UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Effect of FTY720 treatment on macrophage polarization and its impact on the alveolar bone repair process

Efeito do tratamento com FTY720 na polarização de macrófagos e seu impacto no processo de reparo ósseo alveolar

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Orientador: Prof. Dr. Gustavo Pompermaier Garlet

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“O verdadeiro cientista aposta sempre em todos os cavalos, e aplaude incondicionalmente o vencedor, qualquer que seja. A isenção não é desinteresse, distanciamento frio: é paixão pela verdade desconhecida.”

Olavo de Carvalho

RESUMO

O processo de reparo ósseo alveolar pode ser influenciado por vários fatores locais e sistêmicos que incluem mediadores e células do sistema imunológico. Dentre essas células, os macrófagos são essenciais para desencadear o processo de reparo, podendo adquirir um perfil inflamatório (M1) ou anti-inflamatório e pró-reparativo (M2). Nesse contexto, avaliamos os efeitos do FTY720 na polarização de macrófagos para o perfil M2 e seus efeitos no processo de reparo ósseo alveolar. Nesse estudo foram utilizados camundongos C57BL/6 (N=5/tempo/grupo), machos, com 8 semanas de idade. Os animais foram divididos em grupo que recebeu o fármaco FTY720 via oral, na dosagem de 3mg/Kg/24h, durante todo o período experimental, e grupo controle que recebeu apenas o veículo em regime equivalente. Todos animais foram submetidos à extração do incisivo superior direito e avaliados nos períodos de 0, 1, 3, 7 e 14 dias pós extração, seguido por análises de tomografia computadorizada (μCT), histomorfométrica, birrefringência, imuno-histoquímica e molecular (PCRArray). Nossos resultados demonstraram que no período de 14 dias, o grupo FTY720 apresentou maior densidade de tecido ósseo, maior volume de tecido ósseo (B.V), maior volume de fração de tecido ósseo pelo tecido total (BV/TV), maior número e espessura de trabéculas (Tb.1 e Tb.Th, respectivamente) ($p<0.05$). Ainda no período de 14 dias, o grupo FTY720 apresentou maior número de osteoblastos e osteoclastos em relação ao grupo controle($p<0.05$). Em concordância, a expressão de vários marcadores de tecido ósseo como, BMP2, BMP7, ALPL, SOST e RANK, tiveram suas expressões de mRNA aumentadas no grupo FTY720. Esse aumento pode estar relacionado com a potencialização na formação do tecido ósseo comparado ao grupo controle. Os níveis de mRNA de FIZZ, ARG2 e IL-10, sofreram aumento no grupo FTY720 em conjunto com a presença de células $CD206^{+}$ no período de 14 dias, podendo sugerir uma participação dos macrófagos M2 na potencialização do processo de reparo ósseo alveolar. O grupo FTY720 também mostrou aumento nos níveis de expressão de mRNA de CCR2, CCR5, CXCR1, CXCL3, CCL20 e CCL25, quimicionas e receptores de quimiocinas envolvidos no recrutamento de células inflamatórias e células mesenquimais indiferenciadas (MSCs) com destaque para o aumento da expressão de CXCL12 ($p<0.05$), quimiocina responsável no recrutamento de MSCs

para o local de reparo. O aumento na expressão de CXCL12 foi acompanhado pelo aumento na expressão de CD34 no período de 14 dias ($p<0.05$) podendo indicar maior presença de MSCs no sitio de reparo. Assim, os nossos resultados demonstram que o FTY720 favoreceu o processo de reparo ósseo alveolar em camundongos C57BL/6, possivelmente por ter aumentado a expressão de marcadores relacionados com o desenvolvimento do tecido ósseo(ALPL, SOST, RANK), no reparo tecidual (TGF-β1, IL-10), no recrutamento de células indiferenciadas (CXCL12, CD34) e células inflamatórias (CCR2, CCR5) e aparentemente na indução de macrófagos para um perfil M2 (ARG2, FIZZ).

Palavras-chaves: Reparo ósseo. FTY720. macrófagos M2.

ABSTRACT

Effect of FTY720 treatment on macrophage polarization and its impact on the alveolar bone repair process

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 FTY720 on macrophage polarization towards the M2 profile and its effects on the alveolar bone repair process. In this study, we used 8 weeks old male C57BL / 6 mice (N = 5 / time / group). The animals were divided in FTY720 group receiving the drug orally at a dose of 3mg / kg / 24h 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, birefringence, immunohistochemical and molecular analyzes (PCRArray). Our results demonstrated that in the 14-day period, the FTY720 group presented higher bone tissue density, higher bone tissue volume (BV), greater tissue volume fraction (BV / TV), greater number and thickness of trabeculae (Tb.1 and Tb.Th, respectively) ($p<0.05$). In the 14-day period, the FTY720 group had a higher number of osteoblasts and osteoclasts than the control group ($p<0.05$). Accordingly, the expression of various bone markers such as BMP2, BMP7, ALPL, SOST and RANK had their mRNA expressions increased in the FTY720 group. This increase may be related to the potentiation in the formation of the bone tissue compared to the control group. The levels of FIZZ, ARG2 and IL-10 mRNA increased in the FTY720 group together with the presence of CD206 + cells in the 14 days period, suggesting a participation of M2 macrophages in the potentiation of the alveolar bone repair process. The FTY720 group also showed increased expression levels of CCR2, CCR5, CXCR1, CXCL1, CXCL3, CCL20 and CCL25 mRNA, chemokines and chemokine receptors involved in the recruitment of inflammatory cells and undifferentiated mesenchymal cells (MSCs) most notably was the up CXCL12 up regulation ($p<0.05$). CXCL12 is responsible in

the recruitment of MSCs to the repair site. The increase in CXCL12 expression was accompanied by an increase in CD34 expression over a period of 14 days ($p<0.05$), indicating a higher presence of MSCs in the repair site. Thus, our results demonstrate that FTY720 favored the process of alveolar bone repair in C57BL / 6 mice, possibly because it increased the expression of markers related to bone tissue development (ALPL, SOST, RANK), tissue repair (CXCL12, CD34) and inflammatory cells (CCR2, CCR5) and apparently in the induction of macrophages to an M2 profile (ARG2, FIZZ).

Key words: Bone repair. FTY720. M2 macrophages.

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
M-CSF	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
S1P	Sphingosine 1-Phosphate
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

The bone tissue is a connective mineralized tissue whose functions includes the protection of vital organs, locomotion and mineral reserve. Bone is constituted by a mineralized protein matrix that associated with characteristic/unique cells that characterize the bone tissue. Osteoblasts are the cells responsible for the synthesis and mineralization of bone matrix, osteocytes are the most abundant cells of the bone tissue and are responsible the matrix maintenance, and osteoclasts are large and multinucleated cells of hematopoietic origin derived from monocytes/macrophages lineage that are responsible for matrix resorption. Despite its rigidity, the bone tissue is endowed with high plasticity and is constantly being remodeled. 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 (DATTA *et al.*, 2008; BUCK and DUMANIAN, 2012)

The maintenance of bone tissue integrity depends on a precise balance between the formation osteoblasts and osteoclasts, implying a precise interrelation between these two cell types. The balance between these cells, and consequently the bone metabolism, can be influenced by several local and systemic factors, such as genetic factors, diet, hormones and mechanical stimuli (ROBLING and TURNER, 2009; ONO and TAKAYANAGI, 2017). Other important factors that are capable of altering the metabolism of bone tissue are the cells and products belonging to the immune system. Osteoimmunology is the branch of immunology that aims to investigate the interaction between the bone and immune systems (GINALDI and DE MARTINIS, 2016). Several evidences indicate that these two systems share a large number of regulatory molecules including cytokines, signaling molecules, receptors, and transcription factors. 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 osteoclast activity and bone loss, such as observed in rheumatoid arthritis, periodontal and periapical lesions (TAKAYANAGI, 2007). The activity of osteoclasts is controlled by the RANK/RANKL/OPG system, where RANKL is the activating factor of osteoclasts, a transmembrane protein expressed by osteoblasts in bone tissue that

binds to RANK, a receptor present in the membrane of osteoclasts precursor. The RANK and RANKL interplay are indispensable in promoting the differentiation and activation of osteoclasts, being such interaction modulated by osteoprotegerin (OPG), which binds to RANKL inhibiting its interaction with RANK (TAKAYANAGI, 2007; GINALDI and DE MARTINIS, 2016).

In normal conditions, the RANKL/OPG balance is controlled by osteoblasts and osteocytes, however, several factors can influence the balance such as 1,25-dihydroxyvitamin D3, prostaglandin E2 (PGE2) and parathyroid hormone (PTH), osteoclastogenic factors which may increase RANKL expression in osteoblasts. In addition to the endocrine control, some types of leukocytes and cytokines may also produce or stimulate the production of RANKL, and consequently alter the RANKL/OPG ratio and may favor bone resorption (TAKAYANAGI, 2007; GARLET, 2010). The influence of cytokines in the immune system and their interaction with bone tissue is well evident in inflammatory and infectious chronic conditions, where proinflammatory cytokines such as IL-1β, IL-6 and TNFα generally exert a stimulatory effect on osteoclasts and an inhibitory effect on osteoblasts. Conversely, 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 have protective and destructive roles in the process of destruction of bone tissue (TAKAYANAGI, 2007; GRAVES, 2008; GARLET, 2010).

However, with the characterization of different patterns of immune response, especially associated with the polarization of T helper (Th) lymphocytes into multiple phenotypes, the simple dichotomy between pro and anti-inflammatory mediators was incomplete to explain the relation between the immune and bone systems facing the variety of cells and mediators involved in the process. With the initial identification of Th1 and Th2 cells, characterized respectively by IFN-γ or IL-4 expression, Th1-type response was found to be predominant in active periapical and/or periodontal osteolytic lesions, whereas Th2 appears to prevail in lesions with suggestive characteristics of inactivity, suggesting that the Th1/Th2 balance could be determinant of the activity status or stability of the lesions. (GARLET *et al.*, 2003; HONDA *et al.*, 2006; GRAVES, 2008; GARLET, CARDOSO*, et al.*, 2010; HIENZ *et al.*, 2015). With subsequent discovery of other Th subtypes, regulatory T cells (Tregs) and Th17 cells have also shown relevant immunoregulatory roles in the pathogenesis of periodontal

and periapical lesions (GARLET, CARDOSO*, et al.*, 2010; GARLET, GIOZZA*, et al.*, 2010; GARLET *et al.*, 2012; GLOWACKI *et al.*, 2013). Similar to Th1 cells, Th17 cells are described as mediators of lesion activity, although it is not known whether Th1 and Th17 cells operate cooperatively or independently (CHENG *et al.*, 2014; MORETTI *et al.*, 2015). On the other hand, there are reports describing the protective role played by Tregs in the development of periodontal lesions (GARLET, CARDOSO*, et al.*, 2010; GARLET, GIOZZA*, et al.*, 2010) which has as one of the key events its chemoattraction by the interaction between CXCL12 and the CCR4 receptor, and which results in a drastic change in the inflammatory microenvironment that determines the conversion of the phenotype from the active to the inactive lesion.

In this context, it is important to highlight that theoretically inactive lesions are characterized by the greater expression of tissue repair markers, demonstrating that even in chronic inflammatory processes, variations in the response pattern may mediate, even partially, the tissue repair (GARLET *et al.*, 2012), which suggests that certain inflammatory processes may presented a 'constructive' action towards bone repair. However, there are still few studies that focus on understanding the regulatory mechanisms involved in the repair and regeneration process, especially regarding bone repair. Studies have shown that the inflammatory immune response, presenting a controlled and self-limited profile, may play important in the repair process contributing possibly as a mediator of the chemoattraction, activation and differentiation of several cell types potentially involved in the repair (MOUNTZIARIS and MIKOS, 2008; THOMAS and PULEO, 2011; MILLS and SIMPSON, 2012).

Histological studies demonstrate that bone defects repair involves the blood clot formation, followed by the influx of inflammatory cells concomitant to the formation of a granulation tissue, later replaced by newformed bone tissue (THOMAS and PULEO, 2011; MILLS and SIMPSON, 2012). While the exact mechanisms involved in such process are still unclear, studies demonstrating that nonspecific blockade of the inflammatory process slows the repair process, including bone repair, support the concept of constructive inflammation (PARK and BARBUL, 2004; Al-AQL *et al.*, 2008). In order to elucidate possible mechanisms involving elements of the immune inflammatory response in the bone repair process, our group developed a model of alveolar bone repair post-exodontia in mice (VIEIRA *et al.*, 2015). In this model, it was observed that the classically described tissue events are indeed 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 *et al.*, 2015). A crucial point to note is the multiplicity of cell types and mediators potentially involved in the interaction between the immune and bone systems in both pathological conditions and tissue repair. In this context, studies conducted with an exclusive focus on individual markers, without a comprehensive simultaneous assessment of several factors, are not necessarily representative of the overall picture of local immunoregulation.

Among the multiple cell types involved in the repair process, macrophages have been considered as potential key elements in the bridge between inflammation and successful repair outcome. Macrophages are the primary 'danger signs' sensors both in infectious conditions and in aseptic inflammation (MATZINGER, 2002; MOSSER and EDWARDS, 2008; MATZINGER, 2012; NICH and GOODMAN, 2014). In the repair process, macrophages are also thought to be involved in the change from the inflammatory phase to the proliferative phase through the secretion of various mediators such as growth factors and cytokines that act by recruiting various cell types such as fibroblasts that contribute to the construction, organization, and vascularization of new tissue matrix (MOSSER and EDWARDS, 2008; DAS *et al.*, 2015). Although classically considered as proinflammatory cells, macrophages may present distinct functional phenotypes called M1 and M2. In general, the 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 and LEIBOVICH, 2012; HUME, 2015; MOTWANI and GILROY, 2015). On the other hand, macrophages may 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, (HUME, 2015; BASHIR *et al.*, 2016). M2 macrophages are theoretically responsible for macrophage activity in the late stages of repair and are characterized by the high expression of CD163, CD206, MDC, MRC1 receptors and factors such as CCL22, CCL18, IL10, TGFβ1, PDGF, TIMPs, as well as the arginase-1 (Arg -1) and Fizz-1

(HUME, 2015; MOTWANI and GILROY, 2015; BASHIR *et al.*, 2016). Importantly, there are still not well-characterized subtypes of macrophages, and M1 and M2 represents only the polarization state extremes. 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 (WANG *et al.*, 2014; DAS *et al.*, 2015).

Regarding the M1 / M2 polarization in osteolytic lesions, studies with arthritis models demonstrate an association of M1 with tissue destruction, and an inverse role for M2 (SIMA and GLOGAUER, 2013; YE *et al.*, 2014). With regard to periodontal lesions, M1 cells are more numerous than M2, and depletion of M1 macrophages results in attenuation of disease progression (LAM *et al.*, 2014). Regarding tissue repair, studies demonstrate that the repair process involves an initial M1 polarization that rapidly evolves to M2 (SINDRILARU and 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 interfere directly or indirectly with differentiation and the presence or absence of macrophages, as well as variations in their M1/M2 phenotype, could potentially interfere with bone repair (GRAVES *et al.*, 2011; SOUZA and LERNER, 2013; BAUM and GRAVALLESE, 2014).

In addition to the classical signaling molecules that may modulate macrophage polarization, contributing to the persistence or resolution of the inflammatory response, small regulatory molecules have been investigated for their ability to regulate various inflammatory processes. In this context, FTY720 has proven to be an effective tool for the treatment of chronic, autoimmune and degenerative diseases (BRINKMANN *et al.*, 2002; BRINKMANN and LYNCH, 2002; ANASTASIADOU and KNOLL, 2016; GROH *et al.*, 2017). FTY720 (2-amino-2- [2- (4-octylphenyl) ethypropane-1,3-diol) is derived from ISP-1 (miriocin), which is a fungal metabolite widely used in Chinese herbal medicine (BRINKMANN *et al.*, 2002; BRINKMANN and LYNCH, 2002). The FTY720 molecule is an analogue and has structural similarity to sphingosine, a sphingolipid mediator that can trigger cellular responses such as apoptosis survival and increased cell migration (BRINKMANN *et al.*, 2002; BRINKMANN and LYNCH, 2002; MORRIS *et al.*, 2005; NOFER *et al.*, 2007; METZLER *et al.*, 2008). Responses to sphingosine and FTY720 are mediated through five G protein-coupled surface receptors (GPCRs)

named $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$ and $S1P_5$. The diverse effects of these mediators are attributed to the activation of one or more of these five receptors (BRINKMANN *et al.*, 2002; BRINKMANN and LYNCH, 2002; MORRIS *et al.*, 2005; SCHAIER *et al.*, 2009; HUANG *et al.*, 2012; DAS *et al.*, 2013; ANASTASIADOU and KNOLL, 2016). In order to become biologically active molecules, FTY720 and sphingosine must undergo phosphorylation in vivo through sphingosine kinases (Sphk), being transformed into FTY720-1-phosphate and sphingosine-1-phosphate (S1P) (BRINKMANN *et al.*, 2002; BRINKMANN and LYNCH, 2002; ANADA *et al.*, 2007; NOFER *et al.*, 2007; DAS *et al.*, 2013; ANASTASIADOU and KNOLL, 2016; ZHAO *et al.*, 2017).

FTY720 has remarkable modulatory action on immunological processes and, unlike immunosuppressive drugs currently in use, FTY720 does not inhibit the activation and proliferation of T cells, not compromising the host defenses (BRINKMANN *et al.*, 2002; BRINKMANN and LYNCH, 2002; MORRIS *et al.*, 2005; NOFER *et al.*, 2007; SCHAIER *et al.*, 2009). FTY720 basically results in the retention of lymphocytes in secondary lymphoid organs, decreasing the amount of circulating effector T cells (BRINKMANN *et al.*, 2002; BRINKMANN and LYNCH, 2002; MORRIS *et al.*, 2005; HUGHES *et al.*, 2008). Several experimental models have demonstrated the FTY720 immunosuppressive roles. In nephrectomized rats, the treatment with FTY720 was effective in decreasing not only glomerular and tubulointerstitial lymphocytes, but also in reducing the number of M1 and M2 macrophages and key mediators of inflammation and renal fibrosis. (SCHAIER *et al.*, 2009). When administered to low-density lipoprotein (LDL) receptor-deficient rats undergoing a high cholesterol diet, FTY720 interfered with lymphocyte activity, decreased expression of M1 macrophage markers, and increased expression of M2 macrophage markers, reducing the formation of atherosclerotic lesions (NOFER *et al.*, 2007).

In addition to its immunoregulatory action, FTY720 also proves to be an excellent alternative in tissue engineering, with very positive results when applied in bone critical defect models. Its use has been advocated because it is an option that can overcome the problems found in the use of other therapeutic alternatives, such as bone morphogenetic proteins (BMPs) that present high cost, short shelf life and skepticism regarding the delivery dose (DAS *et al.*, 2013). Local delivery of FTY720 in a critical mandibular bone defect in rats increases blood vessel growth and recruitment of M2 macrophages, leading to increased bone tissue growth in the defect area (DAS

et al., 2013). Similar results were found when FTY720 is administered in a critical cranial bone defect model in rats, leading to increased bone tissue formation and increased microvasculature density in the bone defect area (PETRIE ARONIN *et al.*, 2010). In addition to these findings, in vitro studies demonstrate that FTY720 also exerts a chemotactic effect recruiting undifferentiated cells, and increasing the expression of osteogenesis-related transcription factors, enhancing the induction of osteoblast differentiation and contributing to the increase bone matrix formation (SATO *et al.*, 2012; DAS *et al.*, 2013).

Considering the importance of the M1/M2 polarization for the immunoregulation and tissue repair processes, and the presence of both M1 and M2 cells in the alveolar bone repair process, it is possible to assume that macrophage polarization may play an active involvement in this process. In this context, this study was designed to test the effects of FTY720 to induce M2 macrophage polarization and to evaluate its participation in the alveolar bone repair process.

2 ARTICLE

2 ARTICLE

Bone

Effect of FTY720 treatment on macrophage polarization and its impact on the alveolar bone repair process

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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 FTY720 on macrophage polarization towards the M2 profile and its effects on the alveolar bone repair process. In this study, we used 8 weeks old male C57BL / 6 mice (N = 5 / time / group). The animals were divided in FTY720 group receiving the drug orally at a dose of 3mg / kg / 24h 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, birefringence, immunohistochemical and molecular analyzes (PCRArray). Our results demonstrated that in the 14-day period, the FTY720 group presented higher bone tissue density, higher bone tissue volume (BV), greater tissue volume fraction (BV / TV), greater number and thickness of trabeculae (Tb.1 and Tb.Th, respectively) ($p < 0.05$). In the 14-day period, the FTY720 group had a higher number of osteoblasts and osteoclasts than the control group ($p < 0.05$). Accordingly, the expression of various bone markers such as BMP2, BMP7, ALPL, SOST and RANK had their mRNA expressions increased in the FTY720 group. This increase may be related to the potentiation in the formation of the bone tissue compared to the control group. The levels of FIZZ, ARG2 and IL-10 mRNA increased in the FTY720 group together with the presence of CD206 + cells in the 14 days period, suggesting a participation of M2 macrophages in the potentiation of the alveolar bone repair process. The FTY720 group also showed increased expression levels of CCR2, CCR5, CXCR1, CXCL1, CXCL3, CCL20 and CCL25 mRNA, chemokines and chemokine receptors involved in the recruitment of inflammatory cells and undifferentiated mesenchymal cells (MSCs) most notably was the up CXCL12 up regulation ($p < 0.05$). CXCL12 is responsible in the recruitment of MSCs to the repair site. The increase in CXCL12 expression was accompanied by an increase in CD34 expression over a period of 14 days ($p < 0.05$), indicating a higher presence of MSCs in the repair site. Thus, our results demonstrate that FTY720 favored the process of alveolar bone repair in C57BL / 6 mice, possibly because it increased the expression of markers related to bone tissue development (ALPL, SOST, RANK), tissue repair (CXCL12, CD34) and inflammatory cells (CCR2, CCR5) and apparently in the induction of macrophages to an M2 profile (ARG2, FIZZ).

Key words: Bone repair, FTY720, M2 macrophages

3 DISCUSSION

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Alveolar bone repair is a complex process comprising several successive and coordinated stages that are initiated shortly after tooth extraction. There is some evidence in the literature that a moderate and transient inflammatory response is necessary to promote the bone repair process after injury, as inflammatory mediators released at the initial moments and for a limited time after the trauma initiate the repair cascade, recruiting mesenchymal cells and other inflammatory cells leading to increased extracellular matrix synthesis and stimulating angiogenesis. In this context, the macrophages are regarded as one of the main cells acting in the tissue repair by coordinating the inflammatory and angiogenesis processes. (AI-AQL *et al.*, 2008; BOYCE and XING, 2008; VIEIRA *et al.*, 2015; EDDERKAOUI, 2017). These cells can respond to different environmental stimuli and can acquire a classic pro-inflammatory activation profile (M1), or an alternative activation profile (M2) with an anti-inflammatory and pro-reparative character. M2 macrophages are associated with assistance in resolving inflammation and promoting tissue formation and remodeling (FERRANTE and LEIBOVICH, 2012; DAS *et al.*, 2015; HUME, 2015; BASHIR *et al.*, 2016). Considering the previous evidences of the potential modulation of M1/M2 phenotype by FTY720, this study aims to evaluate the impact of the use of FTY720 on the polarization of macrophages to the M2 profile and the process of alveolar bone repair in C57BL/6 mice.

After the upper central incisor extraction, there is the incitement of an inflammatory response triggering a cascade of temporally controlled signs and events. The first post-extraction event is the filling of the alveolus by blood clot, which has mediators responsible for the recruitment of inflammatory cells and undifferentiated cells (MOUNTZIARIS and MIKOS, 2008; VIEIRA *et al.*, 2015). Our results show a change in the initial period of 0h in the blood clot organization in the FTY720 group. The clot was concentrated in separate portions around the alveolar walls, thus leaving "blanks" between these clot portions. In the control group, the clot was distributed in a widespread manner occupying the entire extension of the alveolus. Thus, histological and histomorphometry analysis of the FTY720 group showed a smaller amount of clot when compared to the control group as well a greater amount of blank spaces in the

alveolar area, registered in the histomorphometry analysis in the "others" parameter counting. Studies present Sphingosine 1-phospate, the natural FTY720 analogue, as an abundant mediator in the plasma being phosphorylated and released by platelets in the clotting process, contributing to platelet aggregation and regulating various cellular processes such as proliferation, modulation of cell migration and cytoskeleton rearrangement, comprising a potent chemoattractant for endothelial cells, and consequently contributing to angiogenesis (ENGLISH *et al.*, 2000; ANADA *et al.*, 2007; MAHAJAN-THAKUR *et al.*, 2015). FTY720, when administered, is also phosphorylated and released by platelets, but even though it is an analog of S1P, FTY720 has been shown to be effective in inhibiting platelet aggregation and other factors that would trigger the clotting process (ZHAO *et al.*, 2017). Although it has caused a change in the clotting arrangement, FTY720 does not appear to have caused any inhibiting effect on the coagulation process since the repair process has proceeded normally. Additional investigation is needed to determine the exact alteration derived from FTY720 administration in the blood clot formed after dental extraction in the animals studied.

Considering the presence of inflammatory cells during the alveolar bone repair process, when the histomorphometry analysis is evaluated, the FTY720 group presents a lower number of inflammatory cells compared to the control group in the 0h period. In the other periods evaluated, there was no significant statistical difference between the groups regarding the number of inflammatory cells during the process. There was also no statistically significant difference between the groups in all periods assessed through immunohistochemical analysis. In the 1d period it is possible to note a peak in the number of inflammatory cells that can be correlated mainly with the high number of GR1 Ly6g + and CD80 + cells, indicating a large number of granulocytes and M1 macrophages, respectively, in the evaluated period. Interestingly, the FTY720 group, despite the absence of a statistically significant difference, had slightly higher numbers of F480 + (M0), CD80 + (M1) and CD206 + (M2) cells in the 7d period.

Our results also demonstrated that the mRNA levels of many inflammatory mediators, such as IL-6 and tumor necrosis factor alpha (TNF-α), were significantly elevated in the first days of the repair process. IL-6 mRNA expression levels were elevated in the FTY720 group compared to the control in the 7 days period. IL-6, is

produced by several cell types, including macrophages (M1), fibroblasts, osteoblasts and stromal cells (BLANCHARD *et al.*, 2009; FERRANTE and LEIBOVICH, 2012) and is a critical inflammatory mediator to trigger osteogenesis since it can regulate both the osteoblasts and osteoclasts activities promoting both deposition and reabsorption of bone tissue (MOUNTZIARIS and MIKOS, 2008; BLANCHARD *et al.*, 2009; THOMAS and PULEO, 2011). The elevation of IL-6 expression levels in the FTY720 group occurred in the 7-day period, coinciding with a high number of macrophages ($CD80^+$ cells, $F4/80^+$ cells) in the same period, being one of the cell types responsible for the secretion of this mediator. Analyzing our results, we can suggest that IL-6 increased levels may derive from increased macrophage presence and activity in the repair site, and possibly acted constructively in the alveolar bone repair process in the FTY720 group, considering that the number of osteoblasts and markers of bone tissue development such as ALPL, SOST and RANK were up regulated.

Another cytokine that had increased mRNA expression in the FTY720 group was IL-10, which has potent effects on the suppression of inflammatory responses and is secreted by several cell types including M2 macrophages (THOMAS and PULEO, 2011; ZHANG *et al.*, 2014). IL-10 can down-regulate the synthesis of pro-inflammatory cytokines, such as IL-6, and promote osteoblast differentiation (ZHANG *et al.*, 2014). The increase in expression of IL-10 mRNA levels occurred over the 14-day period coinciding with the down-regulation of IL-6. It is important to note that in the same period we can observe the increase in ARG2 and FIZZ mRNA expressions, both markers expressed by M2 macrophages (THOMAS and PULEO, 2011; MARTINEZ and GORDON, 2014; WANG *et al.*, 2014; DAS *et al.*, 2015). The CD206 + cell count in the 14-day period was higher than the other markers, indicating a strong correlation with the participation of M2 macrophages in the production of IL-10 resulting in the bone repair improvement in the FTY720 group in relation to the control group.

The recruitment of inflammatory cells or undifferentiated cells to the repair site is also mediated through chemokines. Chemokines can promote inflammation, angiogenesis, migration of steam cells and plays important roles in bone metabolism (XING *et al.*, 2010; EDDERKAOUI, 2017). The chemokine CXCL3, which has inflammatory cell recruitment effects controlling monocytes adhesion and migration, had its mRNA expression increased in the FTY720 group over the 3 and 7 days

periods. The mRNA expression for CCR2 (chemokine receptor 2) and CCR5 (chemokine receptor 5) were higher in the FTY720 group compared to the control in the 14 days and 7 and 14 days periods, respectively. CCR2 and CCR5 are involved in the recruitment of macrophages to the repair site (BIGUETTI *et al.*, 2018). The increase in the expression of these two chemokine receptors may be correlated with the presence of macrophages (F4/80 + CD80 + and CD206 +) at the repair site in the 7 and 14 days periods.

The CCL20 chemokine, which is related to the recruitment of $CCR6^{+}$ mononuclear cells, had increased mRNA expression in the FTY720 group within 7 days. The IL-8 receptor, or CXCR1, involved in neutrophil migration to the repair site, also had increased mRNA expression in the FTY720 group over the 3 days period (EDDERKAOUI, 2017). Stroma cell-derived factor 1, also known as CXCL12, is an important chemokine in the bone repair process because of its ability to recruit osteoblastic progenitors by promoting new bone formation (MYERS *et al.*, 2015; EDDERKAOUI, 2017). Its expression was regulated upwards in the periods of 3 and 7 days in the FTY720 group in comparison to the control group. The literature shows that FTY720 is able to recruit CD34 + cells through the CXCL12 / CXCR4 axis (KIMURA *et al.*, 2004). Our results evidenced an increase in the expression of CD34 mRNA in the FTY720 group in relation to the control in the 14 days period. Thus, increase in these chemokines and their receptors shows that FTY720 may exert a potentiation in recruitment of hematopoietic stem cells progenitors (HPCs) and osteoblasts precursors (A DAS 2015, KIMURA 2004).

The FTY720 group showed a decrease in TNF-α mRNA expression within 7 days compared to the control group. Tumor necrosis factor α (TNF-α) is an important mediator in the recruitment of inflammatory cells to the site of injury and in the bone repair process (KARNES *et al.*, 2015). The decrease of TNF-α and other proinflammatory mediators due to the use of FTY720 agrees with other findings in the literature (NOFER *et al.*, 2007; HUGHES *et al.*, 2008; YU *et al.*, 2015). The decrease in TNF-α levels did not appear to have a negative effect on the repair process.

Analyzing the connective tissue features along the bone repair process, the FTY720 group presented some changes in relation to the control group. The density of collagen fibers was lower in the FTY720 group compared to the control group in the

3 days period. The total analysis of the area of collagen fibers also shows a smaller amount of fibers in the FTY720 group. Still in the FTY720 group related to the control, the number of fibroblasts was higher in the period of 7 days and lower in the period of 14 days. FTY720 may alter fibroblast functions in some study models (SHI et al 2017 however, its current action on these cells in the bone repair process still requires further investigation. The density of blood vessels was lower in the FTY720 group in the 7 and 14 days periods. This difference is probably due to the fact that the granulation tissue and later the bone tissue occupied most of the alveolus, consequently reducing the incidence of other structures. FTY720 shows angiogenic effects in other studies in the literature (PETRIE ARONIN *et al.*, 2010; DAS *et al.*, 2013). To further clarify these effects other analyzes evidencing the vasculature development should be conducted. Finally, the FTY720 group showed an increase in MMP1 mRNA expression within 14 days in relation to the control group. MMP1 is a matrix metalloproteinase responsible for the degradation and remodeling of the extracellular matrix (ECM) and is extremely important for the osteogenesis process (HENLE *et al.*, 2005). This finding may be related to the lower amount of yellow birefringent fibers in the FTY720 group compared to the control group in the 14 days period. These evidences suggest a greater collagen matrix remodeling favoring bone deposition.

Gradually, the granulation tissue undergoes remodelation, giving space for the new bone matrix secretion. The impact of FTY720 treatment on the alveolar bone repair process was positive. The FTY720 group presented an augmented bone tissue formation at the end of the process in the evaluated period of 14 days. The histomorphometry and μCT analysis showed more bone tissue formation with higher trabeculae number and thickness. This result agrees with other studies where the FTY720 also potentiated the repair of critical bone defects. There was a greater formation of bone tissue and greater filling of the alveolus in the group treated with FTY720 in relation to the control group. This result agrees with other studies where the FTY720 also potentiated the repair of critical bone defects (PETRIE ARONIN *et al.*, 2010; HUANG *et al.*, 2012; DAS *et al.*, 2013). According to the results cited above, the molecular analysis of PCR shows high expressions of several markers involved in the osteogenesis process during alveolar bone repair in the animals of the FTY720 group.

The bone morphogenetic proteins (BMPs) and Transforming growth factor-beta 1 (TGF-β1) are central factors in the differentiation of osteoblasts during physiological or repair osteogenesis (BANDYOPADHYAY *et al.*, 2006; VIEIRA *et al.*, 2015). TGF-β1, in addition to having direct effects on osteoblasts, may intensify the effect of other bone growth factors such as BMPS (ZIMMERMANN *et al.*, 2005). The levels of mRNA expression of TGF-β1 in the FTY720 group compared to the control were down-regulated over the 7 days period and up-regulated over the 14 days period. About the BMPs, FTY720 animals showed increased mRNA expression for BMP2 and BMP7. BMP7 had its expression increased in the FTY720 group compared to the control group in the 7 days periods. BMP2 had an increase in its expression in the 3 days period in both groups, decreasing in the control group after this period. In the FTY720 group their expression continued to increase until reaching a peak in the 14 days period. The BMPs mentioned above have a very potent osteogenic capacity and BMP2 is more related to the mineralization potentiation in the final stages of the repair process (HUGHES-FULFORD e LI, 2011; Chen *et al.*, 2012) and BMP7 also responsible for inducing the expression of osteoblastic differentiation markers such as alkaline phosphatase gene activity (ALPL), also up-regulated in the FTY720 group compared to the control group. Runx2, the main transcription factor involved in osteoblast differentiation (VIEIRA *et al.*, 2015), despite the absence of a statistically significant difference, had a slight increase in its expression in the FTY720 group. Accompanying this finding, we can also observe the highest number of osteoblasts in the 14-day period in the FTY720 group compared to the control group. This increase in the number of osteoblasts is also related to the increase in ALPL expression, and may contribute to the greater formation of mineralized tissue in the FTY720 group in relation to the control group.

Sclerostin is a small protein encoded by the SOST gene that is present in osteocytes, indicating advanced osteoblastic differentiation (LEWIECKI, 2014; VIEIRA *et al.*, 2015). This protein is associated with bone remodeling regulation, with inhibitory effects on matrix deposition by osteoblasts (LEWIECKI, 2014). The levels of SOST mRNA expression were up-regulated in the FTY720 group relative to the control group over the 14 days period. It is interesting to note in the FTY720 group, that in the same period, RANK levels were also elevated in relation to the control group. RANK, receptor activator of NF-κB, is present in osteoclasts and their precursors and their binding to

RANKL triggers the reabsorption process (AI-AQL *et al.*, 2008; BOYCE E XING, 2008; MENEZES *et al.*, 2008). The elevation of RANK mRNA is suggestive of higher numbers in the osteoclast count. In agreement with this finding, we have the increase in the number of osteoclasts in the FTY720 group in relation to the control group in the periods of 7 and 14 days. Thus, increases in SOST and RANK levels may be compatible with a higher rate of/ remodeling of bone tissue. In summary, our results demonstrate that FTY720 favored the process of alveolar bone repair in C57BL / 6 mice, possibly because it increased the expression of markers related to bone tissue development (ALPL, SOST, RANK), tissue repair (CXCL12, CD34) and inflammatory cells (CCR2, CCR5) and apparently in the induction of macrophages to an M2 profile (ARG2, FIZZ).

4 CONCLUSION

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In summary, our data suggest that FTY720 caused a potentiation in the alveolar bone repair process in C57BL / 6 mice compared to the control group with the possible involvement of M2 macrophages in the process

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ANNEX

ANNEX

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 016/2016

Bauru, 6 de setembro de 2016.

Senhor Professor,

Informamos que a proposta intitulada ***Papel da polarização de macrófagos para um perfil M2 no processo de reparo ósseo alveolar****, registrada sob* ***CEEPA-Proc. Nº 016/2016,*** tendo Vossa Senhoria como Pesquisador Responsável, que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi analisada e considerada APROVADA a sua execução nas dependências da FOB-USP, *ad referendum* da Comissão de Ética no Ensino e Pesquisa em Animais (CEEPA).

Finalidade	*( ) Ensino (X) Pesquisa Científica*
Vigência da autorização:	*Julho/2016 a Maio/2018*
Espécie/linhagem/raça:	*Camundongo isogênico/ C57Bl/6*
Nº de animais:	*n=90*
Peso/Idade	*25g/8 semanas*
Sexo:	*Machos*
Origem:	*Biotério da Faculdade de Medicina de Ribeirão Preto (FM/USP)*

Esta CEEPA solicita que ao final da pesquisa seja enviado um 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 Ensino e Pesquisa em Animais

Prof. Dr. Gustavo Pompermaier Garlet
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