Universidade do Porto

FEUP Faculdade de Engenharia

TETRACYCLINE EFFECTS ON DIABETIC BONE REGENERATION

José Carlos Osório Rodrigues da Silva

Dissertação para obtenção do grau de Mestre em Engenharia Biomédica

Orientadores

Professor Doutor Pedro Sousa Gomes

Professora Doutora Maria Helena Raposo Fernandes

Faculdade de Engenharia da Universidade do Porto

Porto, Julho de 2012

To my Brother

Acknowledgements

The present dissertation could not be done without the contribute of several persons and entities to which I want to express my gratitude, specially thanking:

my supervisors, Prof. Doctor Pedro Sousa Gomes and Prof. Doctor Maria Helena Fernandes, for their constant support, incentive and knowledge transmitted, essential to development of this project and also for the opportunities to integrate other projects.

to the Laboratory of Pharmacology and Biocompatibility of FMDUP, in the person of Prof. Doctor Maria Helena Fernandes and Prof. Doctor Pedro Sousa Gomes that were always available to help me in any circumstance, Dr. Mónica Garcia who helped me with all laboratory procedures.

to all my friends and family for the support and incentive during the development and concretization of this thesis, specially:

to Nuno Magalhães, Rui Maia, Hélder Martinho and Raquel Marques for their friendship, support and incentive during the development and concretization of this thesis.

to my Parents for the unconditional support and incentive in every moments of my life.

to my Brother, who gave me the greatest support and the highest strength at all times and circumstances.

Abstract

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia yielded by abnormalities in the secretion or response of cells to insulin. DM seems to negatively affect bone tissue metabolism and healing processes, giving rise to a diabetes-mediated osteopenic condition.

In this project, an animal model, representative of the human diabetic condition, and broadly used in experimental research (streptozotocin-induced diabetic rat) was used. Following the successful STZ-mediated hyperglycemia induction, rat bone marrow-derived osteoblastic cultures, from both control and diabetic rats were established and characterized in terms of proliferative and osteogenic capabilities. Cultures established from STZ animals revealed an increased expression of ALP and a broadly impairment of the cell proliferation and osteogenic capability.

Following, and due to the known catabolic action of doxycycline in bone metabolism, a tentative approach to enhance the osteogenic activity of diabetic-derived cell cultures was conducted and thoroughly characterized. Doxycycline was able to induce the cell proliferation and the functional activity of both control and diabetic-derived cultures.

Accordingly, doxycycline presents here as a candidate osteogenic agent for both conditions of physiologic bone metabolism, as well as for situations in which the osteoblastic function is impaired, such as the diabetes-mediated osteopenic condition.

Abbreviations

AGEs	Advanced glycation end products
ALP	Alkaline phosphatase
αMEM	Alpha Minimum Essential Medium
BMP-2	Bone morphogenic protein-2
BSP-1	Osteopontin, Bone sialoprotein-1
BSP-2	Bone sialoprotein-2
CLSM	Confocal laser scanning microscopy
CMTs	Chemically modified tetracyclines
COL I	Collagen type I
CT	Calcitonin
DCCT	Diabetes Control and Complications Trial
DGAV	Direção Geral de Alimentação e Veterinária
DM	Diabetes mellitus
ECM	Extracellular matrix
FBS	Fetal bovine serum
FPG	Fasting plasma glucose
GH	Growth hormone
GLUT2	Glucose transporter 2
GMD	Gestational diabetes mellitus

IDD	Insulin-dependent diabetes
IGF-I	Insulin-like growth factor-I
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
MMP-9	Matrix metalloproteinase-9
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NOD	Non-obese diabetic
OGTT	Oral glucose tolerance test
PKC	Protein kinase C
PTH	Parathyroid hormone
RUNX2	Runt-related transcriptional factor 2
SDD	Subantibacterial dose doxycycline
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes

Content

1: BACKGROUND AND LITERATURE REVIEW

1.1 – BONE PHYSIOLOGY AND FUNCTION

The bone tissue is a specialized form of connective tissue that can be both coped as a tissue and an organ system. It is a component of the skeleton, being committed to the protection, support and motion of the entire organism. It possesses specific and individual properties such as a high flexibility and elasticity – which thus allows for the protection of vital organs. It must be able to resist bending and yet needs to be flexible enough to absorb energy without breaking. The stiffness of the bone also underwrites to the conservation of the structural support and mechanical action, allowing for the precise and controlled muscular movement. At the cellular level, the bone tissue is a fecund and active biological entity, being considered a dynamic tissue that achieves its distinct features through its material composition and structural properties.

1.1.1 – Macrostructure

Bones of the skeletal system can be broadly classified according to their shape in long (e.g. femur, tibia, and humerus), short (e.g. tarsus and carpus), flat (e.g. ribs and cranial bones), and irregular (e.g. vertebrae of the spine). Despite these differentiated clusters, all bones are macrostructurally similar in that densely packed or compact bone – i.e., cortical bone – is found on the exterior, whilst the interior is comprised of a tridimensional structure of trabecular tissue, i.e., cancellous bone (1).

Cortical bone is found on the outer surface of individual bones and surrounds the trabecular structure. Approximately 80% of the skeleton corresponds to cortical bone, with trabecular bone making up the remaining 20%. Despite the architecture and relative amount of the cortical tissue, at any given anatomical location, it seems to be essentially related to its function at that specific site (2). The mechanical and protective functions of bone are mainly provided by the compact structure of the cortical bone, whereas the majority of metabolic functions are due to the trabecular bone, such that it is more responsive to disturbances in metabolic homeostasis (1).

Although both cortical and cancellous bone are made up of the same extracellular matrix (ECM) and cell types, they are structurally and functionally different (Table 1.1).

Table 1.1 - Differences between human cortical and cancellous bone. Adapted from (3).

Attributes	Cortical	Trabecular
Skeletal mass	80%	20%
Bone surface	33%	67%
Surface/volume ratio (mm^{-1})	2.5	20
Soft tissue	10%	65%
Adult tissue	Secondary osteon (Haversian systems) Interstitial lamellae Circumferential lamellae	Curved plates, rods Bone structural units Interstitial lamellae
Porosity	Low	High
Marrow	Fat	Hematopoietic
Main soft tissue	Viscera	Marrow
Developmental	Intramembranous ossification	Endochondral ossification
Turnover	Slow	Rapid
Function	Mainly biomechanical, also supportive and protective	Mainly mineral haemostasis, also supportive

Cortical bone is made up of a collection of cylindrical units named Haversian systems, or osteons, which run parallel to the outer surface of the bone. Each Haversian system is constituted by a central canal – through which blood vessels, lymphatic nodes, connective tissue and poorly myelinated fibers pass – surrounded by concentric lamellae of bony tissue. These lamellae are interspersed by small voids designated lacunae, which are thus interconnected by small channels, i.e. canaliculi (4). Osteocytes are found within the lacunae and extend cytoplasmic processes into the canaliculi. These canaliculi permit the nutritional support and oxygenation of the cellular constituents of the tissue and are likewise responsible for the removal of the waste products originating from metabolic actions. The gaps between the Haversian systems are made up of interstitial bone which consists of similar tissue elements, but

in a less organized pattern. Each Haversian system is separated from its neighboring structures by interstitial lamellae, and form a cement line which are strongly basophilic and have a high content of inorganic matrix (4).

Cancellous bone consists of an irregular network of thin calcified trabeculae, made up of irregular osteon fragments, which are surrounded by marrow space. The structure of the trabeculae can be classified as being rod-like or plate-like, the proportion of which relies on the magnitude and distribution of loading. Further, this type of bone is not generally infiltrated by large blood vessels, being the medullar cavities, filled with hematopoietic marrow, responsible for the metabolic support (4).

1.1.2 – Microstructure

Cortical and cancellous bone can comprise either woven or lamellar bone. Woven (or primary) bone is broadly found during the embryonic stage of bone development and is later resorbed and replaced by lamellar bone (5). Primary bone can also be found in epiphysial plates, ear ossicles, during fracture healing processes and during the closure of cranial sutures. Comparing to the lamellar bone tissue, woven bone has a higher metabolic rate, which substantiates an increased turnover during the remodeling phase. It is also characterized by an anisotropic distribution of cells and collagen fibers, which discloses its scattered and irregular display, and biomechanical fragility (5). Lamellar (or secondary) bone, on the other hand, is produced much more slowly and is characterized by a highly and ordered arrangement whereas the cells are broadly uniform in size, shape and orientation, supporting an improved biomechanical performance, in comparison to woven bone (5).

1.1.2.1 - Bone extracellular matrix

From a compositional point of view, the bone tissue may be regarded as a composite material composed of inorganic mineral (65%), organic matrix (35%), cells

and water (20% of wet weight of bone). Structural organization of the bone tissue is depicted in Figure 1.1.

The overall extracellular structure of the bone acts as an interconnected network for cells, in close interaction. The organic part of this matrix is broadly constituted by collagen fibers, predominantly collagen type I. The adequate and ordered deposition of collagen in sufficient amounts is required to accomplish the formation of optimal bone mass and mineral density during skeletal development, since bone mineral crystals deposition is aligned with the long axis parallel to the collagen axis. As so, collagen fibers are responsible for bone elasticity, flexibility and the organization of the matrix. Several non-collagenous proteins can also be found among the extracellular organic component. Among the several, osteopontin (or bone sialoprotein-1, BSP-1), bone sialoprotein-2 (BSP-2), osteocalcin and osteonectin play an important role in bone metabolic functions. Their exact biological role is far from completely fulfilled but several functions have already been adequately established in osteoblastic and osteoclastic cell function and interaction. Bone matrix also contains proteoglycans (e.g., decorin and biglycan, which seem to modulate cell activity, specially the collagen fibrillogenesis process) and glycoproteins (e.g., fibronectin and vitronectin, with a distinctive role in cell signaling) (4).

The inorganic component of the extracellular matrix plays an important role in the strengthening of the tissue and in ion storing. The main constituent is a highly substituted and crystalline calcium phosphate apatite, $Ca_{10}(PO_4)_6(OH)_2$,. Ionic substituents, such as magnesium, strontium, carbonate, citrate, and fluoride are broadly present and seem to modulate the biological response in the local microenvironment (4).

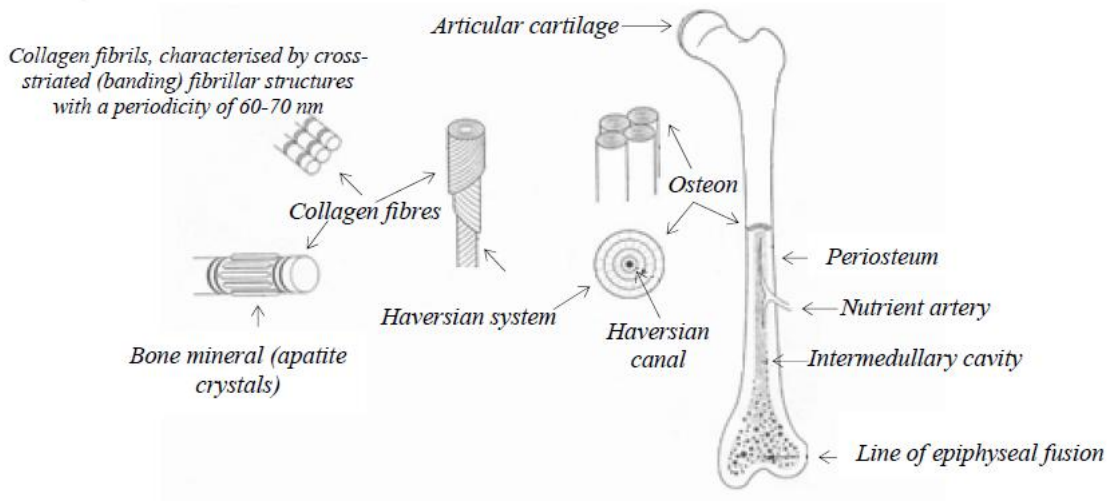


Figure 1.1 – Structural organization of the bone tissue. Adapted from (6).

1.1.2.2 – Bone Cells

The bone tissue is, in essence, composed of three main types of cells: osteoclasts, osteoblasts and osteocytes.

Osteoclasts are large, multi-nucleated cells (with 3 to 20 nuclei), derived from circulating hematopoietic progenitors that differentiate along the monocyte/macrophage lineage (Figure 1.2). They are commonly found in areas of bone resorption and reveal a high mobility (7). These cells mediate the resorption process via the release of powerful lysosomal enzymes and acids which digest protein and mineral components of the bone matrix (8). The key family of proteinases involved in osetoclastic bone degradation are cathepsins (i.e., cysteine proteinases) and matrix metalloproteinases (7).

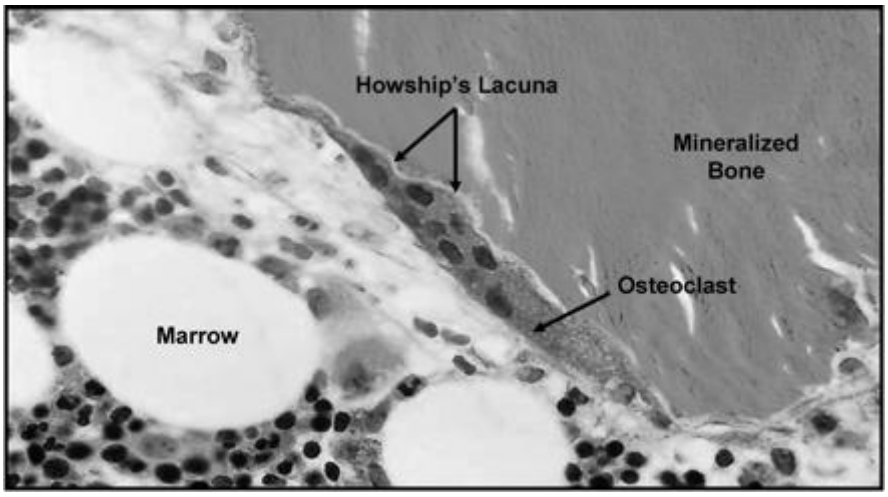


Figure 1.2 – Histological analysis of human bone revealing multinucleated osteoclasts forming resorption pits, i.e., Howship's lacunae (9).

Osteoblasts are derived from undifferentiated mesenchymal cells which can be found, among other locations, in the bone marrow, endosteum and periosteum (Figure 1.3). Osteoblasts, found on the bone surfaces undergoing remodeling events, are in charge for the bone formation, via the deposition of the extracellular matrix rich in collagenous and non-collagenous proteins, and by its subsequent mineralization process (10). Their morphology is cuboidal when they are active and becomes progressively flattened out when the metabolic index decreases (10). Within the cell, the cytoplasmic membrane is rich in alkaline phosphatase, an important enzyme for the initiation of the mineralization process (11). The expression of high levels of this enzyme is associated with a shift to a more differentiated state of the osteoblastic differentiation and usually determines the beginning of the mineralization process of an *in vitro* osteoblastic population (11). Following activation, osteoblasts can follow one of three pathways: they can (1) remain active cells, (2) become embedded by the extracellular matrix that is subsequently mineralized – turning to osteocytes or, (3) become relatively inactive and, eventually, develop into bone lining cells.

Osteocytes are estimated to comprise more than 90% of the bone cells in the adult skeleton. These cells derive, essentially, from mature osteoblasts trapped within the mineralized bone matrix and are responsible for its maintenance. They communicate with other bone cells, in the local microenvironment, through cannaliculi channels and gap junctions (10). Besides allowing cell-mediated exchanges of minerals

and nutrients, this network is also believed to sense mechanical deformation within bone that modulates the bone formation or resorption processes (12). The specific mechanical stimuli to which bone cells respond *in vivo* may be related to strain changes itself as well as strain generated changes to their fluid environment, which consequently affects the release of signaling molecules and growth factors that seem to regulate cell proliferation and differentiation (13).

Bone lining cells are broadly elongated and cover the vast majority of the bone surface within areas that are not being remodeled. They establish intercellular contacts by thigh junctions or cytoplasmic extensions, being interconnected with each other and also with osteocytes (14). Due to their abridged metabolic activity, they present fewer organelles than osteoblasts. Their activity within the bone metabolism has been a focus of debate, despite they seem to, on one way, prepare the bone microenvironment for osteoclast-mediated resorption and, on another way, regulate the deposition and mineralization of the extracellular matrix enrolling osteoblastic-like functions (14).

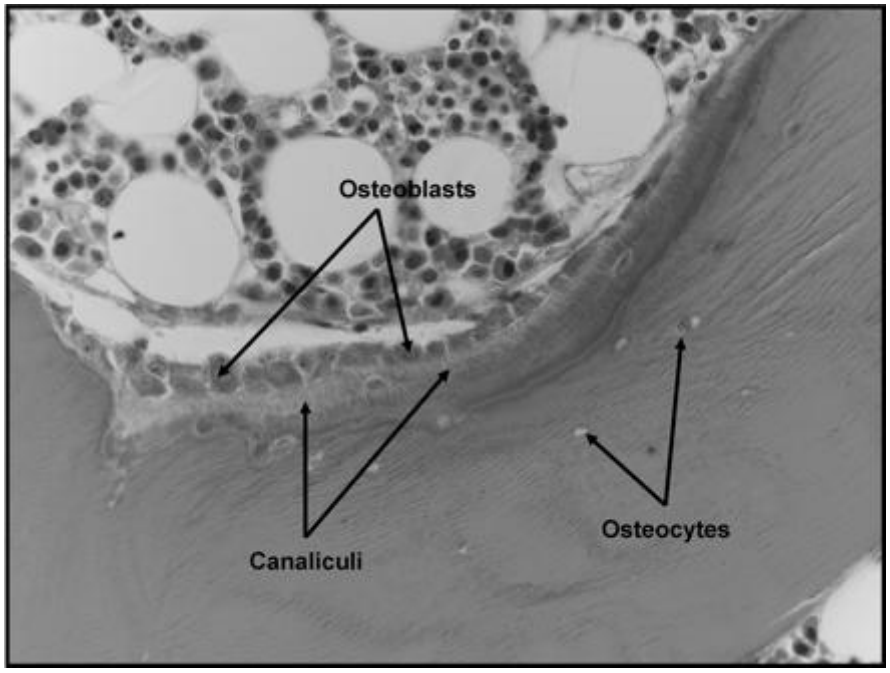


Figure 1.3 – Histological analysis of human bone revealing osteoblasts and osteocytes (9).

1.1.3 – Bone remodeling

Bone is a dynamic and metabolic active specialized connective tissue that is characterized by a continuous process of tissue renewal, i.e., remodeling (Figure 1.4). In this highly regulated and complex process, old bone is removed and new bone is produced in order to replace it. Each remodeling cycle is balanced such that the bone resorption rate equals the bone formation rate, and usually lasts about 90 to 130 days (15). The remodeling process is dependent upon the cellular interaction between osteoclasts and osteoblasts. These are further modulated by specific bio-molecular factors and signaling pathways which are crucial for bone metabolism and are of major interest in metabolic bone diseases (15).

The main purpose of bone remodeling is to prevent the accumulation of fatigue and repair microdamage and is initiated under certain environmental stimuli. Once the stimulus is present, the remodeling process can be divided into three main stages (16). The first stage of remodeling involves bone resorption that is mediated by osteoclasts. Osteoclast activation is ordinarily initiated by an interaction of hematopoietic precursors with cells of the osteoblastic lineage but may also be initiated by inflammatory cells, particularly T cells (16). Once osteoclasts are formed, there is a resorption phase of limited duration and a brief reversal phase (second phase), during which the bone surface is covered by mononuclear cells but bone formation does not begun immediately (16). Following, the formation phase (third phase) is then initiated, possibly by factors produced by the osteoclast or reversal cells, or released from the bone matrix. The formation phase, which is substantially longer than the first phases, involves the production and mineralization of matrix by progressive waves of osteoblasts. These then become flat lining cells, are embedded in the bone as osteocytes, or undergo apoptosis (16).

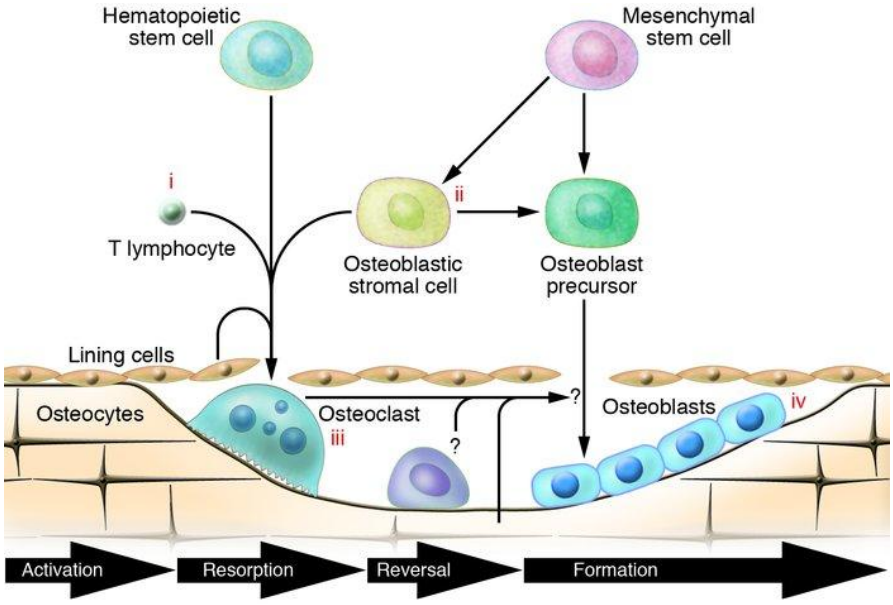


Figure 1.4 – Schematic representation of the bone remodeling cycle (16).

There are a number of local and systemic factors that regulate normal skeletal homeostasis. Bone metabolism and turnover are influenced by systemic hormones such as parathyroid hormone (PTH), calcitonin (CT), growth hormone (GH), 1,25-dihydroxyvitamin D3, thyroxine, estrogens, androgens and glucocorticoids, as well as mechanical stress (17). The refered hormones are of particular significance as they exert their effects on the bone via production of locally produced growth factors. Bone metabolism is also mediated by various local cytokines and growth factors, which are produced by bone cells, where other cells in the microenvironment act in an autocrine or paracrine manner to regulate the proliferation and differentiation of bone cell precursors, and to a lesser extent, the activity of mature osteoclasts and osteoblasts (17).

1.1.4 – Bone healing

Despite the intrinsic capacity of bone to regenerate and self-repair, this ability is limited to small fractures and therapeutic solutions need to be applied to promote bone healing in case of defects of crucial size (18). Moreover, bone replacement

therapies are needed to obviate bone deficiencies associated with reconstruction of congenital and traumatic skeletal defects, cosmetic procedures, degenerative disorders, surgical resection following neoplastic transformation, and chronic infection (18, 19). The worldwide market for bone replacement and repair therapies was estimated to be approximately €300 million in 2003, with a number of bone grafting procedures reaching 2.2 million in 2006 (19). Especially considering the burden of nonunion fractures, the need for bone tissue substitutes is constantly increasing due to the rapid growth of human population and extension of life expectancy. Today, the number of elderly reporting age-related fractures is estimated to be nearly 100 million per year worldwide, and this number is projected to massively increase over the next decades, with the number of elders estimated to be about 2 billion by 2050 (20).

In several clinical cases associated with bone deficiency, patient comfort and bone functionality can only be restored by surgical reconstruction. Current treatments for these patients are based on the transplantation of autogeneic and/or allogeneic bone grafts, or implantation of graft materials with osteoconductive and osteoinductive properties. Nonetheless, developed strategies display a reduced integration and lack of biological functionality and mechanical compliance, which usually lead to medium-to-long term implant failure and substitution, especially in patients with poor bone quality, associated for example with degenerative disorders. Therefore, there is therefore an urgent need to find alternative and effective solutions for the design of optimal bone regenerative therapies, which display high osteoinductive and angiogenic potential, biological functionality, large-scale availability, safety and reasonable cost.

1.2 - DIABETES

Diabetes mellitus (DM) encompasses a group of metabolic disorders of multiple etiologies, clinically characterized by chronic hyperglycemia, with disturbances of carbohydrate, fat and protein metabolism (21). Established effects seem to result from alteration in insulin secretion, action, or both (21). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (22).

Several pathogenic processes are involved in the development of diabetes. These seem to range from immune-mediated processes of autoimmune destruction of the β-cells of the pancreas with consequent insulin deficiency, to abnormalities that result in resistance to insulin action (21). Deficient insulin action results from inadequate insulin production and/or diminished tissue responses. Generally, these mechanisms may coexist in the same patient, and it may be unclear which abnormality, if either alone, is the primary cause of the hyperglycemia (23).

Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. Impairment of growth and susceptibility to certain infections may also accompany chronic hyperglycemia. Acute, life-threatening consequences of uncontrolled diabetes are hyperglycemia with ketoacidosis or the nonketotic hyperosmolar syndrome, which can progress to coma and, if untreated, death.

Long-term complications of diabetes include retinopathy, nephropathy, peripheral and autonomic neuropathy which may induce gastrointestinal, genitourinary, and cardiovascular symptoms. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial, and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes.

The vast majority of cases of diabetes can be divided into two broad etiopathogenetic categories (Figure 1.5). In type 1 diabetes, the cause is an absolute deficiency of insulin secretion, broadly associated with an autoimmune process, while

in type 2 diabetes, the cause is a combination of resistance to insulin action and an inadequate compensatory insulin secretory response (24, 25). In the latter category, a degree of hyperglycemia sufficient to cause pathologic and functional changes in various target tissues, but without clinical symptoms, may be present for a long period of time before clinical detection (25).

1.2.1 – Diabetes classification

1.2.1.1 - Type 1 diabetes (T1D)

This clinical form of diabetes, which accounts for around 5–10% of diabetes-affected individuals, previously encompassed by the terms insulin-dependent diabetes (IDD), type I diabetes, or juvenile-onset diabetes, results from a cell-mediated autoimmune destruction of the β-cells of the pancreas (26). Markers of β-cell immune destruction, broadly found in association with fasting hyperglycemia, include autoantibodies against islet cells, insulin, glutamic acid decarboxylase and tyrosine phosphatases IA-2 and IA-2β (26). Despite autoimmunity seem to be the predominant effector mechanism of T1D, its primary cause is not yet fully established. T1D seems to precipitate in genetically susceptible individuals, very likely as a result of an environmental trigger. Further, the disease has strong HLA associations, with linkage to the DQA and DQB genes, and is influenced by the DRB genes, which can either reveal a predisposing or protective phenotype (27).

In this form of diabetes, the rate of β-cell destruction is quite variable, being rapid in some individuals (mainly infants and children) and slow in others (mainly adults). Some patients, particularly children and adolescents, may present with ketoacidosis as the first manifestation of the disease. Others have modest fasting hyperglycemia that can rapidly change to severe hyperglycemia and/or ketoacidosis in the presence of infection or other stress. Still others, particularly adults, may retain residual β-cell function sufficient to prevent ketoacidosis for many years (28).

Some forms of type 1 diabetes have no known etiologies. Some of these patients have permanent insulinopenia and are prone to ketoacidosis, but have no evidence of autoimmunity. Although only a minority of patients with type 1 diabetes fall into this category, of those who do, most are of African or Asian ancestry. Individuals with this form of diabetes suffer from episodic ketoacidosis and exhibit varying degrees of insulin deficiency between episodes (29).

1.2.1.2 - Type 2 diabetes (T2D)

This form of diabetes, which accounts for around 90% of those patients affected with diabetes, previously referred to as non-insulin-dependent diabetes, type Ⅱ diabetes, or adult-onset diabetes, encompasses individuals who have insulin resistance and usually have relative insulin deficiency (30). Although the specific etiologies are not known, autoimmune destruction of β-cells does not seem to occur in these patients. Despite the fact, most affected individuals with this form of diabetes are obese, and obesity itself causes some degree of insulin resistance (31). This form of diabetes is rarely associated with ketoacidosis and it may go undiagnosed for many years, due to the gradually development of hyperglycemia that may not be severe enough for the patient to notice any of the classic symptoms (30). In this patients, insulin secretion is defective and usually insufficient to compensate for insulin resistance – which may improve with weight reduction and/or pharmacological treatment of hyperglycemia, but is seldom restored to normal (32). The risk of developing this form of diabetes increases with age, obesity, and lack of physical activity and it seems to occurs more frequently in individuals with hypertension or dyslipidemia, and its frequency varies in different racial/ethnic subgroups (33). It is often associated with a strong genetic predisposition, more so than is the autoimmune form of type 1 diabetes. However, the genetics of this form of diabetes are complex and not clearly defined (27).

1.2.1.3 – Other forms of diabetes

Several other forms of diabetes have been described and etiologically classified independently. Genetic defects of β cells originate a condition referred to as maturity-onset diabetes of the young and characterized by impaired insulin secretion with minimal or no defects in insulin action. They are inherited in an autosomal dominant pattern (34). Genetic defects in insulin action, essentially associated with the insulin receptor, have also been identified and proven to induce conditions that range from hyperinsulinemia and modest hyperglycemia to severe diabetes (34). Severe diseases of the exocrine pancreas, i.e., pancreatitis, trauma, infection, pancreatectomy, and pancreatic carcinoma may induce a significant reduction in β-cell mass that develops into a diabetic state (35). Diabetes may also develop secondary to several endocrinopathies, usually associated with an increased level of hormones that antagonize insulin action. This is the case of acromegaly, Cushing's syndrome, glucagonoma, pheochromocytoma, in which the growth hormone, cortisol, glucagon and epinephrine, respectively, are elevated (36). Many drugs were also shown to affect both insulin secretion and action. These drugs may not cause diabetes by themselves, but they may precipitate diabetes in individuals with insulin resistance (37). Viral infections have been associated with β-cell destruction. Congenital rubella, coxsackievirus B, cytomegalovirus, adenovirus, and mumps have been implicated in inducing certain cases of the disease (38).

1.2.1.4 - Gestational diabetes mellitus

Gestational diabetes mellitus (GMD) is defined as any degree of glucose intolerance condition, with onset or first recognition, during pregnancy (39). This definition applies regardless of whether insulin or only diet modification is used for treatment, or whether the condition persists after pregnancy. GMD is detected through the screening of pregnant women for clinical risk factors and, among at-risk women, testing for abnormal glucose tolerance that is usually mild an asymptomatic (39). Its etiology seems to share the common background of physiological and genetic abnormalities that characterize diabetes outside of pregnancy. In fact, women with

GDM are at high risk for having or developing diabetes when they are not pregnant (40).

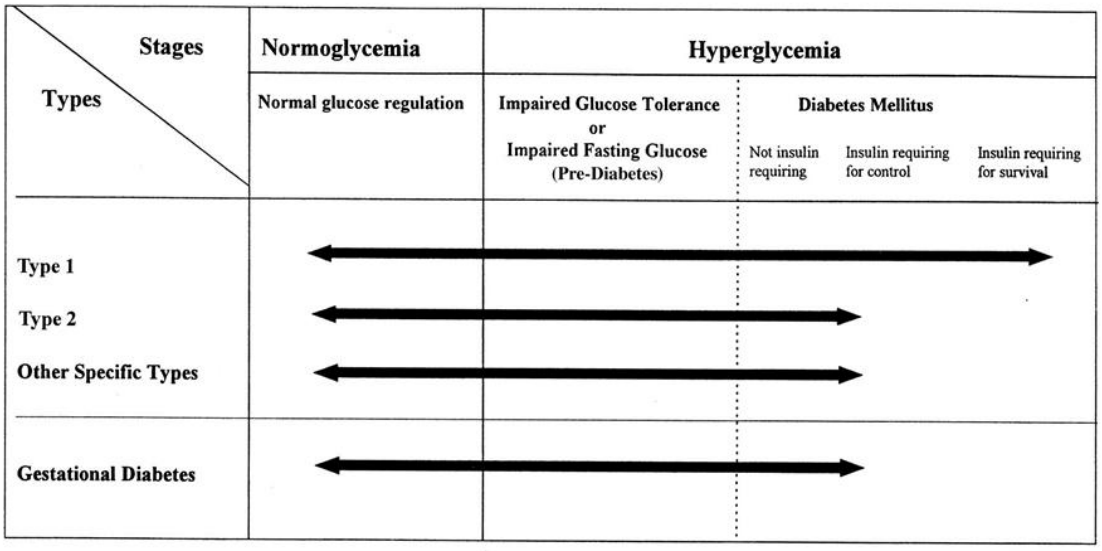


Figure 1.5 – Etiologic types and stages of glycemic disorders. Adapted from (41).

Some patients cannot be clearly classified as having type 1 or type 2 diabetes. Clinical presentation and disease progression vary considerably in every form of the disease. For instance, patients who otherwise have type 2 diabetes may present with ketoacidosis. Similarly, patients with type 1 may have a late onset and a slow (but relentless) progression of disease despite having features of autoimmune disease. Such difficulties in diagnosis may occur in children, adolescents, and adults. The true diagnosis may become more obvious over time.

1.2.2 – Diabetes diagnosis

For decades, the diagnosis of diabetes has been broadly based on the assessment of the plasma glucose level, either by the fasting plasma glucose (FPG) or by the 2-h value in the 75-g oral glucose tolerance test (OGTT) (42). More recently, several international organizations recommended the use of the A1C test to diagnose

diabetes, with a threshold of ≥6.5% (42). The A1C has several advantages to the FPG and OGTT, including greater convenience (since fasting is not required), evidence to suggest greater preanalytical stability, and less day-to-day perturbations during periods of stress and illness (43). These advantages must be balanced by greater cost, the limited availability of A1C testing in certain regions of the developing world, and the incomplete correlation between A1C and average glucose in certain individuals (42, 43). The currently accepted criteria for the diagnosis of diabetes are presented on Table 1.2.

Table 1.2 - Criteria for the diagnosis of diabetes. Adapted from (43)

A1C ≥6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
OR
FPG ≥126 mg/dL (7.0 mmol/L). Fasting is defined as no caloric intake for at least 8 h.*
OR
2-h plasma glucose ≥200 mg/dL (11.1 mmol/L) during an OGTT. The test should be performed as described by the WHO, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
OR
In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L)

*In the absence of unequivocal hyperglycemia, result should be confirmed by repeat testing.

1.2.3 – Diabetes complication

The clinical course and prognosis for diabetes-affected individuals is mostly influenced by duration of the condition and by the degree of metabolic control. This

relation was first established in the Diabetes Control and Complications Trial (DCCT) where strict control of blood glucose levels improved prognosis of type 1 diabetes through the reduction of the severity and occurrence of microvascular-related complications (44). Several subsequent clinical reports revealed similar data (45, 46).

Major microvascular complications of diabetes include retinopathy, nephropathy, and neuropathy. Retinopathy may apparent of the time of diagnosis, especially in type 1 diabetes. Furthermore, diabetes is the most commonly cause of adult blindness in developed countries, either due to retinopathy-related disease, cataracts development or even glaucoma (47). Also, individuals affected by diabetes are 17 times more prone to develop kidney disease and diabetes seems to be the major cause of end-stage renal failure in developed countries (48). Diabetic neuropathy is the most common complication of diabetes reaching 45–50% prevalence compared to 25–30% of retinopathy and 20% of nephropathy (49). Signs and clinical symptoms can mostly be attributed to functional and structural alterations detected in peripheral nerves.

Macrovascular disease, including coronary heart disease, cerebrovascular disease or stroke, and peripheral vascular disease are common causes of morbidity and mortality in diabetic patients, being these individuals 2 to 8 more prone to develop cardiovascular-related complications (50). Furthermore affected individuals have about twice the prevalence of hypertension and the incidence of stroke, compared to non-diabetic individuals (50).

Infection, ulceration, and limb amputation are serious and costly complications of diabetes, although many may be timely preventable.

These pathological alterations seem to develop by a direct damage to a particular subset of cells. While most cells are able to reduce the transport of glucose inside the cell when they are exposed to hyperglycemia - so that their internal glucose concentration stays constant – the cells damaged by hyperglycemia are those that cannot do this efficiently (51). Thus, diabetes selectively damages cells, like endothelial cells and mesangial cells, whose glucose transport rate does not decline rapidly as a result of hyperglycemia, leading to high glucose inside the cell. This is important in the

way that the explanation for what causes complications must involve mechanisms going on inside these cells, rather than outside.

The first such mechanism that was discovered was the polyol pathway and increased polyol pathway flux, described in peripheral nerve (52). Later on, the increased formation of advanced glycation end products (AGEs) was found do significantly contribute to cellular damage (53). The hyperglycemia-induced activation of protein kinase C (PKC) isoforms was also found to contribute to cellular disfunction (54), as well as the increased hexosamine pathway flux and consequent overmodification of proteins by N-acetylglucosamine (55). An overview of the cellular damage mediated by hyperglycemia is presented on Figure 1.6.

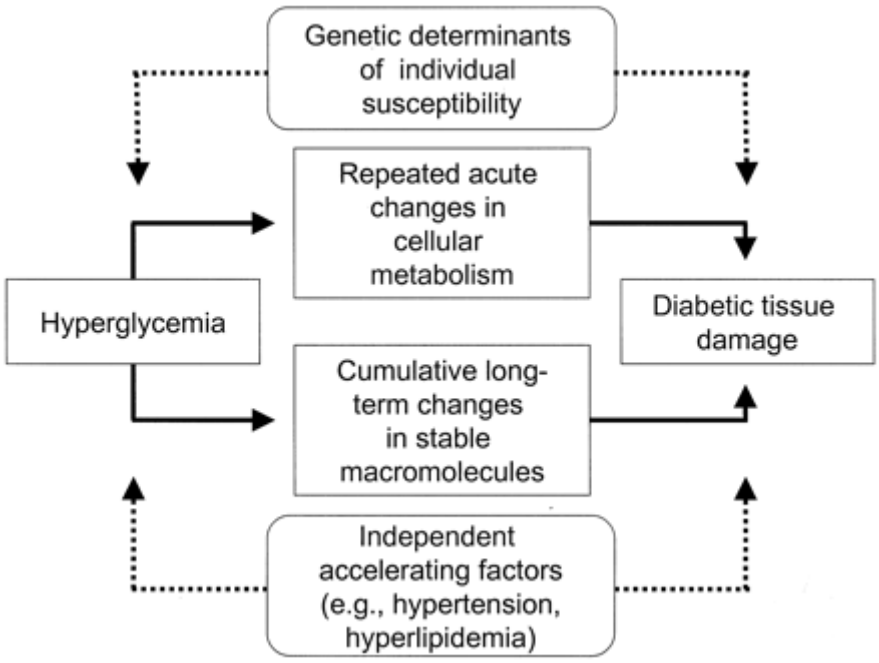


Figure 1.6 – Main characteristics of hyperglycemia-induced tissue damage. Adapted from (56).

1.2.4 – Diabetic osteopenia

The effect of diabetes mellitus on bone healing and metabolism has been reviewed extensively in literature (57-59). Nonetheless, current knowledge of the

mechanisms associated with the attained findings is rather incomplete. Reports are contradictory at times, depending on the type of diabetes studied, the metabolic control, the onset of the disease, in the case, the animal models, and time points used. For example, type 2 diabetes is a multidimensional condition and experimental models available to mimic it are far from representing the complete spectrum of the disease. Thus more contradictory findings are present when evaluating the effects of type 2 diabetes on bone metabolism and regeneration. Reports on the effect of this condition on bone mineral density, geometry and biomechanical properties have been sketchily unreliable, probably due to differences in the specific bone characteristics, as well as points in the disease progression, looked at throughout various studies (60). In contrast, type 1 diabetes is undoubtedly associated with bone loss. Studies have shown that about 20% of type 1 diabetic patients between ages 20-65 are osteoporotic and up to 40% are osteopenic (61-63). Type 1 diabetes is thus a simpler model to study the effects of hyperglycemia on bone.

The changes in mechanical and physical properties in bone under diabetic conditions are most likely correlated with bone turn over change (58, 64). Speculation on whether diabetes affects the bone resorption process is vastly discussed in literature. In that regard, systemic acidosis usually associated with diabetes and its renal complications, decreases blood and extracellular pH and thus could elicit a response in bone (48). However, even though decreased extracellular pH activates osteoclast activity and promotes bone resorption it does not necessarily imply that the overall osteoclast activity is increased in the diabetic bone. While some studies report increased bone destruction under hyperglycemic conditions there is a significant amount of evidence showing an overall decrease in osteoclast activity portrayed by reduced osteoclastogenesis and resorption rate in experimental diabetes (58, 65).

Contrary to bone resorption, bone formation seems to be the process in bone turnover that may be affected the most. Particularly, alterations in bone mineralization phase have been found consistently under hyperglycemia, despite the inconsistent findings attained with different experimental protocols. Regardless of this fact, there is an agreement that hyperglycemia reduces proliferation of osteoblasts and seems to alter the functional activity of these cells (66, 67).

In vivo studies support that bone turnover and mineralization are affected by diabetes. Bone mineralization was found to be reduced in experimentally induced diabetic animals (68). Complimentary studies examining bone turnover in humans found this process to be slower in type 2 diabetes-affected individuals, as demonstrated by decreased bone turnover markers in the serum (69). Curiously, decreased bone mineral apposition was reported to be accompanied by increased glomerular filtration rate (+70%) and calcium output (+568%), as well as reduced calcium reabsorption, possibly associated with a decrease in calcium-sensing receptors in the distal kidney (69). Serum osteocalcin levels are usually used as a bone turnover marker; these were broadly found to be decreased in diabetic conditions, as well as the levels of serum bone specific alkaline phosphatase (70, 71). Taken as a whole, these findings suggest that the bone formation process in particular, rather than bone resorption, is affected in diabetes.

In this way, osteoblastic cells seem to be affected at the genetic level in studies of uncontrolled diabetes. Osteoblast development master regulator runt-related transcription factor 2 (RUNX2) and some of its target genes such as matrix metalloproteinase-9 (MMP-9) that are important in matrix turnover, as well as osteocalcin, a known osteoblastic marker, were found to be significantly down regulated in diabetic-derived osteoblastic cells (67, 72).

1.2.5 – Animal models of diabetes

A variety of diabetes rodent models exist and share the common property with the attained manifestations of the human disease (73, 74). Some animal models become spontaneously diabetic, such as the non-obese diabetic (NOD) rat (75), while other diabetic models are pharmacologically induced by compounds such as alloxan, streptozotocin (STZ), vacor, dithizone, and 8-hydroxyquinone (76-78).

The streptozotocin-induced diabetes model has been extensively used, making it particularly useful for building upon and comparing results of other studies. STZ is a nitrosurea, derived from the microorganism *Streptomyces achromogenes*. It was first

introduced as an antibiotic and a drug for cancer treatment. Following absorption, It enters pancreatic β cells through glucose transporter 2 (GLUT2) channels in the plasma membrane and causes cellular toxicity and local immune responses that lead to hypoinsulinemia and hyperglycemia in animals (14). It is known to alkylate DNA by transferring its methyl group to the 6^{th} oxygen of the guanine base, causing damage to the molecule. Additionally, poly(ADP)-ribose polymerase is overactivated to repair STZ-caused DNA breaks, which results in the depletion of NAD^+, and subsequently ATP, within the cell. The drop in these molecules may result in inhibition of normal functions of the cell such as insulin production (79). In some models, especially rats, a single dose of STZ is effective at inducing T1 diabetes.

Depending on the length of the study, and thus the time for which the animals are hyperglycemic, STZ-induced rats exhibit several pathologies representative of chronic complications associated with DM. STZ-induced animals show a predictable blood glucose increase in response to the drug, due to the selective up-taken by pancreatic β-cells. Unfortunately other organs such as kidney and liver, that possess the GLUT2 receptor can also be affected (80).

Blood glucose response to STZ follows a predictable triphasic pattern of hyperglycemia. Beginning as early as 1 hour of administration of the drug, hypoinsulemia caused hyperglycemia develops. This stage lasts 2-4 hours and is characterized by morphological changes in the β-cells, such as swelling of the mitochondria and decrease in Golgi body area. After 4-8 hours, hypoglycemia sets in. This response can last for a few hours, is irreversible and can be severe enough to cause convulsions and even death. It is caused by an extreme release of insulin into circulation upon expulsion of vesicles and bursting of the cell membranes. Permanent hyperglycemia occurs 12-48 hours after STZ administration. It is characterized by degranulated and destroyed β-cells, debris of which is cleaned up by non-activated macrophages. Non β-cells remain intact, further demonstrating the specificity of the drug (76).

In addition to hyperglycemia, STZ-induced show symptoms of diabetic retinopathy, neuropathy, nephropathy-associated renal lesions, and as well,

demonstrate bone osteopenia and have been used in studies examining bone formation in diabetic conditions (69, 73, 81, 82).

1.3 - TETRACYCLINES

Tetracyclines were first discovered in 1948 as a natural fermentation product of *Streptomyces aureofaciens*. The first chemically purified tetracycline was chlortetracycline, developed around the 1950's (83). Currently, 3 groups of tetracyclines are available: natural-derived products, semisynthetic compounds, and chemically modified tetracyclines (CMTs) (83). Around three decades ago, this class of antimicrobial agents was unexpectedly found to inhibit collagenases and other host-derived matrix metalloproteinases (MMPs) in a mechanism independent of their antimicrobial activity (84). Accordingly, tetracyclines were thought to be of therapeutic use in a wide variety of conditions in which pathologically elevated MMPs activity and concomitant extracellular matrix proteins degradation are the hallmark of the disease pathogenesis (85). Several clinical conditions may be adequate targets for tetracyclines action including periodontitis, osteoporosis/osteopenia, rheumatoid arthritis, cancer invasion and metastasis, corneal ulceration, abdominal aortic aneurysms, inflammatory skin diseases and other immune-inflammatory conditions (85).

Early works have shown that collagenases and other MMPs may participate in the degradation of type I collagen, the major constituent of the organic matrix of bone, as well as the destruction of other connective tissue constituents. In this way, conducted preliminary i*n vivo* research has shown that tetracyclines were able to inhibit bone loss through inhibition of osteoclast-mediated bone resorption, but also by enhancing osteoblast activity, upregulation of type I collagen expression and increased bone formation, essencially in conditions of induced bone loss (86, 87).

Apart from the in vivo observations, later studies aimed to address the mechanisms associated with the net induced catabolism of bone metabolism. It was found that tetracyclines were able to increase the bone formation process via: (1) the enhancement of the expression of type I procollagen mRNA and collagen synthesis; (2)

partially restoring osteoblast activity and bone matrix formation and mineralization depressed during impaired conditions; and (3) increasing the number of recruited active osteoblasts (88). These actions, at least in part, seem to be mediated by the tetracyclines' ability to inhibit already-active collagenase and MMPs in the extracellular matrix – a mechanism that seems to be dependent of its Ca^{++} and Zn^{++} chelating ability; their capacity to prevent the conversion of latent procollagenase/pro-MMPs into active forms; and the down-regulation of the expression of procollagenase/pro MMPs, which comes in line with their inhibition of pro-inflammatory cytokines such as the interleukin-1, tumor necrosis factor alpha and interleukin-6, as well as phospholipase A2/prostaglandin E2 and inducible nitric oxide synthase (83, 89).

Because of the clinical applicability of these drugs, several translational studies aimed to demonstrate the clinical validity of this approach. A study in ovariectomized, osteopenic aged rats revealed the catabolic effects of minocycline in a model of high-turnover bone loss, representative of the changes naturally occurring in humans, soon after the menopause. Dynamic histomorphometric analysis revealed an increase osteoid surface, mineralizing surface, mineral apposition rate and bone formation rate, while a reduction in the eroded surface was also identified (90). Minocycline was found to promote an increase in the femoral bone mineral density and to induce positive effects on trabecular bone, in a process believed rely both on the increase in bone formation and a decrease in bone resorption. Another study using an animal model of ovariectomy-induced, estrogen-deficiency osteoporosis, in which CMT-8 (the chemically modified, nonantimicrobial analog of doxycycline), the increase of hitomorphometric parameters of both the alveolar bone the trabecular bone in the tibia, were verified (91).

These research approaches lead to the development of a low dose therapeutic regimen of doxycycline – a semi-synthetic tetracycline - shown to be non-antimicrobial, effective and safe. Within this therapeutic regimen, doxycycline was found to attain peak plasma levels of around 1 μg/mL, and to maintain mean plasma levels of around 0.5 μg/mL for several hours. These aforementioned properties made a compelling case for SDD as a novel pharmaceutical approach for the management of local, as well as systemic, osteoporosis or osteopenia conditions. A large two-year

human multi-institutional, double-blinded, placebo-controlled clinical trial was enrolled and demonstrated the beneficial therapeutic effects of SDD in postmenopausal women exhibiting both periodontitis (local bone loss) and osteopenia (systemic bone loss). In this long-term clinical trial, SDD was clearly anti-resorptive, as it was able to reduced alveolar bone height loss and alveolar bone density loss and reduced serum ICTP and CTX, biomarkers of bone resorption (92, 93). These data suggest the potential of this therapeutic approach to reduce the risk of conversion of bone loss associated with osteopenia into the more serious bone deficiency disease, osteoporosis.

2: RESEARCH HYPOTHESYS AND OBJECTIVES

2.1. – Research Hypothesis

It was hypothesized that due to the known effects of diabetes in the metabolism of the bone tissue, the induction of the diabetic condition could further impair the osteogenic potential of bone marrow-derived precursor cells. Furthermore, and since tetracycline were found to ameliorate the ostegenic function of osteoblastic cells, it was further hypothesized that the hindrances associated with the osteogenic capability of precursor cells could be lessen by doxycycline, in concentration attained in a low-dose regimen, with no antibacterial activity.

2.2 – Objectives

1) To establish STZ-induced hyperglycemia in rats, in order to institute an experimental diabetic model, representative of the human condition of diabetes mellitus;

2) To establish and characterize bone marrow-derived osteoblastic cultures from STZ and control animals, in the absence of exogenous osteogenic stimuli;

3) To address the effects of doxycycline, in concentrations representative of those attained in a SDD regimen, in the proliferation and functional activity of established control- and STZ-derived cultures;

4) To identify the most significant signaling pathways that modulate the doxycycline-mediated effects in the established osteoblastic cultures.

3: MATERIALS AND METHODS

3.1 - Materials

All cell culture chemicals and supplies were purchased from Merck and Sigma-Aldrich (St. Louis, MO) unless otherwise noted. All tissue culture flasks and plates were obtained from Corning (Corning, NY). Doxycycline was purchased from Sigma-Aldrich.

3.2 - Methods

3.2.1 - Animals

The animal study was performed under the authorization of the DGAV - *Direcção Geral de Alimentação e Veterinária* and observed the technical standards of protection of experimental animals, according to the Portuguese (Decree No. 1005/92) and European (Directive 2010/63) legislation.

In this study, 10 Wistar rats (Charles River, Wilmington, MA), 7-8 weeks old, with a body weight of around 250–300 g, were used. Animals were allowed to acclimatize for 1 week before the beginning of the study. Rats were housed in groups, in conventional type Ⅱ cages, on a controlled environment of temperature and humidity, in a 12h light/dark cycle. All animals were clearly identified using indelible ink. Dry feed and water was supplied ad libitum.

Diabetes was induced by a single intraperitoneal injection of streptozotocin (60 mg/kg, dissolved freshly in 10 mM citrate buffer, pH 4.5) to rats fasted for 12 hours (n=5); control rats received citrate buffer alone (n=5). Hyperglycemia was confirmed by measuring tail vein blood glucose levels with a glucometer (Accu-Chek GO, Roche Diagnostics, Portugal) 72 h after streptozotocin or buffer injection and expressed as milligrams per deciliter. Rats with blood glucose levels ≥300 mg/dl were considered to be diabetic.

6 weeks following STZ or vehicle administration, rats were euthanized by exsanguination following induction of general anesthesia by intracardiac puncture

under pentobarbital anesthesia (intraperitoneal injection of pentobarbital sodium 35 mg/kg).

3.2.2 – Establishment of the bone-marrow derived osteoblastic cultures

Cells were isolated using a modification of the method described by Maniatopoulus (94). Briefly, bone marrow was obtained from the tibias and femurs of male Wistar rats. Bones were excised aseptically, cleaned of soft tissues, and decontaminated in a solution of culture medium (α-MEM) containing 1000 $UI.mL^{-1}$ penicillin and 1000 $\mu g.mL^{-1}$ streptomycin, 30 minutes plus 30 minutes. Following, femoral or tibial epiphyses were cut off and diaphyses flushed out using with α-MEM supplemented with 10% FBS, ascorbic acid (50 $\mu g.mL^{-1}$), fungizone (2.5 $\mu g.mL^{-1}$), penicillin-streptomycin (100 $UI.mL^{-1}$ and 100 $mg.mL^{-1}$). Cell suspension was prepared by repeatedly pipetting up and down, and plated onto conventional tissue culture plates. After 7 days, the medium was changed to remove the non-adherent cells and, subsequently renewed every 2 days. On reaching around 75% confluence (~15 days), cells were released with trypsin 0,04% in 0.25% EDTA solution and plated at $2x10^4$ $cells/cm^2$ in culture plates.

Cultures were maintained in the previously described conditions for 12 days. Were applicable, doxycycline (0.5 or 1 $\mu g.ml^{-1}$) were added to the culture medium that was renewed every 2 days. Cell cultures were characterized for viability, proliferation and differentiation events.

3.2.3 – Characterization of bone-marrow derived osteoblastic cultures

3.2.3.1 – Optical microscopy

Cell cultures were regularly monitored by phase contrast optical microscopy, for the assessment of cell morphology and proliferation.

3.2.3.2 – Cell viability/proliferation (MTT assay)

Cell viability/proliferation of established cultures was estimated by the MTT assay, at days 1, 5, 8 and 12. This method is based in the reduction of the MTT salt [3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma®)] by the mitochondrial succinic dehydrogenase of proliferating cells, to a purple formazan product that accumulates in the cytoplasm. Briefly, 10 µl of MTT solution (5 mg/ml) were added to each 96-well culture dish, which contained 100 µl of complete medium, and incubated at 37 ^{o}C, in a humidified atmosphere of 95% air and 5% CO_2, for 4 hours. Following, the culture medium was decanted and the formazan salts were dissolved in 200 µL of dimethylsulphoxide. The absorbance was measured at 550 nm on an ELISA reader (Synergy HT, Biotek).

3.2.3.3 – Assessment of cell morphology

Established cell cultures morphology were characterized by confocal laser scanning microscopy (CLSM) following staining of cytoskeleton and nucleus counterstaining.

For CLSM assessment, cell cultures were fixed in 3.7% paraformaldehyde (15 minutes) and permeabilized with Triton 0.1%. Following, cultures were incubated with albumin (10 $mg.ml^{-1}$), in order to reduce non-specific staining. Cell cytoskeleton filamentous actin (F-actin) was visualized treating the cells with Alexa Fluor 488®-conjugated phalloidin (1:20 dilution in PBS, 20 minutes) and counterstained with propidium iodide (1 $\mu g.ml^{-1}$, 10 min) for cell nuclei labeling. Labeled cultures were mounted in Vectashield® and examined with a Leica SP2 AOBS (Leica Microsystems) microscopy.

3.2.3.4 – Total protein content

The total protein content was determined according to the Lowry method. In this method, the peptide bonds of proteins react with copper under alkaline conditions to produce the ionized form of copper (Cu^{+}), which then reacts with the Folin reagent - a mixture of phosphotungstic acid and phosphomolybdic acid in phenol. The product becomes reduced to molybdenum/tungsten blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue colour, which depends partly on the tyrosine and tryptophan content, and it can be detected colorimetrically by absorbance at 750 nm. The protocol for protein determination included the use of the following reagents:

A. 20 g $Na_2CO_3.L^{-1}$ 0.1 N NaOH

B. 0.1 g Na Tartrate + 0.05 g $CuSO_{4.5}$ H_2O + 10 mL dH_2O + 1 drop H_2SO_4

C. 50 mL reagent A + 1 mL reagent B

E. Phenol Reagent - 1 part Folin-Ciocalteu 2 N : 1 part water

Briefly, cultures were washed with PBS and incubated with 0.1% Triton, 1 h at 37ºC. 200 µL of the cell lysates were treated with 1.5 mL of reagent C, vortexed and incubated for 10 min. Afterward, 150 µL of reagent E was added and the samples were vortexed and incubated in the dark for 1 hour at room temperature. Finally, the absorbance was measured at 750 nm in 1 cm cuvettes, in a spectrophotometer (Jenway 6405).

A series of dilutions of 0.5 $mg.mL^{-1}$ bovine serum albumin in 0.1% Triton was used as standard. Results were expressed as micrograms per square centimeters ($\mu g.cm^{-2}$).

3.2.3.5 – Alkaline Phosphatase activity

Alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphate esters in an alkaline environment, resulting in the formation of an organic radical and inorganic phosphate. ALP activity can be determined by the hydrolysis of p-nitrophenyl phosphate into a yellow colored product, i.e., p-nitrophenol, which has a maximal absorbance at 405 nm, in an alkaline buffer solution. The rate of the reaction is directly proportional to the enzyme activity.

ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton) and assayed by colorimetric determination of the product p-nitrophenol at λ=405 nm in an ELISA reader (Synergy HT, Biotek). The hydrolysis of p-nitrophenyl phosphate (pH 10.3) was carried out for 30 min at 37°C. The reaction was stopped by the addition of NaOH 5M. The ALP activity of each sample was normalized to its protein concentration and the results were expressed as nanomoles of p-nitrophenol produced per minute per µg of protein (nmol/min^{-1} µg $protein^{-1}$).

3.2.3.6 – Histochemical assays

For the histochemical staining, cultures were first fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate buffer and rinsed with distilled water.

3.2.3.6.1 – Alkaline phosphatase staining

The presence of alkaline phosphatase was demonstrated by the enzymatic hydrolysis of sodium-naphthyl acid phosphate. The product reacted with a diazonium salt (Fast Blue RR) producing a highly colored insoluble azo dye, which precipitated at the site of enzyme activity. A positive reaction was identified by a staining, ranging from yellow, brown or black, according to an increase in the quantity of the enzyme.

At adequate time points, fixed cultures were first rinsed with water and then covered with a solution containing α-Napthyl acid phosphate and Fast Blue RR (2 mg. mL^{-1}) prepared in Tris 0.1M, pH 10. The cultures were kept in the dark for 1 h and then washed again to discontinue the reaction.

3.2.3.6.2 – Collagen staining

Collagen assay is based on the binding of a dye, siriusred F3BA (BDH, UK), to the triple helical collagen fibril.

The staining of the fixed cells was performed with 100 µl/well of 0.1% siriusred F3BA in saturated picric acid, for 1 h, at room temperature, under mild shaking. Thereafter, the dye solution was removed by suction and the stained cell layers extensively washed with 0.01 N hydrochloric acid to remove all non-bound dye. The cell morphology was photodocumented before dissolving the stain.

Following, the stained material was dissolved in 0.2 ml 0.1 N sodium hydroxide using a microplate shaker for 30 min at room temperature. The absorbance of the dyed solution was measured at 550 nm on an ELISA reader (Synergy HT, Biotek), against 0.1 N sodium hydroxide as a blank.

3.2.3.7 – Gene expression analysis

RT-PCR analysis was done on control and diabetic-derived cultures at days 5 and day 8. These were evaluated for the expression of GAPDH, alkaline phosphatase (ALP), bone morphogenic protein-2 (BMP-2), collagen type I (COL I), osteopontin, osteocalcin and osteoprotegerin. Total RNA was extracted using the NucleoSpin® RNA Ⅱ Kit (Macherey-Nagel) according to the manufacturer´s instructions. The concentration and purity of total RNA in each sample were assessed by UV spectrophotometry at 260 nm and by calculating the A260nm/A280nm ratio, respectively. RT-PCR was done using the Titan One Tube RT-PCR system (Roche® Applied Science), according to the manufacturer's instructions, for 28 cycles. RT

reaction mixtures consisted of extracted RNA, Titan RT-PCR buffer, dithiothreitol (DTT), deoxynucleoside triphosphate (dNTP), primers for each tested gene, avian myeloblastosis virus RT (AMV-RT) and water, in a total volume of 25 µl. Total RNA was reverse transcribed with to cDNA (30 minutes at 50ºC), which was then amplified with recombinant Taq-DNA polymerase at different annealing temperatures. For all the genes, the annealing temperature was 60ºC. The primers used are listed on Table 3.1. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide and semi-quantitatively assessed by densitometry with Image J® software. Data were expressed as normalized ratios by comparing the integrated density values for all tested genes with those for GAPDH.

Table 3.1 – Primers used on RT-PCR analysis.

Gene	5' Primer sequence	3' Primer sequence
GAPDH	CTGAGTATGTCGTGGAGTCTACTG	CATACTTGGCAGGTTTCTCCAG
ALP	CTCTCCAAGACGTACAACACCAAC	GGCTCAAAGAGACCTAAGAGGTAG
BMP-2	CCAGACTATTGGACACCAGGTTAG	CACAGAGTTTACCAGAGTCTGCAC
COL I	CTCAAGGTATTCGAGGTGACAAGG	CTTTCTAGAGCCTTCAGGAGTGAG
Osteopontin	GCTAGCTCATTGTCCTGCAGATAG	GACTAGAAGACTAGAAAGCGGCTG
Osteocalcin	AGAACAGACAAGTCCCACACAG	ATACTTTCGAGGCAGAGAGAGG
Osteoprotegerin	CCCTTGCCCTGACTACTCTTATAC	CCTCTCTACACTCTCTGCATTCAC

3.2.3.8 – Evaluation of the doxycycline-mediated effect on osteoblastic signaling pathways

First subcultured cells, of control and STZ animals, were maintained in the previously described conditions for 5 or 8 days. Were applicable, doxycycline (0.5 or 1 $\mu g.ml^{-1}$) were added to the culture medium that was renewed every 2 days.

For the assessment of the doxycycline-mediated effects on significant osteogenic pathways, specific inhibitors of each pathway were added to the culture medium, 24 hours previous to the culture endpoint. Inhibitors of the following pathways were used: mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinases (JNK), p38, nuclear factor kappa-light-chain-enhancer of activated B cells

(NF-kB), intracellular calcium, Wnt/β-Catenin and protein kinase C. Respectively, U0126, SP600125, SB202190, PDTC, KN-93, IWR-1 and GO6983, were used at 5 μM.

Effect of signaling pathways inhibition was assessed the time points in which the effects of doxycycline were more significant. Accordingly, changes associates with viability/proliferation were assessed at day 5, and modifications induced in the ALP activity were addressed at day 8.

3.2.4 – Statistical analysis

Data presented in this work are the result of three separate experiments performed in cell cultures established from different animals. For biochemical data (MTT reduction, total protein content and ALP activity) each point represents the mean ± standard error of 6 independent assays. Statistical analysis was done by one-way analysis of variance (ANOVA). P values ≤ 0.05 were considered significant.

4: RESULTS

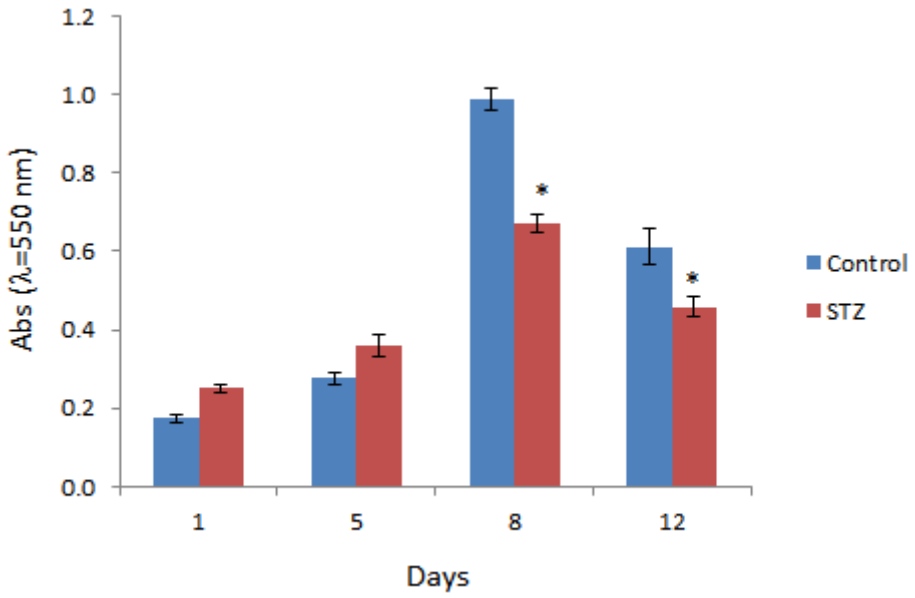
4.1. ESTABLISHMENT OF BONE MARROW-DERIVED OSTEOBLASTIC CULTURES FROM DIABETIC AND CONTROL ANIMALS

In the first part of this study, we aimed to establish a bone marrow-derived osteoblastic culture from diabetic and control animals and to compare the established cultures. Cultures established from control animals were referred as control and cultures established from streptozotocin-induced diabetic animals were refered as STZ.

Cells were cultured in control medium, in the absence of known osteogenic inducers, e.g., dexamethasone and β-glycerophosphate, in order to access the intrinsic ostegenic potential of each cellular population in the absence of any differentiation stimuli. First sub-cultured cells were assessed for cell morphology, cell viability/proliferation and osteoblastic differentiation throughout the 12 days of culture time.

4.1.1 – Cell viability/proliferation

The MTT assay was the method used for assessing cell viability/proliferation (Graphic 4.1). Cultures established from control animals presented an increasing MTT value from de first day of the culture till day 8, diminishing afterwards. Cultures established from STZ animals revealed a similar time-course growth pattern - MTT reduction values increased during the first week of the culture, diminishing subsequently. STZ cultures revealed a slight increase of the MTT reduction values at day 1 and 5, comparing to control, although no significantly differences were found. At day 8 and 12, MTT reduction values for STZ cultures were significantly reduced, in comparison to control.



Graphic 4.1 – Cell viability/proliferation of rat bone marrow-derived cell cultures, established for 12 days, from control and STZ-induced diabetic animals. * - significantly different from control ($p < 0.05$).

4.1.2 – Cell morphology

Cell morphology was addressed by confocal laser scanning microscopy and representative images are shown in Figure 4.1. For control cultures, at day 3, an elongated fibroblast-like morphology, characteristic of osteoblastic cells in culture, was evident, with expanded cytoplasm and an adequate nuclear organization. Furthermore, several cell-to cell contacts were established. The cells proliferated adequately and, at day 5, a large area of the culture surface was already covered by cells. At day 8, an organized flattened sheet of continuous cell layers were verified, a behavior similar to the one of the cultures established at day 12. The cellular behavior of the STZ-derived cultures in this time course was similar.

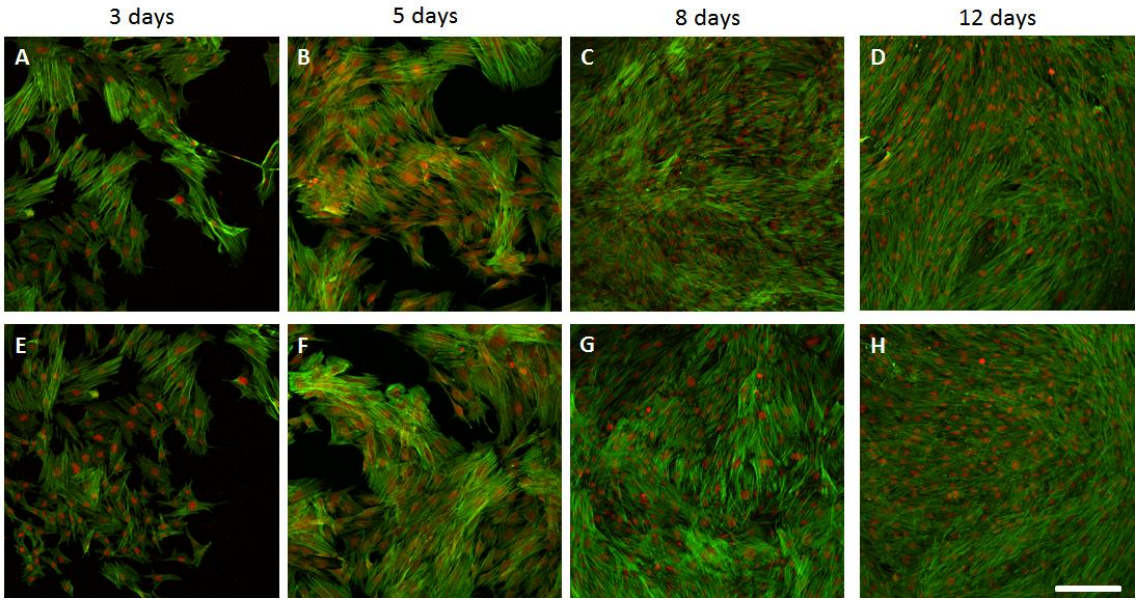
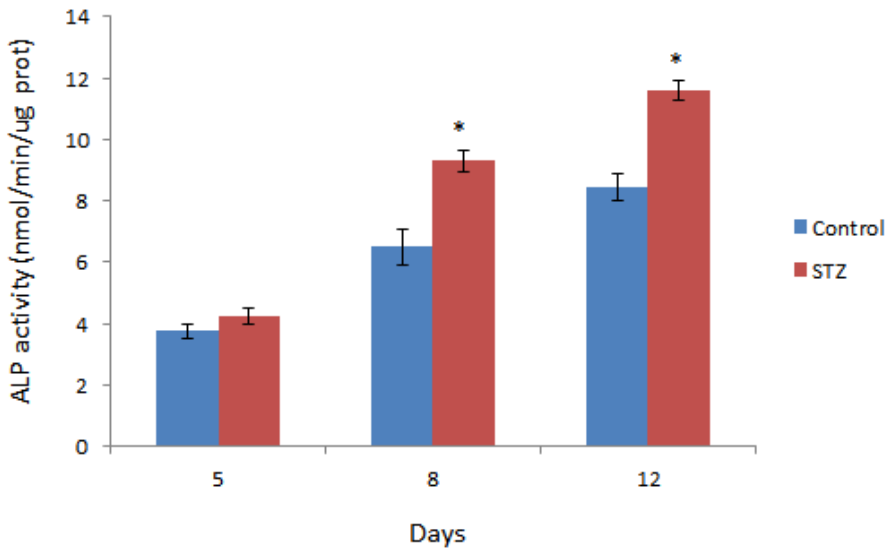


Figure 4.1 – Confocal laser scanning microscopy imaging of rat bone marrow-derived cell cultures, established for 12 days, from control and STZ-induced diabetic animals. Cytoskeleton was stained in green and nucleus counterstained in red. A to D corresponds to representative control cultures and E to H corresponds to representative STZ cultures. Scale bar corresponds to 200 μm.

4.1.3 – Alkaline phosphatase activity

Results concerning ALP activity, normalized by total protein content, are shown in Graphic 4.2. Within these experimental conditions, alkaline phosphatase activity increased gradually with culture time in all the experimental conditions. Despite the similar activity verified at day 5 of culture for both control and STZ cultures, significant differences were attained at day 8 and 12 of culture, with a significant increase of the ALP activity in STZ.



Graphic 4.2 – Alkaline phosphatase activity of rat bone marrow-derived cell cultures, established for 12 days, from control and STZ-induced diabetic animals. * - significantly different from control ($p < 0.05$).

4.1.4 – Alkaline phosphatase staining

Both control and STZ cultures presented a similar behavior regarding the production and distribution of ALP. In Figure 4.2, a representative appearance of alkaline phosphatase staining and distribution, through the 12 days of culture, is shown. Grown cells show a homogenous ALP staining at day 5, in which a tendency for the formation of high density staining clusters can already be acknowledged. This organization is more easily identified at day 8 and 12, in which aggregates of dark brown/black staining can be recognized.

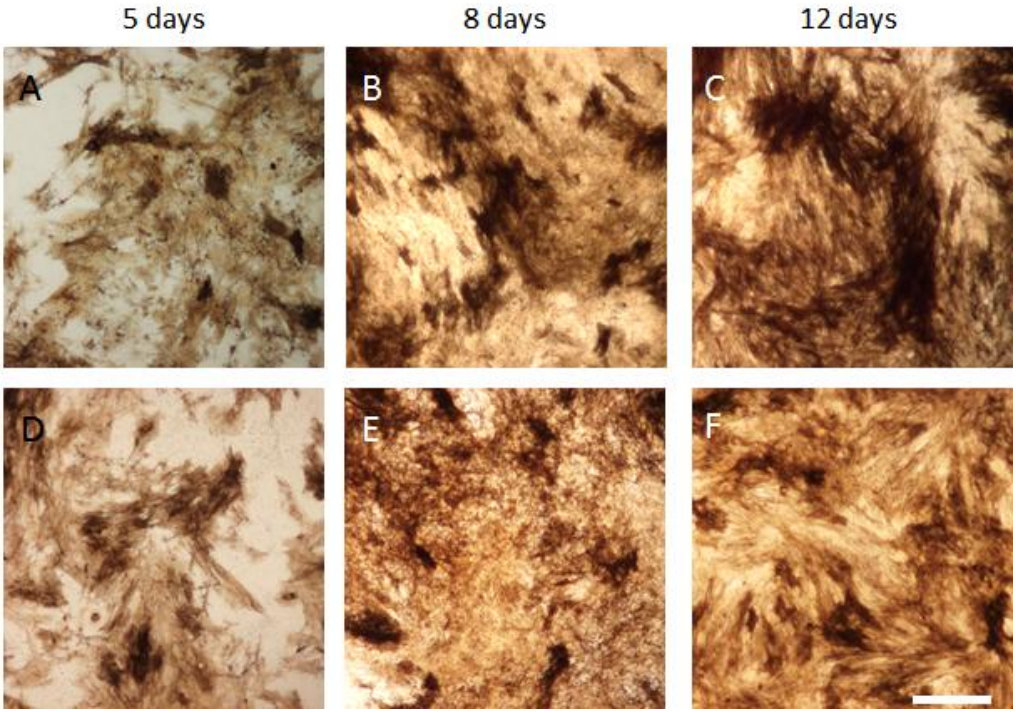


Figure 4.2 - Alkaline phosphatase staining of rat bone marrow-derived cell cultures, established for 12 days, from control and STZ-induced diabetic animals. A to C corresponds to representative control cultures and D to F corresponds to representative STZ cultures. Scale bar corresponds to 300 μm.

4.1.5 – Collagen staining

Regarding collagen content of the established control and STZ cultures, representative histochemical staining and quantitative data are presented on Figure 4.3 and Graphic 4.3, respectively. In control cultures, an increasing staining intensity is verified throughout the assayed culture period. At day 8 and 12, discrete areas of increased staining, arranged in clusters, is verified. In STZ cultures, an increasing intensity of the staining is also verified throughout the culture period but there are no evidences of the organization in clusters. Qualitative data reveal a similar collagen content for both cultures at day 5, and a tendency for a reduced collagen production in comparison to control for STZ cultures, which attained statistical significance at day 12.

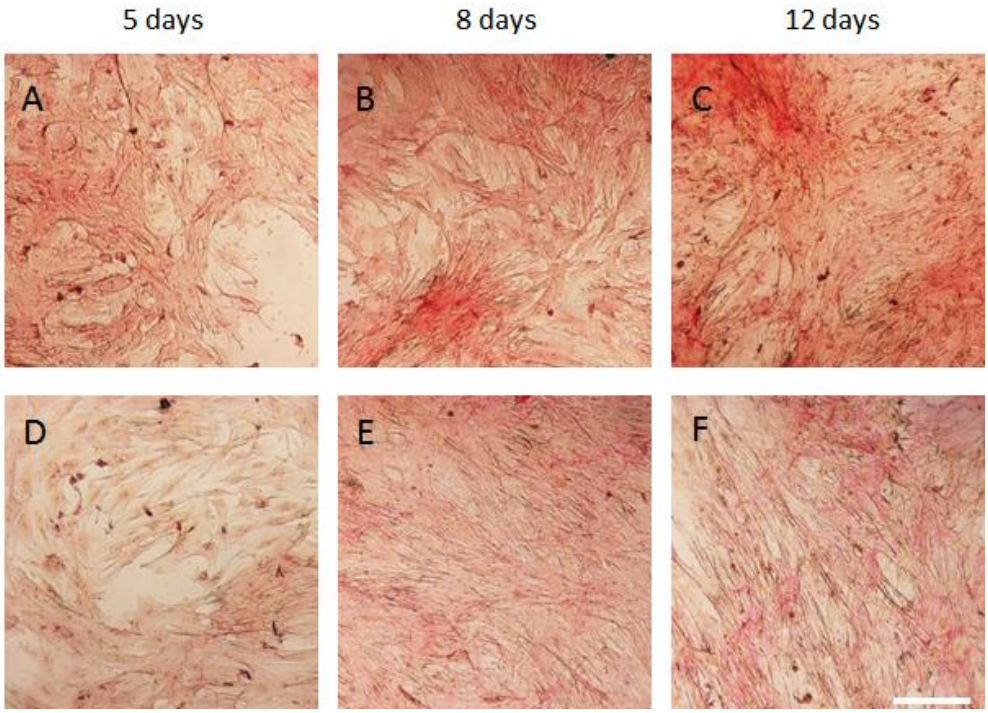
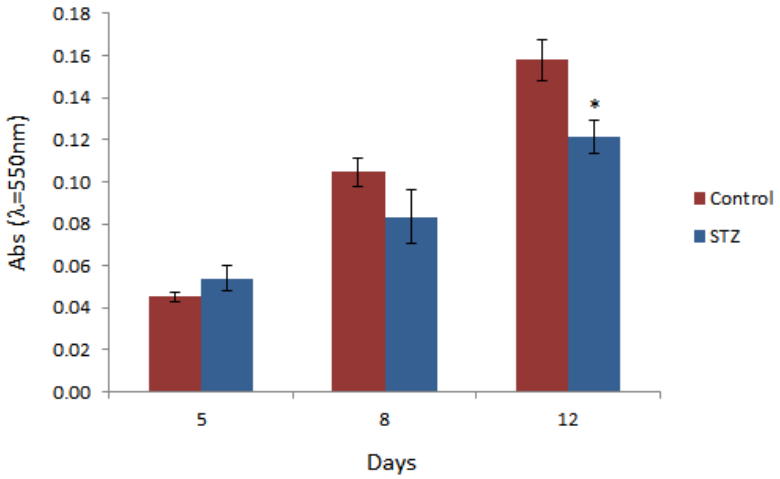


Figure 4.3 – Collagen staining of rat bone marrow-derived cell cultures established for 12 days, from control and STZ-induced diabetic animals. A to C corresponds to representative control cultures and D to F corresponds to representative STZ cultures. Scale bar corresponds to 750 μm.

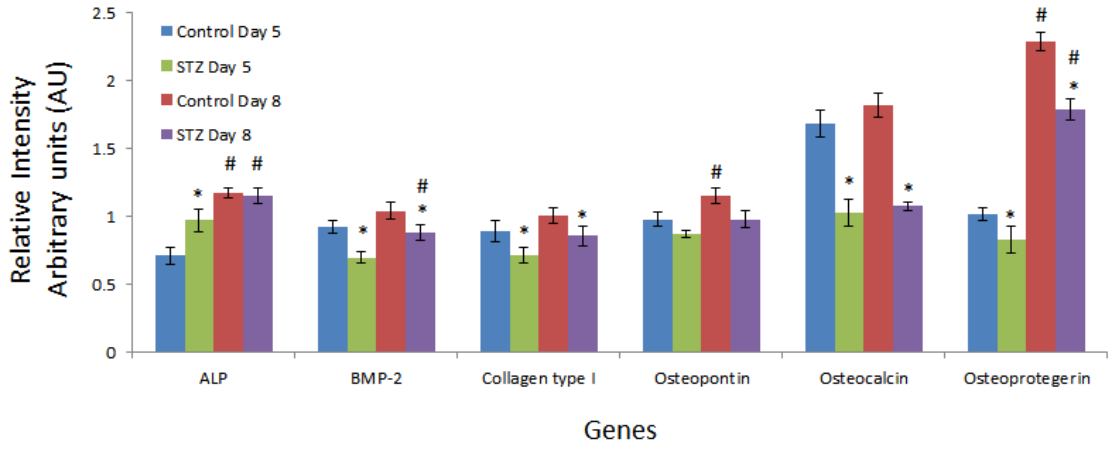


Graphic 4.3 – Total collagen content of rat bone marrow-derived cell cultures established for 12 days, from control and STZ-induced diabetic animals. * - significantly different from control ($p < 0.05$).

4.1.6 – Expression of osteogenic-related markers

RT-PCR analysis showed that both cultures expressed high levels of significant osteogenic markers. Results are shown in Graphic 4.4.

Several differences were attained at both day 5 and day 8 of cultures. At day 5, control cultures expressed reduced levels of alkaline phosphatase, while the levels of of BMP-2, collagen type I, osteocalcin and osteoprotegerin were elevated. There were no differences found regarding the expression of osteopontin at this time point. At day 8, control cultures expressed higher levels of BMP-2, collagen type I, osteocalcin and osteoprotegerin, as comparing to STZ cultures, while there were no differences found between the expression of ALP and osteopontin.



Graphic 4.4 – RT-PCR gene expression of ALP, BMP-2, collagen type I, osteopontin, osteocalcin and osteoprotegerin, in rat bone marrow-derived cell cultures established at day 5 and 8, from control and STZ-induced diabetic animals. The PCR products were subjected to a densitometric analysis and normalization to the corresponding GAPDH value. * - significantly different from control; # - significantly different from day 5 ($p < 0.05$).

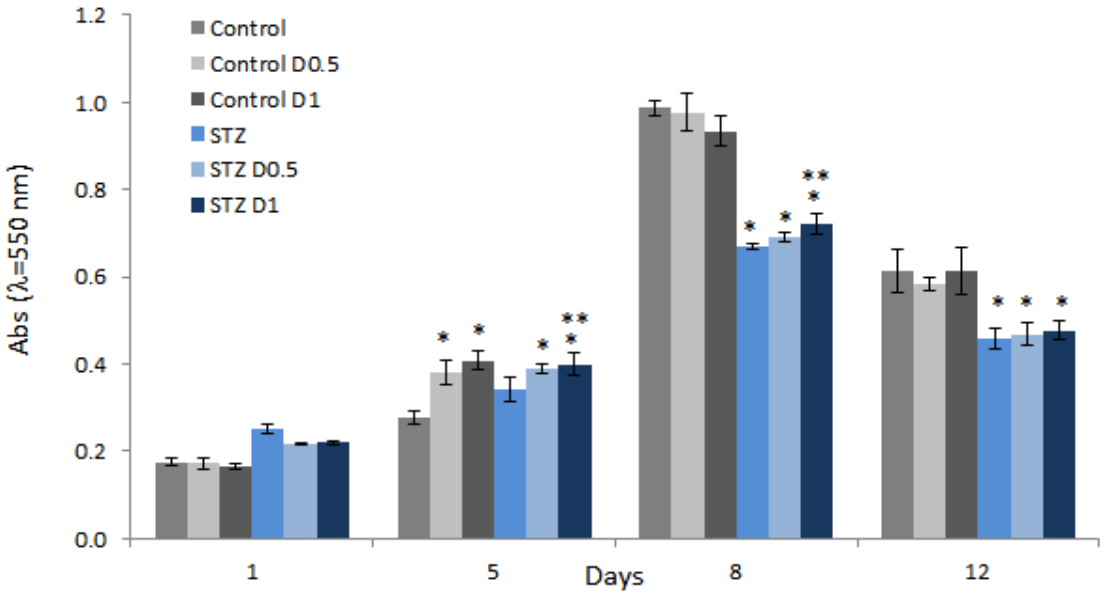
4.2 – ASSESSMENT OF THE EFFECT OF DOXYCYCLINE IN THE FUNCTIONAL ACTIVITY OF BONE MARROW-DERIVED OSTEOBLASTIC CULTURES FROM DIABETIC AND CONTROL ANIMALS

In the second part of this study, we aimed to address the effects of doxycycline in the proliferation and differentiation events of established bone marrow-derived osteoblastic culture from diabetic and control rats.

Cells were cultured in control medium, in the absence of known osteogenic inducers, in the presence of doxycycline at 0.5 μg/ml and 1 μg/ml. First sub-cultured cells were characterized throughout the 12 days of culture time.

4.2.1 - Cell viability/proliferation

The MTT assay was the method used for assessing cell viability/proliferation (Graphic 4.5). Cultures established from control animals presented an increasing MTT value from de first day of the culture till day 8, diminishing afterwards. Cultures established from STZ animals revealed a similar time-course growth pattern - MTT reduction values increased during the first week of the culture, diminishing subsequently. The addition of doxycycline to the culture medium, either at 0.5 μg/ml or 1 μg/ml, induced significantly the viability/proliferation of the control and STZ cultures at day 5, as comparing to the established cultures in the absence of doxycycline. At day 8 and 12, STZ cultures presented MTT reduction values inferior to control. The addition of doxycycline to the culture medium did not modify, significantly, this cellular behavior.



Graph 4.5 – Cell viability/proliferation of rat bone marrow-derived cell cultures established for 12 days, from control and STZ-induced diabetic animals. Culture were grown in the absence (Control, STZ) and presence of doxycycline 0.5 μg/ml (Control D0.5, STZ D0.5) or doxycycline 1 μg/ml (Control D1, STZ D1). * - significantly different from control ($p \leq 0.05$). ** - significantly different from STZ ($p < 0.05$).

4.2.2 – Cell morphology

The time course behavior of control (Figure 4.4) and STZ-derived cultures (Figure 4.5.), grown in the absence or presence of doxycycline was addressed by CLSM and is represented on Figure 4.4 At early time points cells reported a characteristic osteoblastic morphology and evident cell-to cell contacts. Cells proliferated actively and attended cell multilayer organization at later time points (day 8 and 12). Doxycycline, at both concentrations did not seem to modulate the cellular behavior. Also, the cellular behavior of the STZ-derived cultures (Figure 4.5), both in the absence and in the presence of doxycycline was found to be similar to control.

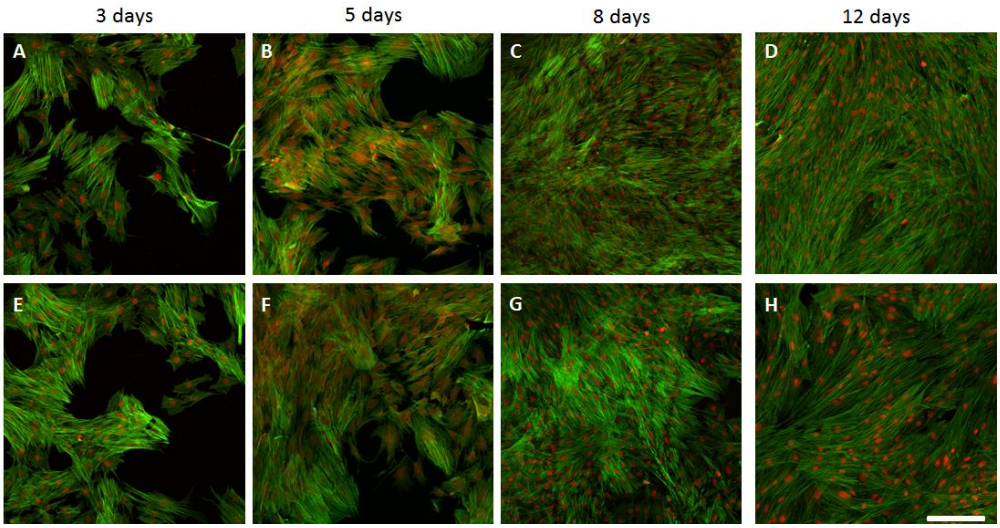


Figure 4.4 – Confocal laser scanning microscopy imaging of rat bone marrow-derived cell cultures, established for 12 days, from control animals. Cytoskeleton was stained in green and nucleus counterstained in red. A to D corresponds to representative control cultures in the absence of doxycycline and E to H corresponds to representative control cultures in the presence of 1 μg/ml doxycycline. Scale bar corresponds to 200 μm.

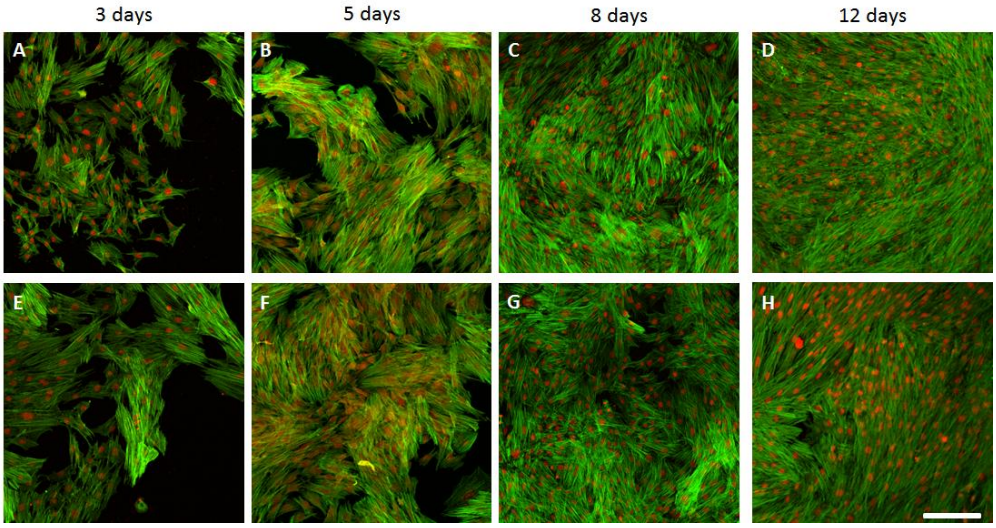
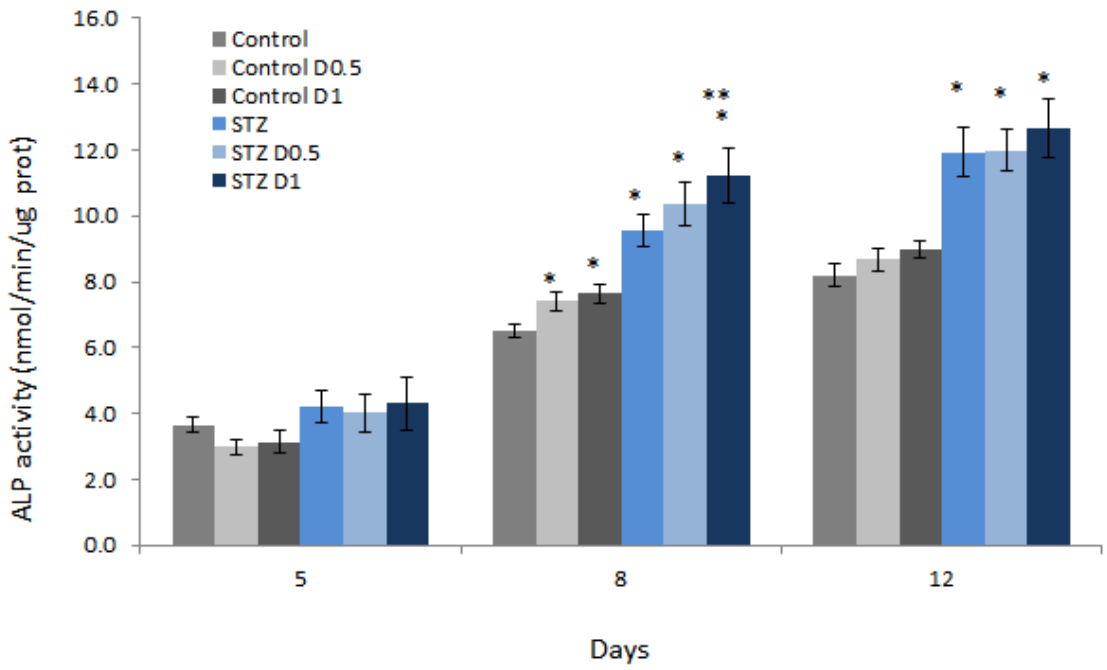


Figure 4.5 – Confocal laser scanning microscopy imaging of rat bone marrow-derived cell cultures, established for 12 days, from STZ animals. Cytoskeleton was stained in green and nucleus counterstained in red. A to D corresponds to representative STZ cultures in the absence of doxycycline and E to H corresponds to representative STZ cultures in the presence of 1 μg/ml doxycycline. Scale bar corresponds to 200 μm.

4.2.3 – Alkaline phosphatase activity

Results concerning the ALP activity of cultures established from control and STZ animals, either in the absence and presence of doxycycline, are shown in Graphic 4.6. Within these experimental conditions, alkaline phosphatase activity increased gradually with culture time in all the experimental conditions. At day 5 of the culture, there were no significant differences between the experimental conditions, in the presence or absence of doxycycline. At day 8, both concentrations of doxycycline, 0.5 μg/ml and 1 μg/ml, induced the ALP activity, as comparing to the respective culture in the absence of tetracyclines. The condition of STZ D1 revealed the highest expression of ALP that was significantly different from STZ alone, at this culture time point. At day 12, significant differences were found between control and STZ cultures, with the later expressing higher ALP activity. Doxycycline, at both concentrations, was not found to significant alter the enzyme activity at this time point.



Graphic 4.6 - Alkaline phosphatase activity of rat bone marrow-derived cell cultures established for 12 days, from control and STZ-induced diabetic animals. Cultures were grown in the absence (Control, STZ) and presence of doxycycline 0.5 μg/ml

(Control D0.5, STZ D0.5) or doxycycline 1 μg/ml (Control D1, STZ D1). * - significantly different from control ($p \leq 0.05$); ** - significantly different from STZ ($p \leq 0.05$).

4.2.4 – Alkaline Phosphatase staining

Representative of ALP staining images are shown in Figure 4.6, 4.7 and 4.8. An increased intensity was verified for all experimental conditions throughout the culture time. At day 5, the appearance of dark nodular aggregates could be identified in all experimental conditions and, at later time points, day 8 and 12, an increased staining, covering the vast majority of the culture could be acknowledged. This is in accordance with the high ALP activity of the established cultures. No significant differences could be qualitatively appreciated for different experimental conditions.

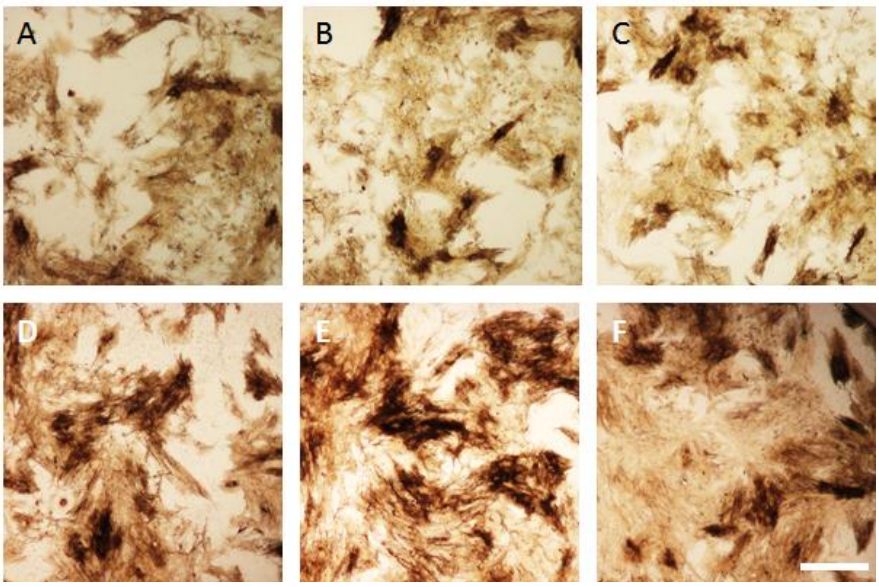


Figure 4.6 – Alkaline phosphatase staining of rat bone marrow-derived cell cultures established at day 5. A – control cultures; B – control cultures with doxycycline 0.5 μg/ml; C – control cultures with doxycycline 1 μg/ml; D – STZ cultures ; E – STZ cultures with doxycycline 0.5 μg/ml; F – STZ cultures with 1 μg/ml. Scale bar corresponds to 300 μm.

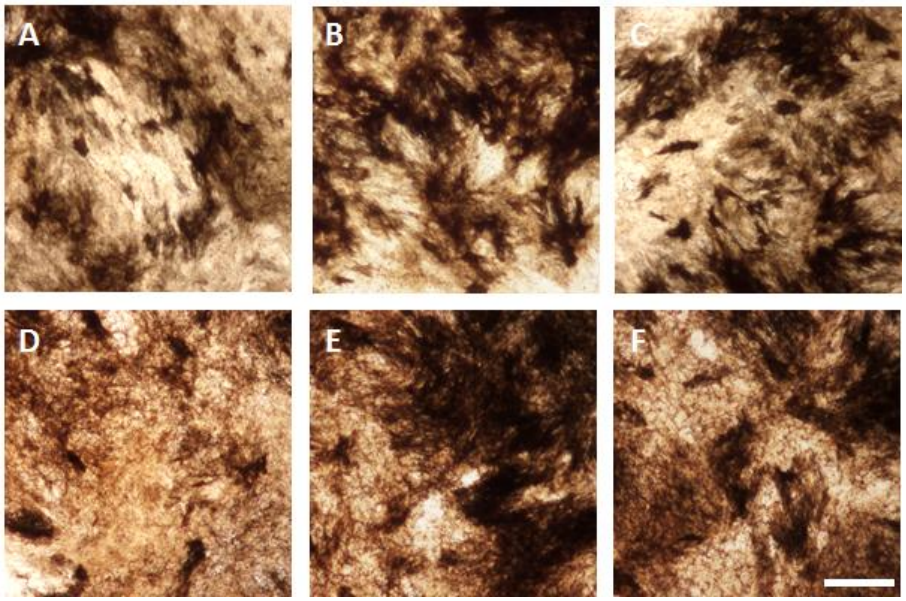


Figure 4.7 – Alkaline phosphatase staining of rat bone marrow-derived cell cultures established at day 8. A – control cultures; B – control cultures with doxycycline 0.5 μg/ml; C – control cultures with doxycycline 1 μg/ml; D – STZ cultures ; E – STZ cultures with doxycycline 0.5 μg/ml; F – STZ cultures with 1 μg/ml. Scale bar corresponds to 300 μm.

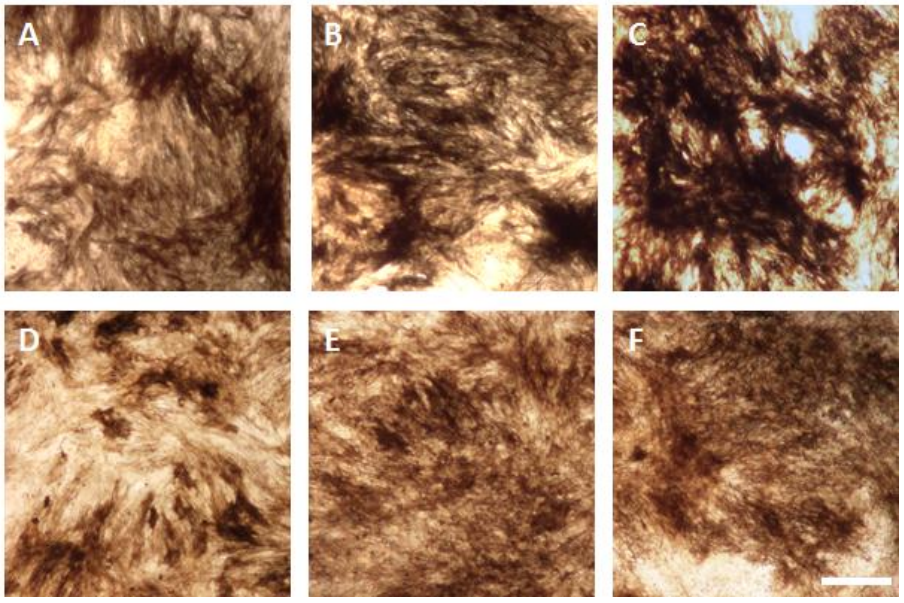


Figure 4.8 – Alkaline phosphatase staining of rat bone marrow-derived cell cultures established at day 12. A – control cultures; B – control cultures with doxycycline 0.5 μg/ml; C – control cultures with doxycycline 1 μg/ml; D – STZ cultures ; E – STZ cultures with doxycycline 0.5 μg/ml; F – STZ cultures with 1 μg/ml. Scale bar corresponds to 300 μm.

4.2.5 – Collagen staining

Regarding the assessment of collagen staining, representative image of all experimental conditions are shown in Figure 4.9, 4.10 and 4.11. Quantitative analysis is shown in Graphic 4.7. At day 5, a tendency for an increased staining could be visualized in STZ cultures, in comparison to control cultures, despite that doxycycline did not seem to significantly alter this parameter. At day 8 and 12, a significantly more intense staining could be attained in all experimental conditions nonetheless evident differences between conditions were difficult to access. Qualitative analysis of the dissolved staining revealed that, at day 5, STZ cultures, both in the presence and absence of doxycycline, had an increased staining comparing to control. At day 8, STZ cultures reported a significantly reduced value, situation that was mitigated by both concentrations of doxycycline. At day 12 of the culture, doxycycline was also shown to increase the collagen staining, in a significant way, in both control and STZ cultures.

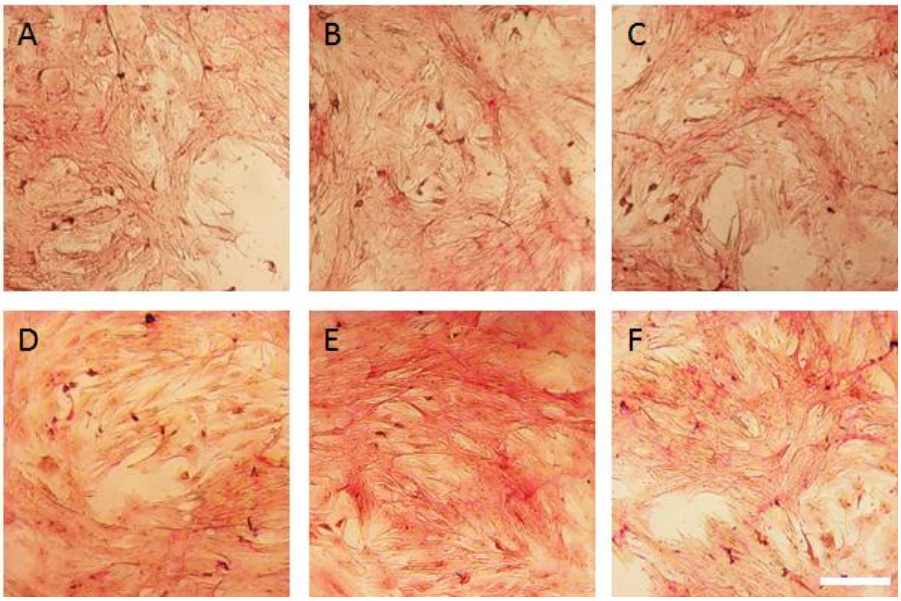


Figure 4.9 – Collagen staining of rat bone marrow-derived cell cultures established at day 5. A – control cultures; B – control cultures with doxycycline 0.5 μg/ml; C – control cultures with doxycycline 1 μg/ml; D – STZ cultures ; E – STZ cultures with doxycycline 0.5 μg/ml; F – STZ cultures with 1 μg/ml. Scale bar corresponds to 750 μm.

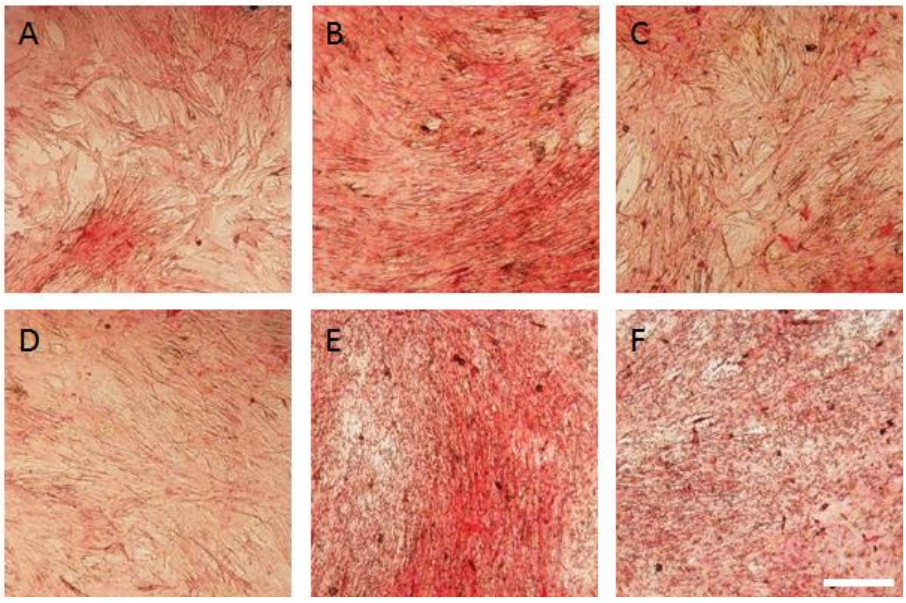


Figure 4.10 – Collagen staining of rat bone marrow-derived cell cultures established at day 8. A – control cultures; B – control cultures with doxycycline 0.5 μg/ml; C – control cultures with doxycycline 1 μg/ml; D – STZ cultures ; E – STZ cultures with doxycycline 0.5 μg/ml; F – STZ cultures with 1 μg/ml. Scale bar corresponds to 750 μm.

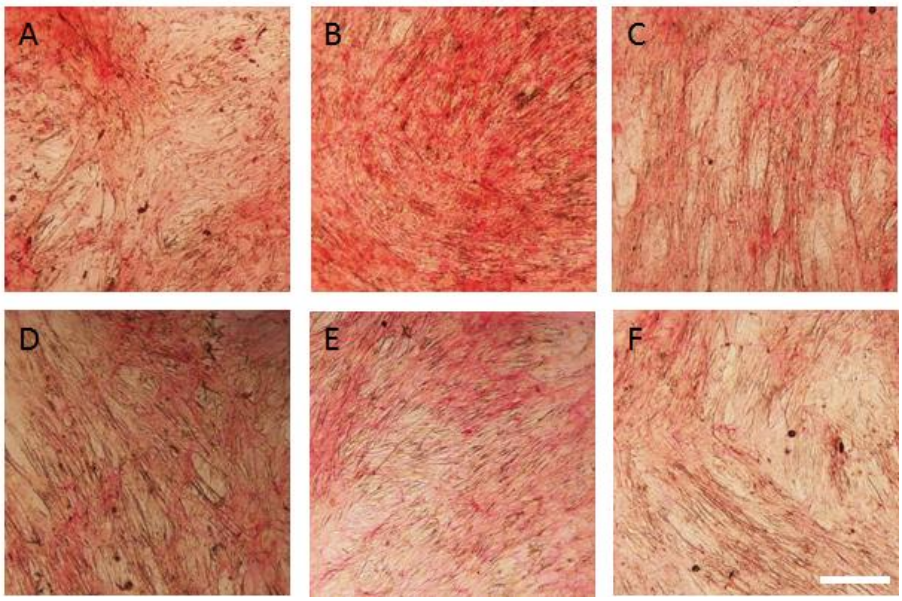
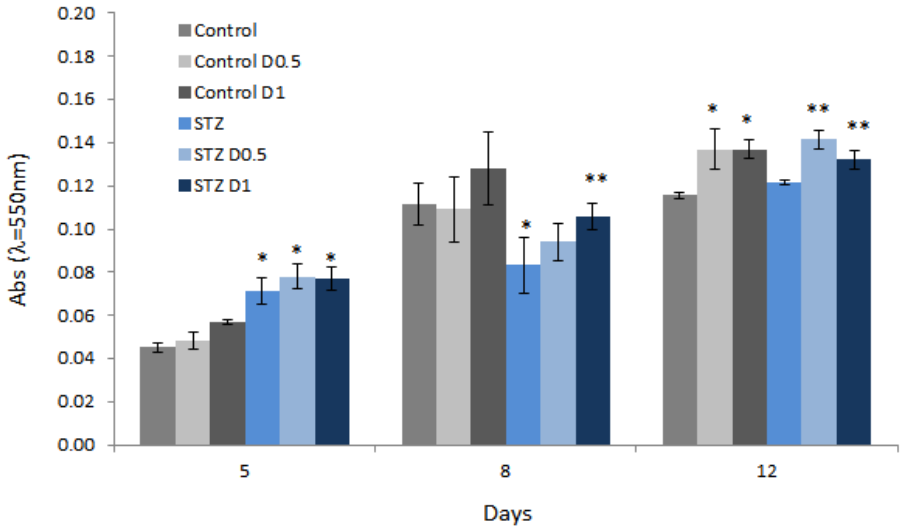


Figure 4.11 – Collagen staining of rat bone marrow-derived cell cultures established at day 12. A – control cultures; B – control cultures with doxycycline 0.5 μg/ml; C – control cultures with doxycycline 1 μg/ml; D – STZ cultures ; E – STZ cultures with doxycycline 0.5 μg/ml; F – STZ cultures with 1 μg/ml. Scale bar corresponds to 750 μm.



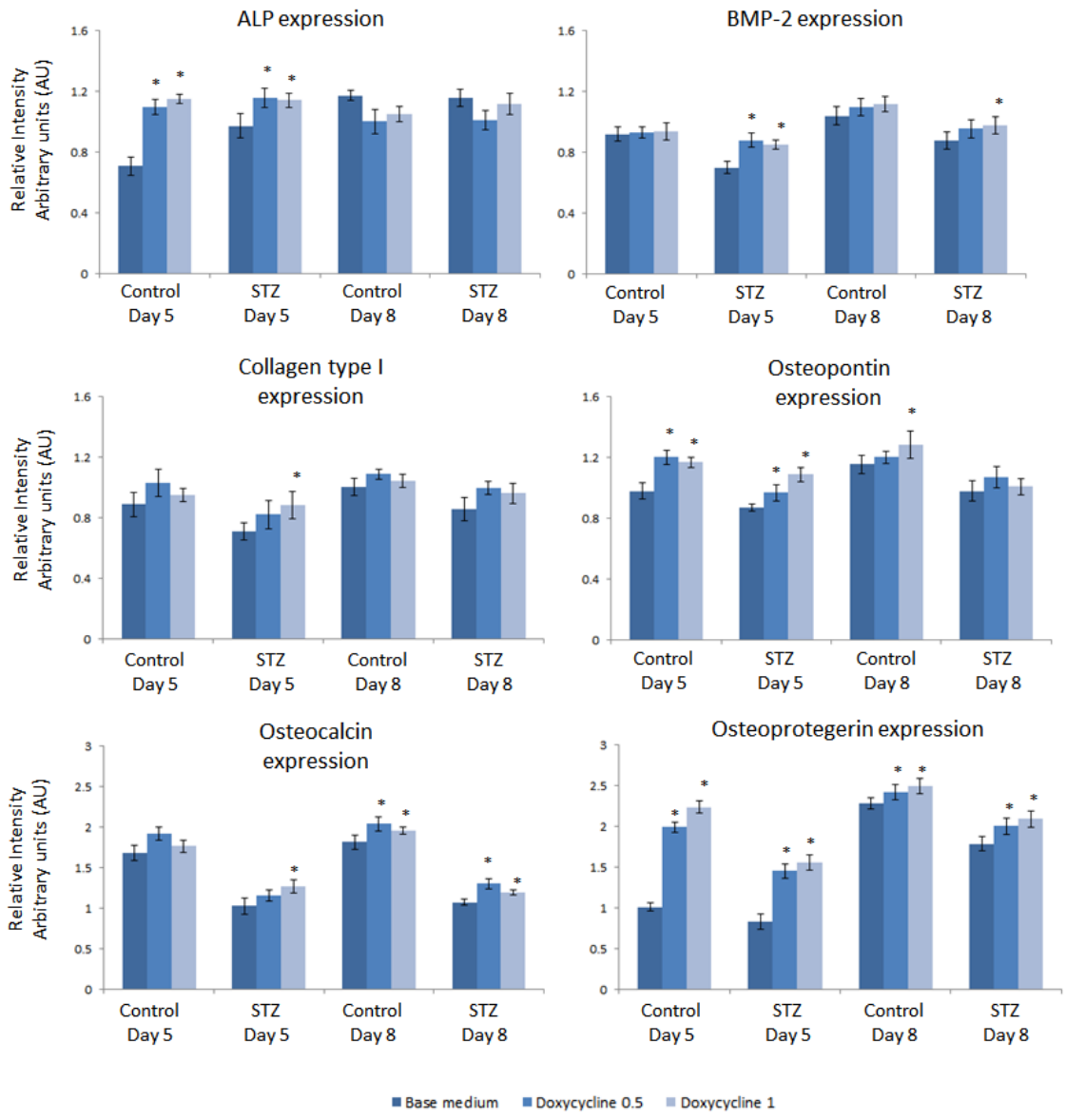
Graphic 4.7 – Total collagen content of rat bone marrow-derived cell cultures established for 12 days, from control and STZ-induced diabetic animals. Cultures were grown in the absence (Control, STZ) and presence of doxycycline 0.5 μg/ml (Control D0.5, STZ D0.5) or doxycycline 1 μg/ml (Control D1, STZ D1). * -

significantly different from control ($p < 0.05$); ** - significantly different from STZ ($p < 0.05$).

4.2.6 – Expression of osteogenic-related markers

Established osteogenic cultures from control and STZ animals, were grown in the absence (base medium) and presence of doxycycline and were analyzed for RT-PCR analysis regarding the expression of significant osteogenic markers. Results are shown in Graphic 4.8.

Doxycycline was able to induce the expression of ALP, at day 5, both in control and STZ cultures. At day 8, no significant effects differences were found between all the experimental conditions. Doxycycline, especially at the 1 μg/ml dosage, was able to induce the expression of BMP-2 in STZ cultures, both at day 5 and 8, while no differences were found in the doxycycline-treated cultures of control animals. Collagen type I expression was barely affected by doxycycline – only the highest dosage was able to induce its expression at day 5 and no significant differences were found in the other experimental conditions or time points. Osteopontin expression was enhanced by both concentrations of doxycycline at day 5, in both control and STZ cultures, while the inductive effect was only maintained at day 8, in control cultures established with 1 μg/ml doxycycline. Regarding osteocalcin expression, both doxycycline concentrations were able to induce its expression at day 8, in STZ and control cultures. In which regards to osteoportegerin, both concentrations of doxycycline were able to enhance its expression in STZ and control cultures, at both 5 and 8 days time points.



Graphic 4.8 – RT-PCR gene expression of ALP, BMP-2, collagen type I, osteopontin, osteocalcin and osteoprotegerin in rat bone marrow-derived cell cultures established at days 5 and 8, from control and STZ-induced diabetic animals, in the absence (base medium) and presence of doxycycline (0.5 and 1 μg/ml). The PCR products were subjected to a densitometric analysis and normalization to the corresponding GAPDH value. * - significantly different from control.

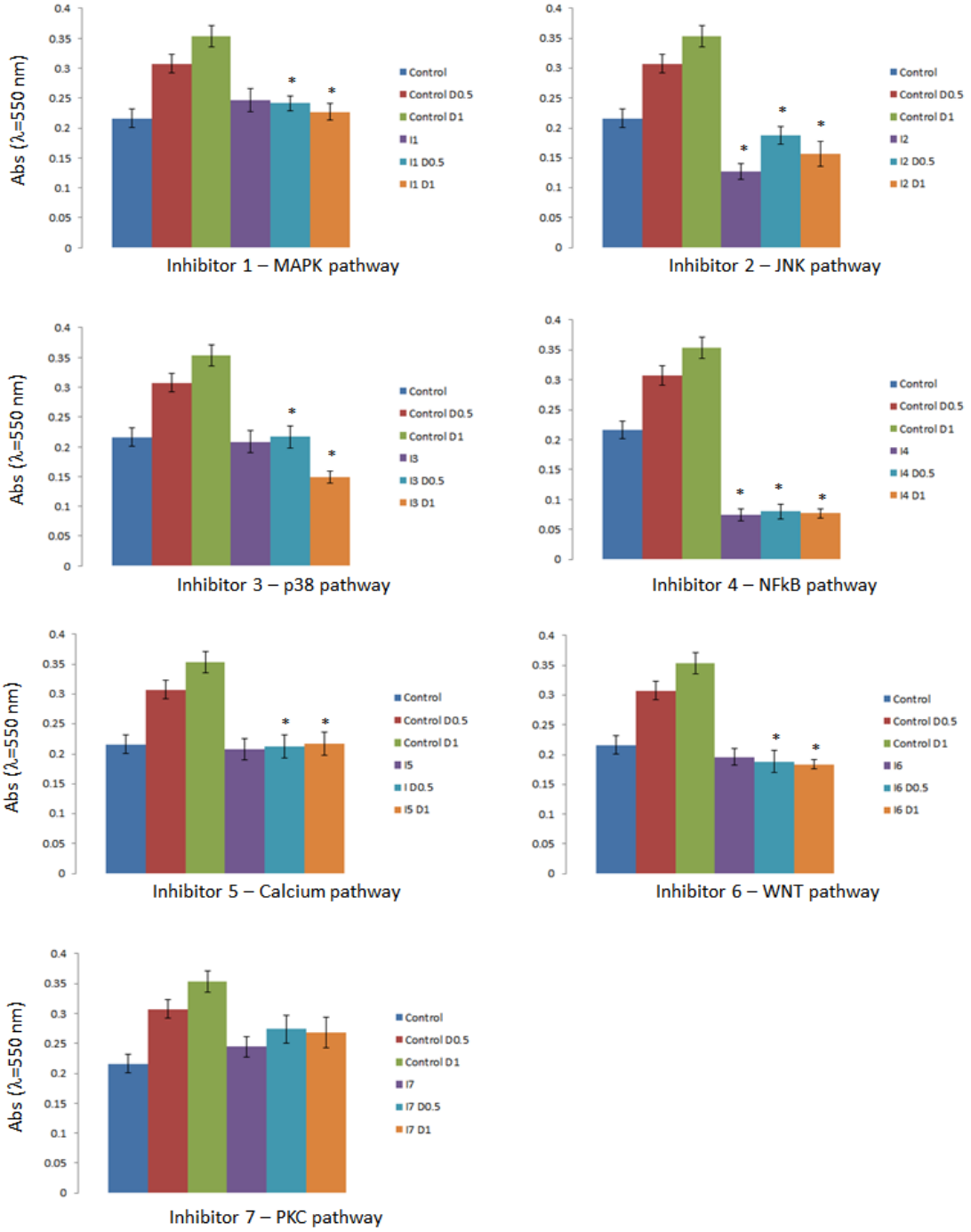
4.3 – EVALUATION OF THE DOXYCYCLINE-MEDIATED EFFECTS ON OSTEOBLASTIC SIGNALING PATHWAYS

Assessment of the doxycycline-mediated effects over relevant osteogenic signalling pathways was established at day 5 and day 8. At day 5, doxycycline was able to induce the viability/proliferation of established cultures, in both control and STZ conditions, and though the assessment of the mechanistic rationale for it was addressed at this time point. At day 8, doxycycline was found to increase the activity of ALP, in both control and STZ cultures. The mechanism associated with this process were though evaluated at this time point.

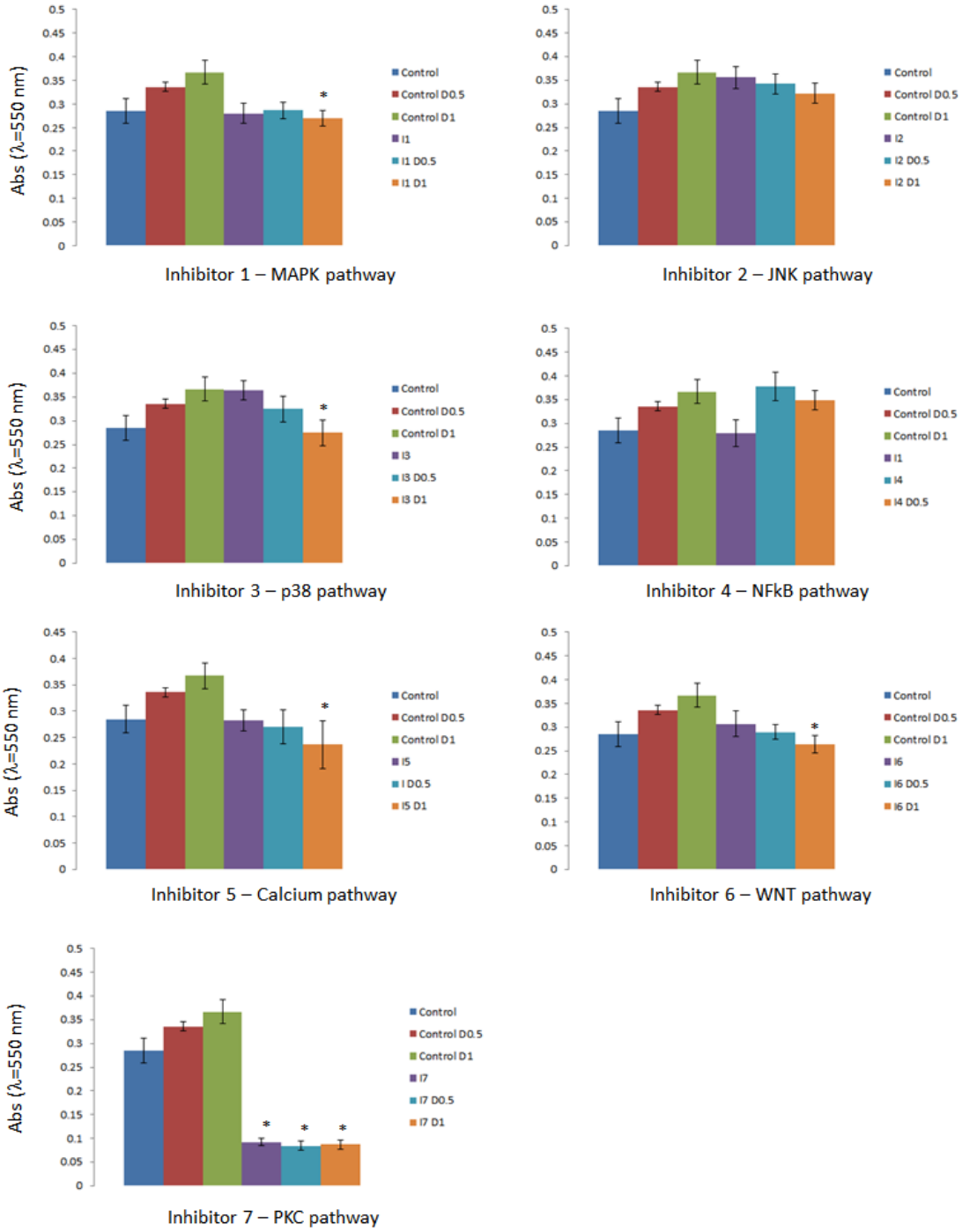
4.3.1 – Effects on cell viability/proliferation

In order to scrutinize the doxycycline-mediated effects on the cell viability/proliferation, at day 5, specific inhibitors of relevant signaling pathways were added to the culture medium for 24 hours (Graphic 4.9 and 4.10). In control cultures, as previously shown, both concentrations of doxycycline induced the MTT reduction value. Within the assayed signalling pathways, MAPK, p38, calcium and WNT were found to be involved in the doxycycline-mediated enhancement of cell proliferation. When the specific inhibitors of these pathways were added, significant differences with paired control (cultures with no added inhibitor) were found while no differences were found for cultures without doxycyclines (Graphic 4.9).

In STZ cultures, the same pathways, i.e., MAPK, p38, calcium and WNT pathways were found to mediate the enhancement of the MTT reduction value, by doxycycline 1 μg/ml (Graphic 4.10).



Graphic 4.9 – Cell viability/proliferation of rat bone marrow-derived cell cultures established from control animals, at day 5, in the absence or presence of a specific inhibitor of a osteogenic signaling pathway (Ix – in which x represents the number of the inhibitor). Cultures were grown in the absence (control, Ix) and presence of doxycycline 0.5 μg/ml (Control D0.5, Ix D0.5) or doxycycline 1 μg/ml (Control D1, Ix D1). * - significantly different from the paired control without the inhibitor ($p \leq 0.05$);.

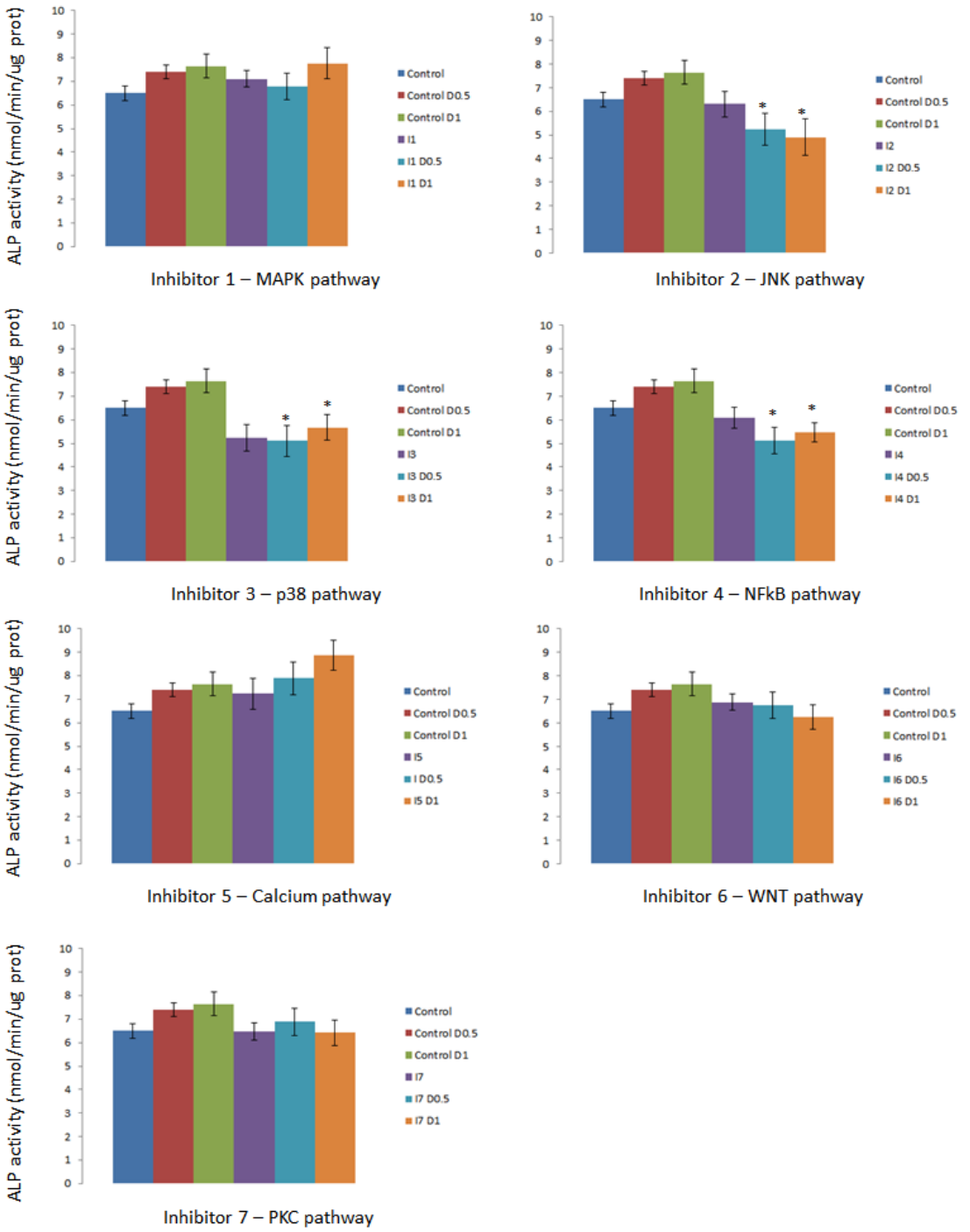


Graphic 4.10 – Cell viability/proliferation of rat bone marrow-derived cell cultures established from STZ animals, at day 5, in the absence or presence of a specific inhibitor of a osteogenic signaling pathway (Ix – in which x represents the number of the inhibitor). Cultures were grown in the absence (control, Ix) and presence of doxycycline 0.5 μg/ml (Control D0.5, Ix D0.5) or doxycycline 1 μg/ml (Control D1, Ix D1). * - significantly different from the paired control without the inhibitor ($p \leq 0.05$).

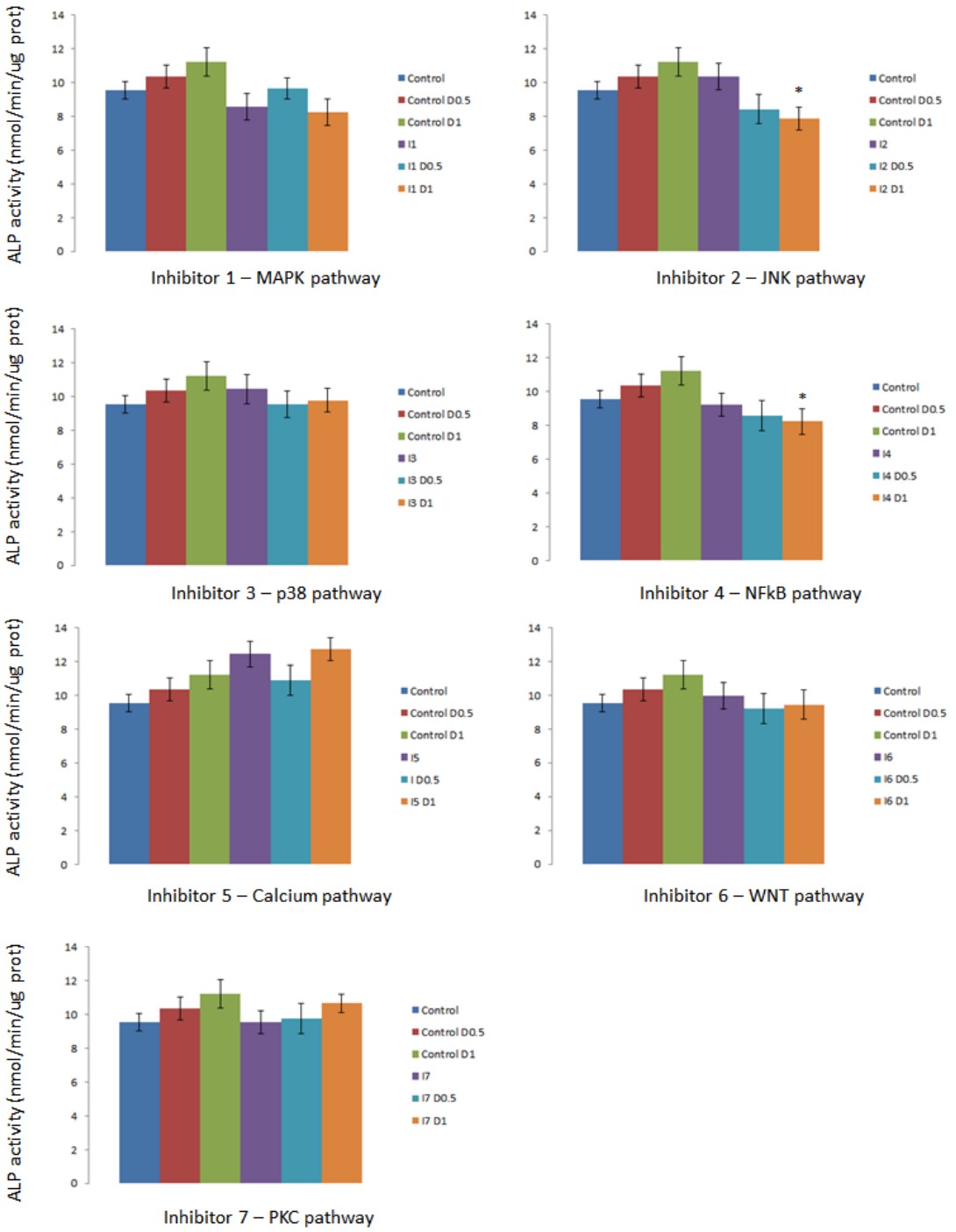
4.3.2 – Effects on the ALP activity

The assessment of the doxycycline-mediated effects on the functional activity (i.e., ALP activity) of established control and STZ cultures was conducted at day 8. Specific inhibitors of relevant osteogenic signaling pathways were added to the culture medium for 24 hours (Graphic 4.11 and 4.12). In control cultures, as previously shown, both concentrations of doxycycline induced the ALP activity at day 8. Within the assayed signaling pathways JNK, p38 and NFkB were found to be involved in the doxycycline-mediated enhancement of the osteogenic cultures functional activity. When the specific inhibitors of these pathways were added, significant differences with paired control (cultures with no added inhibitor) were found while no differences were found for cultures without doxycyclines (Graphic 4.11).

In STZ cultures, the JNK and NFkB pathways were found to be the most significant to mediate the enhancement of the ALP activity in cultures treated with doxycycline 1 μg/ml (Graphic 4.12).



Graphic 4.11 – Alkaline phosphatase activity of rat bone marrow-derived cell cultures established from control animals, at day 8, in the absence or presence of a specific inhibitor of a osteogenic signaling pathway (Ix – in which x represents the number of the inhibitor). Cultures were grown in the absence (control, Ix) and presence of doxycycline 0.5 μg/ml (Control D0.5, Ix D0.5) or doxycycline 1 μg/ml (Control D1, Ix D1). * - significantly different from the paired control without the inhibitor ($p \leq 0.05$).



Graphic 4.12 – Alkaline phosphatase activity of rat bone marrow-derived cell cultures established from STZ animals, at day 8, in the absence or presence of a specific inhibitor of a osteogenic signaling pathway (Ix – in which x represents the number of the inhibitor). Cultures were grown in the absence (control, Ix) and presence of doxycycline 0.5 μg/ml (Control D0.5, Ix D0.5) or doxycycline 1 μg/ml (Control D1, Ix D1). * - significantly different from the paired control without the inhibitor ($p \leq 0.05$).

5: DISCUSSION

Of worldwide relevance, diabetes is one of the most established systemic conditions with increasing epidemiological importance, affecting both life expectancy and quality of life. Type 1 diabetes mellitus, characterized by insulinopenia, and type 2 diabetes mellitus, characterized by a resistance to the metabolic actions of insulin, are both associated with the affection of several organs and tissues, including reported consequences over skeletal health (57). Overall, bone related alterations verified in conjunction with T1DM include diminished bone growth (especially during pubertal growth), decreased adult bone density, and increased risk for adult osteopenia and osteoporosis (95). Moreover, in type 2 diabetes mellitus, which is broadly characterized by hyperinsulinemia and peripheral insulin resistance, a normal to increased bone density is verified, yet decreased bone strength is attained, which contributes to bone fragility and impairment of tissue healing (96). In this regard, specific abnormalities in the bone micro-architecture have been recognized, although no definitive remarks have been established (97). The etiology of the increased BMD in T2DM remains unclear, as evidence of decreased bone resorption (98), increased bone resorption (99), decreased bone formation (58), and increased bone formation (100) have all been reported. Further, large prospective clinical studies have demonstrated that history of T1DM or T2DM were associated with an increased risk of fracture of upper extremity and hip bones (62). Also, regarding both clinical types of diabetes, impairment of the fracture healing has been registered in large clinical trials (101). The diabetic fracture callus has been associated with reduced biomechanical properties, impaired cellular proliferation in the early callus, diminished collagen synthesis and, at late stages of healing, deficits in the mineralization process (102).

Several investigations have addressed the bone healing and regeneration using animal models of diabetes. Due to their availability, easy of manipulation, relative low cost and biological significance, rodent models have found widespread utilization. The spontaneous diabetic BB (BioBreeding) rat has been the most studied model of spontaneous diabetes, while the other model most commonly studied is the chemically induced, i.e., the streptozotocin-induced T1DM model. Studies using these models suggest that several potential underlying mechanisms

may contribute to bone pathology in insulin-deficiency. STZ-induced diabetes in rats, as in poorly controlled T1DM in humans, causes non-osmotic hypercalciuria, which can lead to a negative Ca^{2+} balance (69). Advanced glycation end products may also contribute to poor bone strength, and increased receptors for AGEs (RAGEs) are manifested in a fracture-healing model in chemically-induced diabetes (103). Alteration in the levels of insulin-like growth factor-I (IGF-I), IGF-I receptors and insulin receptors were also noted in bone growth plates (104), while alterations in the expression of collagenous proteins were addressed in fracture healing of diabetic rats (105).

In the first part of the conducted study we aim to establish and characterize bone marrow-derived osteoblastic cell cultures from either control and STZ animals. First subcultured cells were maintained in culture medium without any ostegenic inducing agent and the viability/proliferation, functional activity, especially in which regards to the osteogenic differentiation pathway, were assessed. In which regards to cell proliferation, during the first 5 days of culture no significant differences were found between STZ and control cultures. At day 8 and 12, a tendency for a reduced value of MTT reduction was attained in STZ cultures. Also, no significant differences were found regarding cell morphology, as assessed by confocal laser scanning microscopy following staining of cell cytoskeleton and nucleus counterstaininig. In terms of functional activity, ALP activity was high and increased throughout the culture time for both experimental conditions. Biochemical determination correlated well with histochemical staining, revealing a high and increased stain intensity and nodular aggregation, within the time of the culture. STZ cultures revealed a tendency for an increased and significant expression of ALP at days 8 and 12 of the culture. On the other hand, the synthesis of collagenous proteins was found to be impaired in STZ conditions, especially at the later time points of the culture (day 8 and 12), as addressed by quantitative and histochemical qualitative data. In terms of gene expression, an increased expression of ALP was found at day 5, in STZ cultures, while a tendency for a decreased expression of relevant ostegenic markers, i.e., BMP-2, collagen type I, osteopontin and osteoprotegerin was found for both day 5 and 8.

These results come in line with reported data from the literature in which several lines of evidence converge to justify the idea that insulin exerts a direct anabolic effect over osteoblastic cells. Human osteoblasts and multiple osteoblast-like cell lines express a significant number of insulin receptors on the cell surface and have a high capacity for insulin binding (106, 107). In response to physiological doses of insulin, cultured osteoblasts show an increased cell proliferation and enhancement of the functional activity regarding collagen synthesis and alkaline phosphatase production (108-110). How insulin signaling might promote osteoblastogenesis is speculative; however, proposed direct approaches by which insulin could act on bone cells include the inactivation of p27, a cyclin-dependent kinase inhibitor that could attenuate cell proliferation in osteoblasts (111); also, a direct signaling sequence from insulin receptors to PI 3-kinase, which seems to, in association with Runx2, regulate osteoblast differentiation and migration (112). Apart from the possible direct actions of insulin on osteoblastic cells, coupled indirect mechanisms may exert synergistic effects with other anabolic agents in bone, such as IGF-I and parathyroid hormone (113).

Apart from the causal insulin effect in biological systems, the effect of hyperglycemia on the behavior of osteoblastic cells or on the osteogenic differentiation of precursor cells has also been addressed by some authors. Gopalakrishnan et al. reported that high concentrations of glucose in the culture medium reduced the cell proliferation, ALP activity, the number of nodules formed, and the area stained for collagen (114). Embryonic stem cells cultured in hyperglycemia conditions gave rise to a reduced number of colonies with a more limited differentiated potential and a decreased expression of proteins associated with the stem cell state (115). Furthermore, osteogenic differentiation of these cells was favored in physiological glucose concentrations, with an increased matrix mineralization, enhanced expression of cell-type-specific mRNAs, and increased activity of alkaline phosphatase, comparing to hyperglycemic concentrations (115). Further, high glucose seems to suppress the cell growth, mineralization, and expression of osteogenic markers (e.g., Runx2, collagen I, osteocalcin, osteonectin), while the expression of adipogenic markers (e.g., PPARγ, aP2, resistin, and adipsin)

were promoted, in human osteoblastic cells. These alterations were shown to be, at least partially, mediated by the activation of the cAMP/PKA/ERK pathway (116). In another approach, Brenner et al addressed the effect of serum from diabetic patients in the proliferation and functional activity of human bone cells (117). Under diabetic serum, the authors verified an impaired cell attachment, spreading and cell proliferation. Furthermore, the expression of collagenous proteins were found to be impaired (117). Furthermore, and despite the general reduction in osteogenic-related markers and cellular calcium uptake, osteoblasts were thought to exhibit at least two responses in hyperglycemia conditions: an early acute response that is hyperosmolality driven and a late acute and chronic response that is predominantly hyperglycemia driven (118). Taken together, it was hypothized that osteoblasts exhibit immediate/early acute changes in gene expression in response to hyperglycemia that are predominantly driven by hyperosmolality; while chronically-mediated alterations seem to induce adaptive measures in these cells to compensate for the hyperosmotic stress, which no longer influence expression of osteoblast marker genes. This was found to substantiate the attained increase of ALP expression and activity in chronic hyperglycemia conditions, as opposing to a general reduction in other osteogenic markers (118). This behavior was similar to the one attained by the established cell cultures and corroborates the idea that long-term hyperglycemia conditions may not only alter the functional activity of osteoblastic cells but also impair the metabolic equilibrium of precursor cells and hinder their differentiation potential. This comes in line and seem to justify, at least in part, the broadly increase concentrations of total ALP in patients affected by diabetes, despite the reduction of other bone turnover/formation markers, e.g., osteocalcin, pyridinoline and deoxypyridinoline (71, 119-122)

Despite these results, in which the fundamental role of close regulation of the insulin and glycemia levels for the modulation of the osteoblastic behavior, the intrinsic osteogenic potential of osteoblastic precursor cells derived from diabetic conditions, has been less thoroughly discussed. In fact, to the best of our knowledge, the assessment of the *in vitro* ostegenic potential of bone marrow-

derived precursor cells, in the absence of exogenous osteogenic supplements, has not been critically evaluated before.

Reported studies in which the osteogenic differentiation of diabetic-derived precursor cells was assessed demonstrate that, *in vivo*, Runt-related transcription factor 2 (RUNX2), and several RUNX2 target genes, including matrix metalloproteinase-9, Akp2, integrin binding sialoprotein, Dmp1, Col1a2, Phex, Vdr, osteocalcin, and osterix, seem to be significantly down-regulated in insulin-deficient, hyperglycemic conditions; while the expression of bone morphogenic protein-2, transcriptional coactivator with PDZ-binding motif, and TWIST2, all important regulators of RUNX2, were not impacted by diabetes. For these authors, this further suggests that the defect in osteogenesis resides at the level of RUNX2 expression and its activity (67). Cell culture studies also revealed that, compared to control marrow cells, diabetic bone marrow cells increased osteoblast caspase 3 activity and the ratio of Bax/Bcl-2 expression, in a direct co-culture model, suggesting a relationship between type I diabetes, bone marrow and osteoblast death (123).

Overall, a clear tendency for the impaired osteogenic behavior and lessened osteogenic potential of precursor cells is clear from the literature from both in *in vitro* and *in vivo* approaches to the study of diabetes-mediated effect in the bone tissue. Also, this seems to converge to the attained modifications in bone metabolism that contribute to the establishment of diabetic osteopenia.

As so, in the second part of the work, a tentative approach for the enhancement of the osteogenic potential of bone marrow-derived osteogenic cultures from diabetic animals was conducted by reaching hand of a low dosage regimen of doxycycline. Tetracyclines, apart from their antibacterial activity, have been found to exert a wide range of catabolic actions over the metabolic balance of the bone tissue, by modulating both the osteoblastic and osteoclastic function (88). As tetracyclines were found to improve the osteogenic function in a wide-ranging variety of models, both in physiologic and pathologic conditions, the rationale behind this approach was set on its ability to improve he osteogenic potential of

osteoblast-precursor cells in diabetic conditions, situation that was addressed and thoroughly characterized on the first part of this work.

As so, doxycycline (0.5 or 1 μg/ml – concentrations representative of the peak and mean serum levels, respectively, of human SDD regimens) was added to established bone marrow-derived osteoblastic cultures of STZ and control animals, in the absence of any ostegenic inducing agents. At both concentrations, doxycycline was found to induce the viability/proliferation of the established cultures at day 5, in both STZ and control cultures. At day 8, doxycycline's highest concentration was able to induce the MTT reduction value in STZ cultures. These effects are believed to be mediated, at least partially, by the activation of MAPK, p38, calcium and WNT pathways, as verified by the subsequent analysis of the effects of specific inhibitors of signalling pathways.

In terms of functional activity, all culture conditions expressed high levels of ALP that further increased with culture time, as assessed by histochemical staining. Quantitative determination of ALP activity revealed that doxycycline was able to induce the enzymatic activity at day 8, in both control and STZ cultures, reporting no significant alterations in the other culture time points. In terms of collagen determination, doxycycline was able to increase the total collagen content of the cultures, at day 8 for the STZ cultures, and especially at late culture time points (day 12) for both control and STZ cultures. In terms of the assessment of relevant osteogenic markers gene expression, doxycycline was able to induce ALP expression, at day 5, for both control and STZ cultures. No differences though were found at day 8. The expression of BMP-2 was also enhanced by tetracyclines but only in STZ cultures, which allowed this condition to express levels similar to those attained in control. The expression of collagen type I was broadly unaffected by doxycycline while osteopontin expression was broadly enhanced by both doxycycline concentrations, at day 5, in both STZ and control cultures. Osteocalcin expression was enhanced by doxycycline at later time points (day 8) in both control and STZ cultures and osteoprotegerin expression was greatly enhanced by both 0.5 μg/ml and 1 μg/mL doxycycline, in all experimental conditions and time points. The attained induction in the functional activity is believed to be mediated, at least

partially, by the activation of JNK and NFkB pathways – the most significantly affected by the addition of specific inhibitors to the culture medium.

These results come in line with previously published research reports. Early *in vivo* works have shown catabolic actions of tetracyclines over the bone metabolism. In STZ rats, bone-lining cells were found to incorporated little 3H-proline or secrete little labeled protein and produce only a very thin osteoid layer. With minocycline administration diabetic animals increased both the incorporation of 3H-proline by osteoblasts and their secretion of labeled protein toward the osteoid matrix (124). Furthermore, in the same animal model, the diabetes-induced suppression of synthesis and secretion of protein by osteoblasts was found to be restored to near-normal levels by the administration of minocycline or CMTs (86). Bone regeneration in a dog model revealed that the administration of tetracycline induced a more regenerative healing and minimized crestal resorption, in comparison to control (125). Also, in a dog model, the administration of doxycycline reduced the severity degree of osteoarthritis, with reduced levels of total collagenase activity and inhibition of the proliferation and hypertrophy of chondrocytes (126). These results were later confirmed in a double-blinded, randomized, placebo-controlled trial which reported a doxycycline-dependent reduction in the rate of joint space narrowing, in knees of obese women with established osteoarthritis (127). Overall, most of the established investigations, with positive results, were based on the evaluation of bone forming activity by tetracyclines over pathological-induced bone diseases in experimental animal models of low or high bone turnover processes of clinical conditions. More scarcely, the assessment of tetracyclines' effect on the normal bone remodeling conditions has been conducted. In this situation and increased osteoblastic activity and osteoid formation was verified in the alveolar bone of the squirrel monkeys, with proven catabolic effects on the osteogenic function (128).

Cell culture studies were also developed to address the effects of tetracyclines in the relevant cell populations for the bone metabolism. Results are in line with the attained evidences in this experimental work. In human bone marrow-derived osteoblastic cells, doxycycline has increased significantly the number of

active osteoblastic cells that yielded a proportional amount of a normal mineralized extracellular matrix (129). This behavior was further confirmed when the human osteoblastic cultures were grown on the surface of two biomaterials for bone regeneration, i.e., hydroxyapatite and a glass-ceramic composite (130). Also in human osteoblasts, doxycycline, rather than minocycline, was found to enhance cell maturation and differentiation rather the proliferation process itself (131). Doxycycline analogs were also found to dose-dependently inhibit the IL-1 beta-induced IL-6 secretion in osteoblastic cells at the post-transcriptional level, affecting IL-6 mRNA stability (132). Recently, doxycycline, at 1 μg/ml, was found to increase the expression of osteocalcin, osteonectine and ALP in human periodontal ligament cells. Moreover, the increase in ALP expression, was found to be more significant than the one induced by BMP-2 (133).

Furthermore, and in terms of bone metabolism relevance, tetracyclines have been shown to effect the modulation of the osteoclastogenic response. These drugs were found to modulate the osteoclasts activity in experimental diabetic conditions, by normalizing their structural and enzymatic features (134). More recently, doxycycline and minocycline were found to inhibit the receptor activator of NF-κB ligand (RANKL)-induced osteoclastogenesis of precursor cells, despite no effects on cell growth and phagocytic activity were attained. Further, these authors also report the absence of effect on cell proliferation or differentiation, in bone-forming osteoblast . Also, i*n vivo*, the injection of tetracyclines into RANKL-injected mice and RANKL-transgenic mice suppressed the RANKL-mediated osteoclastogenesis process and promoted the concomitant appearance of $CD11c^+$ cells (135).

Tetracyclines, and specifically doxycycline, seem to be effective osteogenic agents and broadly induce the expression of osteogenic factors in several cellular populations of osteoblasts and precursor cells. In this work we have shown that doxycycline, in a concentration representative of the SDD regimen, was able to induce the osteogenic properties of bone marrow derived cell cultures, both in control and in STZ-induced experimental diabetic condition, in the absence of other ostegenic agents. This process was found to be, at least in part, mediated by the activation of several osteogenic signaling pathways, affecting both the cell

proliferation and functional activity. Apart from this evidence, other non-antimicrobial properties of tetracyclines may not be disregarded and their ability to inhibit members of the MMPs family (136) and to scavange reactive oxygen species (137), their anti-apoptotic (138) and anti-inflammatory activity (89) may also contribute to attained enhancement of the osteogenic function.

6: CONCLUSIONS

Successful STZ-mediated hyperglycemia induction allowed for establishment of bone marrow-derived cell cultures that were thoroughly characterized and compared to the ones established form control animals. Despite the increased expression of ALP in STZ-derived cultures, a broadly impairment of the cell proliferation and osteogenic capability was achieved, as assessed by collagen expression and RT-PCR evaluation of significant osteogenic markers, i.e., ALP, BMP-2, collagen type I, osteopontin, osteocalcin and osteoprotegerin.

The addition of doxycycline, in concentrations representative of those attained in SDD, was found to increase the cell proliferation and, essentially, the functional activity of both STZ and control cultures. MAPK, p38, calcium and WNT pathways were found to participate in the doxycycline-mediated enhancement of the cell proliferation, while JNK and NFkB pathways were found to be the most related to the enhancement of the functional activity.

Accordingly, doxycycline presents here as a candidate osteogenic agent for both conditions of physiologic bone metabolism, as well as for situations in which the osteoblastic function is impaired, such as the diabetes-mediated osteopenic condition.

7: BIBLIOGRAPHY

1. Weiner S, Wagner H. The material bone: structure-mechanical function relations. Annu Rev Mater Sci 1998;28:271-98.

2. Weiner S, Traub W, Wagner H. Lamellar Bone: Structure–Function Relations. J Structure Biol. 1999;126:241-55.

3. Bronner F, Worrell R, editors. Orthopaedics: Principles of Basic and Clinical Science. Boca Raton: CRC Press; 1999.

4. Bilezikian J, Raisz L, Martin T, editors. Principles of Bone Biology. Vol. 1. Boca Raton: Academic Press; 2008.

5. Gorski J. Is all bone the same? Distinctive distributions and properties of non-collagenous matrix proteins in lamellar vs. woven bone imply the existence of different underlying osteogenic mechanisms. Crit Rev Oral Biol Med. 1998;9:201-23.

6. O'Driscoll S, Fitzsimmons J. The role of periosteum in cartilage repair. Clin Orthop. 2001;391 Suppl:S190-207.

7. Teitelbaum S. Bone Resorption by Osteoclasts. Science. 2000;289:1504-8.

8. Rodan G, Martin T. Role of osteoblasts in hormonal control of bone resorption—A hypothesis. Calcified Tissue International. 1981;33:349-51.

9. Clarke B. Normal Bone Anatomy and Physiology. Clinical Journal of the American Society of Nephrology. 2008;3(Supplement 3):S131-S9.

10. Hall B, editor. Bone: The osteoblast and osteocyte. New Jersey: The Telford Press; 1990.

11. Coelho M, Fernandes M. Human bone cell cultures in biocompatibility testing. Part Ⅱ: effect of ascorbic acid, beta-glycerophosphate and dexamethasone on osteoblastic differentiation. Biomaterials. 2000;21:1095-102.

12. Holtrop M. Light and electron microscopic structure of bone-forming cells. In: Hall B, editor. The Osteoblasts and Osteocytes. New Jersey: Telford Press Inc; 1990. p. 1-39.

13. Han Y, Cowin S, Schaffler M, Weinbaum S. Mechanotransduction and strain amplification in osteocyte cell processes. PNAS. 2004;101(47):16689-94.

14. Parfitt A. The Bone Remodeling Compartment: A Circulatory Function for Bone Lining Cells. Journal of Bone and Mineral Research. 2001;16:1583-5.

15. Lanyon L. Osteocytes, strain detection, bone modeling and remodeling. Calcified Tissue International. 1993;53:S102-S7.

16. Raisz L. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. The Journal of Clinical Investigation. 2005;115:3318-25.

17. Robling AG, Castillo AB, Turner CH. Biomechanical and Molecular regulation of bone remodeling Annual Review of Biomedical Engineering. 2006;8:455-98.

18. Petite H, Viateau V, Bensaid W, Meunier A, de Pollak C, Bourguignon M, et al. Tissue-engineered bone regeneration. Nat Biotech. [10.1038/79449]. 2000;18(9):959-63.

19. Rose F, Oreffo R. Bone Tissue Engineering: Hope vs Hype. Biochemical and Biophysical Research Communications. 2002;292:1-7.

20. Reichert J, Hutmacher D. Bone Tissue Engineering. In: Pallua N, Suscheck C, editors. Tissue Engineering: Springer Berlin Heidelberg; 2011. p. 431-56.

21. Kuzuya T, Nakagawa S, Satoh J, Kanazawa Y, Iwamoto Y, Kobayashi M, et al. Report of the Committee on the classification and diagnostic criteria of diabetes mellitus. Diabetes Research and Clinical Practice. 2002;55(1):65-85.

22. Heydari I, Radi V, Razmjou S, Amiri A. Chronic complications of diabetes mellitus in newly diagnosed patients. International Journal of Diabetes Mellitus. 2010;2:61-3.

23. Guyton AC, Hall JE. Insulin, Glucagon, and Diabetes Mellitus. Textbook of Medical Physiology. 11ª ed. Philadelphia, Pennsylvania: Saunders Elsevier; 2006. p. 961-77.

24. Bach J-F. Insulin-Dependent Diabetes Mellitus as an Autoimmune Disease. Endocrine Reviews. 1994;15:516-42.

25. Gunter MJ, Hoover DR, Yu H, Wassertheil-Smoller S, Rohan TE, Manson JE, et al. Insulin, Insulin-Like Growth Factor-I, and Risk of Breast Cancer in Postmenopausal Women. Journal of the National Cancer Institute. 2009 January 7, 2009;101(1):48-60.

26. Van Belle TL, Coppieters KT, Von Herrath MG. Type 1 Diabetes: Etiology, Immunology, and Therapeutic Strategies. Physiological Reviews. 2011;91(1):79-118.

27. Nejentsev S, Howson JMM, Walker NM, Szeszko J, Field SF, Stevens HE, et al. Localization of type 1 diabetes susceptibility to the MHC class I genes HLA-B and HLA-A. Nature. 2007;450:887-92.

28. Kitabchi AE, Umpierrez GE, Murphy MB, Barrett EJ, Kreisberg RA, Malone JI, et al. Management of Hyperglycemic Crises in Patients With Diabetes. Diabetes Care. 2001;24:131-53.

29. Imagawa A, Hanafusa T, Miyagawa J-i, Matsuzawa Y. A Novel Subtype of Type 1 Diabetes Mellitus Characterized by a Rapid Onset and an Absence of Diabetes-Related Antibodies. New England Journal of Medicine. 2000;342:301-7.

30. Saltiel AR. New Perspectives into the Molecular Pathogenesis and Treatment of Type 2 Diabetes. Cell. 2001;104:517-29.

31. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, et al. Hypoadiponectinemia in Obesity and Type 2 Diabetes: Close Association with Insulin Resistance and Hyperinsulinemia. Journal of Clinical Endocrinology & Metabolism. 2001;86(5):1930-5.

32. Rother KI. Diabetes Treatment — Bridging the Divide. New England Journal of Medicine. 2007;356:1499-501.

33. Narayan KMV, Boyle JP, Geiss LS, Saaddine JB, Thompson TJ. Impact of Recent Increase in Incidence on Future Diabetes Burden. Diabetes Care. 2006;29:2114-6.

34. Florez JC, Hirschhorn J, Altshuler D. The inherited basis of diabetes mellitus: Implications for the Genetic Analysis of Complex Traits. Annual Review of Genomics and Human Genetics. 2003;4(1):257-91.

35. Go V. The Exocrine pancreas: Biology, pathobiology, and diseases. New York: Raven Press 1986.

36. Resmini E, Minuto F, Colao A, Ferone D. Secondary diabetes associated with principal endocrinopathies: the impact of new treatment modalities. Acta Diabetologica. 2009;46:85-95.

37. Luna B, Feinglos M. Drug-induced hyperglycemia. JAMA: The Journal of the American Medical Association. 2001;286(16):1945-8.

38. Fairweather D, Rose N. Type 1 diabetes: virus infection or autoimmune disease? Nat Immunol. 2002;3:338-40.

39. Jovanovic L, Pettitt D. Gestational diabetes mellitus. JAMA: The Journal of the American Medical Association. 2001;286(20):2516-8.

40. Buchanan TA, Xiang AH. Gestational diabetes mellitus. The Journal of Clinical Investigation. 2005;115(3):485-91.

41. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care. 2004;27(suppl 1):s5-s10.

42. The American Diabetes Association - Position Paper - Standards of Medical Care in Diabetes—2012. Diabetes Care. 2012;35(Supplement 1):S11-S63.

43. Sacks DB, Arnold M, Bakris GL, Bruns DE, Horvath AR, Kirkman MS, et al. Position Statement Executive Summary: Guidelines and Recommendations for

Laboratory Analysis in the Diagnosis and Management of Diabetes Mellitus. Diabetes Care. 2011 June 1, 2011;34(6):1419-23.

44. Strowig S, Raskin P. Glycemic control and complications of diabetes: after the Diabetes Control and Complications Trial. Diabetes Rev 1993;3:237–57.

45. Ohkubo Y, Kishikawa H, Araki E, Miyata T, Isami S, Motoyoshi S, et al. Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study. Diabetes Research and Clinical Practice. 1995;28(2):103-17.

46. UK Prospective Diabet Study Grp - Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes. Lancet. 1998;352:837 - 53.

47. Saaddine J, Honeycutt A, Narayan K, Zhang X, Klein R, Boyle J. Projection of diabetic retinopathy and other major eye diseases among people with diabetes mellitus: United states, 2005-2050. Archives of Ophthalmology. 2008;126(12):1740-7.

48. Atkins R, Zimmet P. Diabetes: Diabetic kidney disease: act now or pay later. Nat Rev Nephrol. 2010;6(3):134-6.

49. Yagihashi S, Yamagishi S-I, Wada R. Pathology and pathogenetic mechanisms of diabetic neuropathy: Correlation with clinical signs and symptoms. Diabetes Research and Clinical Practice. 2007;77(3, Supplement):S184-S9.

50. Ray K, Seshasai S, Wijesuriya S, Sivakumaran R, Nethercott S, Preiss D, et al. Effect of intensive control of glucose on cardiovascular outcomes and death in patients with diabetes mellitus: a meta-analysis of randomised controlled trials. Lancet.373(9677):1765-72.

51. Kaiser N, Sasson S, Feener E, Boukobza-Vardi N, Higashi S, Moller D, et al. Differential regulation of glucose transport and transporters by glucose in vascular endothelial and smooth muscle cells. Diabetes. 1993;42:80-9.

52. Gabbay K, Merola L, Field R. Sorbitol pathway: presence in nerve and cord with substrate accumulation in diabetes. Science. 1966;151:209–10.

53. Brownlee M, Cerami A, Vlassara H. Advanced Glycosylation End Products in Tissue and the Biochemical Basis of Diabetic Complications. New England Journal of Medicine. 1988;318(20):1315-21.

54. Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes. 2000 November 1, 2000;49(11):1939-45.

55. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Are Oxidative Stress−Activated Signaling Pathways Mediators of Insulin Resistance and β-Cell Dysfunction? Diabetes. 2003 January 1, 2003;52(1):1-8.

56. Brownlee M. The Pathobiology of Diabetic Complications. Diabetes. 2005;54(6):1615-25.

57. Schwartz A. Diabetes Mellitus: Does it Affect Bone? Calcif Tissue Int. 2003;73:515-9.

58. Krakauer J, McKenna M, Burderer N, Rao D, Whitehouse F, Pafitt A. Bone loss and bone turnover in diabetes. Diabetes. 1995;44:775–82.

59. Ivers RQ, Cumming RG, Mitchell P, Peduto AJ. Diabetes and Risk of Fracture. Diabetes Care. 2001;24(7):1198-203.

60. Prisby RD, Swift JM, Bloomfield SA, Hogan HA, Delp MD. Altered bone mass, geometry and mechanical properties during the development and progression of type 2 diabetes in the Zucker diabetic fatty rat. Journal of Endocrinology. 2008;199(3):379-88.

61. Campos-Pastor MM, López-Ibarra PJ, Escobar-Jiménez F, Serrano-Pardo MD, García-Cervigón A. Intensive Insulin Therapy and Bone Mineral Density in Type 1 Diabetes Mellitus: A Prospective Study. Osteoporosis International. 2000;11(5):455-9.

62. Janghorbani M, Van Dam R, Willett W, Hu F. Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol. 2007:495-505.

63. López-Ibarra P, Pastor M, Escobar-Jiménez F, Pardo M, González A, Luna J, et al. Bone mineral density at time of clinical diagnosis of adult-onset type 1 diabetes mellitus. Endocr Pract. 2001;7(5):346-51.

64. Shyng YC, Devlin H, Sloan P. The effect of streptozotocin-induced experimental diabetes mellitus on calvarial defect healing and bone turnover in the rat. Oral & Maxillofacial Surgery. 2001;30:70-4.

65. He H, Liu R, Desta T, Leone C, Gerstenfeld LC, Graves DT. Diabetes Causes Decreased Osteoclastogenesis, Reduced Bone Formation, and Enhanced Apoptosis of Osteoblastic Cells in Bacteria Stimulated Bone Loss. Endocrinology. 2004;145(1):447-52.

66. Thrailkill KM, Liu L, Wahl EC, Bunn RC, Perrien DS, Cockrell GE, et al. Bone Formation Is Impaired in a Model of Type 1 Diabetes. Diabetes. 2005 October 1, 2005;54(10):2875-81.

67. Fowlkes JL, Bunn RC, Liu L, Wahl EC, Coleman HN, Cockrell GE, et al. Runt-Related Transcription Factor 2 (RUNX2) and RUNX2-Related Osteogenic Genes Are Down-Regulated throughout Osteogenesis in Type 1 Diabetes Mellitus. Endocrinology. 2008;149(4):1697-704.

68. Yoshino T, Yamaguchi I. Bone mineral density at the metaphysis is specifically reduced in STZ-treated diabetic rats. Life Sci. 1996;9(2):161-16.

69. Ward DT, Yau SK, Mee AP, Mawaer EB, Miller CA, Garland HO, et al. Functional, Molecular, and Biochemical Characterization of Streptozotocin-Induced Diabetes. Journal of the American Society of Nephrology. 2001;12(4):779-90.

70. Lumachi F, Camozzi V, Tombolan V, Luisetto G. Bone Mineral Density, Osteocalcin, and Bone-specific Alkaline Phosphatase in Patients with Insulin-

dependent Diabetes Mellitus. Annals of the New York Academy of Sciences. 2009;1173:E64-E7.

71. Bouillon R, Bex M, Van Herck E, Laureys J, Dooms L, Lesaffre E, et al. Influence of age, sex, and insulin on osteoblast function: osteoblast dysfunction in diabetes mellitus. Journal of Clinical Endocrinology & Metabolism. 1995;80(4):1194-202.

72. Lu H, Kraut D, Gerstenfeld L, Graves D. Diabetes Interferes with the Bone Formation by Affecting the Expression of Transcription Factors that Regulate Osteoblast Differentiation. Endocrinology. 2003;144:346–52.

73. McNeill J. Experimental models of diabetes. Boca Raton: CRC Press LLC. 1999.

74. Rees D, Alcolado J. Animal models of diabetes mellitus. Diabetic Medicine. 2005;22(4):359-70.

75. Nakhooda A, Like A, Chappel C, Murray F, Marliss E. The spontaneously diabetic Wistar rat. Metabolic and morphologic studies. Diabetes. 1977;26:100-12.

76. Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia. 2008;51(2):216-26.

77. Weiss R. Streptozotocin: a review of it pharmacology, efficacy and toxicity. Cancer Treat Rep. 1982;66:427-38.

78. Weide L, Lacy P. Low-dose streptozocin-induced autoimmune diabetes in islet transplantation model. Diabetes. 1991;40:1157-62.

79. Yamamoto H, Uchigata Y, Okamoto H. Streptozotocin and alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. Nature. [10.1038/294284a0]. 1981;294(5838):284-6.

80. Shevalye H, Stavniichuk R, Xu W, Zhang J, Lupachyk S, Maksimchyk Y, et al. Poly(ADP-ribose) polymerase (PARP) inhibition counteracts multiple

manifestations of kidney disease in long-term streptozotocin-diabetic rat model. Biochemical Pharmacology. 2010;79(7):1007-14.

81. Schnedl WJ, Ferber S, Johnson JH, Newgard CB. STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes. 1994 November 1, 1994;43(11):1326-33.

82. Deeds M, Anderson J, Armstrong A, Gastineau D, Hiddinga H, Jahangir A, et al. Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models. Laboratory Animals. 2011 July 1, 2011;45(3):131-40.

83. Sapadin AN, Fleischmajer R. Tetracyclines: Nonantibiotic properties and their clinical implications. Journal of the American Academy of Dermatology. 2006;54(2):258-65.

84. Golub LM, Lee HM, Lehrer G, Nemiroff A, McNamara TF, Kaplan R, et al. Minocycline reduces gingival collagenolytic activity during diabetes. Journal of Periodontal Research. 1983;18(5):516-26.

85. Golub LM, Ramamurthy NS, McNamara TF, Greenwald RA, Rifkin BR. Tetracyclines Inhibit Connective Tissue Breakdown: New Therapeutic Implications for an Old Family of Drugs. Critical Reviews in Oral Biology & Medicine. 1991 January 1, 1991;2(3):297-321.

86. Sasaki T, Ramamurthy N, Golub L. Tetracycline administration increases collagen synthesis in osteoblasts of streptozotocin-induced diabetic rats: a quantitative autoradiographic study. Calcif Tissue Int 1992;50:411-9.

87. Golub L, Ramamurthy N, Llavaneras A, Ryan M, Lee H, Liu Y. A chemically modified nonantimicrobial tetracycline (CMT-8) inhibits gingival matrix metalloproteinases, periodontal breakdown, and extra-oral bone loss in ovariectomized rats. An NY Acad Sci. 1999;878:290-310.

88. Payne JB, Golub LM. Using tetracyclines to treat osteoporotic/osteopenic bone loss: From the basic science laboratory to the clinic. Pharmacological Research. 2011;63(2):121-9.

89. Webster G, Del Rosso JQ. Anti-Inflammatory Activity of Tetracyclines. Dermatologic Clinics. 2007;25(2):133-5.

90. Williams S, Wakisaka A, Zeng Q, Barnes J, Martin G, Wechter W. Minocycline prevents the decrease inBMDand trabecular bone in ovariectomized aged rats. Bone. 1996;19:637–44.

91. Golub L, Ramamurthy N, Llavaneras A, Ryan M, Lee H, Liu Y. chemically modified nonantimicrobial tetracycline (CMT-8) inhibits gingival matrix metalloproteinases, periodontal breakdown, and extra-oral bone loss in ovariectomized rats. An NY Acad Sci. 1999;878:290–310.

92. Payne J, Stoner J, Nummikoski P, Reinhardt R, Goren A, Wolff M. Subantimicrobial dose doxycycline effects on alveolar bone loss in postmenopausal women. J Clin Periodontol. 2007;34:776-87.

93. Golub L, Lee H, Stoner J, Reinhardt R, Sorsa T, Goren A. Doxycycline effects on serum bone biomarkers in postmenopausal women. J Dent Res. 2010;89:644–9.

94. Maniatopoulos C, Sodek J, Melcher A. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. Cell and Tissue Research. 1988;254(2):317-30.

95. Thrailkill KM, Lumpkin CK, Bunn RC, Kemp SF, Fowlkes JL. Is insulin an anabolic agent in bone? Dissecting the diabetic bone for clues. American Journal of Physiology - Endocrinology And Metabolism. 2005;289(5):E735-E45.

96. de Liefde I, van der Klift M, de Laet C, van Daele P, Hofman A, Pols H. Bone mineral density and fracture risk in type-2 diabetes mellitus: the Rotterdam Study. Osteoporosis International. 2005;16(12):1713-20.

97. Carnevale V, Romagnoli E, D'Erasmo E. Skeletal involvement in patients with diabetes mellitus. Diabetes Metab Res Rev. 2004;20:196-204.

98. Espallargues M, Sampietro-Colom L, Estrada M, Sola M, del Rio L, Setoain J, et al. Identifying bone-mass-related risk factors for fracture to guide bone densitometry measurements: a systematic review of the literature. Osteoporosis International. 2001;12:811–22.

99. Isaia GC, Ardissone P, Di Stefano M, Ferrari D, Martina V, Porta M, et al. Bone metabolism in type 2 diabetes mellitus. Acta Diabetologica. 1999;36(1):35-8.

100. el Miedany Y, el Gaafary S, el Baddini M. Osteoporosis in older adults with non-insulin-dependent diabetes mellitus: is it sex related? Clin Exp Rheumatol. 1999;17:561-7.

101. Herskind A, Christensen K, Norgaard-Andersen K, Andersen J. Diabetes mellitus and healing of closed fractures. Diabetes Metab. 1992;18:63–4.

102. Gandhi A, Beam H, O'Connor J, Parsons J, Lin S. The effects of local insulin delivery on diabetic fracture healing. Bone. 2005;37:482-90.

103. Santana RB, Xu L, Chase HB, Amar S, Graves DT, Trackman PC. A Role for Advanced Glycation End Products in Diminished Bone Healing in Type 1 Diabetes. Diabetes. 2003;52(6):1502-10.

104. Hock J, Centrella M, Canalis E. Insulin-Like Growth Factor I Has Independent Effects on Bone Matrix Formation and Cell Replication. Endocrinology. 1988;122(1):254-60.

105. Spanheimer RG, Umpierrez GE, Stumpf V. Decreased Collagen Production in Diabetic Rats. Diabetes. 1988;37(4):371-6.

106. Fulzele K, Riddle RC, DiGirolamo DJ, Cao X, Wan C, Chen D, et al. Insulin Receptor Signaling in Osteoblasts Regulates Postnatal Bone Acquisition and Body Composition. Cell. 2010;142(2):309-19.

107. Thomas DM, Hards DK, Rogers SD, Ng KW, Best JD. Insulin receptor expression in bone. Journal of Bone and Mineral Research. 1996;11(9):1312-20.

108. Levy J, Murray E, Manolagas S, Olefsky J. Demonstration of Insulin Receptors and Modulation of Alkaline Phosphatase Activity by Insulin in Rat Osteoblastic Cells. Endocrinology. 1986;119(4):1786-92.

109. Yano H, Ohya K, Amagasa T. Effects of insulin on in vitro bone formation in fetal rat parietal bone. Endocr J. 1994;41:293-300.

110. Hahn TJ, Westbrook SL, Sullivan TL, Goodman WG, Halstead LR. Glucose transport in osteoblast-enriched bone explants: Characterization and insulin regulation. Journal of Bone and Mineral Research. 1988;3(3):359-65.

111. Drissi H, Hushka D, Aslam F, Nguyen Q, Buffone E, Koff A, et al. The Cell Cycle Regulator p27kip1 Contributes to Growth and Differentiation of Osteoblasts. Cancer Research. 1999;59(15):3705-11.

112. Fujita T, Azuma Y, Fukuyama R, Hattori Y, Yoshida C, Koida M, et al. Runx2 induces osteoblast and chondrocyte differentiation and enhances their migration by coupling with PI3K-Akt signaling. The Journal of Cell Biology. 2004;166(1):85-95.

113. Zoidis E, Ghirlanda-Keller C, Schmid C. Stimulation of glucose transport in osteoblastic cells by parathyroid hormone and insulin-like growth factor I. Molecular and Cellular Biochemistry. 2011;348(1):33-42.

114. Gopalakrishnan V, Vignesh RC, Arunakaran J, Aruldhas MM, Srinivasan N. Effects of glucose and its modulation by insulin and estradiol on BMSC differentiation into osteoblastic lineages. Biochemistry and Cell Biology. 2006;84(1):93-101.

115. Dienelt A, zur Nieden N. Hyperglycemia Impairs Skeletogenesis from Embryonic Stem Cells by Affecting Osteoblast and Osteoclast Differentiation. Stem Cells and Development. 2011;20:465-74.

116. Wang W, Zhang X, Zheng J, Yang J. High glucose stimulates adipogenic and inhibits osteogenic differentiation in MG-63 cells through cAMP/protein kinase A/extracellular signal-regulated kinase pathway. Molecular and Cellular Biochemistry. 2010;338(1):115-22.

117. Brenner RE, Riemenschneider B, Blum W, Mörike M, Teller WM, Pirsig W, et al. Defective stimulation of proliferation and collagen biosynthesis of human bone cells by serum from diabetic patients. Acta Endocrinologica. 1992;127(6):509-14.

118. Botolin S, McCabe L. Chronic hyperglycemia modulates osteoblast gene expression through osmotic and non-osmotic pathways. J Cell Biochem. 2006;99:411-24.

119. Kanazawa I, Yamaguchi T, Yamamoto M, Yamauchi M, Kurioka S, Yano S, et al. Serum Osteocalcin Level Is Associated with Glucose Metabolism and Atherosclerosis Parameters in Type 2 Diabetes Mellitus. Journal of Clinical Endocrinology & Metabolism. 2009 January 1, 2009;94(1):45-9.

120. Sosa M, Dominguez M, Navarro MC, Segarra MC, Hernández D, de Pablos P, et al. Bone mineral metabolism is normal in non-insulin-dependent diabetes mellitus. Journal of Diabetes and its Complications. 1996;10(4):201-5.

121. Olmos J, Perez-Castrillon J, Garcia M, Garrido J, Amada J, Gonzales-Macias J. Bone densitometry and biochemical bone remodeling markers in type 1 diabetes mellitus. Bone Miner. 1994;26:1-8.

122. Miazgowski T, Czekalski S. A 2-year Follow-up Study on Bone Mineral Density and Markers of Bone Turnover in Patients with Long-standing Insulin-Dependent Diabetes Mellitus. Osteoporosis International. 1998;8(5):399-403.

123. Coe LM, Irwin R, Lippner D, McCabe LR. The bone marrow microenvironment contributes to type I diabetes induced osteoblast death. Journal of Cellular Physiology. 2011;226(2):477-83.

124. Sasaki T, Kaneko H, Ramamurthy N, Golub L. Tetracycline administration restores osteoblast structure and function during experimental diabetes. Anat Rex. 1991;231:25-34.

125. Waleed A-A, Bissada N, Greenwell H. The effect of local doxycycline with and without Tricalcium phosphate on the regenerative healing potencial of periodontal osseous defects in dogs. J Periodontol. 1989;60:582-9.

126. Yu L, Smith G, Brandt K, Myers S, O'Connor B, Brandt D. Reduction of the severity of canine osteoarthritis by prophylactic treatment with oral doxycycline. Arthritis Rheum. 1992;35:1150-9.

127. Brandt K, Mazzuca S, Katz B, Lane K, Buckwalter K, Yocum D. Effects of doxycycline on progresion of osteoarthritis. Arthritis & Rheumatism. 2005;52:2015-25.

128. Polson A, Bouwsma O, McNamara T, Golub L. Enhancement of Alveolar Bone Formation after Tetracycline Administration in Squirrel Monkeys. J Appl Res Clin Dentist. 1995;2:32-42.

129. Gomes P, Fernandes M. Effect of therapeutic levels of doxycycline and minocycline in the proliferation and differentiation of human bone marrow osteoblastic cells. Arch Oral Biol. 2007;52:251-9.

130. Gomes P, Santos J, Fernandes M. Cell-induced response by tetracyclines on human bone marrow colonized hydroxyapatite and Bonelike. Acta Biomater. 2008;4:630-7.

131. Almazin S, Dziak R, Andreana S, Ciancio S. The effect of doxycycline hyclate, chlorhexidine gluconate, and minocycline hydrochloride on osteoblastic proliferation and differentiation in vitro. J Periodontol. 2009;80:999-1005.

132. Kirkwood K, Martin T, Andreadis S, Kim Y. Chemically modified tetracyclines selectively inhibit IL-6 expression in osteoblasts by decreasing mRNA stability. Biochem Pharmacol. 2003;66:1809-19.

133. Muthukuru M, Sun J. Doxycycline Counteracts Bone Morphogenic Protein-2 Induced Osteogenic Mediators. J Periodontol. 2012;in press.

134. Kaneko H, Sasaki T, Ramamurthy N, Golub L. Tetracycline administration normalizes the structure and acid phosphatase activity of osteoclasts in streptozotocin-induced diabetic rats. Anat Rec. 1990;227:427-36.

135. Kinugawa S, Koide M, Kobayashi Y, Mizoguchi T, Ninomiya T, Muto A, et al. Tetracyclines convert the osteoclastic-differentiation pathway of progenitor cells to produce dendritic cell-like cells. J Immunol. 2012;188:1772-81.

136. Bench T, Jeremias A, Brown D. Matrix metalloproteinase inhibition with tetracyclines for the treatment of coronary artery disease. Pharmacol Res. 2011;64:561-6.

137. Kraus R, Pasieczny R, Lariosa-Willingham K, Turner M, Jiang A, Trauger J. Antioxidant properties of minocycline: neuroprotection in an oxidative stress assay and direct radical-scavenging activity. J Neurochem. 2006;94:819-27.

138. Chen M, Ona V, Li M, Ferrante R, Fink K, Zhu S, et al. Minocycline inhibits caspase-1 and caspase-3 expression and delays mortality in a transgenic mouse model of Huntington disease. Nat Med. 2000;6:797-801.