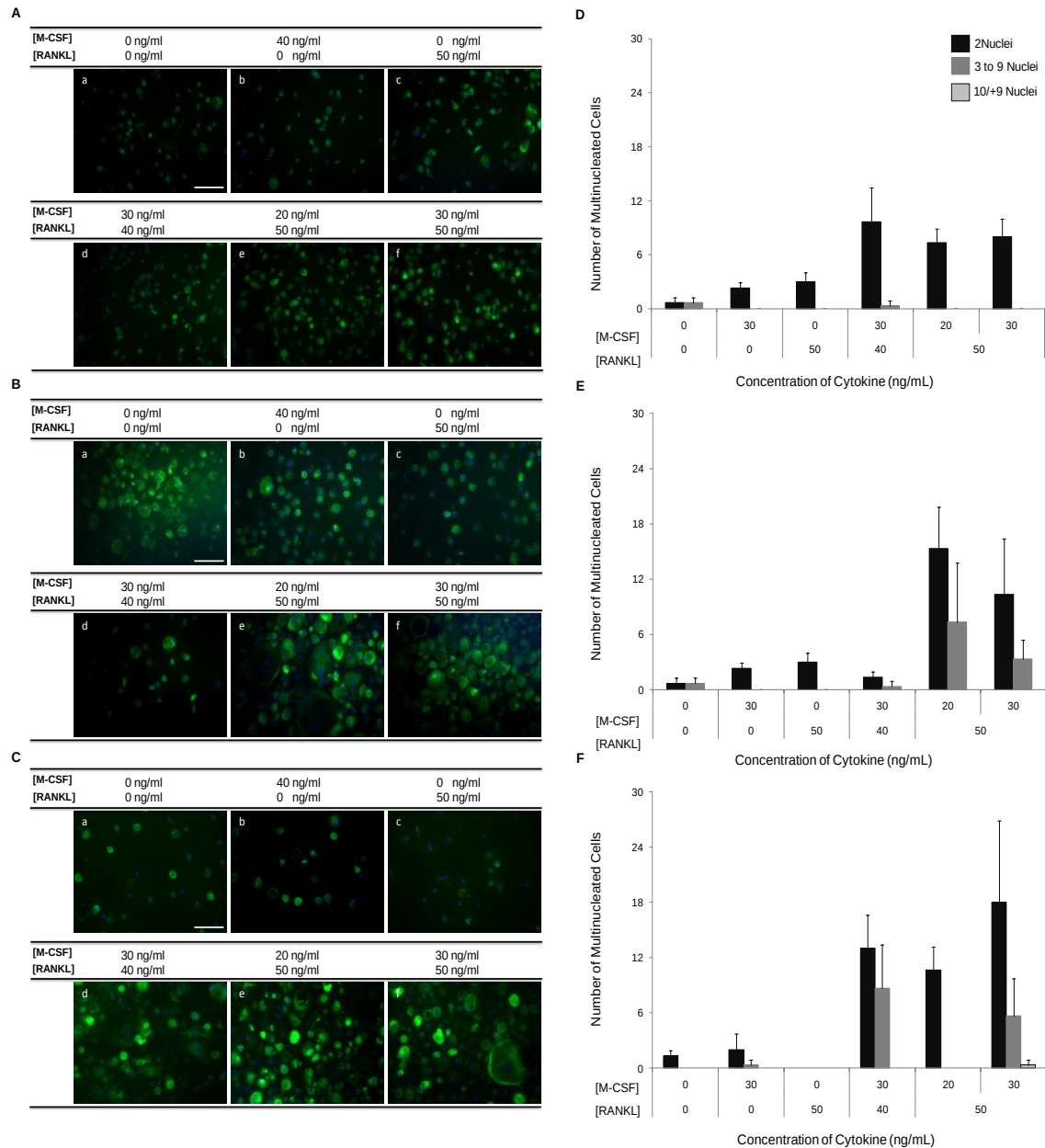


Figure 10 – Osteoclast-like cells differentiated in the presence of higher concentrations of RANKL with M-CSF have more nuclei per cell. Monocytes were allowed to differentiate into OC for: 7 (A), 14 (B) or 21 (C) days, in presence of the indicated concentrations of M-CSF and RANKL (a to f). The presence of multinucleated cells (formation of characteristic actin rings, surrounding 2 or more nuclei) was evaluated by actin cytoskeleton staining, using Alexa Fluor® 488 conjugated phalloidin (green), and DAPI for nuclei staining (blue). Scale bar corresponds to 100 μ m. Quantification of results for days 7 (D), 14 (E) and 21 (F) was performed by counting all multinucleated cells and dividing them into 3 categories: 2 nuclei (black bars), 3 to 9 nuclei (dark grey bars) and 10 or more nuclei (light grey bars). The average of 4 experimental replicates from 1 biological replicate is shown. Errors bars represent standard deviation. M-CSF: Macrophage Colony-Stimulating Factor; RANKL: Receptor Activator of Nuclear Factor κ B.

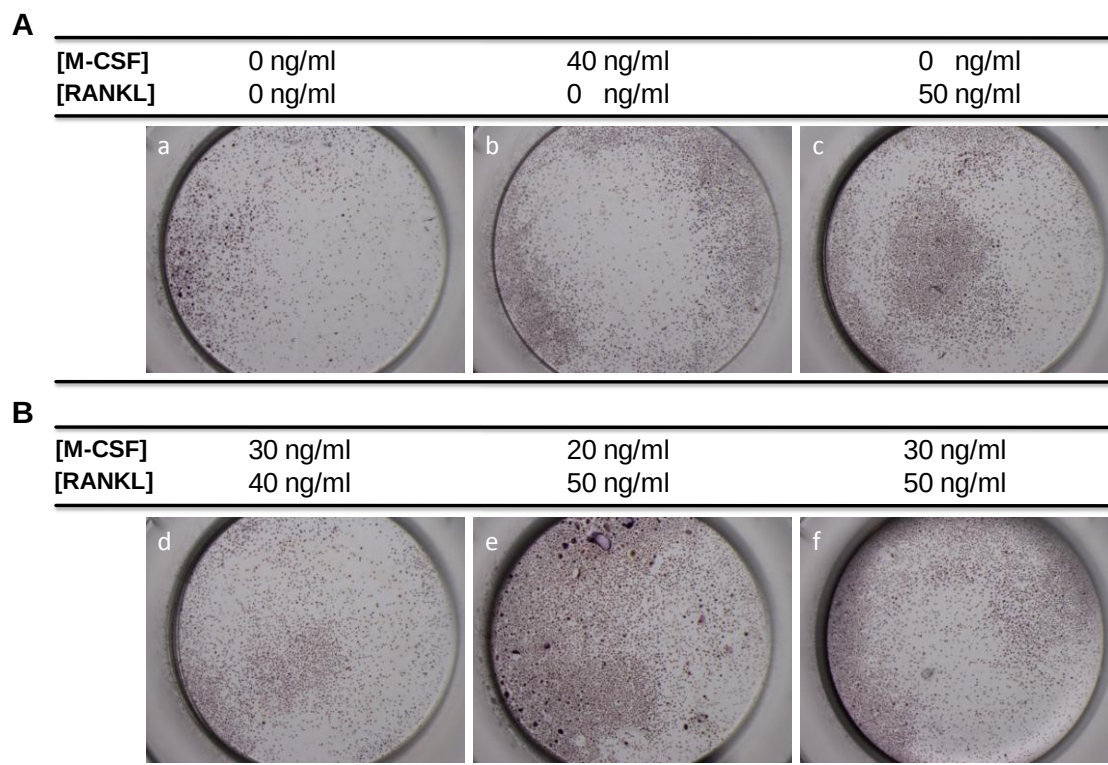


Figure 11 – Osteoclast-like cells differentiated in the presence of RANKL and M-CSF for 21 days, are TRAP producers. Monocytes were cultured: **A:** without cytokines or in the presence of M-CSF or RANKL alone or **B:** in presence of the indicated concentrations of osteoclast differentiating cytokines. Images were obtained using a Stereomicroscope coupled to a digital camera. The full well of a 96-well plate is shown (diameter = 0.36 cm). M-CSF: Macrophage Colony-Stimulating Factor; RANKL: Receptor Activator of Nuclear Factor κ B; TRAP: Tartrate Resistant Acid Phosphatase.

1.2. IDENTIFICATION OF FUNCTIONAL OSTEOCLASTS

As TRAP staining is not specific for OC, also detecting the TRAP isoform produced by macrophages, OC-like cells were tested for their ability to degrade dentine substrates. Dentine slices are commonly used to evaluate OC resorptive behavior *in vitro*, in which cultured OC excavate resorption lacunae on the mineralized substrate [95]. This assay has been widely used to investigate the effects of different factors, such as cell contact, cytokines, among others, on bone resorption *in vitro*, being one of the most used methods [96, 97]. So, to confirm that OC-like cells obtained in our culture conditions were in fact functional, cells were cultured for 21 days on top of dentine slices, in presence of the selected combination of OC differentiating cytokines (50 ng/mL of RANKL and 30 ng/mL of M-CSF). Cells cultured without cytokines or slices without cells were used as controls. Results illustrated in Figure 12, show that OC have the



ability to adhere to dentine slices in much higher numbers than cells cultured without differentiating cytokines (Figure 12, compare images b and c). Also, OC cultured on dentine slices are functional, as they were able to degrade the dentine surface, forming resorption pits, which stain a lighter shade of pink on the hematoxylin–eosin staining (Figure 12, white arrows in images c and f).

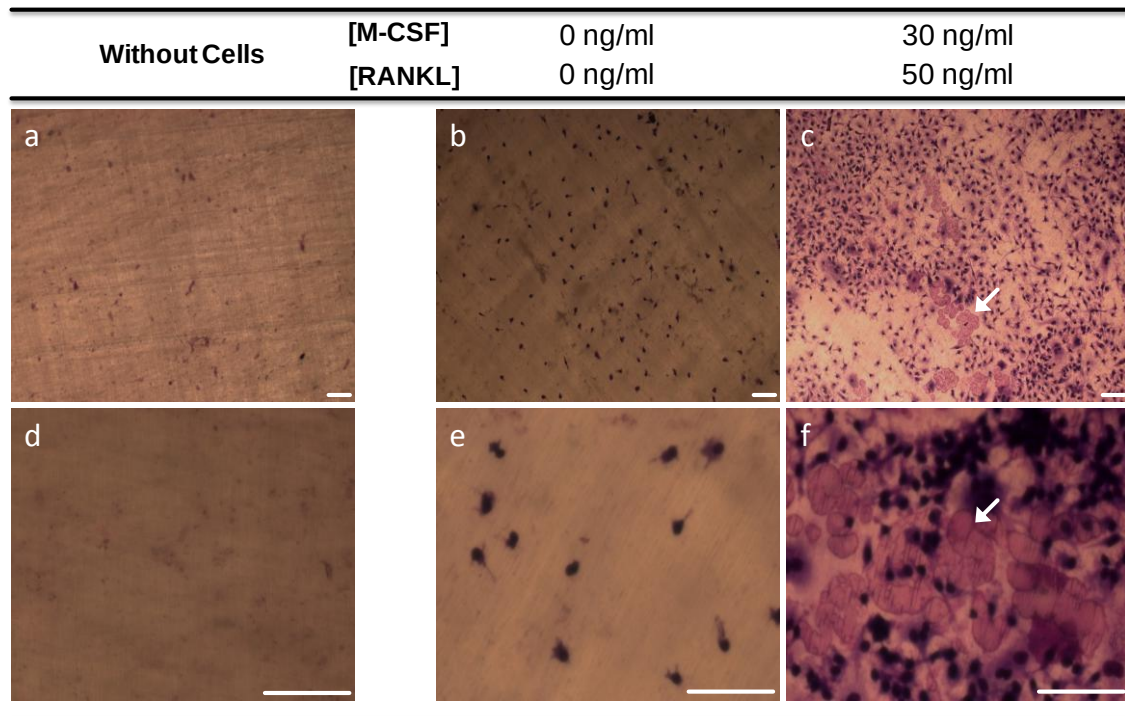


Figure 12 – Osteoclasts attach to dentine slices and are able to degrade its surface, forming lacunae. Slices without cells (a and d), or with monocytes cultured for 21 days without cytokines (b and e) or in presence of 50 ng/mL of RANKL with 30 ng/mL of M-CSF, to induce osteoclast formation (c and f). Resorption pits stain light pink, are indicated (white arrows) and are only visible when dentine slices were cultured with osteoclasts (visualized in dark violet). Scale bar corresponds to 100 μ m. M-CSF: Macrophage Colony-Stimulating Factor; RANKL: Receptor Activator of Nuclear Factor κ B.

To further confirm the resorption pits formed by OC on dentine slices, cells were analyzed at much higher resolution, using scanning electron microscopy (SEM). Figure 13 shows the formation of resorption pits on dentine, just in the case of cells cultured with OC differentiating cytokines (Figure 13, compare images d and e). The structure of dentine slices without cells and for so intact is shown as a control (Figure 13a). Also, cells cultured without cytokines show very little adhesion while OC adhere in much higher numbers to the dentine slices (Figure 13 compare b and c). This is in agreement with the results presented above (Figure 12). These data are also in agreement with

those obtained by others, since OC are reported as the only cells able to perform excavations on top of mineralized substrates [82].

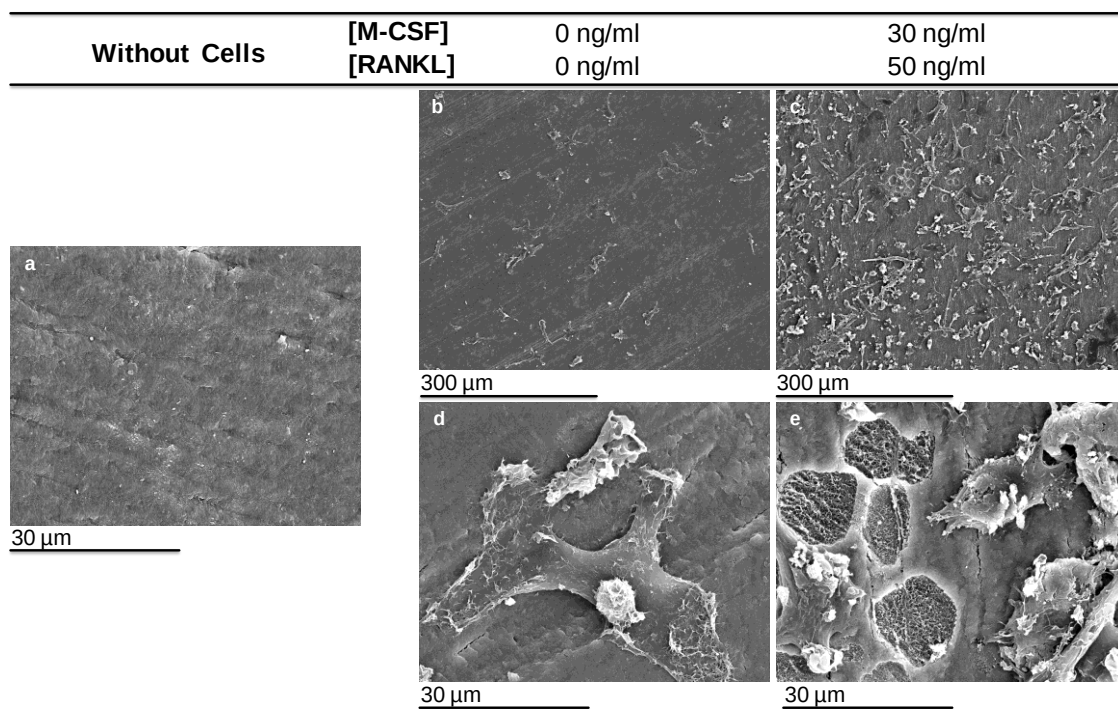


Figure 13 – Osteoclasts are the only cells with the ability to perform excavation pits on top of dentine slices. Dentine slices without cells were used as a control (a). Monocytes were cultured for 21 days: without cytokines (b and c) or in presence of osteoclast differentiating cytokines (d and e). M-CSF: Macrophage Colony-Stimulating Factor; RANKL: Receptor Activator of Nuclear Factor κ B.

1.3. EXPRESSION OF PHENOTYPIC MARKERS BY OSTEOCLAST-LIKE CELLS GENETIC MARKERS

To further characterize the population of OC obtained in our culture conditions, after 17 days of differentiation RNA was isolated and gene expression of TRAP, VNR, Cath K, CNR and RANK examined by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The expression of these genes is reported to be specific of OC [29, 73]. Upon extraction, RNA integrity was confirmed by gel electrophoresis (Figure 14A), where bands corresponding to 18S and 28S are visible. Expression of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GADPH) was used as positive control for



the PCR. The number of amplification cycles performed for each gene is indicated in Figure 14B and was based in the literature [29, 73].

The gene expression analysis at the mRNA level confirmed the expression of OC characteristics markers (Figure 14B) [29, 73]. As can be seen in Figure 14B, all bands have the expected size (Table 2). However, the amplification efficiency differed among genes, particularly in the case of VNR and RANK. Although a band of the expected size was obtained for VNR, two additional non-specific bands could also be detected. Regarding RANK, a band was clearly seen, but not of the correct size. Still, a more thorough analysis reveals a faint band slightly above the 250bp band of the DNA ladder, which possibly corresponds to the expected product. This indicates that the amplification conditions for these genes are not ideal and would need to be optimized in future studies. Finally, the intensity of the bands obtained for GAPDH differed according to the number of cycles performed. This was anticipated since the same amount of RNA was used for all conditions and reflects a higher number of amplified copies with more cycles.

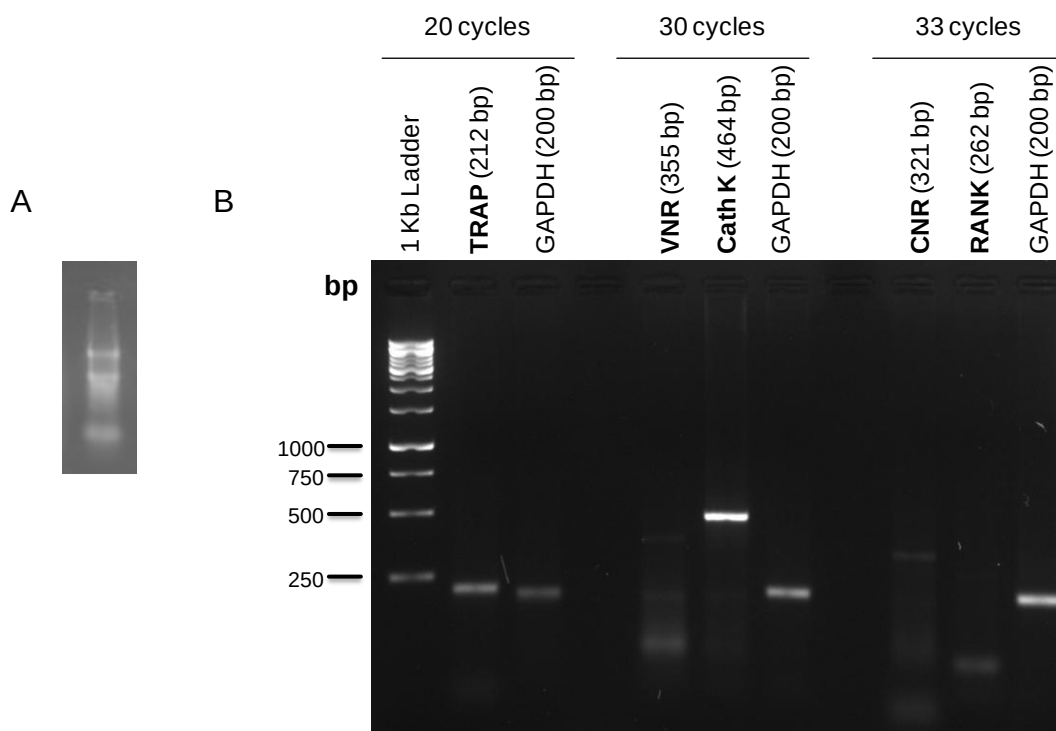


Figure 14 – Osteoclasts differentiated on TCPS express their characteristic markers. Monocytes were culture for 17 days and **A**: their total RNA was extracted and its integrity analyzed and **B**: expression of 5 osteoclast genes was evaluated by RT-PCR. bp: Base Pair; TRAP: Tartrate Resistant Acid Phosphatase; VNR: Vitronectin Receptor; Cath K: Cathepsin K; CNR: Calcitonin Receptor; RANK: Receptor Activator of Nuclear Factor κ B; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; TCPS: Tissue Culture Polystyrene; RNA: Ribonucleic Acid; RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction.

2. OSTEOCLASTOGENESIS ON CHITOSAN SUBSTRATES

One of the goals of this work was to investigate the influence of modified chitosan substrates on the formation of OC. Thus, after optimizing OC culture conditions, cells were cultured on chitosan substrates modified or not with HFG. Chitosan modified with collagen type I was used as a control, as collagen modification was described in the literature as potentiating osteoclastogenesis, when compared to chitosan alone [73]. However, since multinucleated cells formed on collagen-modified chitosan were very similar to those obtained on chitosan, in terms of number of multinucleated cells and number of nuclei per cell, these results will not be discussed here (please see Annex 1 for further details concerning multinuclear OC formation on chitosan substrates modified with collagen).

The first step was to test the ability of cells to adhere to the different substrates; so, monocytes were isolated and induced to differentiate into OC, in presence of the selected concentrations of RANKL and M-CSF. Culture was performed for a period of 7 days and then all nuclei were counted. It was assumed that each nucleus corresponds to one cell that had adhered to the substrate. Results illustrated in Figure 15 show that cells adhered to both chitosan substrates in much higher numbers than to TCPS, but there was no statistically significant difference between unmodified or HFG-modified chitosan substrates.

Therefore, monocytes were collected after the adherence step, counted and seeded at 0.63×10^6 cells/cm² on the different substrates. This step was performed in an attempt to reduce the variability in the initial number of cells in the different conditions. Cells were then induced to form OC by culture in the presence of 50 ng/mL of RANKL and 30 ng/mL of M-CSF. Cultures were analyzed at days 7, 14 and 21, for the presence of multinucleated cells, by staining the actin cytoskeleton and the nuclei. Cells were observed under a fluorescence microscope and four images from each condition were taken. These images were used to count all cells and divided into previously defined categories: 2 nuclei, 3 to 9 nuclei and 10 or more nuclei (please see Annex 2 for analysis of the results treatment performed after counting all the cells).



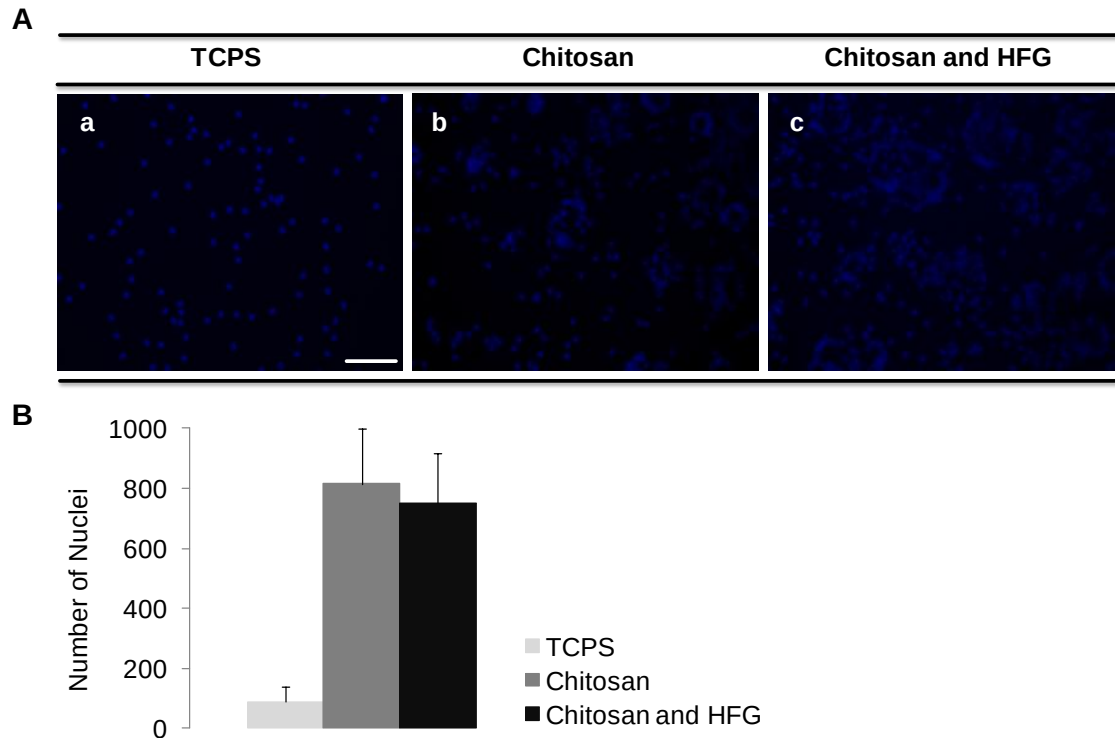


Figure 15 – Cell adhesion to modified or unmodified chitosan substrates. **A:** Monocytes were induced to differentiate into osteoclasts with RANKL and M-CSF, on the indicated substrates for 7 days before nuclei staining with Dapi (blue). Scale bar corresponds to 100 μm . **B:** Quantification of results illustrated in A (total area of 357 mm^2 per image). The average of 12 experimental points obtained in three different experiments (3 individuals, 4 points per experiment) is shown. Error bars represent standard deviation. TCPS: Tissue Culture Polystyrene; HFG: Human Fibrinogen.

Results illustrated in Figure 16 show that as early as day 7, the number of multinucleated cells is significantly higher on chitosan substrates, when compared to TCPS. After 14 days of culture it is visible an increase in the size of the cells cultured on both chitosan substrates (Figure 16A, images e and f), with the majority of cells cultured on these substrates exhibiting more than 2 nuclei (Figure 16B, middle row). Interestingly, there are significantly more cells with 3 to 9 nuclei on chitosan modified with HFG, when compared to chitosan alone. As early as day 14, some cells with 10 or more nuclei are already visible on chitosan substrates. At day 21, the number of multinucleated cells with 3 to 9 nuclei is still significantly higher on chitosan substrates than on TCPS. With the increase of the time of culture, it is visible an increase in size of the cells and number of multinucleated cells, since at day 21 there are more cells with 3 to 9 nuclei, when compared to day 7. These differences are notorious, particularly on chitosan substrates. For all the days of analysis, chitosan modified with HFG is the substrate where there are bigger cells, with more nuclei per cell.

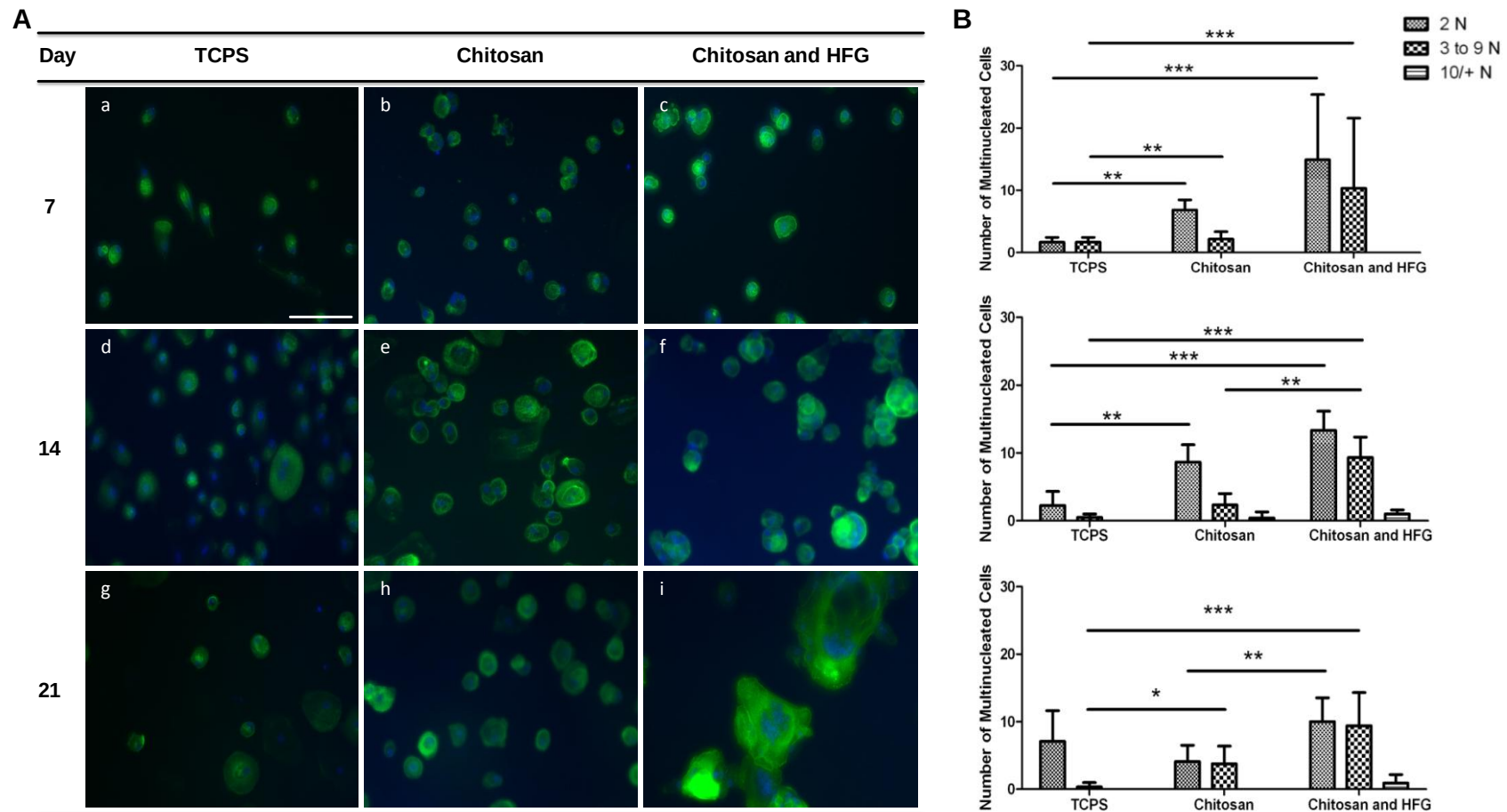


Figure 16 – Osteoclasts differentiated on HFG-modified chitosan are the most multinucleated cells. **A:** Monocytes isolated by the adherence method were differentiated into OC in presence of M-CSF and RANKL for 7 (a to c), 14 (d to f) or 21 (g to i) days, on TCPS (a, d and g), chitosan (b, e and h) and HFG modified chitosan (c, f and i). The presence of multinucleated cells (formation of characteristic actin rings, surrounding 2 or more nuclei) was evaluated by actin cytoskeleton staining, using Alexa Fluor® 488 conjugated phalloidin (green), and DAPI for nuclei staining (blue). Scale bar corresponds to 100 μ m. **B:** Quantification of the results illustrated in A, for 7 (top), 14 (middle) and 21 (bottom) days. Multinucleated cells were divided into 3 categories: 2 nuclei (small checked bars), 3 to 9 nuclei (checked bars) and 10 or more nuclei (stripped bars). The average of 12 experimental points obtained in three independent experiments (3 different individuals with 4 points per experiment) is shown. Errors bars represent standard deviation. Kruskal-Wallis followed by Dunns comparison test was performed and asterisks indicate statistical significance: * p<0.05; ** p<0.01; *** p<0.001.

Most authors choose to plate PBMC instead of pure populations of monocytes [29, 44, 91]. A study by Nicholson and others [93], proved that the ability of 10^6 PBMC to originate TRAP⁺ MNC cells is similar to a population formed by 10^5 monocytes, which is in agreement with the estimated 8-10% monocytes in the PBMC population. However, the contamination of the monocytic population separated by the adherence method with dominant lymphocytes, implicated that the number of monocytes actually available to differentiate into OC varied in different experiments. Moreover, the effect of differentiating cytokines could be influenced by cellular crosstalk, particularly with lymphocytes, which are known RANKL producers [29, 93]. Thus, a negative selection method, which allows the isolation of monocytes with a higher degree of purity, was tested as an alternative to the adherence method.

Monocytes were isolated by negative selection, plated at 0.63×10^6 cells/cm², and induced to form OC by culture in presence of 50 ng/mL of RANKL and 30 ng/mL of M-CSF. Cultures were analyzed at days 7, 14 and 21, for the presence of multinucleated cells. As above, cells were observed under a fluorescence microscope and four images from each condition were taken. These images were used to count all cells and divide them into previously defined categories: 2 nuclei, 3 to 9 nuclei and 10 or more nuclei per cell. As shown in Figure 17, as early as day 7, most of the cells cultured on chitosan substrates have already merged, they are bigger, and show already multiple nuclei per cell, in contrast to cells cultured on TCPS the same period of time. As illustrated in Figure 17A, at day 7, the majority of cells are organized into clusters on chitosan substrates, which does not occur on TCPS, where cells are homogeneously distributed; this may be a consequence of the number of cells that adhered to each substrate and might have repercussions on the number of multinucleated cells that form. When the results illustrated in Figure 17A were quantified (Figure 17B), significantly higher numbers of cells with 2 nuclei and 3 to 9 nuclei had formed already at day 7, between cells cultured on TCPS and on chitosan substrates, both unmodified or HFG modified. These differences were maintained at day 14, where a considerable number of cells with 10 or more nuclei could already be observed. Finally, at day 21 for both chitosan substrates most of the multinucleated cells have 3 or more nuclei. Importantly, for cells with 10 or more nuclei results are also significantly different between chitosan and HFG-modified chitosan. For all substrates there is an increase on the size of cells and number of multinucleated cells along the culture time. However, this is more evident on chitosan substrates, especially when these have been modified with HFG.



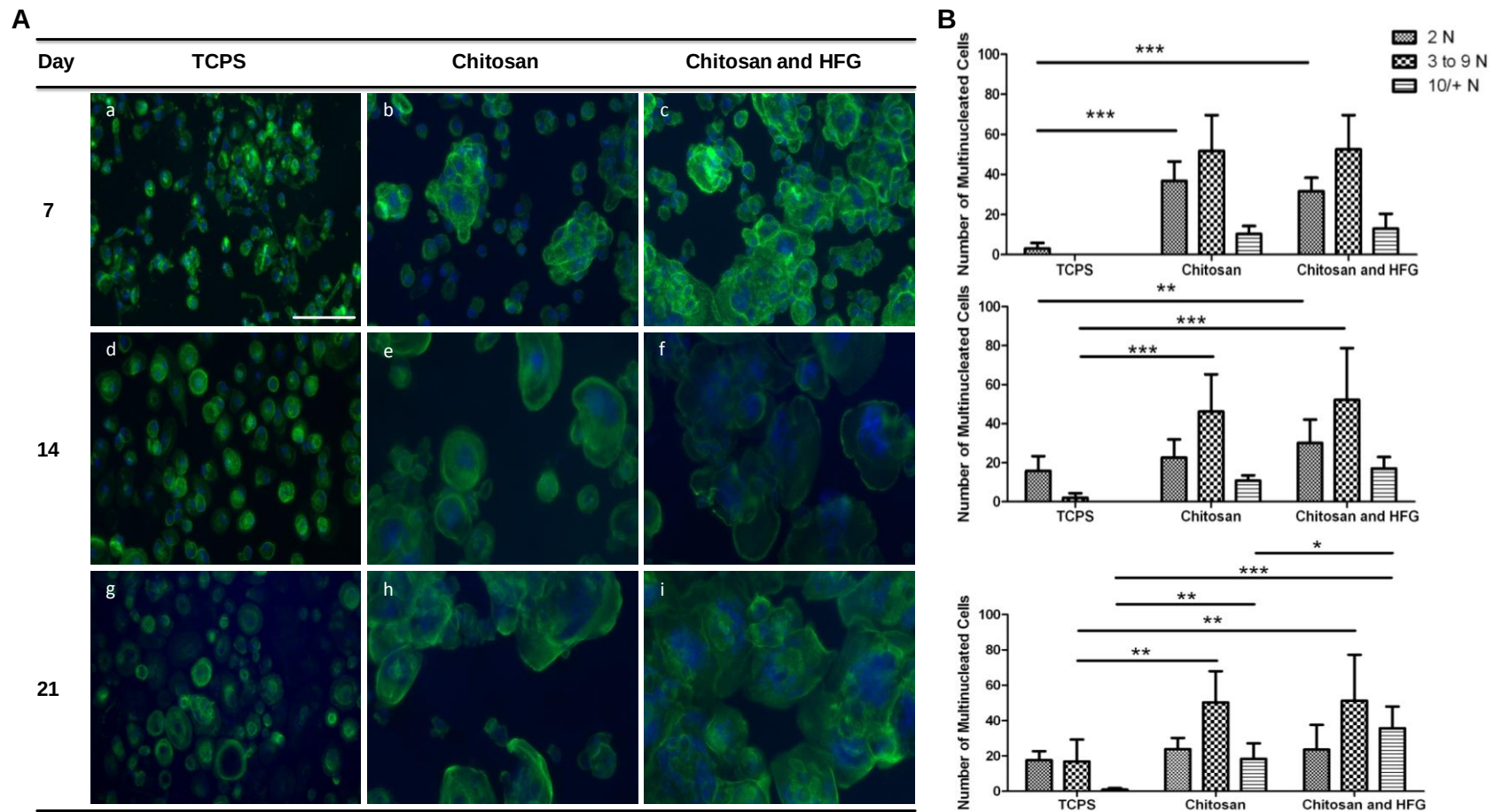


Figure 17 – Osteoclasts differentiated on HFG-modified chitosan are the most multinucleated cells. **A:** Monocytes isolated by the negative selection method were differentiated into OC in presence of M-CSF and RANKL for 7 (a to c), 14 (d to f) or 21 (g to i) days, on TCPs (a, d and g), chitosan (b, e and h) and HFG modified chitosan (c, f and i). The presence of multinucleated cells (formation of characteristic actin rings, surrounding 2 or more nuclei) was evaluated by actin cytoskeleton staining, using Alexa Fluor® 488 conjugated phalloidin (green), and DAPI for nuclei staining (blue). Scale bar corresponds to 100 μ m. **B:** Quantification of the results illustrated in A, for 7 (top), 14 (middle) and 21 (bottom) days. Multinucleated cells were divided into 3 categories: 2 nuclei (small checked bars), 3 to 9 nuclei (checked bars) and 10 or more nuclei (stripped bars). The average of 12 experimental points obtained in three independent experiments (3 different individuals with 4 points per experiment) is shown. Errors bars represent standard deviation. Kruskal-Wallis followed by Dunns comparison test was performed and asterisks indicate statistical significance: * p<0.05; ** p<0.01; *** p<0.001.

Analysis of the results obtained by the two monocyte isolation methods revealed that on chitosan substrates there is an enhancement of osteoclastogenesis, when compared to TCPS. The cells formed are bigger, with more nuclei per cell, and this is even more obvious when chitosan is modified with HFG. A previous study showed that collagen modified chitosan enhanced osteoclastogenesis [73]. However, in our experimental conditions we did not observe a significant difference between collagen modified and unmodified chitosan substrates (please see Annex 1). This is contrary to the significant difference, particularly at later differentiation times, observed for cells cultured on HFG modified chitosan, when compared with unmodified chitosan substrates. Furthermore, on modified and unmodified chitosan substrates OC formed defined actin rings, surrounding the multiple nuclei, which indicates good adhesion to the substrate [82]. Also, actin rings, are usually part of the sealing zone, formed to prevent acid leakage from the area that is being resorbed, so their formation is usually associated with OC resorbing their substrate.

Our results also indicate that a much higher number of bigger and multinucleated OC, with more nuclei per cell, were obtained from a highly pure monocytic population (isolation by the negative selection method), probably because a more precise number of monocytes can be plated without contaminating lymphocytes. Yet, independently of the isolation method used, osteoclastogenesis was always achieved at a higher rate on chitosan substrates than on TCPS, especially after HFG-modification. In fact, results obtained for chitosan substrate modified with HFG are better than those ones obtained for collagen-modified chitosan (Annex 1).

2.1. EVALUATION OF TRAP PRODUCTION ON CHITOSAN

OC are described as TRAP-producing cells [98]. Some authors argue that TRAP is secreted onto the bone surface, being consecutively endocytosed and transported to the basolateral side of the OC [99, 100]. As shown above, OC cultured on TCPS were TRAP positive at all time points analysed (Figures 9 and 11). So, we decided to investigate whether OC were also functional on chitosan substrates by looking into TRAP production. OC were isolated by the negative selection method and cultured for 21 days on top of chitosan substrates, modified or not with HFG. TRAP staining results



indicate that OC are also functional on these substrates, as cells have some granules with a characteristic dark brown color (Figure 18A).

In order to have a more quantitative perspective, a quantification of the enzyme being produced in a 24-hour period, in unmodified or HFG modified chitosan substrates was conducted. At day 20, culture medium was changed and analysis was performed at day 21. Results illustrated in Figure 18B indicate that cells cultured on chitosan substrates produced slightly more TRAP than the ones cultured on TCPS. Since TRAP secretion by OC has been reported during bone resorption, these results suggest that OC might maintain a resorptive behavior even when cultured on a non-bone substrate such as chitosan [101].

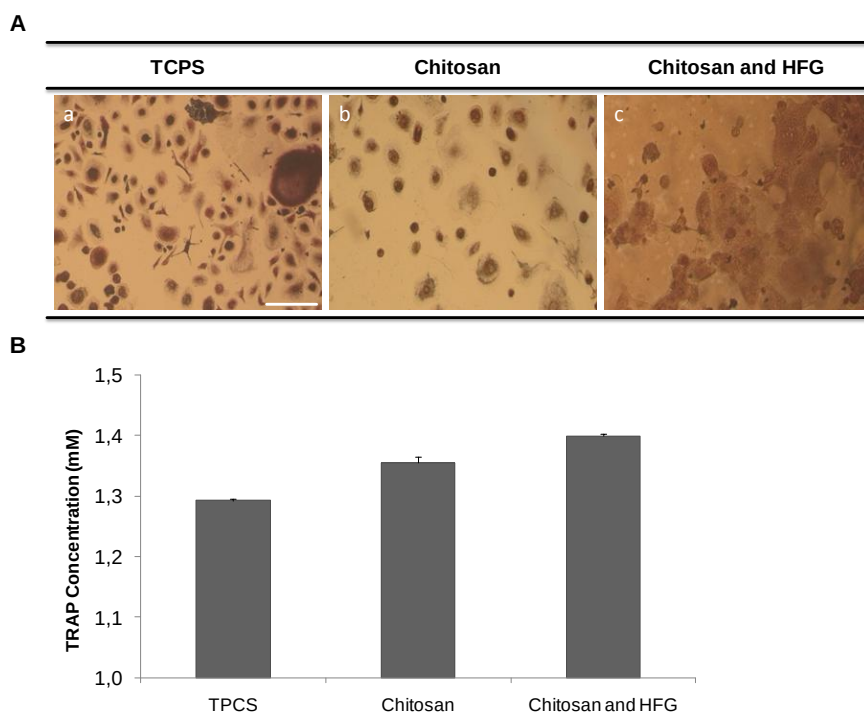


Figure 18 – Osteoclasts cultured on chitosan substrates are TRAP producers. **A:** Monocytes were cultured for 21 days in the presence of 50 ng/mL of RANKL and 30 ng/mL of M-CSF in TCPS (a), chitosan (b) and HFG modified chitosan (c) substrates. Scale bar corresponds to 100 μ m. **B:** Quantification of TRAP production in 24h. The activity of TRAP was measured in the supernatant and lysate of cells and enzyme activity released into the supernatant was calculated as a percentage of total enzyme activity. The average of three independent readings for three experimental replicates and one biological sample is shown. Error bars represent standard deviation. TCPS: Tissue Culture Polystyrene; HFG: Human Fibrinogen.

3. CHITOSAN DEGRADATION

Having found that chitosan substrates, particularly after HFG modification, potentiate the formation of multinucleated, TRAP producing OC-like cells, we proceeded to investigate whether OC were able to degrade these substrates.

For that monocytes were isolated by the negative selection method, and plated in a 24-well plate at a density of 0.25×10^6 cells/cm² on top of chitosan films, modified or not with HFG. The decrease in the cell density is due to the fact that for this kind of studies cells plated at higher density would be confluent and so preclude the film degradation follow up. Cells were cultured for 21 days, in OC differentiating conditions and monitored at the indicated time points. Cells were stained with Hematoxylin-Eosin, which stains cells with different shades of purple and chitosan red, in order to visualize both OC morphology and possible degradation areas on chitosan films. As shown in Figure 19, cells are very small at day 1 and their size increases with time of culture. Interestingly, at days 14 and 21, some degradation areas appear to have formed around the cells as indicated by the arrow in Figure 19i.

Since Hematoxylin Eosin staining is performed on fixed cells, degradation follow up could not be evaluated over time. Therefore, a different method was carried out in order to investigate the resorptive behavior of OC on chitosan substrates. Thus, chitosan was labeled with FITC, to obtain a fluorescent substrate that would allow film degradation to be monitored during the entire culture period. FITC-labeled chitosan films were prepared by spincoating, which allows ultrathin and homogeneous films. However, the fluorescence of the films with only one layer was not detectable under the microscope, so we produced films with 4 layers.

Monocytes were isolated by the negative selection method, and plated at a density of 0.25×10^6 cells/cm², on FITC-labeled chitosan substrates, modified or not with HFG, for 17 days, in the presence of OC differentiating cytokines. Cultures were analyzed at days 3, 7, 10, 14 and 17, for the existence of possible degradation areas, by fluorescence microscopy analysis (qualitative analysis) and fluorimetry, using an area scan analysis (quantitative analysis).



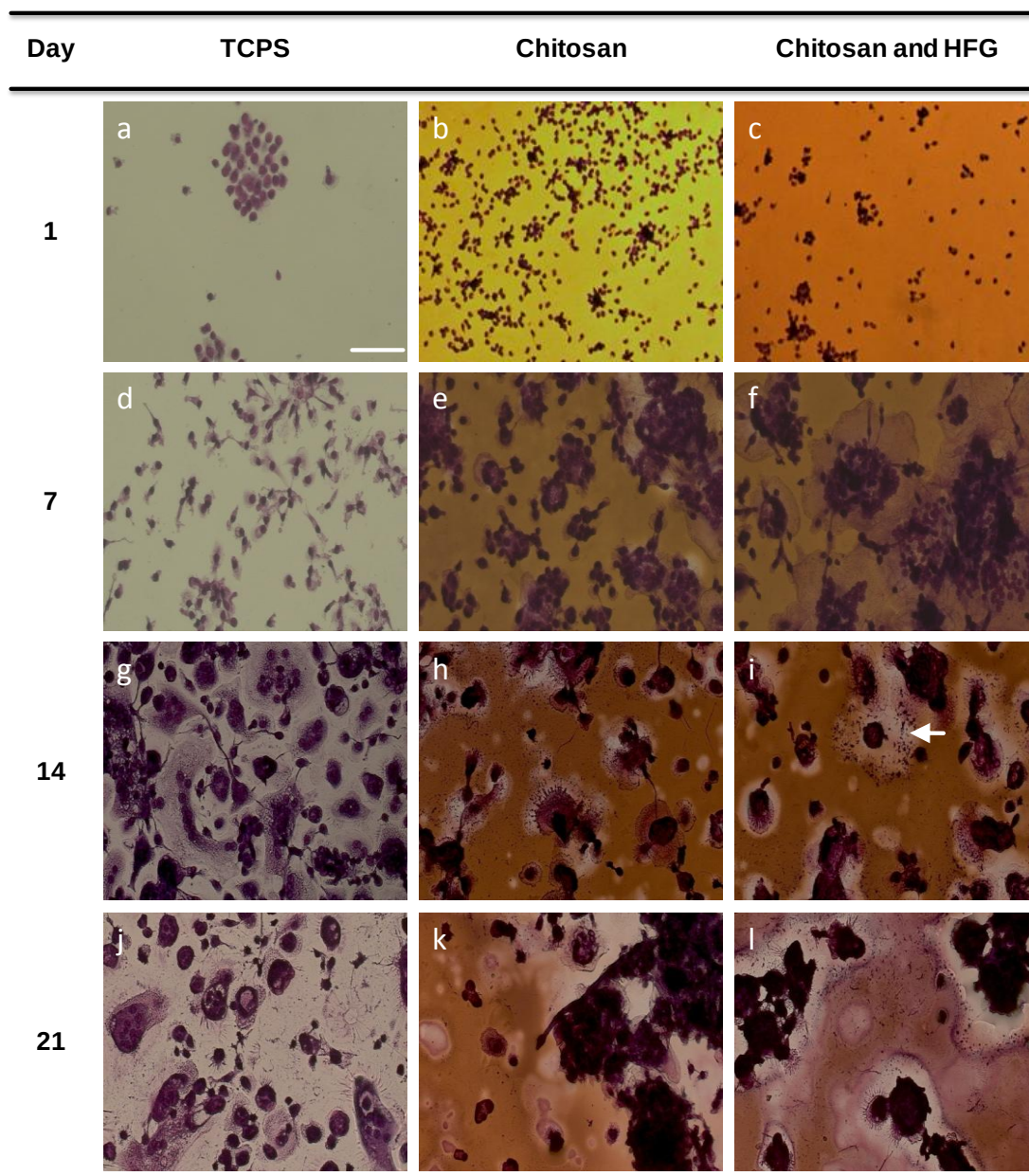


Figure 19 – Osteoclasts are able to degrade unmodified and modified chitosan substrates. Monocytes cultured in the presence of 30 ng/ml of M-CSF and 50 ng/ml of RANKL. Cells were cultured for 1, 7, 14 and 21 days in TCPS (a, d, g and j), in chitosan (b, e, h and k) and in HFG modified chitosan (c, f, i and l). Hemacolor staining reveals chitosan in orange and cells in dark brown. Arrow points at a potential degradation area. Scale bar corresponds to 100 μ m. TCPS: Tissue Culture Polystyrene; HFG: Human Fibrinogen.

As illustrated in Figure 20, the number and size of the degradation areas (black holes) significantly increased over time. In the majority of cases, cells are close to the holes, which may indicate that they were degrading by the time that the image was taken. Apparently, the rate of degradation increases by the seventh day of culture, which corresponds to OC precursor's formation [44]. Moreover, comparing the degradation

areas on both chitosan substrates and HFG modified chitosan substrates it seems that degradation is higher on the latter.

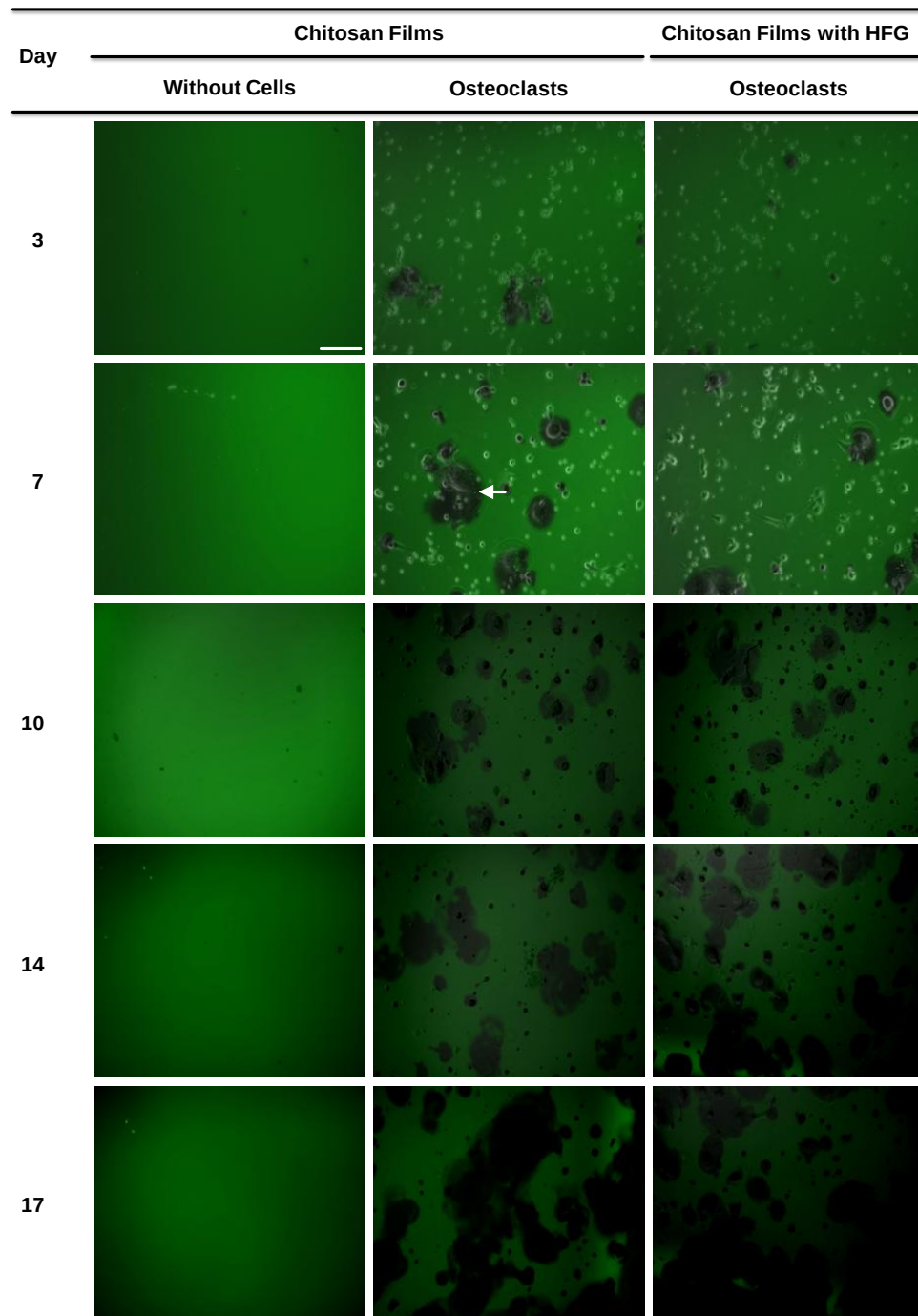


Figure 20 – Activity of osteoclasts on modified and unmodified FITC-chitosan films. Monocytes were cultured on FITC-labeled chitosan films. Chitosan films without cells were used as a negative control (left column). Osteoclasts were cultured for 17 days, in the presence of 30 ng/ml of M-CSF and 50 ng/ml of RANKL, on chitosan (middle column) or on HFG modified chitosan (right column). Osteoclast cultures were followed over time to investigate substrate degradation. The presence of dark areas on the green background (chitosan film) indicates film degradation. Arrow points to a lacuna formed on chitosan film. Scale bar corresponds to 100 μ m.

Interestingly, chitosan appeared to be incorporated into vesicles inside OC, as illustrated in Figure 21. Thus, it is tempting to speculate that chitosan may be transported along the OC, after its degradation, by a process that might be similar to what happens when OC are degrading bone matrix – transcytosis. To confirm this, different tests were performed, namely staining with a red lysotracker dye, which is incorporated inside acidic vesicles [102], and by acid wash, washing the film but preserving chitosan inside the cells. Unfortunately, both assays were unsuccessful and no confirmation could be obtained.

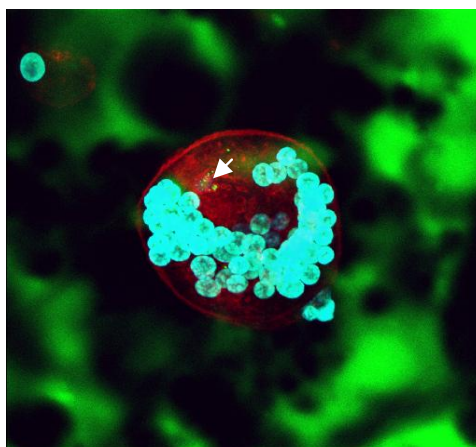


Figure 21 – Osteoclasts appear to internalize FITC-labeled chitosan, after its degradation. Monocytes were cultured for 17 days on fibrinogen-modified FITC-labeled chitosan. At day 17 actin cytoskeleton staining with Alexa Fluor® 594 conjugated phalloidin (red), and nuclei staining with DAPI (blue) was performed. Chitosan (green) is visible inside cellular vesicles (red) (arrow). Original Magnification: 20x (4x Zoom).

Furthermore, we decided that it was necessary not only to observe the formation of the resorption areas but also, and more importantly, to quantify the degradation performed by OC on both chitosan and HFG modified chitosan films. So, quantification of chitosan degradation was performed by fluorimetry. For this, fluorescence was measured across all of the well, resulting in a matrix of values and a color scale figure (for an example see Figure 22A). In the color scale figure, results are displayed ranging from blue (low fluorescence) to red (high fluorescence) (Figure 22A). After each measurement, for each well, a color scale figure was obtained. In this figure, blue areas (Figure 22A) may be interpreted as film degradation. In addition, the matrix of values allows calculating the average of fluorescence for each time point of analysis, helping to understand the evolution of the fluorescence loss and, consequently, the global degradation of the film.



It is necessary to consider the natural loss of film fluorescence, so, films incubated in culture medium were used as controls. The quantification of chitosan degradation was performed with cells from three different donors, three replicates per experiment, and the resulting average is presented on Figure 22B.

The results presented in Figure 22B suggest that OC may be degrading chitosan films as fluorescence intensity is lower when cells are present. Although, there is a gradual loss of fluorescence over the culture period, on both chitosan and HFG modified chitosan films, it is higher with OC. Surprisingly, the results obtained for the two different substrates were similar.

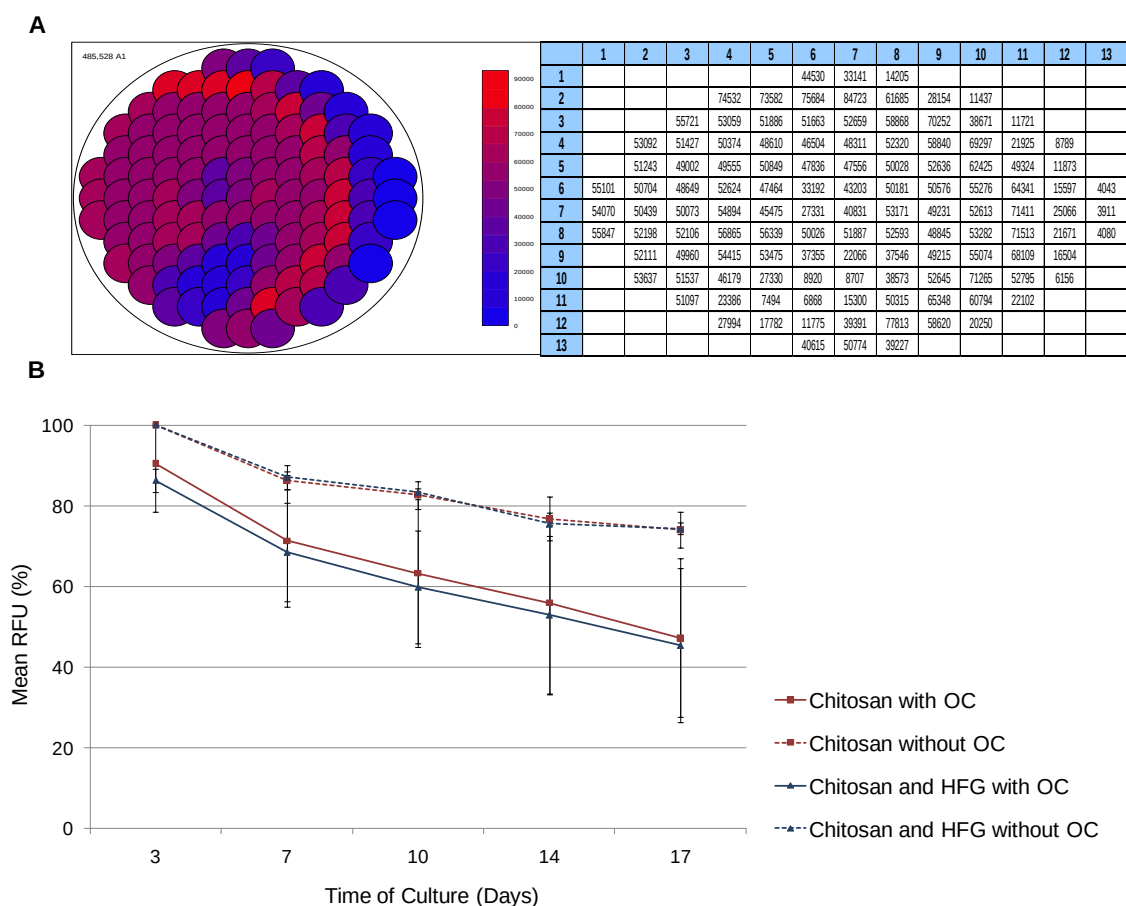


Figure 22 – Quantification of Chitosan and HFG modified chitosan degradation. **A:** Output of an area scan reading. Left image is a color scale image, where the results are displayed ranging from blue (low RFU) to red (High RFU), generated from the RFU matrix obtained (right image). **B:** Quantification of chitosan and HFG modified chitosan degradation by OC over the culture time. OC were cultured on FITC-labeled chitosan and FITC-labeled chitosan films with HFG for 17 days, in the presence of 50 ng/mL of RANKL and 30 ng/mL of M-CSF. Films without cells were used as a control of the loss of films fluorescence. Error bars represent Standard Deviation. HFG: Human Fibrinogen; OC: Osteoclast; RFU: Relative Fluorescence Units.

Aiming at clarifying these data, another approach was pursued. Instead of analyzing directly OC cultures, the corresponding images taken at different time points were treated, with the ImageJ 1.44 Software using the particle analysis tool. Analysis of Figure 24, which summarizes all quantified data (3 donors with 3 experimental replicates), reveals that the degradation area increases with the differentiation time of OC, in agreement with the fluorimetry results. In most days of analysis, the degradation rate is quite similar in chitosan and chitosan and HFG; however, at day 17, when mature OC are present, there is a bigger increase in the degraded area of the HFG-modified chitosan films, when compared to chitosan alone. Although further confirmation is required, as results are not statistically significant, it is tempting to speculate that when chitosan is modified with HFG there is an enhancement on the OC ability to degrade the substrate.

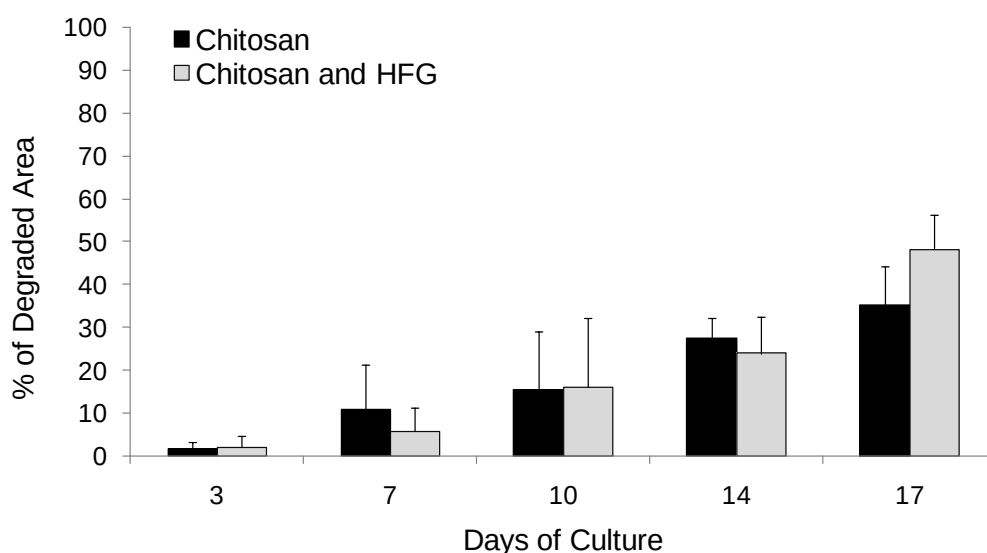


Figure 23 – Quantification of Chitosan and HFG modified Chitosan degradation by ImageJ analysis.

Quantification of chitosan and HFG modified chitosan degradation by OC over the culture time. OC were cultured on FITC-labeled chitosan and FITC-labeled chitosan films with HFG for 17 days, in the presence of 50 ng/mL of RANKL and 30 ng/mL of M-CSF. Films without cells were used as a control for the loss of films fluorescence. Error bars represent Standard Deviation. HFG: Human Fibrinogen. OC: Osteoclasts.



4. OC CULTURE ON 3D HFG MODIFIED CHITOSAN SCAFFOLDS

As *in vivo* cells live in 3D microenvironment and 3D culture have been reported to influence cell behavior, the next step was to culture OC in 3D chitosan scaffolds, modified or not with HFG, and with or without M-CSF and RANKL. At day 21, cells were fixed, stained for the actin cytoskeleton and nuclei, and observed under Confocal Microscopy. As Figure 24 illustrates, OC cultured on chitosan scaffolds are able to attach to the substrate, although cells are small (Figure 24a), when compared to the OC cultured on chitosan scaffolds modified with HFG (Figure 24b). A higher magnification image shows the morphology of a cell with, at least, 5 nuclei cultured on chitosan modified with HFG (Figure 24c).

To better analyze the morphology of OC on 3D chitosan scaffolds SEM analysis was performed. Figure 25A illustrates OC attached to chitosan scaffolds with their characteristic spread morphology [82]. When monocytes were cultured on chitosan scaffolds without OC differentiating cytokines they gave rise only to macrophages, visible in Figure 25B, with a very different morphology from OC.

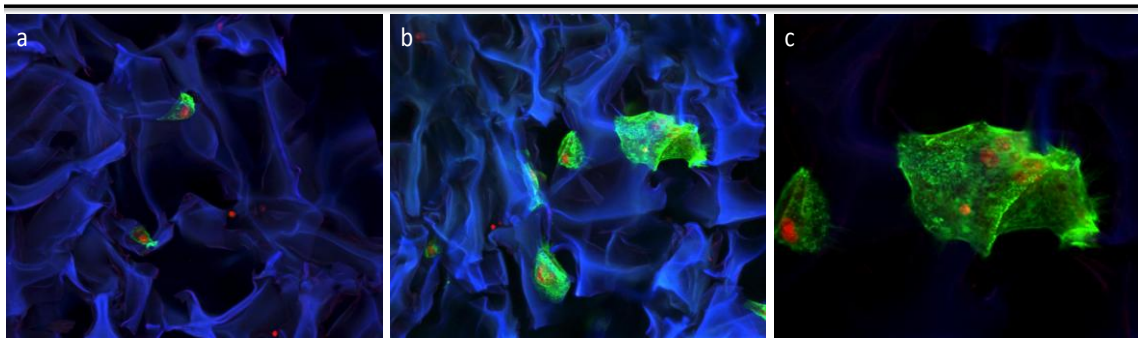


Figure 24 – 3D Modified Chitosan Scaffolds induce the formation of multinuclear OC. Monocytes were cultured for 21 days, in the presence of 50 ng/mL of RANKL and 30 ng/mL of M-CSF, in chitosan scaffolds (a) or in chitosan scaffolds modified with HFG (b and c). Actin cytoskeleton was labeled using Alexa Fluor® 488 conjugated phalloidin (green) and nuclei stained with propidium iodide (red) followed by confocal microscopy analysis. Chitosan is represented in blue due to the autofluorescence signal. Original Magnification: 20x. Image c shows a 4x magnification of the selected area of b. M-CSF: Macrophage Colony-Stimulating Factor; RANKL: Receptor Activator of Nuclear Factor κ B; OC: Osteoclasts.

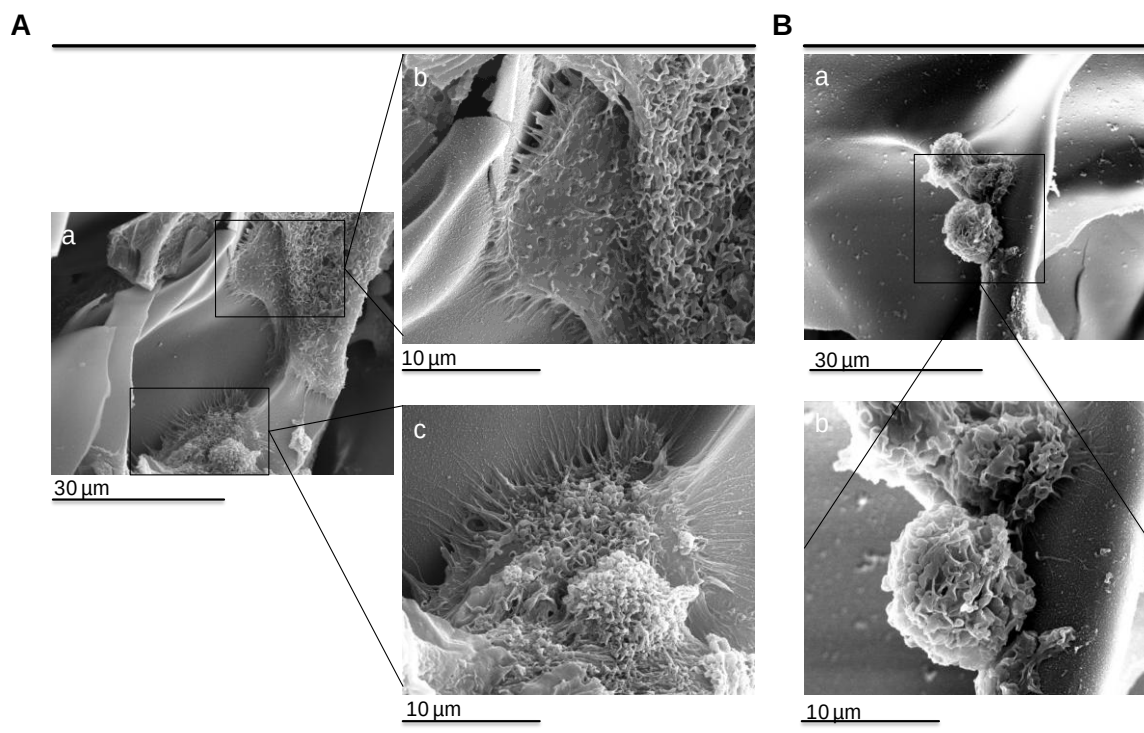


Figure 25 – Analysis of OC formed on 3D chitosan scaffolds by SEM. Monocytes were cultured for 21 days in chitosan scaffolds, **A**: in the presence of 50 ng/mL of RANKL and 30 ng/mL of M-CSF (a to c), or **B**: without OC differentiating cytokines (a and b). Cells were treated to be analyzed by SEM as follows: cells were fixed, dehydrated, critical point dried, mounted onto appropriate supports with araldite glue, sputtered-coated with gold and examined by Scanning Electron Microscopy (SEM). M-CSF: Macrophage Colony-Stimulating Factor; RANKL: Receptor Activator of Nuclear Factor κ B. OC: Osteoclasts.



CHAPTER IV

CONCLUDING REMARKS





In this study we started by testing OC differentiation conditions from monocytes, as to cell density, concentrations of OC differentiating cytokines and time points for analysis. Importantly, mature osteoclasts were shown to be functional.

OC cultured on chitosan revealed that this biomaterial has the capacity to support the formation of functional OC. Our results showed that much higher number of bigger and more multinuclear OC were obtained when we have a population of monocytes with a higher degree of purity (monocytes isolated by the negative selection method). Independently of the method used to isolate monocytes chitosan modified with HFG always induced a higher number of multinucleated cells, particularly with 10 or more nuclei at 21 days of culture. TRAP quantification indicates that when OC are cultured on chitosan substrates, especially when this is modified with HFG, more TRAP is released.

An analysis of chitosan degradation was performed and the obtained data indicates that unmodified and HFG-modified chitosan substrates are degraded by OC. Two different approaches were used to investigate OC capacity to degrade chitosan films and it was observed that chitosan modification with HFG led to a higher area of film degradation.

OC cultures were also performed for 3D culture systems, where OC differentiated on chitosan scaffolds were able to attach to the scaffold and to merge, forming multinucleated cells. Though, osteoclastogenesis on 3D scaffolds was more evident when the scaffolds were modified with HFG, since cells were bigger and presented multiple nuclei per cell.

In summary, it is possible to say that we were able to establish OC culture on HFG-modified chitosan substrates and also to confirm the ability of OC to degrade a non-mineralized substrate. Aiming to contribute for bone tissue regeneration, it is now important to confirm these results *in vivo*, investigating the potential role of OC in modified chitosan scaffold degradation, upon implantation.





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ANNEXES





ANNEX 1: OC CULTURED ON COLLAGEN-MODIFIED CHITOSAN

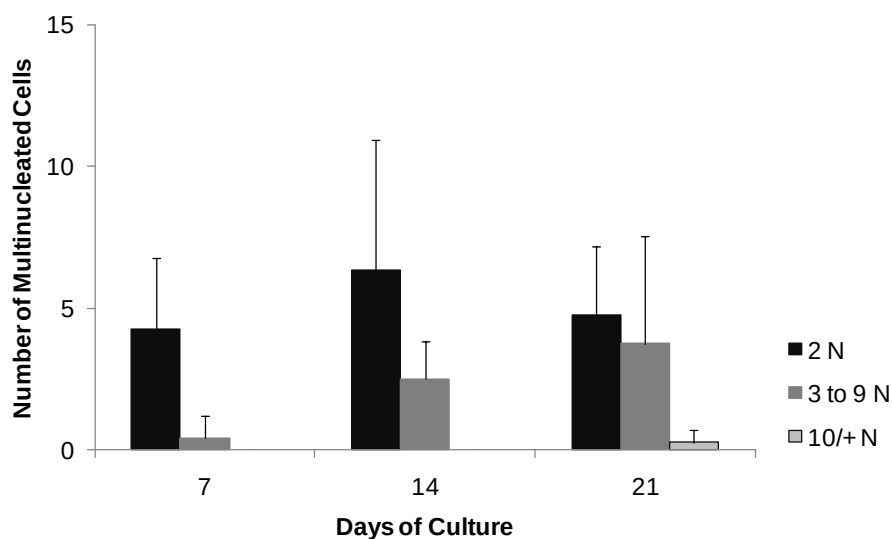


Figure 1 – Osteoclasts were differentiated on Collagen-modified chitosan. Monocytes isolated by the adherence method were differentiated into OC in presence of M-CSF and RANKL for 7, 14 or 21 days, on collagen modified chitosan and results concerning the number of multinucleated cells were quantified. Multinucleated cells were divided into 3 categories 2 nuclei, 3 to 9 nuclei and 10 or more nuclei. The average of 12 experimental points obtained in three independent experiments (3 different individuals with 4 points per experiment) is shown. Errors bars represent standard deviation.

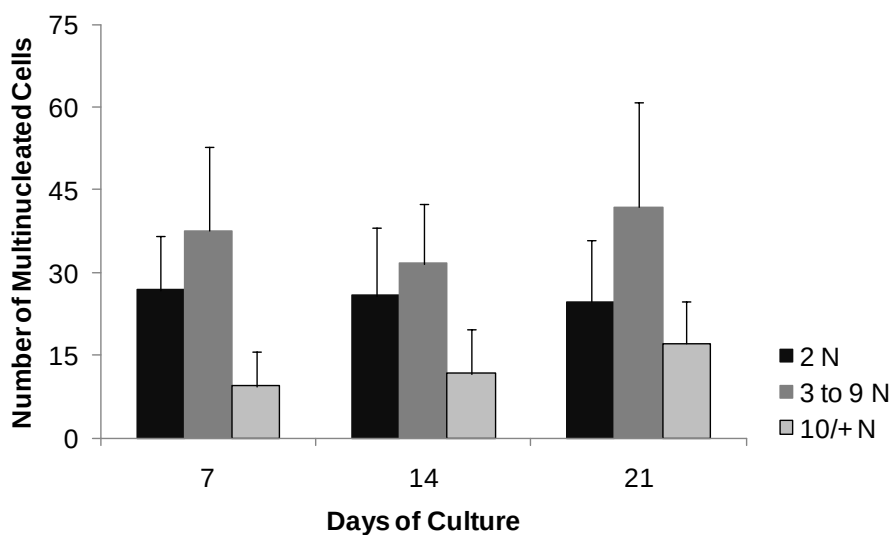


Figure 2 – Osteoclasts were differentiated on Collagen-modified chitosan. Monocytes isolated by the negative selection method were differentiated into OC in presence of M-CSF and RANKL for 7, 14 or 21 days, on collagen modified chitosan and results concerning the number of multinucleated cells were quantified. Multinucleated cells were divided into 3 categories 2 nuclei, 3 to 9 nuclei and 10 or more nuclei. The average of 12 experimental points obtained in three independent experiments (3 different individuals with 4 points per experiment) is shown. Errors bars represent standard deviation.





ANNEX 2: TREATMENT OF RESULTS

Table 1 – Result of cell count at day 7, on TCPS.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D7_R+M.1	10,00	1,00	0,00	0,00	1,00	2,00	12,00	11,00	1,09
E7_D7_R+M.2	19,00	1,00	0,00	0,00	1,00	2,00	21,00	20,00	1,05
E7_D7_R+M.3	17,00	3,00	0,00	0,00	3,00	6,00	23,00	20,00	1,15
E7_D7_R+M.4	9,00	1,00	0,00	0,00	1,00	2,00	11,00	10,00	1,10
Average	13,75	1,50	0,00	0,00	1,50	3,00	16,75	15,25	1,10
St. Dev.	4,99	1,00	0,00	0,00	1,00	2,00	6,13	5,50	0,04
E7_D7_RANKL1	3,00	0,00	0,00	0,00	0,00	0,00	3,00	3,00	1,00
E7_D7_RANKL2	4,00	0,00	0,00	0,00	0,00	0,00	4,00	4,00	1,00
E7_D7_RANKL3	5,00	0,00	0,00	0,00	0,00	0,00	5,00	5,00	1,00
E7_D7_RANKL4	6,00	0,00	0,00	0,00	0,00	0,00	6,00	6,00	1,00
Average	4,50	0,00	0,00	0,00	0,00	0,00	4,50	4,50	1,00
St. Dev.	1,29	0,00	0,00	0,00	0,00	0,00	1,29	1,29	0,00
E7_D7_MCSF.1	4,00	0,00	0,00	0,00	0,00	0,00	4,00	4,00	1,00
E7_D7_MCSF.2	8,00	1,00	0,00	0,00	1,00	2,00	10,00	9,00	1,11
E7_D7_MCSF.3	12,00	1,00	0,00	0,00	1,00	2,00	14,00	13,00	1,08
E7_D7_MCSF.4	4,00	0,00	0,00	0,00	0,00	0,00	4,00	4,00	1,00
Average	7,00	0,50	0,00	0,00	0,50	1,00	8,00	7,50	1,05
St. Dev.	3,83	0,58	0,00	0,00	0,58	1,15	4,90	4,36	0,06
E7_D7_None.1	6,00	0,00	0,00	0,00	0,00	0,00	6,00	6,00	1,00
E7_D7_None.2	3,00	0,00	0,00	0,00	0,00	0,00	3,00	3,00	1,00
E7_D7_None.3	3,00	0,00	0,00	0,00	0,00	0,00	3,00	3,00	1,00
E7_D7_None.4	4,00	0,00	0,00	0,00	0,00	0,00	4,00	4,00	1,00
Average	4,00	0,00	0,00	0,00	0,00	0,00	4,00	4,00	1,00
St. Dev.	1,41	0,00	0,00	0,00	0,00	0,00	1,41	1,41	0,00

Table 2 – Result of cell count at day 7, on Chitosan.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D7_R+M.1	22,00	7,00	2,00	0,00	9,00	21,00	43,00	31,00	1,39
E7_D7_R+M.2	19,00	6,00	1,00	0,00	7,00	16,00	35,00	26,00	1,35
E7_D7_R+M.3	21,00	5,00	4,00	0,00	9,00	24,00	45,00	30,00	1,50
E7_D7_R+M.4	20,00	4,00	2,00	0,00	6,00	14,00	34,00	26,00	1,31
Average	20,50	5,50	2,25	0,00	7,75	18,75	39,25	28,25	1,39
St. Dev.	1,29	1,29	1,26	0,00	1,50	4,57	5,56	2,63	0,08
E7_D7_RANKL1	14,00	0,00	0,00	0,00	0,00	0,00	14,00	14,00	1,00
E7_D7_RANKL2	9,00	1,00	0,00	0,00	1,00	2,00	11,00	10,00	1,10
E7_D7_RANKL3	8,00	0,00	0,00	0,00	0,00	0,00	8,00	8,00	1,00
E7_D7_RANKL4	9,00	0,00	0,00	0,00	0,00	0,00	9,00	9,00	1,00
Average	10,00	0,25	0,00	0,00	0,25	0,50	10,50	10,25	1,03
St. Dev.	2,71	0,50	0,00	0,00	0,50	1,00	2,65	2,63	0,05
E7_D7_MCSF.1	19,00	1,00	0,00	0,00	1,00	2,00	21,00	20,00	1,05
E7_D7_MCSF.2	13,00	2,00	0,00	0,00	2,00	4,00	17,00	15,00	1,13
E7_D7_MCSF.3	14,00	1,00	0,00	0,00	1,00	2,00	16,00	15,00	1,07
E7_D7_MCSF.4	6,00	2,00	0,00	0,00	2,00	4,00	10,00	8,00	1,25
Average	13,00	1,50	0,00	0,00	1,50	3,00	16,00	14,50	1,13
St. Dev.	5,35	0,58	0,00	0,00	0,58	1,15	4,55	4,93	0,09
E7_D7_None.1	20,00	1,00	0,00	0,00	1,00	2,00	22,00	21,00	1,05
E7_D7_None.2	8,00	2,00	0,00	0,00	2,00	4,00	12,00	10,00	1,20
E7_D7_None.3	12,00	1,00	0,00	0,00	1,00	2,00	14,00	13,00	1,08
E7_D7_None.4	22,00	2,00	0,00	0,00	2,00	4,00	26,00	24,00	1,08
Average	15,50	1,50	0,00	0,00	1,50	3,00	18,50	17,00	1,10
St. Dev.	6,61	0,58	0,00	0,00	0,58	1,15	6,61	6,58	0,07



Table 3 – Result of cell count at day 7, on Chitosan and HFG.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D7_R+M.1	28,00	4,00	1,00	0,00	5,00	13,00	41,00	33,00	1,24
E7_D7_R+M.2	34,00	10,00	4,00	0,00	14,00	34,00	68,00	48,00	1,42
E7_D7_R+M.3	29,00	5,00	3,00	0,00	8,00	21,00	50,00	37,00	1,35
E7_D7_R+M.4	34,00	8,00	4,00	0,00	12,00	32,00	66,00	46,00	1,43
Average	31,25	6,75	3,00	0,00	9,75	25,00	56,25	41,00	1,36
St. Dev.	3,20	2,75	1,41	0,00	4,03	9,83	12,97	7,16	0,09
E7_D7_RANKL.1	17,00	1,00	0,00	0,00	1,00	2,00	19,00	18,00	1,06
E7_D7_RANKL.2	16,00	0,00	0,00	0,00	0,00	0,00	16,00	16,00	1,00
E7_D7_RANKL.3	12,00	1,00	0,00	0,00	1,00	2,00	14,00	13,00	1,08
E7_D7_RANKL.4	16,00	1,00	0,00	0,00	1,00	2,00	18,00	17,00	1,06
Average	15,25	0,75	0,00	0,00	0,75	1,50	16,75	16,00	1,05
St. Dev.	2,22	0,50	0,00	0,00	0,50	1,00	2,22	2,16	0,03
E7_D7_MCSF.1	42,00	4,00	0,00	0,00	4,00	8,00	50,00	46,00	1,09
E7_D7_MCSF.2	28,00	5,00	0,00	0,00	5,00	10,00	38,00	33,00	1,15
E7_D7_MCSF.3	15,00	3,00	0,00	0,00	3,00	6,00	21,00	18,00	1,17
E7_D7_MCSF.4	24,00	4,00	0,00	0,00	4,00	8,00	32,00	28,00	1,14
Average	27,25	4,00	0,00	0,00	4,00	8,00	35,25	31,25	1,14
St. Dev.	11,24	0,82	0,00	0,00	0,82	1,63	12,09	11,64	0,03
E7_D7_None.1	10,00	2,00	0,00	0,00	2,00	4,00	14,00	12,00	1,17
E7_D7_None.2	11,00	3,00	0,00	0,00	3,00	6,00	17,00	14,00	1,21
E7_D7_None.3	14,00	2,00	0,00	0,00	2,00	4,00	18,00	16,00	1,13
E7_D7_None.4	14,00	3,00	0,00	0,00	3,00	6,00	20,00	17,00	1,18
Média	12,25	2,50	0,00	0,00	2,50	5,00	17,25	14,75	1,17
Desvio Padrão	2,06	0,58	0,00	0,00	0,58	1,15	2,50	2,22	0,04

Table 4 – Result of cell count at day 7, on Chitosan and Collagen.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D7_R+M.1	18,00	4,00	2,00	0,00	6,00	14,00	32,00	24,00	1,33
E7_D7_R+M.2	18,00	4,00	0,00	0,00	4,00	8,00	26,00	22,00	1,18
E7_D7_R+M.3	12,00	9,00	0,00	0,00	9,00	18,00	30,00	21,00	1,43
E7_D7_R+M.4	10,00	4,00	0,00	0,00	4,00	8,00	18,00	14,00	1,29
Average	14,50	5,25	0,50	0,00	5,75	12,00	26,50	20,25	1,31
St. Dev.	4,12	2,50	1,00	0,00	2,36	4,90	6,19	4,35	0,10
E7_D7_RANKL.1	18,00	2,00	0,00	0,00	2,00	4,00	22,00	20,00	1,10
E7_D7_RANKL.2	21,00	2,00	0,00	0,00	2,00	4,00	25,00	23,00	1,09
E7_D7_RANKL.3	19,00	3,00	0,00	0,00	3,00	6,00	25,00	22,00	1,14
E7_D7_RANKL.4	18,00	3,00	0,00	0,00	3,00	6,00	24,00	21,00	1,14
Average	19,00	2,50	0,00	0,00	2,50	5,00	24,00	21,50	1,12
St. Dev.	1,41	0,58	0,00	0,00	0,58	1,15	1,41	1,29	0,03
E7_D7_MCSF.1	7,00	1,00	0,00	0,00	1,00	2,00	9,00	8,00	1,13
E7_D7_MCSF.2	20,00	2,00	0,00	0,00	2,00	4,00	24,00	22,00	1,09
E7_D7_MCSF.3	8,00	2,00	0,00	0,00	2,00	4,00	12,00	10,00	1,20
E7_D7_MCSF.4	13,00	3,00	0,00	0,00	3,00	6,00	19,00	16,00	1,19
Average	12,00	2,00	0,00	0,00	2,00	4,00	16,00	14,00	1,15
St. Dev.	5,94	0,82	0,00	0,00	0,82	1,63	6,78	6,32	0,05
E7_D7_None.1	11,00	0,00	0,00	0,00	0,00	0,00	11,00	11,00	1,00
E7_D7_None.2	19,00	2,00	0,00	0,00	2,00	4,00	23,00	21,00	1,10
E7_D7_None.3	19,00	0,00	0,00	0,00	0,00	0,00	19,00	19,00	1,00
E7_D7_None.4	16,00	3,00	0,00	0,00	3,00	6,00	22,00	19,00	1,16
Average	16,25	1,25	0,00	0,00	1,25	2,50	18,75	17,50	1,06
St. Dev.	3,77	1,50	0,00	0,00	1,50	3,00	5,44	4,43	0,08

Table 5 – Result of cell count at day 14, on TCPS.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D14_R+M.1	32,00	4,00	1,00	0,00	5,00	11,00	43,00	37,00	1,16
E7_D14_R+M.2	30,00	3,00	0,00	0,00	3,00	6,00	36,00	33,00	1,09
E7_D14_R+M.3	35,00	0,00	1,00	0,00	1,00	3,00	38,00	36,00	1,06
E7_D14_R+M.4	44,00	3,00	1,00	0,00	4,00	9,00	53,00	48,00	1,10
Média	35,25	2,50	0,75	0,00	3,25	7,25	42,50	38,50	1,10
Desvio Padrão	6,18	1,73	0,50	0,00	1,71	3,50	7,59	6,56	0,04
E7_D14_RANKL.1	17,00	3,00	0,00	0,00	3,00	3,00	20,00	20,00	1,00
E7_D14_RANKL.2	65,00	2,00	0,00	0,00	2,00	2,00	67,00	67,00	1,00
E7_D14_RANKL.3	75,00	6,00	1,00	0,00	7,00	6,00	81,00	82,00	0,99
E7_D14_RANKL.4	72,00	3,00	0,00	0,00	3,00	4,00	76,00	75,00	1,01
Média	57,25	3,50	0,25	0,00	3,75	3,75	61,00	61,00	1,00
Desvio Padrão	27,16	1,73	0,50	0,00	2,22	1,71	27,94	28,01	0,01
E7_D14_MCSF.1	21,00	1,00	0,00	0,00	1,00	2,00	23,00	22,00	1,05
E7_D14_MCSF.2	12,00	1,00	0,00	0,00	1,00	2,00	14,00	13,00	1,08
E7_D14_MCSF.3	12,00	1,00	0,00	0,00	1,00	2,00	14,00	13,00	1,08
E7_D14_MCSF.4	20,00	1,00	0,00	0,00	1,00	2,00	22,00	21,00	1,05
Média	16,25	1,00	0,00	0,00	1,00	2,00	18,25	17,25	1,06
Desvio Padrão	4,92	0,00	0,00	0,00	0,00	0,00	4,92	4,92	0,02
E7_D14_None.1	11,00	0,00	0,00	0,00	0,00	0,00	11,00	11,00	1,00
E7_D14_None.2	10,00	0,00	0,00	0,00	0,00	0,00	10,00	10,00	1,00
E7_D14_None.3	7,00	0,00	0,00	0,00	0,00	0,00	7,00	7,00	1,00
E7_D14_None.4	11,00	0,00	0,00	0,00	0,00	0,00	11,00	11,00	1,00
Média	9,75	0,00	0,00	0,00	0,00	0,00	9,75	9,75	1,00
Desvio Padrão	1,89	0,00	0,00	0,00	0,00	0,00	1,89	1,89	0,00

Table 6 – Result of cell count at day 14, on Chitosan.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D14_R+M.1	13,00	6,00	2,00	0,00	8,00	20,00	33,00	21,00	1,57
E7_D14_R+M.2	1,00	4,00	4,00	0,00	8,00	22,00	23,00	9,00	2,56
E7_D14_R+M.3	7,00	10,00	5,00	3,00	18,00	99,00	106,00	25,00	4,24
E7_D14_R+M.4	6,00	7,00	2,00	0,00	9,00	22,00	28,00	15,00	1,87
Média	6,75	6,75	3,25	0,75	10,75	40,75	47,50	17,50	2,56
Desvio Padrão	4,92	2,50	1,50	1,50	4,86	38,84	39,21	7,00	1,19
E7_D14_RANKL.1	2,00	1,00	0,00	0,00	1,00	2,00	4,00	3,00	1,33
E7_D14_RANKL.2	1,00	1,00	0,00	0,00	1,00	2,00	3,00	2,00	1,50
E7_D14_RANKL.3	2,00	1,00	0,00	0,00	1,00	2,00	4,00	3,00	1,33
E7_D14_RANKL.4	1,00	1,00	0,00	0,00	1,00	2,00	3,00	2,00	1,50
Média	1,50	1,00	0,00	0,00	1,00	2,00	3,50	2,50	1,42
Desvio Padrão	0,58	0,00	0,00	0,00	0,00	0,00	0,58	0,58	0,10
E7_D14_MCSF.1	7,00	0,00	1,00	0,00	1,00	3,00	10,00	8,00	1,25
E7_D14_MCSF.2	7,00	1,00	0,00	0,00	1,00	2,00	9,00	8,00	1,13
E7_D14_MCSF.3	10,00	1,00	0,00	0,00	1,00	2,00	12,00	11,00	1,09
E7_D14_MCSF.4	8,00	1,00	0,00	0,00	1,00	2,00	10,00	9,00	1,11
Média	8,00	0,75	0,25	0,00	1,00	2,25	10,25	9,00	1,14
Desvio Padrão	1,41	0,50	0,50	0,00	0,00	0,50	1,26	1,41	0,07
E7_D14_None.1	17,00	2,00	0,00	0,00	2,00	4,00	21,00	19,00	1,11
E7_D14_None.2	15,00	3,00	0,00	0,00	3,00	6,00	21,00	18,00	1,17
E7_D14_None.3	14,00	1,00	0,00	0,00	1,00	2,00	16,00	15,00	1,07
E7_D14_None.4	14,00	4,00	0,00	0,00	4,00	8,00	22,00	18,00	1,22
Média	15,00	2,50	0,00	0,00	2,50	5,00	20,00	17,50	1,14
Desvio Padrão	1,41	1,29	0,00	0,00	1,29	2,58	2,71	1,73	0,07



Table 7 – Result of cell count at day 14, on Chitosan and HFG.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D14_R+M.1	25,00	14,00	9,00	1,00	24,00	82,00	107,00	49,00	2,18
E7_D14_R+M.2	21,00	11,00	4,00	1,00	16,00	53,00	74,00	37,00	2,00
E7_D14_R+M.3	20,00	8,00	8,00	1,00	17,00	57,00	77,00	37,00	2,08
E7_D14_4	12,00	12,00	11,00	1,00	24,00	83,00	95,00	36,00	2,64
Média	19,50	11,25	8,00	1,00	20,25	68,75	88,25	39,75	2,23
Desvio Padrão	5,45	2,50	2,94	0,00	4,35	15,97	15,56	6,18	0,29
E7_D14_RANKL.1	14,00	4,00	0,00	0,00	4,00	8,00	22,00	18,00	1,22
E7_D14_RANKL.2	11,00	2,00	0,00	0,00	2,00	4,00	15,00	13,00	1,15
E7_D14_RANKL.3	9,00	0,00	0,00	0,00	0,00	0,00	9,00	9,00	1,00
E7_D14_RANKL.4	13,00	2,00	0,00	0,00	2,00	4,00	17,00	15,00	1,13
Média	11,75	2,00	0,00	0,00	2,00	4,00	15,75	13,75	1,13
Desvio Padrão	2,22	1,63	0,00	0,00	1,63	3,27	5,38	3,77	0,09
E7_D14_MCSF.1	19,00	5,00	0,00	0,00	5,00	10,00	29,00	24,00	1,21
E7_D14_MCSF.2	20,00	4,00	2,00	0,00	6,00	16,00	36,00	26,00	1,38
E7_D14_MCSF.3	24,00	6,00	0,00	0,00	6,00	12,00	36,00	30,00	1,20
E7_D14_MCSF.4	18,00	2,00	0,00	0,00	2,00	4,00	22,00	20,00	1,10
Média	20,25	4,25	0,50	0,00	4,75	10,50	30,75	25,00	1,22
Desvio Padrão	2,63	1,71	1,00	0,00	1,89	5,00	6,70	4,16	0,12
E7_D14_None.1	18,00	4,00	0,00	0,00	4,00	8,00	26,00	22,00	1,18
E7_D14_None.2	15,00	2,00	0,00	0,00	2,00	4,00	19,00	17,00	1,12
E7_D14_None.3	10,00	2,00	0,00	0,00	2,00	4,00	14,00	12,00	1,17
E7_D14_None.4	14,00	0,00	0,00	0,00	0,00	0,00	14,00	14,00	1,00
Média	14,25	2,00	0,00	0,00	2,00	4,00	18,25	16,25	1,12
Desvio Padrão	3,30	1,63	0,00	0,00	1,63	3,27	5,68	4,35	0,08

Table 8 – Result of cell count at day 14, on Chitosan and Collagen.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D14_R+M.1	13,00	4,00	3,00	0,00	7,00	17,00	30,00	20,00	1,50
E7_D14_R+M.2	7,00	3,00	3,00	0,00	6,00	25,00	32,00	13,00	2,46
E7_D14_R+M.3	5,00	3,00	4,00	0,00	7,00	13,00	18,00	12,00	1,50
E7_D14_R+M.4	6,00	2,00	3,00	0,00	5,00	15,00	21,00	11,00	1,91
Média	7,75	3,00	3,25	0,00	6,25	17,50	25,25	14,00	1,84
Desvio Padrão	3,59	0,82	0,50	0,00	0,96	5,26	6,80	4,08	0,46
E7_D14_RANKL.1	6,00	0,00	0,00	0,00	0,00	0,00	6,00	6,00	1,00
E7_D14_RANKL.2	10,00	0,00	0,00	0,00	0,00	0,00	10,00	10,00	1,00
E7_D14_RANKL.3	9,00	2,00	0,00	0,00	2,00	4,00	13,00	11,00	1,18
E7_D14_RANKL.4	8,00	1,00	0,00	0,00	1,00	2,00	10,00	9,00	1,11
Média	8,25	0,75	0,00	0,00	0,75	1,50	9,75	9,00	1,07
Desvio Padrão	1,71	0,96	0,00	0,00	0,96	1,91	2,87	2,16	0,09
E7_D14_MCSF.1	10,00	0,00	0,00	0,00	0,00	0,00	10,00	10,00	1,00
E7_D14_MCSF.2	9,00	2,00	1,00	0,00	3,00	7,00	16,00	12,00	1,33
E7_D14_MCSF.3	10,00	1,00	0,00	0,00	1,00	2,00	12,00	11,00	1,09
E7_D14_MCSF.4	9,00	0,00	0,00	0,00	0,00	0,00	9,00	9,00	1,00
Média	9,50	0,75	0,25	0,00	1,00	2,25	11,75	10,50	1,11
Desvio Padrão	0,58	0,96	0,50	0,00	1,41	3,30	3,10	1,29	0,16
E7_D14_None.1	3,00	0,00	0,00	0,00	0,00	0,00	3,00	3,00	1,00
E7_D14_None.2	4,00	0,00	0,00	0,00	0,00	0,00	4,00	4,00	1,00
E7_D14_None.3	7,00	1,00	0,00	0,00	1,00	2,00	9,00	8,00	1,13
E7_D14_None.4	7,00	1,00	1,00	0,00	2,00	5,00	12,00	9,00	1,33
Média	5,25	0,50	0,25	0,00	0,75	1,75	7,00	6,00	1,11
Desvio Padrão	2,06	0,58	0,50	0,00	0,96	2,36	4,24	2,94	0,16

Table 9 – Result of cell count at day 21, on TCPS.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D21_R+M.1	48,00	6,00	0,00	0,00	6,00	12,00	60,00	54,00	1,11
E7_D21_R+M.2	57,00	9,00	0,00	0,00	9,00	18,00	75,00	66,00	1,14
E7_D21_R+M.3	72,00	11,00	0,00	0,00	11,00	22,00	94,00	83,00	1,13
E7_D21_R+M.4	48,00	11,00	0,00	0,00	11,00	22,00	70,00	59,00	1,19
Média	56,25	9,25	0,00	0,00	9,25	18,50	74,75	65,50	1,14
Desvio Padrão	11,32	2,36	0,00	0,00	2,36	4,73	14,27	12,66	0,03
E7_D21_RANKL.1	96,00	9,00	0,00	0,00	9,00	18,00	114,00	105,00	1,09
E7_D21_RANKL.2	93,00	11,00	0,00	0,00	11,00	22,00	115,00	104,00	1,11
E7_D21_RANKL.3	37,00	4,00	0,00	0,00	4,00	8,00	45,00	41,00	1,10
E7_D21_RANKL.4	67,00	6,00	0,00	0,00	6,00	12,00	79,00	73,00	1,08
Média	73,25	7,50	0,00	0,00	7,50	15,00	88,25	80,75	1,09
Desvio Padrão	27,45	3,11	0,00	0,00	3,11	6,22	33,34	30,38	0,01
E7_D21_MCSF.1	72,00	6,00	2,00	0,00	8,00	18,00	90,00	80,00	1,13
E7_D21_MCSF.2	56,00	4,00	0,00	0,00	4,00	8,00	64,00	60,00	1,07
E7_D21_MCSF.3	67,00	8,00	1,00	0,00	9,00	19,00	86,00	76,00	1,13
E7_D21_MCSF.4	34,00	4,00	0,00	0,00	4,00	8,00	42,00	38,00	1,11
Média	57,25	5,50	0,75	0,00	6,25	13,25	70,50	63,50	1,11
Desvio Padrão	16,88	1,91	0,96	0,00	2,63	6,08	22,17	19,07	0,03
E7_D21_None.1	36,00	2,00	0,00	0,00	2,00	4,00	40,00	38,00	1,05
E7_D21_None.2	26,00	2,00	0,00	0,00	2,00	4,00	30,00	28,00	1,07
E7_D21_None.3	55,00	4,00	0,00	0,00	4,00	8,00	63,00	59,00	1,07
E7_D21_None.4	35,00	4,00	0,00	0,00	4,00	8,00	43,00	39,00	1,10
Média	38,00	3,00	0,00	0,00	3,00	6,00	44,00	41,00	1,07
Desvio Padrão	12,19	1,15	0,00	0,00	1,15	2,31	13,83	12,99	0,02

Table 10 – Result of cell count at day 21, on Chitosan.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D21_R+M.1	22,00	4,00	2,00	0,00	6,00	16,00	38,00	28,00	1,36
E7_D21_R+M.2	6,00	1,00	5,00	0,00	6,00	23,00	29,00	12,00	2,42
E7_D21_R+M.3	25,00	7,00	4,00	0,00	11,00	25,00	50,00	36,00	1,39
E7_D21_R+M.4	23,00	6,00	5,00	0,00	11,00	28,00	51,00	34,00	1,50
Média	19,00	4,50	4,00	0,00	8,50	23,00	42,00	27,50	1,67
Desvio Padrão	8,76	2,65	1,41	0,00	2,89	5,10	10,49	10,88	0,50
E7_D21_RANKL.1	4,00	2,00	2,00	1,00	5,00	25,00	29,00	9,00	3,22
E7_D21_RANKL.2	2,00	1,00	4,00	1,00	6,00	37,00	39,00	8,00	4,88
E7_D21_RANKL.3	6,00	3,00	0,00	1,00	4,00	17,00	23,00	10,00	2,30
E7_D21_RANKL.4	11,00	1,00	4,00	0,00	5,00	15,00	26,00	16,00	1,63
Média	5,75	1,75	2,50	0,75	5,00	23,50	29,25	10,75	3,01
Desvio Padrão	3,86	0,96	1,91	0,50	0,82	9,98	6,95	3,59	1,41
E7_D21_MCSF.1	6,00	1,00	0,00	0,00	1,00	2,00	8,00	7,00	1,14
E7_D21_MCSF.2	5,00	0,00	1,00	0,00	1,00	14,00	19,00	6,00	3,17
E7_D21_MCSF.3	8,00	0,00	3,00	0,00	3,00	14,00	22,00	11,00	2,00
E7_D21_MCSF.4	9,00	6,00	1,00	0,00	7,00	16,00	25,00	16,00	1,56
Média	7,00	1,75	1,25	0,00	3,00	11,50	18,50	10,00	1,97
Desvio Padrão	1,83	2,87	1,26	0,00	2,83	6,40	7,42	4,55	0,87
E7_D21_None.1	5,00	1,00	2,00	0,00	3,00	14,00	19,00	8,00	2,38
E7_D21_None.2	12,00	2,00	2,00	0,00	4,00	13,00	25,00	16,00	1,56
E7_D21_None.3	7,00	2,00	0,00	1,00	3,00	21,00	28,00	10,00	2,80
E7_D21_None.4	8,00	1,00	2,00	0,00	3,00	19,00	27,00	11,00	2,45
Média	8,00	1,50	1,50	0,25	3,25	16,75	24,75	11,25	2,30
Desvio Padrão	2,94	0,58	1,00	0,50	0,50	3,86	4,03	3,40	0,52



Table 11 – Result of cell count at day 21, on Chitosan and HFG.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D21_R+M.1	20,00	6,00	9,00	1,00	16,00	56,00	76,00	36,00	2,11
E7_D21_R+M.2	19,00	6,00	4,00	0,00	10,00	27,00	46,00	29,00	1,59
E7_D21_R+M.3	23,00	11,00	3,00	0,00	14,00	32,00	55,00	37,00	1,49
E7_D21_R+M.4	16,00	10,00	3,00	0,00	13,00	30,00	46,00	29,00	1,59
Média	19,50	8,25	4,75	0,25	13,25	36,25	55,75	32,75	1,69
Desvio Padrão	2,89	2,63	2,87	0,50	2,50	13,33	14,15	4,35	0,28
E7_D21_RANKL.1	6,00	2,00	0,00	0,00	2,00	4,00	10,00	8,00	1,25
E7_D21_RANKL.2	2,00	1,00	1,00	0,00	2,00	5,00	7,00	4,00	1,75
E7_D21_RANKL.3	3,00	0,00	1,00	0,00	1,00	7,00	10,00	4,00	2,50
E7_D21_RANKL.4	3,00	1,00	0,00	0,00	1,00	2,00	5,00	4,00	1,25
Média	3,50	1,00	0,50	0,00	1,50	4,50	8,00	5,00	1,69
Desvio Padrão	1,73	0,82	0,58	0,00	0,58	2,08	2,45	2,00	0,59
E7_D21_MCSF.1	4,00	3,00	0,00	0,00	3,00	6,00	10,00	7,00	1,43
E7_D21_MCSF.2	10,00	2,00	0,00	0,00	2,00	4,00	14,00	12,00	1,17
E7_D21_MCSF.3	3,00	3,00	0,00	0,00	3,00	6,00	9,00	6,00	1,50
E7_D21_MCSF.4	2,00	3,00	2,00	0,00	5,00	12,00	14,00	7,00	2,00
Média	4,75	2,75	0,50	0,00	3,25	7,00	11,75	8,00	1,52
Desvio Padrão	3,59	0,50	1,00	0,00	1,26	3,46	2,63	2,71	0,35
E7_D21_None.1	6,00	1,00	1,00	0,00	2,00	5,00	11,00	8,00	1,38
E7_D21_None.2	6,00	3,00	0,00	0,00	3,00	6,00	12,00	9,00	1,33
E7_D21_None.3	6,00	4,00	1,00	0,00	5,00	13,00	19,00	11,00	1,73
E7_D21_None.4	9,00	3,00	0,00	0,00	3,00	6,00	15,00	12,00	1,25
Média	6,75	2,75	0,50	0,00	3,25	7,50	14,25	10,00	1,42
Desvio Padrão	1,50	1,26	0,58	0,00	1,26	3,70	3,59	1,83	0,21

Table 12 – Result of cell count at day 21, on Chitosan and Collagen.

Conditions	Mono Cells	Multinucleated Cells			Total of MNC	MNC Nuclei	Total Nuclei	Total Number of Cells	Average Nuclei/Cell
		2	3 to 9	10/+					
E7_D21_R+M.1	10,00	3,00	0,00	0,00	3,00	6,00	16,00	13,00	1,23
E7_D21_R+M.2	16,00	3,00	0,00	0,00	3,00	6,00	22,00	19,00	1,16
E7_D21_R+M.3	9,00	4,00	0,00	0,00	4,00	8,00	17,00	13,00	1,31
E7_D21_R+M.4	7,00	4,00	0,00	0,00	4,00	8,00	15,00	11,00	1,36
Média	10,50	3,50	0,00	0,00	3,50	7,00	17,50	14,00	1,26
Desvio Padrão	3,87	0,58	0,00	0,00	0,58	1,15	3,11	3,46	0,09
E7_D21_RANKL.1	9,00	1,00	0,00	0,00	1,00	2,00	11,00	10,00	1,10
E7_D21_RANKL.2	7,00	0,00	1,00	0,00	1,00	5,00	12,00	8,00	1,50
E7_D21_RANKL.3	7,00	0,00	4,00	0,00	4,00	19,00	26,00	11,00	2,36
E7_D21_RANKL.4	5,00	1,00	1,00	0,00	2,00	5,00	10,00	7,00	1,43
Média	7,00	0,50	1,50	0,00	2,00	7,75	14,75	9,00	1,60
Desvio Padrão	1,63	0,58	1,73	0,00	1,41	7,63	7,54	1,83	0,54
E7_D21_MCSF.1	10,00	1,00	0,00	1,00	2,00	12,00	22,00	12,00	1,83
E7_D21_MCSF.2	10,00	5,00	2,00	0,00	7,00	16,00	26,00	17,00	1,53
E7_D21_MCSF.3	8,00	4,00	0,00	0,00	4,00	8,00	16,00	12,00	1,33
E7_D21_MCSF.4	9,00	3,00	0,00	0,00	3,00	6,00	15,00	12,00	1,25
Média	9,25	3,25	0,50	0,25	4,00	10,50	19,75	13,25	1,49
Desvio Padrão	0,96	1,71	1,00	0,50	2,16	4,43	5,19	2,50	0,26
E7_D21_None.1	6,00	2,00	1,00	0,00	3,00	8,00	14,00	9,00	1,56
E7_D21_None.2	8,00	6,00	2,00	0,00	8,00	19,00	27,00	16,00	1,69
E7_D21_None.3	11,00	4,00	0,00	0,00	4,00	8,00	19,00	15,00	1,27
E7_D21_None.4	14,00	4,00	1,00	0,00	5,00	11,00	25,00	19,00	1,32
Média	9,75	4,00	1,00	0,00	5,00	11,50	21,25	14,75	1,46
Desvio Padrão	3,50	1,63	0,82	0,00	2,16	5,20	5,91	4,19	0,20

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