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Effects of antihypertensive drugs in the differentiation and activation of human bone cells

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EFFECTS OF ANTIHYPERTENSIVE DRUGS IN THE DIFFERENTIATION AND ACTIVATION OF HUMAN BONE CELLS

Dissertação submetida à Escola Superior de Tecnologia da Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Tecnologia Bioquímica em Saúde, realizada sob a orientação científica do Professor Doutor João Miguel Silva e Costa Rodrigues da Faculdade de Medicina Dentária da Universidade do Porto e co-orientação da Professora Doutora Cristina Prudêncio e Professor Ricardo Ferraz da Escola Superior de Tecnologia da Saúde do Porto.

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Resumo

O osso é um tecido metabolicamente ativo e a sua remodelação é importante para regular e manter a massa óssea. Esse processo envolve a reabsorção do material ósseo por ação dos osteoclastos e a síntese de novo material ósseo mediado pelos osteoblastos.

Vários estudos têm sugerido que a pressão arterial elevada está associada a alterações no metabolismo do cálcio, o que leva ao aumento da perda de cálcio e da remoção de cálcio do osso. Embora as alterações no metabolismo ósseo sejam um efeito adverso associado a alguns fármacos antihipertensores, o conhecimento em relação a este efeito terapêutico ligado com os bloqueadores de canais de cálcio é ainda muito escasso. Uma vez que os possíveis efeitos no osso podem ser atribuídos à ação antihipertensiva dessas moléculas, ou através de um efeito direto nas atividades metabólicas ósseas, torna-se necessário esclarecer este assunto.

Devido ao facto de que as alterações no metabolismo ósseo são um efeito adverso associado a alguns fármacos antihipertensores, o objetivo deste trabalho é avaliar o efeito que os bloqueadores dos canais de cálcio exercem sobre as células ósseas humanas, nomeadamente osteoclastos, osteoblastos e co-culturas de ambos os tipos celulares.

Verificou-se que os efeitos dos fármacos antihipertensores variaram consoante o fármaco testado e o sistema de cultura usado. Alguns fármacos revelaram a capacidade de estimular a osteoclastogénese e a osteoblastogénese em concentrações baixas. Independentemente da identidade do fármaco, concentrações elevadas revelaram ser prejudiciais para a resposta das células ósseas. Os mecanismos intracelulares através dos quais os efeitos foram exercidos foram igualmente afetados de forma diferencial pelos diferentes fármacos.

Em resumo, este trabalho demonstrou que os bloqueadores dos canais de cálcio utilizados possuem a capacidade de afetar direta- e indiretamente a resposta de células ósseas humanas, cultivadas isoladamente ou co-cultivadas. Este tipo de informação é crucial para compreender e prevenir os potenciais efeitos destes fármacos no tecido ósseo, e também para adequar e eventualmente melhorar a terapêutica antihipertensora de cada paciente.

Palavras-chave: metabolismo ósseo, osteoblastogénese, osteoclastogénese, fármacos antihipertensores, bloqueadores de canais de cálcio, co-culturas

Abstract

Bone is a metabolically active tissue and its remodeling is important to regulate and maintain bone mass. That process involves the resorption of bone material by action of osteoclasts, and synthesis of new bone material, mediated by osteoblasts.

Several studies have suggested that high blood pressure is associated with changes in the calcium metabolism, which leads to increased calcium loss and increased calcium removal from bone. Although changes in bone metabolism are one side-effect associated with some AHDs, the knowledge regarding this effect associated with calcium channel blockers therapeutics is still very scarce. Since possible bone effects can be attributed to the antihypertensive action of those molecules, or by a direct effect on bone metabolic activities, a clarification about this issue is of the utmost importance.

Due to the fact that changes in bone metabolism are one side-effect associated with some AHDs, the aim of this work is to assess the effects that calcium channel blockers exert on human bone cells, namely, osteoclasts, osteoblasts and co-cultures of both cell types.

It was observed that the effects of AHDs varied among the different tested molecules and the used cell culture system. Some drugs revealed the ability to stimulate osteoclastogenesis and osteoblastogenesis at low doses. Despite the identity of the molecule, high concentrations of AHDs were negative for the bone cells response. The intracellular mechanisms through which the effects were exerted were also differentially affected by the different AHDs.

In summary, this work demonstrated that calcium channel blockers have the ability to affect directly and indirectly the behavior of human bone cells, cultured isolated or co-cultured. This information is crucial to understand and prevent the potential effects of AHDs in bone tissue and also to adjust and eventually improve the antihypertensive therapy given to each patient.

Keywords: bone metabolism, osteoblastogenesis, osteoclastogenesis, antihypertensive drugs, calcium channel blockers, co-cultures

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Abbreviations

AHDs – antihypertensive drugs

ALP – alkaline phosphatase

BMP-2 – bone morphogenetic protein 2

CATK – cathepsin K

Cbfa 1 – core binding factor a1

CLSM – confocal laser scanning microscopy

COL 1 – collagen type 1

CTR – calcitonin receptor

EDTA – ethylenediamine tetraacetic acid

JNK – c-Jun N-terminal kinase

M-CSF – macrophage colony stimulating factor

MEK – methyl ethyl ketone

MTT – (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)

NF-kB – nuclear factor kappa B

OPG – osteoprotegerin

OSX – osterix

PBMC – peripheral blood mononucleated cells

PBS – phosphate-buffered saline

PKC – protein kinase C

*p*NPP – *para*-nitrophenilphoshate

RANK – receptor activator of nuclear factor kappa B

RANKL – receptor activator of nuclear factor kappa B ligand

TRAP – tartarate resistant acid phosphatase

VNR – vitronectin receptor

α-MEM – α minimal essential medium

Figure Index

Introduction

Bone is a characteristic tissue of all vertebrates, essential for the protection of inner organs and functioning as a support for body structures. It provides rigidity and shape, allows the cerebral and pulmonary function, locomotion, supports haematopoiesis in the bone marrow, fixation of the muscles and storage of minerals (calcium, phosphate), having the capacity to mobilize mineral reserves quickly, if necessary, in order to maintain calcium homeostasis (1,2). Bone is a metabolically active tissue, highly vascularized, able to adapt its structure to mechanical stimuli and to repair structural damage through the remodeling process. Bone remodeling is important to regulate and maintain bone mass, and involves the resorption of bone material by action of osteoclasts and synthesis of new bone material, mediated by osteoblasts (1–3). Thus, large changes in their structure due to injury or disease can alter the equilibrium between both activities in the body and, consequently, compromise the quality of life of individuals. The rate of bone turnover, structure and composition of the collagen matrix, size, structure, density and geometry combine to determine the general mechanical properties of bone. Defects in these parameters will result in pathologies. In order to ensure an adequate bone strength, the process of bone turnover should be regulated carefully (4,5).

Several studies have suggested that high blood pressure is associated with abnormalities in the calcium metabolism, which leads to increased calcium loss and increased calcium removal from bone. Since various antihypertensive agents are used for the treatment of hypertension, and despite the fact that it has not been fully clarified if the arterial hypertension is a risk factor for low bone mass and / or fractures, it is important to investigate the interference of these drugs on bone metabolism (6–9). Some categories of antihypertensive drugs (AHDs) have been reported to increase bone mineral density and reduce the risk of fractures (7,10). Though, the question is whether these molecules have a specific direct or indirect effect on bone tissue, due to the blood pressure decrease and the consequential effects on calcium metabolism associated with hypertension. In this context, it has been shown that people using certain AHDs have a lower fracture risk than the general population, which favors the hypothesis that AHDs may have a direct effect on bone cells (7,10–19).

Due to the high incidence of hypertension in the world and the high frequency of use of AHDs, particularly calcium channel blockers, it is necessary to study their influence on bone metabolism. Being bone disease very common and fractures a problem particularly relevant in the elderly, and given the incidence of hypertension, a detailed knowledge of the association of the two is imperative in order to improve the quality of life of patients.

CHAPTER I

State of Art

1.1 Bone

The bone is a specialized connective tissue with a biphasic structure consisting of an extracellular inorganic phase and an organic phase comprising bone cells and the extracellular organic matrix. The inorganic phase is mainly composed of calcium and phosphate, being the hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] form the most important one. It has also other ions in smaller quantities, such as silicon, for example (2,20). The inorganic phase has two major biological functions: the ability to resist to external and internal forces which the bone is subjected, preserving the shape of the body as a whole and protecting vital organs and it is also a reservoir of ions. The organic phase of bone matrix comprises 25-30% of the total bone mass (21,22) and performs a variety of functions, deeply influencing the structure and also the mechanical and biochemical properties of the tissue. The main structural component of the organic phase is collagen type I (COL1) (fibrous), representing about 90% of the proteins of the bone (2); the remaining 10% consist in a variety of smaller non-structural proteins, including osteonectin, osteocalcin, phosphoproteins, sialoprotein, growth factors and blood proteins (2,20).

As the load support structure of the body, the bone is formed from a combination of compact and cancellous bone, which is strengthened in pressure points (20,21). The compact bone is responsible for the stability of the skeleton, with a solid, essentially non-porous structure (3). It is present in long, short and flat bones and contains many units in the form of cylinders, called osteons (5,21). The osteocytes (bone cells) are found in small gaps between the concentric layers of the matrix, called lamellae. The matrix contains collagen fibers and mineral deposits, especially calcium and phosphate. In contrast, the cancellous bone is arranged in a porous pattern. It contains numerous bars and plates called trabeculae, which follow lines of stress. The cancellous bone ensures the elasticity and stability of the skeleton and contributes with about 70% of the total bone metabolism (2). This type of bone houses a large proportion of bone marrow and is essentially present in the metaphysis of long bones, vertebral and iliac crest (2,5,21).

1.2 Bone cells

The bone has three main types of cells: osteoblasts and osteoclasts, whose functions are closely linked, and osteocytes (23). The osteoblasts and osteoclasts work in a coordinated way, with their activities of bone formation and resorption, respectively, to

carry out the growth, repair and remodeling of bone tissue (5,24). In order to the balance formation and bone resorption in healthy individuals, osteoblasts secrete factors that regulate the differentiation of osteoclasts and osteocytes secrete factors that regulate the activity of both osteoblasts and osteoclasts (5,24,25). Bone responds to calcium-dependent signals from the parathyroid gland and via vitamin D metabolites. Osteoblasts deposit calcium phosphate crystals by mechanisms including phosphate and calcium transport associated with alkalinization, in order to neutralize the acid liberated by mineral deposition. Calcium mobilization by osteoclasts is mediated by acid secretion. Both bone forming and bone resorbing cells utilize calcium signals as regulators of differentiation and activity (26,27).

1.2.1 Osteoblasts

Osteoblasts descend from mesenchymal progenitor cells and have a major role in creating and maintaining the architecture of the skeleton. They are specialized mononuclear cells responsible for the deposition of bone matrix and for the regulation of the differentiation and function of osteoclasts (23,28). Histologically, the active osteoblasts appear as large cuboidal cells on the bone surface, with a visible prominent rough endoplasmic reticulum and Golgi complex. When active, they express high levels of alkaline phosphatase (ALP), which is thought to be important in the bone mineralization process (20,23,25,28). These cells express receptors for many hormones including 1,25-dihydroxyvitamin D, sex steroids and corticosteroids (20). In the process of osteoblasts maturation, several genes, such as core binding factor a1 (Cbfa1), also known as Runx2, and Osterix (OSX) play an important role (23). Besides these, the Wnt / β-catenin pathway plays a role not only in the osteoblasts differentiation, but also in their proliferation (1,2,4,20,24,25). After termination of the bone matrix synthesis, osteoblast cells may be subjected to cell death by apoptosis (programmed cell death) or differentiate into osteocytes or bone lining cells (23).

Osteoblasts are also a key element in the regulation of bone resorption by the expression of several modulators of osteoclastogenesis, in particular the receptor activator of nuclear factor kappa B (NF-kB) ligand (RANKL), which binds to its receptor RANK, expressed on the surface of pre-osteoclastic cells, inducing their differentiation and fusion. The importance of this RANKL-RANK interaction in the osteoclastogenic process is

highlighted by the fact that in combination with macrophage colony stimulating factor (M-CSF), it is sufficient to promote osteoclastogenesis *in vitro* (23). Some hormones, cytokines and humoral factors may affect calcium homeostasis and bone density by inducing the expression of RANKL inside the osteoblasts. Among the factors that activate bone remodeling, vitamin D is known to induce the increase of bone resorption and hypercalcemia in serum. The response of osteoblasts to vitamin D may vary depending on the stage of differentiation of these cells: immature cells respond in a pro-osteoclastic resorbing activity, evidenced by an increase in the expression of RANKL; on the other hand, mature cells are mainly associated with an osteogenic response. Both osteoblastic cellular behaviors can be regulated by circulating levels of vitamin D *in vivo* (26,27).

Moreover, osteoblasts secrete a dimeric glycoprotein (osteoprotegerin - OPG), which acts as a soluble decoy receptor for RANKL, blocking RANKL-RANK interaction and thereby inhibiting osteoclastogenesis and bone resorption (23).

Overall, the major osteoblastic markers include ALP, COL1, osteocalcin, bone sialoprotein, bone morphogenetic protein 2 (BMP-2), RANKL and OPG (5,23,25).

1.2.2 Osteocytes

The quiescent bone surfaces are covered by an almost continuous single layer of inactive flattened osteoblasts, which are found dispersed throughout the bone matrix, often referred as bone lining cells (5). The osteocytes are osteoblasts that became sequestered during bone matrix synthesis, being characterized by its inability to secrete osteoid. The osteocytes are the most abundant cells in the bone, comprising more than 90% of the bone cells (5). They communicate with each other and with the surroundings through extensions of their plasma membrane. The bone matrix isolates osteocytes from each other and in order to circumvent that situation, osteocytes interact with other osteocytes and bone cells by an elaborate network of dendritic processes, which run inside lacunar cannaliculi (5,20,23).

Despite their relative inactivity compared with osteoblasts, osteocytes play a central role in the determination and maintenance of bone structure. Those processes may have the potential to stimulate either bone resorption or synthesis. The osteocytes act as stress sensors, detect mechanical stress and respond to biochemical stimuli, instructing osteoclasts in bone resorption and osteoblasts in bone formation (5,20,23).

1.2.3 Osteoclasts

Osteoclasts are mobile large multinucleated cells that play an essential role in bone homeostasis. They descended from the monocyte / macrophage CD14+ lineage, responsible for the function of resorption of bone matrix (5,29,30). These cells are formed by fusion of mononuclear precursors derived from pluripotent hematopoietic stem cells and subsequent differentiation along the surface of the bone, a process designated osteoclastogenesis. These precursors are found not only in bone marrow, but also in the peripheral blood circulation (5,20,30–33). The osteoclasts form resorption sites, adhering firmly to the bone surface, where they secrete protons to dissolve the mineral phase and proteolytic enzymes, especially cathepsin K (CATK), and tartrate-resistant acid phosphatase (TRAP) to degrade the collagenous matrix (5,33). The mature osteoclasts express receptors for various signaling molecules, including calcitonin and prostaglandins (20).

The osteoclastogenesis is a complex process dependent on the action of various hormones, growth factors, and others, involving multiple intracellular signaling pathways, and is regulated by many other cytokines and by intracellular signals, including Ca^{2+} (30,32). Vitamin D induces osteoclast differentiation, which occurs mainly as a consequence of the binding of RANKL to RANK, expressed on the membrane of hematopoietic cells, in the presence of M-CSF. Osteoclasts act on bone remodeling, in addition to acting in resorption and release of calcium into the organism. Bone remodeling occurs continuously throughout life, and in order to maintain proper serum calcium concentrations, it is necessary to assure that the mechanism of bone resorption will be controlled, and vitamin D has an important role in that process (26,27,34).

The mature osteoclast is activated by signals, which leads to the beginning of bone remodeling. The osteoclast cell body is polarized, and in response to its activators, it undergoes internal structural changes that prepare the bone to be reabsorbed, such as actin cytoskeletal rearrangements and the formation of a tight junction between the surface of bone and the basement membrane, in order to create a sealed compartment. This external vacuole is then acidified by the export of hydrogen ions and the lytic enzymes TRAP and CATK are secreted to the resorption lacunae. Through this process the osteoclast erodes and degrades the underlying bone (5,29–33).

As noted above, there are two factors that play a central role in osteoclast development, whose are sufficient to promote osteoclastogenesis **in vitro**, the M-CSF and RANKL. The M-CSF is critical for the survival and proliferation of the progenitors of osteoclasts, while RANKL directly controls the differentiation process, interacting and activating RANK. The main osteoclastic markers include TRAP, CATK, carbonic anhydrase 2, vitronectin receptors (VNR), calcitonin receptors (CTR) and actin rings (5,29,31,33,35,36).

1.3 Bone remodeling

During the adult life, the skeleton suffers a continuous process of repair and renewal. Once formed, the bone is submitted to a process called bone remodeling, which involves resorption and synthesis of bone. This process occurs in micro scale along the skeleton, in the basic microscopic multicellular, in response to a variety of stimuli (2,4,20). Imbalances in the process of bone remodeling can result in serious disturbances in skeletal structure and function, potentially increasing morbidity and shortening life. Most bone diseases in adults are due to excessive osteoclast activity leading to an imbalance in the process that promotes resorption (1,2,4,20,24,37,38). As mentioned above, osteocytes have a central role in triggering and regulating bone remodeling. These cells detect microfissures, mechanical pressure and changes in the hormonal environment of bone and communicate with bone cells, which initiate bone resorption and formation (24).

A bone remodeling cycle consists of four distinct and sequential phases: activation, resorption, reversal and formation (25). During the activation phase, osteoclastic precursors are recruited from the bloodstream and bone marrow, adhere to bone and differentiate into active osteoclasts that begin the process of resorption. The action of osteoclasts is closely related to its interaction with bone matrix proteins, including osteopontin and bone sialoprotein, which were secreted by osteoblasts during the previous cycle of bone formation (25). When resorption is complete, the reversal phase begins: the osteoclasts secrete signals that promote migration and differentiation of osteoblastic precursors, and die by apoptosis. Osteoblastic precursors proliferate locally and differentiate into mature osteoblasts. They migrate to the resorption lacunae formed by the osteoclasts. The formation phase continues with the synthesis of new bone material by osteoblasts until the resorbed tissue is completely replaced (1,2,4,20,24,38).

1.4 Hypertension

Arterial hypertension is a major risk factor for coronary heart disease, heart failure, stroke and nephroangiosclerosis. Adopting a healthy lifestyle can prevent the onset and the development of the disease and its risk of incidence can be reduced by early detection and monitoring. The arterial hypertension corresponds to all situations where it is present increased values of blood pressure. Thus, values of systolic blood pressure greater than or equal to 140 mm Hg (millimeters of mercury) and / or diastolic blood pressure values above 90 mmHg are considered for the characterization of this condition (39). In most cases, there is no known cause for the arterial hypertension, although in some situations it is possible to identify an associated disease which is the true cause of the hypertension. Generally, the older the person is, the greater the probability of developing hypertension (39,40).

Presently, it is thought that the worldwide prevalence of hypertension in the adult population is about 25 to 30% (about 1000 million people), increasing in individuals aged over 60 years, and accounting for about 7.6 million deaths per year and 7 million premature deaths. Directly associated with high blood pressure are several risk factors, such as age, race, obesity, tobacco use, excessive alcohol or a sedentary lifestyle. In turn, the arterial hypertension appears as a modifiable risk factor associated with cardiovascular disease (41).

In Portugal, there are approximately two million of hypertensive people. Of these, only half is aware of having high blood pressure, only a quarter are medicated and only 16% have the situation under control (39).

In this context, antihypertensive molecules represent an important class of drugs for the prevention of the high morbidity and mortality associated with arterial hypertension. Currently there are many drugs effective in reducing the blood pressure (39,40).

1.5 Antihypertensive drugs

Adopting healthy lifestyles is an important factor for the decrease of blood pressure values. However, these changes are not always enough, and therefore the use of AHDs is often inevitable. The aim of antihypertensive therapy is to make the diastolic pressure to remain below 90 mmHg, without compromising renal, myocardial or cerebral function, without producing adverse reactions (42).

The AHDs can be classified, according to its main mechanism of action, in six major groups: diuretics, angiotensin renin modifiers, calcium channel blockers, depressants of adrenergic activity, direct vasodilators and others (42).

After studying the possible causes and factors of comorbidity, treatment of a hypertension should start with a single drug in low initial dose, which may be progressively increased until obtaining the desired effect or the appearance of adverse reactions. The initial choice of an AHDs should fall on one considered as a first line drug. First-line therapy refers to the initial or first treatment given when someone is diagnosed with a particular disease or condition. It is considered the most effective treatment. If it is needed to associate two drugs, the association still must focus, when possible, on two first line molecules. The individual situation of the patient influences the choice of the appropriate AHD (42).

1.5.1 Calcium channel blockers

The calcium channel blockers are considered one of the first line AHDs. Metabolic significant adverse effects do not occur with their use, especially in the lipid profile. These drugs act preventing the entry of calcium into cells, inhibiting the flow of calcium into the cardiac smooth muscle cells and competitively blocking the entry of calcium through the slower channels present in the cytoplasmic membranes (43). Their main actions are conducted in smooth cells of blood vessels (by inhibiting the contraction of vascular smooth muscle), cardiac cells (inhibition of cardiac conduction, particularly in the sinoatrial and atrioventricular nodes, where conduction is dependent calcium flux) and hemodynamics (42–44).

There are essentially three types of calcium channel blocker drugs, which present pharmacodynamic differences with significant clinical translation: dihydropyridines, fenilalquilamines and benzothiazepines. The dihydropyridines have higher affinity to the vessels resulting in systemic and coronary vasodilation, the fenilalquilamines decrease heart rate and atrioventricular conduction and benzothiazepines are in an intermediate position between them (42–44).

1.6 Antihypertensive drugs and bone metabolism

Several changes in calcium metabolism have been reported in hypertension. The final effect of such changes is an alteration of bone mass and bone quality. A decrease of bone mineral density is related to hypertension. In fact, it appears as one of the major cardiovascular risk factors for fractures (10,45–47). AHDs can have an indirect impact on osteoporosis by improving the negative effects of high blood pressure as well as direct effects on bone metabolism, strength, and density. Calcium has a central role in bone strength and in the balance between osteoblastic and osteoclastic activities, the basis of the bone remodeling process. (10).

Previous studies demonstrate an increase in bone mineral density and reduced fracture risk in patients taking antihypertensive diuretics (9,11). These may act directly on the cells involved in bone metabolism or indirectly by decreasing calcium excretion in urine. In the kidney, diuretics block sodium and chloride resorption. This effect causes an exchange of sodium and calcium, by promoting calcium influx and efflux of sodium (7–9,11,12).

The antihypertensive beta-blockers exert an effect on bone remodeling through the sympathetic nervous system. Most studies have shown a decreased risk of fracture, but there are also studies that show the existence of a neuronal network initiated by leptin that inhibits osteoblast activity and increases the function of osteoclasts (8,9). Also, contradictory results have been reported about the effects on bone mineral density (7–9).

Angiotensin II is able to act on cells involved in bone metabolism, indirectly by controlling the flow on capillaries in the bone marrow or directly via receptors located in osteoblasts, which promote the release of mediators that in turn activate osteoclastic cells (9,15). At the level of osteoblasts, it stimulates DNA synthesis, cell proliferation, collagen synthesis by the precursors of the osteoblasts and decreases the mineralization promoted by mature osteoblasts (15). The use of inhibitors of angiotensin-converting enzyme has been associated with a decreased risk of fracture or improving bone metabolism (7–9,15,16).

1.6.1 *In vitro* studies using calcium channel blockers

Results of *in vitro* studies have shown that calcium channel blockers may also affect bone cells (8), but few data are currently available about this issue. Calcium channel blockers inhibit calcium influx through calcium channel L-type voltage-dependent at the

level of vascular smooth muscle, thus interrupting the contraction process. As mentioned above, during their differentiation, osteoblasts express ALP, COL1 and other bone matrix proteins, forming a mineralized bone matrix. During the process, osteoblasts rely in come mechanisms that involve the action of voltage-dependent calcium channels (6). In addition, several bone regulatory factors, such as vitamin D3, parathyroid hormone and prostaglandin E2 cause an increase in the concentration of intracellular calcium, part of which is decreased by dihydropyridine, one type of calcium channel blocker (6). These factors also promote or inhibit differentiation of osteoblasts. Therefore, it is noted that signaling through calcium channel L-type may be important for the function of osteoblasts (6–9). With respect to osteoclasts, the molecular and cellular effect of calcium channel blockers remains unclear.

The effect of calcium channel blockers on bone metabolism was examined in the 1990s in a few studies with a small number of experimental subjects ($10 \leq n \leq 20$) (10). The collected data have not revealed any considerable effect of calcium antagonists on bone metabolism. However, in view of the fact that the number of experimental subjects was small, it is not possible to exclude an effect of calcium channel blockers on the indicators of bone tissue homeostasis. Regarding this, one of the assumptions is that long-term use of calcium channel blockers can be associated with osteoporosis, due to a decline of bone marrow perfusion, observed in healthy volunteers (10,19). On the other hand, no evidence was found regarding an altered fracture risk in users of calcium channel blockers, in a case–control fracture study (10,18). However, in a pharmacoepidemiological study, conducted in the general population with a larger number of experimental subjects, calcium channel blockers reduced the risk of fractures by 6 % (adjusted OR = 0.94, 95 % CI 0.91–0.96). The decreased risk was higher with the use of calcium channel blockers that are not related to dihydropyridine (10,17).

Some studies addressed the effect of amlodipine on bone metabolism in male albino Wistar rats (13) and the effects of different amlodipine and lacidipine doses on ovariectomized rat femurs' calcium and phosphor content (14). In the first one, exerted an effect on bone metabolism. The results indicate that amlodipine treatment in the long-term administration of amlodipine at a dose of 0.3 mg/100 g body weight may induce deceleration of bone metabolism. The expression of BMP-2 in proximal tibia was investigated by means of immunoblotting, and bone mineral density was measured by Dual-energy X-ray Absorptiometry in lumbar and caudal vertebrae and in femoral areas.

Mechanical properties of the femurs were measured by three-point bending of the shaft and compression testing of the femoral neck. The experiment was performed on outbred rats (13). The second one highlighted the potential beneficial effects of calcium channel blockers in the prevention of bone loss induced by ovariectomy. For that, the study investigated the effects of different doses of amlodipine and lacidipine on ovariectomized rat femurs' calcium and phosphor content. These drugs decreased the bone loss in an ovariectomy-induced osteopenic rat model. Bone calcium and phosphor concentration was measured by a Wavelength Dispersive Spectrometer. The findings in this study suggest that potent calcium channel blockers, such as amlodipine and lacidipine, may have a beneficial effect on bone metabolism, in addition to their well-established antihypertensive effect (14).

Due to the scarcity of the available information, the effects of calcium channel blockers on bone can not be ruled out. Furthermore, the data available was essentially obtained by clinical studies, without information regarding the molecular and cellular processes that are the basis of bone metabolism. Finally, the published reports present some limitations that further impair the establishment of precise considerations about this issue. Namely, the experimental subjects were heterogeneous in terms of patient age, sex, comorbidities, and ethnic origin. In addition, the effect of a particularly antihypertensive on bone may depend on the time of their use. Finally, the articles reviewed included different types of studies ranging from randomized clinical trials to pharmacoepidemiological studies (10).

Undoubtedly, more research is required in order to characterize the possible positive or negative effects of calcium channel blockers on bone metabolism.

On the basis of the previous considerations, the plan of the present project was to study the effects of antihypertensive calcium channel blockers (amlodipine, felodipine, diltiazem, lercanidipine and nifedipine) in human bone cells and it has focused on the influence of some factors on osteoclastogenic and osteoblastogenic process. It aimed to characterize how AHDs affect the differentiation and function of cultured human osteoblastic and osteoclastic cells, cultured isolated and co-cultured. Finally, it was also intended to analyze the influence of several signaling pathways important for osteoclastogenesis and osteoblastogenesis in the cellular responses to the tested drugs. For that, the minimum concentration of each AHD that elicited a significant effect on analyzed parameters was chosen.

CHAPTER II

Material and Methods

2.1 Cell Cultures

2.1.1 Osteoclast cell cultures

Peripheral blood mononuclear cells (PBMC), used as osteoclast precursor cells, were isolated from blood of 25-35 years old healthy male donors, after informed consent. After dilution with phosphate-buffered saline (PBS) (2:1), blood was applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences). Samples were centrifuged at 400 g for 30 minutes and PBMC were collected at the interface between Ficoll-Paque and PBS. Cells were washed twice with PBS. On average, for each 90 mL of processed blood about $450x10^6$ PBMC were obtained. PBMC, seeded at $5x10^5$ cells/cm^2, were maintained in α-minimal essential medium (α-MEM) supplemented with 30% human serum (from the same donor from which PBMC were collected), 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine, and 25 ng/mL M-CSF and 40 ng/mL RANKL (osteoclastogenic enhancers, (29,33,35,36)). Cell cultures were maintained in the absence (control) or in the presence of 10^{-8}-10^{-4} M of different AHDs (which include plasma concentration) and they were characterized throughout a 21 day period, at days 7, 14 and 21, for TRAP activity, total protein content, number of TRAP+ multinucleated cells, presence of cells with actin rings and expressing vitronectin and calcitonin receptors and apoptosis rate. Following, PBMC were treated with the minimum concentration of each AHD that elicited a significant effect on those parameters, and were further characterized for the expression of osteoclast-related genes, calcium phosphate resorbing ability and for the involvement of some osteoclastogenesis-related signalling pathways on cellular response. The AHDs tested - amlodipine, felodipine, diltiazem, lercanidipine and nifedipine - were renewed at each medium change, once a week. Cell cultures were incubated in a 5% CO_2 humidified atmosphere at 37ºC.

2.1.2 Osteoblast cell cultures

Osteoblasts were obtained from femur heads of patients (25-45 years old) undergoing orthopaedic surgery procedures, after informed consent. Briefly, bone was broken in small pieces, which were maintained in α-MEM supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 μg/mL ascorbic acid. After reaching about 70% of confluence, cells were detached with

0.05% trypsin and 0.5 mM ethylenediamine tetraacetic acid (EDTA), and were seeded at 10^4 cells/cm^2. Cell cultures, performed in the same culture medium mentioned above, were treated with 10 mM β-glycerophosphate and were maintained in the absence (control) or in the presence of 10^{-8}-10^{-4} M (which include plasma concentration) of different AHDs. Cells were characterized throughout a 21 day period, at days 7, 14 and 21, for cellular proliferation/viability, ALP activity, total protein content, histochemical staining of ALP and phosphate deposits, staining of actin and nuclei and apoptosis rate. Following, osteoblasts were treated with the minimum concentration of each AHD that elicited a significant effect on those parameters, and were further characterized for the expression of osteoblast-related genes and for the involvement of some osteoblastogenesis-related signalling pathways on cellular response. Cultures were incubated at 37ºC in a 5% CO_2 humidified atmosphere and the culture medium was replaced once a week.

2.1.3 Co-cultures of osteoblasts and osteoclasts

Osteoblasts were seeded at 10^3 cells/cm^2 and were incubated for 24h in α-MEM, supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 50 μg/ml ascorbic acid. After that, PBMC were added at $5x10^5$ cells/cm^2 to the osteoblasts and co-cultures were maintained in α-MEM supplemented with 20% human serum (from the same donor where PBMC were obtained), 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B and 2 mM L-glutamine. Cell cultures were maintained in the absence (control) or in the presence of 10^{-8}-10^{-4} M (which include plasma concentration) of different AHDs and they were characterized throughout a 21 day period, at days 7, 14 and 21. Cellular response was assessed through the same osteoclast and osteoblast parameters mentioned above for the corresponding isolated cell cultures. Cultures were maintained in a 5% CO_2 humidified atmosphere at 37ºC for 21 days. Culture medium was replaced once a week and the AHDs were renewed at each medium change.

2.2 Characterization of cell cultures

2.2.1 Total protein content

Cellular protein content was quantified by Bradford's method (48). Cell cultures were washed twice with PBS and solubilized with 0.1 M NaOH. After addition of Coomassie® Protein Assay reagent (Fluka), the samples were homogeneized and the absorbance was quantified at 595 nm in an ELISA plate reader (Synergy HT, Biotek). Results were expressed as mg/mL.

2.2.2 TRAP activity quantification

TRAP activity was assayed by *para*-nitrophenilphosphate (*p*NPP) hydrolysis method (22,49–52). Cell cultures were washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100. Cellular extracts were incubated with 22.5 mM *p*NPP in 0.225 M sodium acetate, 0.3375 M KCl, 0.1% Tx-100, 22.5 mM sodium tartarate and 0,225 mM iron chloride (pH = 5.8) for 1 hour at 37ºC. After stopping the reaction with 5 M NaOH, the absorbance of the samples was measured at 400 nm in an ELISA plate reader (Synergy HT, Biotek). Results were normalized with the corresponding total protein content values, and were expressed as nmol/min/$\mu g_{protein}$.

2.2.3 ALP activity quantification

ALP activity was assayed by *p*NPP hydrolysis method (52). Cell cultures were washed twice with PBS and solubilized with 0.1% (V/V) Triton X-100. Cellular extracts were incubated with 22.5 mM *p*NPP in 0.15 M bicarbonate buffer (pH 10.3) for 1 hour at 37ºC. After stopping the reaction with 5 M NaOH, the absorbance of the samples was measured at 400 nm in an ELISA plate reader (Synergy HT, Biotek). Results were normalized with the corresponding total protein content values, and were expressed as nmol/min/$\mu g_{protein}$.

2.2.4 Cellular proliferation/viability

The cellular proliferation/viability assessment was performed by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Cell cultures were incubated with 5mg/mL MTT, for 3 hours at 37ºC. After incubation, the culture medium was removed and dimethyl sulfoxide (DMSO) was added. The absorbance was quantified at 550 nm in an ELISA plate reader (Synergy HT, Biotek).

2.2.5 Histochemical staining of TRAP

Cell cultures were fixed with 3.7% formaldehyde for 15 minutes and then washed with distilled water. The cells were stained for TRAP with Acid Phosphatase, Leukocyte kit (Sigma), according to manufacturer's instructions. Shortly, after fixation the cells were incubated with 0.12 mg/mL naphtol AS-BI, in the presence of 6.76 nM tartrate and 0.14 mg/mL Fast Garnet GBC, for 1 hour at 37ºC in the dark. Cell layers were washed and stained with hematoxilin. After being washed with water, cells were visualized by light microscopy. Cells multinucleated and positive for TRAP were counted (Nikon TMS phase contrast microscope).

2.2.6 Histochemical staining of ALP

Cell cultures were histochemically stained for the activity of ALP. The method used was based on the hydrolysis of α-naphtyl phospate by ALP and the precipitation of phosphate liberated by reaction with a salt, giving rise to the formation of a coloured product. The cell cultures were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer for 20 minutes and then washed with cacodylate buffer. Then, samples were incubated at 37ºC in the dark for 1 hr in 0.1 M Tris buffer, pH 10 containing 2 mg / ml Na-α-naphtyl phospatase and 2 mg / ml of Fast blue RR salt. The reaction was stopped by rinsing the samples with distilled water. A positive reaction is identified by the presence of a brown to black colour, in accordance with the amount of the enzyme. Cells were visualized under a Nikon TMS phase contrast microscope.

2.2.7 Histochemical staining for the presence of phosphate deposits

Phosphate deposits present in the matrix were identified by the method of von Kossa. After fixation with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer, the cultures were washed with cacodylate buffer and were overlaid with a solution of silver nitrate 1% and subjected to UV radiation for 1 h (UV lamp, Vilber Lourmat , VL model 4L). After washing with distilled water, cells were incubated with a solution of sodium thiosulfate 5% for 2 min, and washed again with distilled water. The positive reaction is reflected by the appearance of black deposits in the cell layer. Cells were visualized under a Nikon TMS phase contrast microscope.

2.2.8 Staining of actin, VNR, CTR and nuclei and visualization by confocal laser scanning microscopy (CLSM)

Cell cultures were washed twice with PBS and fixed with 3.7% *para*-formaldehyde for 15 minutes. Cells were permeabilized with 0.1% (V/V) Triton X-100 for 5 minutes and stained for F-actin with 5 U/mL Alexa Fluor® 647-Phalloidin (Invitrogen), for VNR and CTR with 50 μg/mL mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems), respectively, and for nuclei with 500 nM propidium iodide, in the case of osteoblasts. Anti-VNR and anti-CTR detection was performed with 2 μg/mL Alexa Fluor® 488-Goat anti-mouse IgGs. Cultures were visualized by CLSM (Leica TCP SP2 AOBS confocal microscope).

2.2.9 Apoptosis quantification

Apoptosis was quantified by measuring the caspase-3 activity. For that, cell cultures were washed twice with PBS and assessed for caspase-3 activity with the EnzCheck® Caspase-3 Assay Kit #2 (Molecular Probes), according to manufacturer's instructions. Fluorescence was analysed at 496/520 nm (excitation/emission) in an ELISA plate reader (Synergy HT, Biotek). Results were presented as a % of activity (normalized with the corresponding total protein content value), compared to the control.

2.2.10 Calcium phosphate resorbing ability

Control and AHDs-treated osteoclast cell cultures and co-cultures preformed on calcium phosphate coated culture plates (BD BioCoat™ Osteologic™ Bone Cell Culture Plates, BD Biosciences). After 21 days of culture, cells were bleached with 6% NaOCl and 5.2% NaCl, and the remaining calcium phosphate layers were visualized by phase contrast light microscopy (Nikon TMS phase contrast microscope). Resorption lacunae were identified and total resorbed area was quantified with ImageJ 1.41software.

2.2.11 RT-PCR analysis

Total RNA from cell cultures was extracted and isolated with RNeasy® Mini Kit (QIAGEN) according to manufacturer's instructions. RNA was quantified by measuring the absorbance of the samples at 260 nm and by calculating the $A_{260nm}/A_{280\ nm}$ ratio, respectively. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and COL I by osteoblasts, GAPDH and CATK by PBMC and GAPDH, COL I and CATK by co-cultures, was assessed by RT-PCR. RNA, 0.5 mg, was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55ºC.The primers used are listed on table 1. RT-PCR products were electrophoretically separated on a 1% (w/v) agarose gel and gel band intensities were quantified with ImageJ 1.41 software. Values were considered as a percentage of the corresponding GAPDH value of each experimental condition.

Table 1: Primers used for characterization of cell cultures

Gene	5' Primer	3' Primer
GADP	5'-CAGGACCAGGTTCACCAACAAGT-3'	5'-GTGGCAGTGATGGCATGGACTGT-3'
CATK	5'-AGGTTCTGCTGCTACCTGTGGTGAG-3'	5'-CTTGCATCAATGGCCACAGAGACAG-3'
COL1	5'-TCCGGCTCCTGCTCCTCTTA-3'	5'-ACCAGCAGGACCAGCATCTC-3'

2.2.12 Assessment of the intracellular mechanisms involved in the osteoclastogenic and the osteoblastogenic cell response

To evaluate the influence of several signaling pathways, involved in the osteoclastogenic and in the osteoblastogenic processes, cell cultures were supplemented with different specific inhibitors of signalling pathways, namely: U0126 1 μM (methyl ethyl ketone (MEK) inhibitor), PDTC 10 μM (NFkB inhibitor), GO6983 5 μM (protein kinase C (PKC) inhibitor) and SP600125 10 μM (c-Jun N-terminal kinase (JNK) inhibitor) (52). Cell cultures were assessed for total protein content and for TRAP and ALP activities.

2.3 Statistical analysis

Data presented in this work are the means of separate experiments performed with cells from different blood donors. Three replicas of each condition were made for each experiment and data are expressed as the mean ± standard deviation. Data were evaluated using a two-way analysis of variance (ANOVA) and no significant differences in the pattern of the cell behavior were observed. Statistical differences found between control and experimental conditions were determined by Bonferroni's method. For values of $p \leq 0.05$, differences were considered statistical significant.

CHAPTER III

Results

3.1 PBMC

3.1.1 TRAP activity quantification

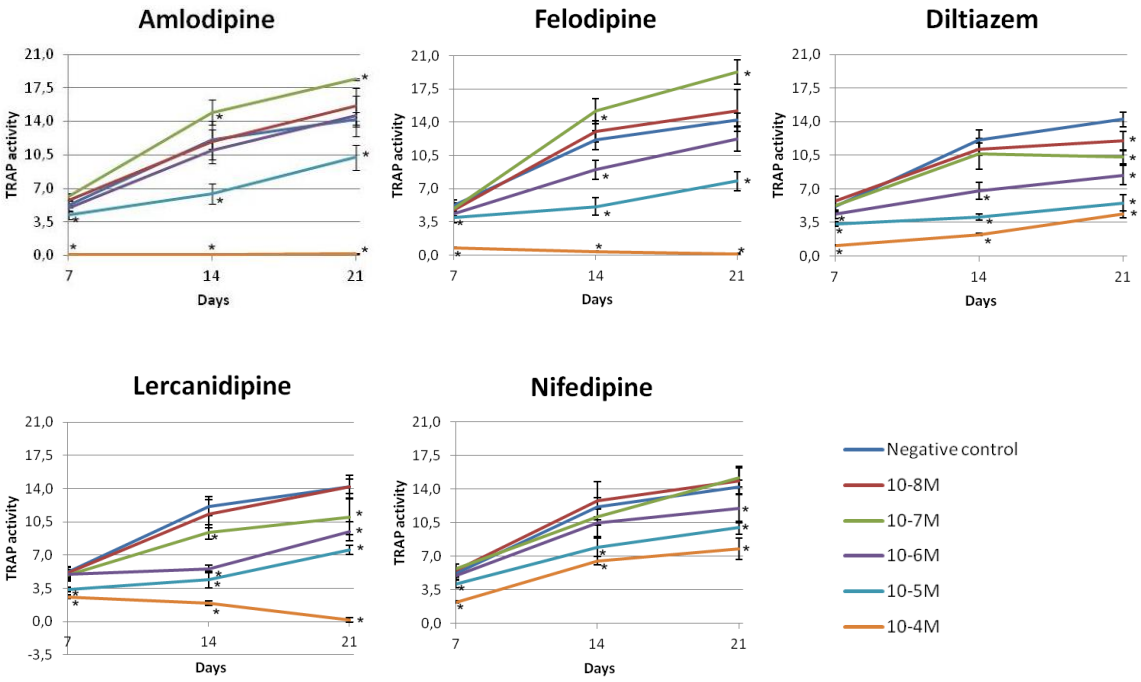


Figure 3.1: TRAP activity of PBMC cultures maintained in the presence of recombinant M-CSF and RANKL and treated with the AHDs.

Figure 3.1 shows the TRAP activity of PBMC cultures maintained in the presence of recombinant M-CSF and RANKL, in the absence (control) or supplemented with different AHDs, cultured for 7, 14 and 21 days. At control conditions, TRAP activity increased until day 14 and stabilized from days 14 to 21. The tested AHDs revealed different abilities to modulate osteoclastogenesis. Supplementation with low doses of amlodipine and felodipine resulted in a significant increase of TRAP activity. The maximum response was achieved with 10^{-7} M, with an increase of about 29% and 35.5% for amlodipine and felodipine, respectively. At concentrations higher than 10^{-6} M, both molecules elicited a decrease on osteoclastic behavior. Compared to the control, the presence of diltiazem, lercanidipine and nifedipine resulted in a dose-dependent decrease of TRAP activity, which became statistically significant (16%, 23% and 16%) for concentrations higher than 10^{-8}, 10^{-7} and 10^{-6} M, respectively.

3.1.2 Histochemical staining of TRAP

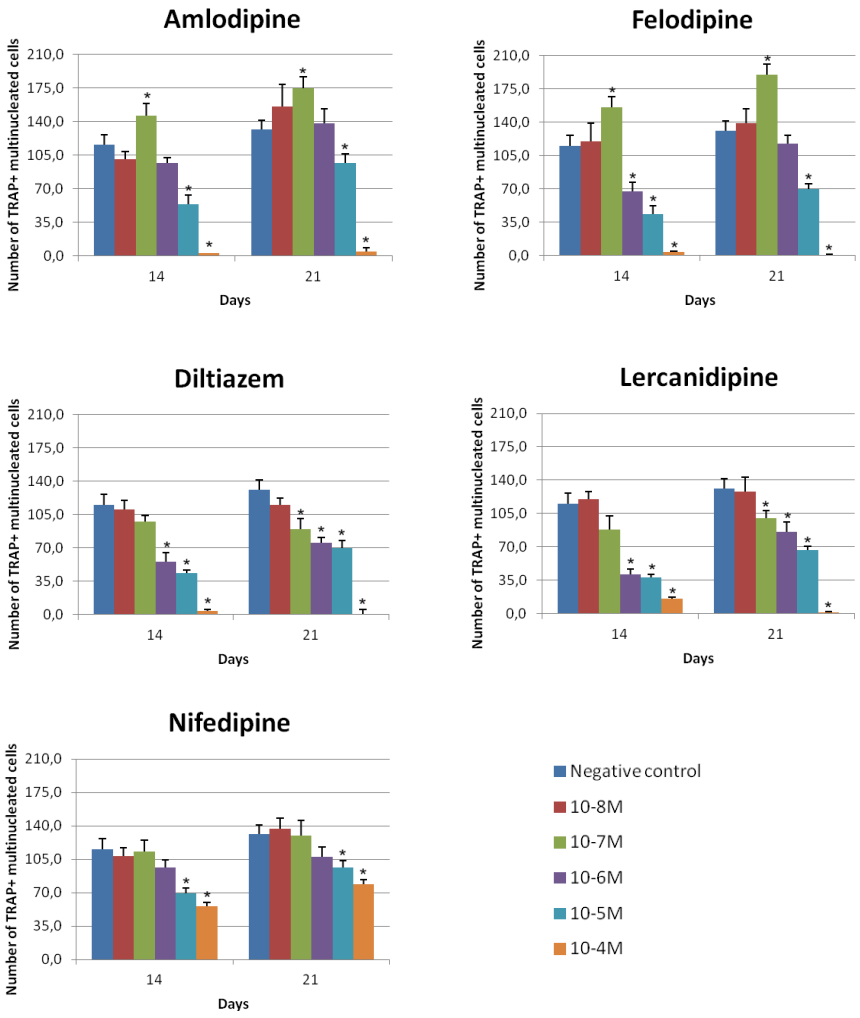


Figure 3. 2: Number of TRAP+ multinucleated cells on PBMC cultures maintained in the presence of recombinant M-CSF and RANKL and treated with the AHDs.

Figure 3.2 shows that, after 14 and 21 days of culture, the profile observed for the number of TRAP-positive multinucleated cells in PBMC cultures followed a pattern similar to that observed in TRAP activity, either in control conditions or in the presence of AHDs. Shortly, supplementation with the AHDs resulted in an increase of number of TRAP-positive multinucleated cells at low doses, in the case of amlodipine and felodipine (25% and 31%, respectively).The presence of the remaining AHDs resulted in a dose-dependent decrease of number of TRAP-positive multinucleated cells.

3.1.3 Staining of actin, VNR, CTR and visualization by CLSM

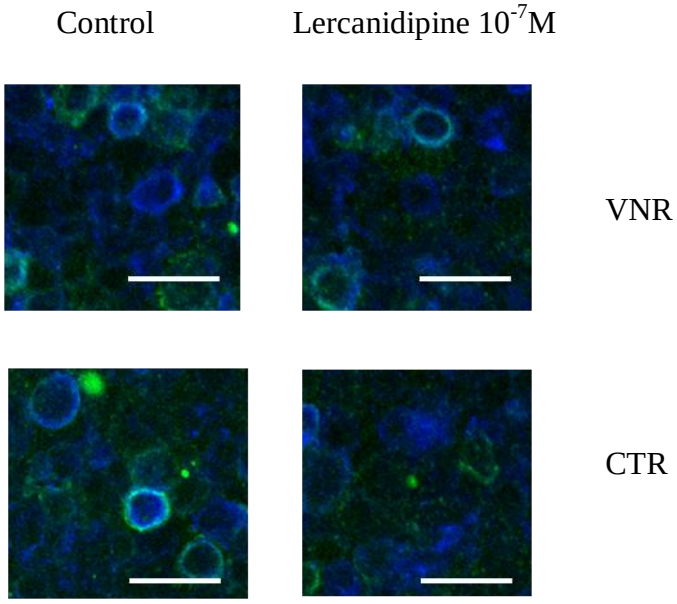


Figure 3.3: Presence of multinucleated cells displaying actin rings and VNR or CTR in PBMC cultures assessed by CSLM. Fluorescence images showing actin rings (blue) and VNR or CTR (green). White bars represent 120 µm.

PBMC cultures were stained for actin and for VNR and CTR and visualized by CLSM (figure 3.3). It was possible to visualize cells displaying the analyzed osteoclast-features, which confirmed the presence of osteoclasts in the tested conditions. The amount of osteoclastic cells in the different conditions was somehow correlated with the qualitative pattern observed for TRAP activity.

3.1.4 Apoptosis quantification

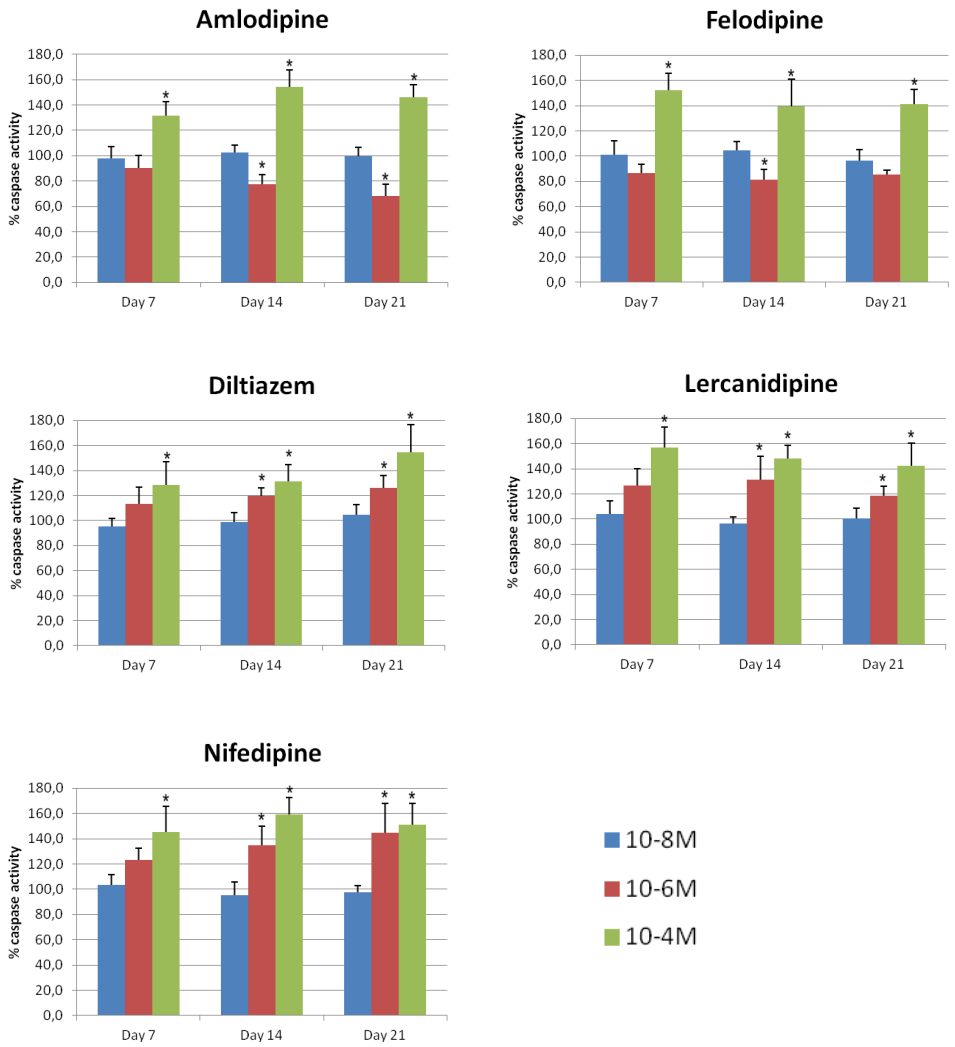


Figure 3.4: Caspase-3 activity on PBMC cultures

Supplementation with amlodipine and felodipine revealed a decrease of caspase-3 activity at concentration 10^{-6} M and an increase of caspase activity at dose 10^{-4} M, compared to concentration 10^{-8} M (figure 3.7). The other drugs elicited a dose-dependent increase of caspase activity.

3.1.5 Calcium phosphate resorbing ability

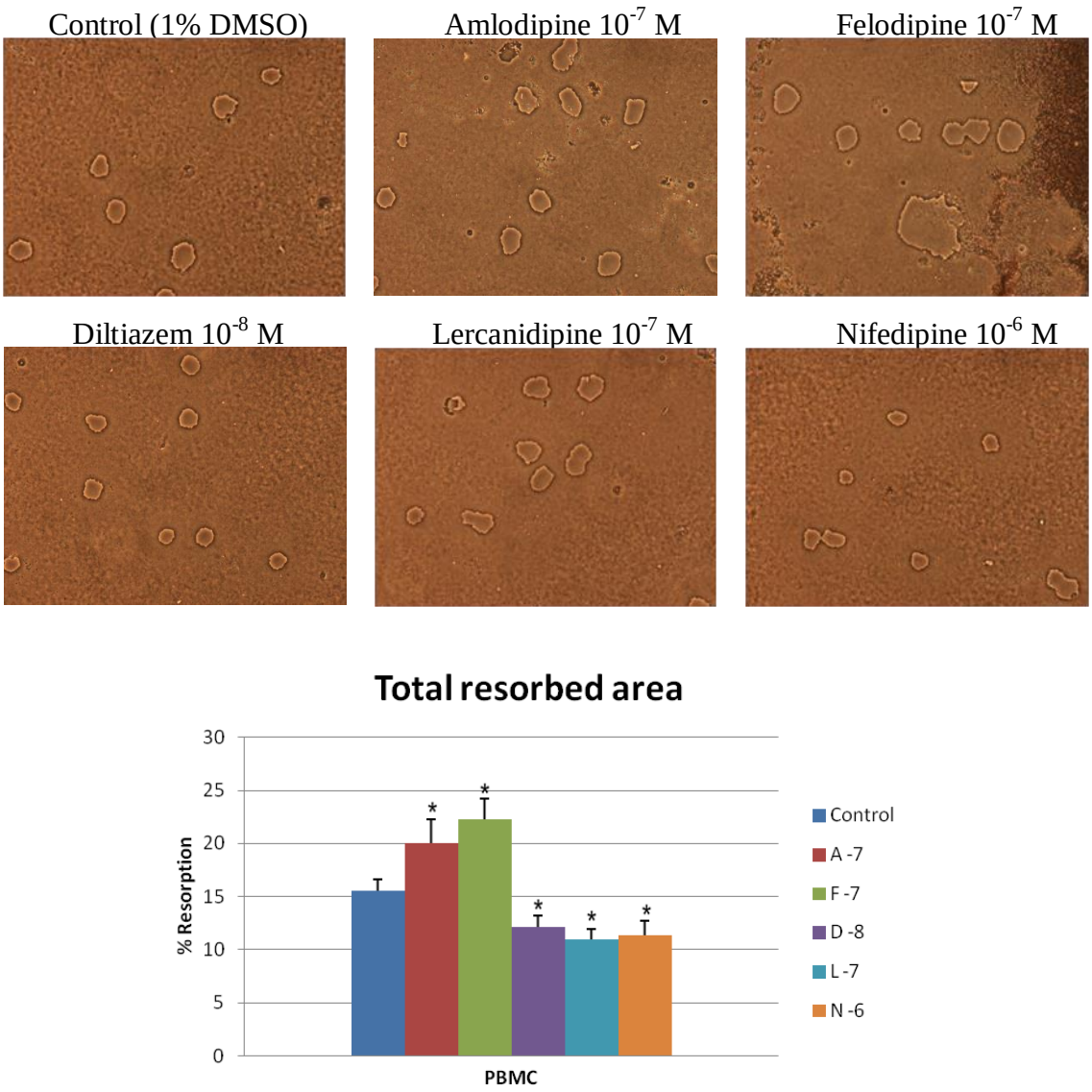


Figure 3.5: Calcium phosphate resorbing ability and total resorbed area of PBMC cultures mantained in the presence of recombinant M-CSF and RANKL and treated with the AHDs.

In figure 3.5, it was observed that the presence of amlodipine and felodipine induced an increase of about 28.5% and 43%, respectively, on the total resorbed area. On the other hand, the other drugs elicited a decrease on the resorbing activity (about 22%, 30% and 27% lower than the control, for diltiazem, lercanidipine and nifedipine, respectively).

3.1.6 Assessment of the intracellular mechanisms involved in the osteoclastogenic cell response

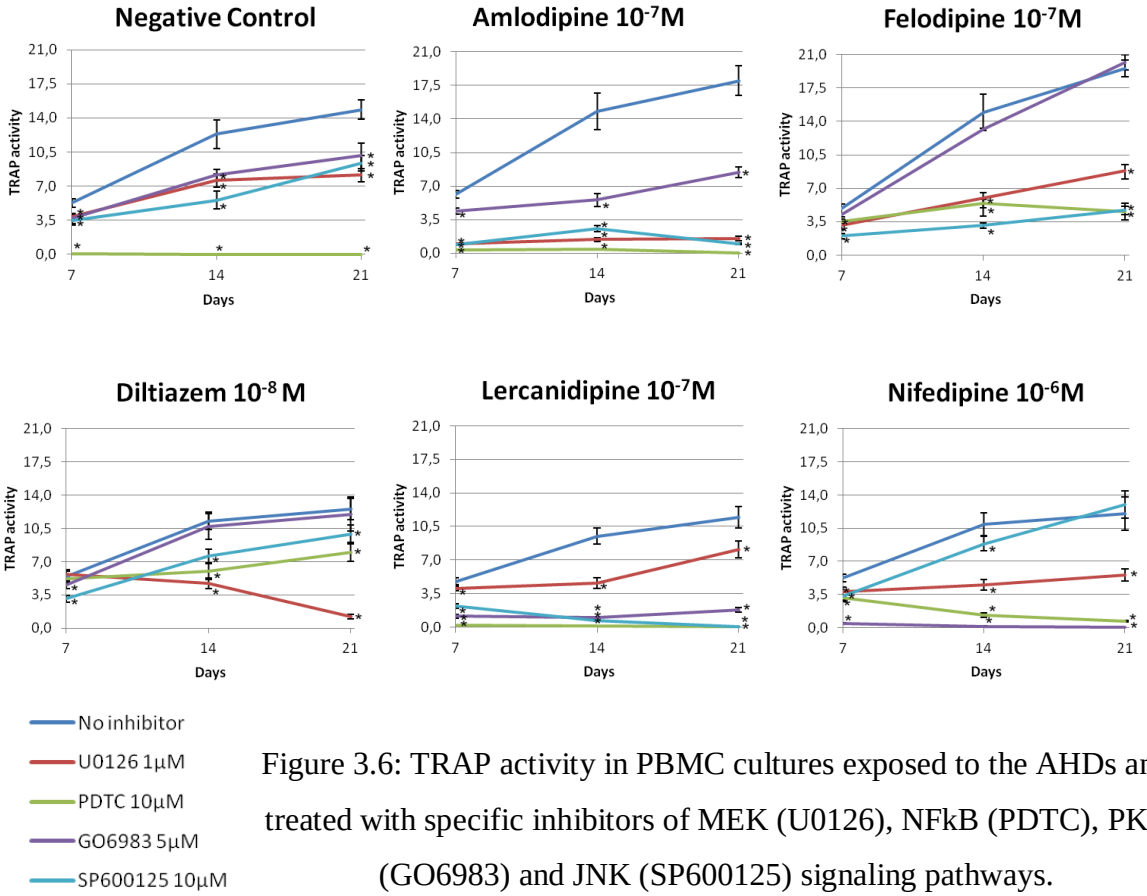


Figure 3.6: TRAP activity in PBMC cultures exposed to the AHDs and treated with specific inhibitors of MEK (U0126), NFkB (PDTC), PKC (GO6983) and JNK (SP600125) signaling pathways.

PBMC cultures were treated with specific inhibitors of several signaling pathways involved in the osteoclastic differentiation and function; TRAP activity measured at days 7, 14 and 21 is presented in figure 3.6.

In control PBMC cultures, NFkB appears to be particularly important, as the presence of PDTC practically abolished the production of TRAP. U0126, GO6983 and SP600125 induced a partial decrease in the production of TRAP. In general, the presence of the AHDs caused some significant changes in the described behavior. A higher inhibition in TRAP activity than the one achieved in the control was seen in the presence of U0126, in cell cultures treated with amlodipine, felodipine and diltiazem. In the presence of PDTC, it was observed a lower inhibition in TRAP activity, compared to the control, especially with felodipine and diltiazem. The presence of GO6983 did not affect the cellular response in cell cultures treated with felodipine and diltiazem, while in the presence of the remaining AHDs it elicited a decrease on TRAP activity higher than the

one observed in the control. Osteoclastic behavior of cell cultures supplemented with diltiazem and nifedipine were not significantly affected by SP600125. On the other hand, the presence of this molecule caused a sharp decrease on TRAP cellular response on PBMC cultures performed in the presence of amlodipine, felodipine and lercanidipine.

3.2 Osteoblasts

3.2.1 Cellular proliferation/viability

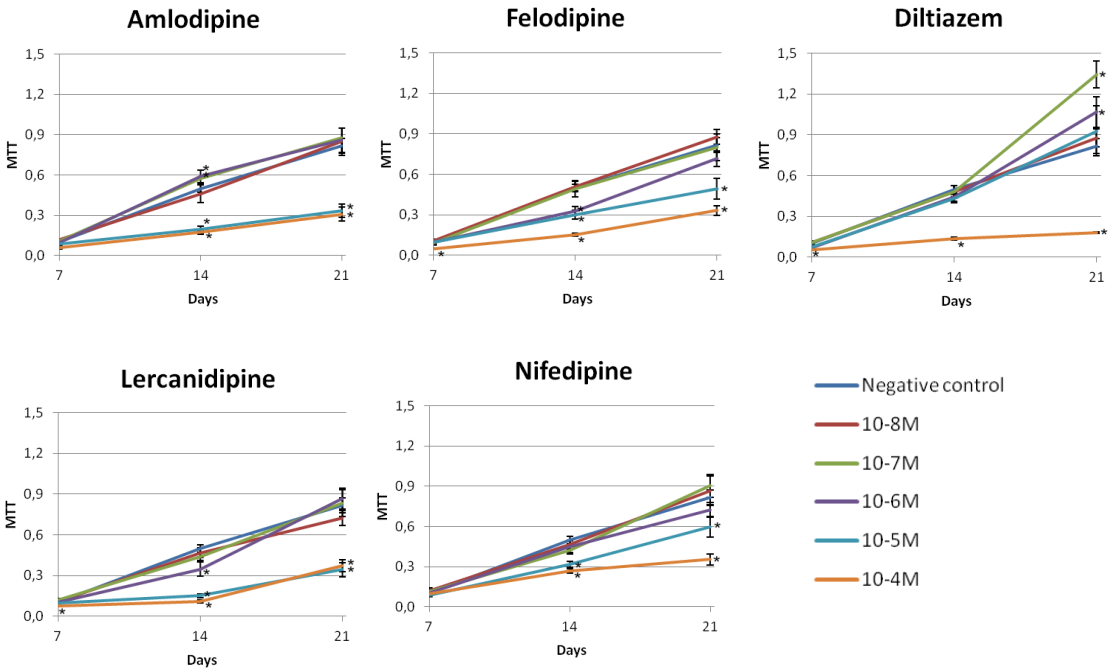


Figure 3.7: Cell proliferation/viability of osteoblasts, evaluated by the MTT assay.

In figure 3.7, it is observed that osteoblast cultures displayed an increase on cell proliferation/viability through the 21 days of culture. The presence of AHDs at low doses didn't alter significantly that behavior, except in the case of diltiazem, which elicited a stimulation of cellular proliferation/viability. The maximum response was achieved at 10^{-7} M, with an increase of about 65% for diltiazem compared to the control. At high doses, all AHDs cause a decrease on cell proliferation/viability.

3.2.2 ALP activity quantification

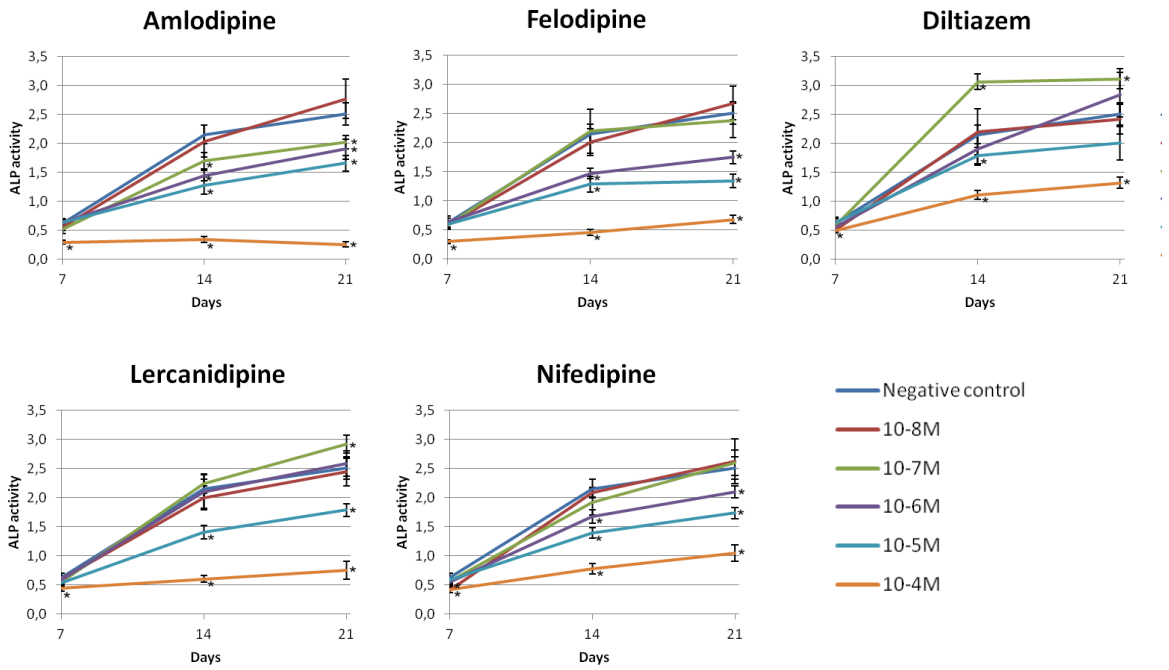


Figure 3.8: ALP activity of osteoblasts treated with the AHDs.

Figure 3.8 demonstrates the ALP activity of osteoblasts maintained in the absence (control) or presence of different AHDs, cultured for 7, 14 and 21 days. In the control, ALP activity increased mostly until day 14 and tended to stabilize afterwards. The tested AHDs revealed diverse abilities to modulate osteoblastogenesis. At low doses, diltiazem and lercanidipine caused an increase on ALP activity. At concentrations higher than 10^{-6} M, both molecules elicited a decrease on osteoblastic behavior. The highest response was achieved with 10^{-7} M, with an increase of about 24% and 16% for diltiazem and lercanidipine, respectively. Compared to the control, the presence of amlodipine, felodipine and nifedipine resulted in a dose-dependent decrease of ALP activity, which became statistically significant (19%, 5% and 16%) for concentrations higher than 10^{-7}, 10^{-7} and 10^{-6} M, respectively.

3.2.3 Staining of actin and nuclei and visualization by CLSM

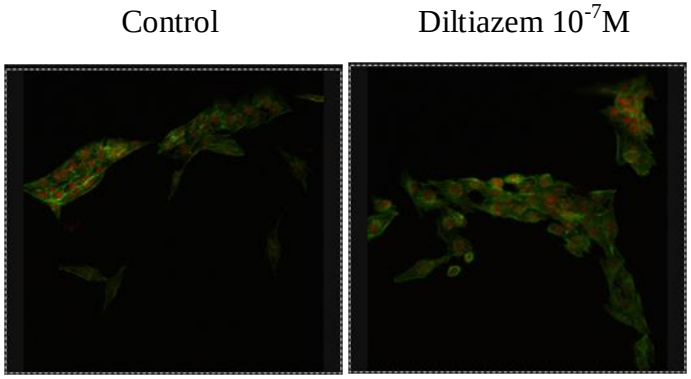


Figure 3.9: Presence of multinucleated cells displaying actin rings and nuclei in osteoblasts cultures assessed by CSLM. Fluorescence images showing actin rings (gree) and nuclei (red). White bars represent 120 µm.

Osteoblasts cultures were stained for actin and nuclei and visualized by CLSM (figure 3.9), which confirmed the presence of osteoblasts in tested conditions. The cells were spread over the bottom of the culture wells and exhibited a proper elongated morphology. The amount of osteoblastic cells in the different conditions was somehow correlated with the qualitative pattern observed for ALP activity.

3.2.4 Apoptosis quantification

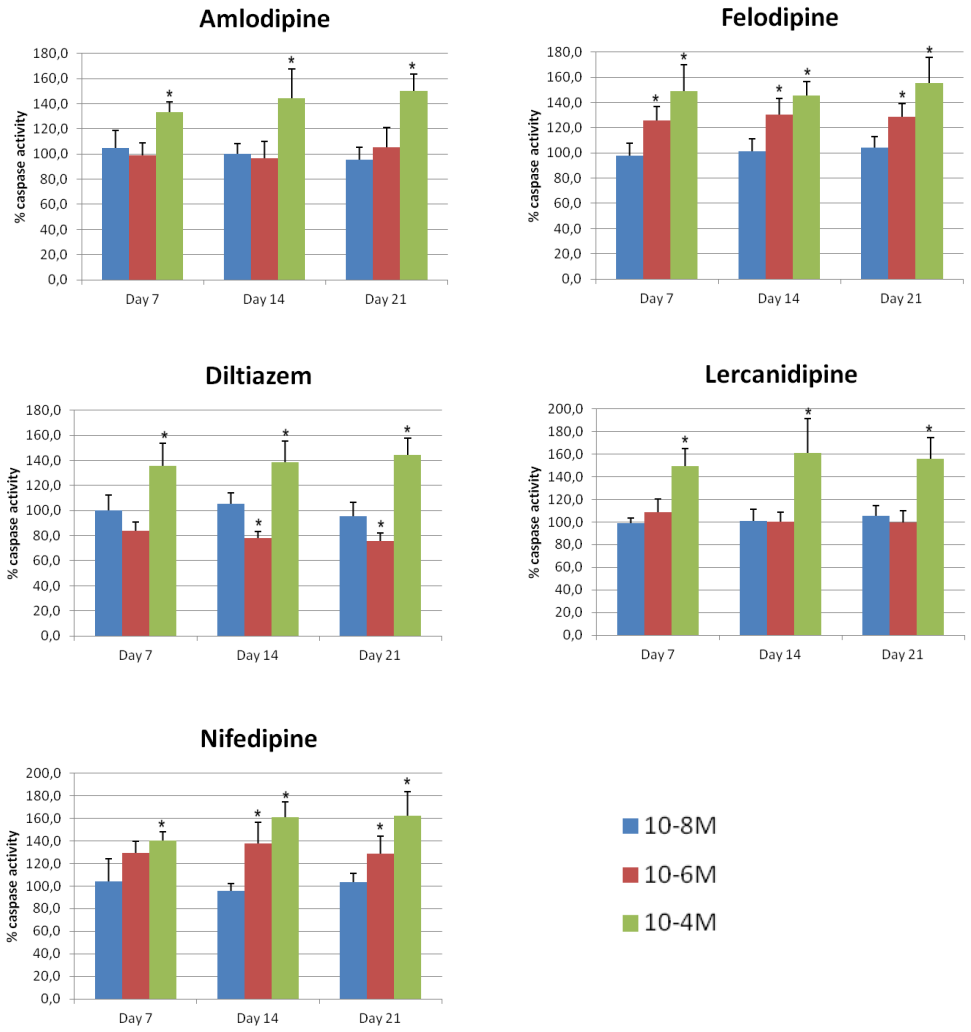


Figure 3.10: Caspase-3 activity on osteoblasts cultures

Figure 3.10 demonstrates that amlodipine and lercanidipine did not affect significantly caspase-3 activity at 10^{-8} M and 10^{-6} M, while at 10^{-4} M it induced an increase on cell response. Felodipine and nifedipine elicited a higher apoptotic behavior at high doses. Supplementation with diltiazem revealed a decreased in caspase activity at concentration 10^{-6} M.

3.2.5 Histochemical staining of ALP

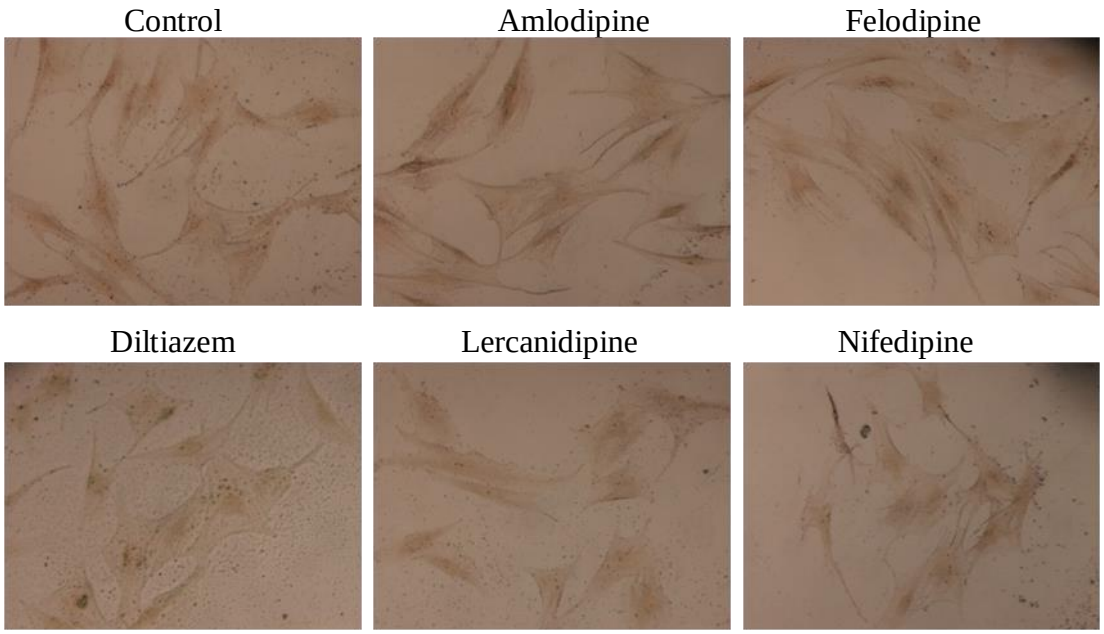


Figure 3.11: Histochemical staining of ALP in osteoblasts cultures.

The osteoblasts cultures showed positive staining reaction in the presence of ALP (figure 3.11). The AHDs treated cultures had a relatively uniform staining, being evidence for areas of high intensity of staining associated with cell groups.

3.2.6 RT-PCR analysis

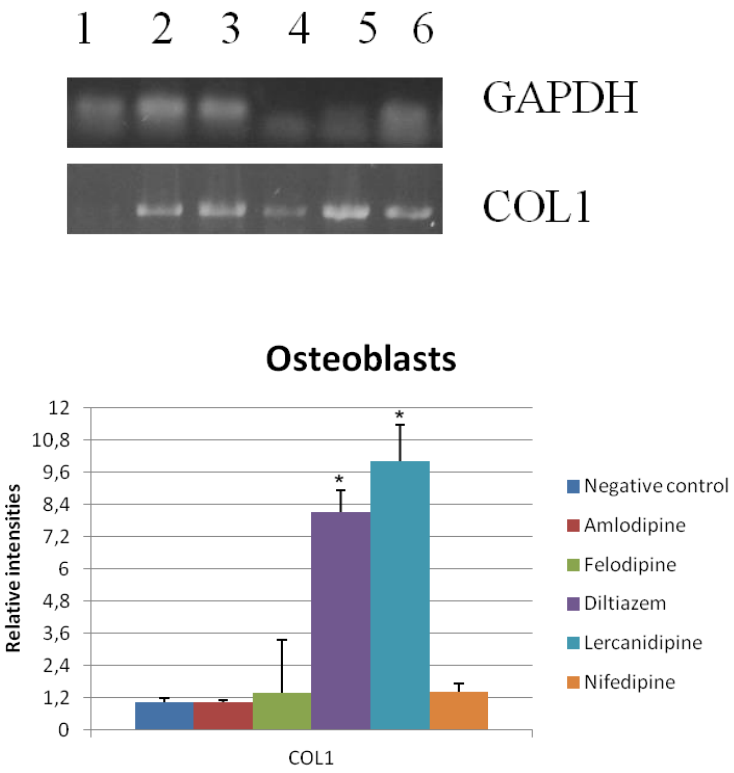


Figure 3.12: Expression of osteoblastic-associated genes, and representative agarose gel. Densitometric analysis of the RT-PCR products, normalized by GAPDH, of osteoblasts cultures. 1 – control; 2 – amlodipine; 3 – felodipine; 4 – diltiazem; 5 – lercanidipine; 6 – nifedipine.

Cell layer was assessed by RT-PCR (figure 3.12), in order to verify the expression of the housekeeping gene GAPDH and the osteoblastic gene COL1. The osteoblasts cultures revealed expression of both genes, in the presence of AHDs. In the control, the expression of COL1 was low, and was not significantly affected by the presence of amlodipine, felodipine and nifedipine. However, when cell cultures were treated with diltiazem and lercanidipine, the expression levels increased significantly about 7x and 9x, respectively.

3.2.7 Assessment of the intracellular mechanisms involved in the osteoblastogenic cell response

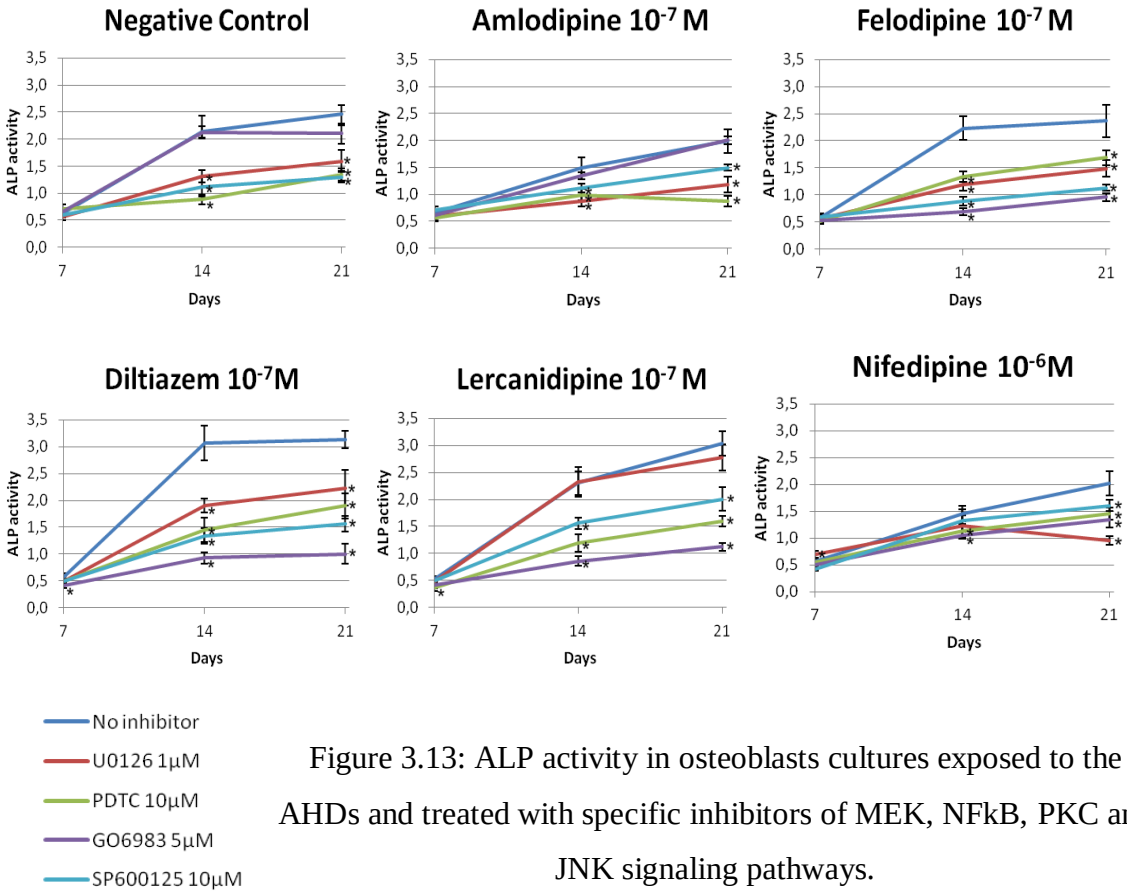


Figure 3.13: ALP activity in osteoblasts cultures exposed to the AHDs and treated with specific inhibitors of MEK, NFkB, PKC and JNK signaling pathways.

Osteoblasts cultures were treated with specific inhibitors of several signaling pathways involved in the osteoblastic differentiation and function; ALP activity was quantified and the results are presented in figure 3.13. In control conditions (absence of the AHDs), the presence of all inhibitors caused a decrease in the levels of this enzyme. Comparatively, the presence of the AHDs showed some significant differences. A higher inhibition in ALP activity was seen in the presence of U0126, when cell cultures were treated with amlodipine and nifedipine. Diltiazem and felodipine-supplemented cell cultures revealed a similar inhibition than the one observed in the absence of any AHDs, while in the presence of lercanidipine U0126 did not significantly affect ALP activity. PDTC caused a lower inhibition in the presence of the AHDs, although with the exception of amlodipine, whose inhibition was higher than in the control. The presence of GO6983 caused a significant decrease in ALP activity in the presence of all drugs. SP600125 elicited a lower inhibition on cell response in the presence of amlodipine, lercanidipine and

nifedipine, while in the presence of the remaining two AHDs (felodipine and diltiazem), the inhibition was similar to the one observed in the control.

3.3 Co-cultures of PBMC and osteoblasts

3.3.1 TRAP activity quantification

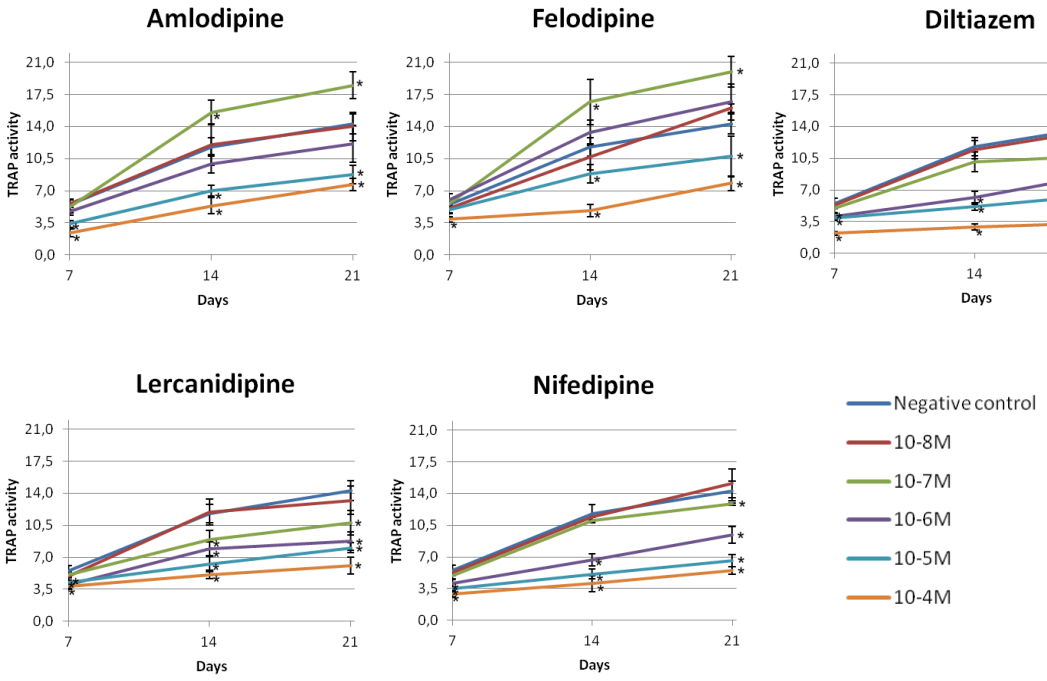


Figure 3.14: TRAP activity of co-cultures treated with the AHDs.

Figure 3.14 demonstrates the TRAP activity of co-cultures, in the absence (control) or supplemented with different AHDs, cultured for 7, 14 and 21 days. TRAP activity increased until day 14 and stabilized after that, at control conditions. Supplementation with low doses of amlodipine and felodipine resulted in a significant increase of TRAP activity. The maximum response was achieved with 10^{-7} M, with an increase of about 29% and 40% for amlodipine and felodipine, respectively. At concentrations higher than 10^{-5} M, both molecules elicited a decrease on osteoclastic behavior. Compared to the control, the presence of diltiazem, lercanidipine and nifedipine resulted in a dose-dependent decrease of TRAP activity, which became statistically significant (24%, 25% and 34%) for concentrations higher than 10^{-7}, 10^{-7} and 10^{-6} M, respectively.

3.3.2 Histochemical staining of TRAP

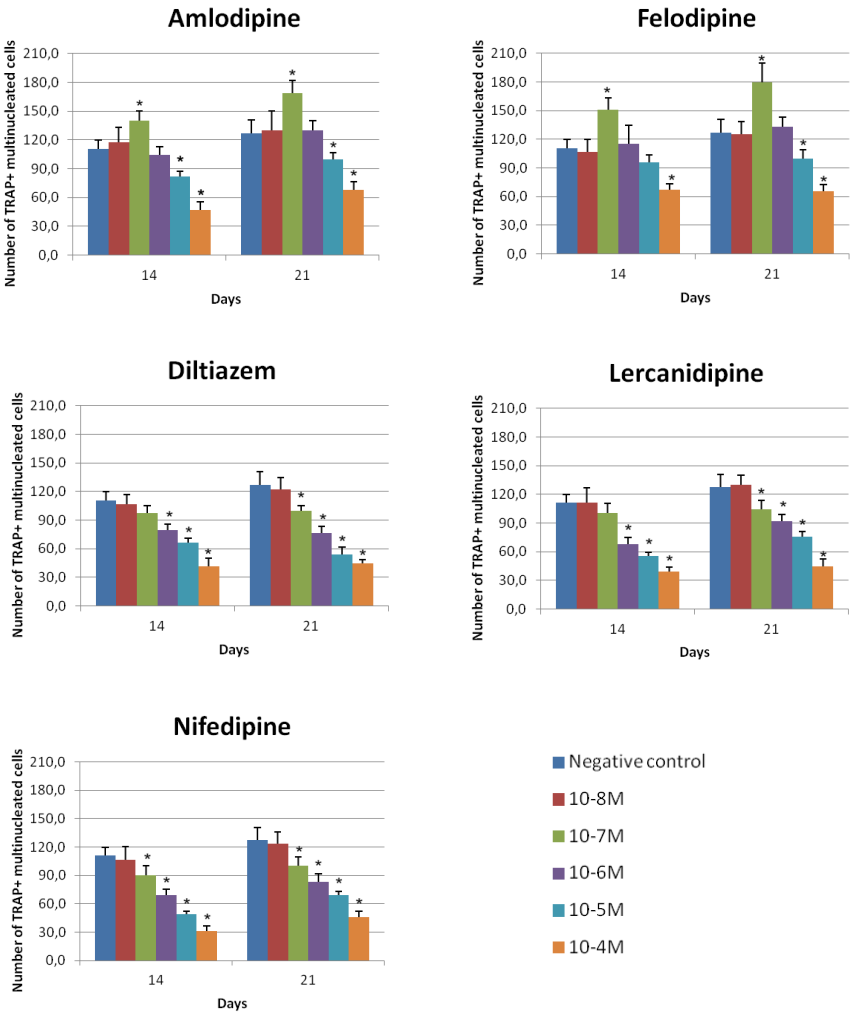


Figure 3.15: Number of TRAP+ multinucleated cells on co-cultures treated with the AHDs.

Figure 3.15 shows that, after 14 and 21 days of culture, the profile observed for the number of TRAP-positive multinucleated cells in co-cultures followed a pattern similar to that observed in TRAP activity, either in control conditions or in the presence of AHDs. Shortly, supplementation with the AHDs resulted in an increase of number of TRAP-positive multinucleated cells at low doses, in the case of amlodipine and felodipine (33% and 41.5%, respectively). The presence of the remaining AHDs resulted in a dose-dependent decrease of number of TRAP-positive multinucleated cells.

3.3.3 Assessment of the intracellular mechanisms involved in the osteoclastogenic cell response

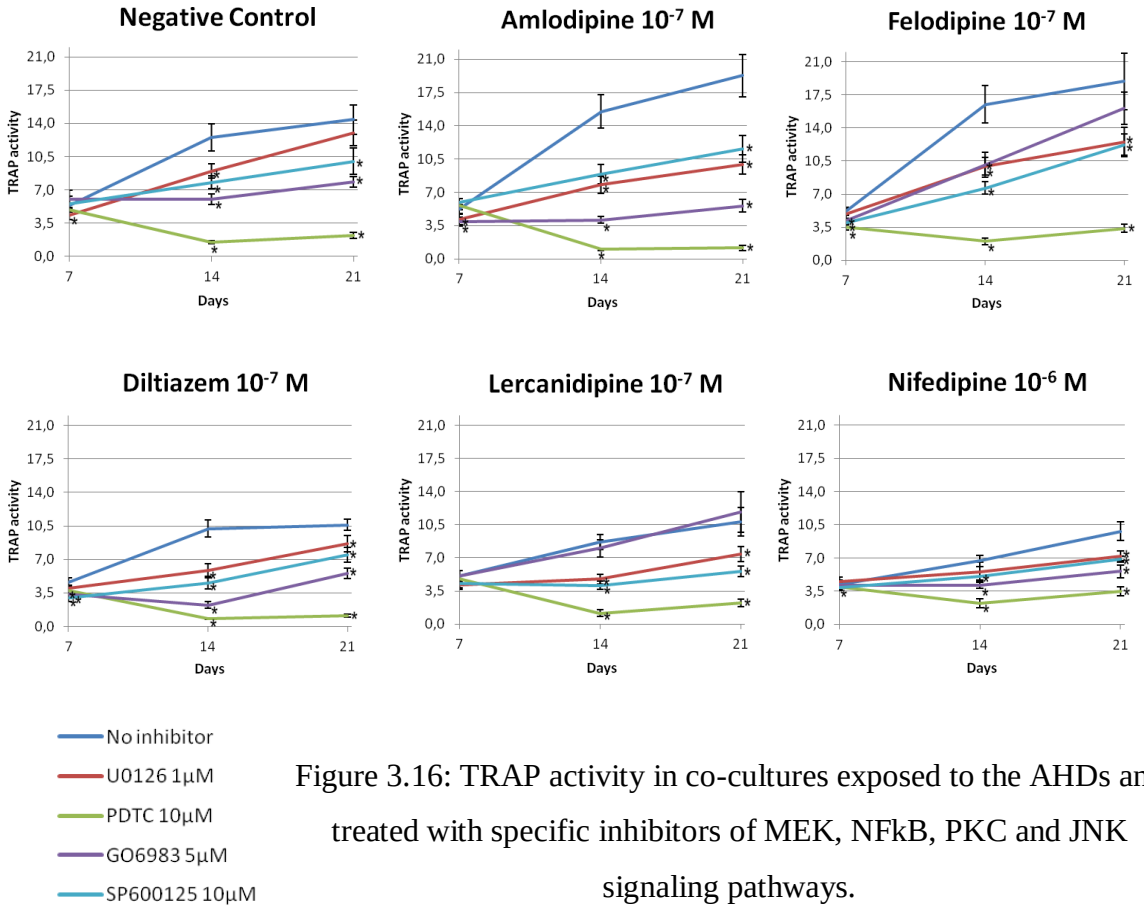


Figure 3.16: TRAP activity in co-cultures exposed to the AHDs and treated with specific inhibitors of MEK, NFkB, PKC and JNK signaling pathways.

Co-cultures were treated with specific inhibitors of several signaling pathways involved in osteoclastic differentiation and function; TRAP activity measured in this culture is presented in figure 3.16. In control co-cultures, all signaling pathway inhibitors induced a decrease in TRAP activity, especially PDTC, which caused an almost total decrease. The one exception was U0126, which did not affect significantly cell behavior. In general, the presence of the AHDs showed some significant alterations on this pattern. Compared to the control, an inhibition in TRAP activity was seen in the presence of U0126. Supplementation with PDTC elicited a similar response in co-cultures treated with all the tested AHDs, although less pronounced in the case of nifedipine. The presence of GO6983 caused a higher inhibition on TRAP activity in the presence of amlodipine, while co-cultures treated with felodipine, lercanidipine and nifedipine were less affected by that molecule. SP600125 caused a lower inhibition in the presence of diltiazem and nifedipine,

while in the presence of the remaining AHDs, its inhibition profile was similar to the control.

3.3.4 ALP activity quantification

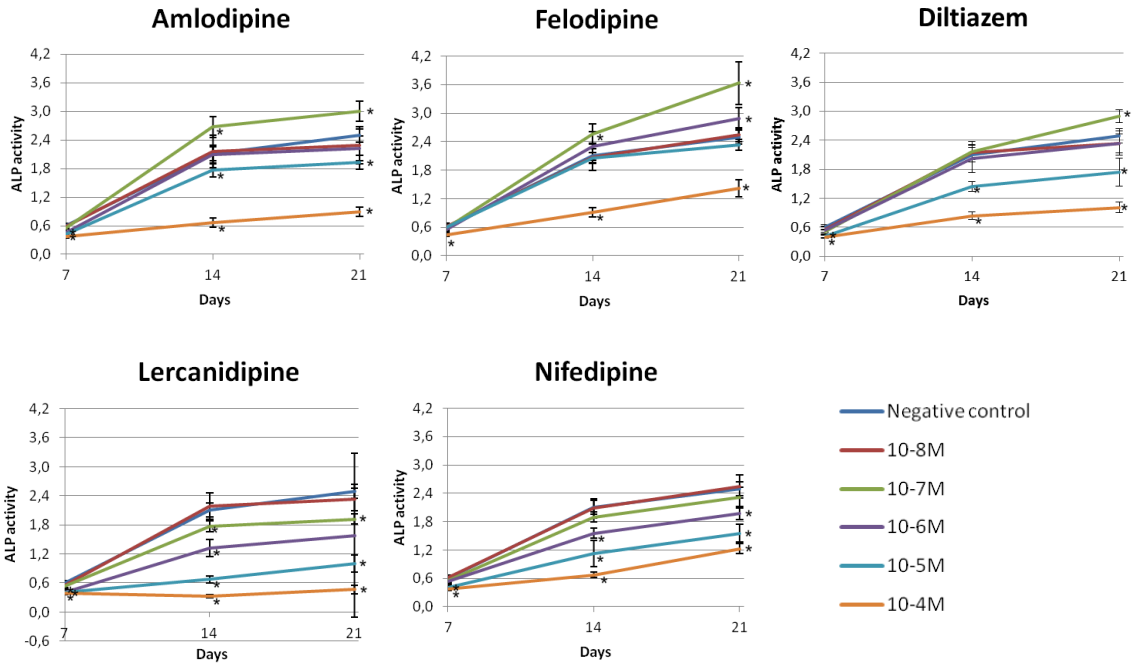


Figure 3.17: ALP activity of co-cultures treated with the AHDs.

Figure 3.17 demonstrates the ALP activity of co-cultures maintained in the absence (control) or presence of different AHDs, cultured for 7, 14 and 21 days. In control, ALP activity increased mostly until day 14 and tended to stabilize afterwards. At low doses, amlodipine, felodipine and diltiazem caused an increase on ALP activity compared to the control. The maximum response was achieved with 10^{-7} M, with an increase of about 20%, 46% and 16% for amlodipine, felodipine and diltiazem, respectively. At concentrations higher than 10^{-5} M, these molecules elicited a decrease on osteoblastic behavior. Compared to the control, the presence of lercanidipine and nifedipine resulted in a dose-dependent decrease of ALP activity, which became statistically significant (23% and 21%) for concentrations higher than 10^{-7} and 10^{-6} M, respectively.

3.3.5 Assessment of the intracellular mechanisms involved in the osteoblastogenic cell response

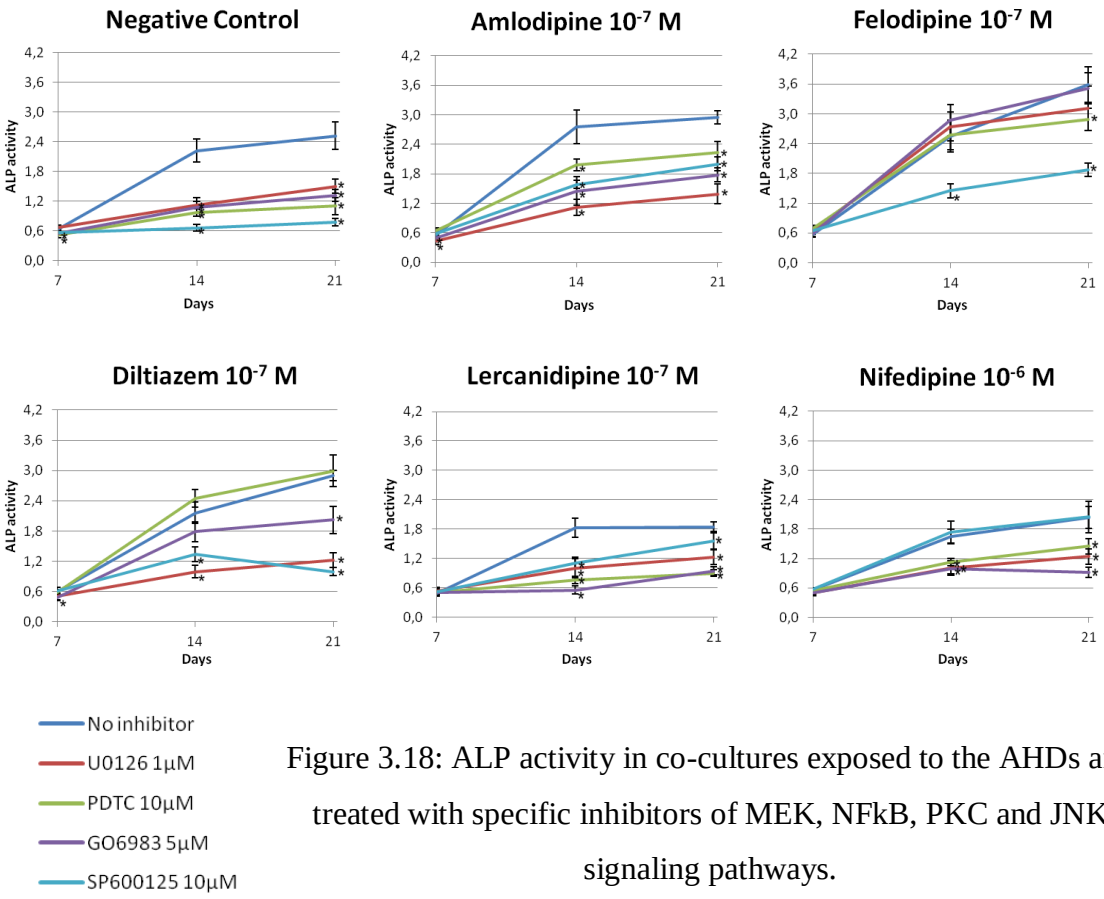


Figure 3.18: ALP activity in co-cultures exposed to the AHDs and treated with specific inhibitors of MEK, NFkB, PKC and JNK signaling pathways.

Co-cultures were treated with specific inhibitors of several signaling pathways involved in osteoblastic differentiation and function; ALP activity measured in this culture is presented in figure 3.18. In the control, all the tested inhibitors caused a decrease in the production of ALP. Comparatively, the presence of the AHDs elicited some important changes. Osteoblastic behavior of cell cultures supplemented with felodipine was not significantly affected by U0126. Supplementation with lercanidipine and nifedipine caused a lower inhibition on ALP activity, while in the presence of the remaining two AHDs the response was identical to the control. PDTC appeared to have a lower inhibitory ability in the presence of the AHDs, particularly in the case of diltiazem, where it did not affect cell response. GO6983 showed a partial inhibitory profile in all tested conditions, except in the case of co-cultures treated with felodipine. SP600125 caused an inhibition (similar to the control) on ALP activity in co-cultures treated with amlodipine, felodipine and diltiazem.

On the other hand, in the presence of lercanidipine and especially nifedipine, that inhibitor had little or no effect on osteoblastic behavior of co-cultures.

3.3.6 Staining of actin, VNR, CTR and visualization by CLSM

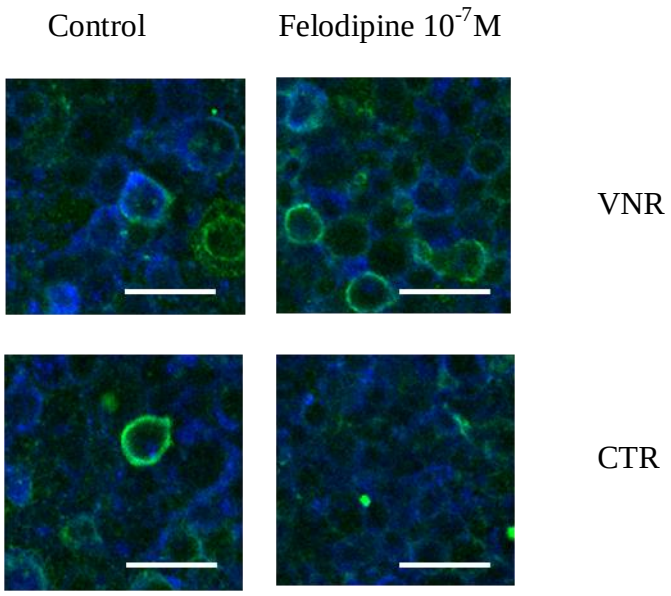
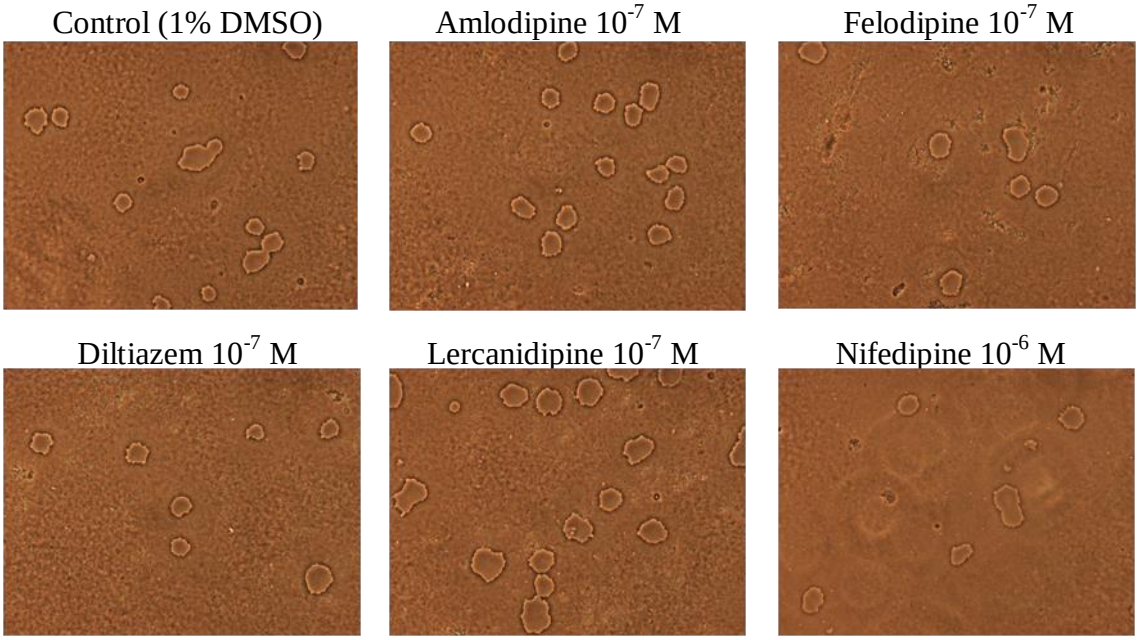


Figure 3.19: Presence of multinucleated cells displaying actin rings and VNR or CTR in co-cultures assessed by CSLM. Fluorescence images showing actin rings (blue) and VNR or CTR (green). White bars represent 120 µm.

Figure 3.19 shows representative images of co-cultures stained for actin and for VNR and CTR and visualized by CLSM. It was possible to visualize cells displaying the analyzed osteoclast-features, which confirms the presence of osteoclasts and osteoblasts in tested conditions. The amount of osteoclastic cells in the different conditions was somehow correlated with the qualitative pattern observed for TRAP. Due to the low amount of osteoblastic cells used in the co-cultures, comparatively to the PBMC amount, osteoblastic cells were not able to be observed by CLSM.

3.3.7 Calcium phosphate resorbing ability



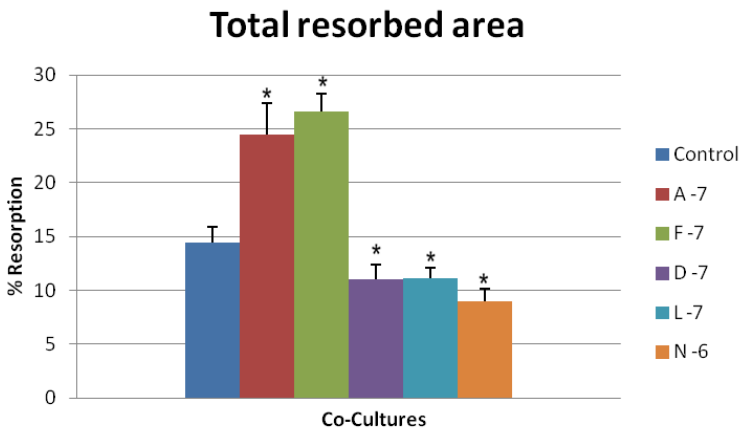


Figure 3.20: Calcium phosphate resorbing ability and total resorbed area of co-cultures treated with the AHDs.

In figure 3.20, it was observed that the presence of amlodipine and felodipine induced a significant increase on the resorbed area on co-cultures (about 69% and 84% higher than the control for amlodipine and felodipine, respectively). In contrast, the other drugs elicited a decrease on the resorbing activity (about 24%, 23% and 38% lower than the control for diltiazem, lercanidipine and nifedipine, respectively).

3.3.8 Histochemical staining of ALP

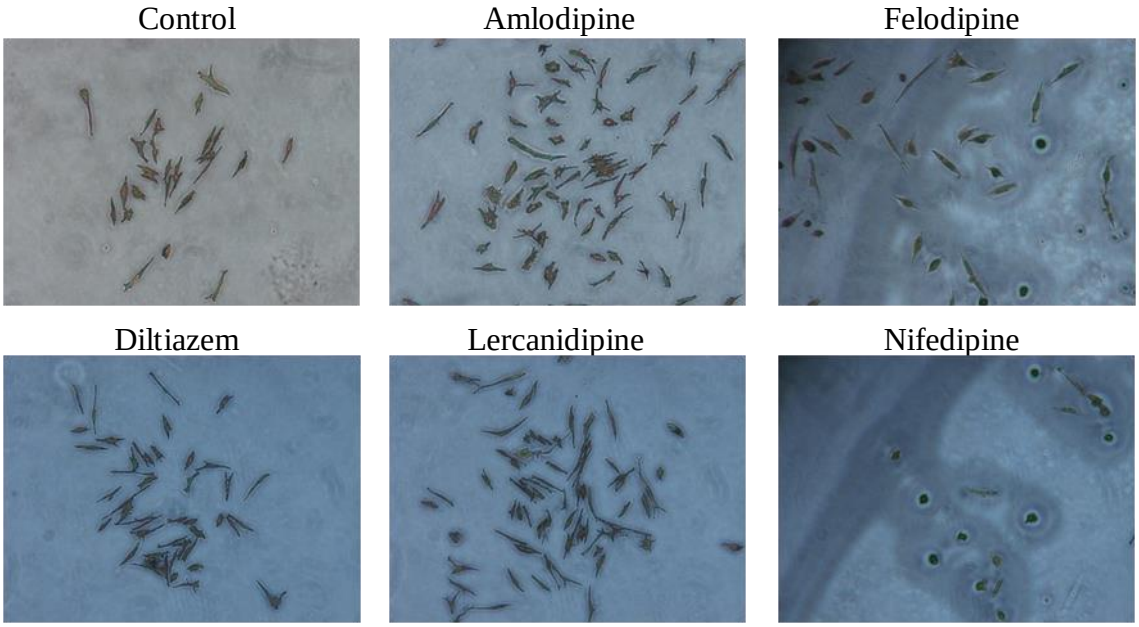


Figure 3.21: Histochemical staining of ALP in co-cultures.

The co-cultures showed positive staining reaction in the presence of ALP (figure 3.21). The AHDs treated cultures had a relatively uniform staining, being evidence for isolated cells and not for areas associated with cell groups.

3.3.9 RT-PCR analysis

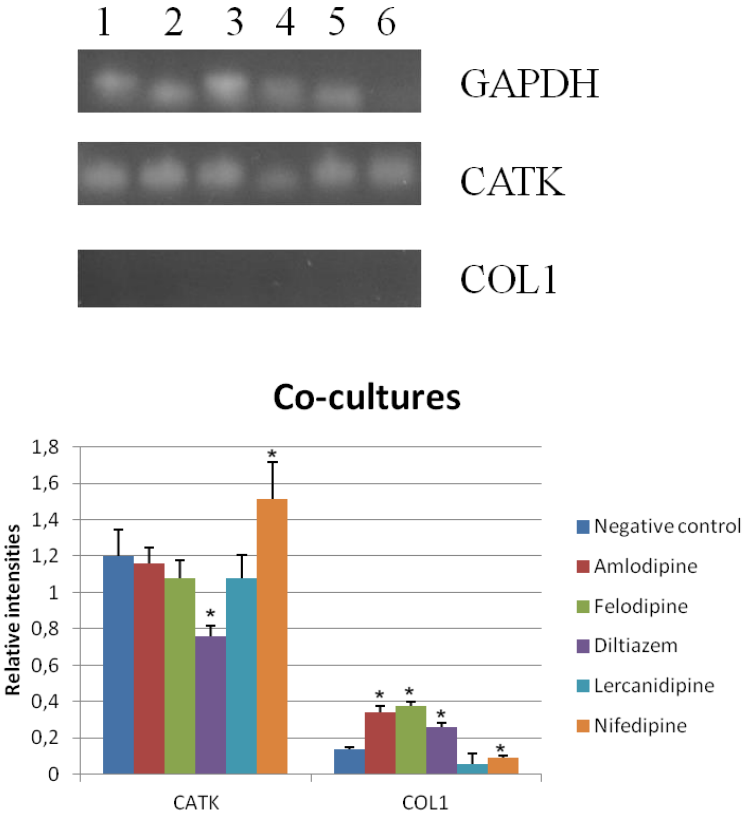


Figure 3.22: Expression of osteoclastic and osteoblastic-associated genes, and representative agarose gel. Densitometric analysis of the RT-PCR products, normalized by GAPDH, of co- cultures. 1 – control; 2 – amlodipine; 3 – felodipine; 4 – diltiazem; 5 – lercanidipine; 6 – nifedipine.

Co-cultures layers were assessed by RT-PCR (figure 3.22), in order to verify the expression of GAPDH, the osteoclastic gene CATK and the osteoblastic gene COL1.

The co-cultures revealed expression of the analyzed osteoclastic gene, in the control and in the presence of AHDs. When co-cultures were treated with nifedipine, the expression levels increased about 26%. With the supplementation with amlodipine, felodipine and lercanidipine the expression levels of the analyzed gene were not significantly affected, while in the presence of diltiazem the value increase about 37%.

The osteoblastic gene analyzed was expressed at very low levels, as revealed by the agarose gel. However, in the densitometric analysis it was possible to determine its expression. When co-cultures were treated with amlodpine, felodipine and diltiazem the

expression levels increased about 148%, 173% and 92%, respectively. With the supplementation with lercanidipine and nifedipine the expression levels of analyzed gene decreased about 57% and 33%, respectively.

CHAPTER IV

Discussion

In the bone microenvironment, the mutual osteoclast-osteoblast communication has a central role in their differentiation and function. The parallel coordinated bone resorption and formation events are highly complex and regulated processes, responsible for the life-long bone remodeling with the aim of ensure a healthy tissue formation and maintenance (52). AHDs can have an indirect impact on osteoporosis as well as direct effects on bone metabolism, strength, and density. Calcium has a central role in bone strength and in the balance between osteoblastic and osteoclastic activities, the basis of the bone remodeling process (46). The present study examined the effects of amlodipine, felodipine, diltiazem, lercanidipine and nifedipine on human osteoclasts and osteoblasts differentiation and activation in three different cell culture models: PBMC, osteoblasts and co-cultures of osteoblasts and osteoclasts.

The effects of AHDs on the osteoclast and osteoblast development are scarcely reported in the literature, with only a few published studies about it. Moreover, these studies have just analysed the effect of two AHDs – amlodipine and nifedipine on bone cells. One experiment was performed on outbred rats and it was observed that amlodipine treatment may induce deceleration of bone metabolism. The expression of BMP-2 in proximal tibia was investigated by means of immunoblotting, and bone mineral density was measured by Dual-energy X-ray Absorptiometry in lumbar and caudal vertebrae and in femoral areas. The results showed induced suppression of bone turnover, in particular by decreasing osteoformation, and to a lesser extent osteoresorption (13). Other study investigated the effects of different amlodipine and lacidipine doses on ovariectomized rat femurs' calcium and phosphor content. Bone calcium and phosphate concentrations were measured by a Wavelength Dispersive Spectrometer. The findings suggested that amlodipine decreased the bone loss, having a beneficial effect on bone metabolism (14). Another work using osteoblastic MC3T3-E1 cells revealed that nifedipine and amlodipine weakly block calcium channels on osteoblastic cells, and also partially elevated ALP activity at higher concentrations, by the quantification of ALP activity, DNA and hydroxyproline content, mineralization and cytosolic calcium measurement (6). In a study conducted with 11 men given nifedipine for three years, no differences in bone mass and bone turnover markers were found as compared to the control group (7). Another study showed decreased plasma calcitonin levels in postmenopausal women treated with nifedipine (7). Finally, the effects of amlodipine were assessed in an 8-week study, which revealed no changes in bone remodeling markers of menopausal women (7). Taken

together, the published studies were performed in different experimental models, of different origins and mainly *in vivo* models, which can help to explain the contradictions found among the data obtained. Moreover, in order to have a more accurate perspective of the effects of calcium channel blockers on human bone metabolism, it is of the utmost importance to assess the way human bone cells behave in the presence of those molecules. Up till now there is no data regarding this issue. Nevertheless, it is important to note that this represents only the first step towards a physiological understanding of how AHDs may affect bone tissue, and a correlation to the clinical situation can not be directly predicted, due to the complex *in vivo* environment.

PBMC cultures were performed in the presence of the osteoclastogenic recombinant growth factors M-CSF and RANKL. Several studies have shown that both M-CSF and RANKL are required for the expression of typical osteoclastic genes, including TRAP, CTR, VNR and CATK (35). TRAP activity is observed as an important cytochemical marker of osteoclasts; its concentration is utilized as a biochemical marker of osteoclast function and, consequently, as an indicator of bone resorption (53). It was observed that the tested AHDs had the ability to differentially affect osteoclastogenesis. At low doses, amlodipine and felodipine caused an increase on osteoclastic differentiation, while the other drugs inhibited it, as observed by TRAP activity, quantification of TRAP+ multinucleated cells, calcium phosphate resorbing ability and expression of CATK gene. At higher doses, all the molecules caused a decrease on the process, which is in agreement with the results obtained previously by other authors (6,13,14). The observed results were at least partially accompanied by a corresponding change on apoptosis rate, determined by caspase-3 activity.

PBMC cultures were also analyzed for the involvement of several signaling pathways, with a well-established role in osteoclastogenesis (52). In general, the presence of the AHDs caused some significant alterations in the relative contribution of the tested pathways on the response of osteoclastic cells. Concerning the intracellular signalling pathways, MEK pathway was not significantly affected by nifedipine; lercanidipine caused a lower influence of the pathway, while it was upregulated by the remaining AHDs. NFkB pathway appeared as a major osteoclastogenic intracellular mechanism, although in the presence of felodipine and diltiazem it seemed to lose some of its relevance. PKC signaling pathway did not contribute to the cellular response in PBMC cultures treated with felodipine and diltiazem, but in the presence of the other AHDs it was obtained a decrease

on TRAP activity higher than the one observed in the control. This reveals the importance of this pathway in the osteoclast differentiation, especially in the presence of nifedipine, where it practically abolished osteoclast differentiation. JNK pathway was not affected by diltiazem and nifedipine, while the remaining AHDs caused a higher involvement of that pathway on osteoclastic response.

Osteoblasts play a crucial role in bone formation through their proliferation, differentiation and bone tissue formation. ALP is a membrane bound enzyme that is often used as a marker for osteogenic differentiation, a complex process of sequential expression of early and late marker proteins (35,37). MTT assay was used to determine the viability/proliferation of the cells. Cell viability can be defined as the number of healthy cells in a sample. Cell viability assays are often useful when primary cells are isolated and maintained in culture to determine optimal culture conditions for these populations. Cell proliferation is the determination of the number of dividing cells (54). It was observed that the tested AHDs had the ability to differentially affect not only cell viability/proliferation but also osteoblastogenesis. At low doses, diltiazem and lercanidipine caused an increase on osteoblastic differentiation, which is compatible with the results obtained in previously studies (6), while the other drugs inhibited it. At higher doses, all the molecules caused a decrease on the process, which can be partly attributed to changes in the apoptotic process in the different experimental conditions, which is in line with results obtained previously (13).

Regarding the intracellular signalling pathways, MEK pathway had a contribution in the osteoblastogenic response. Cultures treated with lercanidipine were not significantly affected by the MEK inhibitor, which suggests that in these conditions MEK pathway may not be as important for osteoblastogenesis as it happens in the control. The treatment with felodipine and diltiazem had a similar inhibitory profile compared to the control. However, ALP activity was marked inhibited in the cultures exposed to the others AHDs, suggesting that these molecules might lead to an upregulation of the pathway. NFkB appeared to be not so involved in osteoblastogenesis, in the presence of AHDs, except in the case of amlodipine, where the observed inhibition was higher than in the control. PKC signaling pathway appeared to affect the cellular response in osteoblasts cultures treated with all drugs, especially in the presence of felodipine, diltiazem and lercanidipine, where its involvement was higher than in the control. JNK pathway appeared to be downregulated by amlodipine, lercanidipine and nifedipine and not affected by the remaining two AHDs.

In co-cultures, osteoclastic response was stimulated by amlodipine and felodipine at low doses. At high doses all AHDs caused a decrease in osteclastogenesis. Co-cultures also showed a high osteoblastic response at low doses of amlodipine, felodipine and diltiazem. At high doses all drugs revealed a decrease in the differentiation of osteoblasts.

Co-cultures were also analyzed for the involvement of several signaling pathways, with a role in osteoclastogenesis and osteoblastogenesis. The presence of the AHDs induced some alterations in the relative contribution of the tested pathways in the response of co-cultured osteoclastic and osteoblastic cells. Regarding the osteoclastic response, MEK pathway seemed to be upregulated by the tested AHDs. NFkB appeared to be a major intracellular mechanism in all the experimental conditions, although with a lower relevance in the presence of nifedipine. PKC signaling pathway seemed to be not involved in osteoclastogenesis in the presence of felodipine and lercanidipine, while amlodipine appeared to upregulate it. In the presence of the remaining AHDs, the involvement of this pathway was similar to the control. JNK pathway was negatively affected by diltiazem and nifedipine, while the other AHDs caused a similar inhibition profile of osteoclastogenesis, compared to the control.

In relation to the intracellular signalling pathways involving osteoblasts, it was observed that in the control the tested inhibitors caused a decrease in ALP activity, which indicates that all signaling pathways influence osteoblastic differentiation. Cultures treated with felodipine were not significantly affected by U0126, which suggests that in these conditions MEK pathway may not be important for osteoblastogenesis. Also, nifedipine- and lercanidipine-treated co-cultures revealed a lower inhibition and in the presence of the other two AHDs the response was similar to the control. NFkB appears to be not so important in cell cultures treated with the AHDs, because the inhibition of osteoblasts differentiation is lower than the observed in the control, particularly in the case of diltiazem, where it was not observed any inhibition. PKC signaling pathway appears to have a partial contribution in all tested conditions, except in the case of co-cultures supplemented with felodipine. JNK pathway appears not to be an important pathway with supplementation with lercanidipine and nifedipine, because the inhibitor had no effect on osteoblasts differentiation. With the remaining drugs a similar inhibition compared to the control was observed.

Taken together, AHDs seemed to cause a direct effect on human osteoclastic and osteoblastic differentiation. Interestingly, some of the tested molecules increased while

others inhibited the processes in a dose-dependent profile. Furthermore, the osteoclastic and osteoblastic response was different when cells were cultured isolated or co-cultured, which suggests that AHDs might also modulate osteoclastogenesis and osteoblastogenesis in an indirect way, that is, by modulating the osteoclastogenic and osteoblastogenic properties of osteoblasts and osteoclasts, respectively. Unraveling the mechanisms beneath these observations might help to explain the adverse effects on bone tissue described for this drug class.

Conclusion

Owing to the high incidence of hypertension and the worldwide high frequency of use of AHDs, in particular the calcium channel blockers, it is necessary to study their influence on bone metabolism. Since bone diseases are very common –fractures are a problem particularly relevant in the elderly –, and given the incidence of hypertension, a detailed knowledge of the association of the two factors is crucial in order to act on the improvement of the quality of life of patients and their health status in general.

Comparing plasma Cmax and the obtained results, in the osteoclasts cultures, amlodipine and felodipine caused an increase, while diltiazem caused a decrease in TRAP activity. Lercanidipine and nifedipine had no effect in its activity. In the osteoblasts cultures, diltiazem induced ALP activity, and the others AHDs had no effect in this activity. In co-cultures, TRAP activity decreased in the presence of diltiazem and nifedipine and was not affected in the presence of the remaining AHDs. ALP activity was increased with diltiazem and not affected with the others AHDs.

The objectives proposed for this study were fulfilled. The effects of AHDs calcium channel blockers in human bone cells were studied, on a first stage, by the focus on the influence of a wide range of concentrations of five different AHDs in the osteoclastogenic and osteoblastogenic process. AHDs caused differential in the osteoclastic and the osteoblastic behavior of PBMC, osteoblasts and co-cultures of human PBMC and osteoblasts. In order to have some insights on the underlying mechanisms of action of AHDs in the cell culture systems used, the influence of several signaling pathways on the observed cellular responses was assessed.

In summary, this work provides new insights on the *in vitro* effects of AHDs on human bone cells, open the doors towards a better understanding about this up till now poorly clarified issue.

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Publications, communications and prizes

Panel Communication

- February 13 – 15, 2013 - Oliveira T, Costa-Rodrigues J, Ferraz R, Prudêncio C, Fernandes M H. Antihypertensive drugs and bone metatoblism: *in vitro* study with human osteoclastic cells. In IJUP'13 – 6º Encontro de Jovens Investigadores da Universidade do Porto
- May 18 – 21, 2013 - Oliveira T, Costa-Rodrigues J, Ferraz R, Prudêncio C, Fernandes M H. Modulation of osteoclastogenesis by antihypertensive drugs. In Congress ECTS'13 – European Calcified Tissue Society