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Response of the periodontal tissues to β -adrenergic stimulation

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ABSTRACT

Aims: Stimulation of β -adrenergic receptors (β AR) in osteoblasts by isoproterenol (ISO) was shown to induce Vascular Endothelial Growth Factor (VEGF) and angiogenesis in long bones. We thus aimed to determine the vascular response of mandibular tissues to β AR stimulation regarding blood vessel formation.

Main methods: Six-week-old wild-type C57BL6 female mice received daily intraperitoneal injections of ISO or PBS for 1 month. Hemimandibles and tibias were collected for immunolocalization of endomucin, tyrosine hydroxylase (TH), neuropeptide Y (NPY) and norepinephrine transporter (NET). Moreover, *Vegfa*, *Il-1 β* , *Il-6*, *Adrb2* and *Rankl* mRNA expression was assessed in mandibles and tibias 2 hours after PBS or ISO treatment.

Key findings: Despite similar sympathetic innervation and *Adrb2* expression between mandibular tissues and tibias, with TH and NPY+ nerve fibers distributed around blood vessels, ISO treatment did not increase endomucin+ vessel area or the total number of endomucin+ vessels in any of the regions investigated (alveolar bone, periodontal ligament, and dental pulp). Consistent with these results, the expression of *Vegfa*, *Il-6*, *Il-1 β* , and *Rankl* in the mandibular molar region did not change following ISO administration. We detected high expression of NET by immunofluorescence in mandible alveolar osteoblasts, osteocytes, and periodontal ligament fibroblasts, in addition to significantly higher *Net* expression by qPCR compared to the tibia from the same animals.

Significance: These findings indicate a differential response to β AR agonists between mandibular and tibial tissues, since the angiogenic potential of sympathetic outflow observed in long bones is absent in periodontal tissues.

keywords: Periodontitis, Sympathetic Nervous System, Blood Vessels, Bone.

INTRODUCTION

The consequences of chronic exposure to elevated endocrine or neural responses resulting from stress (allostatic overload) include increased risk of cardiovascular disease, metabolic syndrome, gastrointestinal disorders and other inflammatory diseases, including periodontitis.¹⁻³ Psychological stress can change the morphology of the healthy periodontal ligament (PDL)⁴ and it also accentuates alveolar bone loss and tissue breakdown in induced periodontitis in rats.^{5,6} Likewise, human studies have shown worsening of periodontitis clinical parameters in patients with stress.⁷⁻⁹ The mechanisms by which allostatic overload contributes to periodontitis are not fully understood. It is proposed that it can stimulate the Hypothalamic-Pituitary-Adrenal (HPA)-Axis to release corticosteroids, which have immunosuppressive action; it can cause behavioral alterations, such as an increase in tobacco usage or poor oral hygiene that can impact biofilm formation; and finally, it stimulates the sympathetic nervous system (SNS) with release

of catecholamines (epinephrine/norepinephrine-NE). The role of SNS in periodontitis pathogenesis is still under investigation, but evidence suggest it may interfere with both pillars of periodontal disease pathogenesis: the host response, acting on periodontal and immune cells; and the microbiota, directly stimulating the growth of several bacterial strains and enhancing the virulence of others such as *Porphyromonas gingivalis*.^{10,11}

Under stress, periodontal tissues are exposed to increasing levels of catecholamines, which bind to specific α - and β -adrenergic receptors (AR) present in gingival and PDL fibroblasts, keratinocytes and alveolar bone.¹²⁻¹⁵ *In vivo* studies have shown the functionality of these receptors in the periodontium/periodontal disease using β AR agonists, antagonists or sympathectomy: SNS blockade led to a reduction in alveolar bone loss induced by periodontitis, with inhibition of osteoclastic differentiation.¹⁶⁻¹⁸ It also reduced the release of stress-induced interleukin (IL)-1 β , IL-6, and IL-8.¹⁵ On the other hand, the stimulation of β AR by isoproterenol (ISO, a β 1/2AR agonist) stimulated alveolar bone loss in induced periodontitis in rats.¹⁹

The SNS is involved in the regulation of blood flow and blood vessel formation. It was previously shown that ISO stimulates the production of Vascular Endothelial Growth Factor (VEGF) in mouse long bones, increasing bone vascular density through the activation of the β 2AR in osteoblasts.²⁰ In periodontitis, it is speculated that an increase in vascularization favors inflammation due to higher recruitment of inflammatory cells and nutrients, thus contributing to the progression from a controlled inflammatory status to an exacerbated one, leading to a dysbiotic oral ecosystem and to the onset of periodontitis.²¹ An increase in microvascular density and crevicular VEGF levels have been linked with disease progression and severity.²²⁻²⁴ Considering the relevance of stress as a risk factor for periodontitis, and the less explored role of SNS activation on periodontal vascularization as a potential host susceptibility factor, the goal of this study was to determine the vascular response of the mandibular tissues to β AR stimulation in term of blood vessel formation and its relation to SNS.

MATERIAL AND METHODS

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine (AN 6979) and reported following ARRIVE guidelines. Wild-type C57BL6/J female mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA) and allowed to acclimate for at least one week before start of the experiments. Animals were housed 2–5 per cage, kept on a 12h light-dark cycle in a temperature-controlled environment (22°C), and had water and standard chow *ad libitum*.

Microvascular density (MVD), SNS markers and norepinephrine transporter (NET) evaluation

Twenty 6-week-old female wild-type (WT) C57BL6/J mice (0.0182 ± 0.0011 g) were randomly divided into two groups: ISO group (n=10) in which mice received daily intraperitoneal (IP) injection of ISO (3 mg/Kg/day),²⁰ and a PBS group (n=10) in which mice received daily IP injections of phosphate buffered saline (PBS), for 4 weeks. At the completion of the treatment period, mice were weighed, anesthetized in an isoflurane chamber, and perfused with 4% paraformaldehyde solution (PFA, 441244, Sigma). Hearts removed, weighed and hemimandibles were collected and fixed in 4% PFA for 24 hours. Specimens were decalcified in ethylenediaminetetraacetic acid (EDTA) solution (0.5M, pH 7.4, ED2SS, Sigma) at 37°C for 10 days. After routine paraffin embedding process, 4 μ m thick serial sagittal sections from right hemimandibles were used for MVD assessment (PBS n=9, because one sample was lost during histological processing/ ISO n=10). The left hemimandibles of PBS group were taken in three different planes (sagittal, coronal, and transversal) to be immunostained for TH, NPY and NET (n=5) in order to evaluate the normal sympathetic innervation pattern and the presence of NET in nontreated animals.

After dewaxing and hydration, antigen retrieval was performed using Tris-EDTA buffer (pH 9) at 80°C for 20 min in a water bath. For TH, NPY and NET sections were permeabilized with 0.1% Triton-X in PBS, then all sections were blocked using 5% normal Goat or Donkey serum in PBS-Tween 20. Immunostaining was performed using a Rat anti-Mouse endomucin (1:100, Sc65495, Santa Cruz Biotechnology), Rabbit anti-NET (1:100 dilution in TBS/1%BSA, PA5-77494, Invitrogen), Rabbit anti-TH (1:200 dilution in blocking solution, AB152, Millipore), or Rabbit-anti-NPY (1:100 dilution in blocking solution, H-049-03, Phoenix Pharmaceuticals) antibodies at 4°C overnight. Slides were then washed and incubated with a Goat anti-Rabbit or Donkey anti-mouse IgG Alexa Fluor 594 conjugated secondary antibody (1:200 for Endomucin, AB_2340859; 1:500 for NET; 1:1000 for NPY and TH, diluted in TBS/1%BSA, 115-587-003, Jackson Immuno) at room temperature for 1-3 hours. The nucleus was counterstained using Hoechst (1:10000, H3569, Thermofisher). Brain sections were used as positive controls (Supplementary Figure 1) and primary antibody was omitted in negative controls.

For SNS markers, images from one slide/animal were acquired using a Nikon A1-Rs laser scanning confocal microscope, with either a 20x Plan apo/0.75NA or a 40x Plan fluor/0.75NA lens. To evaluate NET density, the slides were automatically scanned at 20x using BioTek Cytation 5 (DAPI and TxRED filters). Three regions of interest (ROIs) in the furcation area of the second molar were defined using FIJI software (fiji.sc): one delimitating the alveolar bone; one in the PDL; and one encompassing the osteoblastic layer. After delineation of the ROIs, the number of NET⁺ cells per total number of cells was determined and a percentage of NET⁺ cells per region was calculated.

MVD was assessed in three sections per animal, which were captured and analyzed using Bioquant software (2017 version, Bioquant Image Analysis Corporation) with a 20x lens in a fluorescent microscope. The ROIs were delineated in the mesial, distal, and furcation areas of the second molar for the alveolar

bone and PDL. For the mesial and distal sites, the ROI was delimited by the cemento-enamel junction (top), the roots of the second and third, or first molar (lateral) to the apex of each tooth (bottom) (Supplementary Figure 2a). In the furcation area, the ROI included the interradicular septum and was limited by the cementum surface in the furcation region (Supplementary Figure 2b). It was also evaluated in the pulp region (Supplementary Figure 2c). Then, endomucin⁺ surfaces were selected and the area of endomucin⁺ cells per total area of the selected ROI was calculated as well as the total number of vessels. Mean values of the alveolar bone, PDL, and pulp ROIs were used in the statistical analysis as the molar region value for each animal.

Gene expression in the mandible and tibia using RT-qPCR

Twelve WT 6-weeks-old female C57BL6/J mice were randomly divided into two groups as described above. Two hours after ISO or PBS injection, mice were euthanized in the isoflurane chamber and the mandibular alveolar bone with the molars and the tibias were dissected out and snap-frozen in liquid nitrogen. All tissues were pulverized using a N₂ cooled mortar and pestle and transferred to TRIzol (15596018, Invitrogen). RNA extraction was performed according to the manufacturer's instruction and RNA quality confirmed by 260/280 ratio between 1.8-2.0 and by the presence of rRNA bands on agarose gel electrophoresis. Samples with low RNA quality were not used in the further transcription process. The isolated RNA was resuspended in ultrapure DNase/RNase free water. RNAs were treated with DNase I (DNase I amplification grade, 18068015, Thermo Fisher) before cDNA synthesis to eliminate DNA contaminants. For cDNA preparation, reverse transcriptase and random primers were used according to the manufacturer's instructions (High-capacity cDNA Reverse Transcription kit, 4368813, Thermo Fisher). The cDNA amplification process was performed with 140ng in a total final volume of 10 μ L in a thermocycler (95°C 3 min, 40 cycles 10sec 95°C, 45sec 60°C) using SYBR green (iQ SYBR Green Supermix, 1725272, BioRad). The specificity of the reaction was verified by melting curve analysis and amplification efficiency was between 90–110% for all the genes. *Gapdh* was used as endogenous control after analysis of *Gapdh*, *Hprt*, and *Tbp* genes using refFinder.²⁵ Primer sequences are summarized in Table 1. Evaluation of gene expression was performed using the $2^{-\Delta\Delta CT}$ method. For *Net* analysis, expression was quantified in the alveolar bone (the teeth were removed) and the tibia from 2-month-old (n=4) mice that received no treatment.

Gene	Forward	Reverse
<i>Gapdh</i>	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
<i>Tbp</i>	CCTTGTAACCTTCACCAATGAC	ACAGCCAAGATTCACGGTAGA
<i>Hprt</i>	TCAGTCAACGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
<i>Vegfa</i>	CACGACAGAAGGAGAGCAGAAG	CTCAATCGGACGGCAGTAGC
<i>Il-6</i>	TAGTCCTTCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
<i>Il-18</i>	GGAGAACCAAGCAACGACAAAATA	TGGGGAAGTCTGCAGACTCAAAC
<i>Rankl</i>	TGCCTACAGCATGGGCTTT	AGAGATGAACGTGGAGTTACTGTTT
<i>Net</i>	AGGCGCTCCTTTCTTGAA	AGAGGGACTTCGGAGGTTCT
<i>Adrb2</i>	TGGGGCCAGTCACATCCTTAT	TGACGCACAACACATCAATGG

Table 1: Primer sequences for RT-qPCR reaction.

Statistical analysis

The sample size was calculated based on the primary outcome of MVD previously published.²⁰ For statistical analysis (experimental unit=animal; $\alpha=0.05$; GraphPad Prism version 6.0), after normality distribution evaluation, Student's t-test was used for MVD, heart weight/body weight ratio, and gene expression analysis between the tibia and mandibular molar region/alveolar bone.

RESULTS

Innervation of the mandibular molar region and impact of β AR stimulation on microvascular density and gene expression

We first determined the pattern of SNS innervation in the mandibular molar region. Sections in three different planes were obtained from five WT mice and stained for the sympathetic markers Tyrosine hydroxylase (TH) and Neuropeptide Y (NPY). Immunofluorescence (IF) signals for TH immunoreactivity were observed as punctuate structures around blood vessels corresponding to transversal sections of sympathetic neurons (Figure 1a, b). These structures were mainly located in the PDL and occasionally in the bone marrow area of the alveolar bone (Figure 1c). On these 2D thin sections, we could not detect any ramification of fibers from vessels towards the marrow or bone surfaces, nor close contact between TH⁺ cell processes and osteoblasts. The TH signal was also observed in the cytoplasm of PDL fibroblasts and odontoblasts (Supplementary Figure 3). Similar to TH immunoreactivity, NPY signals were observed around blood vessels, thus confirming the sympathetic origin of these fibers. Signal for NPY was also observed in PDL cells, in osteoblasts lining alveolar bone and osteocytes embedded into the alveolar bone (Figure 1d). Nerves of sympathetic origin thus innervate mandibular molar region tissues and are associated with blood vessels, similarly to what was observed in long bones.^{26,27}

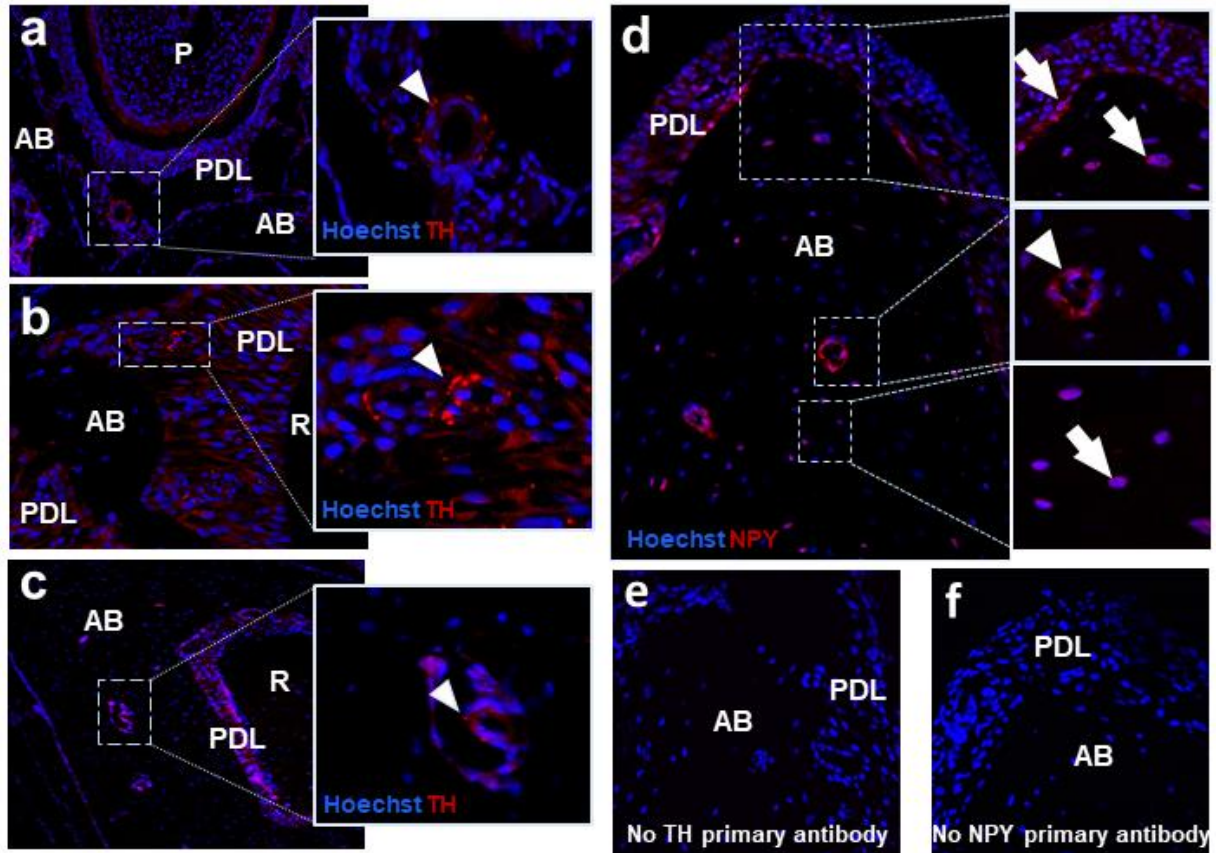


Figure 1: Sympathetic nervous system markers in the molar region (immunofluorescence, 6 weeks-old wild-type C57BL/6 mice, n=5, 2 sections per animal). a) Coronal section of the first molar region, showing puncta structures around blood vessels (arrow head), positive for TH (red). b) Transversal section showing the same pattern of distribution of the TH positive fibers. Insert from the transversal section showing TH fibers and scattered dots in the proximity, probably sprouting nerves (arrow head). c) Transversal section showing that positive fibers for TH were sparsely seen in the alveolar bone, always associated with blood vessels (arrow head). d) Neuropeptide Y (NPY) immunostaining in the furcation area, in sagittal sections, around blood vessels (arrow head), and positivity for osteoblasts and osteocytes (arrows) was also observed. e-f) Negative control where the primary antibodies for TH (e) and NPY (f) were omitted. Original magnification of 20x and 40x on the inserts. Periodontal ligament (PDL), Alveolar bone (AB), Root (R).

To determine if β AR stimulation enhances periodontium vascularization, WT mice were treated daily for 4 weeks with ISO or PBS, and microvascular density (MVD) was quantified histologically. ISO was used to mimic sympathetic activation during stress response, without interfering with the HPA axis, and the 4-weeks timeframe of ISO daily injections was chosen based on our previous findings where such regimen and dose increased MVD in long bones²⁰. Unexpectedly, ISO treatment did not increase endomucin+ vessel area nor the total number of endomucin+ vessels in any of the regions investigated (molar region, alveolar bone, PDL and dental pulp), when compared with the PBS group (Figure 2a), despite similar mRNA expression of *Adrb2*, the gene coding for the β 2AR, between molar region and long bone tissues (Figure 2b). The increased heart weight/body weight ratio in the ISO group also confirmed drug

activity (Figure 2c). Consistent with these results, the expression of direct β 2AR target genes including *Vegfa*, *Il-6*, *Il-1 β* , and *Rankl* in the molar region did not change 2h following ISO administration, whereas the expression of *Vegfa*, *Il-1 β* , and *Rankl* increased in the tibia of these animals (Figure 3a-d), as we reported previously.²⁰ The 2-hrs timepoint was chosen to assess direct β 2AR target genes in response to ISO, as signaling for most G-protein coupled receptors is tightly regulated at multiple downstream levels to maintain homeostasis. This is the case for the β 2AR and expression of *Vegf*, which returns to baseline after 24hrs.^{20,28}

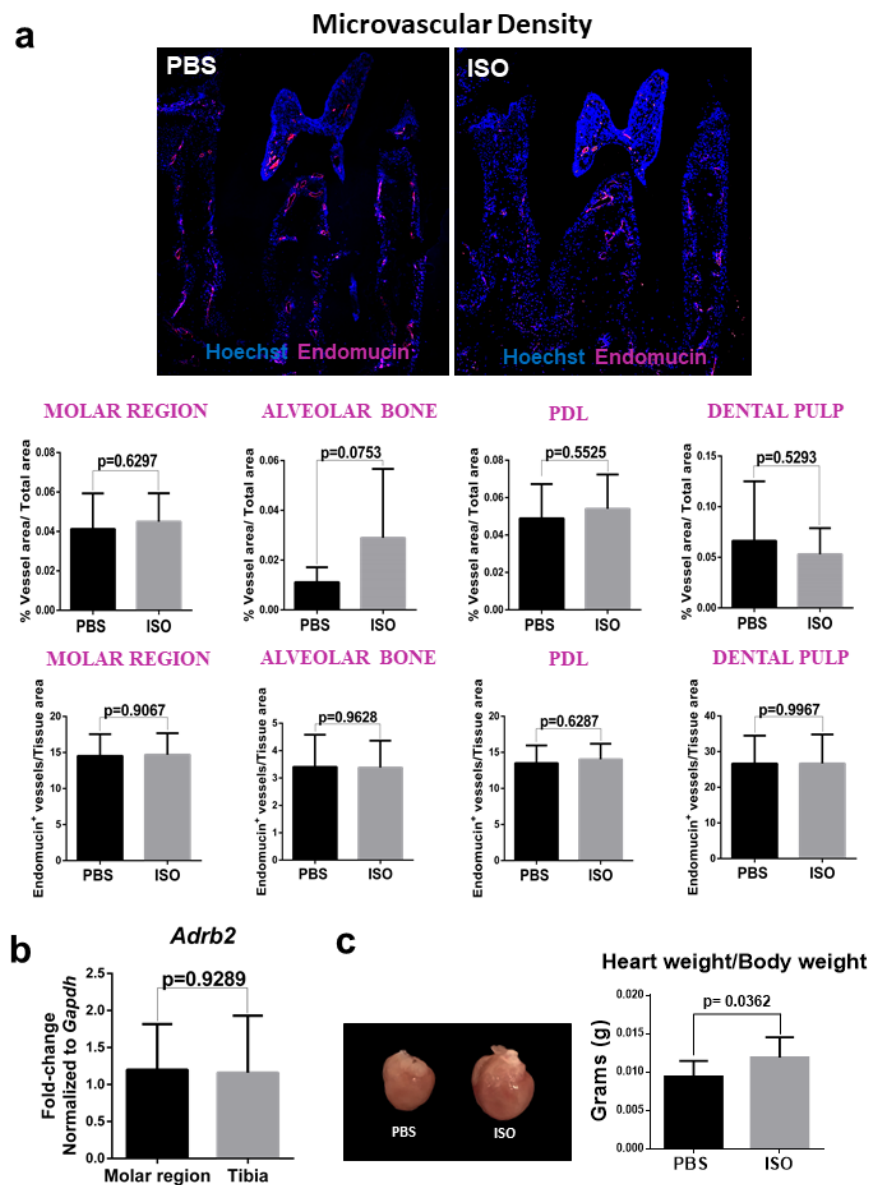


Figure 2: Blood vessel density in the mandibular molar region in response to β -AR stimulation by isoproterenol (ISO). Wild-type 6 weeks-old C57BL/6 mice were treated with ISO or PBS for 4 weeks. a) Graphs shows means $\pm$ standard deviation of the ratio Endomucin+ area/ Total area and Total number

of endomucin+ vessels/ Tissue area in the molar region and separately in the alveolar bone, PDL and dental pulp (T-test, PBS n=9, ISO n=10; mean of 3 slides per animal), showing no significant effect of ISO treatment on microvascular density. On the upper portion of the figure, photomicrographs of second inferior molar furcation area from a representative section from each group. Original magnification of 20x. b) Graph of mean $\pm$ standard deviation of *Adrb2* mRNA expression in the molar region and tibia, showing similar expression levels (T-test, Molar region n=6, Tibia n=5). c) Graph of mean $\pm$ standard deviations of heart weight/body weight ratio after one-month ISO treatment, demonstrating the drug activity (T-test, PBS and ISO n=10).

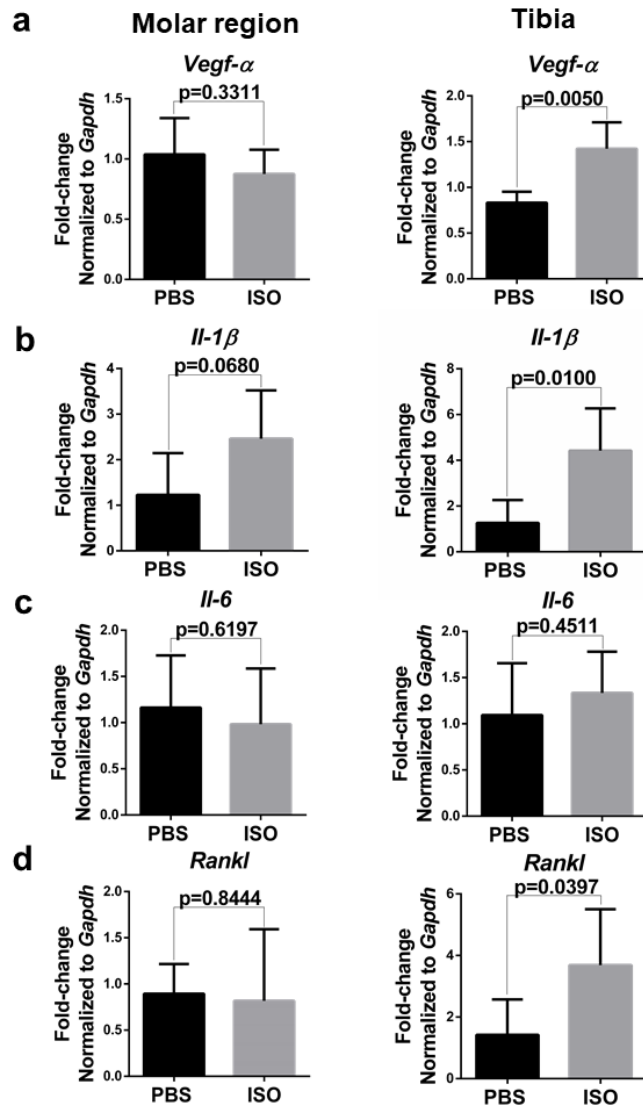


Figure 3: Expression of vascular endothelial growth factor (*Vegfa*), interleukin-1 β (*Il-1 β*) and -6 (*Il-6*) and *Rankl* in response to isoproterenol (ISO) treatment. Wild-type 6 weeks-old C57BL/6 mice were treated with ISO or PBS for 2 hours. Graphs of means $\pm$ standard-deviation for Molar region (on the

right) and Tibia (on the left) for: a) *Vegfa*; b) *Il-1 β* ; c) *Il-6* and d) *Rankl* (t-test, Molar region: PBS n=5, ISO n=5; Tibia: PBS n=4-5, ISO n=5-6).

NET is expressed in osteoblasts, osteocytes, and PDL cells of the mandibular molar region

The differential response of mandibular tissues and long bone to the exogenous β AR agonist ISO in term of β AR target gene expression and MVD led us to investigate other components involved in the regulation of sympathetic outflow. SNS signaling involves post-synaptic β ARs but also a number of mechanisms that regulate the release of NE from central and peripheral sympathetic nerves.²⁹ Among them is NE reuptake by NET, which is highly expressed in presynaptic sympathetic neurons and responsible for the clearance of NE from the extracellular space and the replenishment of NE stores. NET has been detected in differentiated osteoblasts and osteocytes from long bones, where it is postulated to contribute to the control of NE bone marrow content and β AR signaling in bone cells.^{26,30} We thus investigated the presence of NET in the mandible alveolar bone using IF. A specific NET IF signal was observed in the osteoblastic layer (Figure 4a), in PDL cells (Figure 4b) and in bone-embedded osteocytes (Figure 4c). The density of NET⁺ osteocytes and osteoblasts in the furcation area (50.79%) was higher than the density of NET⁺ PDL (30.65%). In addition, *Net* mRNA expression was higher in the alveolar bone than in the tibia from the same animals (Figure 4e).

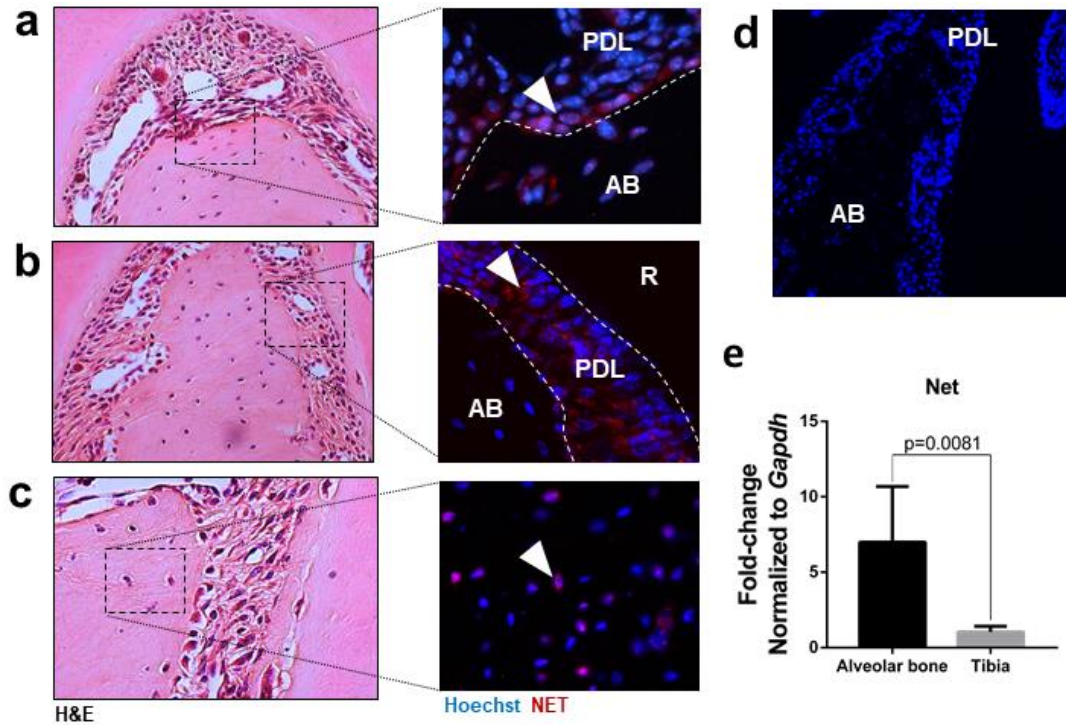


Figure 4: NET protein expression in the molar region and tibia. a-d) Immuno-positive staining for NET was observed in the osteoblastic layer (arrowhead, a), periodontal ligament cells (arrowhead, b) and bone-embedded osteocytes (arrowhead, c). Original magnification of 20x (left, H&E staining) and 40x

(right, immunofluorescence). Primary antibody omission was used as negative control (d), original magnification of 20x (Wild-type 6 weeks-old C57BL/6 mice, n=5, 4 sections per animal). e) *Net* mRNA expression in the alveolar bone compared to tibia (qPCR, t-test, Wild-type C57BL/6 mice, n=4. Teeth were removed for this analysis).

DISCUSSION

We have previously shown that β 2AR stimulation by ISO promotes VEGF expression and blood vessel formation in adult mouse long bones, where it contributes to skeletal breast cancer metastasis.²⁰ High VEGF levels are associated with periodontitis severity, since vascularization is critical to the presence of inflammatory cells in the periodontal tissue and is the conduit of nutrients for periodontal pathogenic bacteria, such as *P. gingivalis*, which uses heme for survival and growth.²²⁻²⁴ Hence, we asked whether the pro-angiogenic action of β AR agonists in long bones would occur in the mandibular molar region. Unexpectedly, we found that ISO did not increase the MVD nor the expression of *Vegfa* in the mandibular molar region, despite similar expression level of *Adrb2* between tibia and mandibular molar region tissues. Although we cannot exclude at that point a reduction in β 2AR protein level or cell surface expression due to challenges in detecting this transmembrane receptor by IF, the differential response of these two tissues may stem from their different embryonic origin. Facial bones originate from the neural crest through intramembranous ossification while the axial skeleton is mesoderm-derived and formed by endochondral mechanisms.³¹ Others have noted differences between these two tissues: mesoderm-derived and neural crest-derived osteoblasts for instance display different osteogenic potentials *in vitro*;³² Mandibular osteoblasts deposit more mineralized matrix and express higher levels of alkaline phosphatase than tibial osteoblasts;³² Others have also shown that different bone envelopes, such as femoral periosteum and different parts of the mandible (alveolar wall, periosteum, retromolar endosteum) respond differently to agonists/antagonists of the catecholaminergic pathway.¹³ The higher expression of VEGF observed in femurs compared to mandible, both in fetus and adults at RNA and protein levels, also support differential mechanisms controlling vessel density between these two tissues.³³

The presence of TH was detected in neurons around blood vessels but also in PDL cells and odontoblasts. Such unexpected immunoreactivity was also reported by others.^{15,34,35} Restrain stress was shown to enhance TH expression in PDL fibroblasts,¹⁵ and TH levels were shown to be high in humans with periodontitis.³⁴ Leptin, a hormone capable of activating the SNS, was also associated with high levels of TH in PDL fibroblasts *in vitro* and *in vivo*.³⁴ The biological and pathophysiological relevance of TH expression in extra-neuronal periodontal tissues remains to be determined.

The influence of adrenergic signaling on periodontitis has been supported by the use of beta-blockers, which block β AR signaling.^{16-19,36} Propranolol led to a decrease in serum levels of C-reactive

protein, TNF- α , IL-6 and to a reduction in osteoclasts number in periodontitis.^{17,18} However, the administration of ISO or propranolol did not affect the number of osteoclasts in the healthy alveolar bone,¹³ indicating that β AR stimulation/inhibition has no effect on alveolar bone in the absence of an inflammatory stimuli. These observations suggest that the SNS influences the periodontal environment mainly by its action on inflammatory cells, possibly orchestrating the inflammatory process associated with periodontitis, and independently of changes in blood vessel density. In fact, the SNS represents the main communication between the central nervous system and immune cells, and a pro-inflammatory effect is expected after adrenergic receptors stimulation in inflammatory cells.³⁷⁻³⁹ The use of immunodeficient models will be critical to address this question.

NET does not uptake pharmacological β AR agonists like ISO and thus the expression of this transporter in alveolar bone osteocytes/osteoblasts and PDL fibroblasts, as well as its higher expression in alveolar bone versus long bones, cannot explain the lack of response of mandibular tissues to ISO. However, it supports the notion that mandibular bone tissues have multiple protective mechanisms to blunt the effect of stress and SNS activation. Further studies using selective NET inhibitors and tissue-specific *Net* loss-of-function experiments are necessary to elucidate the functionality of this transporter in periodontal tissues and periodontitis pathogenesis.

CONCLUSION

β AR stimulation by the pharmacological β AR agonist ISO does not increase MVD in the mouse mandibular molar region, as opposed to tibias. Periodontium tissues also express NET, whose role is to reuptake endogenous NE released by sympathetic nerves. These findings demonstrate that the periodontium is innervated but, has a high capacity to limit β AR signaling.

AUTHOR CONTRIBUTIONS

- RMM: Contributed to conception, study design, data acquisition and interpretation, performed all statistical analyses, drafted and critically revised the manuscript.
- ALA and FE: Contributed to funding acquisition, study design and data analysis, drafted and critically revised the manuscript.

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