UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Role of the gene *Slc11a1* and selected genotypes for minimum and maximum inflammatory reactivity in the process of alveolar bone healing in mice

Papel do gene *Slc11a1* e de genótipos selecionados para mínima e máxima reatividade inflamatória no processo de reparo ósseo alveolar em camundongos

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Orientador: Prof. Dr. Gustavo Pompermaier Garlet

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FOLHA DE APROVAÇÃO

A ciência é o nutriente da inteligência, enquanto a fé é o alimento da alma. A ciência exige pesquisa, a fé exige contemplação e estudo. Onde termina o limite estreito de alcance da ciência, começa o horizonte infinito da fé. Ambas se completam.

Prof. Felipe Aquino

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ABSTRACT

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Role of the gene *Slc11a1* and selected genotypes for minimum and maximum inflammatory reactivity in the process of alveolar bone healing in mice

The process of alveolar bone healing can be influenced by several local and systemic factors, which include the immune system and healing related genes. However, the exact role of host inflammatory responsiveness and genetic background in bone healing process remains unclear. In this context, we evaluated the influence inflammation in alveolar bone healing taking advantage of mice strains genetically selected for maximum (AIRmax) or minimum (AIRmin) acute inflammatory response, as well AIR strains homozygous for RR/SS *Slc11a1* genotypes. Experimental groups (N=5/time/group) comprised 8-week-old male or female AIRmax and AIRmin; and substrains AIRminRR, AIRminSS and AIRmaxRR and AIRmaxSS; submitted to extraction of upper right incisor and evaluated at 0, 3, 7, 14 and 21 days after upper incision extraction by micro-computed tomography (μCT), histomorphometry, birefringence, immunohistochemistry and molecular (PCRArray) analysis. Initially, our results demonstrated that AIRmin mice presented an early increase ($p<0.05$) in bone volume, hyperdense regions, density of bone matrix and osteoblasts, increased ($p<0.05$) expressed of BMP4, BMP7 and RUNX2 when compared to AIRmax strain. AIRmin mice also presented lower counts of $GR1^+$ and $CD80^+$ cells, and higher counts of $F4/80^+$ and $CD206^+$ cells, in parallel with higher mRNA expression of CX3CL1, CCL5, CCR5 and ARG when compared to AIRmax animals. In late repair stages, the AIRmin strain presented a decreased ($p<0.05$) density of osteoclast and blood vessels than AIRmax, along lower RANKL and Catepk and higher PHEX and SOST mRNA expression, but the healing outcome at the endpoint was similar in AIRmin and AIRmax strains. When analyzed the effect of RR/SS *Slc11a1* genotypes was evaluated in parallel with the influence AIRmin/AIRmax background, we initially observed that the AIRmax strain, associated with both RR and SS *Slc11a1* genotypes, presented a more effective bone healing, characterized by increased ($p<0.05$) of bone volume and predominance of red fiber in analysis in contrast to AIRmin strains. AIRmaxRR presented increased ($p<0.05$) $F4/80^+$ and decreased $CD80^+$ e $CD206^+$ cells count,

while AIRmaxSS presented increased ($p<0.05$) $GR1^+$, $F4/80^+$ and $CD80^+$ and decreased $CD206^+$ cells. When the analysis was performed in order to address the influence *Slc11a1* variants, AIRmaxSS strain presented a bone healing delay when compared to AIRmaxRR; characterized by decreased ($p<0.05$) of bone volume, trabecular number and red collagen fibers, increased ($p<0.05$) $GR1^+$ and $CD80^+$ and decreased $F4/80^+$ and $CD206^+$. Conversely, AIRminSS presented a more effective healing when compared with AIRminRR mice; characterized by increased ($p<0.05$) of bone volume, trabecular number/separation and red birefringence, increased $GR1^+$ and decreased $CD206^+$ cells count. In conclusion, while AIRmin and AIRmax strains presents a similar healing outcome at the endpoint, the early repair in AIRmin strain was associated with decreased presence of neutrophils and M1 macrophages, and increased M2 macrophages. Additionally, our while results showed that AIRmax inflammatory background was associated to a more effective bone healing process irrespective of the presence of RR/SS *Slc11a1* genotypes, RR genotype favors the healing in AIRmax background and SS genotype was found to favor the healing in the AIRmin background.

Keywords: Bone repair. Inflammation. *Slc11a1*

RESUMO

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Papel do gene *Slc11a1* e de genótipos selecionados para mínima e máxima reatividade inflamatória no processo de reparo ósseo alveolar em camundongos

O processo de reparo ósseo alveolar pode ser influenciado por vários fatores locais e sistêmicos, que incluem o sistema imunológico e os genes relacionados ao reparo. No entanto, o exato papel da resposta inflamatória do hospedeiro e genético background no processo de reparo ósseo ainda não está claro. Neste contexto, avaliamos a influência da inflamação no reparo óssea alveolar, em camundongos selecionadas geneticamente para uma resposta inflamatória aguda máxima (AIRmax) ou mínima (AIRmin), como também em camundongos AIR homozigoto para os alelos RR/SS do gene *Slc11a1*.Neste estudo foram utilizados camundongos machos e fêmeas (N=5/tempo/grupo), das linhagens selecionados para máxima e mínima (AIRmax e AIRmin) reação inflamatória, e também as sublinhagens AIRminRR, AIRminSS, AIRmaxRR e AIRmaxSS com idade aproximada de 8 semanas. Todos foram submetidos à extração do incisivo superior direito e avaliados nos períodos de 0, 3, 7, 14 e 21 dias pós extração, seguido pela análise tomografia computadorizada (µCT), análise histomorfometria, análise de birrefringência, análise imuno-histoquímica e análise molecular (PCRArray). Inicialmente, nossos resultados demonstraram que a linhagem AIRmin, no período inicial, apresentou um aumento ($p<0.05$) no volume ósseo, nas regiões hiperdensas, na densidade de matriz óssea e osteoblastos, seguido pelo aumento ($p<0.05$) na expressão de BMP4, BMP7 e RUNX2 quando comparado a linhagem AIRmax. Camundongos AIRmin também apresentou uma menor contagem de células $GR1^+$ e $CD80^+$ e aumento da contagem de células $F4/80^+$ e $CD206^+$, em paralelo com aumento da expressão de mRNA de CX3CL1, CCL5, CCR5 e ARG quando comparado aos camundongos AIRmax. Nos períodos tardios, a linhagem AIRmin apresentou uma diminuição ($p<0.05$) na densidade de osteoclastos e vasos sanguíneos em comparação AIRmax, seguido por uma diminuição na expressão de mRNA de RANKL e Catepk e aumento de PHEX e SOST, mas o processo de reparo ósseo alveolar, no período final foi semelhante entres as linhagens AIRmin e AIRmax. Quando analisamos o efeito dos alelos RR/SS do gene *Slc11a1*

em paralelo com a influência do background AIRmin/AIRmax, nós inicialmente observamos que a linhagem AIRmax associada com ambos os alelos RR/SS do gene *Slc11a1* apresentaram um processo de reparo mais efetivo, caracterizado pelo aumento ($p<0.05$) volume ósseo e predominância de fibras vermelhas em comparação com a linhagem AIRmin. Camundongos AIRmaxRR apresentaram aumento ($p<0.05$) na contagem de células $F4/80^+$ e diminuição na contagem de células $CD80^+$ e $CD206^+$, enquanto, camundongos AIRmaxSS apresentou um aumento ($p<0.05$) na contagem de células $GR1^+$, $F4/80^+$, $CD80^+$ e diminuição na contagem de células $CD206^+$. Quando analisamos a influência dos alelos do gene *Slc11a1*, a linhagem AIRmaxSS apresentaram um atraso no reparo óssea quando comparado ao AIRmaxRR; caracterizado pela diminuição ($p<0.05$) do volume ósseo, número trabecular e fibras colágenas vermelhas, seguido pelo aumento ($p<0.05$) da contagem de células $GR1^+$ e $CD80^+$ e diminuição de células $F4/80^+$ e $CD206^+$. Por outro lado, camundongos AIRminSS apresentaram um reparo ósseo mais efetivo quando comparada com AIRminRR; caracterizada pelo aumento ($p<0.05$) do volume ósseo, número / separação trabecular e birrefringência das fibras colágenas no espectro vermelho, seguido pelo aumento da contagem de células $GR1^+$ e diminuição das células $CD206^+$. Diante disso, os nossos resultados demonstraram que as linhagens AIRmin e AIRmax apresentaram um processo de reparo ósseo alveolar semelhantes no período final do reparo, enquanto no reparo inicial a linhagem AIRmin estava associada com a diminuição de neutrófilos e macrófagos M1 e aumento dos macrófagos M2. Além disso, nossos resultados demonstraram que background AIRmax estava associado a um processo de reparo mais efetivo, independentemente da presença de genótipos RR/SS *Slc11a1*, o genótipo RR favorece o reparo no background AIRmax e o genótipo SS favoreceu a reparo no background AIRmin.

Palavras-chaves: Reparo ósseo. Inflamação. *Slc11a1*

LIST OF ABREVIATIONS

AIR	Acute inflammatory response
BMP	Bone morphogenic protein
CCL	C-C motif chemokine
CCR	C-C chemokine receptor type
COL1A2	Collagen type I alpha 2 chain
COL2A1	Collagen type II alpha 1 chain
CXCL	C-X-C motif chemokine
CX3CR	CX3C chemokine receptor
CD80	M1 macrophages
CD206	M2 macrophages
F4/80	Macrophages
GR1	Granulocytes
IL	Interleukin
KO	Knockouts mice
M1	Macrophages exhibit high levels of pro-inflammatory cytokines
M2	Macrophages exhibit high levels of anti-inflammatory cytokines
M-CSF	Macrophage colony-stimulating factor
MCP-1	Monocyte Chemoattractant Protein-1
MIP–1 α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
NFATc1	Nuclear factor of activated T-cells
Nramp 1	Natural resistence-associated macrophage protein 1
NF-kB	Factor nuclear kappa β
OPG	Osteoprotegerin
OSX	Osteoblast-specific transcription factor Osterix
PDGF	Platelet-derived growth fator
QTLs	Quantitative Trait Loci
RANK	Receptor activator of nuclear factor kappa β
RANKL	Receptor activator of nuclear factor kappa-B ligand
RUNX2	Runt-related transcription factor 2
Slc11a1	Solute carrier family 11 a member 1
TGF- β	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TRAF6	TNF receptor associated factor 6
VEGF	Vascular endothelial growth fator
WT	C57Bl/6 wild-type mice
μCT	Micro-computed tomography

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1 INTRODUCTION

1 INTRODUCTION

The bone tissue is a mineralized tissue of connective nature that dispose itself forming the bones, the rigid structures and resistant that form the skeleton, having the functions of support, protection, motion as well as the reserve of minerals, (RIDDLE; CLEMENS, 2017). The bone tissue is composed by a bone matrix that is responsible for the syntheses of phosphate, calcium and collagen fibers and by different cell types that participate of the bone formation and maintenance, such as osteoblasts, osteocytes and osteoclasts (GHIASI *et al.*, 2017; RIDDLE;CLEMENS, 2017).

In the oral cavity, the bone tissue has an important role in the teeth support, forming the alveolar bone, a dynamic tissue were the fibers of the periodontal ligament are inserted, and that respond rapidly to mechanical stimuli with bone formation or resorption (GHIASI *et al.*, 2017). Indeed, despite being a rigid and resistant structure, the bone tissue presents a significant elasticity, and is also characterized by periodical remodeling. The process of bone remodeling accounts for the maintenance of its anatomical and structural integrity, as well for that adaptation to the microenvironment modifications (DATTA *et al.*, 2008; ZHAO *et al.*, 2016). This process is directly related to the dynamic balance of the bone formation by the osteoblasts and resorption by the osteoclasts (KARSENTY; WAGNER, 2002; TAKAYANAGI, 2005). Such cellular dynamics also confers to bone tissue exhibits a high capacity of regeneration when injured, thus, bone fractures and small defects produced by pathology or trauma are repaired with the production of a new bone tissue, with morphofunctional characteristics similar to the original bone tissue (TSIRIDIS *et al.*, 2007; AI-AQL *et al.*, 2008). However, despite such capacity, more extensive defects or associated with certain systemic alterations, present a delayed repair, or are not repaired even after long periods.

The control of bone metabolism can be influenced by different factors, such as local and systemic factors, which include genetic factors, diet, and stimuli from physical activity (ONO; TAKAYANAGI, 2017). In addition to these factors, recently a ramification of immunology, called osteoimmunology, have been attracting increasing attention, derived from studies focused in the relation between the immune and the

bone systems. However, studies in the emerging osteoimmunology field have a major focus in the bone destruction caused by the abnormal activity of the immune system in different pathological conditions, mainly associated with the RANK/RANKL/OPG system. In fact, such association was initially identified in the study of rheumatoid arthritis, a pathology, in which the maintenance of the chronic inflammatory process leads to the joint bone resorption. Interestingly, in molecular and pathological terms, rheumatoid arthritis presents a series of similarities to that observed in bone loss associated with periodontitis, whose immunopathogenesis has been the subject of study by our research group (GARLET, CARDOSO, SILVA*, et al.*, 2006; SILVA *et al.*, 2007; GARLET *et al.*, 2008; TROMBONE, FERREIRA*, et al.*, 2009; TROMBONE *et al.*, 2010; CARDOSO *et al.*, 2011).

The key to the understanding of osteoimmunology may be the presence of common regulatory molecules, such as cytokines, receptors of transcription factors, shared by bone and immune systems (TAKAYANAGI, 2005). In this context, generally inflammatory mediators inhibit the osteoblastic activity and enhance osteoclast activity, while anti-inflammatory mediators have the opposite effect (GARLET, CARDOSO, CAMPANELLI*, et al.*, 2006; GARLET *et al.*, 2007). Part of the modulation exerted by inflammatory/immunological mediators on bone cells involves the modulation of RANK/RANKL/OPG system. In physiological conditions, osteoblasts and osteocytes play a major role in the regulation of the RANK/RANKL/OPG system, however the interference of leukocytes on such system can disturb the homeostasis of bone tissue (TAKAYANAGI, 2005; GARLET, CARDOSO, CAMPANELLI*, et al.*, 2006; LORENZO *et al.*, 2008). In fact, RANKL originally cloned from T cells, is characteristically produced by activated T lymphocytes, which destabilize the balance between RANKL and OPG in inflammatory environments resulting in bone resorption (TAKAYANAGI, 2005).

However, despite recent advances in osteoimmunology, details of the interaction between the immune and bone systems in bone formation and repair context are still scarce, and most studies have focused on the influence of cells and immunological mediators on osteoclast activation and on the bone resorption process, mainly in models of rheumatoid arthritis, myeloma and periodontal disease (LI *et al.*, 2007; ANG *et al.*, 2009). Thus, little is known about the possible influences of the interactions

between the bone and the immune system in the formation process and bone repair, as well as the molecular mechanisms involved in this process still unknown.

The bone healing process involves an injury-triggered local inflammatory immune reaction, whose extent and intensity are thought to influence the outcome of the healing process (RODRIGUES *et al.*, 2011; SCHMIDT-BLEEK *et al.*, 2014). While chronic and exacerbated inflammatory response are usually associated with impaired healing, a transitory and moderate inflammatory process are assumed contributes to healing mediating the chemotaxis of cells associated with the repair process and promoting local production/release of growth factors classically associated with bone anabolic processes (EMING *et al.*, 2009; KONNECKE *et al.*, 2014).

The process of alveolar bone repair has been researched in different species such as dogs, rats, monkeys and in humans, using densitometric, radiographic, histochemical and histological methods (SILVA *et al.*, 2007; CHIU *et al.*, 2013). As an alternative study of alveolar bone repair, we standardized in our laboratory a model of alveolar bone repair post extraction in mice, which allows the use of genetically modified animals for the expression/absence of determined factors that allow the establishment of direct relations of cause effect between this factors and the biological / pathological events allowing the clarification of the mechanisms involved in the regulation of bone tissue during the alveolar repair in homeostatic conditions (KORPI *et al.*, 2009). Previous results (histological, histomorphometric and molecular) of this experimental model (VIEIRA *et al.*, 2015) demonstrated that the alveolar bone repair process presents the same sequence of events previously described in humans and mice (CARDOSO *et al.*, 2011; RODRIGUES *et al.*, 2011) presenting only one variation in the chronology (but not in the sequence of events) of the repair process, characteristic of murine metabolism (ELSUBEIHI; HEERSCHE, 2004; KORPI, 2009).The process of alveolar repair depends initially of the formation of a blood clot within the alveolus, followed by the formation of a fibrin net (ELSUBEIHI; HEERSCHE, 2004; CARDOSO *et al.*, 2011; RODRIGUES *et al.*, 2011).

Gradually this clot suffers invasion of fibroblasts associated with proliferation of endothelial cells, originating new blood vessels; being replaced by a highly organized granulation tissue, with fibroblasts in intense proliferation and activity of extracellular matrix components syntheses. In the sequence, the osteoblasts found inside the

alveolus synthetize a mineralized osteoid matrix that forms the bone trabecular, so the granulation tissue is gradually replaced by bone tissue (VIEIRA *et al.*, 2015). The alveolar repair will be concluded when the alveolus is completely filled with newly formed bone tissue and the crest alveolar is remodeled, providing a dynamic osteoclast-osteoblastic balance and the new bone will be able to support new stimuli. In humans this occurs around the seventh month (210 days) after the extraction of the upper right incisor, which in experimental models equivalent to 28 days in rats and 21days in mice (VIEIRA *et al.*, 2015).

With the effective characterization of the model of the alveolar bone repair in mice (VIEIRA *et al.*, 2015), our research group performed cause-and-effect analyzes using genetically deficient mice (knockout, KO) for TNF p55 receptor, responsible for the inflammatory effects of this cytokine. In general, our results demonstrate that the absence of TNF-α results in a delay of the repair process, that involves the modulation of several genes associated to the recruiting and survival of leukocyte, and the differentiation and activity of osteoblasts (VIEIRA *et al.*, 2015) demonstrating an important role for the inflammatory mediators in the process of the alveolar bone repair. It is important to state that even without TNF-α, other important inflammatory mediators, such as IL-1b and IL-6 were present in significant levels throughout the alveolar bone repair process (VIEIRA *et al.*, 2015), suggesting that other inflammatory mediators can be responsible for the phenotype of partial alterations of the kinetics of repair presented in animals TNFp55 knockout.

At this point, it is mandatory to consider that while models involving knockout mice are an extremely important tool for elucidating the individual roles of such mediators (in which a determined cytokine is completely absent), the response presented by these animals does not mimic properly the potential effects of genetic variations that could explain the individual differences present in physiological processes and in human diseases once the variations described in humans (usually SNPs single nucleotide polymorphism) modulate the response changing the levels of certain factors, and not excluding completely the host response (TROMBONE, CARDOSO*, et al.*, 2009).

To study the effects of different inflammatory genotypes/phenotypes, bidirectional selective breeding allowed the generation of mice strains genetically selected for

maximal (AIRmax) or minimal (AIRmin) inflammatory reactions (IBANEZ *et al.*, 1992; DE FRANCO *et al.*, 2007; CANHAMERO *et al.*, 2011). These strains were obtained initially from a heterogeneous founding population (F0) produced through the intercrossing of eight inbred mouse strains (A/J, DBA/2J, P/J, SWR/J, SJL/J, CBA/J, BALB/cJ e C57BL/6J) (STIFFEL *et al.*, 1990).The intercrossing of these strains was made based in the intensity of the inflammatory reaction created by the injection of the agent Biogel in the subcutaneous tissue of the animal, and mating between animals with higher and lower response in relation to the normal distribution of the mice population resulting in each generation. From the 20ª generation of the selective mating it's admitted that the strains reached the maximum of phenotype separation (called the limit of selection), in which the allele that check the maximum and minimum inflammatory response are fixed in homozygosis on AIRmax and AIRmin strains (IBANEZ *et al.*, 1992).

In fact, the AIRmax and AIRmin strains present significant differences in the capacity of inflammatory response to many inflammatory agents (BORREGO *et al.*, 2006; PETERS *et al.*, 2007); forming an adequate model for the study of the mechanism of inflammatory/immune response in deferent infectious models (ARAUJO *et al.*, 1998; BIOZZI *et al.*, 1998; DE FRANCO *et al.*, 2007; PETERS *et al.*, 2007). The AIRmax and AIRmin strains were used successfully in previous studies of our research study group (TROMBONE, FERREIRA*, et al.*, 2009; TROMBONE *et al.*, 2010) in experimental periodontitis models and models of comorbidity arthritis/periodontitis, in which the dichotomy inflammatory phenotypes were confirmed; reinforcing that the variations presented by the AIRmax and AIRmin animals are due to an inflammatory profile distinct that involves the modulation of the expression of many inflammatory mediators simultaneously as TNF-α, IL-1b e IL-6 (TROMBONE, FERREIRA*, et al.*, 2009; Trombone *et al.*, 2010). Besides that, it is important to highlight that the AIRmax and AIRmin strains also present a differed profile in relation to the healing response and tissue repair (DE FRANCO *et al.*, 2007). These evidences show a possible modulation of the bone repair process due to of the intensity of the immune/ inflammatory process (using AIRmax and AIRmin mice strain).

Genetic studies indicated that the contrasting inflammatory responsiveness of AIRmix and AIRmax strains involves at least 11 QTLs (Quantitative Trait Loci) (BIOZZI *et al.*, 1998). Subsequent studies identified six inflammatory QTLs associated with the

tissue regeneration phenotype in AIR strains were located on chromosomes 1, 7, 8, 12, 14, and 16 (GORIAINOV *et al.*, 2014). These loci harbor several candidate genes involved in tissue regeneration and in determining sensitivity to experimentally induced diseases. The *Slc11a1* (solute carrier family 11a member 1) gene, locate on chromosome 1, is the most likely candidate, and its regulatory role was demonstrated in the early inflammatory events along ear tissue regeneration (CANHAMERO *et al.*, 2011).

Study that have characterized the genetic bases that result the differential phenotypes between the AIRmax and AIRmin strain, demonstrated that the gene *Slc11a1* ("solute carrier family 11a member 1") it is one of the genes responsible for the deferential response between the strains. *Slc11a1* alleles are named alleles R or S once they demonstrate resistance (R) or susceptibility (S) of determined infections/diseases (ARAUJO *et al.*, 1998; RIBEIRO *et al.*, 2003). In fact, subsequent studies have demonstrated significant frequency differences of these alleles in the AIRmax and AIRmin strains, the R allele being predominate AIRmax animals, while the presence of the allele S is characteristic of the AIRmin starin (ARAUJO *et al.*, 1998).

It is important to say that the gene *Slc11a1* (before nominated Nramp1 ["natural resistance-associated macrophage protein-1"]) (FORBES; GROS, 2003), has pleiotropic functions, such as Fe^{+2} protons and other divalent cations (Zn^{+2} e Mn^{+2}) (FRITSCHE *et al.*, 2007), as well as in the regulation of the macrophage activity, reflecting in its activation and consequently in the production of nitric oxide TNF-α (BARTON *et al.*, 1995), IL-1 (KITA *et al.*, 1992). It is important to mention that such molecules, individually or in combination, are potentially associated to the modulation processes of alveolar bone repair, as direct determiners of the nature and intensity of the immune/ inflammatory response.

Besides the variants R and S of *Slc11a1*, other loci were identified as responsible for the control of dichotomist inflammatory responsiveness of the strains AIRmin and AIRmax, independent of the gene *Slc11a1* (BORREGO *et al.*, 2006; DE FRANCO *et al.*, 2007; CANHAMERO *et al.*, 2014). These loci are determined QTLs (Quantitative Trait Loci) and are chromosomal regions associated to quantitative characters, therefore these regions can be detected by genetic markers dispersed by the genome

and its association with phenotype studied can be analyzed by QTL mapping programs. Through the mapping of the QTL it is possible to estimate the location of the genes that control the phenotypic variation of a character, the magnitude of its effects and the interactions with other QLT (PATERSON *et al.*, 2003; PATERSON *et al.*, 2013). Different studies have already detected that the frequency of the allele S of the gene *Slc11a1* in the initial population (F0) was 25% however after 30 generations of selected crossbreeds there was a displacement of this allele to 60%in AIRmin and 9% in AIRmax (ARAUJO *et al.*, 1998). Regarding the QTL of the gene *Slc11a1* studies detected 3 polymorphic regions in chromosomes 6, 11 and 13 that are involved in the regulation of acute inflammation (BORREGO *et al.*, 2006). To this, it's believed that the frequency deviation has occurred due to the selection process and that this gene and many others very close are participating of the control of the intensity of the acute inflammatory reaction (ARAUJO *et al.*, 1998).

In this way, to understand better the behavior of the AIRmax and AIRmin animals in relation to the allelic imbalance of the gene *Slc11a1* and its R and S alleles and the loci responsible for the inflammatory control, assisted genotype breeding were performed for the production of four substrains of homozygous mice for the R and S alleles of the *Slc11a1*. The substrains were called AIRmaxRR, AIRmaxSS, AIRminRR and AIRminSS (BORREGO *et al.*, 2006; DE FRANCO *et al.*, 2007; PETERS *et al.*, 2007).

In this context, studies demonstrate that the allele RR/SS of the gene *Slc11a1* as well as the general inflammatory background (derivate form QTLs) have influence on the inflammatory phenotype of sub strains (PETERS *et al.*, 2007). In relation to tissue regeneration the S allele of the gene *Slc11a1* in maximum inflammatory reaction favors the tissue repair, whereas in the minimal inflammatory reaction there is no tissue regeneration (DE FRANCO *et al.*, 2007). Recently, researchers investigated the inflammatory profile and the genetic expression in mice AIRmaxRR and AIRmaxSS in the initial tissue regeneration phase and observed that the susceptibility of the allele gives the animals an increase of the edema and also there is a different gene expression in each strain (CANHAMERO *et al.*, 2011). However, any study was performed at different substrains AIRmin and AIRmax with the aim of studying the alveolar bone repair process.

In this context, we hypothesize that AIRmin and AIRmax strains, due its distinct inflammatory responsiveness and healing potential, can also present distinct bone healing outcomes, and therefore, its comparative analysis can provide important information regarding the link between inflammation and bone healing. Therefore, in this study, mice genetically selected for maximum (AIRmax) or minimum (AIRmin) acute inflammatory response were submitted the extraction of the upper right incisor and comparatively evaluated regarding to the intensity and nature of the inflammatory response along alveolar bone healing, as well regarding the healing outcome by means of microtomographic, histological/histomorphometric and molecular analysis.

Also, we hypothesize that the model using AIRmax and AIRmin mice with the R and S allele of the *Slc11a1* gene is extremely interesting as an experimental study model for the alveolar bone repair process, since it will clarify the effect of the R and S alleles in relation to the intensity inflammatory. Therefore, in this study, substrains of homozygous mice for the R and S alleles of the *Slc11a1* were submitted the extraction of the upper right incisor and comparatively evaluated regarding to the intensity, nature of the inflammatory response and effect dos alleles along alveolar bone healing, as well regarding the healing outcome by means of microtomographic, histological/histomorphometric and molecular analysis.

2 ARTICLES

2 ARTICLES

The articles presented is this thesis were written according to the guidelines for article submission.

- Articles 2.1 – Influence of inflammatory response in alveolar bone healing in mice genetically selected in the maximum (Airmax) or minimum (Airmin) inflammatory reaction (Submitted in January 2018).

- Article 2.2- The role of the gene *Slc11a1* and selected genotypes for minimum and maximum inflammatory reactivity in the process of alveolar bone healing in mice (To be submitted)

Articles 2.1

Influence of inflammatory response in alveolar bone healing in mice genetically selected in the maximum (Airmax) or minimum (Airmin) inflammatory reaction

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Conflicts of interest: The authors deny any conflicts of interest.

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ABSTRACT

The exact role of inflammatory immune response in bone healing process is still unclear, but the success of the alveolar bone healing process seems to be associated with a moderate and transitory inflammatory response, while insufficient or exacerbated responses seems to have a detrimental influence in the healing outcome. In this context, we evaluated the influence inflammation in alveolar bone healing taking advantage of mice strains genetically selected for maximum (AIRmax) or minimum (AIRmin) acute inflammatory response. Experimental groups (N=5/time/group) comprised 8-week-old male or female AIRmax and AIRmin submitted to extraction of upper right incisor and evaluated at 0, 3, 7, 14 and 21 days after upper incision extraction by micro-computed tomography (μCT), histomorphometry, birefringence, immunohistochemistry and molecular (PCRArray) analysis. Our results demonstrate that in the early periods (3 and 7 days) the AIRmin strain presented an increased ($p<0.05$) bone volume and hyperdense regions, increased ($p<0.05$) density of bone matrix and osteoblasts, increased ($p<0.05$) expressed of BMP4, BMP7 and RUNX2 when compared to AIRmax strain. The analysis of the inflammatory infiltrate demonstrated that AIRmin mice presented lower counts of $GR1^+$ and $CD80^+$ cells, and higher counts of $F4/80^+$ and $CD206^+$ cells, in parallel with higher mRNA expression of CX3CL1, CCL5, CCR5 and ARG when compared to AIRmax animals. In late (14 and 21 days) repair stages, the AIRmin strain presented a decreased ($p<0.05$) density of osteoclast and blood vessels than AIRmax, along lower RANKL and Catepk and higher PHEX and SOST mRNA expression. Our results also demonstrate a similar healing outcome in both strains at the endpoint. In conclusion, despite the similar endpoint healing, our results demonstrate that the early repair observed in the AIRmin strain was associated with decreased presence of neutrophils and M1 macrophages, and increased counts and activity of M2 macrophages. Further studies are required to clarify the underlying mechanisms that lead to the differential response of AIRmin and AIRmax strains along bone repair.

Keywords: Bone. Inflammation. Cytokine

Articles 2.2

The role of the gene *Slc11a1* and selected genotypes for minimum and maximum inflammatory reactivity in the alveolar bone healing process in mice

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ABSTRACT

The process of alveolar bone healing can be influenced by several local and systemic factors, which include the immune system and healing related genes. However, the exact role of host inflammatory responsiveness and genetic background in bone healing process remains unclear. In this context, we evaluated the influence inflammation in alveolar bone healing taking advantage of mice strains genetically selected for maximum (AIRmax) or minimum (AIRmin) acute inflammatory response, as well for homozygous RR/SS genotypes of *Slc11a1* gene. Experimental groups (N=5/time/group) comprised 8-week-old male or female AIRminRR, AIRminSS and AIRmaxRR and AIRmaxSS submitted to extraction of upper right incisor and evaluated at 0, 3, 7 and 14 days after upper incision extraction by micro-computed tomography (μCT), histomorphometry, birefringence, immunohistochemistry and molecular (PCRArray) analysis. Evaluating the influence AIRmin/AIRmax inflammatory background, observed that the AIRmax strain, associated with both RR and SS *Slc11a1* genotypes, demonstrate a more effective bone healing, characterized by progressive increase ($p<0.05$) of bone volume and predominance of red fiber in analysis in contrast to AIRmin strains. AIRmax strain also presented increased ($p<0.05$) $F4/80^+$ cells counting, and decreased $CD80^+$ e $CD206^+$ in the AIRmaxRR cells when compared to AIRminRR mice, while AIRmaxSS presented increased ($p<0.05$) $GR1^+$ $F4/80^+$, $CD80^+$ cells counting and decreased $CD206^+$ cells when compared to AIRminSS mice. On the other hand, when the analysis is performed in order to address the influence RR/SS *Slc11a1* alleles, observed that SS allele was associated with significant alveolar bone healing delay when AIRmaxSS and AIRmaxRR strains were compared. This delay was characterized by decreased ($p<0.05$) of bone volume and trabecular number, decreased ($p<0.05$) red collagen fibers, increased ($p<0.05$) $GR1^+$ and $CD80^+$ cell counts and decreased ($p<0.05$) counts of $F4/80^+$ and $CD206^+$ cells when AIRmaxSS and AIRmaxRR strains were compared. In the AIRmin strain, SS genotype was associated with a more effective healing when compared with RR; characterized by increased ($p<0.05$) bone volume, trabecular number and trabecular separation, increased red collagen birefringence, increased $GR1^+$cells and decreased $CD206^+$ cells count when compared with AIRminRR. Taken together, our results showed that AIRmax inflammatory background was associated to a more effective bone healing process irrespective of the presence of RR or SS *Slc11a1* genotypes. Interestingly, while the association of RR genotype with AIRmax background favor bone healing, the SS genotype was found to favor the healing in the AIRmin background.

Keywords: Bone repair. Inflammation. *Slc11a1*

3 DISCUSSION

3 DISCUSSION

While a transitory and moderate host response allegedly contributes to healing, exacerbated responses seems to have a detrimental role in the healing outcome. However, the exact role of inflammatory immune response in bone healing process is still unclear. In this context, this study took advantage of mice strains genetically selected for maximum (AIRmax) or minimum (AIRmin) acute inflammatory response to study the relationship between inflammatory responsiveness and bone healing outcome.

Our data demonstrated that bone healing process occurs in both strains, but we observed in the early periods (3 and 7 days) there is a delay in the AIRmax repair when compared to the AIRmin mice, while in the late periods (14 and 21 days) both strains present similar characteristics in the alveolar bone healing. In the view of extreme inflammatory phenotypes previously described to these mice strains (i.e., maximal or minimal acute inflammatory response), as well in the view of the dichotomic response to ear lesions healing, it is possible to consider that the slight delay and similar bone healing outcome are an unpredicted phenotype.

In this context, it is mandatory to consider that the details of the interaction between the immune and bone systems in bone formation and repair context are still scarce, and most studies have focused on the influence of cells and immunological mediators on osteoclast activation and on the bone resorption process (LI *et al.*, 2007; ANG *et al.*, 2009). Therefore, despite the lack of the divergent healing phenotypes projected, we performed a comparative analysis between AIRmin and AIRmax strains in order to address the possible reasons for the initial delay in the repair, and the subsequent events that lead to a similar healing outcome at the endpoint.

In our study with AIRmax and AIRmin, we observed that the sequence of events for the repair in the early periods differs between the strains (Figures 1 and 5), but subsequently follows the events describe in the literature (VIEIRA *et al.*, 2015).In summary, after tooth extraction, histological analysis demonstrate the formation of a blood clot, which is succeeded by the formation of a fibrin network, followed by formation of a granulation tissue and subsequent replacement by new bone tissue filling the alveolus. The early histological event, we observed that AIRmin mice

presence of a denser blood clot and with a more amount of inflammatory exudate in the comparison the AIRmax mice (Figures 1A and 1B), as well as, the AIRmin presented a higher density of bone matrix and osteoblast in comparison to AIRmax (Figures 1A, 1B, 6), following increase expressed of BMP4, BMP7, responsible by induction of mesenchymal cells in cartilage and bone and RUNX2 responsible by osteoblastic differentiation (Figures 8 and 9) (VIEIRA *et al.*, 2015). Before that, we believed that the difference between blood clot formed after tooth extraction, plus the inflammatory background of the animal, interfered in the initial alveolar bone repair process, influencing the delay in AIRmax when compared AIRmin.

The delay in the bone healing process in the AIRmax mice in the early periods can be associated with the increased counts cells of $GR1^+$ and $CD80^+$, as well as, increased mRNA expression of CXCL1, CXCL2, CXCR1 and MMP8 (Figures 3A, 3C, 8 and 9) followed by decreased mRNA expression of CCL5/CCR5 and ARG1. This associated can be confirmed by other studies that shown in the AIRmax mice a higher neutrophils production in the bone medulla, a higher concentration of neutrophils in blood and an increased resistance of locally infiltrated neutrophils to spontaneous apoptosis (RIBEIRO *et al.*, 2003; FERNANDES *et al.*, 2016). A strong expression of CXCL1, CXCL2 was also developed in the inflammatory skin process of AIRmax compared to AIRmin mice. These chemokines are known for their strong neutrophil chemotactic activity and for binding to CXCR2 (IL8RB) protein (ARAUJO *et al.*, 1998).

However, in the general aspect, the inflammatory process of the study model analyzed presented a lower counts neutrophil in site inflammatory when compared to other experimental studies of chronic diseases and transient inflammation that demonstrating that AIRmax mice were more susceptible periodontitis, arthritis, lung inflammatory and also preseted an increased regenerative capacity, followed by higher expresses CXCL1, CXCl9, CXCL5, CXCL13 and higher levels IL-1b, RANKL and MMP-13 when compared AIRmin figures 3, 8 and 9 (MARIA *et al.*, 2003; DE FRANCO *et al.*, 2007; FRITSCHE *et al.*, 2007; TROMBONE *et al.*, 2010). Also observed a lower counts cells $GR1^+$ in comparison to the other markers, $F4/80^+$, $CD80^+$ and $CD206^+$. We believe that the lowest counts of neutrophil occur due to the study model, bone repair, and also by difference that the strains present in relation the gene *Slc11a1* (solute carrier family 11 member 1).

In addition, we also believe that the delay in the early periods can be occurs due to the allelic unbalance of the gene *Slc11a1* (Nramp1) in the AIRmin and AIRmax mice. The o gene *Slc11a1* presents different functions, among them can cite functions that are related to the repair process, such as, modulation and activation of macrophages (FRITSCHE *et al.*, 2007; CORREA *et al.*, 2017). In the bone repair process the unbalance of the gene *Slc11a1* can have influenced modulation and activation of macrophages, for AIRmin mice demonstrated a higher counts cells F4/80^{+} and CD206^{+}, followed by increased mRNA expressed CXBl11, CCL5/CCR5 and ARG1 than AIR max (Figures 3B and 3D). However, when comparing counts cells the F4/80^{+} with counts cell the CD80^{+} and CD206^{+} was observed a lower counts cells F4/80^{+}, which that the expected was a higher counts cells F4/80^{+}, for this marker theoretically would mark all types of macrophages. We believe that this difference occurred due to the inefficiency of the antibodies used.

It is important remember that macrophages are involved in the removal of apoptotic cells (ADEREM; UNDERHILL, 1999), microorganism ingestion and elimination (GORDON, 2002; TAYLOR *et al.*, 2005), antigen presentation and consequent T cell activation (VILLACRES-ERIKSSON, 1995; MARTINEZ-POMARES, GORDON, 2007), the resolution of inflammatory processes, angiogenesis, and tissue repair (ALIBERTI, 2005; SICA; MANTOVANI, 2012; MANTOVANI *et al.*, 2013). Recruitment of monocytes/macrophages to injury sites occurs through the binding of chemokines, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL8, CXCL9 and CXCL10 and CCL2, CCL3, CCL4, CCL5, CCL11, CCL17 and CCL22, with specific chemokines receptors expressed in the cell surface, being CCR2 and CCR5 are the most important during inflammatory responses (PALOMINO; MARTI, 2015). The macrophages are classically considered as proinflammatory cells, but these cells may present distinct functional phenotypes, called M1 and M2. Macrophages can be classically activated M1 (stimulated by TLR ligands and IFN-Y) or alternatively activated M2 (stimulated by IL-4/IL-13) in response to various signals; these states mirror the Th1–Th2 polarization of T cells (BISWAS; MANTOVANI, 2010; SICA; MANTOVANI, 2012; MANTOVANI *et al.*, 2013).

The M1 macrophages exhibit high levels of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6) and inducible nitric oxide synthase (PAPAIOANNOU *et al.,* 2015). In the

infectious conditions, studies associated M1 cells with Th1 and / or Th17 type responses (BASHIR *et al.*, 2015; HUME, 2015; MOTWANI;GILROY, 2015). While the M2 macrophages develop a regulatory and/or repair phenotype with action in the response control and tissue repair (BASHIr *et al.*, 2015; HUME, 2015; MOTWANI; GILROY, 2015). This phenotype regulatory occurs due to high expression of CD163, CD206, MDC, MRC1 receptors and factors such as CCL22, CCL18, IL10, TGFβ1, PDGF, TIMPs, as well as the markers arginase-1 (Arg-1) and Fizz-1 (BASHIR *et al.*, 2015; HUME, 2015; MOTWANI; GILROY, 2015). Experimental models of arthritis and periodontal lesions characterized the role of M1 and M2 and demonstrated the association of M1 with tissue destruction and an inverse role for M2 (SIMA; GLOGAUER, 2013; LAM *et al.*, 2014; YE *et al.*, 2014; HE *et al.*, 2015). On the other hand, studies related to tissue repair have shown that the repair process involves an initial M1 polarization that rapidly evolves to M2 (SINDRILARU; SCHARFFETTER-KOCHANEK, 2013). Although such studies do not specifically involve bone repair, these studies corroborate with our findings, we believed that the presence of M1 ($CD80^+$) and neutrophils in early bone repair may be contributed to delay repair, while the presence of M2 macrophages ($CD206^+$) in the local of alveolar bone repair is involved in the early bone repair, being reinforced by the intense expression of the Arg-1 in the repair sites, as well as CCL5 chemokines and its CCR5 receptor, associated with macrophage migration (not yet polarized) during the repair of different tissues (Figures 3D, 4D, 8 and 9).

In relation to the late bone repair process, we observed that AIRmax presenting a gradual decrease of the neutrophil, while in the AIRmin presented opposite effect (Figure 3A), but in the period both strain presented the alveolar bone repair. Although bone repair was observed in the lines, we observed that AIRmax mice presente a high density of blood vessels and osteoclast (Figure 2F, 2I) followed by a high expression of mRNA RANKL and CATEPk, MMP9 and MMP13, VEGFa and VEGFb (Figures 8 AND 9) and high birefringence of collagen fibers in the red color (Figure 7). These data suggest that there was healing with the production of a new bone tissue in both I strains, but AIRmin demonstrated decreased RANKL, that is the principal osteoclastogenic factor that acts via binding RANK receptors present on the surface of preosteoclasts, inducing their differentiation and activation e also decreased density of

blood vessels, suggest the formed bone tissue is still immature, while AIRmax can be considered as mature bone tissue with presence of bone marrow.

Comparing the two periods, early and late, we observed that in the early period the presence of neutrophil e M1 macrophage at the site of the injury influenced a delay in the beginning of the bone healing, in this way, the inflammatory process mediated by the neutrophils un favor the bone repair at moment. On the other hand, in the period late the presence of macrophages M2 was essential for the repair process, because in the period of 14 days we observed a peak in the counts of macrophages M2 in AIRmax (Figure 3D), suggesting therefore that the initial delay of the repair is "corrected" during activation M2 in the period late.

Some studies have already been performed using the AIRmin and AIRmax mice, among all the studies, the model of ear healing process, have demonstrated that the AIRmax strain presents a high regenerative capacity when compared to AIRmin. Genetic studies demonstrated that the gene *Slc11a1* (“solute carrier family 11a member 1”) it is one of the genes responsible for the deferential response between the strains (CANHAMERO *et al.*, 2014). And its alleles are named alleles R or S once they demonstrate resistance (R) or susceptibility (S) of determined infections/diseases (ARAUJO *et al.*, 1998; RIBEIRO *et al.*, 2003). In fact, subsequent studies have demonstrated significant frequency differences of these alleles in the AIRmax and AIRmin strains, the R allele being predominate AIRmax animals (91%RR – 9%SS), while the presence of the allele S is characteristic of the AIRmin strain (60%SS – 40%RR) (ARAUJO *et al.*, 1998).

Due to the AIRmin and AIRmax strains to be heterozygous for the *Slc11a1* (Nramp1) R and S alleles, new *Slc11a1* homozygous AIR sublines were produced by genotyping heterogeneous AIRmax and AIRmin, which were called of AIRmaxRR, AIRmaxSS, AIRminRR, and AIRminSS strains (BORREGO *et al.*, 2006; PETERS *et al.*, 2007). Indeed, several studies demonstrated that the both RR/SS *Slc11a1* alleles and the AIRmin/AIRmax background have influence on the inflammatory phenotype of substrains AIRminRR, AIRminSS, AIRmaxRR and AIRmaxSS (PETERS *et al.*, 2007; CANHAMERO *et al.*, 2011; CANHAMERO *et al.*, 2014).

Therefore, the analysis of AIR substrains homozygous for *Slc11a1* alleles allows the simultaneous analysis of the influence of R and S alleles in a given biological

process (such as bone repair), but also allow the analysis of the influence of other genetic factors composing the ‘min’ and ‘max’ genetic backgrounds (CANHAMERO *et al.*, 2014). Indeed, additional genetic studies identified some/multiple/numerous QTLs associated with the distinct inflammatory responsiveness derived from AIRmin and AIRmax backgrounds, independently of the *Slc11a1* alleles (BIOZZI *et al.*, 1998). Among them, have already been identified six inflammatory QTL involved in the tissue regeneration phenotype in AIRmax and AIRmin mice were found to be located on chromosomes 1, 7, 8, 12, 14, and 16 (CANHAMERO *et al.*, 2011; CANHAMERO *et al.*, 2014).

In this way, performing a comparative analysis of alveolar bone repair features in substrains AIRminRR, AIRminSS, AIRmaxRR and AIRmaxSS strains, it is possible to analyze and discuss the data obtained from two viewpoints, one focused in the influence AIRmin/AIRmax inflammatory background (when AIRminRR vs AIRmaxRR and AIRminSS vs AIRmaxSS comparisons are performed), and other focused in the influence of the both R and S *Slc11a1* alleles (via the AIRminRR vs AIRminSS and AIRmaxRR vs AIRmaxSS comparisons) in the bone healing process.

First of all, evaluating the influence AIRmin/AIRmax inflammatory background, we observed that the AIRmax inflammatory background, associated with both RR and SS *Slc11a1* genotypes, contribute to a more effective bone healing process when compared to AIRmin background. Indeed, our results demonstrate a progressive increase of bone volume, bone volume fraction, trabecular thickness, trabecular number and trabecular separation (Figure 1, 2), following by increased birefringence of collagen fibers in the red color and area of collagen, (Figure 4). Studies that analyzed the ear hole regeneration capacities are similares as our results, since ear regeneration were more effective on the AIRmax background when comparation AIRmin background. While in the background AIRmin did not present ear hole regeneration capacities (DE FRANCO *et al.*, 2007; CANHAMERO *et al.*, 2011; CANHAMERO *et al.*, 2014).

Importantly, such results must be confirmed by the histomorphometric analysis, molecular analysis and enzyme-linked immunosorbent assay (ELISA). However, uCT data have been found to be reliable to address bone formation along process of alveolar bone healing in mice (VIEIRA *et al.*, 2015).

On the other hand, when the analysis is performed in order to address the influence RR/SS *Slc11a1* alleles, we observed that SS *Slc11a1* allele demonstrated a delay to the alveolar bone healing in AIRmax mice when compared to AIRmaxRR mice. This delay was characterized by decreased of bone volume and trabecular number (Figure 3), following by decreased birefringence of collagen fibers in the red color and area of collagen, (Figure 4). However, in the AIRmin strain, the SS *Slc11a1* allele was associated with an opposing repair outcome, i.e. a more effective healing when compared with AIRminRR mice; characterized by increased of bone volume, trabecular number and trabecular separation (Figure 1, 2), following by increased birefringence of collagen fibers in the red color and area of collagen, (Figure 4). In relation the SS *Slc11a1* allele our results are different when compared ear hole regeneration capacities. In the ear regeneration in the AIRmin background mice with both RR/SS *Slc11a1* alleles did not regenerate ear holes. While, the AIRmax background mice with SS *Slc11a1* allele regenerate ear holes, the opposing effect was associate in the AIRmax strain with RR *Slc11a1* allele, i.e. no regenerated ear (DE FRANCO *et al.*, 2007; CANHAMERO *et al.*, 2011; CANHAMERO *et al.*, 2014). We believe that it is difference occur due to feature of regenerating tissues are different (cartilage / bone).

Importantly, such results must be confirmed by the histomorphometric analysis, molecular analysis and enzyme-linked immunosorbent assay (ELISA). However, uCT data have been found to be reliable to address bone formation along process of alveolar bone healing in mice. We believe that with the results presented plus the other pending results would make it possible to understand the opposite effect of R and S alleles in association with the different inflammatory backgrounds (AIRmin and AIRmax).

In order to determine the factors responsible for the modulation of bone repair outcome in AIRminRR, AIRminSS, AIRmaxRR and AIRmaxSS, we performed a immunohistochemical analysis of inflammatory infiltrate to detect the presence of Ly6g-Gr1^{+} (granulocytes), F4/80^{+} (macrophages), CD80^{+} (M1 macrophages) and CD206^{+} (M2 macrophages) positive cells in the repair at periods of 3, 7 and 14 days after tooth extraction.

First of all, evaluating the influence AIRmin/AIRmax inflammatory background, we observed that the AIRmax inflammatory background, associated with RR *Slc11a1* genotype presented increased counts cells F4/80$^+$ and decreased CD80$^+$ e CD206$^+$ cells when compared to AIRminRR mice (Figures 6B, 6C, 6D). While, AIRmax inflammatory background, associated with SS *Slc11a1* genotype presented increased counts cells GR1$^+$, F4/80$^+$, CD80$^+$ and following by increased and decreased CD206$^+$ cells e when compared to AIRminSS mice (Figures 6A, 6B, 6C and 6D). On the other hand, when the analysis is performed in order to address the influence RR/SS *Slc11a1* alleles, we observed that SS *Slc11a1* allele demonstrated increased counts cells GR1$^+$ and decreased counts cells F4/80$^+$, following by increased and decreased counts cells CD80$^+$ and CD206$^+$ in AIRmax mice when compared to AIRmaxRR mice (Figures 6A, 6B, 6C and 6D). However, in the AIRmin strain, the SS *Slc11a1* allele demonstrated increased counts cells GR1$^+$, following by decreased counts cells CD206$^+$ in AIRmin mice when compared to AIRminRR mice (Figures 6A, 6D).

Surprisingly, it was not possible to stablish a clear association between the immunohistochemical data and the repair outcome analyzed by µCT and birefringence of collagen fibers. Our results differ from other findings literature, primarily due to the study model, i.e., alveolar bone repair process. In addition, after the extraction of the tooth, a blood clot is formed, which is of extreme importance for the repair process, serving as a provisional outline for invasion of constitutive cells, leukocytes and release of several growth factors and cytokines (NURDEN, 2011; BURNOUF *et al.*, 2013). The triggered inflammatory response is transient, initiated by damage-associated molecular patterns (DAMPs) with strong counts macrophage in the healing. While in other models, there is not formation blood clot, the inflammatory response is chronic, initiated by pathogen-associated molecular pattern with high neutrophil expression (DE FRANCO *et al.*, 2007; CANHAMERO *et al.*, 2011; CANHAMERO *et al.*, 2014; CORREA *et al.*, 2017). Furthermore, we still need the histomorphometric data, molecular and ELISA analysis to be able to clarify the results obtained in the process of alveolar bone repair.

Our current hypothesis is that for the success of alveolar bone repair is necessary a moderate inflammatory process, that is not, insufficient or not exacerbated. We believed that the delay in alveolar bone repair in the AIRmin inflammatory background mice can be associated to inflammatory insufficient process, that is, this strain does

not achieve the point considered necessary for repair. While, AIRmax inflammatory background presents a repair closer to the ideal point. On the other hand, in the analysis of the RR/SS alleles, we believe that the SS allele in both background provides an increase in the inflammatory process, thus, AIRminSS is closer to the ideal point for repair, while AIRmaxSS presents an exacerbation of the inflammatory process, exceed from the point ideal for repair.

We believe that our results could contribute considerably to characterize the influence of the magnitude of the maximum or minimum inflammatory response in the early and late periods of the alveolar bone repair process in homeostatic conditions. And we also believe that after the inclusion of the pending data our results could contribute considerably to characterize the role of the gene *Slc11a1* and selected genotypes for minimum and maximum inflammatory reactivity in the process of alveolar bone healing in mice in homeostatic conditions.

4 CONCLUSION

4 CONCLUSION

In summary, the present study demonstrated that despite the similar endpoint healing, our results demonstrate that the early repair observed in the AIRmin strain was associated with decreased presence of neutrophils and M1 macrophages, and increased counts and activity of M2 macrophages. As also demonstrated that AIRmax inflammatory background was associated to a more effective bone healing process irrespective of the presence of RR or SS *Slc11a1* genotypes. Interestingly, while the association of RR genotype with AIRmax background favor bone healing, the SS genotype was found to favor the healing in the AIRmin background.

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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 Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 003/2014

Bauru, 14 de maio de 2014.

Senhor Professor,

O projeto de pesquisa encaminhado a esta Comissão de Ética no Ensino e Pesquisa em Animais, denominado **Estudo do papel do gene Slc11a1 e de genótipos selecionados para mínima e máxima reatividade inflamtória no processo de reparo ósseo alveolar em camundongos,** de sua autoria e Priscila Maria Colavite Machado com a colaboração de Andreia Espindola Vieira, Carlos Eduardo Repeke, Cláudia Cristina Biguetti e Franco Cavallafoi enviado ao relator para avaliação e considerado APROVADO *ad referendum* desta Comissão, em 10 de março de 2014.

Solicitamos que ao final da pesquisa seja enviado, para avaliação desta Comissão, 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,

Prof. Dr Luís Antônio de Assis Taveira
Vice-Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

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

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-101 – C.P. 73
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Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 003/2014

Bauru, 31 de agosto de 2016.

Senhor Professor,

Em atenção à solicitação de alterações na pesquisa intitulada ***Estudo do papel do gene Slc11a1 e de genótipos selecionados para mínima e máxima reatividade inflamtória no processo de reparo ósseo alveolar em camundongos***, registrada sob ***CEEPA-Proc. Nº 003/2014***, tendo Vossa Senhoria como Pesquisador Responsável, foram analisadas, com base nos dados

- correção da vigência do protocolo de *01/04/2012 a 30/12/2014* para ***04/2014 a 04/2017***;

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

Docente do Departamento de Ciências Biológicas

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Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 003/2014

Bauru, 31 de agosto de 2016.

Senhor Professor,

Em atenção à solicitação de alterações na pesquisa intitulada ***Estudo do papel do gene Slc11a1 e de genótipos selecionados para mínima e máxima reatividade inflamatória no processo de reparo ósseo alveolar em camundongos***, registrada sob **CEEPA-Proc. Nº 003/2014**, tendo Vossa Senhoria como Pesquisador Responsável, foram analisadas, com base nos dados:

- *correção da vigência do protocolo de 01/04/2012 a 30/12/2014 para* ***04/2014 a 04/2017***;
- *acréscimo de 160 animais, das linhagens AIRmnSS, AIRmnRR, AIRmaxSS, AIRmaxRR, totalizando 368 (208 animais já foram utilizados) pois nas análises iniciais evidenciou-se a necessidade de aumento do "n"*.

Considerando que o estudo 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, em reunião ordinária da Comissão de Ética no Ensino e Pesquisa em Animais (CEEPA), realizada no dia **26 de agosto de 2016**.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigência da autorização	*Abril/2014 a Abril/2017*
Espécie/linhagem/raça	*Camundongo heterogênico AIRmnSS, AIRmnRR, AIRmaxSS, AIRmaxRR*
Nº de animais	*368 (208 + 160 animais)*
Peso/Idade	*25g/8 semanas*
Sexo	*Machos e Fêmeas*
Origem	*Instituto Butantan, São Paulo*

Prof. Dr. Gustavo Pompermaier Garlet

Docente do Departamento de Ciências Biológicas

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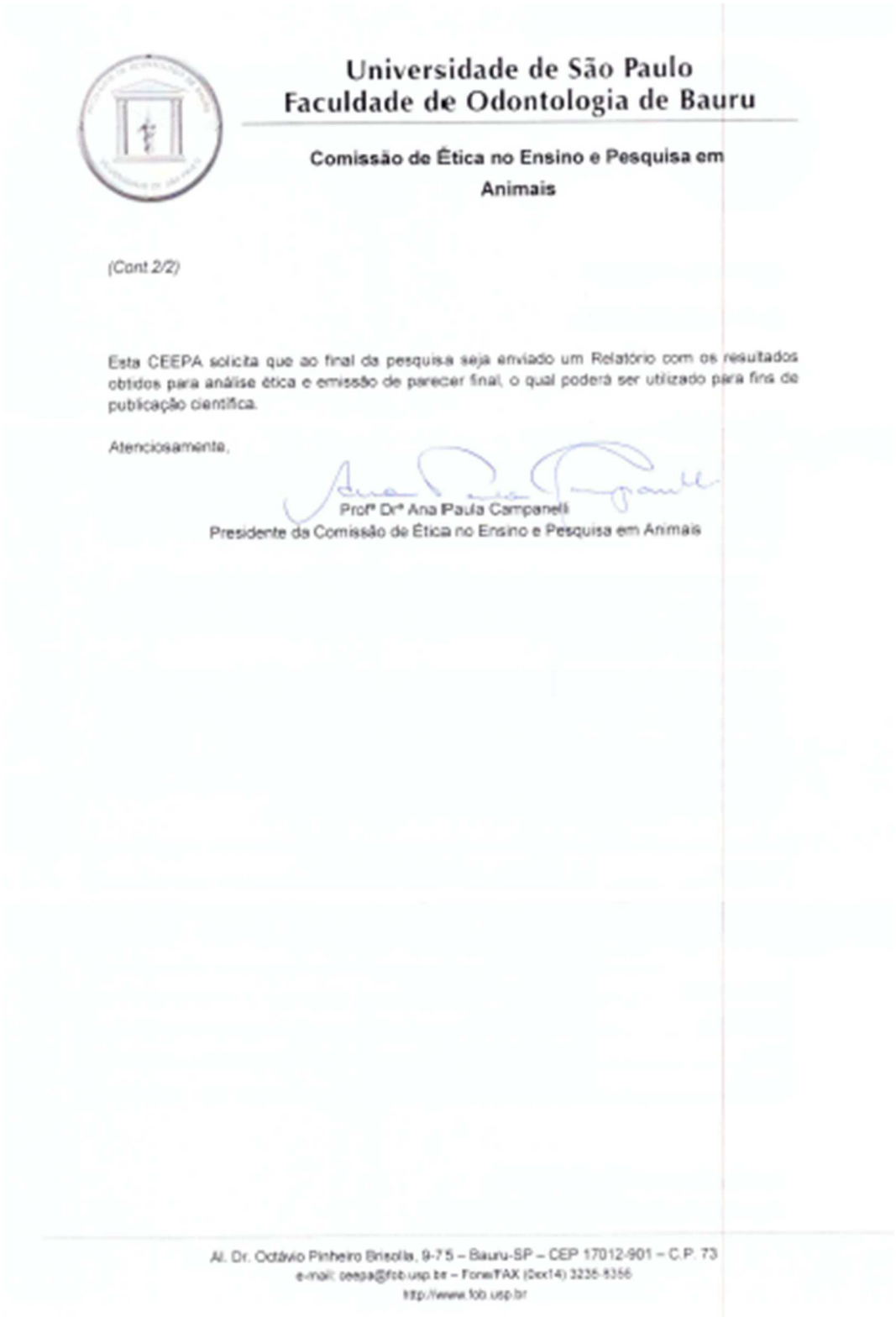

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

(Cont.2/2)

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

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
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Anexo B – Submissão do artigo “Influence of inflammatory response in alveolar bone healing in mice genetically selected in the maximum (Airmax) or minimum (Airmin) inflammatory reaction” ao periódico Journal of Applied Oral Science.

Journal of Applied Oral Science - Manuscript ID JAOS-2018-0028

Journal of Applied Oral Science <onbehalfof@manuscriptcentral.com>

sáb 13/01/2018 12:34

Para:colavitepm@hotmail.com <colavitepm@hotmail.com>;

Cc:colavitepm@hotmail.com <colavitepm@hotmail.com>; garletgp@usp.br <garletgp@usp.br>;

13-Jan-2018

Dear Mrs. Machado:

Your manuscript entitled "Influence of inflammatory response in alveolar bone healing in mice genetically selected in the maximum (Airmax) or minimum (Airmin) inflammatory reaction" has been successfully submitted online and is presently being given full consideration for publication in the Journal of Applied Oral Science.

Your manuscript ID is JAOS-2018-0028.

Please mention the above manuscript ID in all future correspondence or when calling the office for questions. If there are any changes in your street address or e-mail address, please log in to ScholarOne Manuscripts at https://mc04.manuscriptcentral.com/jaos-scielo and edit your user information as appropriate.

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Thank you for submitting your manuscript to the Journal of Applied Oral Science.

Sincerely,
Journal of Applied Oral Science Editorial Office