UNIVERSIDADE DE SÃO PAULO
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Macrophage polarization dynamics and the impact of its modulation on the alveolar bone repair process

Dinâmica da polarização de macrófagos e o impacto de sua modulação no processo de reparo ósseo alveolar

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Orientador: Prof.Dr.Gustavo Pompermaier Garlet

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Dedico este trabalho,

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“Também na minha casa, hoje, nenhuma cadeira continua como estava ontem, pois eu já não sou o mesmo”

Fiódor Mikhailovitch Dostoiévski

RESUMO

Este estudo avaliou a dinâmica da polarização de macrófagos e o impacto de sua modulação, por drogas e pelo processo de envelhecimento, no resultado do processo de reparo ósseo alveolar em camundongos. Os animais C57Bl-6 foram submetidos à extração do incisivo superior e tratados com Paclitaxel (nas doses de 1 mg/kg/semana, 10 mg/kg/semana e 10 mg/kg/48h), previamente descrito como um composto indutor do perfil M1. No geral, os grupos tratados com PTX apresentaram maior densidade de fibroblastos e fibras, com menor densidade de tecido ósseo no período de 7 dias ($p<0,05$). Os grupos PTX também apresentaram maior densidade de vasos. O tratamento com PTX na dose de 1 e 10 mg/kg/semana mostrou principalmente uma diminuição na densidade de células inflamatórias, enquanto a PTX na dose de 10 mg/kg/48h mostrou uma maior contagem de células inflamatórias. Apesar dessas alterações, o reparo ósseo alveolar ocorreu de forma adequada, em todos os grupos tratados com PTX. Por outro lado, quando testado em um contexto inflamatório distinto (ou seja, lesões periapicais experimentais inflamatórias crônicas), a administração de PTX foi associada a um aumento na progressão/gravidade da lesão, associada a menor número de células M2(CD206+), menor expressão de ARG1 ($p<0,05$) e maior contagem de células M1(CD80+), associada à maior expressão de vários mediadores pró-inflamatórios ($p<0,05$), sugerindo que a capacidade da PTX em promover o perfil pró-inflamatório M1 pode depender da natureza do microambiente inflamatório. O próximo passo foi a avaliação dos efeitos do Ibuprofeno (IBU, 40 mg/kg e 200 mg/kg) na dinâmica da polarização dos macrófagos e no resultado do reparo ósseo alveolar. Nossos resultados mostraram que os grupos IBU apresentaram diminuição na contagem de células inflamatórias, que foi mais pronunciada na dose de 200 mg/kg ($p<0,05$). Além disso, o grupo IBU, na dose de 40 mg/kg, apresentou menor contagem de células CD206+ e menor contagem de células GR1+ no período de 3 dias ($p<0,05$). Apesar de tais alterações, a terapia com ibuprofeno não demonstrou prejudicar o processo de cicatrização óssea, como comumente referido na literatura. Por fim, avaliamos a possível influência das alterações associadas ao envelhecimento precoce na resposta imune inflamatória e seu impacto no reparo ósseo. Nossos resultados demonstraram que o grupo AGING apresentou aumento da expressão de mRNA de interleucinas pró-inflamatórias e diminuição da expressão de mRNA de interleucinas regulatórias. Níveis mais baixos de fatores de crescimento

como níveis de FGF1, Tfgb1 e Vegfa mRNA também estavam presentes no grupo AGING. Marcadores relacionados a macrófagos, como iNOS, ARG e FIZZ, apresentaram regulação negativa no grupo AGING. Marcadores de células-tronco também se mostraram diminuídos no grupo AGING em vários períodos experimentais, em comparação com o grupo controle ($p<0,05$). O grupo AGING também apresentou aumento da densidade de fibras e fibroblastos de acordo com o aumento de Col1A2, Col1A1 e MMP8 ($p<0,05$) e diminuição geral nas BMPs 2, 4 e 7 e em vários marcadores ósseos ($p<0,05$). Consequentemente, a densidade óssea no grupo AGING foi menor com menor contagem de osteoblastos ($p<0,05$). Nossos resultados apontam para um possível perfil pró-inflamatório de baixo grau e diferenciação retardada de células progenitoras ósseas. No entanto, tais alterações não parecem prejudicar o processo de reparo ósseo, uma vez que o alvéolo foi preenchido com novo osso ao final do período experimental.

Palavras-chave: Reparo ósseo, macrófagos M1, polarização

ABSTRACT

Macrophage polarization dynamics and the impact of its modulation on the alveolar bone repair process

This study evaluated the dynamics of macrophage polarization and the impact of its modulation, by drugs and by the aging process, on the outcome of the alveolar bone repair process in mice. Initially, C57Bl-6 submitted to upper incisor extraction was treated with Paclitaxel (PTX, at 1 mg/kg/week, 10 mg/kg/week and 10 mg/kg/48h doses), previously described as a M1 polarizing compound. Overall, PTX treated groups tendent to show greater density of fibroblasts and fibers, showing lower density of bone tissue in the 7-days period ($p<0.05$). PTX groups also showed higher density of vessels. PTX at 1 and 10 mg/kg/week dose, showed mostly a downregulation in inflammatory cells, while PTX at 10 mg/kg/48h dose showed a higher count in the inflammatory cells. Despite of these alterations, alveolar bone repair proceeded appropriately, in all PTX treated groups. Conversely, when tested in a distinct inflammatory context (i.e. chronic inflammatory experimental periapical lesions) PTX administration was associated with increased lesion progression/severity, associated with less M2(CD206+) cells numbers, lower ARG1 expression ($p<0.05$)and M1(CD80+) cells count associated with higher expression of several pro-inflammatory mediators ($p<0.05$), suggesting that PTX capacity to promote M1 pro-inflammatory profile may depend on the nature of inflammatory microenvironment. The next step was the evaluation of of Ibuprofen (IBU, 40 mg/kg and 200 mg/kg) effects on macrophage's polarization dynamics and alveolar bone repair outcome. Our results showed that the IBU groups showed /decreased (?) inflammatory cells count, which was more pronounced in the at the 200 mg/kg dose ($p<0.05$). Also, the IBU group, at 40 mg/kg, showed lower count of CD206+ and lower count of GR1+ cells at the 3-days period ($p<0.05$). Despite such alterations, Ibuprofen therapy didn't show to impair the bone healing process, as commonly mentioned in the literature. Finally, we evaluated the possible influence of early aging associated changes in inflammatory immune response and its impact in bone repair. Our results demonstrated that the AGING group presented increased mRNA expression of pro-inflammatory interleukins and downregulated mRNA expression of the regulatory interleukins. Lower levels of growth factors as FGF1, Tfgb1 and Vegfa mRNA levels were also present in AGING group.

Macrophage related markers, such iNOS, ARG and FIZZ showed downregulation in AGING group. Stem cell markers also showed downregulation in the AGING group in several experimental periods, compared with the control group ($p<0.05$). The AGING group also showed increased density in fibers and fibroblasts accordingly with the upregulation in Col1A2, Col1A1 and MMP8 ($p<0.05$) and general decrease in BMPs 2, 4 and 7 and in a several bone markers ($p<0.05$). Accordingly, bone density in the AGING group was lower with lower counts of osteoblasts ($p<0.05$). Our results points to a possible low grade pro-inflammatory profile and delayed differentiation of bone progenitor cells. However, such alterations didn't seem to impair bone healing process, since the as the alveolar socket was filled with new bone at the end of the experimental period.

Keywords: Bone repair. M1 macrophages. polarization

LIST OF ABREVIATIONS

BMP	Bone morphogenic protein
CCL	C-C motif chemokine
CXCL	C-X-C motif chemokine
CX3CR	CX3C chemokine receptor
CXCL12	Stromal cell derived factor-1
CD80	M1 macrophages
CD206	M2 macrophages
F4/80	Macrophages
FTY720	**Fingolimod hydrochloride**
GR1	Granulocytes
IL	Interleukin
M1	Macrophages exhibit high levels of pro-inflammatory cytokines
M2	Macrophages exhibit high levels of anti-inflammatory cytokines
IBU	Macrophage colony-stimulating factor
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NF-kB	Factor nuclear kappa β
OPG	Osteoprotegerin
RANK	Receptor activator of nuclear factor kappa β
RANKL	Receptor activator of nuclear factor kappa-B ligand
RUNX2	Runt-related transcription factor 2
TGF- β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
PTX	Paclitaxel
SOST	gene that provides instructions for making the protein sclerostin
ALPL	gene that provides instructions for making alkaline phosphatase
WT C57Bl/6	wild-type mice
µCT	Micro-computed tomography

SUMMARY

1- Introduction

1 INTRODUCTION

Bone tissue is a specialized mineralized connective tissue whose functions include protection of vital organs, locomotion, and mineral reserve. Bone is constituted of a mineralized protein matrix that is associated with characteristic cells such as osteoblasts, responsible for the synthesis and mineralization of the bone matrix, osteocytes, the most abundant cells in bone tissue, which are responsible for maintaining the matrix and, finally, osteoclasts, which are large, multinucleated cells of hematopoietic origin derived from the monocyte/macrophage lineage, are responsible for matrix resorption. Despite its rigidity and inert appearance, bone tissue is endowed with high plasticity and is constantly being renewed in response to various stimuli. The remodeling process consists of cyclic bone resorption by osteoclasts followed by the deposition of a new matrix by osteoblasts, resulting in tissue renewal and maintenance of its structural and functional integrity (BUCK e DUMANIAN, 2012b, a; DATTA e colab., 2008). The remodeling property also offers the bone tissue a high regeneration capacity when there is an injury, producing new bone tissue, with morpho functional characteristics like the original (Z.S. e colab., 2008)

Thus, the maintenance of tissue integrity, as well as the repair process, depend on a precise balance between the formation of new matrix by osteoblasts and its reabsorption by osteoclasts, implying a precise interrelationship between these cells and their mediators. This balance can be affected by several local and systemic factors, such as genetic factors, diet, hormones, and mechanical stimuli (ONO e TAKAYANAGI, 2017; ROBLING e TURNER, 2009; TAKAYANAGI, 2007). Among the various important factors in modulating the activity of bone tissue cells and, consequently, in their metabolism, the components of the immune system seem to play a very relevant role, evidenced in studies in the field of osteoimmunology, a branch of immunology that aims to investigate the interaction between the bone and immune systems (GINALDI e DE MARTINIS, 2016; TAKAYANAGI, 2007). Several evidence indicate that these two systems share many regulatory molecules, including cytokines, signaling molecules, receptors, and transcription factors. Such interaction is based on modulation in the remodeling process by the action of mediators produced by cells of the immune system and, in short, the prevalence of pro-inflammatory mediators causes inhibition in the function of osteoblasts and potentiates the action of

osteoclasts, in turn, anti-inflammatory mediators show exactly opposite effect (GARLET, G. P., 2010; GARLET, Gustavo Pompermaier e colab., 2012; GRAVES, Dana T. e colab., 2011). The most characteristic example of the interaction between the bone and immune systems is the exacerbated and prolonged activation of the immune system associated with chronic inflammation, which leads to increased osteoclastic activity and bone loss, as observed in rheumatoid arthritis, periodontal and periapical lesions (TAKAYANAGI, 2007).

Osteoclast activity is controlled by the RANK/RANKL/OPG system, where RANKL is the osteoclast activating factor, a transmembrane protein expressed by osteoblasts in bone tissue that binds to RANK, a receptor present on the membrane of the osteoclast precursor. RANK and RANKL interactions are indispensable in promoting the differentiation and activation of osteoclasts, and this interaction is modulated by osteoprotegerin (OPG), which binds to RANKL, inhibiting its interaction with RANK (GINALDI e DE MARTINIS, 2016; TAKAYANAGI, 2007). Under normal conditions, the RANKL/OPG balance is controlled by osteoblasts and osteocytes, however some types of leukocytes and cytokines can also produce or stimulate the production of RANKL and, consequently, alter the RANKL/OPG ratio and favor bone resorption (GARLET, G. P. e colab., 2006; MENEZES e colab., 2008; TAKAYANAGI, 2007).

The influence of cytokines on the immune system and their interaction with bone tissue is well evident in chronic inflammatory and infectious conditions, where pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα generally exert a stimulatory effect on osteoclasts and an inhibitory effect on osteoblasts. On the other hand, cytokines with anti-inflammatory properties, such as IL-4 and IL-10, exert a stimulatory effect on osteoblasts and inhibitory effects on osteoclasts. Thus, cytokines may play a protective and destructive role in the process of bone tissue destruction (GARLET, G. P. e colab., 2006; GRAVES, Dana, 2008; GRAVES, Dana T. e colab., 2011; TAKAYANAGI, 2007). However, with the characterization of different patterns of immune response, especially associated with the polarization of T helper (Th) lymphocytes in multiple phenotypes, the simple dichotomy between pro- and anti-inflammatory mediators is incomplete to explain the relationship between the immune and bone systems considering the variety of cells and mediators involved in the process. With the initial identification of Th1 and Th2 cells, characterized respectively by the expression of IFN-γ or IL-4, the Th1 type response was predominant in active

periapical and/or periodontal osteolytic lesions, while Th2 seems to predominate in lesions with characteristics of inactivity, suggesting that the Th1 / Th2 balance could be determinant of the activity status or stability of the lesions. (CLAUDINO e colab., 2010; GARLET, G. P., 2010; GARLET, G. P. e colab., 2006; GARLET, Gustavo Pompermaier e colab., 2010; GRAVES, Dana T. e colab., 2011; HONDA e colab., 2006)

In this context, it is important to emphasize that theoretically inactive lesions are characterized by greater expression of tissue repair markers, demonstrating that, even in chronic inflammatory processes, variations in the response pattern can mediate, even partially, tissue repair (GARLET, G. P., 2010; GARLET, G. P. e colab., 2006; GARLET, Gustavo P. e colab., 2010), which suggests that certain inflammatory processes may have a 'constructive' action in relation to bone repair. Despite great advances in the field of osteoimmunology, showing cells and mediators responsible for the activity and paralysis of chronic lesions, there are still few studies that focus on understanding the regulatory mechanisms involved in the repair and regeneration process, especially with regard to bone repair. It is important to emphasize that even under conditions considered physiological, the immune system is extremely relevant for tissue repair, with a controlled and self-limited inflammatory response being extremely important to orchestrate the various phases of this complex process. (MILLS, L A e SIMPSON, 2012; MOUNTZIARIS e MIKOS, 2008; THOMAS e PULEO, 2011). Histological studies demonstrate that the repair of bone defects involves the formation of blood clots, followed by the influx of inflammatory cells concomitant with the formation of a granulation tissue, later replaced by newly formed bone tissue (MILLS, L A e SIMPSON, 2012; THOMAS e PULEO, 2011). Although the exact mechanisms involved in such a process are still unclear, studies demonstrating that nonspecific blockade of the inflammatory process slows down the repair process, including bone repair, support the concept of constructive inflammation (PARK e BARBUL, 2004; Z.S. e colab., 2008). To elucidate possible mechanisms involving elements of the inflammatory immune response in the bone repair process, our group developed a post-extraction alveolar bone repair model in mice (VIEIRA e colab., 2015). In this model, it was observed that the tissue events classically described are in fact accompanied by a series of events associated with the immune and inflammatory response; including temporally coordinated expression of repair markers and growth factors (BMPs, TGF-β, VEGF); bone markers (RUNX2, ALP, OCN, DMP1,

PHEX, RANKL, OPG, RANK, CTSK); matrix remodeling markers (MMP-2, MMP-9, TIMP1, TIMP3, COL1A2); (CCR1, CCR2 and CCR5), especially during the early stages of alveolar bone repair, suggesting a regulatory role for inflammatory molecules (VIEIRA e colab., 2015).

A crucial point to be observed is the multiplicity of cell types and mediators potentially involved in the interaction between the immune and bone systems in pathological conditions of tissue repair. In this context, studies carried out with an exclusive focus on individual markers, without a comprehensive simultaneous assessment of several factors, are not necessarily representative of the general picture of local immunoregulation. Among the various cell types involved in the repair process, macrophages have been considered as possible key elements in the bridge between inflammation and successful repair outcomes. Macrophages are key "danger signal" sensors in both infectious and aseptic inflammation conditions (MARTINEZ e GORDON, 2014; MATZINGER, 2002, 2012; MILLS, C D e colab., 2000; MOSSER e EDWARDS, 2008). In the repair process, macrophages are also believed to be involved in shifting from the inflammatory phase to the proliferative phase through the secretion of various mediators, such as growth factors and cytokines, which act by recruiting various types of cells, such as fibroblasts that contribute to repair. the construction, organization, and vascularization of new tissue matrix (DAS e colab., 2015a; MOSSER e EDWARDS, 2008). Although classically considered as pro-inflammatory cells, macrophages can present distinct functional phenotypes called M1 and M2.

In general, activation of macrophages via receptors such as TLR and RAGE developed in the presence of IFN-γ, TNF-α or LPS results in an inflammatory phenotype called M1, also called classical activation; expressing TNFα, IL-6, IL-1β, IL-6 and iNOS (FERRANTE e LEIBOVICH, 2012; HUME, 2015; MOTWANI e GILROY, 2015). On the other hand, macrophages can also undergo an alternative activation in response to IL-4 and/or IL-10 and, consequently, develop a regulatory and/or reparative phenotype called M2 (BASHIR e colab., 2016; HUME, 2015). M2 macrophages are theoretically responsible for macrophage activity in late stages of repair and are characterized by high expression of CD163, CD206, MDC, MRC1 receptors and factors such as CCL22, CCL18, IL10, TGFβ1, PDGF, TIMPs, as well as arginase-1 (Arg-1) and Fizz-1 (BASHIR e colab., 2016; HUME, 2015; MOTWANI e GILROY, 2015). It is important to emphasize that there are still no well-characterized

subtypes of macrophages, and M1 and M2 represent only the extremes of the polarization state. Macrophages and their activation states are characterized by plasticity and flexibility and, depending on the stimuli of the environment in which they are present, can assume a wide range of functions (DAS e colab., 2015a; WANG, Nan e colab., 2014)

Regarding the M1/M2 polarization in osteolytic lesions, studies with models of arthritis demonstrate an association of M1 with tissue destruction and an inverse role for M2 (SIMA e GLOGAUER, 2013). Regarding periodontal lesions, M1 cells are more numerous than M2, and the depletion of M1 macrophages results in attenuation of disease progression (LAM e colab., 2014, 2016). Regarding tissue repair, studies demonstrate that the repair process involves an initial M1 polarization that rapidly evolves to M2 (SINDRILARU e SCHARFFETTER-KOCHANEK, 2013). Although these studies do not specifically involve bone repair, cytokines and growth factors differentially produced by M1 and M2 (such as TNF-α, IL-10 and TGF-β) may directly or indirectly interfere with differentiation and the presence or absence of macrophages, as well as variations in its M1 / M2 phenotype, could interfere with bone repair (BAUM e GRAVALLESE, 2014; GRAVES, Dana T. e colab., 2011; SOUZA e LERNER, 2013)

While the role in tissue destruction exerted by M1 macrophages is relatively well known in several study models, especially in contexts of chronic inflammation, the constructive/beneficial role of these cells in the repair process still does not seem clear. Generally, M1 macrophages contribute to the initiation of the 'constructive inflammation' process, which enables cell migration to the repair site. Repair models in muscle tissue show that the initial response to injury is predominantly pro-inflammatory with the presence of M1 macrophages and their pro-inflammatory cytokines TNF-α and IL1β with the aim of removing necrotic/damaged tissues (MOTWANI e GILROY, 2015). Basically, tissue repair goes through phases involving coagulation, initial inflammatory response, granulation tissue formation which is followed by regeneration/repair. Within a few hours after injury, the tissue is invaded by polymorphonuclear cells (PMN) and later by macrophages that will exercise the function of eliminating organisms or cell debris. By phagocytosing apoptotic PMNs, macrophages prevent the release of toxic proteases and reactive oxygen species, preventing injury amplification (SINDRILARU e SCHARFFETTER-KOCHANEK, 2013). When the phagocytic functions of macrophages are compromised in animals, there is a marked delay in the repair process after injury (SINDRILARU e

SCHARFFETTER-KOCHANEK, 2013). However, the participation of M1 macrophages and inflammatory mediators in the bone repair process is still unclear and seems to be limited to fracture models. Another point to be highlighted concerns the factors that potentially induce the M1/M2 polarization in the context of tissue repair, and how to experimentally modulate this process. In chronic and infectious conditions, it is believed that IFNg and IL4, characteristic products of Th1 and Th2 cells, are mainly responsible for the polarization of macrophages towards the M1 and M2 phenotypes (respectively). However, previous studies demonstrate that such cytokines are absent from the repair sites, a condition characterized by the absence of specific antigens and which in theory happens without a direct influence of T cells. Therefore, factors other than IFNg and IL4, still to be identified, would in theory be responsible for controlling the M1/M2 temporal balance at the repair sites. In addition to the probable 'natural' non-involvement of IFNg and IL4 in this process, if the experimental M1/M2 modulation were performed through the administration of such cytokines, we would have as a complicating factor their action on bone cells, as previously described (MILLS, L A e SIMPSON, 2012; MOUNTZIARIS e MIKOS, 2008; THOMAS e PULEO, 2011).

We must also consider the absence of models derived from genetically deficient mice in which it is possible to modulate the M1/M2 balance in the context of tissue repair. Thus, we opted for the pharmacological route to potentiate the M1 response in the models proposed in this project. Thus, it is imperative to highlight that Paclitaxel at low doses can induce the polarization of macrophages to the M1 profile, without causing negative effects on cell division processes described at high doses (YAMAGUCHI e colab., 2017), showing a potential regulator of the M1 function applicable to the proposed models. Considering that there is little information in the literature regarding the participation of M1 macrophages in bone tissue repair, we intend to investigate the role of these cells using the model of alveolar bone repair in C57Bl/6 mice advocated by our group (VIEIRA e colab., 2015). For this, we will induce the polarization of macrophages to the M1 profile using the drug Paclitaxel/Taxol (PTX), which is an antineoplastic agent capable of reprogramming M2 macrophages to the M1 profile in a TLR4-dependent manner, as a potential alternative to enhance the repair process. (WANDERLEY e colab., 2018).

Besides the current and sophisticated mediators used to modulate the inflammatory response, there is many classic drugs that are still used for this purpose and, by far the most common is the non-steroidal anti-inflammatory drugs (NSAIDs),

used for treatment of both acute and chronic inflammation (HALEY e VON RECUM, 2019). its mechanism of action involves the inhibition of the enzyme cyclooxygenase (COX), responsible for regulating the releasing of prostanoids, such prostaglandins, prostacyclin (PG12) and thromboxane A2 (TXA2) and arachidonic acid metabolites released from cells in certain circumstances. Such mediators play key roles in injury response, generally released in the inflammatory phase, having vasodilatory properties and being able to activate, or sensitize nociceptors that normally innervate the area, clinically causing the painful sensation (LISOWSKA e colab., 2018; MITCHELL e colab., 2018) That way, non-steroidal anti-inflammatory drugs (NSAIDs) are potent mediators, which can cause great relief in pain and control inflammation, with enzymatic inhibition of COX enzymes. (POUNTOS e colab., 2012, 2021) Nonetheless, apart from the traditional COX-related actions, NSAIDs could influence other aspects in the several phases of the repair process, such alterations in cytokine production and cells adhesion (DÍAZ-GONZÁLEZ e SÁNCHEZ-MADRID, 2015; RAAIJMAKERS e colab., 2022) There are even reports showing that the administration of NSAIDs in a model of muscle injury showed an increase in the tissue concentration of macrophages, reducing the number of necrotic fibers, suggesting a possible induction of the M1 profile (Cheung & Tidball, 2003). Thus, it would be pertinent to investigate the possible influences of NSAIDs on the different aspects and phases of bone repair, such as the balance of mediators, cell differentiation, matrix deposition and also the kinetics of inflammatory cells and the induction of macrophage polarization. However, several reports suggest that NSAIDs are correlated with various levels of impairment to the bone healing process, such as decreased mineral content, decreased density and strength, and delayed healing (POUNTOS e colab., 2008) Considering that most studies correlating NSAIDs to bone repair process occurs in bone fracture models, our study could have some different approach in this theme.

To clarify these issues, we also carried out a study to elucidate the influence in the modulation of the inflammatory process by NSAIDs in an intramembranous ossification repair model, after dental extraction (VIEIRA e colab., 2015), trying to highlight the NSAIDs influence on several alveolar bone repair steps, without focusing on prostaglandin modulation. For this, we administered ibuprofen (IBU) in C57BL/6 mice, following the evolution of the alveolar bone repair process in the periods of 0h, 1, 3, 7 and 14 days after extraction, through microtomographic, histological,

immunological analyses. -histochemical and molecular, and the outcome of the repair process will be compared with C57BL/6 mice in a control group.

It is evident how complex and important the interactions between the bone system and the immune system are, both in physiological and pathological conditions, however, it is still important to keep in mind the possible results of this interaction when both systems are altered or, in some way, compromised. Such a scenario can occur due to the aging of the organism, in this context, it is important to address the effects of this process on the bone and immune systems and, again, during the interaction of both. Generally, the aging process increases the amount of cellular damage, impairing the intracellular clearance and control mechanisms, together with the unhealthy organelles (DILORETO e MURPHY, 2015), there are also profound changes in gene expression, metabolism, and chromatin organization, developing a pro-inflammatory secretory phenotype, contributing for chronic and low-grade inflammatory profile, with increased levels of pro-inflammatory mediators, such IL-6, TNF-α and IL1-B. (AIELLO e colab., 2019; CLARK, Dan e colab., 2017; DILORETO e MURPHY, 2015; FARR e KHOSLA, 2019; FRANCESCHI e colab., 2000). Such an accumulation of inflammatory mediators could affect several important physiological processes or, in some cases specific microenvironments, like repair sites. Such disbalance could affect, for instance, macrophage polarization, playing a critical role in modulating what genes are expressed. Macrophages are heterogeneous cells, and their various phenotypes are still being elucidated and currently, there is not much understanding of how the polarization spectrum of these cells changes with age (YARBRO e colab., 2020). As macrophages are vital cells for the repair process, a better understanding of their alterations in the face of the aging process is extremely important to develop therapeutic methods that aim to circumvent these problems.

In turn, the age-related changes in bone tissue seems to be related with an impairment in the remodelation balance, due to osteocytes functions alterations, eventually leading to bone loss (FARR e KHOSLA, 2019). Consequently, the bone tissue's remarkable repair ability also gets impaired with age, since important cells of the immune system, like macrophages, T-cells, and mesenchymal stem cells (MSCs) have demonstrated intrinsic age-related changes that could impact bone healing (CLARK, Dan e colab., 2017). In summary, MSCs show decreased quantity and osteoblastic and chondrogenic differentiation potential, macrophages demonstrate decreased proliferation and increased activation, and T-cells are negatively affected

by age-related changes in bone marrow. Finally, aged animals show decreased vascular density within the callus, due to decreased levels of key angiogenic factors. Nonetheless, the aging effects on bone repair are mainly highlighted in fracture models (CLARK, Dan e colab., 2017; MEINBERG e colab., 2019), where an endochondral type of repair takes place, there is still a gap of information about age-related changes in the intramembranous bone repair outcome. Another fact the deserves to be highlighted, since most studies aim the elderly populations. is the relative lack of information aiming to elucidate when exactly the age-related changes start to manifest and how much they could impair the physiological processes (LU e colab., 2008; MEINBERG e colab., 2019). To address this gap, we proposed apply your intramembranous bone repair model, after tooth extraction (VIEIRA e colab., 2015) to elucidate possible age-related changes and their influence through all important repair phases and participating cells and mediators, during the experimental periods. Instead of using elderly animals, we used middle-aged C57/BL6 mice (32 weeks-old), compared to control mice (8 weeks-old) to address the possible initial physiological changes due to the aging process.

Thereby, the first article presents the Paclitaxel influences in the alveolar bone repair outcome. The second article presents the influence of Ibuprofen therapy in the alveolar bone healing process. Finally, the third article presents the analysis of early aging associated changes also in alveolar bone repair.

2- Articles

2 ARTICLES

The articles presented in this thesis were written according to the instructions and guidelines for article submission of the corresponding journals.

- **Article 1 -** Analysis of modulation of M1/M2 polarization transition by paclitaxel in transitory and chronic inflammatory environments and its influence on bone tissue
- **Article 2 -** Impact of ibuprofen on M1/M2 polarization dynamics and its impact in alveolar bone repair after tooth extraction.
- **Article 3 -** Analysis of early aging associated changes in inflammatory immune response and its impact in alveolar bone repair outcome

Article 1

Analysis of modulation of M1/M2 polarization transition by paclitaxel in transitory and chronic inflammatory environments and its influence on bone tissue

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ABSTRACT

The alveolar bone repair process may be influenced by several local and systemic factors that include mediators and immune system cells. Among these cells, macrophages are essential to trigger the repair process, and may acquire an inflammatory (M1) or anti-inflammatory and pro-reparative profile (M2). In this context, we evaluated the effects of Paclitaxel on macrophage polarization towards the M1 profile and its effects on the alveolar bone repair process and in periapical chronic lesions. We used 8 weeks old male C57BL / 6 mice (N = 5 / time / group). The animals were divided in Paclitaxel group receiving the drug intraperitonially at a dose of 1mg / kg / weekly, 10mg / kg / weekly and, lately, 10mg/ kg/ 48 hours, during the whole experimental period, and the control group receiving only the equivalent vehicle. All animals were submitted to extraction of the right upper incisor and were evaluated at 0, 1, 3, 7 and 14 days after extraction, followed by computed tomography (μCT), histomorphometry, immunohistochemical. 8 weeks-old-male C57Bl/6 mice (N=10/time periods), treated with PTX at dose of 10mg/ were submitted to an experimental periapical lesion, followed by molecular and protein assay (RT-PCR) and (ELISA). Our results showed that PTX treatment didn't promote great changes in alveolar bone repair, however, induced some alterations in some repair steps.

PTX treated groups tendent to show greater density of fibroblasts and fibers, showing lower density of bone tissue in the 7-days period ($p<0.05$). PTX groups also showed higher density of vessels. PTX at 1 and 10 mg/kg/week dose, showed mostly a downregulation in inflammatory cells, while PTX at 10 mg/kg/48h dose showed a higher count in the inflammatory cells. Despite of these alterations, alveolar bone repair proceeded appropriately, in all PTX treated groups. However, in periapical lesions, PTX therapy was associated with lesion progression and dimension increase. The PTX group showed increased lesion dimension, less M2(CD206+) cells numbers, and lower ARG1 expression ($p<0.05$). Also, the PTX group showed and M1(CD80+) cells count associated with higher expression of several pro-inflammatory mediators ($p<0.05$). These results point to the PTX capacity in promote the macrophages activation

towards an M1 pro-inflammatory profile, however, such regulation is better observed in the periapical lesion than alveolar bone repair.

Keywords: Bone repair. M1 macrophages. polarization

INTRODUCTION

Bone is a specialized mineralized connective tissue whose functions include protection of vital organs, locomotion, and mineral reserve. Bone is constituted up of a mineralized protein matrix that is associated with characteristic cells, osteoblasts, osteocytes, and osteoclasts(1). Bone tissue is extremely dynamic and is constantly being remodeled in response to physiological needs. The remodeling process consists of cyclic bone resorption by osteoclasts followed by the deposition of a new matrix by osteoblasts, resulting in tissue renewal and maintenance of its structural and functional integrity, implying a precise interplay between this cell types. Such plasticity gives such plasticity also guarantees bone tissue an excellent capacity for regeneration (1,2). However, bone metabolism, under physiological or pathological conditions, can be influenced by several local and systemic factors, such cells and products belonging to the immune system, being important to highlight the great interplay between these two systems (3–6). Such interaction is evident in the bone repair process, which involves complex and well-orchestrated steps, which are initiated in response to tissue damage (7). Repair depends on a transient inflammatory process, where cells of the innate immune system migrate to the site of injury to remove cellular or matrix debris and to regulate tissue healing through the secretion of mediators such as cytokines and growth factors (8). Although there are several cell types participating in this process, studies point to macrophages as important elements in tissue repair, as they are present in all its phases, including inflammation, formation of granulation tissue and deposition of new matrix (9–11). Although they are classically considered pro-inflammatory cells, their ability to adapt to the environment they are in is currently recognized, acquiring different phenotypes.(11,12). Although the microenvironmental stimuli may be diverse, macrophages have been classified into two principal phenotypes, based on the type 1 helper T-cell (Th1) and type 2 helper T-cell (Th2) polarization, since pro-inflammatory cytokines such IFN-γ and tumor necrosis factor-α (TNF-α), predominantly secreted by Th1, induces macrophages to a classic activated macrophage profile (M1). However, Th2 related cytokines such as IL-4 and IL-10, also

known as regulatory mediators, are known to inhibit macrophages classic activation, shifting these cells to acquire an alternative activation, also known as anti-inflammatory M2 macrophages (13,14). It's important to highlight the fact that T-cells are part of the adaptative immune system, being related to other types of injuries, such chronic lesions, and fractures, in turn, they did not participate in alveolar bone repair, which has a relatively fast outcome (15–18). Therefore, the regulation on macrophages polarization may be predominantly due to other factors that need to be elucidated.

In general, the beginning of repair is marked by the predominance of the M1 phenotype, responsible for the release of pro-inflammatory mediators (such as TNF-α and IL1-β), promoting phagocytosis of cell and matrix debris, as well as recruiting more leukocytes for the repair. site in question, through chemoattraction. As the process progresses, macrophages acquire the M2 phenotype, secreting growth and pro-angiogenic factors, responsible for tissue healing, and this transition is important for the proper resolution of the process (11,19–21). In this context, the participation of the M2 phenotype and its importance in the resolution of the bone repair process has been relatively well elucidated, through models that promote the induction of this cell type (22–25). Since macrophages tend to acquire initially a M1 phenotype in the repair process, induced by mostly by damage-associated molecular patterns (DAMPs), they are responsible for orchestrate the initial inflammatory response, producing amounts of cytokines and recruiting and inducing other leukocytes to remove cells and tissue debris in the injury site (11,13,14), therefore, it would be of great therapeutic interest to potentiate such functions in order to obtain better outcomes in bone healing and other repair processes. So, manipulation of immune system cells can be a positive tool to enhance various processes in the body, such as promoting repair or resolution of chronic inflammation.

Recent studies describe several molecules that could be used to modulate macrophage polarization, such as Paclitaxel. Paclitaxel is an antineoplastic agent widely used in the treatment of various types of tumors whose main mechanism of action is the paralysis of the cell cycle via microtubule stabilization, however, it can also act by reprogramming tumor-associated macrophages (TAM), usually with an M2 profile, for an M1 profile, contributing to the antitumor effect of the drug (26). As the administration of Paclitaxel seems to induce the macrophages to an M1-like profile and, consequently, induce a greater production of their pro-inflammatory mediators (26), we decided to use these immunoregulatory properties to demonstrate the

probable impact of these cells in the bone repair process. Thus, the aim of this study was to try to induce the polarization of macrophages to the M1 phenotype, using Paclitaxel, to evaluate the impact of this modulation in our model of alveolar bone repair. We also evaluated such immunoregulatory role of Paclitaxel in the balance and progression of experimental chronic endodontic lesions and their subsequent outcome, to better understand the role of M1 macrophages in these processes.

MATERIALS AND METHODS

Animals and experimental groups

The experimental groups consisted of 8-week-old male C57bl/6 wild type (WT) mice, comprising the control (CTL) and Paclitaxel treated animals (PTX) groups. The experimental groups were treated with PTX at 1mg/kg and 10mg/kg once a week, in the first experiment, and 10mg/kg/48hrs in the second experiment. The animals were acquired from Ribeirão Preto Medical School (FMR/USP) breeding facility, maintained during the experimental period in the facility of Department of Biological Sciences of FOB/USP. Throughout the period of the study, the mice were fed with sterile standard solid mice feed (Nuvital, Curitiba, PR, Brazil) and sterile water. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use following the Guide for the Care and Use of Laboratory Animals principles.

Experimental protocol and mice tooth extraction model

Male C57Bl/6 (WT) 8-week-old mice (*N* = 5 group/time point) were anesthetized by intramuscular administration of 80 mg/kg of ketamine chloride (Dopalen, Agribrans Brasil LTDA) and 160 mg/kg of xylazine chloride (Anasedan, Agribrands Brasil LTDA) in the proportion 1:1 determined according to the animal body mass, were submitted to the extraction of upper right incisor as previously described (17). Considering the potential interference of pain medication in the bone repair process, and the previous observation of the absence of abnormal behavior of mice after the tooth extraction, no additional drugs were administered to both control and experimental groups. In addition, it is important to emphasize that adverse effects of the different treatments on the health and behavior of the animals of both groups were not observed. At the end of the experimental periods (0 h, 1-, 3-, 7- and 14- days post tooth extraction), the animals were euthanized with an excessive dose of anesthetic and

the maxillae samples were collected. The maxillae samples were analyzed by micro-computed tomography (μCT), after the same samples were dissected and prepared for histomorphometry and collagen birefringence analysis or molecular analysis.

Micro-computed tomography (μCT) assessment

The maxillae samples were scanned by the Skyscan 1174 System (Skyscan, Kontich, Belgium), at 50 kV, 800 μA, with a 0.5 mm aluminum filter and 15 % beam hardening correction, ring artifacts, reduction, 180 degrees of rotation and exposure range of 1°. Images were captured with 1304 × 1024 pixels and a resolution of 14 μm pixel size. Projection images were reconstructed using the NRecon software and three-dimensional images obtained by the CT-Vox software. Morphological parameters of trabecular bone microarchitecture were assessed using the CTAn software following recommended guidelines. A cylindrical region of interest (ROI) with an axis length of 2 mm (200 slices) and diameter of 1 mm was determined by segmenting the trabecular bone located from the coronal to apical thirds. Trabecular measurements analyzed included the tissue volume (TV), bone volume (BV) bone volume fraction (BV/TV, %), trabecular thickness (Tb. Th, mm), trabecular number (Tb.N, mm), and trabecular separation (Tb.Sp), as previously described (17).

Histomorphometry analysis

Serial sections (8 semi-serial sections of each maxilla, with a 5 μm thickness for each section) were obtained using a microtome (Leica RM2255, Germany) and stained with HE (hematoxylin and eosin). Morphometric measurements were performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100× immersion objective and a Zeiss kpl 8× eyepiece containing a Zeiss II integration grid (Carl Zeiss Jena GmbH, Jena, Germany) with 10 parallel lines and 100 points in a quadrangular area. The grid image was successively superimposed on approximately 13 histological fields per histological section, comprised of all tooth sockets from the coronal limit adjacent to the gingival epithelium until the lower apical limit. For each animal/socket, sections from the medial were evaluated. In the morphometric analysis, points were counted coinciding with the images of the following components of the alveolar socket: clot, inflammatory cells, blood vessels, fibroblasts, collagen fibers, bone matrix, osteoblasts, osteoclasts and other components (empty space left by the inflammatory exudate or intercellular liquid

and bone marrow); as previously described (17). The results were presented as the volume density (mean) for each evaluated structure.

Immunohistochemistry analysis

Histological sections from 1, 3, 7, and 14 days were deparaffinized following standard procedures. The material was pre-incubated with 3 % Hydrogen Peroxidase Block (Spring Bioscience Corporation, CA, USA) and subsequently incubated with 7 % NFDM to block serum proteins. The histological sections from of all groups were incubated with and anti-Ly6g-Gr1 polyclonal antibody - sc-168490 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), antiF4/80 polyclonal primary antibodies - sc-26642 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD80 monoclonal antibody - sc-9091 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD206 polyclonal antibody - sc-34577 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:50 concentrations for 1 h at room temperature. The identification of antigen-antibody reaction was performed using 3-3′-diaminobenzidine (DAB) and counter-staining with Mayer's hematoxylin. Positive controls were performed in the mouse spleen for positive Ly6gGr1, F4/80, CD80 and CD206 receptors. The analysis of immunolabeled cells was performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100× immersion objective. The quantitative analysis for the different markers was performed throughout the alveolar extension, as previously described (17). The absolute number of immunolabeled cells was obtained to calculate the mean for each section.

RealTime PCR array reactions

RealTime PCR array reactions were performed as previously described. The extraction of total RNA from the remaining alveolus was performed with the RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturers' instructions, as previously described (17). The integrity of the RNA samples was verified by analyzing 1 mg of total RNA in a 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions, and the complementary DNA was synthesized using 1 mg of total RNA through a reverse transcription reaction (Superscript III, Invitrogen Corporation, Carlsbad, CA, USA). RealTime PCR array was performed in a Viia7 instrument (LifeTechnologies, Carlsbad, CA) using a custom panel containing targets

Wound Healing (PAMM-121), Inflammatory cytokines and receptors (PAMM-011) and Osteogenesis (PAMM-026) (SABiosciences, Frederick, MD) for gene expression profiling. An initial analysis performed with pooled samples from all time points was performed to triage the factors with a significant variation in the expression profile, followed by the analysis of the individual time points expression levels of the factors differentially expressed in the initial analysis. RealTime PCR array data were analyzed by the RT2 profiler PCR Array Data Analysis online software (SABiosciences, Frederick, MD), normalized initially using the geometric mean of three constitutive genes (GAPDH, ACTB, Hprt1) and subsequently normalized by a control bone sample (comprising normal alveolar bone), and expressed as fold change relative to this normalizing control, as previously described.

Statistical analysis

Data were presented as means ± SD; initially, the data distribution was tested by the Shapiro-Wilk normality test. The statistical significance comparing control and AGING groups in each time point was analyzed by Student's *t*-tests. Both were performed with Graph-Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). PCR array data were analyzed by the Mann-Whitney test followed by Benjamini-Hochberg test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

First experiment, animals were treated with Paclitaxel (PTX) at 1 and 10mg/kg once a week.

Micro-computed tomography (µCT) analysis

The three-dimensional analysis from the µCT of the maxillae scanned in MicroCT (CT-Vox) showed the alveolar bone healing process comparatively in the Placlitaxel treated mice (in different concentrations: 1mg/Kg and 10 mg/Kg) and control mice (CTL) over time from 3, 7 and 14 days after tooth extraction. In the initial period, 3-days, was observed, in both experimental groups, the presence of initial hyperdense areas. Posteriorly, in the 7-days period, in the three experimental groups were observed more presence of hyperdense areas that evidenced the beginning of new bone formation, centripetal from the lateral and apical walls of the extraction sockets towards the center

and the coronal region of the alveolus. At 14-days period, the evolution in the bone formation process is evident due to the large area of the alveolar socket filled by hyperdense regions, indicating the presence of mineralized tissue (Figures 1-1 and 2-1).

Comparing the PTX treated mice (1mg/Kg and 10mg/Kg) and the CTL groups in a qualitative analysis, through the samples three-dimensional reconstruction, there are no significant differences in the repair process between the studied groups (Figures 1-1 and 2-1). Data observed by the three-dimensional analysis were confirmed when the bone microarchitecture features were quantitatively evaluated. Both groups showed that throughout the periods, there was a progressive increase in bone volume, bone fraction in relation to the total volume, the thickness of trabeculae and the number of trabeculae and decrease in the trabecular separation at 14 days period.

When both groups were compared, the PTX threated animals (at 10mg/Kg concentration) showed less Bone Volume (BV) and Bone volume in relation to tissue volume (BV/TV) in the 3-days period, when compared to control group (Figure 1-3). In the other PTX threated group (1mg/Kg concentration), an increase in the Trabecular Separation (Tb.Sp), when compared to control group, was observed in the 3-days period. Other parameters analyzed did not show significant differences when the PTX and CTL groups were compared.

Histological and histomorphometrical evaluation

The histological and histomorphometric analyses were performed for the qualitative and quantitative analysis of the characteristic elements of distinct bone healing stages, namely blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts and other structures, demonstrating an overall similarity between the bone healing features and kinetics between Paclitaxel treated groups (PTX 1mg/Kg and PTX 10mg/Kg) and its control (CTL) counterpart. Both groups showed the presence of fiber, fibroblasts, and inflammatory cells in the alveolus margin region, demonstrating the onset of the repair process. At 7-days period, the blood clot was gradually substituted by highly vascularized granulation tissue, characterized by many of fibers and fibroblasts and the start of new bone formation from the margins of the socket, in both groups. In the 14-day period, in both groups the regions previously occupied by granulation tissue was gradually substituted by bone tissue (Figures 4-1 and 5-1). Concerning the quantitative analysis, organized

structures present in bone repair in categories within different histological parameters, such as blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures; besides this parameter, we also analyzed the data in relation to connective tissue and bone tissue. Initially, the PTX treated mice, with the 1mg/Kg concentration, showed greater density of fibroblasts in the periods of 7-days and 14-days, when compared to the control group (CTL). Vessel density was lower in the PTX 1mg/Kg group, when compared to the CTL group, in the 3-days period. The PTX 1mg/Kg group had lower bone density in the 7-day period, and an increased number of osteoclasts in the 14-day period, when compared to the control group. As for the number of inflammatory cells, the PTX 1mg/Kg group, when compared to the CTL group, showed an increase in its count in the 3-day period and, subsequently, a decrease in the 7-days and 14-days periods. The “Other” parameter in the PTX 1mg/Kg group had a greater density in the 3-days period and lower density in the 14-days period, when compared to the CTL group (Figures 6-1).

Regarding the PTX treated mice, with the 10mg/Kg concentration, there was a higher density of fibers in the 7-days and 14-days periods when compared to the CTL group. The fibroblasts, in the PTX 10mg/Kg, had higher count as well, in the 3-days and 7-days periods, when compared with the CTL group (FIG.6). The vessels density was higher in the PTX 10mg/Kg in the 7-days period when compared to the CTL group. The bone tissue density was lower in the 7-day period, when compared to the CTL group. No differences in others bone tissue components were identified. The inflammatory cells density was lower in the PTX 10mg/Kg treated mice in the 7-days and 14-days when compared to the CTL group (FIG.6). Finally, when comparing the PTX 10mg/Kg with the CTL group, the blood cloth density was higher in the 7-days period and the “Other” parameter density was higher in the 3-day period and lower in the 7-day period (Figures 6-1).

Immunohistochemistry analysis of Ly6g-GR1+ and CD206+ cells

Aiming the quantification of macrophages and other inflammatory cells in the healing sites, the immunolabeling was performed to Ly6gGR1+ (granulocytes), and CD206+ (M2 macrophages) targets in the alveolar bone repair process in the CTL and PTX treated mice, at periods of 3-, 7- and 14-days after tooth extraction (Figure 7-1). The GR1+ cells were abundant at 1-day and decreased after 3 days

in both groups. No statistical difference was observed between the evaluated groups.

Second experiment, animals were treated with Paclitaxel (PTX) at 10mg/kg/48hrs

Histological and histomorphometrical evaluation

The histological and histomorphometric analyses were performed for the qualitative and quantitative analysis of the characteristic elements of distinct bone healing stages, namely blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures, demonstrating an overall similarity between the bone healing features and kinetics between Paclitaxel (PTX) treated group and its control (CTL) counterpart. Both groups showed the presence of fiber, fibroblasts, and inflammatory cells in the alveolus margin region, demonstrating the onset of the repair process. At 7-days period, the blood clot was gradually substituted by highly vascularized granulation tissue, characterized by many of fibers and fibroblasts and the start of new bone formation from the margins of the socket, in both groups. In the 14-day period, in both groups the regions previously occupied by granulation tissue was gradually substituted by bone tissue (Figure 12-1).

Concerning the quantitative analysis, organized structures present in bone repair in categories within different histological parameters, such as blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures; besides this parameter, we also analyzed the data in relation to connective tissue and bone tissue. Initially, the PTX mice showed higher density of fibers in the 1-day period and 14-days period, when compared to the control group (CTL). The PTX group also showed a higher number of fibroblasts in the 1-days, and 3-days periods, when compared with the CTL group (Figure 13-1). Vessel density was higher in the PTX group, when compared to then CTL group, in the 3-days and 7-days periods, when compared to CTL. The PTX group also showed higher count on the inflammatory cells in the 1-day and 7-days periods, when compared to CTL group. PTX group also showed lower bone density and osteoblast density in the 7-days period and lower osteoclast count in the 14-days period, compared with the control group. Finally, The PTX group had a lower blood cloth density among tissue with higher counts in the

1-day, period, when compared to the CTL group. (Figure 14-1). The “Other” parameter showed higher density in the 1-day period.

Experimental periapical lesion experiment

The experimental periapical lesion begins to gain volume as early as the 3-day period after pulp exposure and infection, reaching its largest size at the 21-day period (Figure 15-1A). The number of macrophages present at the site of injury begins to increase soon after exposure and pulpal infection. General macrophages (F4/80+) and M1 macrophages (CD80+) seem to have their numbers concomitantly increased, from the 0-hour period, increasing and remaining relatively stable, in large quantities at the lesion site until the 21- days period (Figure 15-1B). The M2(CD206+) macrophages, in turn, begin to have their numbers elevated from the 3-day period onwards, increasing steadily until reaching their peak at the 21-day period (Figure 15-1B). However, M1 macrophage activity, as assessed by iNOS expression, appears to be more prominent until the 7-day period when it reaches its peak and starts to become less intense. Concomitantly, in the 7-day period, the activity of the M2 macrophages, assessed by ARG1, begins to become more intense, reaching its peak in the 14-day period, remaining practically constant until the 21-day period (Figure 15-1D). The levels of the total STAT1 transcription factor (STAT1t), present in macrophages, begin to rise along with the number of these, right from the 0-hour period. Levels of phosphorylated STAT1 (STAT1p), indicating the active form of the transcription factor, participating in the polarization of macrophages to an M1 profile, also begin to increase along with STAT1t, however, in a smaller amount (Figure 15-1E).

Finally, the increase in the lesion seems to be involved with the higher prevalence of M1 macrophages and, consequently, higher levels of STAT1p expression (Figure 15-1E). The results also show that periapical lesions acquire larger dimensions when PTX is administered (Figure 16-1A), showing a lower number of M2 macrophages (CD206+) in the 14- and 21-day periods (Figure 16-1B). At the same time, and ARG1 (M2-CD206+), are down-regulated, accompanying the decrease in the number of M2 macrophages (Figure 16-1C). Late administration of PTX, in the 21-day period, seems to cause an increase in the periapical lesion, compared to the control (lesion), again accompanied by changes in the number of subtypes of macrophages, with the number

M1(CD80+) increasing in the 32- days period and the number of M2(CD206+), decreased in the 25- and 32-day periods (Figure 16-1 E, F).STAT1 levels were higher at 7-, 14- and 21-days in the SchizB group, where STAT1 was induced (Figure 17-1B). In turn, the group treated with FTY720, targeting STAT1 inhibition, showed its downregulation only in the 7-day period (Figure 17-1B). However, more expressively, the dimensions of the periapical lesions were larger in the SchizB group, in the 14- and 21-day periods, compared to the control group (lesion). In contrast, the FTY720 group showed smaller lesion dimensions, also in the 14- and 21-day periods (Figure 17-1A). The numbers of M1(CD80+) macrophages at the lesion site were increased with the induction of STAT1, in the SchizB group, in the 7-, 14- and 21-periods. In turn, with STAT1 inhibition, the FTY720 group showed down-regulation in the numbers of M2 macrophages (CD206+) in the 7-, 14- and 21-periods. Also, FTY720 therapy induced downregulation in pro-inflammatory mediators during lesion evolution and, on the other way, SchizB therapy induced upregulation in pro-inflammatory mediators during lesion progression (Figure 17-1A).

PTX treatment showed more prominent pro-inflammatory mediators' upregulation when administered in a late 21-days period (Figure 18-1B). Finally, in human active lesions, M1 macrophages mean were upregulated, as the pro-inflammatory markers, cytokines, and other osteoclastic mediators (iNOS, TNFa, IL1b, IL6, IL12, IFN and RANKL), and, in the other hand, regulatory markers and cytokines were downregulated (IL23, IL10, TGF, ARG1, FIZZ1), compared with control group (Figure 19-1).

DISCUSSION

Alveolar bone repair is a complex process that consists of several coordinated and subsequent phases, where a transient inflammatory process is necessary for a good evolution in tissue repair (7,17). There is a very strong relationship between the cells and products of the immune system and the bone tissue, which can impact on several pathological processes, such as periodontal and endodontic lesions (16,27), as well as on physiological processes, such as turnover and repair process (6). Despite a limited understanding of the contribution of various inflammatory cells and their mediators to bone repair, recent studies tend to point to macrophages and their polarization as important participants in the process (28). In fact, macrophages stimulated towards a M2 phenotype are related with potentiation in repair processes (DAS; BARKER;

BOTCHWEY,; DAS et al., 2013; TABANEZ et al., 2022). Since macrophages tend to acquire initially a M1 phenotype in the repair process, they are responsible for orchestrate the initial inflammatory response, producing amounts of cytokines and recruiting and inducing other leukocytes to remove cells and tissue debris in the injury site (11,13,14), therefore, it would be of great therapeutic interest to potentiate such functions in order to obtain better outcomes in bone healing and other repair processes. So, manipulation of immune system cells can be a positive tool to enhance various processes in the body, such as promoting repair or resolution of chronic inflammation. Therefore, for a better understanding of the participation of M1 subtype macrophages in the bone repair process, we performed our experimental model of intramembranous bone repair (17) in a group of C57BL/6 mice with Paclitaxel (PTX), a drug capable of reprogramming macrophages alternately activated/M2 to the classic/M1 profile.

Initially, our results showed that treatment with PTX at both concentrations (1mg/Kg and 10mg/kg) did not negatively impact new bone formation at the end of the 14-day period, as can be seen in the qualitative analysis through micro tomography reconstructions (Figures 1-1 and 1-2). However, some lesser alterations could be noticed in the bone microarchitecture between the experimental PTX groups and the control group. The group treated with PTX 10mg/Kg/week showed a decrease in the percentage of bone volume in relation to total tissue volume (BV/TV) and bone volume (BV) in the 3-day period (Figure 3-1). The group of animals treated with PTX at a concentration of 1mg/Kg/week showed an increase in trabecular separation (Tb.Sp) in the 3-day period (Figure 3-1). When the microtomographic aspects are compared with the microscopic aspects, analyzing the slides of the evaluated periods, we can conclude that the repair process did not undergo major changes, consolidating itself at the end of the experiment (Figures 4-1 and 5-1). However, in a quantitative analysis, we observed some changes in the density of certain cell types or products during the evaluated experimental periods (Figure 6-1). When compared to the control group, the PTX groups at both evaluated concentrations showed less bone tissue formation in the 7-days period (Figure 6-1). Still evaluating changes in bone tissue and its constituents, the PTX 1mg/Kg group showed a higher density of osteoclasts in the last period compared to the control group (Figure 6-1). No group treated with PTX showed differences in osteoblast density when compared to the control group. Paclitaxel is a drug used in the treatment of neoplasms of the breasts, bladder, lungs, and some

metastases (31) and acts as an inhibitor of microtubule depolymerization, which may affect the proliferation and differentiation of some cell types (32). There are reports in the literature that show a probable negative influence of PTX on osteogenesis for a certain period and that this effect seems to disappear with the passage of time after administration (33), thus, these reports seem to agree with the results obtained in our experiment. As studies that associate PTX with bone tissue generally investigate its use to combat metastases in this tissue, it is difficult to draw a parallel with our experimental model.

Prior to the formation of new bone tissue, histologically, the repair process is marked by sequential and overlapping phases, such as clot formation, migration, and proliferation of MSCs, formation of granulation tissue, angiogenesis, proliferation of fibroblasts and secretion of collagen (17). Considering that PTX affects cell proliferation and differentiation with action on microtubules (32,34), it would be prudent to expect that the use of this drug could affect fibroblast recruitment and, consequently, collagen production. In fact, there are some reports that relate the use of Paclitaxel with the inhibition of growth, migration, and production of collagen by fibroblasts (35,36). However, our results conflict with these findings, since the groups treated with PTX showed an increase in fiber and fibroblast density in some periods (Figure 6-1).

Successfully bone repair is dependent on adequate vascularization of the involved site, requiring the development of new blood vessels (7). As there are reports that point to a possible inhibitory effect in the promotion of angiogenesis, via downregulation of VEGF (37), some alteration in this parameter was expected. Following the most common findings in the literature, the group treated with PTX 1mg/Kg exhibited a decrease in vessel density in the 3-day period compared to the control group. However, controversially, the PTX 10mg/Kg group showed an increase in vessel density in the 7-day period when compared to the control group (Figure 6-1). Therefore, tests are needed for better understanding the influence of Paclitaxel in the angiogenesis process in bone healing.

In addition to the characteristic antineoplastic effect, Paclitaxel also exerts effects on the components of the host's immune system, for example, being able to enhance the response against tumor cells (38). Such immunoregulation attributed to PTX is due to the induction of genes responsible to produce pro-inflammatory mediators, which recruit and activate effector cells, such as macrophages, NKs, dendritic cells and lymphocytes (38,39). Thus, some alteration in the dynamics of migration of

inflammatory cells was expected in the evaluated periods. In fact, when compared to the control group, the PTX 1mg/Kg group showed an increase in inflammatory cell density in the 3-day period, followed by a down-regulation in the 7- and 14-day periods (Figure 6-1). In turn, the PTX 10mg/Kg group showed a decrease in the density of inflammatory cells in the 7- and 14-day periods (Figure 6-1). When we perform the immunohistochemical analysis of the collected specimens, we can notice the prevalence of Ly6gGR1+ cells (granulocytes), in the 3-day period, decreasing in density in the following periods (Figure 7-1). CD206+ cells (Macrophage M2), in turn, are less present in the 3-day period, increasing in the 7- and 14-day periods (Figure 7-1). However, no statistical difference was evidenced between the evaluated groups.

Considering that the administration of PTX (at dosages of 1mg/kg and 10mg/kg, once a week) did not negatively affect the repair, we decided to carry out a new experiment with a group of animals that received a dosage of 10mg/kg, administered every 48 hours, along with a control group. Even with the administration of PTX in shorter periods, there were, again, no major changes in the repair process, with the dental alveolus being satisfactorily filled with bone tissue at the end of the 14-day period, as shown in the qualitative histological analysis (Figure 12-1). The changes in the quantitative histological analysis tended to follow the changes shown in the groups with the weekly PTX administration regimen with a few alterations (Figure 13-1). Maybe, there seems to be a greater delay in the differentiation of osteoblasts and secretion of new matrix, as well as its subsequent remodeling, in the group that received PTX 10mg every 48 hours. Another notable alteration was the presence of inflammatory cells in the new PTX group, which showed greater density in the 1- and 7-day period compared to the control group, suggesting a higher prevalence or longer prevalence of these cells in the repair site (Figure 13-1)

In general, treatment with Paclitaxel was able to change some aspects in certain phases of the repair process, however, without compromising or improving it. The increase in the density of certain components, in our view, can be understood as a greater prevalence of these in the tissue, due to the probable imbalance in the balance of the mediators present during the evaluated periods and, in the end, this balance seems to be reestablished, enabling adequate tissue repair. Although the kinetics of macrophage migration could not have been fully investigated due to the inability to perform the comparison for markers CD80 (Macrophages M1) and F4/80 (Macrophages general) due to problems related to the necessary inputs for the

reaction, we can suggest that the treatment with Paclitaxel apparently did not influence the migration of M2 macrophages, considering that their densities in the experimental groups did not show alterations in relation to the control group, when such comparison is made through the immunohistochemical analysis of CD206+ cells. However, it should be noted that the reprogramming effect on macrophages (for an M1 profile) exerted by Paclitaxel was evident in tumor environments (26,40), which have completely different characteristics from a site undergoing repair. The tumor microenvironment presents local mediators that can influence tumor-associated macrophages (TAM) to adopt an M2-type profile, which contribute to the escape of immune surveillance, angiogenesis, matrix remodeling, contributing to tumor development. In this context, PTX administration reprograms existing TAMs to an M1-type, pro-inflammatory profile, contributing to an antitumor effect (26).

Contrary to the conditions mentioned above, bone under normal conditions and in response to injuries tends to regenerate its damaged matrix through the repair process, which comprises several stages, very well-orchestrated and regulated by complex mechanisms involving systemic and local factors, interacting with several cells that are recruited to the site of injury (7). While the process evolves, the clot is reabsorbed, giving way to a granulation tissue that plays the role of a provisional matrix, which is finally replaced by new bone matrix by osteoblasts, originating from progenitor cells recruited to the repair site (17). In this way, the process shows self-regulation and resolution, unlike what happens in pathological conditions, such as tumor environments or chronic injuries. Chronic inflammatory processes such as rheumatoid arthritis, periodontitis and endodontic lesions usually lead to loss of bone close to the compromised site, not resolving over time (41). Endodontic lesions develop mainly by infection of the pulp tissue by oral bacteria that cause an inflammatory process that invariably evolves to necrosis, reaching the tooth apex (16). As the host's defenses cannot efficiently fight the bacteria and their products that are found inside the root canal, they end up creating a granulation tissue in the periapex, rich in innate and adaptive immunity cells, to try to serve as a barrier to the invasion of microorganisms and their products from the dental canal (16). Thus, the predominance of pro-inflammatory or anti-inflammatory mediators in this environment can accelerate or paralyze osteolysis, characterizing progression or stabilization of the lesion (42,43).

Recently our group evaluated the role of the immunoregulatory molecules VIP (Vasoactive intestinal peptide) and PACAP (Pituitary adenylate cyclase activating

polypeptide) in the alveolar bone healing, and despite of the effectiveness of VIP or PACAP in modulating host response, by the early dominance of M2 macrophages, there were no great impact on the alveolar repair outcome (8) However, in an experimental periapical lesion model, VIP therapy resulted in attenuation in the lesion progression, associated with immunoregulation involving T-cells (44). In this context, we decided to use the same model of induced periapical lesion to evaluate the possible influence of Paclitaxel administration on the balance of a chronic lesion.

Our results show that paclitaxel can be associated with lesion evolution, since PTX treated groups show lesions with bigger dimensions (Figure 15-1). Remarkably PTX, when administered lately (21-days period) also seems to induce a shift in the macrophages present in the lesion site towards a M1 phenotype, since there is a notable upregulation in the iNOS mRNA, and CD80+ cells numbers (Figure 15-1), concomitantly with downregulation in ARG1 and CD206+ cells numbers (Figure 15-1). STAT1p, indicating M1 polarization, was also upregulated in PTX treated group, suggesting these cells activity (Figure 15-1). These alterations are associated with the increase in the lesion dimensions (Figure 15-1). Such lesion's increase in PTX group was associated with the upregulation of pro-inflammatory cytokines such IL-1 and TNF (Figure 16-1) and downregulation in the regulatory mediators, such IL-10 (Figure 16-1). Summary, PTX treatment seems to indeed induce macrophages towards M1 polarization, up regulating M1 markers such iNOS and STAT1p, also increasing CD80+ cells numbers, most likely for reprograming the cells present in the repair/lesion site. However, such induction was more evident in a chronic lesion microenviroment than in the alveolar bone repair.

FIGURES AND FIGURES CAPTIONS

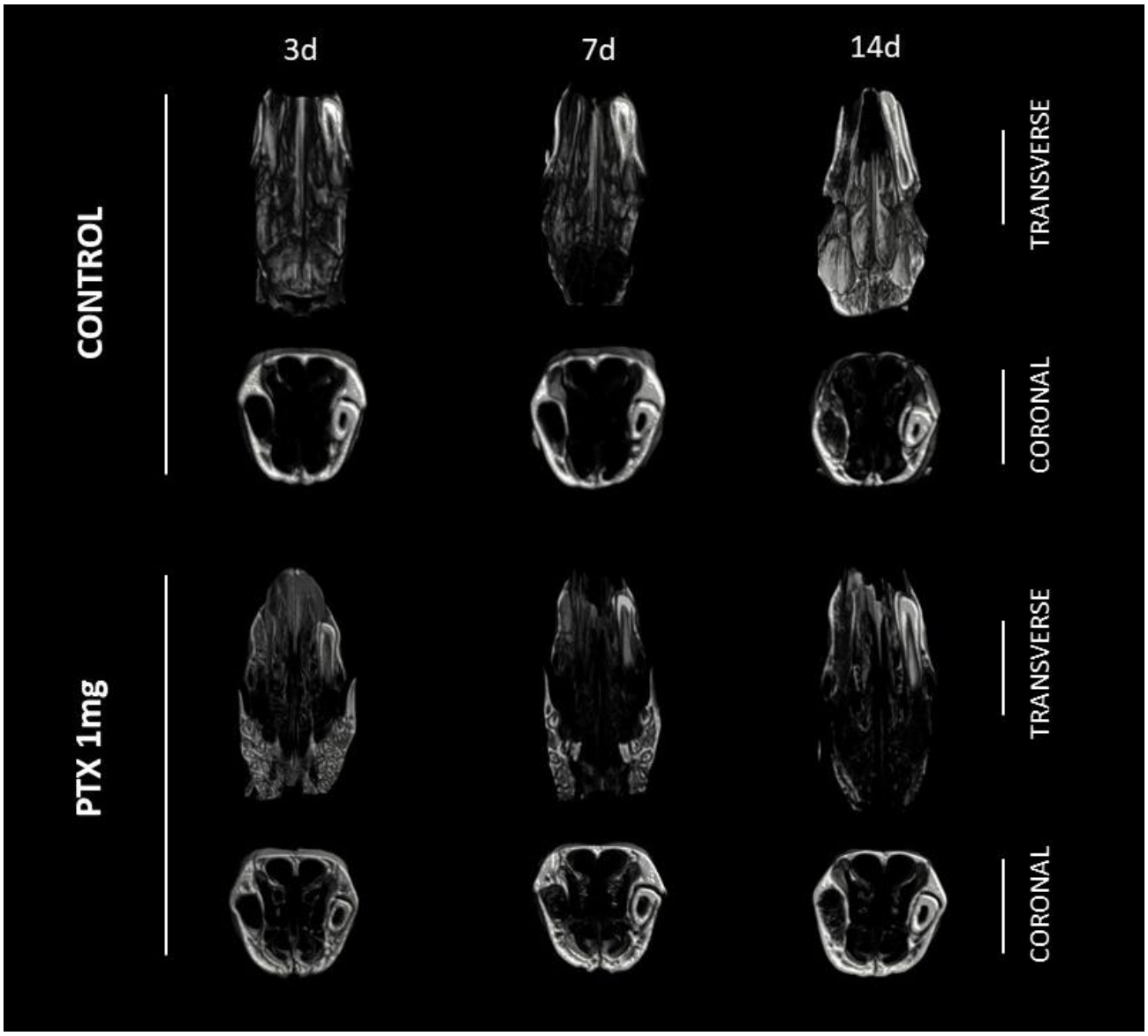

FIGURE 1-1 - Micro-computed tomography (µCT) analysis of bone healing process kinetics in Control (CTL) and Paclitaxel treated (1mg/Kg) mice (PTX). Samples from 8-week-old male mice were scanned with the µCT System (Skyscan 1174; Skyscan, Kontich, Belgium) 3-, 7-, and 14-days post tooth extraction time points for the kinetics evaluation of the bone healing process. Images were reconstructed using the NRecon software and three-dimensional images obtained with the CT-Vox software. The sectioned maxilla is represented at the transverse and coronal planes.

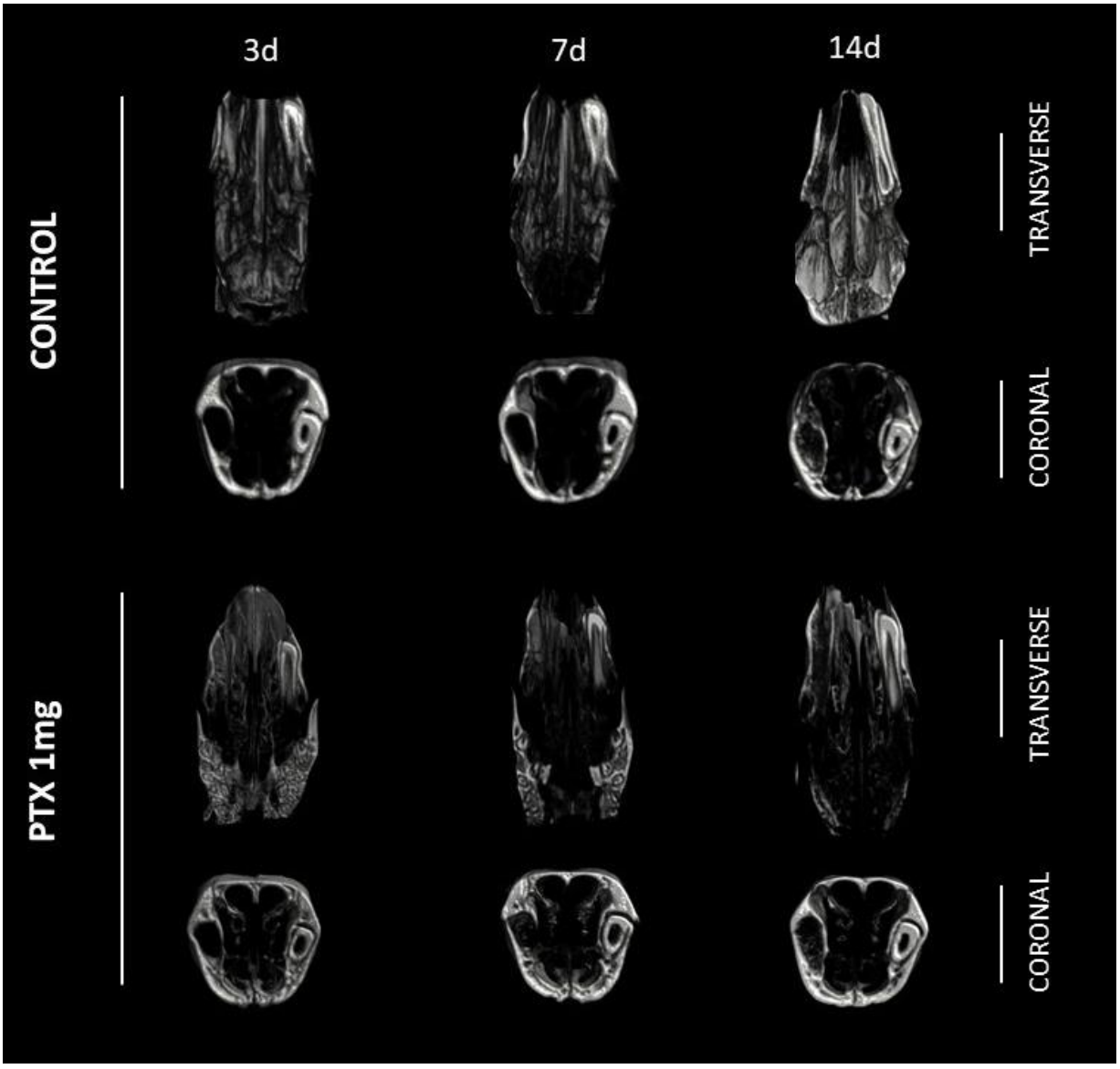

FIGURE 1-2 - Micro-computed tomography (μCT) analysis of bone healing process kinetics in Control (CTL) and Paclitaxel treated (10mg/Kg) mice (PTX). Samples from 8-week-old male mice were scanned with the μCT System (Skyscan 1174; Skyscan, Kontich, Belgium) 3-, 7-, and 14-days post tooth extraction time points for the kinetics evaluation of the bone healing process. Images were reconstructed using the NRecon software and three-dimensional images obtained with the CT-Vox software. The sectioned maxilla is represented at the transverse and coronal planes.

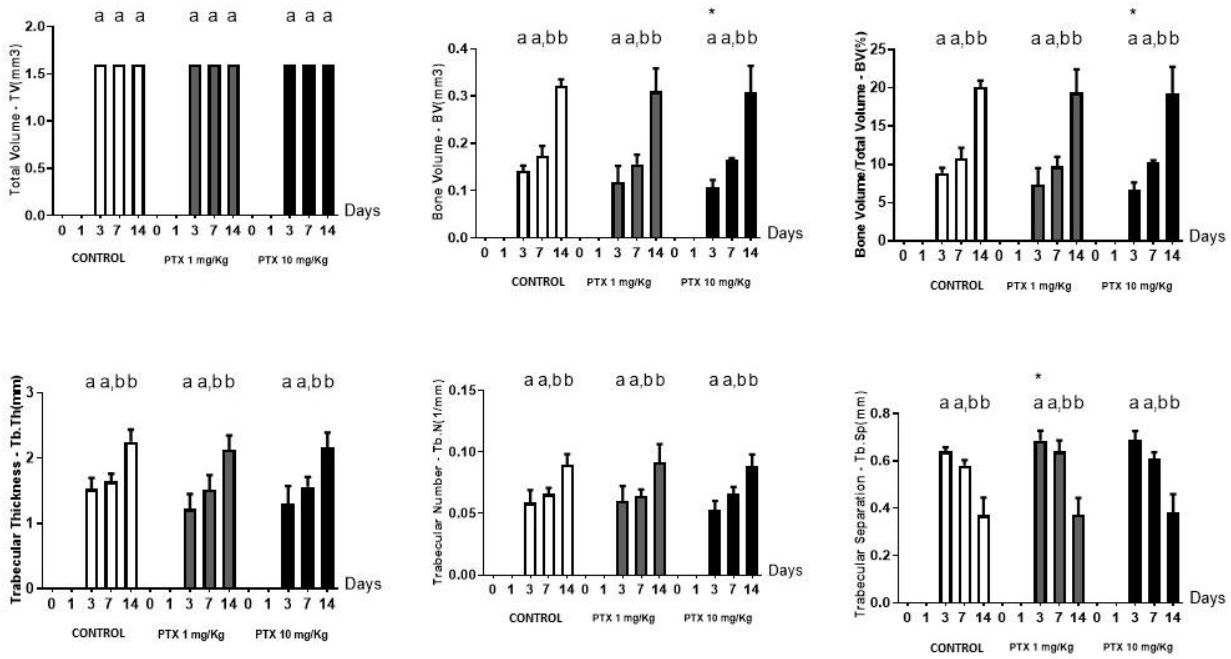

FIGURE 3-1 - Analysis of the morphological parameters of the trabecular bone microarchitecture in the dental alveoli of the control mice (CTL) and PTX threated mice (PTX 1mg/Kg and PTX 10mg/Kg) in the periods of 3-, 7- and 14-days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by microtomographic methods for the characterization mineralized tissue features at healing sites. The morphological parameters of the bone microarchitecture were evaluated using the CTAn software through a cylindrical region of interest (ROI) determined by the segmentation of the trabecular bone located from the coronal to the apical region. The analysis of bone parameters (A–F) included: Tissue volume (mm3) (A), Bone volume (mm3) (B), Bone volume in relation to tissue volume (%) (C), Trabeculae thickness (mm) (D), Number of trabeculae (1/mm) (E), Separation of trabeculae (mm) (F). The results represent the values of the mean and standard deviation in each of the periods analyzed (*Represent the differences between the control and PTX groups in each time point).

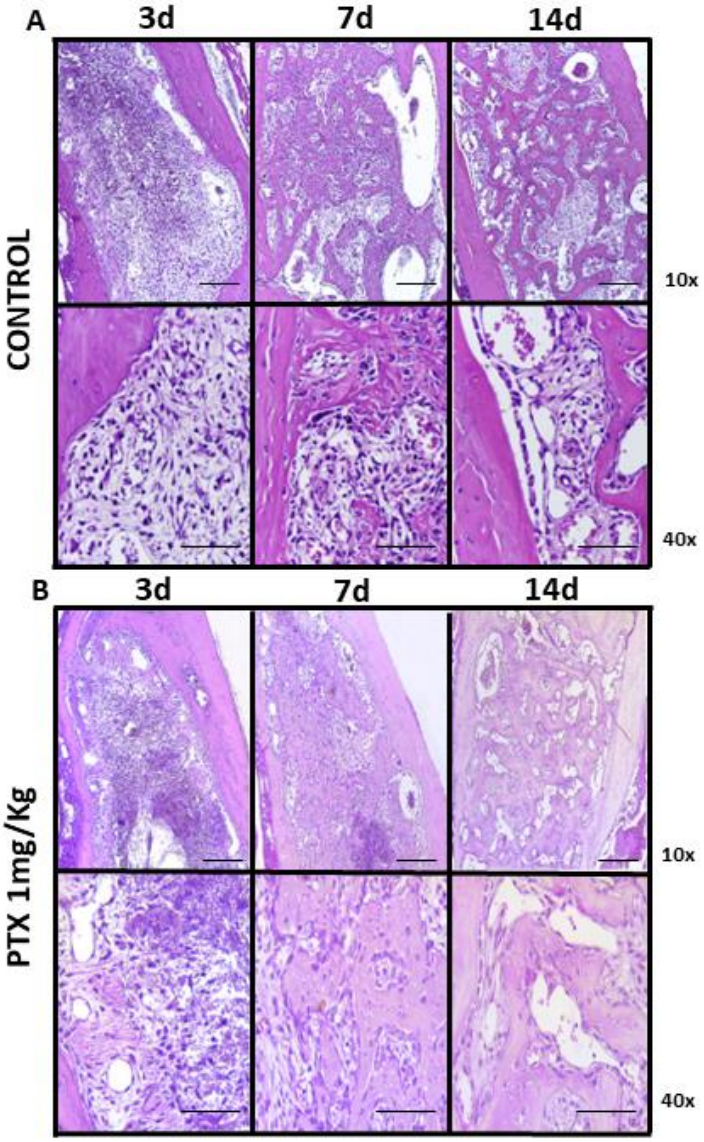

FIGURE 4-1 - Histological analysis of the alveolar healing process in control (CTL) mice (A) and in Paclitaxel treated mice (PTX 1mg/kg) mice (B), in periods of 3 -, 7 - and 14 -days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histological means. Photomicrographs representing the average region of the dental alveolus in 10× and 40× magnifications.

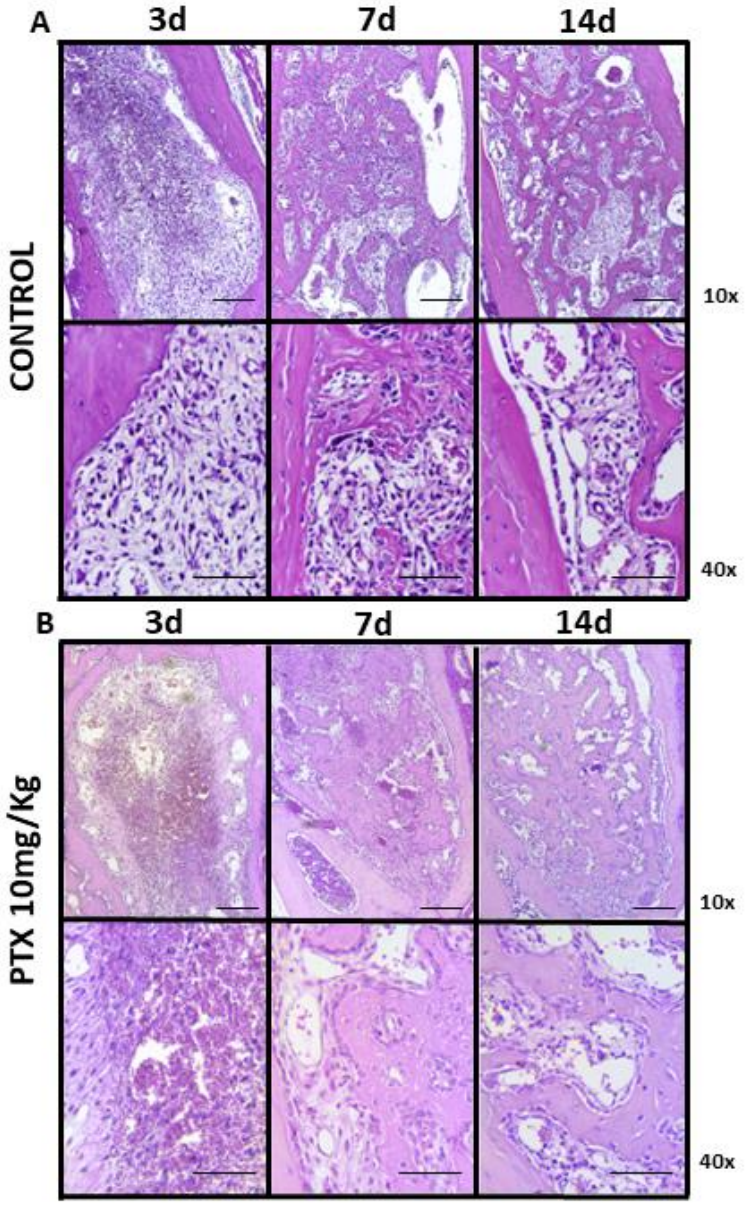

FIGURE 5-1 - Histological analysis of the alveolar healing process in control (CTL) mice (A) and in Paclitaxel treated mice (PTX 10mg/kg) mice (B), in periods of 3 -, 7 - and 14 -days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histological means. Photomicrographs representing the average region of the dental alveolus in 10× and 40× magnifications.

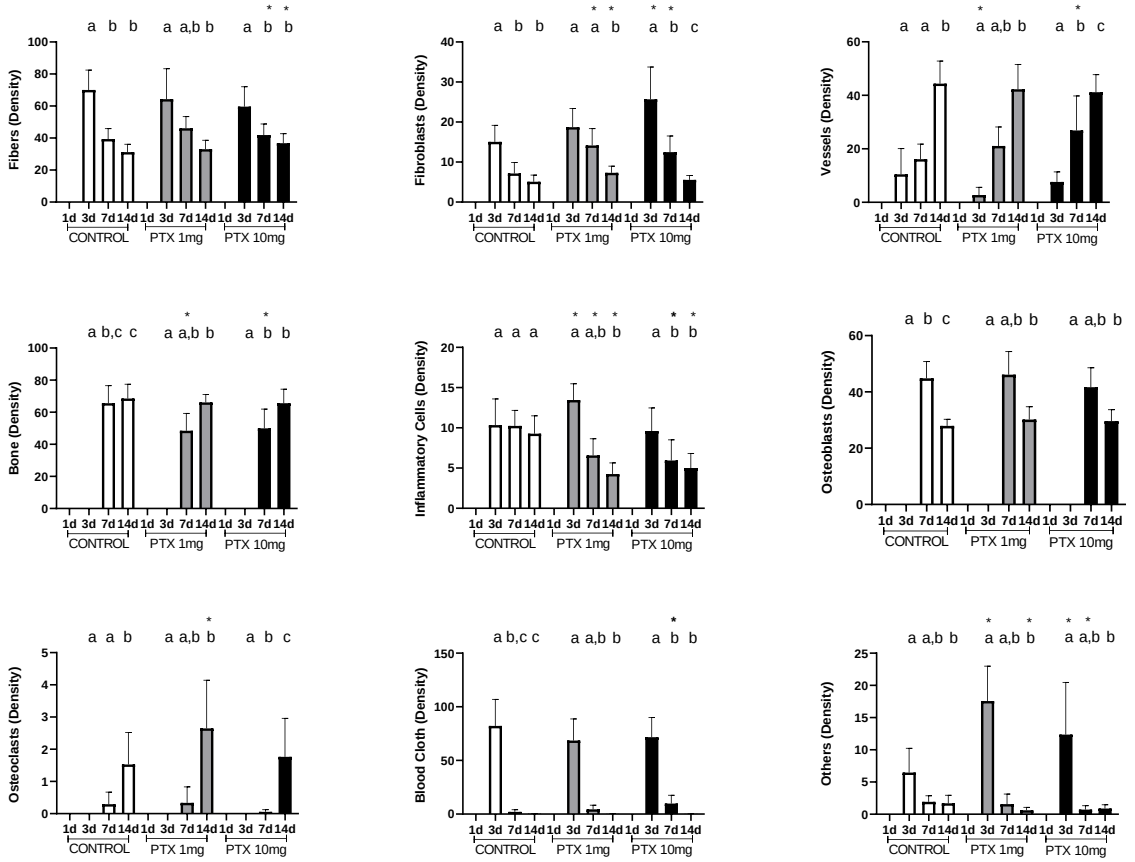

FIGURE 6-1 - Comparative density analysis (%) of connective tissue and its components present in the dental alveolus of control mice (CTL) and Paclitaxel treated mice in different concentrations (PTX 1mg/Kg and PTX 10mg/Kg) in periods of 3-, 7- and 14-days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histomorphometric methods for the characterization of cellular and tissue components at healing sites. Results are presented as the means of density for each structure of the alveolar socket: collagen fibers, fibroblasts, blood vessels, inflammatory cells, clot, and other components (empty space left by intercellular liquid and spaces). Also, the results depict the total density of connective tissue (represented by the sum of collagen fibers, fibroblasts, blood vessels and inflammatory cells). The results represent the values of the mean and standard deviation in each of the periods analyzed (*Represent the differences between the CTL and PTX groups in each time point).

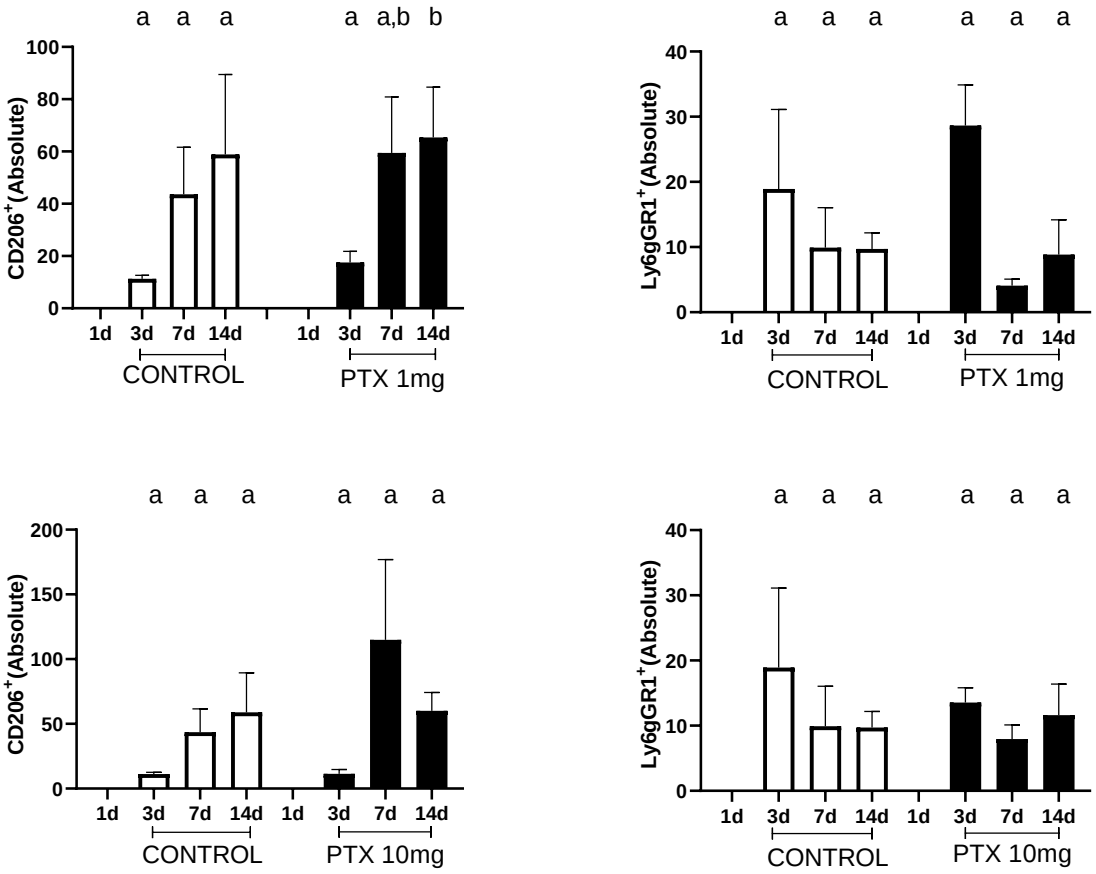

FIGURE 7-1 - Analysis of inflammatory cells in the alveolar bone healing kinetics after tooth extraction in control, and Paclitaxel treated mice. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Immunohistochemistry quantification corresponding CD206+ and Ly6gGr1+ cell at healing sites (*Represent the differences between the control and PTX groups in each time point).

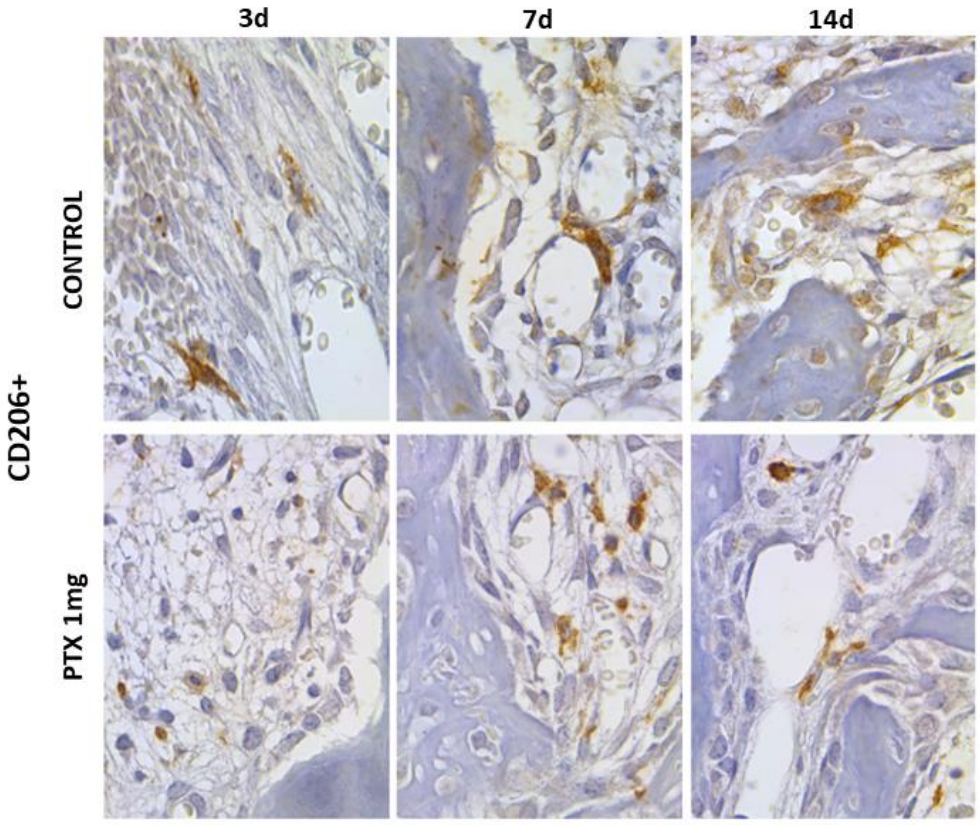

FIGURE 8-1 - Immunohistochemistry analysis for CD206+, cells present in the bone repair process in control, and PTX treated mice (1mg/Kg). Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Representative sections from medial thirds of the socket at days 3, 7 and 14 days after tooth extraction. Anti-staining Mayer's hematoxylin; objective of 100×.

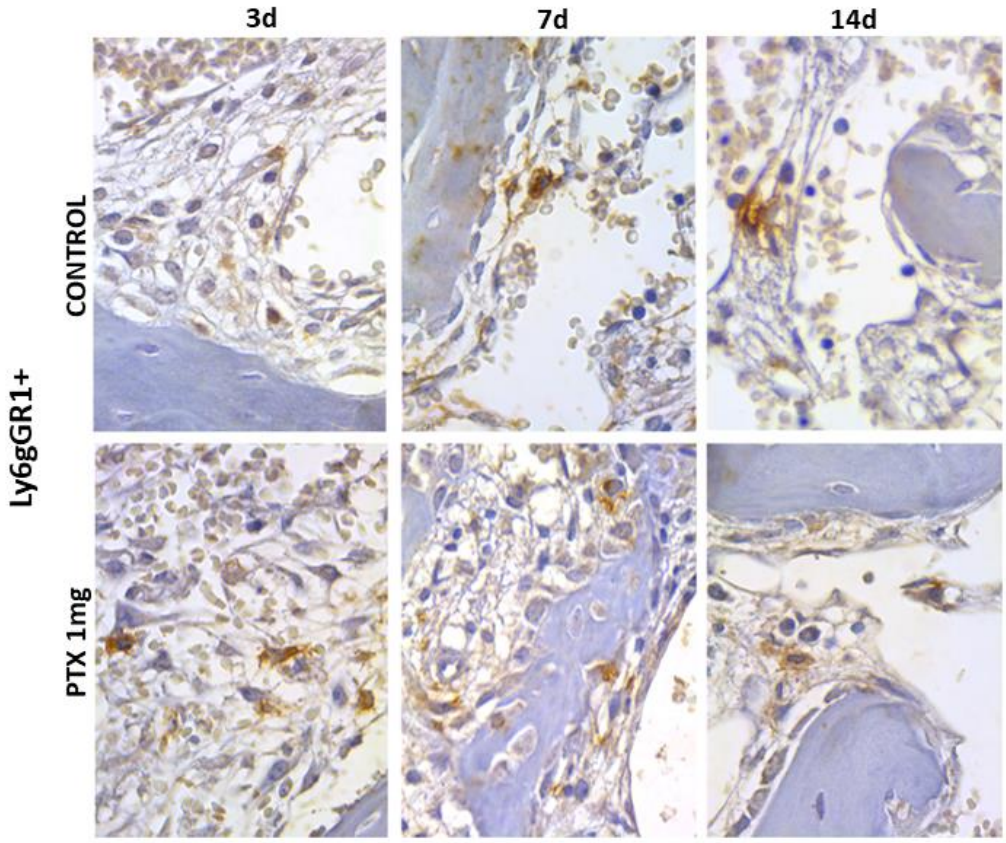

FIGURE 9-1 - Immunohistochemistry analysis for Ly6gGR1+ cells present in the bone repair process in control, and PTX treated mice (1mg/Kg). Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Representative sections from medial thirds of the socket at days 3, 7 and 14 days after tooth extraction. Anti-staining Mayer's hematoxylin; objective of 100×.

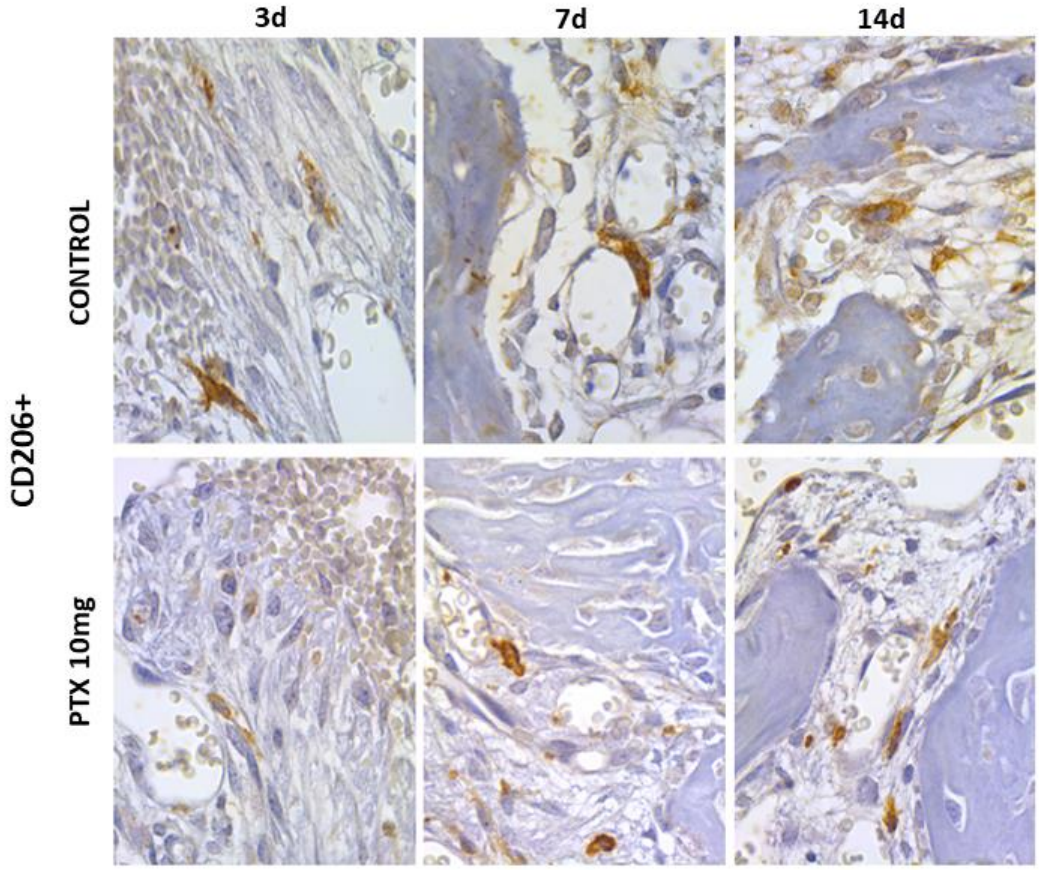

FIGURE 10-1 - Immunohistochemistry analysis for CD206+, cells present in the bone repair process in control, and PTX treated mice (10mg/Kg). Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Representative sections from medial thirds of the socket at days 3, 7 and 14 days after tooth extraction. Anti-staining Mayer's hematoxylin; objective of 100×.

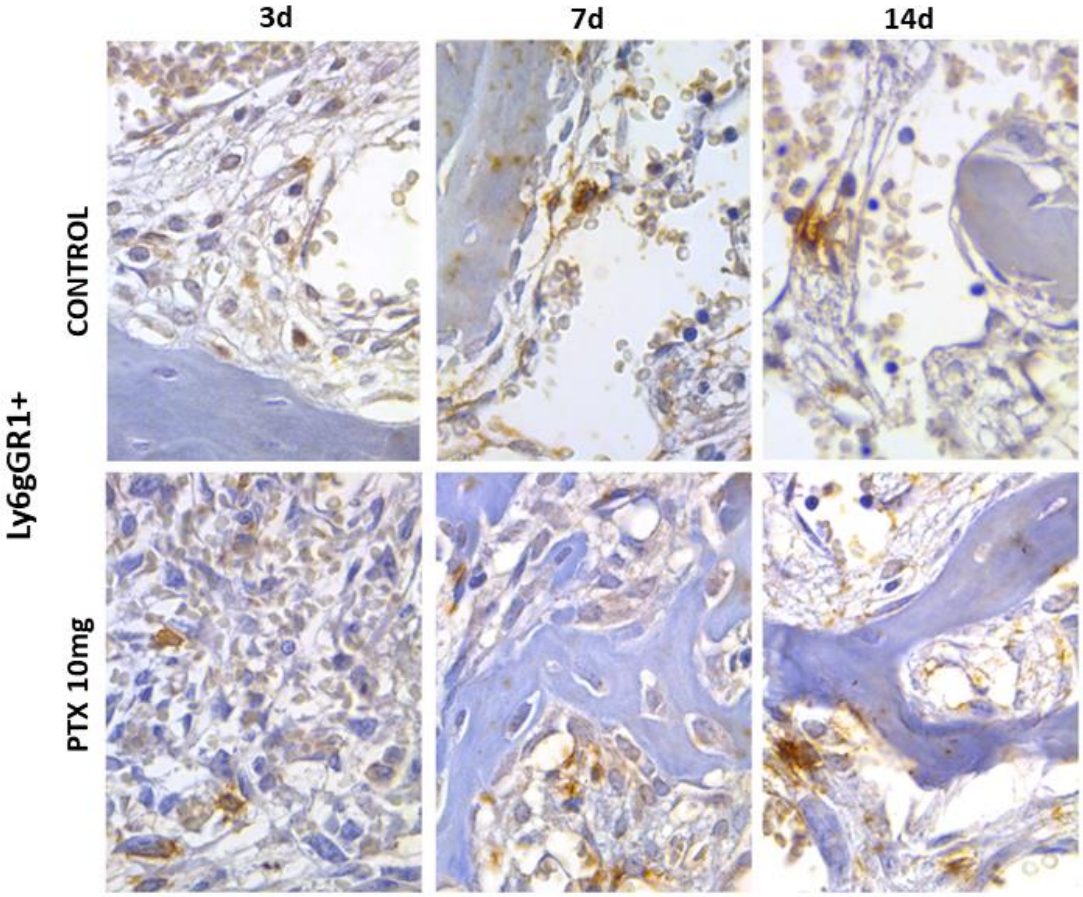

FIGURE 11-1 - Immunohistochemistry analysis for Ly6gGR1+ cells present in the bone repair process in control, and PTX treated mice (10mg/Kg). Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Representative sections from medial thirds of the socket at days 3, 7 and 14 days after tooth extraction. Anti-staining Mayer's hematoxylin; objective of 100×.

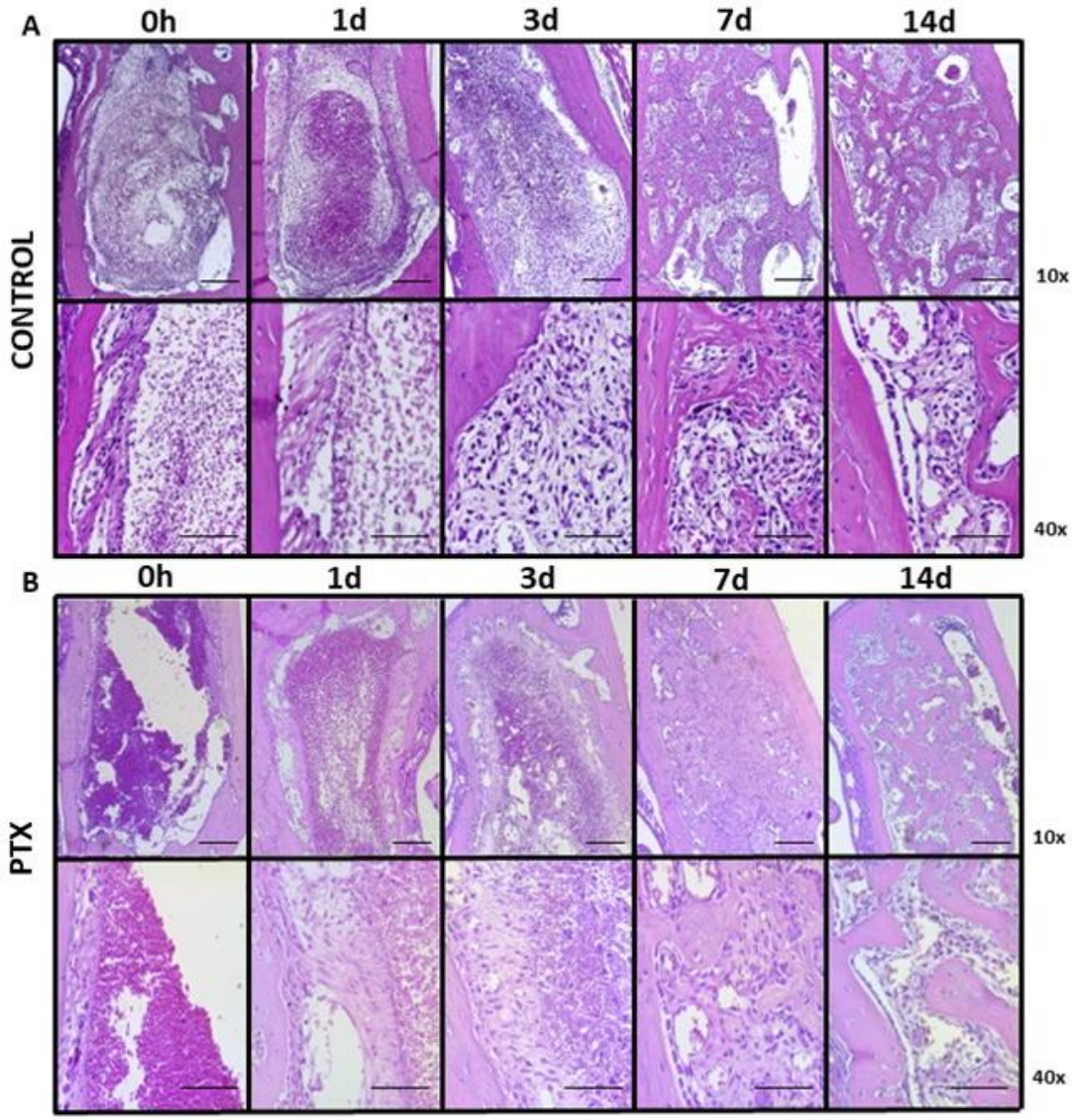

FIGURE 12-1 - Histological analysis of the alveolar healing process in control (CTL) mice (A) and in Paclitaxel treated mice, 10mg/kg/48h (PTX) (B), in periods of 0, 1 -, 3 -, 7 - and 14- days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histological means. Photomicrographs representing the average region of the dental alveolus in 10× and 40× magnifications.

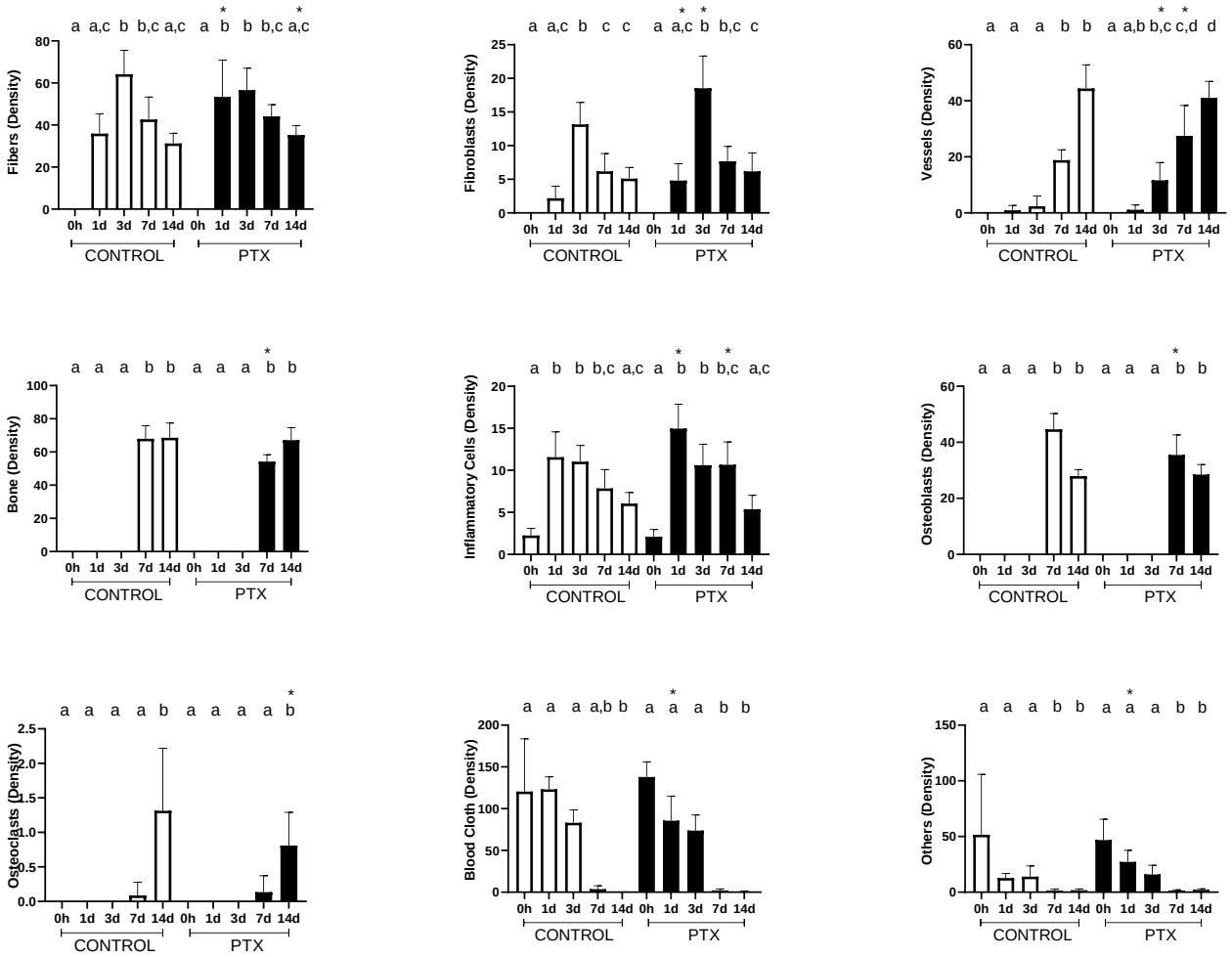

FIGURE 13-1 - Comparative density analysis (%) of connective tissue and its components present in the dental alveolus of control mice (CTL) and Paclitaxel treated mice, 10mg/kg/48h (PTX) in periods of 0, 1-, 3-, 7- and 14-days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histomorphometric methods for the characterization of cellular and tissue components at healing sites. Results are presented as the means of density for each structure of the alveolar socket: collagen fibers, fibroblasts, blood vessels, inflammatory cells, cloth, and other components (empty space left by intercellular liquid and spaces). Also, the results depict the total density of connective tissue (represented by the sum of collagen fibers, fibroblasts, blood vessels and inflammatory cells). The results represent the values of the mean and standard deviation in each of the periods analyzed (*Represent the differences between the CTL and PTX groups in each time point).

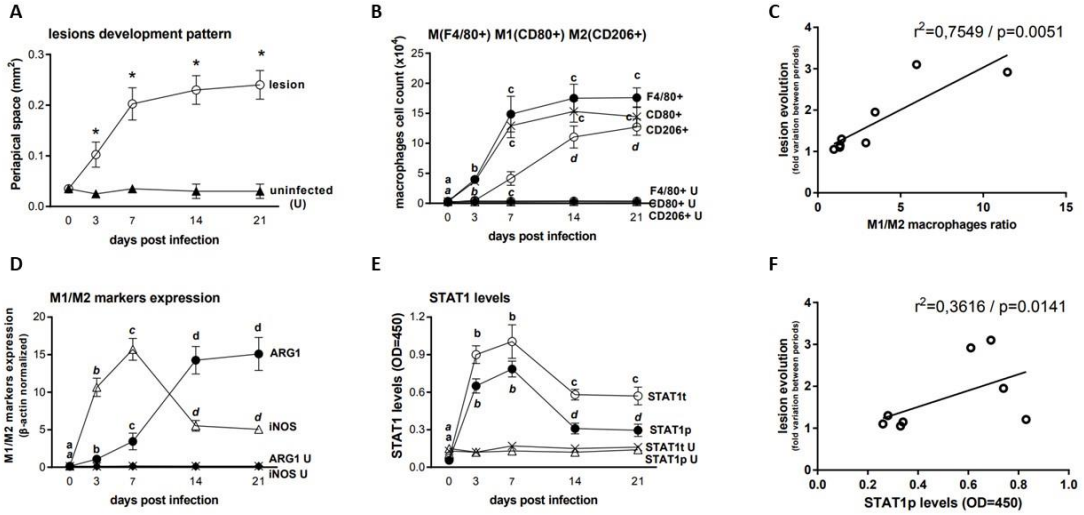

FIGURE 14-1 - Macrophages expression kinetics and its impact on periapical bone loss in experimental periapical lesions in mice. **(A)** Periapical lesion induction (pulp exposure and bacterial inoculation) was performed in C57Bl/6 mice; experimental groups were divided according to each treatment as follows: control (no lesion induction and no VIP treatment), lesion (lesion induction and no PTX treatment), Paclitaxel (lesion induction and treated with 10 mg/kg/week [Sigma-Aldrich] injected intraperitoneally 21 days after pulp exposure. Samples from the experimental and control groups were collected for histomorphometric (n 5 5/group/time point) and molecular analysis (n 5 5/group/time point) and evaluated for **(B)** General macrophages (F4/80+), M1(CD80+) and M2(CD206+) expression in during lesion evolution, measured quantitatively by the RT-PCR TaqMan system; **(C)** M1/M2 macrophages ratio related to the lesion evolution; (D) kinetics of macrophages markers expression iNOS(M1) and ARG1 (M2) from 0 to 21 days; **(E)** levels of expression of endogenous STAT1t (total) and STAT1(p) during the lesion evolution, measured quantitatively by the RT-PCR TaqMan system; **(F)** STAT1p levels correlated with the lesion evolution.

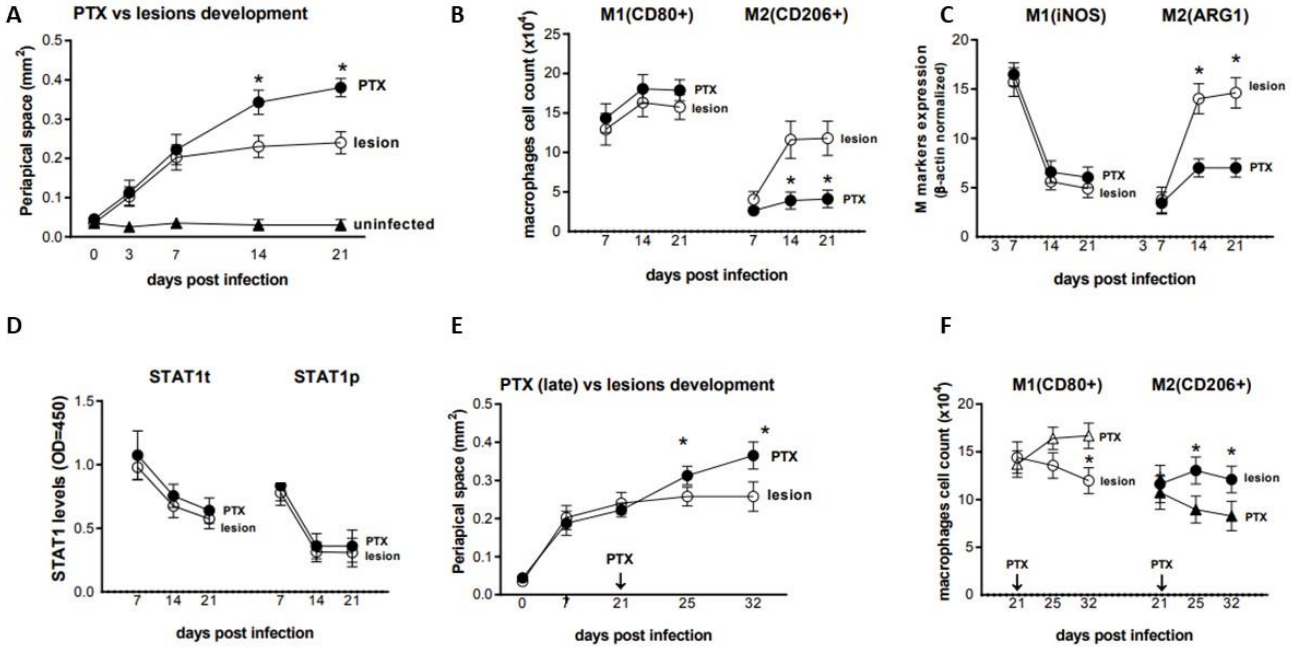

FIGURE 15-1 -: Macrophages expression kinetics and its impact on periapical bone loss in experimental periapical lesions in mice. (A) Periapical lesion induction (pulp exposure and bacterial inoculation) was performed in C57Bl/6 mice; experimental groups were divided according to each treatment as follows: control (no lesion induction and no VIP treatment), lesion (lesion induction and no PTX treatment), Paclitaxel (lesion induction and treated with 10 mg/kg/week [Sigma-Aldrich] injected intraperitoneally 21 days after pulp exposure. Samples from the experimental and control groups were collected for histomorphometric (n 5 5/group/time point) and molecular analysis (n 5 5/group/time point) and evaluated for (B) M1(CD80+) and M2(CD206+) expression in PTX treated and untreated mice measured quantitatively by the RT-PCR TaqMan system; (C) kinetics of macrophages markers expression iNOS(M1) and ARG1 (M2) from 0 to 21 days in the control and PTX treated mice; (D) level of expression of endogenous STAT1t (total) and STAT1(p) in PTX and control group measured quantitatively by the RT-PCR TaqMan system; and (E) the correlation between PTX administration at 21-days period and the lesion evolution index, compared with control(evolution of lesion development was calculated as the fold increase in the periapical space area within the 0–3, 3–7, 7–14, and 14–21 time point intervals).

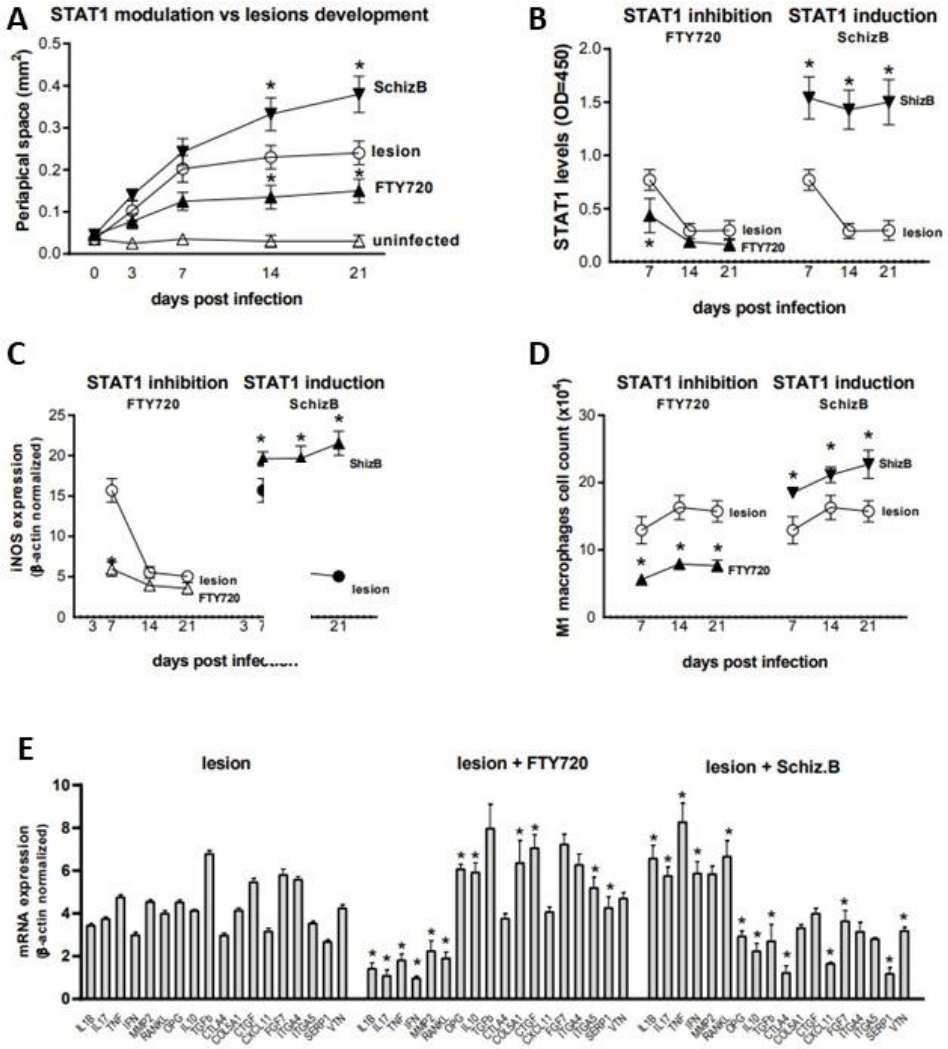

FIGURE 16-1 - STAT1 modulation and its impact in the lesion evolution and macrophages polarization during 0, 3-, 7-, 14- and 21-days periods: **(A)** Lesion progression occupying the periapical space in different groups. SchizB treated group (inducer of STAT1 expression and phosphorylation), FTY720 treated group (inhibitor of STAT1 expression and phosphorylation), lesion group (induced lesion only) and uninfected group; **(B)** STAT1 levels in the FTY720 and SchizB treated groups compared with the lesion group; **(C)** iNOS expression levels after STAT1 inhibition (FTY720) and induction (SchizB); **(D)** M1 macrophages cells count after STAT1 inhibition (FTY720) and induction (SchizB)**; (E)** mRNA expression of several growth factors, bone tissue markers, chemokines, cytokines and matrix markers in the SchizB treated group (induction of STAT1 expression and phosphorylation), FTY720 treated group (inhibition of STAT1 expression and phosphorylation), lesion group (induced lesion only).

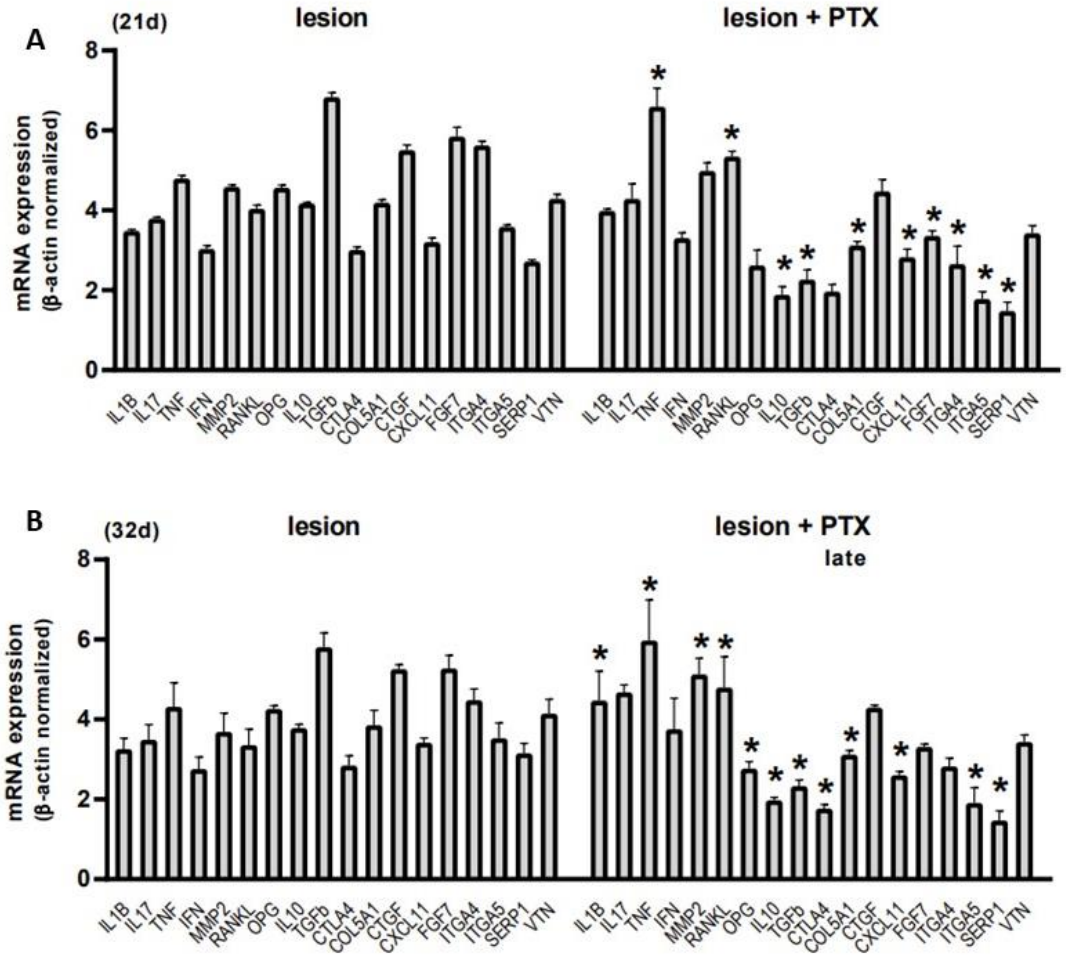

FIGURE 17-1 - mRNA expression of several growth factors, bone tissue markers, chemokines, cytokines, and matrix markers during lesion progression**. (A)** mRNA expression in the lesion group and the PTX treated lesion group since the beginning of the experiment; **(B)** mRNA expression in the lesion group and the PTX treated lesion group at 21 days period(late).

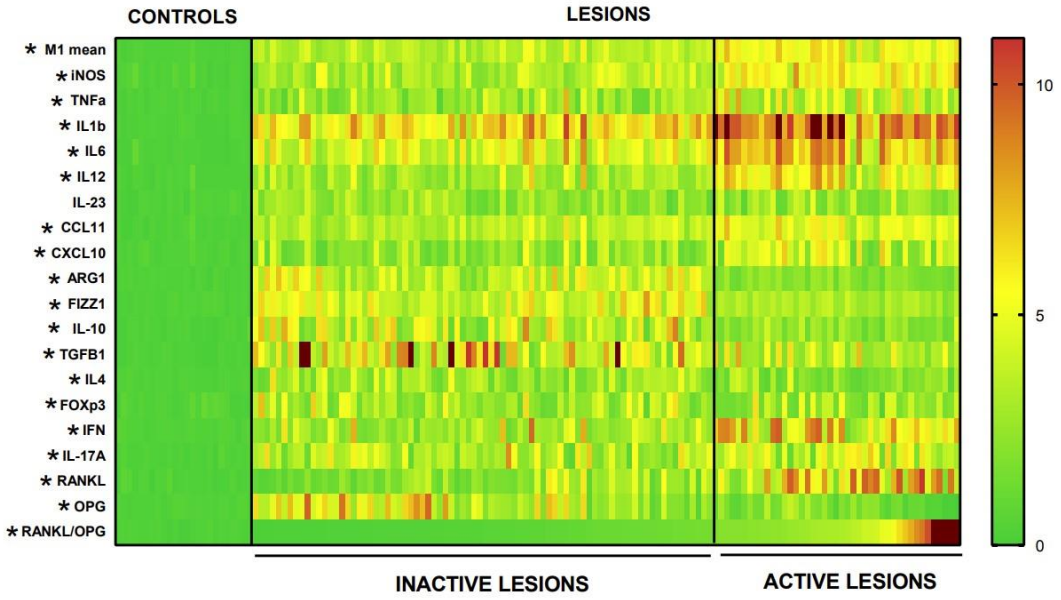

FIGURE 18-1 - Patterns of M1 macrophages and immunologic, bone, and healing marker expression in human periapical lesions. Total RNA was extracted from periapical granulomas (n 5 124) and periodontal ligament control samples (n 5 48), and immunologic, bone, and healing markers were quantitatively measured by the RT-PCR TaqMan system. The control samples and periapical granulomas were comparatively evaluated regarding the M1 macrophages and immunologic, bone, and healing markers. *P, .05 (unpaired t test), control versus lesions.

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Article 2

Impact of ibuprofen on M1/M2 polarization dynamics and its impact in alveolar bone repair after tooth extraction.

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ABSTRACT

The alveolar bone repair process may be influenced by several local and systemic factors that include mediators and immune system cells. Of the many therapeutic alternatives, NSAIDs are widely used as an alternative to control the inflammatory response and pain sensation. Since most reports about the influence of NSAIDs on bone healing come from fracture models, we decided to evaluate the effects of Ibuprofen on alveolar bone repair and their possible impact on the macrophage's polarization dynamics. In this study, we used 8 weeks old male C57BL / 6 mice (N = 5 / time / group) divided in Ibuprofen (IBU) group receiving the drug orally at a dose of 40mg / kg / daily and 200mg / kg / daily, during the whole experimental period, and the control group not being treated. All animals were submitted to extraction of the right upper incisor and were evaluated at 0, 1, 3, 7 and 14 days after extraction, followed by computed tomography (μCT), histomorphometry and immunohistochemical analysis. Our results showed that the IBU group showed lower density of fibers and fibroblasts at 40 mg/kg dose and, controversy, upregulated in the 200 mg/kg treated animals ($p<0.05$). Vessel density were predominantly lower in both IBU treated groups ($p<0.05$). As for inflammatory cells count, both IBU groups showed downregulation, which was more pronounced in the at the 200 mg/kg dose ($p<0.05$). Also, the IBU group, at 40 mg/kg, showed higher count of CD206+ and lower count of GR1+ cells at the 3-days period ($p<0.05$). Finally, bone density was lower in the IBU group (40 mg/kg) in the 7-days period, when compared with control. Despite such alterations, Ibuprofen therapy didn't show to impair the bone healing process, as commonly mentioned in the literature, since the alveolar socket was filled with new bone formed in the end of the experiment, without notable difference compared with the control group.

Keywords: Bone repair. NSAID. Intramembranous ossification

INTRODUCTION

Bone tissue is a specialized connective tissue, characterized by being in constant remodeling, which consists of cyclic bone reabsorption by osteoclasts followed by the deposition of a new matrix by osteoblasts, resulting in tissue renewal and maintenance of its structural and functional integrity. Such integrity depends on a precise balance between osteoblasts, osteocytes, and osteoclasts, implying a precise relationship between these cell types, that are also important participants in the healing process when the tissue is injured (1–3). There is a very strong communication and interrelationship between bone tissue cells and the cells and products of the immune system, which share several mediators, receptors, and transcription factors among themselves. This interplay is mostly evident during bone healing where a complex cascade of events leading to repair of injured site without scar formation. (4–8). Repair depends on a transient inflammatory process, where cells of the innate immune system migrate to the site of injury and remove cellular or matrix debris and regulating tissue healing through the mediator's secretion such as cytokines and growth factors (9).

So, proper communication between all these components is essential to obtain a good repair and restore the integrity of damaged tissues (10,11). Understanding the details of this environment is of vital importance for the creation of therapeutic tools that aim to improve the outcome in the healing process. Most of the repair studies concerning bone tissue in the literature are present in works on bone fracture, which occurs by the combination of endochondral and intramembranous ossifications (2,10). Regardless of the type of ossification, bone healing begins with the infiltration of immune cells within the formed hematoma, releasing cytokines and stimulating the inflammatory phase (5). About the cells participating in this process, it is worth highlighting the presence of macrophages, recognized as important elements in tissue repair, as they are present in all its phases, including inflammation, formation of granulation tissue and deposition of new matrix (11–13). The main mediators released in the inflammatory phase by macrophages and other inflammatory cells are interleukins (mainly IL-1 and IL-6) and tumor necrosis factor (TNF-α), which play an important role in initiating the repair cascade, recruiting other inflammatory cells, and inducing matrix synthesis and angiogenesis (5,10).

Prostaglandins, prostacyclin (PG12) and thromboxane A2 (TXA2) are known as prostanoids and arearachidonic acid metabolites released from cells and are regulated

by the cyclooxygenase (COX) isoenzymes. Prostaglandins play key roles in injury response, generally released in the inflammatory phase, having vasodilatory properties and being able to activate, or sensitize nociceptors that normally innervate the area, clinically causing the painful sensation (14,15). In the clinical context, such inflammation mediators release could cause painful sensation and its modulation or control, have a great therapeutic interest. In this direction, non-steroidal anti-inflammatory drugs (NSAIDs) are potent mediators, which can cause great relief in pain and control inflammation, with enzymatic inhibition of COX enzymes. (16,17). However, there are several reports that the administration of NSAIDs can negatively interfere with the process of repairing bone fractures, since prostaglandins (mainly PGE-2 and PGF-2α) inhibited by them stimulate new bone formation. Several publications suggest that NSAIDs are correlated with various levels of impairment to the bone healing process, such as decreased mineral content, decreased density and strength, and delayed healing (18). Nonetheless, apart from the traditional COX-related actions, NSAIDs could influence other aspects in the several phases of the repair process, such alterations in cytokine production and cells adhesion (19,20).

Thus, the focus of this study is to elucidate the influence in the modulation of the inflammatory process by NSAIDs in an intramembranous ossification repair model, after dental extraction (VIEIRA et al), trying to highlight the NSAIDs influence on several bone repair steps, such as blood cloth, cells migration and differentiation, matrix production and remodelation. Considering that, as mentioned earlier, most studies correlating NSAIDs to bone repair process occurs in bone fracture models, our study could have some different approach in this theme. For this, we administered ibuprofen (IBU) in C57BL/6 mice, following the evolution of the alveolar bone repair process in the periods of 0h, 1, 3, 7 and 14 days after extraction, through microtomographic, histological, immunological analyses. -histochemical and molecular, and the outcome of the repair process will be compared with C57BL/6 mice in a control group.

MATERIALS AND METHODS

Animals and experimental groups

The experimental groups consisted of 8-week-old male C57bl/6 wild type (WT) mice, comprising the control (CTL) and Ibuprofen treated animals (IBU) groups. The experimental group were treated with ibuprofen at 40mg/kg/24hrs, in the first experiment, and 200mg/kg/24hrs in the second experiment. The animals were acquired from Ribeirão Preto Medical School (FMR/USP) breeding facility, maintained during the experimental period in the facility of Department of Biological Sciences of FOB/USP. Throughout the period of the study, the mice were fed with sterile standard solid mice feed (Nuvital, Curitiba, PR, Brazil) and sterile water. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use following the Guide for the Care and Use of Laboratory Animals principles.

Experimental protocol and mice tooth extraction model

Male C57Bl/6 (WT) 8-week-old mice (*N* = 5 group/time point) were anesthetized by intramuscular administration of 80 mg/kg of ketamine chloride (Dopalen, Agribrans Brasil LTDA) and 160 mg/kg of xylazine chloride (Anasedan, Agribrands Brasil LTDA) in the proportion 1:1 determined according to the animal body mass, were submitted to the extraction of upper right incisor as previously described (21). Considering the potential interference of pain medication in the bone repair process, and the previous observation of the absence of abnormal behavior of mice after the tooth extraction, no additional drugs were administered to both control and experimental groups. In addition, it is important to emphasize that adverse effects of the different treatments on the health and behavior of the animals of both groups were not observed. At the end of the experimental periods (0 h, 1-, 3-, 7- and 14- days post tooth extraction), the animals were euthanized with an excessive dose of anesthetic and the maxillae samples were collected. The maxillae samples were analyzed by micro-computed tomography (μCT), after the same samples were dissected and prepared for histomorphometry and collagen birefringence analysis or molecular analysis.

Micro-computed tomography (μCT) assessment

The maxillae samples were scanned by the Skyscan 1174 System (Skyscan, Kontich, Belgium), at 50 kV, 800 μA, with a 0.5 mm aluminum filter and 15 % beam hardening correction, ring artifacts, reduction, 180 degrees of rotation and exposure range of 1°. Images were captured with 1304 × 1024 pixels and a resolution of 14 μm pixel size.

Projection images were reconstructed using the NRecon software and three-dimensional images obtained by the CT-Vox software. Morphological parameters of trabecular bone microarchitecture were assessed using the CTAn software following recommended guidelines. A cylindrical region of interest (ROI) with an axis length of 2 mm (200 slices) and diameter of 1 mm was determined by segmenting the trabecular bone located from the coronal to apical thirds. Trabecular measurements analyzed included the tissue volume (TV), bone volume (BV) bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm), and trabecular separation (Tb.Sp), as previously described(21).

Histomorphometry analysis

Serial sections (8 semi-serial sections of each maxilla, with a 5 μm thickness for each section) were obtained using a microtome (Leica RM2255, Germany) and stained with HE (hematoxylin and eosin). Morphometric measurements were performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100× immersion objective and a Zeiss kpl 8× eyepiece containing a Zeiss II integration grid (Carl Zeiss Jena GmbH, Jena, Germany) with 10 parallel lines and 100 points in a quadrangular area. The grid image was successively superimposed on approximately 13 histological fields per histological section, comprised of all tooth sockets from the coronal limit adjacent to the gingival epithelium until the lower apical limit. For each animal/socket, sections from the medial were evaluated. In the morphometric analysis, points were counted coinciding with the images of the following components of the alveolar socket: clot, inflammatory cells, blood vessels, fibroblasts, collagen fibers, bone matrix, osteoblasts, osteoclasts and other components (empty space left by the inflammatory exudate or intercellular liquid and bone marrow); as previously described (21). The results were presented as the volume density (mean) for each evaluated structure.

Immunohistochemistry analysis

Histological sections from 1, 3, 7, and 14 days were deparaffinized following standard procedures. The material was pre-incubated with 3 % Hydrogen Peroxidase Block (Spring Bioscience Corporation, CA, USA) and subsequently incubated with 7 % NFDM to block serum proteins. The histological sections from of all groups were incubated with and anti-Ly6g-Gr1 polyclonal antibody - sc-168490 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), antiF4/80 polyclonal primary antibodies - sc-

26642 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD80 monoclonal antibody - sc-9091 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD206 polyclonal antibody - sc-34577 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:50 concentrations for 1 h at room temperature. The identification of antigen-antibody reaction was performed using 3-3′-diaminobenzidine (DAB) and counter-staining with Mayer's hematoxylin. Positive controls were performed in the mouse spleen for positive Ly6gGr1, F4/80, CD80 and CD206 receptors. The analysis of immunolabeled cells was performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100× immersion objective. The quantitative analysis for the different markers was performed throughout the alveolar extension, as previously described (21). The absolute number of immunolabeled cells was obtained to calculate the mean for each section.

RealTime PCR array reactions

RealTime PCR array reactions were performed as previously described. The extraction of total RNA from the remaining alveolus was performed with the RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturers' instructions, as previously described (21). The integrity of the RNA samples was verified by analyzing 1 mg of total RNA in a 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions, and the complementary DNA was synthesized using 1 mg of total RNA through a reverse transcription reaction (Superscript III, Invitrogen Corporation, Carlsbad, CA, USA). RealTime PCR array was performed in a Viia7 instrument (LifeTechnologies, Carlsbad, CA) using a custom panel containing targets Wound Healing (PAMM-121), Inflammatory cytokines and receptors (PAMM-011) and Osteogenesis (PAMM-026) (SABiosciences, Frederick, MD) for gene expression profiling. An initial analysis performed with pooled samples from all time points was performed to triage the factors with a significant variation in the expression profile, followed by the analysis of the individual time points expression levels of the factors differentially expressed in the initial analysis. RealTime PCR array data were analyzed by the RT2 profiler PCR Array Data Analysis online software (SABiosciences, Frederick, MD), normalized initially using the geometric mean of three constitutive genes (GAPDH, ACTB, Hprt1) and subsequently normalized by a control bone sample (comprising normal alveolar bone), and expressed as fold change relative to this normalizing control, as previously described (21).

Statistical analysis

Data were presented as means ± SD; initially, the data distribution was tested by the Shapiro-Wilk normality test. The statistical significance comparing control and AGING groups in each time point was analyzed by Student's *t*-tests. Both were performed with Graph-Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). PCR array data were analyzed by the Mann-Whitney test followed by Benjamini-Hochberg test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

First experiment, animals were treated with ibuprofen (IBU) at 40mg/kg/24hr

Micro-computed tomography (µCT) analysis

The three-dimensional analysis from the µCT of the maxillae scanned in MicroCT (CT-Vox) showed the alveolar bone healing process comparatively in the ibuprofen treated mice (IBU) and control mice (CTL) over time from 1, 3, 7 and 14 days after tooth extraction. In the initial period (0 h), in both groups, the alveolar socket was completely empty, thus, the alveolar sockets did not present hyperdense areas. Nonetheless, in the 3-day period, was observed, in both experimental groups, the presence of initial hyperdense areas (Figure 1-3).

In the 7-day period, in both experimental groups were observed the presence of hyperdense areas that evidenced the beginning of new bone formation, centripetal from the lateral and apical walls of the extraction sockets towards the center and the coronal region of the alveolus. At 14-day period, the evolution in the bone formation process is evident due to the large area of the alveolar socket filled by hyperdense regions, indicating the presence of mineralized tissue. Comparing the IBU and the CTL groups in a qualitative analysis, through the samples three-dimensional reconstruction, there are no significant differences in the repair process between the studied groups (Figure 1-3). Data observed by the three-dimensional analysis were confirmed when the bone microarchitecture features were quantitatively evaluated. Both groups showed that throughout the periods, there was a progressive increase in bone volume, bone fraction in relation to the total volume, the thickness of trabeculae and the number of trabeculae and decrease in the trabecular separation at 14 days period. When both

groups were compared, the ibuprofen treated animals showed more bone volume (BV) and Bone volume in relation to tissue volume (BV/TV) in the 7-day period, when compared to control group (Figure 2-3). The other parameters analyzed did not show significant differences when the IBU and CTL groups were compared.

Histological and histomorphometrical evaluation

The histological and histomorphometric analyses were performed for the qualitative and quantitative analysis of the characteristic elements of distinct bone healing stages, namely blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures, demonstrating an overall similarity between the bone healing features and kinetics between Ibuprofen treated group and its control (CTL) counterpart. Both groups showed the presence of fiber, fibroblasts, and inflammatory cells in the alveolus margin region, demonstrating the onset of the repair process. At 7-days period, the blood clot was gradually substituted by highly vascularized granulation tissue, characterized by many of fibers and fibroblasts and the start of new bone formation from the margins of the socket, in both groups. In the 14-day period, in both groups the regions previously occupied by granulation tissue was gradually substituted by bone tissue (Figure 3-3).

Concerning the quantitative analysis, organized structures present in bone repair in categories within different histological parameters, such as blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures; besides this parameter, we also analyzed the data in relation to connective tissue and bone tissue. Initially, the ibuprofen treated mice (IBU) showed lower density of fibers and fibroblasts in the 3-day period, when compared to the control group (CTL). Vessel density was lower in the IBU group, when compared to then CTL group, in the 3-days and 14-days periods and, higher in the 7-day period. The IBU group had lower bone density in the 7-days period and higher bone density in the 14-days period, when compared with the control group. The IBU group also showed lower count on the inflammatory cells in the 14-days period, compared to CTL group. The IBU group had a higher blood cloth density among tissue and a greater presence of the “others” parameter, in the 7-days and 3-days periods, respectively (Figure 4-3).

Immunohistochemistry analysis of Ly6g-GR1+ and CD206+ cells

Aiming the quantification of macrophages and other inflammatory cells in the healing sites, the immunolabeling was performed to Ly6gGR1+ (granulocytes), and CD206+ (M2 macrophages) targets in the alveolar bone repair process in the IBU and CTL mice, at periods of 1-, 3-, 7- and 14-days after tooth extraction (Figure 7-3). The GR1+ cells were abundant at 1-day and decreased after 3 days in both groups. The number of CD206+ cells started were abundant at 1-day with a decrease at the 3-days period and a further increase in the 7-days and 14-days periods (Figure 7-3). When both groups are compared, IBU treated group had a downregulation in the CD206+ and Ly6gGR1+ cells count, both in the 1-day period (Figure 7-3).

Second experiment, animals were treated with ibuprofen (IBU) at 200mg/kg/24hrs

Histological and histomorphometrical evaluation

The histological and histomorphometric analyses were performed for the qualitative and quantitative analysis of the characteristic elements of distinct bone healing stages, namely blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures, demonstrating an overall similarity between the bone healing features and kinetics between Ibuprofen treated group and its control (CTL) counterpart. Both groups showed the presence of fiber, fibroblasts, and inflammatory cells in the alveolus margin region, demonstrating the onset of the repair process. At 7-days period, the blood clot was gradually substituted by highly vascularized granulation tissue, characterized by many of fibers and fibroblasts and the start of new bone formation from the margins of the socket, in both groups. In the 14-day period, in both groups the regions previously occupied by granulation tissue was gradually substituted by bone tissue (Figure 8-3). Concerning the quantitative analysis, organized structures present in bone repair in categories within different histological parameters, such as blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures; besides this parameter, we also analyzed the data in relation to connective tissue and bone tissue. Initially, the ibuprofen treated mice (IBU) showed higher density of fibers in the 3-days period, when compared to the control group (CTL). The IBU group also showed a higher amount of fibroblast in the 1-days, 3-days

and 14-days periods, when compared with the CTL group (Figure 9-3). Vessel density was higher in the IBU group, initially, in the 3-days period, however, becoming lower the 14-days period, when compared to CTL. The IBU group also showed lower count on the inflammatory cells in the 1-days, 3-days and 14-days periods, when compared to CTL group. Finally, The IBU group had a lower blood cloth density among tissue with higher counts in the 1-days, 3-days, 7-days and 14-days periods, when compared to the CTL group. (Figure 9-3).

DISCUSSION

Bone tissue is a specialized connective tissue, characterized by being in constant remodeling, which consists of cyclic bone reabsorption by osteoclasts followed by the deposition of a new matrix by osteoblasts, resulting in tissue renewal and maintenance of its structural and functional integrity. This dynamic present in bone tissue reflects on its remarkably regeneration capacity when it suffers some type of injury (1–3). Bone healing comprises a complex cascade of events leading to repair of injured tissue without scar formation(8). Such a process requires complex and well-orchestrated phases, performed by various cell types that are, in turn, influenced by inflammatory mediators, such as cytokines and growth factors. Some inflammatory mediators, such as prostaglandins (PGEs), mainly PGE-2 and PGF-2α, participate in stimulating new bone formation. Nonetheless, apart from the traditional COX-related actions, NSAIDs could influence other aspects in the several phases of the repair process, such alterations in cytokine production and cells adhesion (19,20).

NSAIDs are widely used inflammatory response modulators for pain control, being associated with impairment in bone healing process, mostly associated with its interference in prostaglandins production(Lisowska et al., 2018; Wheatley et al., 2019a). Bearing in mind that most of the studies correlating NSAIDs to the bone repair process take place in fracture models, focusing on PGEs interference, this study tries to elucidate the influence of the modulation in the inflammatory process by NSAIDs in an intramembranous ossification model, highlighting its effects during several repair phases, after tooth extraction (21).

Overall, our results demonstrate that sustained treatment with ibuprofen (IBU) did not negatively affect the bone repair process in the studied C57BL/6 mice. The analysis of the three-dimensional reconstruction of the maxillae derived from the µCT shows that

the bone repair was carried out adequately and similarly in the control (CTL) and IBU groups (Figure 1-3). However, when bone microarchitecture parameters are evaluated, the IBU group showed an increase in bone volume (BV) and bone volume to total volume fraction (BV/TV) in the 7-day period (Figure 2-3). This result follows the same direction, with few changes, when the histological aspect of the repair is analyzed. The histological slides studied, in a qualitative analysis of the evaluated periods (1d, 3d, 7d and 14d) show the normal kinetics of repair in both groups, similarly, with the presence of blood clot, inflammatory cells, granulation tissue with the formation of a new provisional collagen matrix, replaced by newly formed bone tissue in the final periods (Figure 3-3).

Against the result observed in bone microarchitecture analysis, in the 7-day period, the IBU group showed a lower density of bone tissue when compared to the control group (Figure 4-3), however, showed an increase in the same parameter in the 14- days period (Figure 4-3). Most studies in the literature associate the use of NSAIDs with a negative effect on the outcome of the bone repair process, usually with lack of fracture union, with this effect being time and dose dependent, (23,24). The most likely cause for the negative effects of NSAIDs on the repair process is the decrease in prostaglandins (mainly PGE-2 and PGF-2α), which seem to regulate the expression of BMP-2 and BMP-7, important mediators in the differentiation of progenitors of bone cells in osteoblasts, affecting the physiology of these cells, (18,25–28). Thus, we can suggest that the lower density of bone tissue in the IBU group in the 7-day period is caused by a delay in the differentiation of osteoblasts, as previously mentioned. In turn, the increase in bone tissue density in the later period, in the IBU group, may be related to the delay in the remodeling of newly formed bone tissue, since NSAIDs seem to act in an inhibitory manner in the differentiation and activation of osteoclasts (26,29).

Bearing in mind that the deleterious effects of NSAIDs are dose dependent, we performed a second test, where the experimental group received a dose of ibuprofen of 200mg/Kg every 24 hours. Again, the histological analysis of the evaluated periods showed normal kinetics in the repair process, without notable differences when compared to the control group, with the with the alveolar socket being filled with new bone tissue at the end of the experiment (Figure 8-3). When the quantitative analysis of the structures is evaluated, the IBU group (200mg/kg) maintained the decrease in bone tissue density in the 7-day period, together with the decrease in osteoblast density in the same period (Figure 8-3). Thus, we can observe that the administration

of ibuprofen, either at concentrations of 40 mg/kg or 200 mg/kg (both every 24 hours), did not cause significant impacts on healing within our model of intramembranous bone repair.

Another important aspect for the adequate outcome in alveolar bone healing is the construction of a provisional collagen matrix by fibroblasts. In this sense, treatment with ibuprofen (40mg/kg) showed a downregulation in fiber and fibroblast density in some periods compared to the control group (Figure 4-3). In turn, controversially, the group medicated with ibuprofen at 200mg/kg showed an upregulation in the fiber's density and fibroblasts (Figure 9-3). In addition to the supposed negative effects already explored by NSAIDs on bone repair, their influence on fibroblast proliferation and collagen production seems uncertain because, in the literature, there are reports that NSAIDs can inhibit or delay the proliferation and fixation of fibroblasts in other experimental models (30), or that do not appear to affect fibroblast proliferation in any way (31). Other models demonstrate, in turn, that NSAIDs (in particular, Ibuprofen) could be involved in the upregulation of collagens type I, II and III, consequently increasing their synthesis (32). Thus, the influence of NSAIDs on the function of fibroblasts may be dose and/or time dependent and may be altered by mediators present in the environment where these cells are found. Anyway, for a better understanding of these conflicting results, more analyzes should be carried out in this aspect of the repair. The greatest density of fibers and fibroblasts occurs in the 1- to 3-day periods, when the provisional matrix begins to be built, replacing the clot present until then (Figures 3-3 and 8-3) The clot, in turn, in the IBU group (40 mg/kg), showed a trend towards an increase in its density, with an upward regulation. An expected result, since NSAIDs are classically associated with platelet aggregation (33). Interestingly, the IBU 200mg/kg group showed a decrease in clot density in the initial periods, with an increase in the 7-day period (Figure 9-3). This result may be more related to the concomitant up-regulation of fibers and fibroblasts in the 1-, 3-day periods, occupying more space in the alveolus (Figures 4-3 and 9-3).

Treatment with ibuprofen also seems to have caused possible alterations in the angiogenesis process, considering that the vessel density was altered in the experimental groups compared to the control group (Figures 4-3 and 9-3). The IBU 40mg/kg group showed a prominent decrease in vessel density in some periods, (Figure 4-3). In the IBU 200 mg/kg group, however, there was an increase in vessel density in the initial periods, followed by a subsequent decrease, also suffering a

downward regulation in the latest period. In general, the literature points out that NSAIDs have a marked angiogenesis inhibition effect, which seems to be multifactorial and probably includes local alterations in growth factors and in important mediators of endothelial growth, such as VEGF. NSAIDs also appear to increase endothelial cell apoptosis and decrease their migration. (34,35). In this context, the decrease in vessel density observed in the experimental groups may be related to some of these mechanisms mentioned in the literature, and the increase in vessel density at specific times, in turn, may be a response to the imbalance caused at the site under repair, showing that even with continuous medication with NSAID, there is an intrinsic regulation of the process. However, further studies would be needed to better elucidate these changes.

Finally, another factor changed during the periods studied in the repair process was the migration of inflammatory cells. During the inflammatory process, leukocytes act in an important way, whether in the defense of the organism against microorganisms or in the cleaning of cellular remains and debris from the damaged matrix (13). The IBU 40 mg/kg group showed a reduction of inflammatory cells in the latest period. The IBU 200 mg/kg group showed a more prominent decrease in inflammatory cell density at the 1-, 3- and 14-day periods, suggesting a dose-dependent response (Figures 4-3 and 9-3). When the periods are evaluated through immunohistochemistry (performed only for the IBU 40 mg/kg group, due to problems with the reagents present at the time), the experimental group showed an decrease in the number of CD206+ cells (M2 macrophages) and a decrease in the number of Ly6gGR1+ cells (granulocytes), both in the 1-day period (Figure 7-3). Indeed, the effect of decreasing inflammatory cell density associated with NSAIDs is well documented in the literature and this effect is credited to drug changes in adhesion molecules on endothelial cells (36,37). Although the main effect of NSAIDs is the inhibition of prostaglandin synthesis by blocking COX, they have other COX-independent mechanisms of action, such as interfering with the functionality of a wide variety of cellular functions, in particular the L-selectin function in neutrophils (19). NSAIDs can cause shedding of the L-selectin molecule in neutrophils, decreasing its expression and, consequently, interfering with the recruitment of this specific cell type (38). Thus, such a mechanism may explain the decrease in the number of Ly6gGR1+ cells observed during the repair process.

The administration of ibuprofen in a model of muscle injury showed an increase in the tissue concentration of macrophages, reducing the number of necrotic fibers,

suggesting a possible induction of the M1 profile (39). In fact, ibuprofen seems to induce the M1 profile in macrophages after consistent COX inhibition (40,41), such reports follow our finding, where we had a tendency of downregulation in CD206+ cells in the initial period, in the experimental group (IBU 40mg/kg), showing an decrease in M2 macrophages, in relation to the control group. For a better understanding of this result, other tests shgould be performed, such as, for example, the labeling of total macrophages (F4/80+) and M1 macrophages (CD80+). Such markings were expected to be carried out in our experiment; however, we had problems with the available immunomarkers that ended up making the experiment unfeasible.

In summary, our experiment showed that the continuous administration of ibuprofen at concentrations of 40 mg/kg and 200 mg/kg, as a possible modulator of the inflammatory process, did not cause a negative impact on bone repair, considering that the alveolar process was normally filled with bone tissue at the end of the 14-day period, with no differences compared to the control group. However, the administration of ibuprofen caused alterations in several aspects involved in the bone repair process, such as production of collagen fibers, angiogenesis, number of inflammatory cells and bone tissue density, however, these alterations seemed to be compensated by a probable self-regulated mechanism in the site repair site.

FIGURES AND FIGURES CAPTIONS

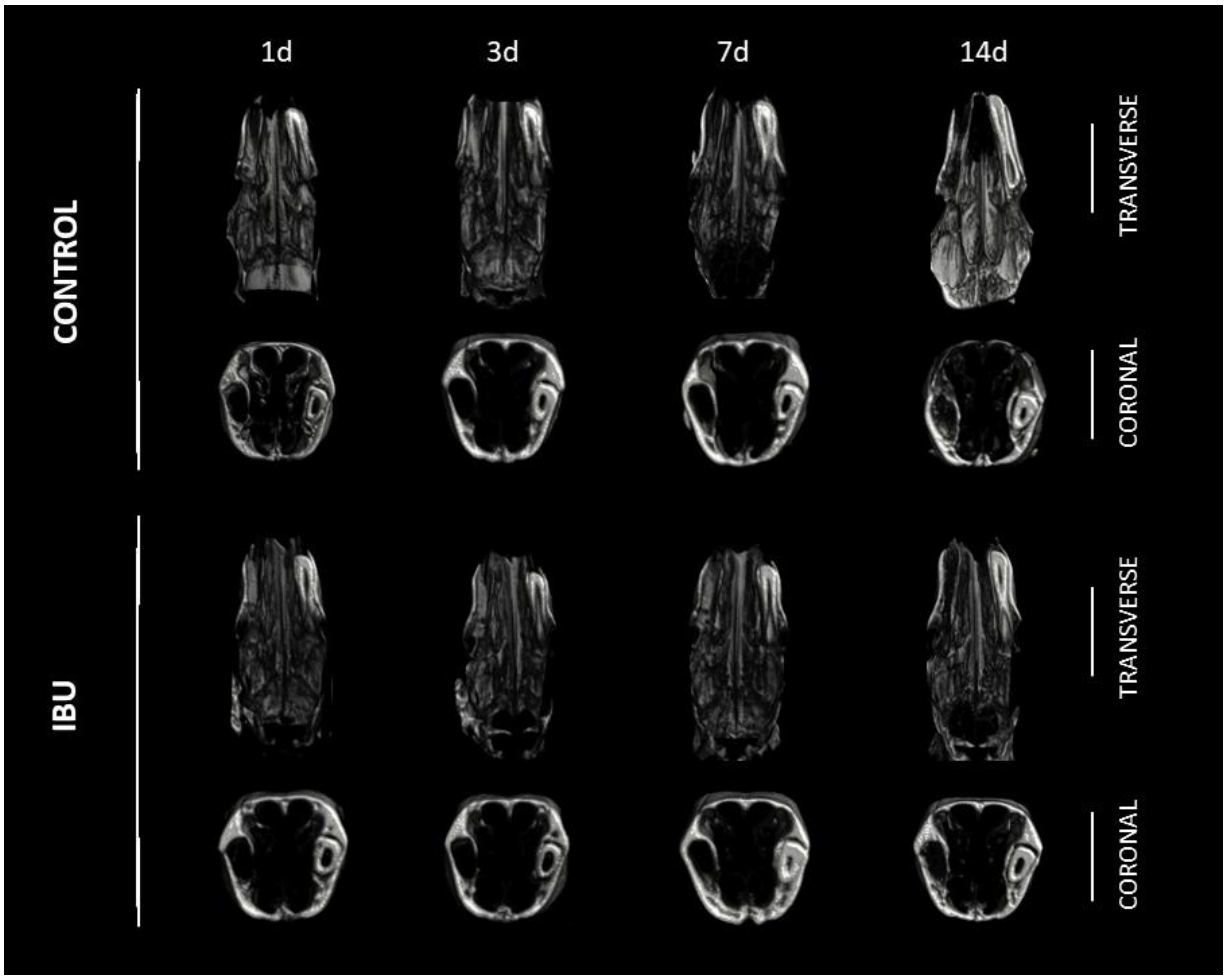

FIGURE 1-3 - Micro-computed tomography (μCT) analysis of bone healing process kinetics in Control (CTL) and Ibuprofen treated mice (IBU). Samples from 8-week-old male mice were scanned with the μCT System (Skyscan 1174; Skyscan, Kontich, Belgium) 1-, 3-, 7-, and 14-days post tooth extraction time points for the kinetics evaluation of the bone healing process. Images were reconstructed using the NRecon software and three-dimensional images obtained with the CT-Vox software. The sectioned maxilla is represented at the transverse and coronal planes.

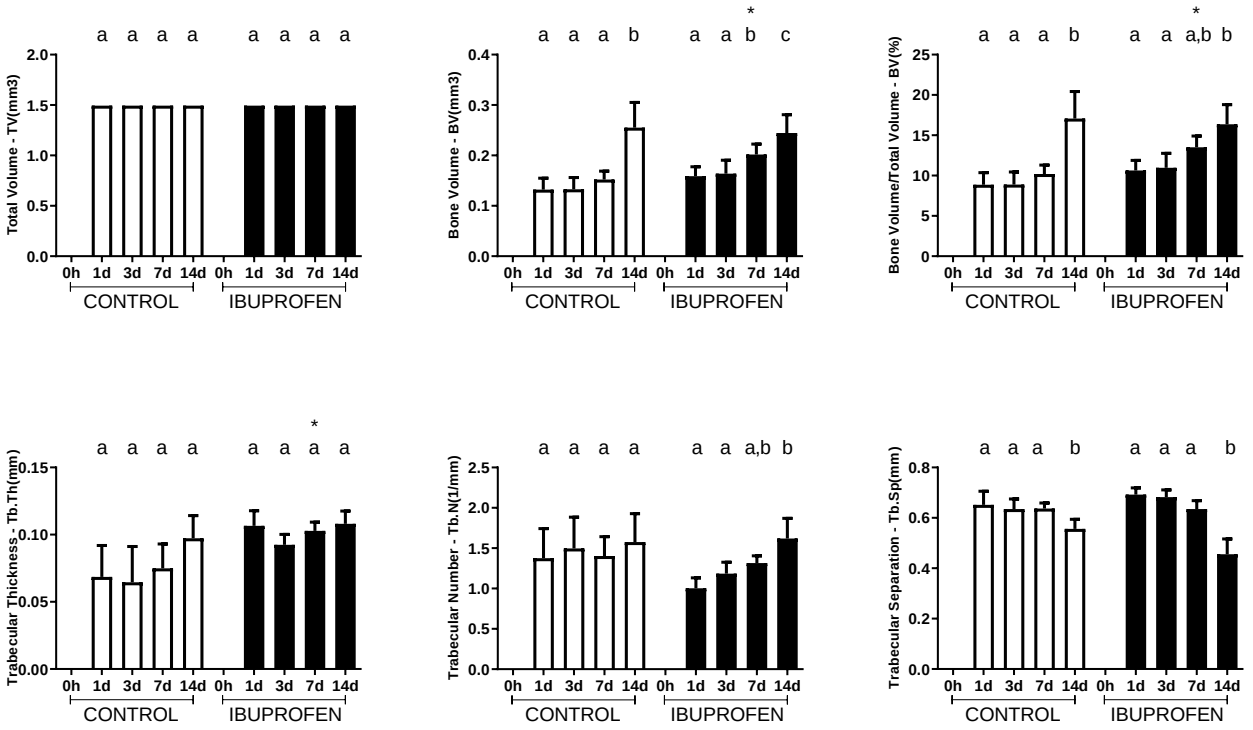

FIGURE 2-3 - Analysis of the morphological parameters of the trabecular bone microarchitecture in the dental alveoli of the control (CTL) mice and Ibuprofen treated mice (IBU) in the periods of 1-, 3-, 7- and 14-days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by microtomographic methods for the characterization mineralized tissue features at healing sites. The morphological parameters of the bone microarchitecture were evaluated using the CTAn software through a cylindrical region of interest (ROI) determined by the segmentation of the trabecular bone located from the coronal to the apical region. The analysis of bone parameters (A–F) included: Tissue volume (mm3) (A), Bone volume (mm3) (B), Bone volume in relation to tissue volume (%) (C), Trabeculae thickness (mm) (D), Number of trabeculae (1/mm) (E), Separation of trabeculae (mm) (F). The results represent the values of the mean and standard deviation in each of the periods analyzed (*Represent the differences between the control and IBU groups in each time point).

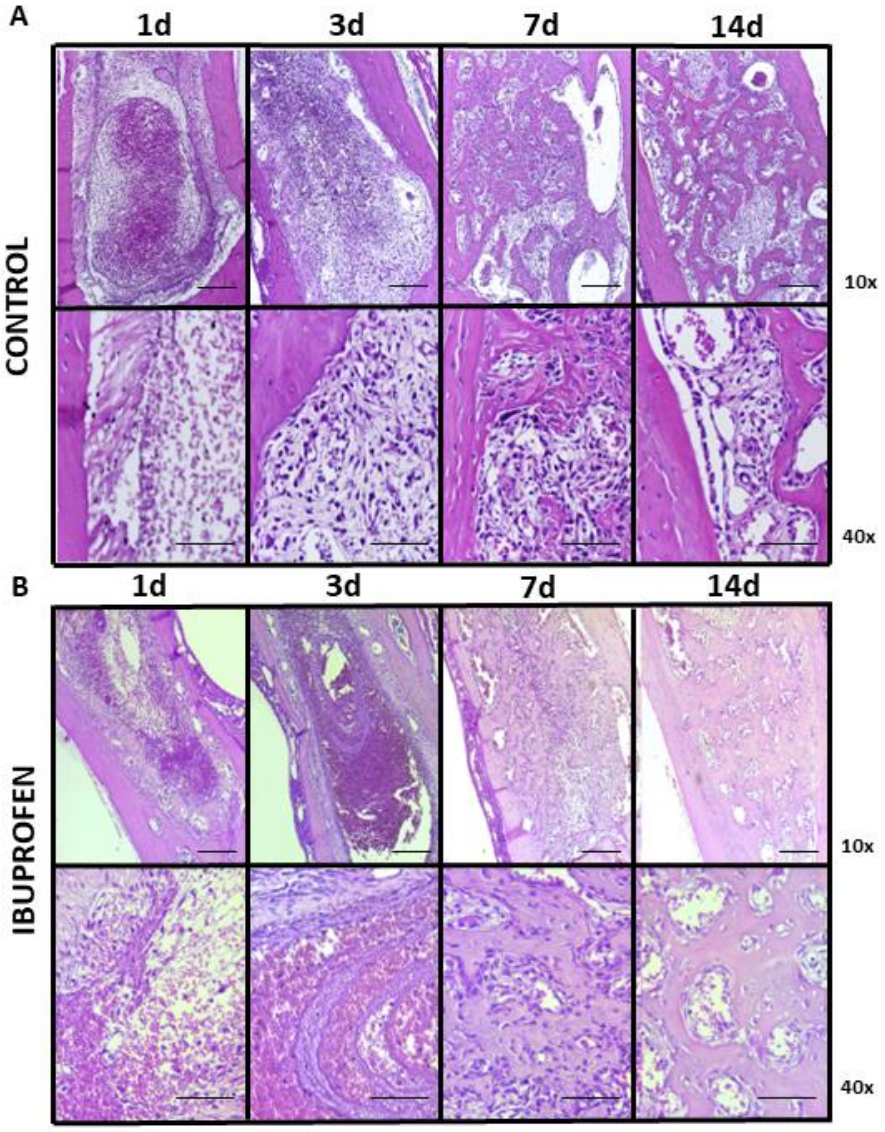

FIGURE 3-3 - Histological analysis of the alveolar healing process in control (CTL) mice (A) and in Ibuprofen treated mice 40mg/kg/24hrs (IBU) (B), in periods of 1 -, 3 -, 7 - and 14- days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histological means. Photomicrographs representing the average region of the dental alveolus in 10× and 40× magnifications.

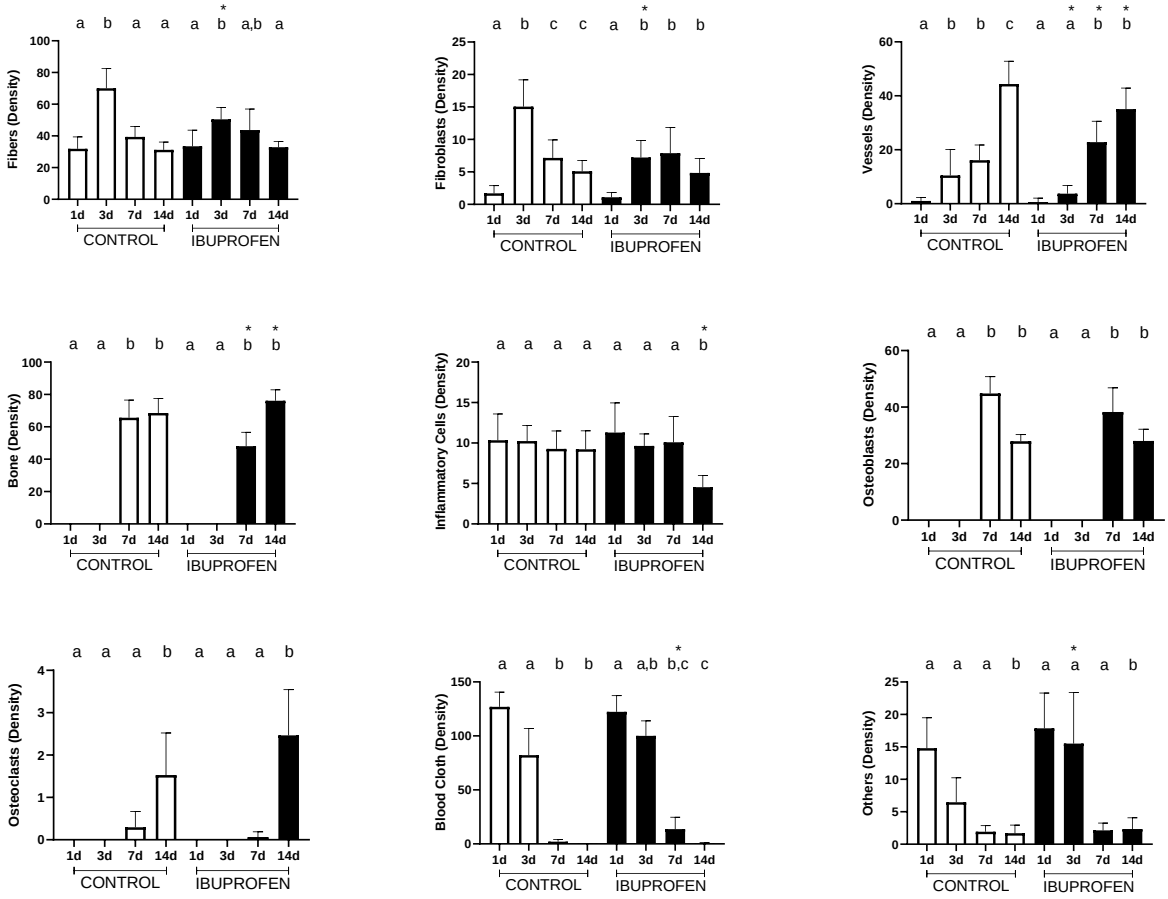

FIGURE 4-3 - Comparative density analysis (%) of connective tissue and its components present in the dental alveolus of control mice (CTL) and ibuprofen treated mice 40mg/kg/24hrs (IBU) in periods of 1-, 3-, 7- and 14-days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histomorphometric methods for the characterization of cellular and tissue components at healing sites. Results are presented as the means of density for each structure of the alveolar socket: collagen fibers, fibroblasts, blood vessels, inflammatory cells, cloth, and other components (empty space left by intercellular liquid and spaces). Also, the results depict the total density of connective tissue (represented by the sum of collagen fibers, fibroblasts, blood vessels and inflammatory cells). The results represent the values of the mean and standard deviation in each of the periods analyzed (*Represent the differences between the CTL and IBU groups in each time point).

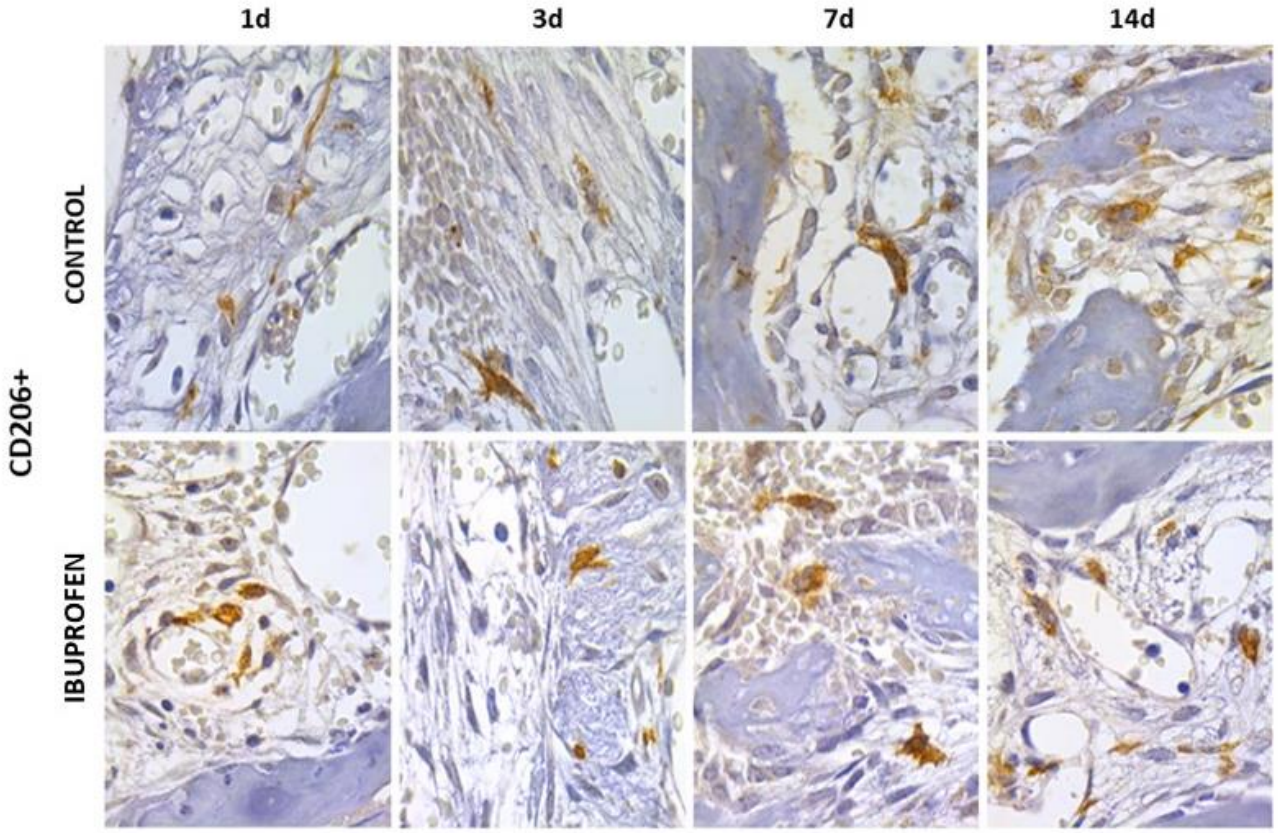

FIGURE 5-3 - Immunohistochemistry analysis for CD206+, cells present in the bone repair process in control and Ibuprofen treated mice 40mg/kg/24hrs. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Representative sections from medial thirds of the socket at days 0, 3, 7 and 14 days after tooth extraction. Anti-staining Mayer's hematoxylin; objective of 100×.

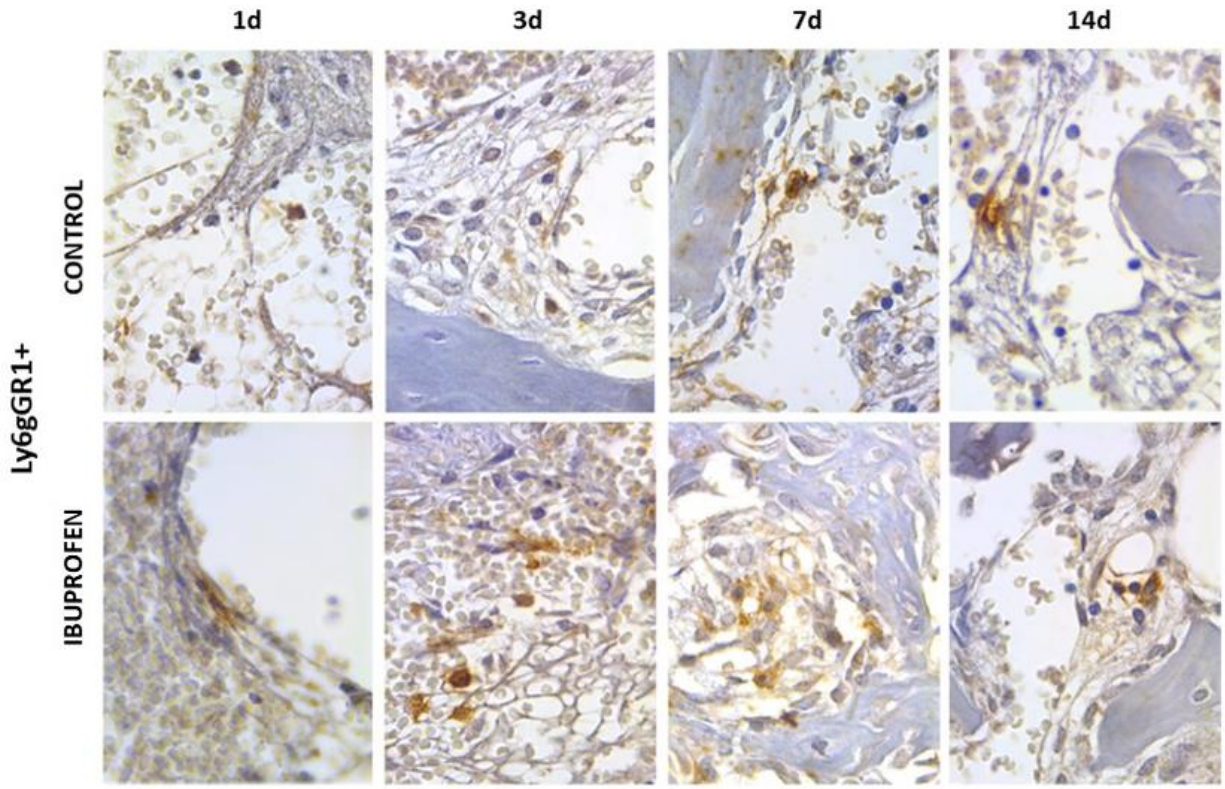

FIGURE 6-3 - Immunohistochemistry analysis for Ly6gGR1+ cells present in the bone repair process in control and Ibuprofen treated mice 40mg/kg/24hrs. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Representative sections from medial thirds of the socket at days 1, 3, 7 and 14 days after tooth extraction. Anti-staining Mayer's hematoxylin; objective of 100×

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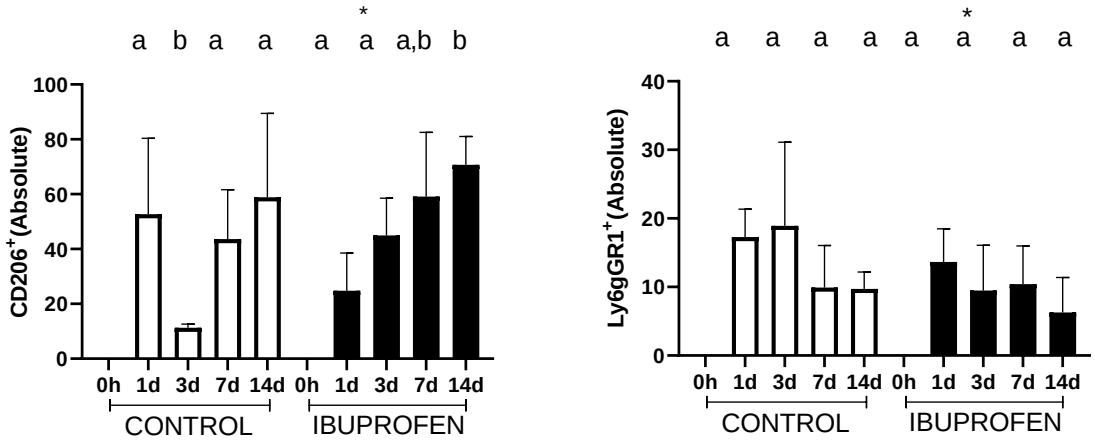

FIGURE 7-3 - Analysis of inflammatory cells in the alveolar bone healing kinetics after tooth extraction in control, and Ibuprofen (40mg/kg) treated mice. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Immunohistochemistry quantification corresponding CD206+ and Ly6gGr1+ cell at healing sites (*Represent the differences between the control and IBU group in each time point).

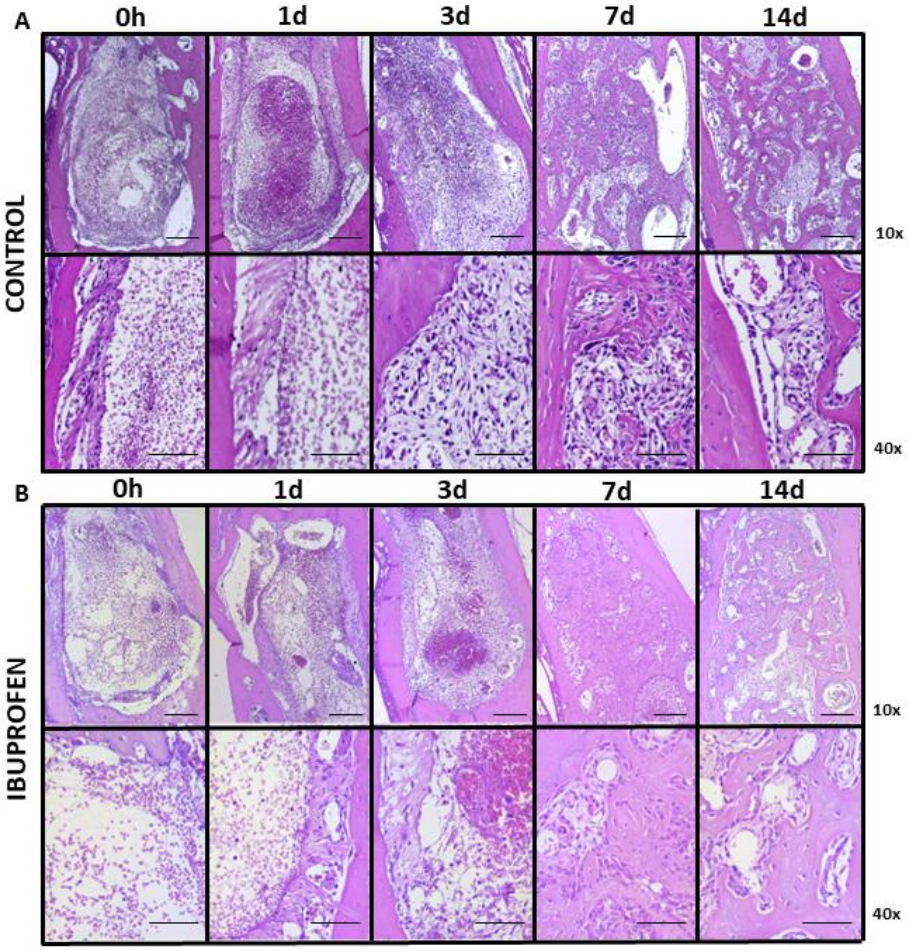

FIGURE 8-3 - Histological analysis of the alveolar healing process in control (CTL) mice (A) and in Ibuprofen treated mice, 200mg/kg/24h (IBU) (B), in periods of 0, 1 -, 3 -, 7 - and 14- days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histological means. Photomicrographs representing the average region of the dental alveolus in 10× and 40× magnifications.

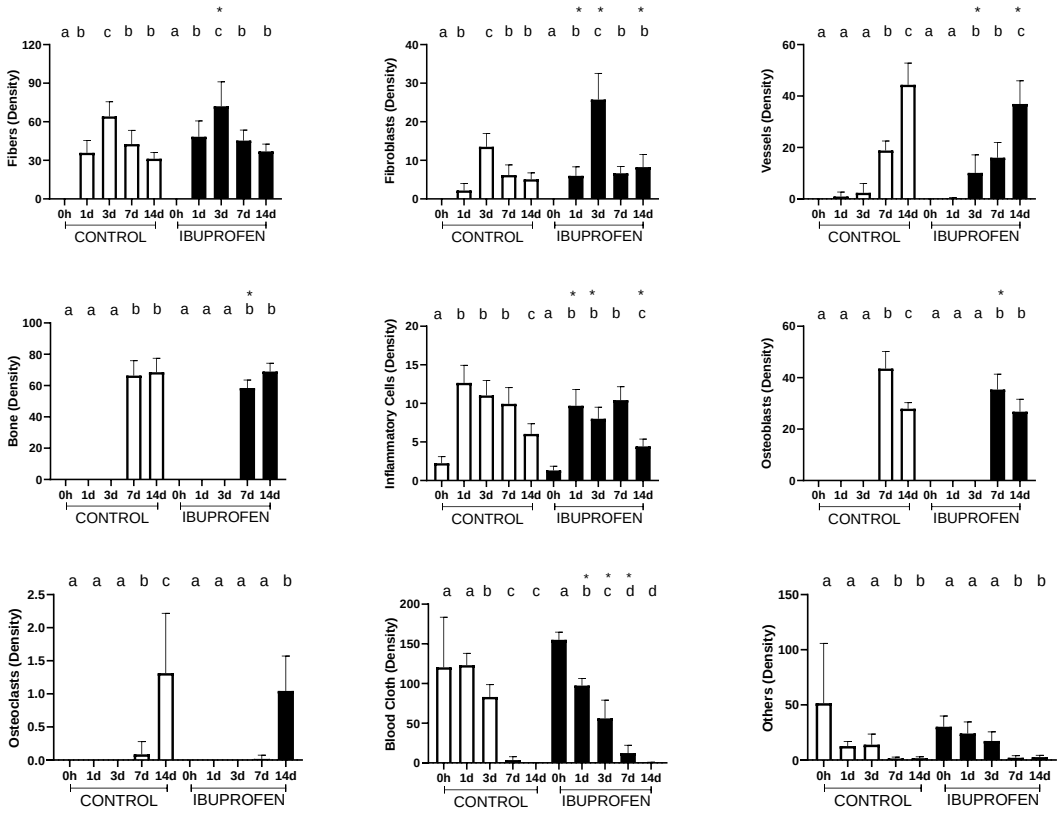

FIGURE 9-3 - Comparative density analysis (%) of connective tissue and its components present in the dental alveolus of control mice (CTL) and ibuprofen treated mice, 200 mg/kg/24hr (IBU) in periods of 0, 1-, 3-, 7- and 14-days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histomorphometric methods for the characterization of cellular and tissue components at healing sites. Results are presented as the means of density for each structure of the alveolar socket: collagen fibers, fibroblasts, blood vessels, inflammatory cells, cloth, and other components (empty space left by intercellular liquid and spaces). Also, the results depict the total density of connective tissue (represented by the sum of collagen fibers, fibroblasts, blood vessels and inflammatory cells). The results represent the values of the mean and standard deviation in each of the periods analyzed (*Represent the differences between the CTL and IBU groups in each time point).

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Article 3

Analysis of early aging associated changes in inflammatory immune response and its impact in alveolar bone repair outcome

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ABSTRACT

The process of alveolar bone healing can be influenced by several local and systemic factors, which include the immune cells and its products. However, the interplay of both systems can be influenced by age-related changes and, such alterations could affect bone repair. Despite of the great number of findings about aging influence in bone healing, information about early aging associated changes in inflammatory immune response and its impact in repair remains unclear. To address such questions, we used 32-week-old male C57BL/6 mice, comprising the AGING group (N=5/time/group) compared with the control group, composed with 8-week-old male C57BL/6 mice (N=5/time/group). alveolar bone repair. All animals were submitted to extraction of the right upper incisor and were evaluated at 0, 1, 3, 7 and 14 days after extraction, followed by computed tomography (μCT), histomorphometry, immunohistochemical and molecular analyzes (PCRArray). Our results demonstrated that the AGING group presented increased mRNA expression of pro-inflammatory interleukins, such IL1B, IL6 and TNF and downregulated mRNA expression of the regulatory interleukin IL10 ($p<0.05$). Lower levels of growth factors as FGF1, Tfgb1 and Vegfa mRNA levels were also present in AGING group. Macrophage related markers, such iNOS, ARG and FIZZ showed downregulation in AGING group. Stem cell markers also showed downregulation such, CD106, CD146, OCT4, NANOG and CD34 in the AGING group in several experimental periods, compared with the control group ($p<0.05$). The AGING group showed general decrease in BMPs 2, 4 and 7 and in a several bone markers (RUNX2, ALPL, DMP1 and PHEX) ($p<0.05$). Accordingly, bone density in the AGING group was lower in 7-day period, with lower counts of osteoblasts ($p<0.05$). The AGING group also showed increased density in fibers and fibroblasts accordingly with the upregulation in Col1A2, Col1A1 and MMP8 ($p<0.05$).Several alterations were seen in the chemokine receptors and chemokines in the AGING group. CCR2, CCR1, CCR5, CCL2, CCL5, CCL12, CCL20, CCL25 and CX3CL1 showed downregulation in mRNA expression levels, while CXCR1, CXCR2 and CXCL12 showed increased mRNA($p<0.05$). Our results points to a possible low grade pro-inflammatory profile, greater prevalence of fibers and fibroblast during the repair, probably associated with a delayed differentiation of bone progenitor cells. However, such alterations didn’t seem to impair bone healing process, since the as the alveolar socket was filled with new bone at the end of the experimental period.

Keywords: Bone repair. early-aging. Osteoimmunology

INTRODUCTION

Bone tissue is a mineralized connective tissue whose functions include protection of vital organs, locomotion, and mineral reserve. Bone constituted of a mineralized protein matrix that is associated with characteristic cells. Osteoblasts are responsible for the synthesis and mineralization of bone matrix, osteocytes, the most abundant cells in bone, are responsible for matrix maintenance and, finally, the osteoclasts, large and multinucleated cells of hematopoietic origin derived from the monocyte/macrophage lineage responsible for matrix resorption (1,2). Bone tissue is endowed with high plasticity being constantly under remodeling process, cycling from bone resorption to bone deposition, resulting in tissue renewal and improvement in its structural and functional integrity. Thereby, such delicate and complex process, needs a precise interplay between these bone cells and their respective products to achieve tissue homeostasis. (1,3,4). Nonetheless, this precise mechanism can be affected or influenced by several local and systemic factors, such the immunological system's cells, and products, since their share several mediators with bone cells (5–7). The influence of cytokines on the immune system and their interaction with bone tissue is well evident in chronic inflammatory and infectious conditions, where pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα generally exert a stimulatory effect on osteoclasts and an inhibitory effect on osteoblasts. On the other hand, cytokines with anti-inflammatory properties, such as IL-4 and IL-10, exert a stimulatory effect on osteoblasts and inhibitory effects on osteoclasts. Thus, cytokines may play a protective and destructive role in the process of bone tissue destruction (5,8–10).

It's evident how complex and important are the interactions between these two systems in both physiological and pathological conditions, however, is important to point the possible outcomes in this interplay when both systems are altered or, somehow compromised. In that context, it's important to address the aging effects on bone and immune systems. Generally, the aging process increases the amount of cellular damage, impairing the intracellular clearance and control mechanisms, together with the unhealthy organelles (11), there are also profound changes in gene expression, metabolism, and chromatin organization, developing a pro-inflammatory

secretory phenotype, contributing for chronic and low-grade inflammatory profile, with increased levels of pro-inflammatory mediators, such IL-6, TNF-α and IL1-B. (11–15). In turn, the age-related changes in bone tissue seems to be related with an impairment in the remodelation balance, due to osteocytes functions alterations, eventually leading to bone loss (12). Consequently, the bone tissue's remarkable repair ability also gets impaired with age, since important cells of the immune system, like macrophages, T-cells, and mesenchymal stem cells (MSCs) have demonstrated intrinsic age-related changes that could impact bone healing (15). In summary, MSCs show decreased quantity and osteoblastic and chondrogenic differentiation potential, macrophages demonstrate decreased proliferation and increased activation, and T-cells are negatively affected by age-related changes in bone marrow. Finally, aged animals show decreased vascular density within the callus, due to decreased levels of key angiogenic factors. Nonetheless, the aging effects on bone repair are mainly highlighted in fracture models (15,16), where an endochondral type of repair takes place, there is still a gap of information about age-related changes in the intramembranous bone repair outcome. Another fact the deserves to be highlighted, since most studies aim the elderly populations. is the relative lack of information aiming to elucidate when exactly the age-related changes start to manifest and how much they could impair the physiological processes (16,17).

To address this gap, we proposed apply your intramembranous bone repair model, after tooth extraction (18) to elucidate possible age-related changes and their influence through all important repair phases and participating cells and mediators, during the experimental periods. Instead of using elderly animals, we used middle-aged C57/BL6 mice (32 weeks-old), compared to control mice (8 weeks-old) to address the possible initial physiological changes due to the aging process.

MATERIAL AND METHODS

Animals and experimental groups

The experimental groups consisted of 8-week-old and 32-week-old male C57bl/6 wild type (WT) mice, comprising the control (CTL) and aging animals (AGING) group. The animals were acquired from Ribeirão Preto Medical School (FMR/USP) breeding facility, maintained during the experimental period in the facility of Department of Biological Sciences of FOB/USP. Throughout the period of the study, the mice were

fed with sterile standard solid mice feed (Nuvital, Curitiba, PR, Brazil) and sterile water. The experimental protocol was approved by the local Institutional Committee for Animal Care and Use following the Guide for the Care and Use of Laboratory Animals principles.

Experimental protocol and mice tooth extraction model

Male C57Bl/6 (WT) 8-week-old and 32-week-old mice (*N* = 6 group/time point) were anesthetized by intramuscular administration of 80 mg/kg of ketamine chloride (Dopalen, Agribrans Brasil LTDA) and 160 mg/kg of xylazine chloride (Anasedan, Agribrands Brasil LTDA) in the proportion 1:1 determined according to the animal body mass, were submitted to the extraction of upper right incisor as previously described(18). Considering the potential interference of pain medication in the bone repair process, and the previous observation of the absence of abnormal behavior of mice after the tooth extraction, no additional drugs were administered to both control and experimental groups. In addition, it is important to emphasize that adverse effects of the different treatments on the health and behavior of the animals of both groups were not observed. At the end of the experimental periods (0 h, 1, 3, 7 and 14 days post tooth extraction), the animals were euthanized with an excessive dose of anesthetic and the maxillae samples were collected. The maxillae samples were analyzed by micro-computed tomography (μCT), after the same samples were dissected and prepared for histomorphometry and collagen birefringence analysis or molecular analysis.

Micro-computed tomography (μCT) assessment

The maxillae samples were scanned by the Skyscan 1174 System (Skyscan, Kontich, Belgium), at 50 kV, 800 μA, with a 0.5 mm aluminum filter and 15 % beam hardening correction, ring artifacts, reduction, 180 degrees of rotation and exposure range of 1°. Images were captured with 1304 × 1024 pixels and a resolution of 14 μm pixel size. Projection images were reconstructed using the NRecon software and three-dimensional images obtained by the CT-Vox software. Morphological parameters of trabecular bone microarchitecture were assessed using the CTAn software following recommended guidelines. A cylindrical region of interest (ROI) with an axis length of 2 mm (200 slices) and diameter of 1 mm was determined by segmenting the trabecular bone located from the coronal to apical thirds. Trabecular measurements analyzed included the tissue volume (TV), bone volume (BV) bone volume fraction

(BV/TV, %), trabecular thickness (Tb. Th, mm), trabecular number (Tb.N, mm), and trabecular separation (Tb.Sp), as previously described (18).

Histomorphometry analysis

Serial sections (8 semi-serial sections of each maxilla, with a 5 μm thickness for each section) were obtained using a microtome (Leica RM2255, Germany) and stained with HE (hematoxylin and eosin). Morphometric measurements were performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100× immersion objective and a Zeiss kpl 8× eyepiece containing a Zeiss II integration grid (Carl Zeiss Jena GmbH, Jena, Germany) with 10 parallel lines and 100 points in a quadrangular area. The grid image was successively superimposed on approximately 13 histological fields per histological section, comprised of all tooth sockets from the coronal limit adjacent to the gingival epithelium until the lower apical limit. For each animal/socket, sections from the medial were evaluated. In the morphometric analysis, points were counted coinciding with the images of the following components of the alveolar socket: clot, inflammatory cells, blood vessels, fibroblasts, collagen fibers, bone matrix, osteoblasts, osteoclasts and other components (empty space left by the inflammatory exudate or intercellular liquid and bone marrow); as previously described (18). The results were presented as the volume density (mean) for each evaluated structure.

Immunohistochemistry analysis

Histological sections from 1, 3, 7, and 14 days were deparaffinized following standard procedures. The material was pre-incubated with 3 % Hydrogen Peroxidase Block (Spring Bioscience Corporation, CA, USA) and subsequently incubated with 7 % NFDM to block serum proteins. The histological sections from of all groups were incubated with and anti-Ly6g-Gr1 polyclonal antibody - sc-168490 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), antiF4/80 polyclonal primary antibodies - sc-26642 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD80 monoclonal antibody - sc-9091 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-CD206 polyclonal antibody - sc-34577 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:50 concentrations for 1 h at room temperature. The identification of antigen-antibody reaction was performed using 3-3′-diaminobenzidine (DAB) and counter-staining with Mayer's hematoxylin. Positive controls were performed in the mouse spleen for positive Ly6gGr1, F4/80, CD80 and CD206 receptors. The analysis of immunolabeled

cells was performed by a single calibrated investigator with a binocular light microscope (Olympus Optical Co., Tokyo, Japan) using a 100× immersion objective. The quantitative analysis for the different markers was performed throughout the alveolar extension, as previously described (18). The absolute number of immunolabeled cells was obtained to calculate the mean for each section.

RealTime PCR array reactions

RealTime PCR array reactions were performed as previously described. The extraction of total RNA from the remaining alveolus was performed with the RNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturers' instructions, as previously described (18). The integrity of the RNA samples was verified by analyzing 1 mg of total RNA in a 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA) according to the manufacturers' instructions, and the complementary DNA was synthesized using 1 mg of total RNA through a reverse transcription reaction (Superscript III, Invitrogen Corporation, Carlsbad, CA, USA). RealTime PCR array was performed in a Viia7 instrument (LifeTechnologies, Carlsbad, CA) using a custom panel containing targets Wound Healing (PAMM-121), Inflammatory cytokines and receptors (PAMM-011) and Osteogenesis (PAMM-026) (SABiosciences, Frederick, MD) for gene expression profiling. An initial analysis performed with pooled samples from all time points was performed to triage the factors with a significant variation in the expression profile, followed by the analysis of the individual time points expression levels of the factors differentially expressed in the initial analysis. RealTime PCR array data were analyzed by the RT2 profiler PCR Array Data Analysis online software (SABiosciences, Frederick, MD), normalized initially using the geometric mean of three constitutive genes (GAPDH, ACTB, Hprt1) and subsequently normalized by a control bone sample (comprising normal alveolar bone), and expressed as fold change relative to this normalizing control, as previously described [5].

Statistical analysis

Data were presented as means ± SD; initially, the data distribution was tested by the Shapiro-Wilk normality test. The statistical significance comparing control and AGING groups in each time point was analyzed by Student's *t*-tests. Both were performed with Graph-Pad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). PCR array

data were analyzed by the Mann-Whitney test followed by Benjamini-Hochberg test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Molecular analysis using Real Time PCR Array

Initially, in the 3- and 7-days periods, when compared to the control group, a decrease in the BMPs 2, 4 and 7 mRNA expression was observed. However, in the period of 14 days, there is an equivalence in the levels of mRNA of BMPs 2 and 4 and, an increase in the expression of BMP 7 in the aging animals when compared to the control group (Figure 2-2). The group of aging mice showed lower expression of FGF1 and Tfgb1 in the 3- and 7-days periods and lower expression of VegfA mRNA levels in 3 days period, when compared to animals in the control group. In the group of aging animals, among the interleukins, an increase in IL1B levels was observed in the periods of 3 and 7 days, IL6 levels were upregulated in the period of 3 days. IL10 levels were downregulated in the 7- and 14-days periods. TNF mRNA levels increased within 3 days and decreased within 7 days (Figure 2-2). The protein components expressed by macrophages iNOS (3-days period), ARG (7- and 14-days periods) and FIZZ (7-days period), had their mRNA expressions decreased in the group of aging mice when compared to the control group. (Figure 2-2).The group of aging animals also showed several alterations in the expression of chemokine receptors, when compared to the control group. CCR1 (7-day period), CCR2 (7- and 14-day periods) and CCR5 (3- and 7-day periods), showed a decrease in their mRNA levels (Figure 2-2). CXCR1 (periods of 3- and 7- days) and CXCR2 (3- days period) showed increased mRNA expression levels. Among the chemokines, CCL2 (3- and 7- days periods), CCL5 (3- days period). CCL12 (3-days period), CCL20 (7-days period), CCL25 (14-days period) and CX3CL1 (7- and 14-days periods), showed decrease in mRNA levels in the group of aging animals when compared to the group control (Figure 2-2). In turn, CXCL12, showed an increase in mRNA levels in the 7- and 14-days periods, when comparing the group of aging animals with the control group (Figure 2-2). Among the components of the connective matrix, we observed an increase in mRNA expression of Col1A2 (7- and 14-days periods), Col1A1 (14-days period) and MMP8 (3-days period) in the aging group, when compared to the control (Figure 2-2). Some markers of mesenchymal stem cells (MSCs) also showed changes in their mRNA expression levels in the aging

group when compared to the control group. CD106 (3-, 7- and 14-days periods), OCT4 (3-days period), NANOG (3-, 7- and 14-days periods), CD34 (7- and 14-days periods). CD146 showed a decrease in mRNA levels within 3-days period and subsequently an increase within 14-days period. Finally, CD105 showed a decrease in mRNA levels within 7-days period and an increase within 14-days period (Figure 2-2). As for the expression of bone tissue markers, the group of aging mice showed a general decrease in the mRNA expression levels of RUNX2 in the 7-days period, ALPL in the 3-, 7- and 14-days periods, DMP1 in the 3-, 7- and 14-days periods and PHEX in 7- and 14-days periods (Figure 2-2).

Immunohistochemistry analysis of Ly6g-GR1+ and CD206+ cells

Aiming the quantification of macrophages and other inflammatory cells in the healing sites, the immunolabeling was performed to Ly6gGR1+ (granulocytes), and CD206+ (M2 macrophages) targets in the alveolar bone repair process in the AGING and CTL mice, at periods of 1-, 3-, 7- and 14-days after tooth extraction (Figure 3-2). The GR1+ cells were abundant at 1-day and decreased after 3 days in both groups. The number of CD206+ cells started were abundant at 1-day with a decrease at the 3-dayS period and a further increase in the 7-days and 14-days periods (Figure 3-2) The CD206+ cell count in the AGING group, was higher in the 3-day period and in the 7-day periods, when compared to the CTL group (Figure 3-2)

Histological and histomorphometrical evaluation

The histological and histomorphometric analyses were performed for the qualitative and quantitative analysis of the characteristic elements of distinct bone healing stages, namely blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures, demonstrating an overall similarity between the bone healing features and kinetics between AGING group and its control (CTL) counterpart. Both groups showed the presence of fiber, fibroblasts, and inflammatory cells in the alveolus margin region, demonstrating the onset of the repair process. At 7-days period, the blood clot was gradually substituted by highly vascularized granulation tissue, characterized by many of fibers and fibroblasts and the start of new bone formation from the margins of the socket, in both groups. In the 14-day period, in both groups the regions previously occupied by granulation tissue

was gradually substituted by bone tissue (Figure 6-2). Concerning the quantitative analysis, organized structures present in bone repair in categories within different histological parameters, such as blood clot, inflammatory cells, fibroblasts, collagen fibers, blood vessels, bone matrix, osteoblasts, osteoclasts, and other structures; besides this parameter, we also analyzed the data in relation to connective tissue and bone tissue. Initially, the Group of 32-week-old mice (AGING) showed greater density of fibers and fibroblast in the periods of 7d and 14d when compared to the control group. Controversially, the AGING group showed lower density of fibroblasts in the 3-days period. Vessel density was lower in the AGING group, when compared to then control group, in the 14-days period. The AGING group had lower bone density and fewer osteoblasts in the 7-day period, and an increased number of osteoclasts in the 14-day period, when compared to the control group (Figure 7-2).

As for the number of inflammatory cells, the AGING group, when compared to the CTL group, showed an increase in its count in the 1-day period and, subsequently, a decrease in the 7-day period. The AGING group showed a greater density of the parameter “Others” in the period of 3 days, when compared to the CTL group (Figure 7-2).

Micro-computed tomography (µCT) analysis

The three-dimensional analysis from the µCT of the maxillae scanned in MicroCT (CT-Vox) showed the alveolar bone healing process comparatively in the aging animals (AGING) and control mice (CTL) over time from 0 hour, 1, 3, 7 and 14 days after tooth extraction. In the initial period (0 h), in both groups, the alveolar socket was completely empty, thus, the alveolar sockets did not present hyperdense areas. Nonetheless, in the 3-day period, was observed, in both experimental groups, the presence of initial hyperdense areas (Figure 8-2). In the 7-day period, in both experimental groups were observed the presence of hyperdense areas that evidenced the beginning of new bone formation, centripetal from the lateral and apical walls of the extraction sockets towards the center and the coronal region of the alveolus. At 14-day period, the evolution in the bone formation process is evident due to the large area of the alveolar socket filled by hyperdense regions, indicating the presence of mineralized tissue. Comparing the Aging and the CTL groups in a qualitative analysis, through the samples three-dimensional reconstruction, there are no significant differences in the repair process between the studied groups (Figure 8-2). Data observed by the three-dimensional

analysis were confirmed when the bone microarchitecture features were quantitatively evaluated. Both groups showed that throughout the periods, there was a progressive increase in bone volume, bone fraction in relation to the total volume, the thickness of trabeculae and the number of trabeculae and decrease in the trabecular separation at 14 days period. When both groups were compared, the aged animals showed more Trabecular thickness in the 0 hour and 3 days periods, when compared to control group (Figure 9-2). The other parameters analyzed did not show significant differences when the Aging and CTL groups were compared.

DISCUSSION

The alveolar bone repair is complex and involves several stages and subsequent and coordinated steps, where a moderate and transient inflammatory reaction is necessary for an adequate outcome of tissue repair (19–21). The influence of cells and components of the immune system on bone tissue is already known, affecting the balance between bone deposition/resorption and, influencing the outcome in activity/remission and in the lesion repair process (10,22). Under normal conditions, bone tissue has excellent repair capabilities, restoring matrix and function impressively. However, it is known that the ability of tissue repair in organisms is impaired with the aging process, as cells are trapped in a permanent state of cell cycle, characterized by specific phenotypic changes, which include changes in gene expression, chromatin structure, cell signaling and the pattern of cell secretion (23). There seems to be agreement in the literature that bone tissue, in aged organisms, responds with a delay to the repair process in case of fracture and is more susceptible to comorbidities and failures in therapeutic processes, such as rehabilitation with osteointegrated implants (16,24,25). Evaluating this scenario, we applied our experimental model of alveolar bone repair (18) in mice during the aging process, not yet considered senile, at 32-weeks-old, to be able to contemplate when and what alterations related to the aging process begin manifest themselves and how they can alter the outcome of the repair process. Briefly, your results showed that the aging animals showed occasional alterations in some variables, like matrix products and density and cells numbers, appearing, initially, that the repair process suffered a delay in some phases but, in the end, was able to overcome these changes, filling the extraction site with new alveolar bone, qualitatively like the control group.

Initially, our results points alterations in the connective matrix and its components in the AGING group, with up regulated expression in the Col1A1 and Col1A2. These genes encode the two alpha chains of type I collagen, which comprises most of the organic matrix of bone tissue (4,26). At the same moment, in the AGING group, fiber density is also upregulated, showing some possible relation with the pointed molecular changes. There was also an increase in MMP8 mRNA, suggesting a possible compensation mechanism due to the apparent collagen matrix components upregulation (Figure 2-2). Finally, in the AGING group, accordingly with these findings, fibroblasts and fibers density appeared to be upregulated in general, together with the fibroblast growth factor 1 (FGF1). At the first impression, these findings go against common reports in the literature on collagen production in aged organisms, which have, lower collagen production(27,28). Nonetheless, these results may suggest, in addition to the increase in density itself, an increase in the permanence time of these structures in the repair site, evidencing a probable delay in the matrix remodelation in the AGING group related to the control animals (18). Furthermore, the most studied bone healing models in aged organisms are those related to fracture repair, where an endochondral bone formation takes place predominantly, showing there is a deficiency in the production of type II and type X collagens, causing a delay in the repair process (15,16). The model proposed in your experiment comprises an intramembranous bone repair and, therefore, it is difficult to draw a parallel between our results and other findings due to the lack of studies in the literature focusing on this type of process. Thus, the alteration in the expression of these genes in question may be a characteristic of the intramembranous bone repair process in aging organisms and, for a better understanding of this finding, further studies should be carried out. Optimal bone healing is vitally dependent on adequate vascularization and, therefore, requires the development of new blood vessels(19). Since angiogenesis is commonly pointed as downregulated in the aging process(29), some alterations in that mechanism were expected to be seen in our AGING experimental group. Indeed, the AGING group presented downregulation in the mRNA expression of the vascular endothelial growth factor (VEGF), at 3-days period, coinciding with the downregulation tendency in the vessel's density, mainly in the 14-days period (Figures 2-2 and 7-2). VEGF is a mediator that plays a central role in the angiogenesis process, and it seems to be downregulated in aged animals (29,30),therefore, your results seem to follow the common findings in the literature. However, the downregulation in VEGF mRNA

expression and in the vessel's density does not appear to have had a negative impact on the repair process. Along with angiogenesis, stem cells recruitment and differentiation play crucial role on bone repair, since these cells will be responsible for the deposition of new mineralized matrix (31). That way, transforming growth factor beta 1 (Tgfb1), a crucial molecule involved in the bone repair process (32), also showed decreased mRNA expression levels in the AGING group (Figure 2-2). TGF-β is a family of growth factors, responsible for regulating cell activity and is crucial in bone formation by activating mesenchymal stem cells (MSCs), fibroblasts, osteoblasts, also promoting the inhibition of osteoclastogenesis (1,19,33). In this sense, the AGING group showed alterations in some stem cells markers of, also participating in the repair process. OCT-4, belonging to the Pou-domain family of transcription factors, has an influence on the expression of NANOG, also a transcription factor present in embryonic stem cells (ECMs) (34,35). These two factors are essential for most of the properties of stem cells, such as self-renewal and pluripotency(35). CD34 and CD105 mRNA levels, markers of hematopoietic and mesenchymal stem cells, respectively, important in promoting angiogenesis (36,37), were also downregulated in the AGING group (Figure 2-2). Finally, CD146 and CD106 are markers expressed in cells with multipotent differentiation potential (Heim 2020, Bragdon et al 2019, Clines et al 2010), showed lower mRNA levels in the AGING group (Figure 2-2). Again, these results, together with the changes in VEGF mRNA levels mentioned above, may be related to the decrease in vessel density in the histomorphometry analysis (Figure 7-2). The general decrease in the mRNA levels of stem cells markers and related transcription factors suggests a decrease in the activity and/or number of these cells and/or possible changes in the mechanisms that aim to maintain their viability of differentiation and, consequently, their performance in the repair process in aging animals (Bragdon & Bahney, 2018; Chen et al., 2022; Meinberg et al., 2019).

The recruitment of inflammatory cells to injury sites is an important phase in the bone healing, since they'll be responsible for producing a complex and controlled cascade several molecules, like growth factors and inflammatory mediators, responsible for orchestrating the repair process(21). The inflammatory cells recruitment and migration is mediated by chemokines and their receptors, which are divided into four different subfamilies (CC, CXC, CX3C and C) (40). The AGING group showed down regulation of mRNA levels of CCR1, CCR2 and CCR5. In turn, the CXCR1 receptor was upregulated initially, suffering subsequently downregulation (Figure 2-2). Finally,

CXCR2 mRNA expression increased in earlier periods (Figure 2-2). CCR2 and CCR5 are receptors involved in the recruitment of the macrophage/monocyte lineage to the site of injury (40,41). Consequently, the decrease in these markers' mRNA levels may be also correlated with the INOS, ARG2 and FIZZ mRNA levels decrease, since they're all macrophage markers (42–44). However, controversially, the immunohistochemical analysis shows an increase in CD206+ cells (M2 macrophages) in the AGING group at certain periods in relation to the control group (Figure 3-2). It was intended to perform the immunohistochemical analysis with the markers F4/80+ (General Macrophages) and CD80+ (M1 macrophages), for a better understanding of the kinetics of inflammatory cells at the repair site, however, due to operational problems with the reagents obtained and available in our laboratory, it was not possible to perform this procedure. Thus, further investigations are needed to elucidate this conflicting aspect. The IL-8 receptors CXCR1 and CXCR2, or also called IL-8 RA/R1 and IL-8 RB/R2, respectively (45), were up-regulated in the AGING group at the initial periods (Figure 2-2). These receptors play an important role in the attraction and migration of hematopoietic progenitor cells and neutrophils to the site of injury (40,45,46). This increase in CXCR1 and CXCR2 mRNA expression could be related to the increase in GR1-Ly6g+ cell count in the immunohistochemical analysis (Figure 3-2). When chemokines are evaluated, the AGING group showed a decrease in CCL2 and CCL5, when compared to the control group (Figure 2-2). CCL2 also known as monocyte chemotactic protein 1 (MCP-1), a ligand of the CCR2 receptor, is one of the earliest and most highly expressed chemokines in response to bone fractures. It is also involved in the regulation of neutrophil migration, angiogenesis, and macrophage infiltration (40). In turn, CCL5, a ligand for CCR1, CCR3, and CCR5, promotes the recruitment and activation of macrophages, lymphocytes, and mast cells (40). Other cytokines involved in the promotion and migration of monocytes/macrophages to the site of injury are CCL12 (47–49) were downregulated in the AGING group during the evolution of the repair process (FD). As for chemokines involved in T cell recruitment and migration, CCL20 and CCL25 (50) were also in the AGING group when compared to the control group. Finally, the AGING group showed increased mRNA levels of CXCL12, (also known as Stroma cell-derived factor 1), (FIG.2). CXCL12 appears to be involved in the repair process by attracting endothelial progenitors and osteoblastic progenitors (40). In general, most chemokine receptors and their respective ligands involved in immune cells migration, especially from the monocyte/macrophage lineage,

had their mRNA expressions decreased in the AGING group compared to the control group, with few exceptions (Figure 2-2). This finding could be related with the decrease in mRNA levels of FIZZ, ARG2 and iNOS, markers for macrophages in tissue repair sites (Figure 2-2).

Considering the inflammatory mediators, as important regulators in bone deposition/resorption balance, and the reported pro-inflammatory profile in aged animals (15,51) the assessment of possible alteration in these variables is extremely important. Thus, the AGING group showed upregulation in the IL-1β, IL-6 and Tumor necrosis factor (TNF) mRNA expression levels (Figure 2-2), all pro-inflammatory cytokines and, generally, potent inducer of the osteoclastogenesis process (5,7,8,42,52,53) . Concomitantly with the upregulation of pro-inflammatory cytokines mRNA expressions, there is a decrease in IL-10 mRNA levels, a cytokine known to be associated with inactivity in chronic osteolytic lesions such as periodontitis due to its action of inhibition of the secretion of pro-inflammatory cytokines and the osteoclastogenesis process (5,8,10,54). These findings are compatible with the common evidence that point a sustained and low-grade inflammatory profile in aged animals (15,16,55). Considering this, the predominance of pro-inflammatory mediators in the repair environment should influence local macrophages to assume an M1 phenotype, also pro-inflammatory (56,57). In fact, a decrease in the mRNA levels of ARG2 and FIZZ, markers of M2 macrophages, involved in the resolving phase of the inflammatory process can be noted (56–58), suggesting a decrease and/or delay in the activity of these cells in the alveolar bone repair process in the AGING group. Such a delay in the resolution of the initial phases of the inflammatory process may be reflected in the tendency towards a decrease in mRNA expression levels of bone maturation indicators, affecting the differentiation of cell groups involved in bone neoformation (Figure 2-2).

As mentioned above, the AGING group showed a decrease in the expression levels of bone morphogenetic proteins (BMP's) and TGFβ. BMP2, BMP4 and BMP7 had down-regulated mRNA expression in several experimental periods (Figure 2-2). BMPs 2 and 7 are mediators recognized for participating widely in the bone formation process, being described as essential factors for the differentiation of osteoblasts and adequate bone formation (3,59) and their expression is possibly the initial trigger for the development of bone tissue mineralization (18). Thus, a decrease in the initial levels of BMPs could impact cell differentiation and, consequently, the deposition and

subsequent mineralization of newly formed bone matrix. Indeed, the AGING group showed a decrease in mRNA expression of RUNX2, the main transcription factor involved in osteoblastic differentiation (3,18) (Figure 2-2). This result seems to impact the histomorphometric analysis in the AGING group, that presented a decrease in the density of osteoblasts and in the density of the bone matrix itself, when compared to the control group (Figure 6-2). Interestingly, there is an increase in the number of osteoclasts present in the 14-day period in the AGING group, suggesting an increase that aims to compensate for a possible delay in remodeling in newly formed bone tissue (Figure 2-2). Following the same pattern, markers of advanced differentiation of osteoblasts and osteocytes, such as PHEX (phosphate-regulating neutral endopeptidase) and DMP1 (dentin matrix protein 1) and ALPL (alkaline phosphatase) also underwent downregulation at 7- and 14- days periods (PHEX) and 3-, 7-, and 14-day periods (DMP1 and ALPL) (Figure 2-2). Despite the decrease in markers, when evaluating the micro tomography of the periods studied in both groups, the animals in the AGING group show an increase in trabecular thickness (TB.Th), in the 3- and 7-day periods (Figure 9-2). This finding differs from what is commonly found in the literature, where a decrease in the number and thickness of trabeculae is expected in bone tissue in older populations (60,61) but, since our experimental group comprises middle aged animals, these changes should be different and not so prominent.

In summary, our data show that, with age, the bone repair process begins to be affected in a discrete manner, with an apparent prolongation of the initial, pro-inflammatory phase. Consequently, the mediators present seem to delay the recruitment and differentiation of osteoblastic precursors, responsible for the production and mineralization of new bone matrix. However, this delay does not seem to negatively affect the outcome of the process, which shows neoformation of bone tissue at the extraction site at the end of the period studied.

FIGURES AND FIGURES CAPTIONS

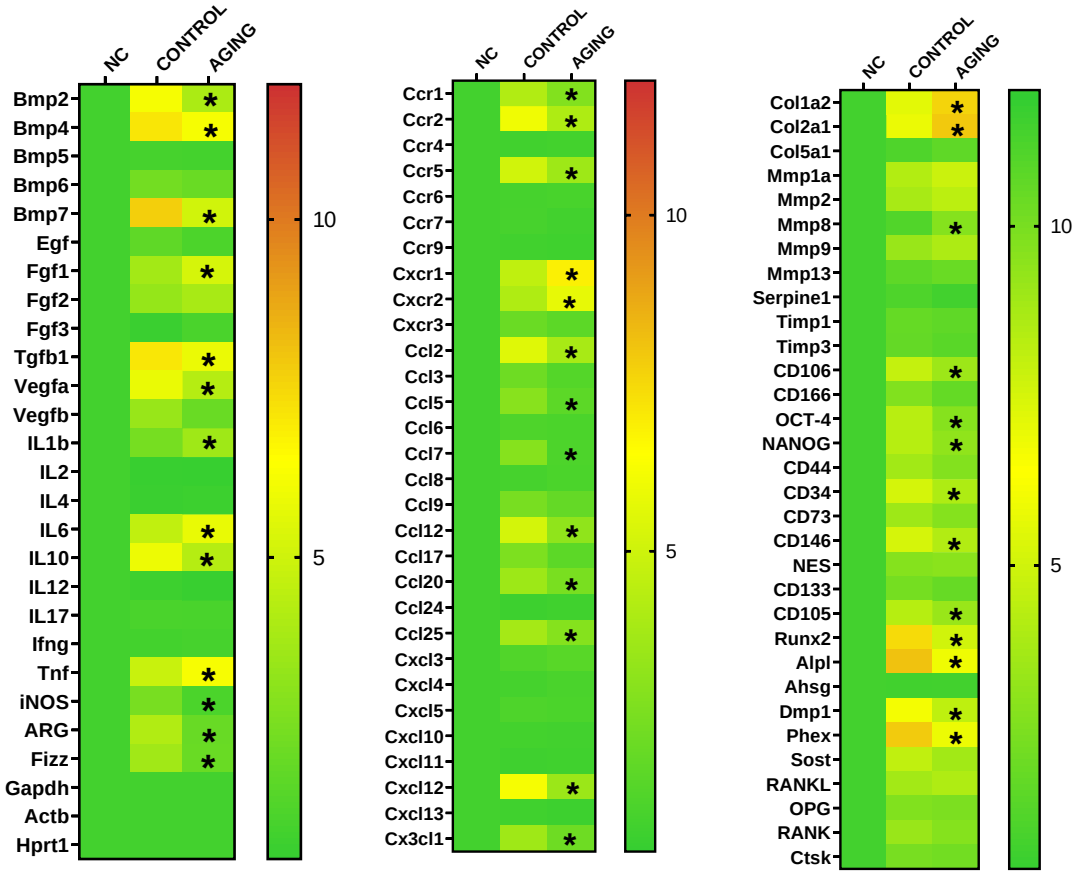

FIGURE 1-2 - Molecular analysis (Real Time PCR Array) using heat map to quantify the expression of the growth factors, extracellular matrix markers, bone markers and cytokines in bone healing process among control, and AGING group. Male C57Bl/6 (WT) mice (*N* = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed for the gene expression profile at healing sites. Gene expression initial analysis was performed with pooled samples from all time points was performed to triage the factors with a significant variation in the expression profile. Results are expressed as fold change relative to a normalizing control (C, i.e., normal alveolar bone) sample and normalized by three constitutive genes (GAPDH, ACTB, Hprt1) (*Represent the differences between the control group and AGING group).

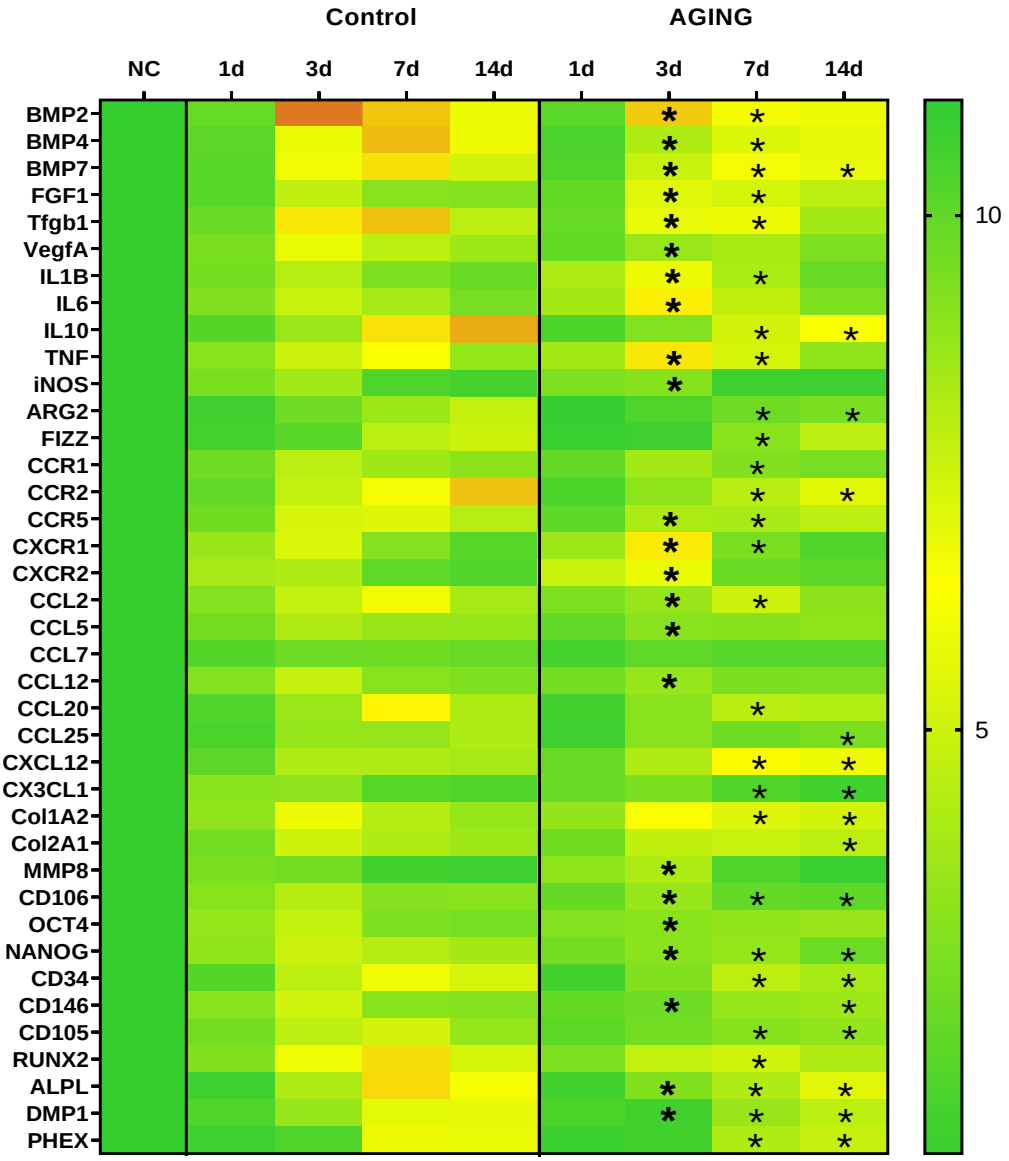

FIGURE 2-2 - Molecular analysis (Real Time PCR Array) using heat map to quantify the expression of the growth factors, extracellular matrix markers, bone markers and cytokines in bone healing process among control, and AGING group. Male C57Bl/6 (WT) mice (*N* = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed for the gene expression profile at healing sites. Following initial gene expression triage, the analysis of all the individual time points expression levels of the factors differentially expressed in the initial analysis was performed. Results are expressed as fold change relative to a normalizing control (C, i.e., normal alveolar bone) sample and normalized by three constitutive genes (GAPDH, ACTB, Hprt1) (*Represent the differences between the control group and AGING group in individual time points).

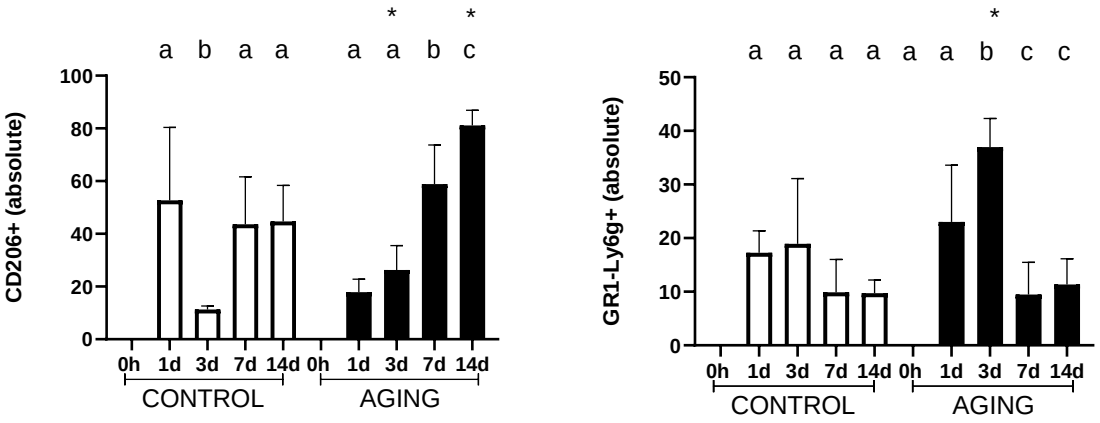

FIGURE 3-2 - Analysis of inflammatory cells in the alveolar bone healing kinetics after tooth extraction in control, and OLD mice. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Immunohistochemistry quantification corresponding CD206+ and Ly6gGr1+ cell at healing sites (*Represent the differences between the control and AGING groups in each time point).

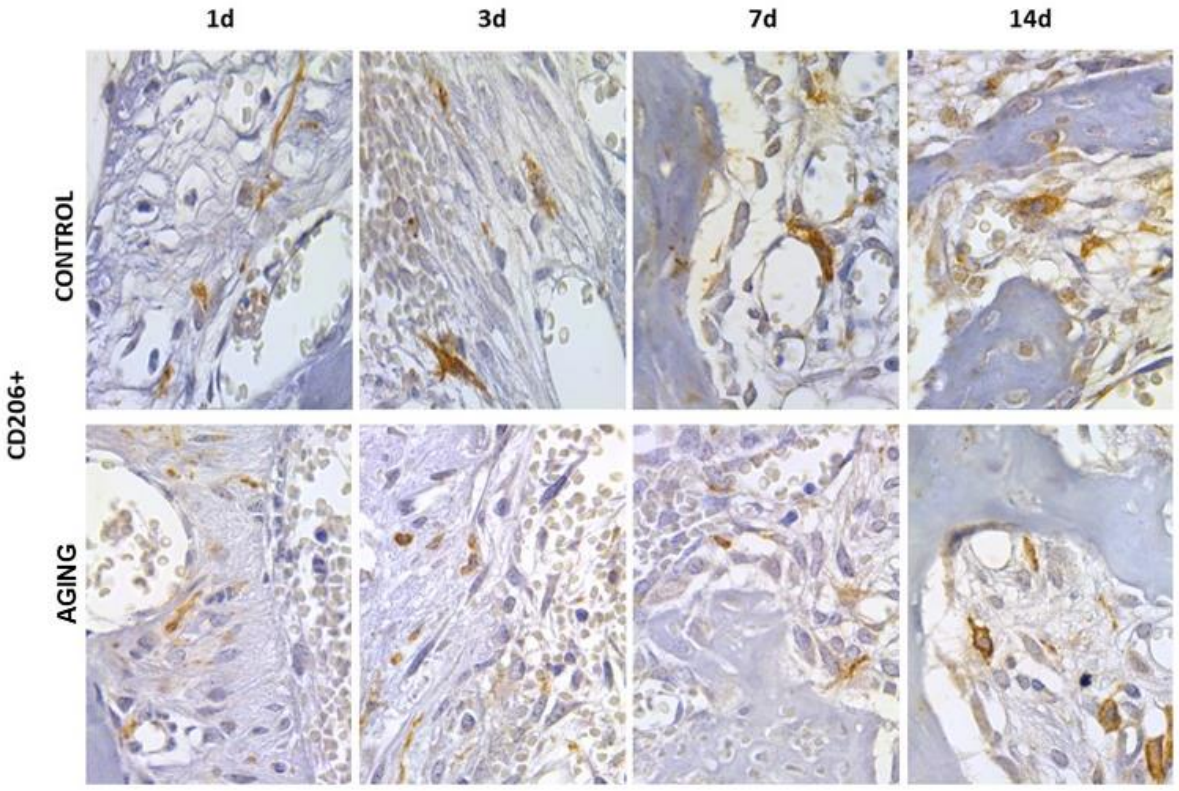

FIGURE 4-2 - Immunohistochemistry analysis for CD206+, cells present in the bone repair process in control, and AGING Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Representative sections from medial thirds of the socket at days 0, 3, 7 and 14 days after tooth extraction. Anti-staining Mayer's hematoxylin; objective of 100×.

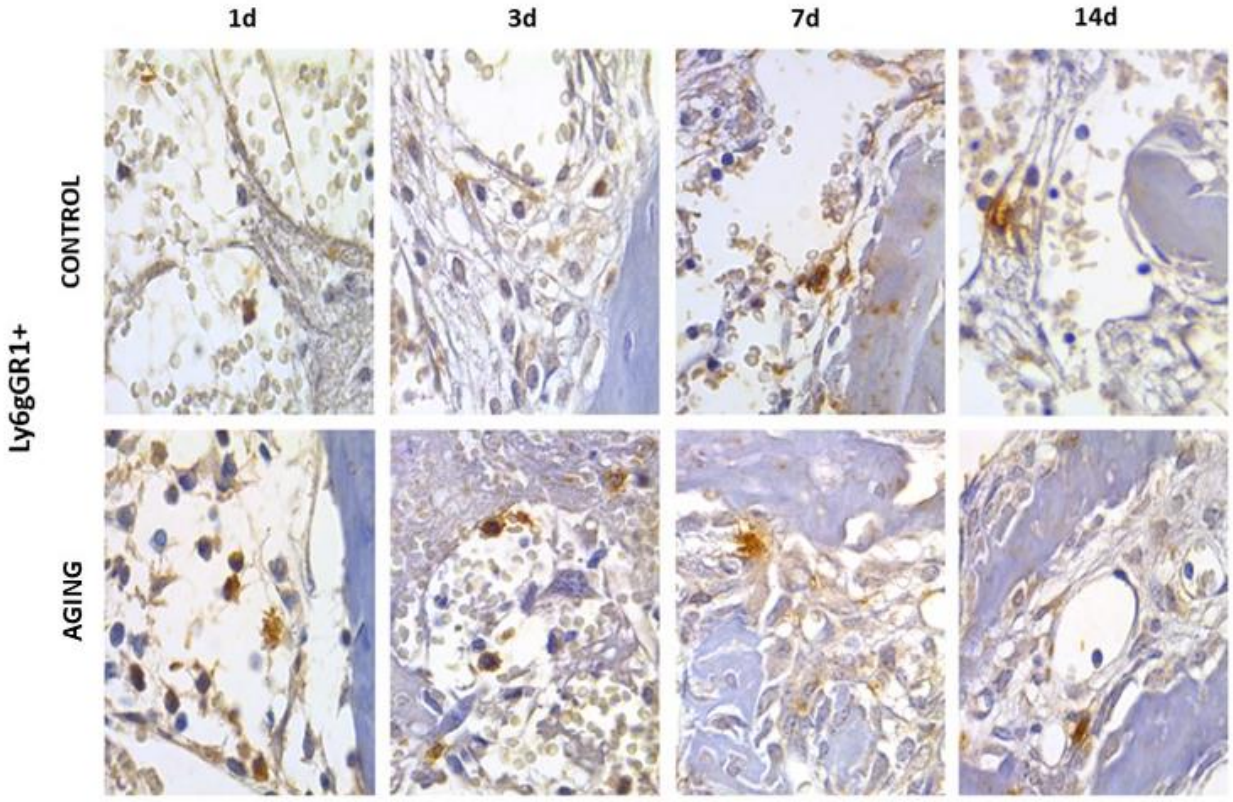

FIGURE 5-2 - Immunohistochemistry analysis for Ly6gGR1+ cells present in the bone repair process in control, and AGING. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by immunohistochemistry for phenotypic profiling of inflammatory infiltrate at healing sites. Representative sections from medial thirds of the socket at days 0, 3, 7 and 14 days after tooth extraction. Anti-staining Mayer's hematoxylin; objective of 100×.

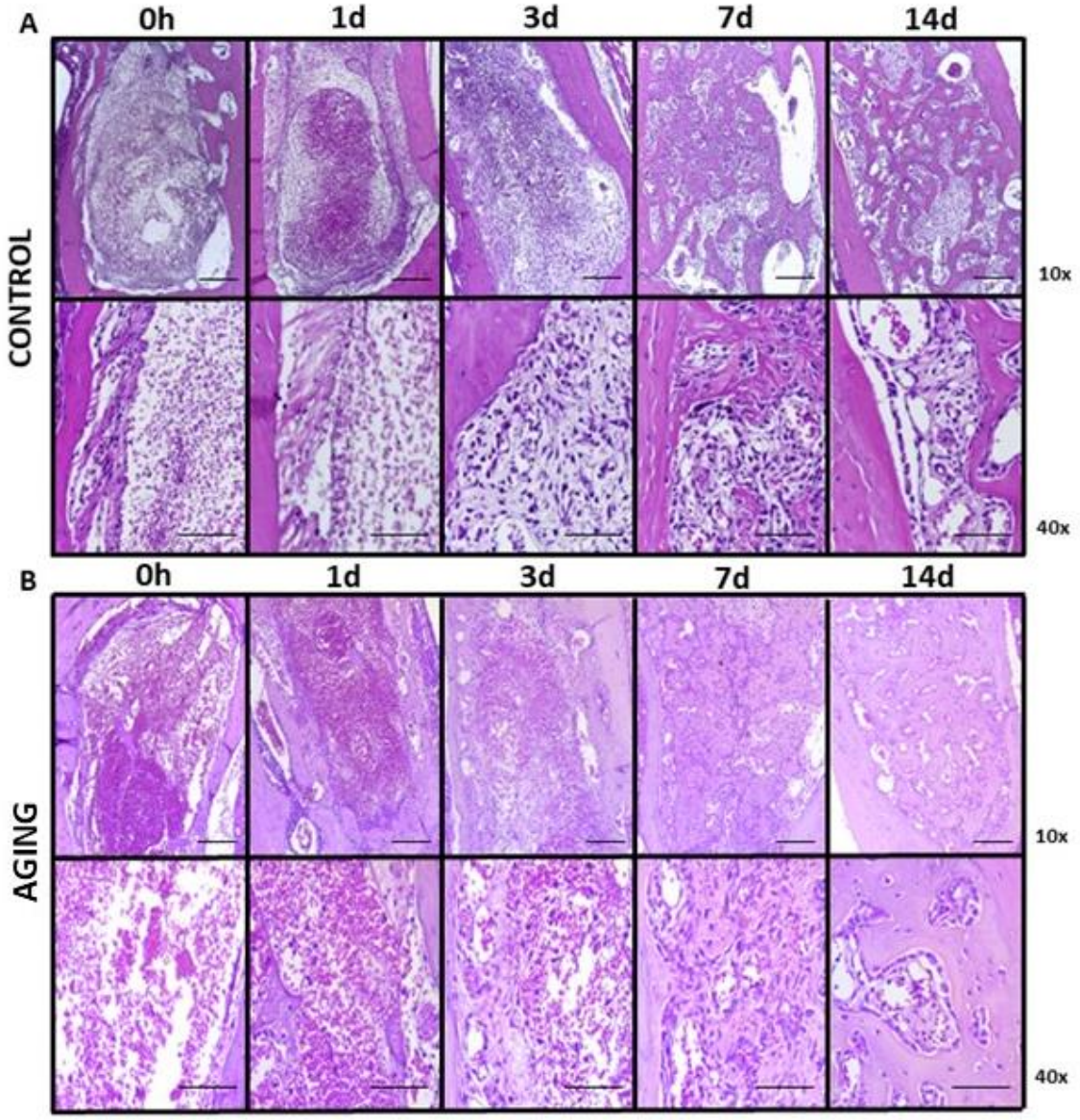

FIGURE 6-2 - Histological analysis of the alveolar healing process in control (CTL) mice (A) and in 32-weeks (AGING) mice (B), in periods of 0 -, 1 -, 3 -, 7 - and 14 -days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histological means. Photomicrographs representing the average region of the dental alveolus in 10× and 40× magnifications.

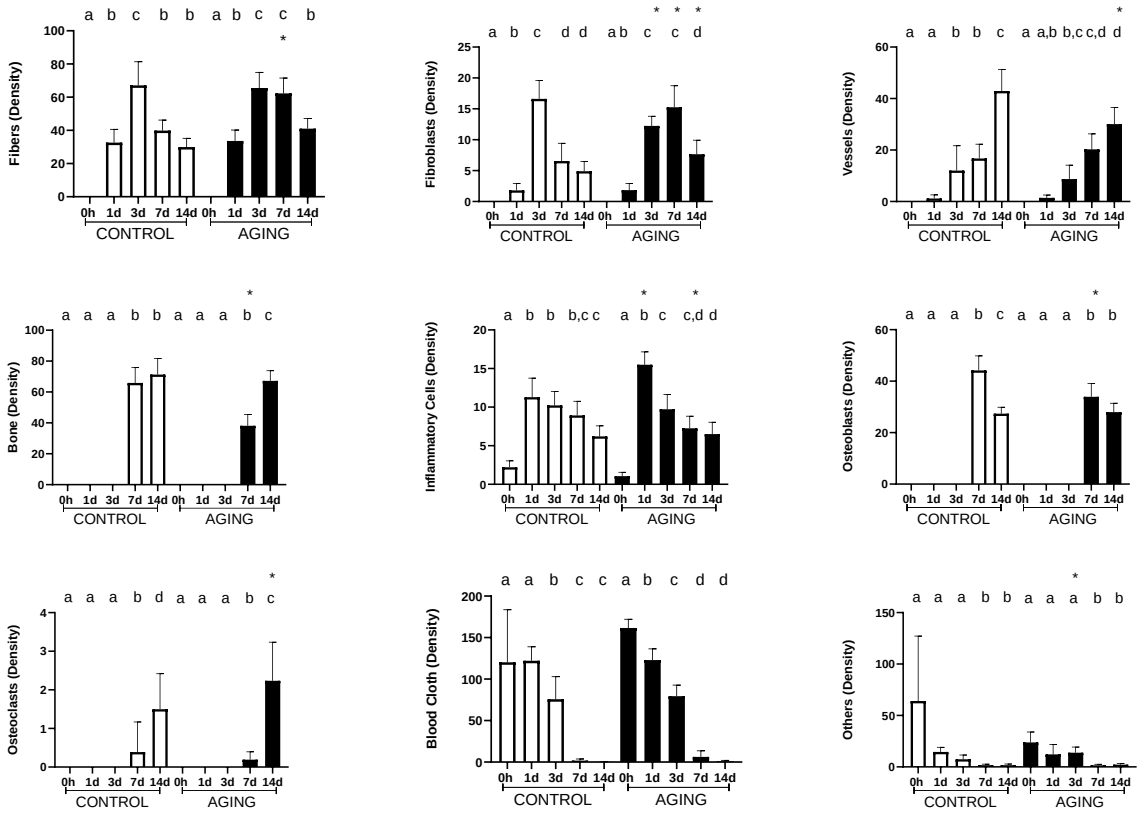

FIGURE 7-2 - Comparative density analysis (%) of connective tissue and its components present in the dental alveolus of control mice (CTL) and 32-weeks old mice (AGING) in periods of 0-, 1-, 3-, 7- and 14-days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by histomorphometric methods for the characterization of cellular and tissue components at healing sites. Results are presented as the means of density for each structure of the alveolar socket: collagen fibers, fibroblasts, blood vessels, inflammatory cells, clot and other components (empty space left by intercellular liquid and spaces). Also, the results depict the total density of connective tissue (represented by the sum of collagen fibers, fibroblasts, blood vessels and inflammatory cells). The results represent the values of the mean and standard deviation in each of the periods analyzed (*Represent the differences between the CTL and AGING groups in each time point).

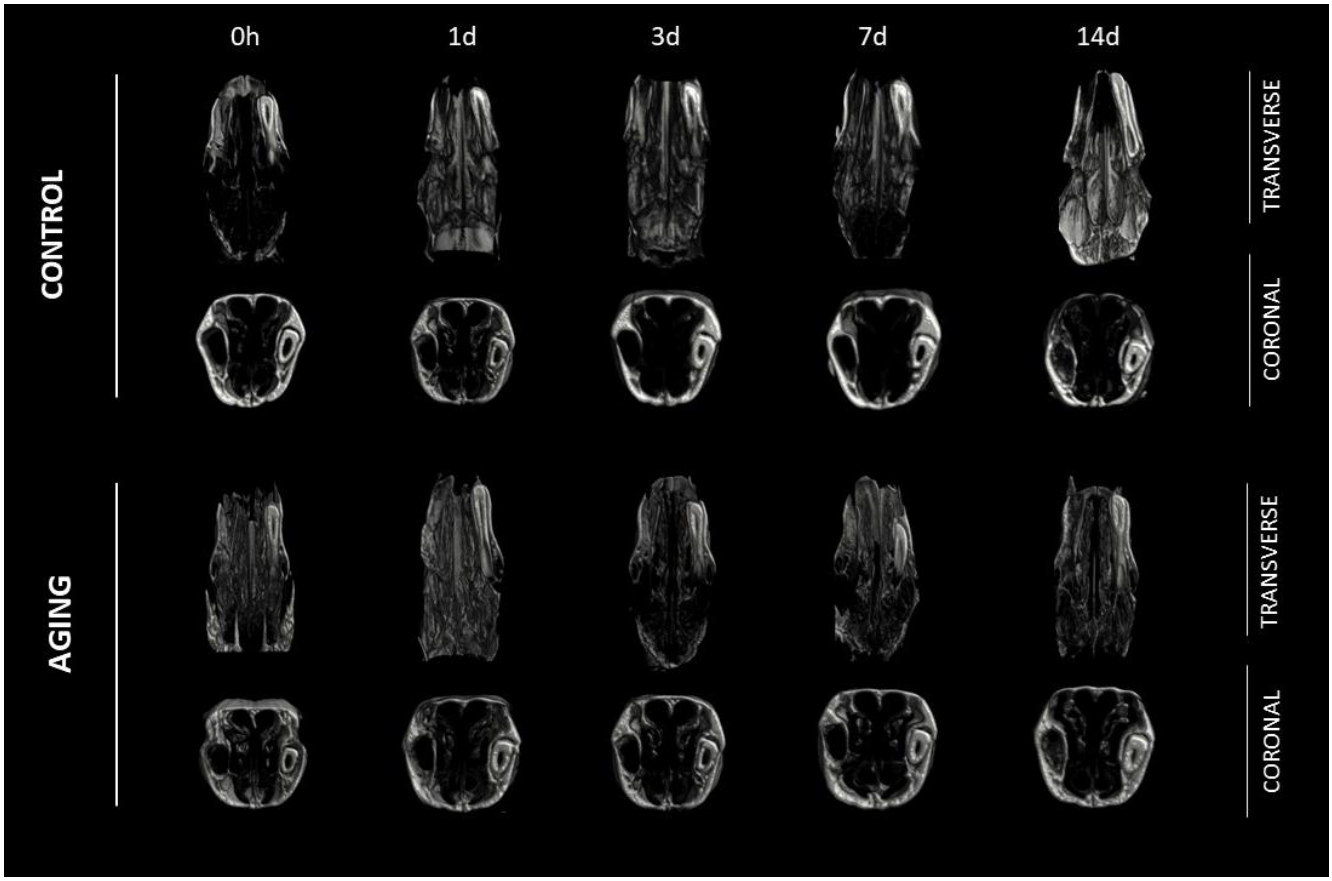

FIGURE 8-2 - Micro-computed tomography (µCT) analysis of bone healing process kinetics in Control (CTL) and (AGING) mice. Samples from 8-week-old male mice (CTL) and 32-week-old male mice (AGING) were scanned with the µCT System (Skyscan 1174; Skyscan, Kontich, Belgium) 0, 1-, 3-, 7-, and 14-days post tooth extraction time points for the kinetics evaluation of the bone healing process. Images were reconstructed using the NRecon software and three-dimensional images obtained with the CT-Vox software. The sectioned maxilla is represented at the transverse and coronal planes.

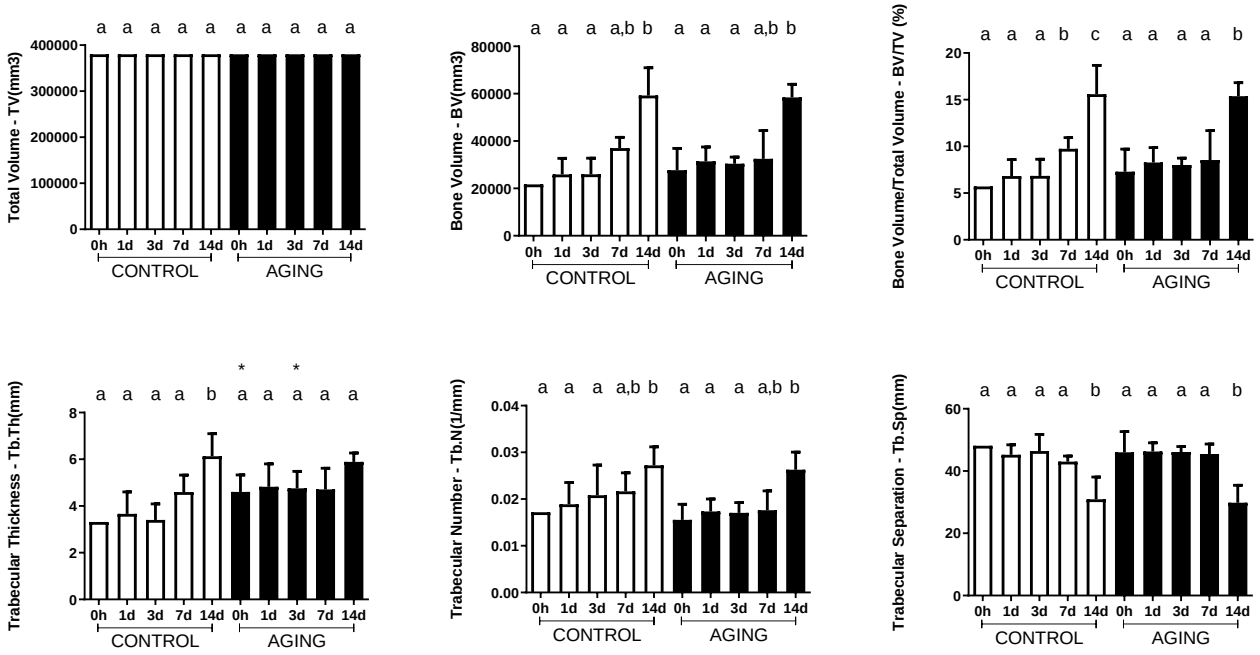

FIGURE 9-2- Analysis of the morphological parameters of the trabecular bone microarchitecture in the dental alveoli of the control mice (CTL) and 32-week aging mice (AGING) in the periods of 0, 1-, 3-, 7- and 14-days post-exodontia. Male C57Bl/6 (WT) mice (N = 6 group/time point) were submitted to the extraction of the upper right incisor and subsequently analyzed by microtomographic methods for the characterization mineralized tissue features at healing sites. The morphological parameters of the bone microarchitecture were evaluated using the CTAn software through a cylindrical region of interest (ROI) determined by the segmentation of the trabecular bone located from the coronal to the apical region. The analysis of bone parameters (A–F) included: Tissue volume (mm3) (A), Bone volume (mm3) (B), Bone volume in relation to tissue volume (%) (C), Trabeculae thickness (mm) (D), Number of trabeculae (1/mm) (E), Separation of trabeculae (mm) (F). The results represent the values of the mean and standard deviation in each of the periods analyzed (*Represent the differences between the control and OLD groups in each time point).

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3- Discussion

3 DISCUSSION

The alveolar bone repair is complex and involves several stages and subsequent and coordinated steps, where a moderate and transient inflammatory reaction is necessary for an adequate outcome of tissue repair (L. A. Mills & Simpson, 2012; Mountziaris & Mikos, 2008; Z.S. et al., 2008). Initially, in response to tissue damage, there are several inflammatory cytokines released in the injury site, such Interleukins (IL-1 and IL-6) and tumor necrosis factor alpha (TNF-α), responsible for recruiting cells of the innate immune system to the site of injury to remove cellular or matrix debris and to regulate tissue healing through the secretion of other mediators such as cytokines and growth factors (Azevedo et al., 2021). Although there are several cell types participating in this process, studies point to macrophages as important elements in tissue repair, as they are present in all its phases, including inflammation, formation of granulation tissue and deposition of new matrix (Morrissette et al., 1999; Park & Barbul, 2004; Sindrilaru & Scharffetter-Kochanek, 2013). Since the initial phases of the repair process mentioned above are mostly orchestrated by classically activated macrophages (Gaffney et al., 2017; Italiani & Boraschi, 2014; Morrissette et al., 1999; Sindrilaru & Scharffetter-Kochanek, 2013), it would be important to consider these cells as possible therapeutic targets to improve the repair outcome, by enhancing their functions performed at the beginning of the process. For this purpose, we used the drug Paclitaxel, which can induce macrophages to assume the M1 polarization.

Initially, our results showed that treatment with PTX, in all concentrations, despite some alterations in the values of bone microarchitecture evidenced in the experimental groups (Figures 1-1, 1-2 and 1-3), did not significantly impact new bone formation at the end of the 14-day period, as can be seen in the qualitative analysis through micro tomography reconstructions (Figure 1-3). The same outcome can be visualized when the histological slides are qualitatively evaluated, showing the formation of new bone tissue filling the alveolar defect at the end of the 14-day experimental period, when comparing the experimental PTX groups with the control group (Figure 4-1). Paclitaxel is a drug used in the treatment of neoplasms of the breasts, bladder, lungs, and some metastases (Martins et al., 2014) acting as an inhibitor of microtubule depolymerization, which may affect the proliferation and differentiation of some cell types (J. Shen et al., 2020), some reports in the literature

show a probable negative influence of PTX on osteogenesis for a certain period and that this effect seems to disappear with the passage of time after administration (Matsumoto et al., 2022), thus, these reports seem to agree with the results obtained in our experiment.

Considering that PTX could affect cell proliferation and differentiation (Choron et al., 2015; J. Shen et al., 2020), it would be prudent to expect that the use of this drug could also affect some parameters through the repair process, marked by sequential and overlapping phases, such as clot formation, migration, and proliferation of MSCs, formation of granulation tissue, angiogenesis, proliferation of fibroblasts and secretion of collagen (Vieira et al., 2015). In fact, PTX treated groups showed an increase in fiber and fibroblast density in some periods (Figures 6-1 and 13-1), conflicting with some reports in the literature that shows that PTX could negatively affect the fibroblasts recruitment and activity (Choritz et al., 2010; Schiff & Horwitz, 1980). in the same direction as the previous finding, PTX reports in the literature show that PTX negatively affects the angiogenesis process, which is important for the repair process (Z.S. et al., 2008). However, once again our results conflict with these reports, showing an increase in vessel density in some evaluated periods, in certain concentrations of PTX. Such results should be analyzed with caution, as they may reflect a delay in the transition between repair phases, caused by changes in factors responsible for coordinating ongoing cellular processes, rather than an increase or decrease in the density of structures in fact. Therefore, more tests are needed for better understanding the influence of Paclitaxel the on these highlighted processes in bone healing.

In addition to the characteristic antineoplastic effect, Paclitaxel also exerts effects on the components of the host's immune system, for example, being able to enhance the response against tumor cells (Javeed et al., 2009). Such immunoregulation attributed to PTX is due to the induction of genes responsible to produce pro-inflammatory mediators, which recruit and activate effector cells, such as macrophages, NKs, dendritic cells and lymphocytes (Javeed et al., 2009; Zaks-Zilberman et al., 2001). Thus, some alteration in the dynamics of migration of inflammatory cells was expected in the evaluated periods. Indeed, when compared to the control group, the PTX treated groups showed an overall decrease in the inflammatory cell’s density in the later periods, in weekly treatment (Figure 6-1). When the immunohistochemical analysis of the collected specimens were performed, we can notice the prevalence of Ly6gGR1+ cells (granulocytes), in the 3-day period,

decreasing in density in the following periods (Figure 7-1). CD206+ cells (Macrophage M2), in turn, are less present in the 3-day period, increasing in the later periods. However, when PTX was administered at 10 mg/Kg in 48 hours regime, there was a notable alteration in the inflammatory cells count, which showed greater density in the 1- and 7-day period compared to the control group, suggesting a higher prevalence or longer prevalence of these cells in the repair site (Figure 13-1).

Despite several minor changes in specific histological parameters over the periods studied, even with the administration of PTX in shorter periods, there were, again, no major changes in the repair process, with the dental alveolus being satisfactorily filled with bone tissue at the end of the 14-day period, as shown in the qualitative histological analysis (Figures 4-1 and 12-1), following the same pattern seen in the PTX groups treated in a weekly regimen. In general, treatment with Paclitaxel was able to change some aspects in certain phases of the repair process, however, without compromising or improving it. Reiterating what was mentioned earlier, the increase in the density of certain components, in our view, can be understood as a greater prevalence of these in the repair site, due to the probable imbalance in the balance of the mediators present during the evaluated periods and, in the end, this balance seems to be reestablished, enabling adequate tissue repair. Although the kinetics of macrophage migration could not have been fully investigated due to the inability to perform the comparison for markers CD80 (Macrophages M1) and F4/80 (Macrophages general) due to problems related to the necessary inputs for the reaction, we can suggest that the treatment with Paclitaxel apparently did not influence the migration of M2 macrophages, considering that their densities in the experimental groups did not show alterations in relation to the control group, when such comparison is made through the immunohistochemical analysis of CD206+ cells. However, it should be noted that the reprogramming effect on macrophages (for an M1 profile) exerted by Paclitaxel was evident in tumor environments (Wanderley et al., 2018; Yamaguchi et al., 2017), which have completely different characteristics from a site undergoing repair. The tumor microenvironment presents local mediators that can influence tumor-associated macrophages (TAM) to adopt an M2-type profile, which contribute to the escape of immune surveillance, angiogenesis, matrix remodeling, contributing to tumor development. In this context, PTX administration reprograms existing TAMs to an M1-type, pro-inflammatory profile, contributing to an antitumor effect (Wanderley et al., 2018).

Contrary to the conditions mentioned above, bone under normal conditions and in response to injuries tends to regenerate its damaged matrix through the repair process, which comprises several stages, very well-orchestrated and regulated by complex mechanisms involving systemic and local factors, interacting with several cells that are recruited to the site of injury (Z.S. et al., 2008). While the process evolves, the clot is reabsorbed, giving way to a granulation tissue that plays the role of a provisional matrix, which is finally replaced by new bone matrix by osteoblasts, originating from progenitor cells recruited to the repair site (Vieira et al., 2015). In this way, the process shows self-regulation and resolution, unlike what happens in pathological conditions, such as tumor environments or chronic injuries. Chronic inflammatory processes such as rheumatoid arthritis, periodontitis and endodontic lesions usually lead to loss of bone close to the compromised site, not resolving over time (Souza & Lerner, 2013). Endodontic lesions develop mainly by infection of the pulp tissue by oral bacteria that cause an inflammatory process that invariably evolves to necrosis, reaching the tooth apex (D. T. Graves, Oates, et al., 2011). As the host's defenses cannot efficiently fight the bacteria and their products that are found inside the root canal, they end up creating a granulation tissue in the periapex, rich in innate and adaptive immunity cells, to try to serve as a barrier to the invasion of microorganisms and their products from the dental canal (D. T. Graves, Oates, et al., 2011). Thus, the predominance of pro-inflammatory or anti-inflammatory mediators in this environment can accelerate or paralyze osteolysis, characterizing progression or stabilization of the lesion (Stashenko et al., 1992, 1994). Recently our group evaluated the role of the immunoregulatory molecules VIP (Vasoactive intestinal peptide) and PACAP (Pituitary adenylate cyclase activating polypeptide) in the alveolar bone healing, and despite of the effectiveness of VIP or PACAP in modulating host response, by the early dominance of M2 macrophages, there were no great impact on the alveolar repair outcome (Azevedo et al., 2021) However, in an experimental periapical lesion model, VIP therapy resulted in attenuation in the lesion progression, associated with immunoregulation involving T-cells (de Campos Soriani Azevedo et al., 2019). In this context, we decided to use the same model of induced periapical lesion to evaluate the possible influence of Paclitaxel administration on the balance of a chronic lesion.

Our results show that paclitaxel can be associated with lesion evolution, since PTX treated groups show lesions with bigger dimensions (Figure 15-1). Remarkably

PTX, when administered lately (21-days period) also seems to induce a shift in the macrophages present in the lesion site towards a M1 phenotype, since there is a notable upregulation in the iNOS mRNA, and CD80+ cells numbers (Figure 14-1), concomitantly with downregulation in ARG1 and CD206+ cells numbers (Figure 15-1). STAT1p, indicating M1 polarization, was also upregulated in PTX treated group, suggesting these cells activity (Figure 14-1). These alterations are associated with the increase in the lesion dimensions (Figure 15-1). Such lesion's increase in PTX group was associated with the upregulation of pro-inflammatory cytokines such IL-1 and TNF (Figure 17-1) and downregulation in the regulatory mediators, such IL-10 (Figure 17-1). Summary, PTX treatment seems to indeed induce macrophages towards M1 polarization, up regulating M1 markers such iNOS and STAT1p, also increasing CD80+ cells numbers, most likely for reprograming the cells present in the repair/lesion site. However, such induction was more evident in a chronic lesion microenvironment than in the alveolar bone repair.

Following the hypothesis of modulation of the initial inflammatory response after tissue injury, we aimed to evaluate the influence of other therapeutic agents on the outcome of the alveolar bone repair process. As previously mentioned, the initial phases of the inflammatory process in response to tissue damage are marked by the expression of various inflammatory mediators, such interleukins (mainly IL-1 and IL-6) and tumor necrosis factor (TNF-α), which play an important role in initiating the repair cascade, recruiting other inflammatory cells, and inducing matrix synthesis and angiogenesis (Ono & Takayanagi, 2017; Z.S. et al., 2008). Acting in conjunction with the mediators mentioned above, prostanoids and arearachidonic acid metabolites released from cells and are regulated by the cyclooxygenase (COX) isoenzymes, exercising key roles in injury response, having vasodilatory properties and being able to activate, or sensitize nociceptors that normally innervate the area, clinically causing the painful sensation (Lisowska et al., 2018; Mitchell et al., 2018). In this clinical context, non-steroidal anti-inflammatory drugs (NSAIDs) are agents that are classically and widely used to modulate the inflammatory response, consequently alleviating the painful sensation after tissue trauma (Pountos et al., 2012, 2021). However, there are several reports that the administration of NSAIDs can negatively interfere with the process of repairing bone fractures. Several publications suggest that NSAIDs are correlated with various levels of impairment to the bone healing process, such as decreased mineral content, decreased density and strength, and delayed healing

(Pountos et al., 2008). Nonetheless, apart from the traditional COX-related actions, NSAIDs could influence other aspects in the several phases of the repair process, such alterations in cytokine production and cells adhesion and even macrophage polarization (Díaz-González & Sánchez-Madrid, 2015; Raaijmakers et al., 2022). Thus, the focus of this study is to elucidate the influence in the modulation of the inflammatory process by NSAIDs in an intramembranous ossification repair model, after dental extraction (Vieira et al., 2015), trying to highlight the NSAIDs influence on several bone repair steps, such as blood cloth, cells migration and differentiation, matrix production and remodelation. Considering that, as mentioned earlier, most studies correlating NSAIDs to bone repair process occurs in bone fracture models, our study could have some different approach in this theme.

Overall, our results demonstrate that sustained treatment with ibuprofen (IBU) did not negatively affect the bone repair process in the studied C57BL/6 mice. The analysis of the three-dimensional reconstruction of the maxillae derived from the µCT shows that the bone repair was carried out adequately and similarly in the control (CTL) and IBU groups (Figure 1-3). This result follows the same direction, with few changes, when the histological aspect of the repair is analyzed. Qualitatively, the repair progressed adequately, with the alveolus being filled with new bone tissue at the end of the experimental period, without marked differences in relation to the control group, as seen in the µCT and histological analysis (Figures 1-3, 2-3 and 3-3).

Most studies in the literature associate the use of NSAIDs with a negative effect on the outcome of the bone repair process, usually with lack of fracture union, with this effect being time and dose dependent, (Al Farii et al., 2021; Wheatley et al., 2019). The most likely cause for the negative effects of NSAIDs on the repair process is the decrease in prostaglandins (mainly PGE-2 and PGF-2α), which seem to regulate the expression of BMP-2 and BMP-7, important mediators in the differentiation of progenitors of bone cells in osteoblasts, affecting the physiology of these cells, (Bandyopadhyay et al., 2006; Kotake et al., 2010; Melguizo-Rodríguez et al., 2018; Pountos et al., 2008; B. Shen et al., 2010). However, even with the treatment with ibuprofen, the repair process seems to self-regulate itself and was able to compensate the possible alterations, showing an excellent outcome at the end of the experiment (Figures 3-3 and 8-3).

Bearing in mind that the deleterious effects of NSAIDs are dose dependent, we performed a second test, where the experimental group received a dose of ibuprofen

of 200mg/Kg every 24 hours. Again, the histological analysis of the evaluated periods showed normal kinetics in the repair process, without notable differences when compared to the control group, with the with the alveolar socket being filled with new bone tissue at the end of the experiment (Figure 8-3). Thus, we can observe that the administration of ibuprofen, either at concentrations of 40 mg/kg or 200 mg/kg (both every 24 hours), did not cause significant impacts on healing within our model of intramembranous bone repair.

Another important aspect for the adequate outcome in alveolar bone healing is the construction of a provisional collagen matrix by fibroblasts. In this sense, treatment with ibuprofen (40mg/kg) showed a downregulation in fiber and fibroblast density in some periods compared to the control group (Figure 4-3). In turn, controversially, the group medicated with ibuprofen at 200mg/kg showed an upregulation in the fiber's density and fibroblasts (Figure 9-3). In addition to the supposed negative effects already explored by NSAIDs on bone repair, their influence on fibroblast proliferation and collagen production seems uncertain because, in the literature, there are reports that NSAIDs can inhibit or delay the proliferation and fixation of fibroblasts in other experimental models (Nguyen & Lee, 1992), or that do not appear to affect fibroblast proliferation in any way (Beata et al., 2005). Other models demonstrate, in turn, that NSAIDs (in particular, Ibuprofen) could be involved in the upregulation of collagens type I, II and III, consequently increasing their synthesis (Ou et al., 2012). Thus, the influence of NSAIDs on the function of fibroblasts may be dose and/or time dependent and may be altered by mediators present in the environment where these cells are found. Anyway, for a better understanding of these conflicting results, more analyzes should be carried out in this aspect of the repair.

The greatest density of fibers and fibroblasts occurs in the initial periods, when the provisional matrix begins to be built, replacing the clot present until then (Figures 3-3 and 9-3) The clot, in turn, in the IBU group (40 mg/kg), showed a trend towards an increase in its density, with an upward regulation. An expected result, since NSAIDs are classically associated with platelet aggregation (Gunaydin & Bilge, 2018). Interestingly, the IBU 200mg/kg group showed a decrease in clot density in the initial periods, with an increase in the 7-day period (Figure 9-3). This result may be more related to the concomitant up-regulation of fibers and fibroblasts in the initial periods, occupying more space in the alveolus (Figure 8-3).

Treatment with ibuprofen also seems to have caused possible alterations in the angiogenesis process, considering that the vessel density was altered in the experimental groups compared to the control group (Figures 3-3 and 9-3). The IBU 40mg/kg group showed a general decrease in vessel density. In general, the literature points out that NSAIDs have a marked angiogenesis inhibition effect, which seems to be multifactorial and probably includes local alterations in growth factors and in important mediators of endothelial growth, such as VEGF. NSAIDs also appear to increase endothelial cell apoptosis and decrease their migration. (Jones et al., 1999; Tarnawski & Jones, 2003). In this context, the decrease in vessel density observed in the experimental groups may be related to some of these mechanisms mentioned in the literature, and the increase in vessel density at specific times, in turn, may be a response to the imbalance caused at the site under repair, showing that even with continuous medication with NSAID, there is an intrinsic regulation of the process. However, further studies would be needed to better elucidate these changes.

Finally, another factor changed during the periods studied in the repair process was the migration of inflammatory cells. During the inflammatory process, leukocytes act in an important way, whether in the defense of the organism against microorganisms or in the cleaning of cellular remains and debris from the damaged matrix (Sindrilaru & Scharffetter-Kochanek, 2013). The IBU groups showed a general reduction of inflammatory cells, with marked downregulation in the 200 mg/kg concentration treated groups, suggesting a dose-dependent response. When the periods are evaluated through immunohistochemistry (performed only for the IBU 40 mg/kg group, due to problems with the reagents present at the time), the experimental group showed an decrease in the number of CD206+ cells (M2 macrophages) and a decrease in the number of Ly6gGR1+ cells (granulocytes), both in the 3-day period (Figure 7-3). Indeed, the effect of decreasing inflammatory cell density associated with NSAIDs is well documented in the literature and this effect is credited to drug changes in adhesion molecules on endothelial cells (Higgs et al., 1980; Hofbauer et al., 1998). Although the main effect of NSAIDs is the inhibition of prostaglandin synthesis by blocking COX, they have other COX-independent mechanisms of action, such as interfering with the functionality of a wide variety of cellular functions, in particular the L-selectin function in neutrophils (Díaz-González & Sánchez-Madrid, 2015). NSAIDs can cause shedding of the L-selectin molecule in neutrophils, decreasing its expression and, consequently, interfering with the recruitment of this specific cell type

(Gómez-Gaviro et al., 2000). Thus, such a mechanism may explain the decrease in the number of Ly6gGR1+ cells observed during the repair process.

The administration of ibuprofen in a model of muscle injury showed an increase in the tissue concentration of macrophages, reducing the number of necrotic fibers, suggesting a possible induction of the M1 profile (Cheung & Tidball, 2003). In fact, ibuprofen seems to induce the M1 profile in macrophages after consistent COX inhibition (Na et al., 2015; Pennock et al., 2018), such reports agree with our finding, where we had a tendency of decrease in CD206+ cells, in the experimental group (IBU 40mg/kg), in relation to the control group. For a better understanding of this result, other tests must be performed, such as, for example, the labeling of total macrophages (F4/80+) and M1 macrophages (CD80+). Such markings were expected to be carried out in our experiment; however, we had problems with the available immunomarkers that ended up making the experiment unfeasible. In summary, our experiment showed that the continuous administration of ibuprofen at different concentrations, as a possible modulator of the inflammatory process, did not cause a negative impact on bone repair, considering that the alveolar process was normally filled with bone tissue at the end of the last period, with no differences compared to the control group.

With the results obtained in the two previous experiments, it is possible to verify that the bone tissue manages to repair defects in its matrix in a formidable way, despite the influences of the applied drugs, which in a certain way act causing an imbalance in the repair site, showing an extraordinary capacity of self-regulation of the cellular and molecular processes involved. Thus, our third experiment aimed to simulate a situation where all functions of the organism begin to undergo theoretically deleterious changes due to the aging process. It is known that the ability of tissue repair in organisms is impaired with the aging process, as cells are trapped in a permanent state of cell cycle, characterized by specific phenotypic changes, which include changes in gene expression, chromatin structure, cell signaling and the pattern of cell secretion (D. Wang & Wang, 2022). There seems to be agreement in the literature that bone tissue, in aged organisms, responds with a delay to the repair process in case of fracture and is more susceptible to comorbidities and failures in therapeutic processes, such as rehabilitation with osteointegrated implants (Compton et al., 2017; Foulke et al., 2016; Meinberg et al., 2019). Nonetheless, the aging effects on bone repair are mainly highlighted in fracture models (Clark et al., 2017; Meinberg et al., 2019), where an endochondral type of repair takes place, there is still a gap of information about

age-related changes in the intramembranous bone repair outcome. Another fact that deserves to be highlighted, since most studies aim the elderly populations. is the relative lack of information aiming to elucidate when exactly the age-related changes start to manifest and how much they could impair the physiological processes (Lu et al., 2008; Meinberg et al., 2019). To address such questions, we applied our experimental model of alveolar bone repair (Vieira et al., 2015) in mice during the aging process, not yet considered senile, at 32-weeks-old, to be able to contemplate when and what alterations related to the aging process begin manifest themselves and how they can alter the outcome of the repair process and, briefly, your results showed that the aging animals showed occasional alterations in some variables, like matrix products and density and cells numbers, appearing, initially, that the repair process suffered a delay in some phases but, in the end, was able to overcome these changes, filling the extraction site with new alveolar bone, qualitatively like the control group.

Our results points alterations in the connective matrix and its components in the AGING group, with up regulated expression in the Col1A1 and Col1A2. These genes encode the two alpha chains of type I collagen, which comprises most of the organic matrix of bone tissue (Buck & Dumanian, 2012a; Pollitt et al., 2006). Also, in the AGING group, fiber density was upregulated, showing some possible relation with the pointed molecular changes. There was an increase in MMP8 mRNA as well, suggesting a possible compensation mechanism due to the apparent collagen matrix components upregulation (Figure 2-2). Finally, in the AGING group, accordingly with these findings, fibroblasts and fibers density appeared to be upregulated in general, together with the fibroblast growth factor 1 (FGF1). At the first impression, these findings go against common reports in the literature on collagen production in aged organisms, which have, lower collagen production(Lago & Puzzi, 2019; Varani et al., 2006). Nonetheless, these results may suggest, in addition to the increase in density itself, an increase in the permanence time of these structures in the repair site, evidencing a probable delay in the matrix remodelation in the AGING group related to the control animals (Vieira et al., 2015). Furthermore, the most studied bone healing models in aged organisms are those related to fracture repair, where an endochondral bone formation takes place predominantly, showing there is a deficiency in the production of type II and type X collagens, causing a delay in the repair process (Clark et al., 2017; Meinberg et al., 2019). The model proposed in your experiment comprises an intramembranous bone repair and, therefore, it is difficult to compare between our results and other findings

due to the lack of studies in the literature focusing on this type of process. Thus, the alteration in the expression of these genes in question may be a characteristic of the intramembranous bone repair process in aging organisms and, for a better understanding of this finding, further studies should be carried out.

Optimal bone healing is vitally dependent on adequate vascularization and, therefore, requires the development of new blood vessels(Z.S. et al., 2008). Since angiogenesis is commonly pointed as downregulated in the aging process(Hodges et al., 2018), some alterations in that mechanism were expected to be seen in our AGING experimental group. Indeed, the AGING group presented downregulation in the mRNA expression of the vascular endothelial growth factor (VEGF), coinciding with the downregulation tendency in the vessel's density, mainly expressed in the later period (Figure 7-2). VEGF is a mediator that plays a central role in the angiogenesis process, and it seems to be downregulated in aged animals (Hodges et al., 2018; Lähteenvuo & Rosenzweig, 2012),therefore, your results seem to follow the common findings in the literature. However, the downregulation in VEGF mRNA expression and in the vessel's density does not appear to have had a negative impact on the repair process.

Along with angiogenesis, stem cells recruitment and differentiation play crucial role on bone repair, since these cells will be responsible for the deposition of new mineralized matrix (Knight & Hankenson, 2013). That way, transforming growth factor beta 1 (Tgfb1), a crucial molecule involved in the bone repair process (Zimmermann et al., 2005), also showed decreased mRNA expression levels in the AGING group (Figure 2-2). TGF-β is a family of growth factors, responsible for regulating cell activity and is crucial in bone formation by activating mesenchymal stem cells (MSCs), fibroblasts, osteoblasts, also promoting the inhibition of osteoclastogenesis (Datta et al., 2008; Ghiasi et al., 2017; Z.S. et al., 2008). In this sense, the AGING group showed alterations in some stem cells markers of, also participating in the repair process. OCT-4, belonging to the Pou-domain family of transcription factors, has an influence on the expression of NANOG, also a transcription factor present in embryonic stem cells (ECMs) (Bais et al., 2012; Matic et al., 2016). These two factors are essential for most of the properties of stem cells, such as self-renewal and pluripotency(Matic et al., 2016). CD34 and CD105 mRNA levels, markers of hematopoietic and mesenchymal stem cells, respectively, important in promoting angiogenesis (Kuroda et al., 2014; Marcianò et al., 2021), were also downregulated in the AGING group (Figure 2-2). Finally, CD146 and CD106 are markers expressed in cells with multipotent

differentiation potential (Bragdon & Bahney, 2018; Heim et al., 2020), showed lower mRNA levels in the AGING group (Figure 2-2). Again, these results, together with the changes in VEGF mRNA levels mentioned above, may be related to the decrease in vessel density in the histomorphometry analysis (Figure 7-2). The general decrease in the mRNA levels of stem cells markers and related transcription factors suggests a decrease in the activity and/or number of these cells and/or possible changes in the mechanisms that aim to maintain their viability of differentiation and, consequently, their performance in the repair process in aging animals (Bragdon & Bahney, 2018; Chen et al., 2022; Meinberg et al., 2019).

The recruitment of inflammatory cells to injury sites is an important phase in the bone healing, since they'll be responsible for producing a complex and controlled cascade several molecules, like growth factors and inflammatory mediators, responsible for orchestrating the repair process(Mountziaris & Mikos, 2008). The inflammatory cells recruitment and migration is mediated by chemokines and their receptors, which are divided into four different subfamilies (CC, CXC, CX3C and C) (Edderkaoui, 2017). The AGING group showed down regulation of mRNA levels of CCR1, CCR2 and CCR5. In turn, the CXCR1 receptor was upregulated initially, suffering subsequently downregulation (Figure 2-2). Finally, CXCR2 mRNA expression increased in earlier periods. CCR2 and CCR5 are receptors involved in the recruitment of the macrophage/monocyte lineage to the site of injury (Brylka & Schinke, 2019; Edderkaoui, 2017). Consequently, the decrease in these markers' mRNA levels may be also correlated with the INOS, ARG2 and FIZZ mRNA levels decrease, since they're all macrophage markers (Italiani & Boraschi, 2014; Ono & Takayanagi, 2017; Zhang et al., 2018). However, controversially, the immunohistochemical analysis shows an increase in CD206+ cells (M2 macrophages) in the AGING group at certain periods in relation to the control group (Figure 3-2). It was intended to perform the immunohistochemical analysis with the markers F4/80+ (General Macrophages) and CD80+ (M1 macrophages), for a better understanding of the kinetics of inflammatory cells at the repair site, however, due to operational problems with the reagents obtained and available in our laboratory, it was not possible to perform this procedure. Thus, further investigations are needed to elucidate this conflicting aspect.

The IL-8 receptors CXCR1 and CXCR2, or also called IL-8 RA/R1 and IL-8 RB/R2, respectively (Baggiolini et al., 1997), were up-regulated in the AGING group at the initial periods. These receptors play an important role in the attraction and migration

of hematopoietic progenitor cells and neutrophils to the site of injury (Baggiolini et al., 1997; Edderkaoui, 2017; Ringe et al., 2007). This increase in CXCR1 and CXCR2 mRNA expression could be related to the increase in GR1-Ly6g+ cell count in the immunohistochemical analysis (Figure 3-2). When chemokines are evaluated, the AGING group showed a decrease in CCL2 and CCL5, when compared to the control group (Figure 2-2). CCL2 also known as monocyte chemotactic protein 1 (MCP-1), a ligand of the CCR2 receptor, is one of the earliest and most highly expressed chemokines in response to bone fractures. It is also involved in the regulation of neutrophil migration, angiogenesis, and macrophage infiltration (Edderkaoui, 2017). In turn, CCL5, a ligand for CCR1, CCR3, and CCR5, promotes the recruitment and activation of macrophages, lymphocytes, and mast cells (Edderkaoui, 2017). Other cytokines involved in the promotion and migration of monocytes/macrophages to the site of injury are CCL12 (Cohen et al., 2022; Helmke et al., 2019; Morteau, 2006) were downregulated in the AGING group during the evolution of the repair process (Figure 2-2). As for chemokines involved in T cell recruitment and migration, CCL20 and CCL25 (Azevedo et al., 2021) were also in the AGING group when compared to the control group. Finally, the AGING group showed increased mRNA levels of CXCL12, (also known as Stroma cell-derived factor 1). CXCL12 appears to be involved in the repair process by attracting endothelial progenitors and osteoblastic progenitors (Edderkaoui, 2017). In general, most chemokine receptors and their respective ligands involved in immune cells migration, especially from the monocyte/macrophage lineage, had their mRNA expressions decreased in the AGING group compared to the control group, with few exceptions. This finding could be related with the decrease in mRNA levels of FIZZ, ARG2 and iNOS, markers for macrophages in tissue repair sites (Figure 2-2).

Considering the inflammatory mediators, as important regulators in bone deposition/resorption balance, and the reported pro-inflammatory profile in aged animals (Clark et al., 2017; Gibon et al., 2017) the assessment of possible alteration in these variables is extremely important. Thus, the AGING group showed upregulation in the IL-1β, IL-6 and Tumor necrosis factor (TNF) mRNA expression levels, all pro-inflammatory cytokines and, generally, potent inducer of the osteoclastogenesis process (Blanchard et al., 2009; Ginaldi & De Martinis, 2016; Ono & Takayanagi, 2017; Palmqvist et al., 2008; Takayanagi, 2005, 2007) . Concomitantly with the upregulation of pro-inflammatory cytokines mRNA expressions, there is a decrease in IL-10 mRNA

levels, a cytokine known to be associated with inactivity in chronic osteolytic lesions such as periodontitis due to its action of inhibition of the secretion of pro-inflammatory cytokines and the osteoclastogenesis process (D. T. Graves, Li, et al., 2011; D. T. Graves, Oates, et al., 2011; Takayanagi, 2005, 2007). These findings are compatible with the common evidence that point a sustained and low-grade inflammatory profile in aged animals (Clark et al., 2017, 2020; Meinberg et al., 2019). Considering this, the predominance of pro-inflammatory mediators in the repair environment should influence local macrophages to assume an M1 phenotype, also pro-inflammatory (Hume, 2015; Mantovani et al., 2013). In fact, a decrease in the mRNA levels of ARG2 and FIZZ, markers of M2 macrophages, involved in the resolving phase of the inflammatory process can be noted (Das et al., 2015b; Hume, 2015; Mantovani et al., 2013), suggesting a decrease and/or delay in the activity of these cells in the alveolar bone repair process in the AGING group. Such a delay in the resolution of the initial phases of the inflammatory process may be reflected in the tendency towards a decrease in mRNA expression levels of bone maturation indicators, affecting the differentiation of cell groups involved in bone neoformation (Figure 2-2).

As mentioned above, the AGING group showed a decrease in the expression levels of bone morphogenetic proteins (BMP's) and TGFβ. BMP2, BMP4 and BMP7 had down-regulated mRNA expression in several experimental periods (Figure 2-2). BMPs 2 and 7 are mediators recognized for participating widely in the bone formation process, being described as essential factors for the differentiation of osteoblasts and adequate bone formation (Bandyopadhyay et al., 2006; Bonewald, 2011) and their expression is possibly the initial trigger for the development of bone tissue mineralization (Vieira et al., 2015). Thus, a decrease in the initial levels of BMPs could impact cell differentiation and, consequently, the deposition and subsequent mineralization of newly formed bone matrix. Indeed, the AGING group showed a decrease in mRNA expression of RUNX2, the main transcription factor involved in osteoblastic differentiation (Bonewald, 2011; Vieira et al., 2015). This result seems to impact the histomorphometric analysis in the AGING group, that presented a decrease in the density of osteoblasts and in the density of the bone matrix itself, when compared to the control group (Figure 7-2). Interestingly, there is an increase in the number of osteoclasts present in the final period in the AGING group, suggesting an increase that aims to compensate for a possible delay in remodeling in newly formed bone tissue. Following the same pattern, markers of advanced differentiation of osteoblasts and

osteocytes, such as PHEX (phosphate-regulating neutral endopeptidase) and DMP1 (dentin matrix protein 1) and ALPL (alkaline phosphatase) also underwent downregulation mostly at the final periods (Figure 2-2). Despite the decrease in markers, when evaluating the micro tomography of the periods studied in both groups, the animals in the AGING group show an increase in trabecular thickness (TB. Th), in some periods (Figure 9-2). This finding differs from what is commonly found in the literature, where a decrease in the number and thickness of trabeculae is expected in bone tissue in older populations (Boskey & Coleman, 2010; Chen et al., 2013) but, since our experimental group comprises middle aged animals, these changes should be different and not so prominent.

In summary, our data show that, with advancing age, the bone repair process begins to be affected in a discrete manner, with an apparent prolongation of the initial, pro-inflammatory phase. Consequently, the mediators present seem to delay the recruitment and differentiation of osteoblastic precursors, responsible for the production and mineralization of new bone matrix. However, this delay does not seem to negatively affect the outcome of the process, which shows neoformation of bone tissue at the extraction site at the end of the period studied. Thus, it is appropriate to state that bone tissue has formidable remodeling and repair capacities, which seem to have a high capacity for self-regulation when the balance between molecular and cellular processes is altered, by drugs or, as seen in the last experiment presented, alterations inherent to aging process. That way, it seems that bone tissue will maintain it’s plastic capacities howsoever, if the organism is not affected by any pathological changes.

4- Conclusion

In summary, the results obtained in the three previous experiments, it is possible to verify that the bone tissue manages to repair defects in its matrix in a formidable way, despite the influences of the applied drugs and the and changes resulting from the aging process, which in a certain way act causing an imbalance in the repair site, showing an extraordinary capacity of self-regulation of the cellular and molecular processes involved. In general, treatment with Paclitaxel was able to change some aspects in certain phases of the alveolar bone repair, however, without causing major changes in the process outcome. Interestingly, in an experimental periapical lesion model, Paclitaxel can be associated with lesion evolution, since PTX treated groups show lesions with bigger dimensions. Remarkably PTX, when administered lately, also seems to induce a shift in the macrophages present in the lesion site towards a M1 phenotype, since there is a notable upregulation in the iNOS mRNA, and CD80+ cells numbers, concomitantly with downregulation in ARG1 and CD206+ cells numbers. Summary, PTX treatment seems to indeed induce macrophages towards M1 polarization, also increasing CD80+ cells numbers, most likely for reprograming the cells present in the repair/lesion site. However, such induction was more evident in a chronic lesion microenvironment than in the alveolar bone repair. In the same way, continuous administration of ibuprofen at different concentrations, as a possible modulator of the inflammatory process, did not cause a negative impact on bone repair, considering that the alveolar process was also normally filled with bone tissue at the end of the last period, with no differences compared to the control group.

Finally, early aging effects seems to affect bone repair process in a discrete manner, with an apparent prolongation of the initial, pro-inflammatory phase. Consequently, the mediators present seem to delay the recruitment and differentiation of osteoblastic precursors, responsible for the production and mineralization of new bone matrix. However, this delay does not seem to negatively affect the outcome of the process, which shows neoformation of bone tissue at the extraction site at the end of the period studied. Thus, it is appropriate to state that bone tissue has formidable remodeling and repair capacities, which seem to have a high capacity for self-regulation when the balance between molecular and cellular processes is altered, by drugs or, as seen in the last experiment presented, alterations inherent to aging process. That way, it seems that bone tissue will maintain it’s plastic capacities howsoever, if the organism is not affected by any pathological changes.

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ANNEXES

Anexo A - Ofício de aprovação do projeto de pesquisa pela Comissão de Ética no Ensino e Pesquisa em Animais da FOB/USP

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Uso de Animais

CEUA-Proc. Nº 011/2019

Bauru, 9 de novembro de 2021.

Senhor Professor,

Em atenção à solicitação de prorrogação na vigência do trabalho de pesquisa intitulada ***"Papel da polarização de macrófagos para um perfil M1 nos processos de reparo ósseo alveolar e de osseointegração em camundongos"***, registrada sob *CEUA-Proc. Nº 011/2019*, tendo Vossa Senhoria como Pesquisador Responsável, que envolve a utilização de animais (roedores), para fins de pesquisa científica, foi analisada e considerada APROVADA em reunião ordinária da Comissão de Ética no Uso de Animais (CEUA), realizada no dia 29 de outubro de 2021.

Vigência do projeto:	*JAN/2020 a JUN/2023*
Espécie/Linhagem:	*Camundongo C57BL/6 (WT)*
Nº de animais:	*1.080 (um mil e oitenta)*
Peso/Idade	*8 semanas/25g (540) / 32 semanas/25 gramas (540)*
Sexo:	*Macho*
Origem:	*Biotério Central da Faculdade de Medicina de Ribeirão Preto FMRP*

Lembramos que esta CEUA solicita: ***1)*** *qualquer alteração na pesquisa seja informada a esta CEUA, por meio de ofício e documentos que se fizerem necessários;* ***2)*** *ao término da pesquisa seja apresentado Relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.*

Atenciosamente,

Profa. Dra. Ana Paula Campanelli
Presidente da Comissão de Ética no Uso de Animais

Prof. Dr. Gustavo Pompermaier Garlet
Docente do Departamento de Ciências Biológicas

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
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Anexo B – Comprovante de submissão de artigo científico “Analysis of modulation of M1/M2 polarization in transition by paclitaxel in transitory and chronic inflammatory environments and its influence on bone tissue” ao periódico JAOS.

Thank you for your submission

Submitted to
Journal of Applied Oral Science

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Title
Analysis of modulation of M1/M2 polarization transition by paclitaxel in transitory and chronic inflammatory environments and its influence on bone tissue

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Anexo C – Declaração de uso exclusivo de artigo em tese.

Pós-Graduação
FOB USP

Assistência Técnica Acadêmica
Serviço de Pós Graduação

DECLARAÇÃO DE USO EXCLUSIVO DE ARTIGO EM DISSERTAÇÃO/TESE

Declaramos estarmos cientes de que o trabalho "Analysis of modulation of M1/M2 polarization transition by paclitaxel in transitory and chronic inflammatory environments and its influence on bone tissue" será apresentado na Tese do aluno André Petenuci Tabanez e que não foi e nem será utilizado em outra dissertação/tese dos Programas de Pós-Graduação da FOB-USP.

Bauru, 26 de julho de 2023.

André Petenuci Tabanez

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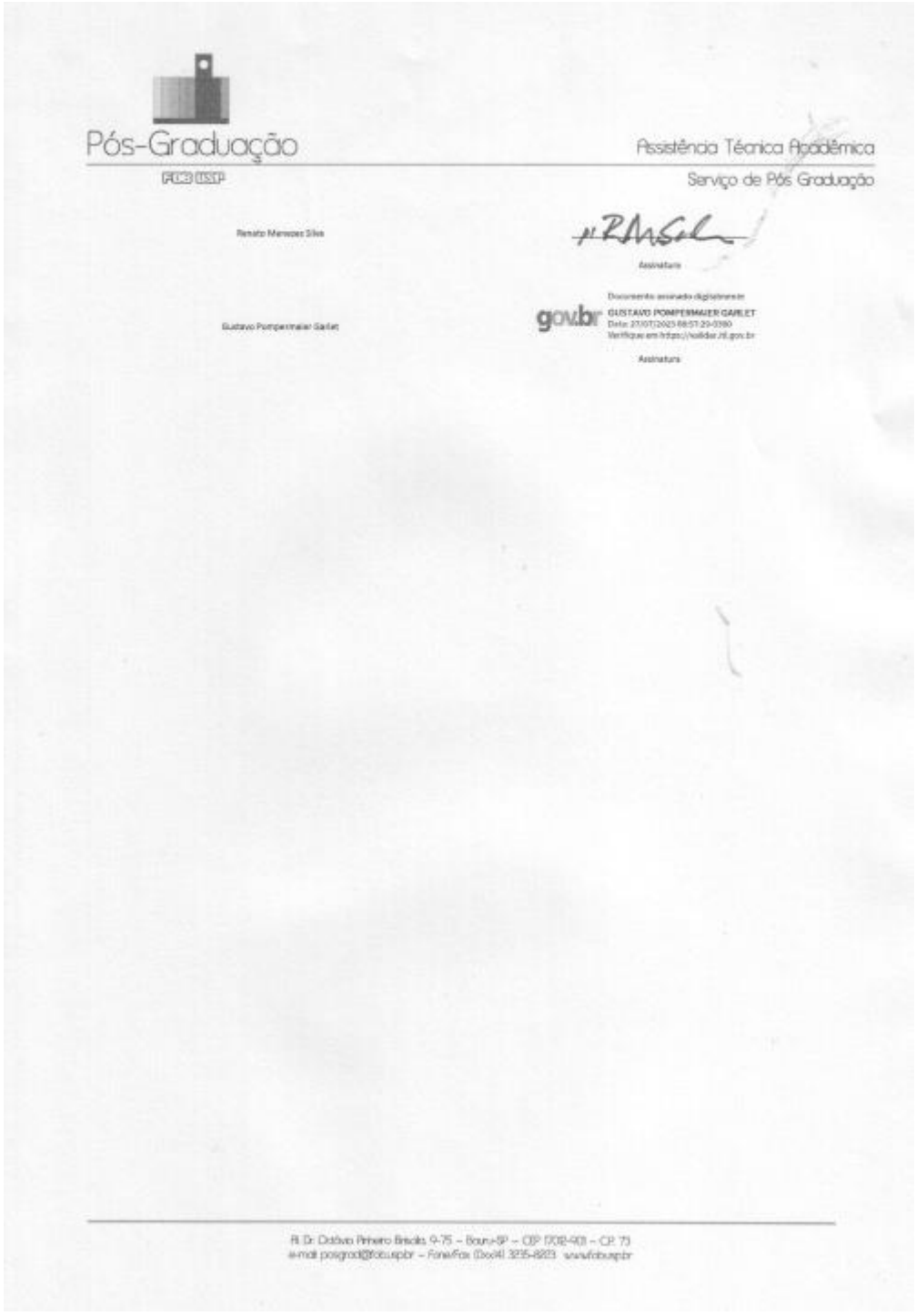

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