UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José dos Campos
Instituto de Ciência e Tecnologia

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ESTRESSE E PERIODONTITE: avaliação da inervação simpática no periodonto e da resposta adrenérgica após infecção por *Porphyromonas gingivalis*

2022

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Tese apresentada ao Instituto de Ciência e Tecnologia, Universidade Estadual Paulista (Unesp), Campus de São José dos Campos, como parte dos requisitos para obtenção do título de DOUTOR, pelo Programa de Pós-Graduação em CIÊNCIAS APLICADAS À SAÚDE BUCAL.

Área: Patologia e Diagnóstico Bucal. Linha de pesquisa: Inflamação, reparação tecidual e patologia do sistema estomatognático

Orientadora: Prof. Assoc. Ana Lia Anbinder

São José dos Campos

2022

Instituto de Ciência e Tecnologia [internet]. Normalização de tese e dissertação [acesso em 2022]. Disponível em http://www.ict.unesp.br/biblioteca/normalizacao

Apresentação gráfica e normalização de acordo com as normas estabelecidas pelo Serviço de Normalização de Documentos da Seção Técnica de Referência e Atendimento ao Usuário e Documentação (STRAUD).

Moraes, Renata Mendonça
Estresse e periodontite: avaliação da inervação simpática no periodonto e da resposta adrenérgica após infecção por Porphyromonas gingivalis / Renata Mendonça Moraes. - São José dos Campos : [s.n.], 2022.
107 f. : il.

Tese (Doutorado em Ciências Aplicadas à Saúde Bucal) - Pós-Graduação em Ciências Aplicadas à Saúde Bucal - Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia, São José dos Campos, 2022.
Orientadora: Ana Lia Anbinder.

1. Periodontite. 2. Sistema nervoso simpático. 3. Estresse. 4. Galleria mellonella. 5. Invertebrados. I. Anbinder, Ana Lia, orient. II. Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia, São José dos Campos. III. Universidade Estadual Paulista 'Júlio de Mesquita Filho' - Unesp. IV. Universidade Estadual Paulista (Unesp). V. Título.

Ficha catalográfica elaborada pela Biblioteca Prof. Achille Bassi e Seção Técnica de Informática, ICMC/USP
com adaptações - STATI, STRAUD e DTI do ICT/UNESP.
Renata Aparecida Couto Martins CRB-8/8376

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São José dos Campos, 25 de Janeiro de 2022.

DEDICATÓRIA

Dedico esta tese a todos os pesquisadores que ousam sonhar e perseveram mesmo quando tudo parece ir contra.

AGRADECIMENTOS

Ao Instituto de Ciência e Tecnologia de São José dos Campos (UNESP) e ao programa de pós-graduação em Biopatologia Bucal e Ciências Aplicadas a Saúde Bucal (CASB), pela aprovação no programa e pela disponibilização das atividades e infraestrutura que possibilitaram que este trabalho fosse executado.

À FAPESP pelo financiamento deste projeto (processo 2018/25933-3), concessão da bolsa regular no país (processo 2017/26461-5) e bolsa de estágio no exterior BEPE (processo 2018/21701-0), assim como à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão de 6 meses de bolsa (01/02/2018 - 31/07/2018) e pelo apoio ao programa CASB.

À minha orientadora, Ana Lia Anbinder, pela longa jornada acadêmica, que me introduziu ao mundo da ciência em 2008 com a minha iniciação científica, seguida pelo meu título de mestre em 2014 e início do doutorado em 2018. Ao longo destes anos aprendi, pelo exemplo, bases fundamentais para o desenvolvimento de uma pesquisa sólida, transparente e que dentro da realidade vivida fosse o mais impactante possível. Obrigada por propiciar um ambiente acolhedor aos questionamentos e fértil ao desenvolvimento. Admiro imensamente a sua paixão pela profissão e seu dom para ensinar, que sempre terei como exemplos de competência. Juntas também aprendemos muito sobre planejamento, trabalho em equipe e acima de tudo resiliência. Quantos momentos nos fizeram mais resilientes! Quando no início quase perdi a submissão de um dos nossos artigos porque não sabia que “major revisions” significava submeter com as correções; Os milhões de pilotos para aquele trabalho da *Bone*; Ficar até altas horas da noite fazendo crachás porque a iniciação esqueceu; Sair para comprar tênis que você nem precisava para agradar a *Lab manager*; Viajar no porta mala do carro para que todos pudessem ir te prestigiar na *UTHealth* e tantos outros momentos que dividimos. Começamos como aluna e professora, mas hoje tenho em você uma amiga!

À minha família que, sem nenhuma dúvida, é meu maior alicerce e sem os quais eu nunca teria chegado até esse momento. Obrigada por me ensinarem o valor dos estudos e o peso de poder honrar com a nossa palavra, qualidades que sem dúvida são imprescindíveis tanto na vida quanto na carreira acadêmica.

To my fiancé Dr. Fabio Stossi, definitely the greatest and happiest surprise of my PhD. Together we fell in love with each other and for our babies (G. mellonella)! First you captivated me with all the wonders of microscopy and the knowledge behind pretty pictures. The imaging classes and the core were my happy place at Baylor! So much fun imaging cleared jaws and embryos. Once we got apart, we somehow became even closer, and your excitement and curiosity about my second project were definitely what got me thought it all! We built together every part of it, and thanks to you I have my amazing heat maps! I will never have words to express how grateful I am for every kind and encouragement word you said, every hug (even virtually) and all the tears you bored with me. This victory is ours!

Aos meus colegas de laboratório Jaqueline Ribeiro, Thaís Aguiar e Carlos Matheus Lescura. Apenas quem trabalha com animais sabe da ligação que se cria com aqueles que te acompanham diariamente na labuta. Eu não poderia ser mais feliz de ter tido vocês como meus companheiros durante esses 4 anos.

A minha querida amiga Bruna, irmã que a vida me trouxe em um dos meus piores momentos, mas que como dizem: "Nos campos solitários e nos cumes desérticos nascem as mais belas flores." E a nossa amizade com certeza é uma das mais belas flores do meu jardim! Sua presença fez com que a infinidade que esse doutorado durou ficasse mais leve e divertida.

A minha amiga/parceira Maíra que sempre entrou nas minhas loucuras que resultaram em infinitas horas contando hemócitos e fazendo leituras no espectrofotômetro! Sem a sua ajuda esse trabalho certamente não seria o mesmo. Acima de tudo, sem você o gosto deste doutorado não seria o mesmo! Muito obrigada por cada bolo e delícia que você cozinhou para a gente, e que acompanharam nossas conversas sobre tudo. Obrigada também pelos momentos off do laboratório, quando

pudemos nos divertir, e por me tirar da zona de conforto me levando para a maior trilha de praias da história e a cachoeira mais gelada!

Aos amigos da microbiologia Patrícia, Evelyn, Rodnei e Felipe. Sempre senti a microbiologia como a minha segunda casa na faculdade e com certeza isso se deve a vocês. Patrícia, minha companheira de viagem, almoços, cafés e PCR! Exemplo de profissional competente e que sempre foi solícita para me ajudar e discutir resultados. Evelyn, minha parceira de almoços, coloração de gram, e principalmente “papo de beleza”. Meus dias não eram os mesmos sem você no laboratório, sempre me fazendo rir e meu cabelo é muito mais hidratado depois que nos conhecemos, rs! Rodnei, amigo de congresso que virou amigo para vida e agora brilha longe da gente, mas que sempre deixará sua marca, inclusive neste trabalho, por ter sido um dos pioneiros do cultivo de *P. gingivalis* no laboratório. Por fim, Felipe, que me ajudou muito com a parte de entomologia e que mesmo longe está sempre respondendo nossas dúvidas sobre tudo.

Aos técnicos de laboratório, em especial ao Sérgio, técnico da patologia, por todo o suporte na parte histológica deste trabalho, mas também pela companhia nos momentos de pandemia, quando éramos os únicos no laboratório e ele sempre me deixava aguada por sobremesas fit.

To my abroad supervisor Florent Elefteriou, for receiving me in his lab with open arms and giving me all the support I needed to conduct my research. Also, for training me to be more critic on my way of doing science and thinking about it. Definitely the weekly lab meetings were a game changer for my career. But, most importantly, thank you for all the barbecues and for liking moussaka and Crème brûlée, forcing Efie to cook for us!

To my wifey/roommate Efie. They say that opposites attract each other, and for us it was definitely true! An outgoing, hyperactive, and joyful person meets the introvert, moody and Netflix-addict Brazilian gal. But this match could not have been better! You made my year abroad one of the best of my life. You thought me cell culture, animal work and we troubleshooted lots of histology together, but it was the crazy moments that will be forever with me. Running from snakes, sunset hunting, garage sales that

ended up in drying clothes on the neighbor, travelling to Chicago to stay in a house full of cats, being stuck in the lab because of storms, waking up in the middle of the night with your "OPA" to get appendix surgery, being the queens of Tropicana, and surviving a pandemic together and creating an even deeper connection during lockdown. Thank you for inspiring me to be the BEST scientist out there!

To my lab mates, Greig, Lingzhen and Maria. Greig, you are one of the best scientists I have ever met, thank you so much for helping me to improve my study design skills. Lingzhen for all the support during mice work, and teaching me how to genotype and transforming me on the RNA extraction queen of the lab. But more than that, thank you for making me feel so welcome in a foreign place, and for all the amazing Chinese food you introduced to me! Maria, the lovely girl from Germany. So precise in every experiment, thank you very much for all the support during Cre assessment troubleshoot and for the talks during lunch and happy hours.

À profa. Juliana Campos Junqueira, por sempre abrir as portas da microbiologia para mim, e por permitir que eu pudesse fazer parte do grupo de pesquisadores envolvidos com a criação de *G. mellonella*. Obrigada por todos os seus comentários durante o meu exame de qualificação, que com certeza elevaram a qualidade desta tese, e também pelo suporte durante a análise histológica e por dividir os cafezinhos diários que nos acordavam depois do almoço!

Aos prof. Estela e Rubens Tango que estiveram no mesmo período que eu em Houston, e me acolheram tão generosamente em sua casa quando fiquei sem teto! Por me lavarem para passear e ouvir todos os meus perrengues e a busca pela bota cowboy rosa perfeita! Por todos os happy hours, almoços, cafés brasileiros divididos e por estarem comigo na minha primeira Black Friday americana, fazendo 3 horas de fila passarem tão rápido!

Aos membros da banca, que disponibilizaram o seu tempo e conhecimento para a leitura desta tese, e certamente possibilitaram que ela atingisse o seu maior potencial.

À profa. Mônica, que disponibilizou o CEBAPE para realização de trabalhos em animais e uso do histotécnico e micrótomo. Sempre muito solícita em ajudar e com uma palavra amiga.

Aos amigos que indiretamente fizeram parte dessa jornada, meu muito obrigada!

"Out of the night that covers me,

Black as the Pit from pole to pole,

I thank whatever gods may be

For my unconquerable soul.

In the fell clutch of circumstance

I have not winced nor cried aloud.

Under the bludgeonings of chance

My head is bloody, but unbowed.

Beyond this place of wrath and tears

Looms but the Horror of the shade,

And yet the menace of the years

Finds and shall find me unafraid.

It matters not how strait the gate,

How charged with punishments the scroll

I am the master of my fate:

I am the captain of my soul."

William Ernest Henley

SUMÁRIO

RESUMO

Moraes RM. Estresse e periodontite: avaliação da inervação simpática no periodonto e da resposta adrenérgica após infecção por *Porphyromonas gingivalis* [tese]. São José dos Campos (SP): Universidade Estadual Paulista (Unesp), Instituto de Ciência e Tecnologia; 2022.

O estresse agrava a doença periodontal por vários mecanismos, sendo a estimulação do sistema nervoso simpático (SNS) um deles. A literatura mostra que a estimulação de receptores β-adrenérgicos (β-AR) aumenta a angiogênese em ossos longos, e a expansão microvascular agrava a periodontite. Ainda, catecolaminas aumentam a virulência de periodontopatógenos e agem na resposta imune. Assim, o objetivo deste trabalho foi avaliar: (1) a inervação simpática no periodonto e a influência da ativação do SNS na vascularização periodontal em camundongos e (2) a influência do sistema adrenérgico nos fatores de virulência de *Porphyromonas gingivalis* (Pg) e na resposta imunológica a este patógeno *in vivo* (*Galleria mellonella*). Na primeira parte, camundongos receberam injeção intraperitoneal de solução salina (PBS) ou isoproterenol (ISO; agonista não seletivo β adrenérgico) por 1 mês, para detecção *in situ* de tirosina hidroxilase, neuropeptídeo Y, transportador de norepinefrina (NET) e endomucina em mandíbulas. Expressão de mRNA de *Vegf-a*, *Il-1β*, *Il-6*, *Adrb2* e *Rankl* foi quantificada 2 h após administração de ISO/PBS em mandíbula e tíbias, que serviram como controle positivo. Diferentemente das tíbias, não houve alteração na expressão dos genes analisados em mandíbula. Por outro lado, NET foi mais expresso no osso alveolar do que na tíbia, sendo detectado nos osteoblastos, osteócitos e células do ligamento. Embora o padrão de inervação e a expressão de *Adrb2* sejam semelhantes entre mandíbula e tíbia, o tratamento com ISO não influenciou no número e área de vasos positivos para endomucina. Na segunda parte, investigamos a influência adrenérgica na resposta imune de *G. mellonella* durante infecção por Pg utilizando norepinefrina (NE; agonista α e β adrenérgico) e ISO. Pg também foi cultivada na presença de ISO (PgISO) ou NE para avaliação da ação direta dos compostos na bactéria. ISO sistêmico protegeu as larvas da infecção por Pg, aumentando o número de hemócitos e reduzindo a contagem de células de Pg na hemolinfa, exclusivamente pelo β-AR. Diferentemente, NE aumentou mortalidade, diminuiu o número de hemócitos. Apenas PgISO aumentou a morte das larvas, apesar de ambos, NE e ISO, terem aumentado a expressão de fatores de virulência na bactéria *in vitro*. ISO circulante, concomitante com PgISO, reduziu parcialmente a mortalidade das larvas. A influência do estresse na doença periodontal envolve diversas vias que alteram os dois pilares da periodontite (microbiota e sistema imune). No entanto, a ação na resposta do hospedeiro parece ser superior, uma vez que a estimulação β-AR em osso alveolar saudável não alterou a produção de citocinas pró-inflamatórias ou microvascularização e a modulação da resposta imune em *G. mellonella* por compostos adrenérgicos foi mais importante para o desfecho da infecção que sua ação direta sobre a bactéria.

Palavras-chave: periodontite; sistema nervoso simpático; estresse; *Galleria mellonella*.

ABSTRACT

Moraes RM. Stress and periodontitis: evaluation of sympathetic innervation in the periodontium and the influence of adrenergic signaling on Porphyromonas gingivalis infection [thesis]. São José dos Campos (SP): São Paulo State University (Unesp), Institute of Science and Technology; 2022.

Stress aggravates periodontitis, and one possible mechanism is the activation of sympathetic nervous system (SNS). The literature shows that stimulation of β-adrenergic receptors (β-AR) induces angiogenesis in long bones, and microvasculature amplification was linked to periodontitis severity. Moreover, catecholamines increase the virulence of some periodontopathogenic bacteria in vitro and influences the innate immunity. Thus, the aim of this study was (1) evaluate the presence and influence of the SNS in the stimulation of periodontal vasculature, and (2) the influence of the adrenergic system on Porphyromonas gingivalis (Pg) virulence and on the immunological response to this pathogen in vivo (Galleria mellonella larvae). For the first part, mice received isoproterenol (ISO, a non-selective β-AR agonist) or saline (PBS) for 1 month, for in situ analysis of tyrosine hydroxylase, neuropeptide Y, norepinephrine transporter (NET) and endomucin in the mandibles. Vegfa, Il-1β, Il-6, Adrb2 and Rankl mRNA expression was assessed 2 hours after PBS/ISO treatment for mandibles and tibia, that served as positive control. We observed that, differently from the tibia, the expression of these genes did not alter on the mandible. However, NET expression was detected in osteoblasts, osteocytes, and periodontal ligament fibroblasts, and were higher expressed when compared to the tibias from the same animals. Although the pattern of sympathetic innervation and Adrb2 expression were similar between tissues, ISO treatment did not increase the area or number of endomucin+ vessels. For the second part, we addressed the adrenergic signaling influence on G. mellonella immune system during Pg infection using norepinephrine (NE, α- and β-AR agonist), ISO and octopamine (insect's endogenous hormone). Pg was also cultivated in the presence of ISO (PgISO) or NE to investigate the direct action of the ligands on bacterial virulence. Systemic administration of ISO protected the larvae from Pg infection by increasing hemocyte density accompanied by reduction of Pg load in hemolymph, in a β-AR manner. In contrast, NE increased mortality, with decreased hemocyte count and no influence on the other parameters. Only PgISO increased larvae death, despite of ISO and NE increased virulence in vitro. The concomitant injection of systemic ISO partially reversed the toxicity of the PgISO. The influence of stress on periodontitis involves different pathways, that alter the two pillars of disease's pathogenesis (microbiota and immune system). However, the influence on the host's inflammatory response seems to overcome the other players, since β-AR activation on healthy alveolar bone didn't alter cytokines production or microvasculature. Besides, the modulation of innate immunity by adrenergic signaling in G. mellonella was more important for the disease's outcome than it's direct action on the bacteria.

Key-words: periodontitis; sympathetic nervous system; stress; Galleria mellonella.

1 INTRODUÇÃO

A periodontite é uma doença altamente prevalente, que acomete até 70% dos adultos com 65 anos ou mais (Eke et al., 2016; Billings et al., 2018). É uma patologia inflamatória crônica multifatorial associada à disbiose da microbiota oral, decorrente da complexa interação entre os micro-organismos e a resposta imunológica do hospedeiro, que leva à destruição dos tecidos de suporte do dente (Papapanou et al., 2018). Algumas condições, conhecidas como fatores de risco, são capazes de modular a progressão da doença. Dentre esses fatores encontram-se: condições genéticas (como polimorfismos, síndromes, predisposição genética a diabetes e outras comorbidades), obesidade, tabagismo, idade, doenças sistêmicas (como diabetes/osteoporose) e estresse (Genco, Borgnakke, 2013).

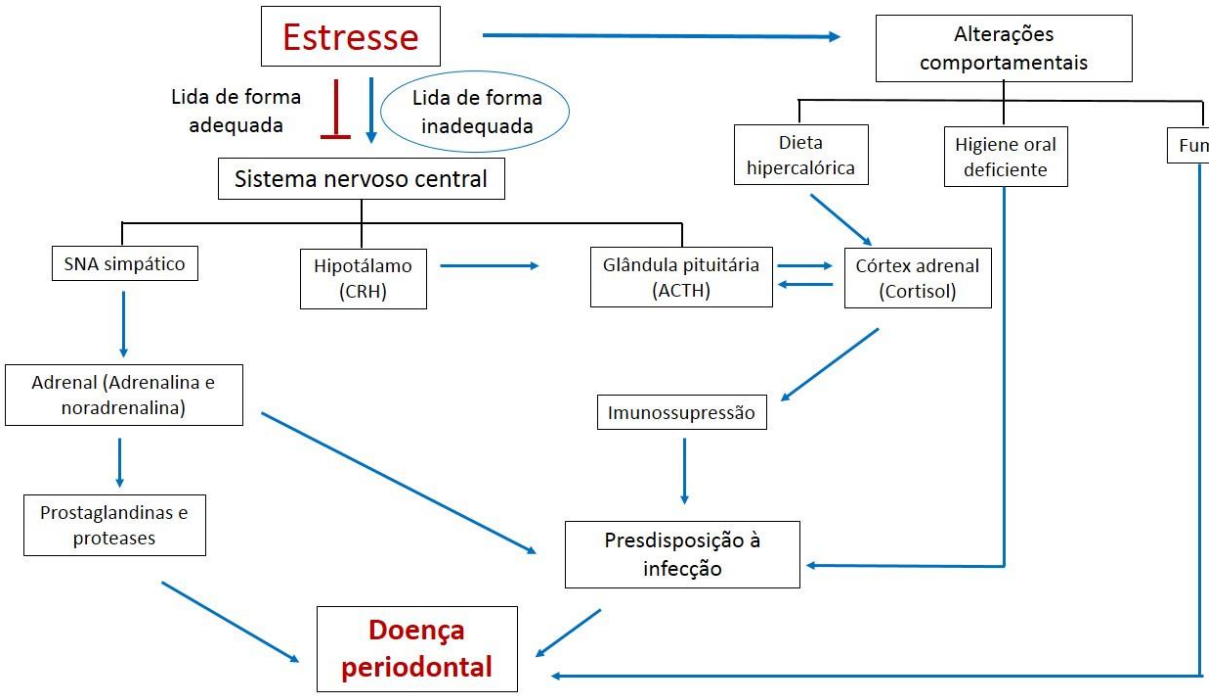
O estresse é definido como um estado em que a homeostase está ameaçada ou prestes a ser ameaçada, e que pode ser desencadeado por uma série de fatores tanto físicos quanto emocionais. O estresse agudo gera prontamente uma resposta fisiológica para atenuar os gatilhos, apresentando uma ação rápida e limitada. Já o estresse crônico se desenvolve após exposição repetida ou prolongada aos estressores (Hering et al., 2015). Uma vez instalado, o estado de estresse ativa o sistema nervoso central (SNC) e periférico, que liberam uma série de neurotransmissores e hormônios. Os principais mediadores periféricos são os glicocorticoides (cortisol), liberados pelo eixo hipotalâmico-pituitário-adrenal (HPA) e as catecolaminas, epinefrina e norepinefrina (NE), liberados pelo sistema nervoso simpático (SNS), tanto pela medula adrenal quanto pelas terminações nervosas periféricas (NE). Durante o estresse crônico há uma liberação sustentada desses mediadores do estresse (epinefrina, NE e cortisol), que deveriam ser liberados em quantidade limitada por um tempo determinado, mas que ficam descontrolados (Chrousos, 2009).

A correlação entre o estresse e a doença periodontal vem sendo evidenciada na literatura. Foi constatado que o estresse leva à congestão e hipertrofia das paredes dos vasos, à hemorragia, e alteração na direção e conformação das fibras colágenas no periodonto saudável de ratos (Antonova, 2008). Ainda, o estresse, combinado à indução de periodontite experimental, pelo método de ligadura ao redor dos molares (Gaspersic et al., 2002) ou por infecção com *Porphyromonas gingivalis* (Nakajima et al., 2006), acentua a perda óssea e de inserção periodontal em ratos. Da mesma

forma, estudos em humanos demonstram piora nos parâmetros clínicos da doença periodontal, como profundidade de sondagem, índice de placa, índice gengival e perda dentária em pacientes estressados (Deinzer et al., 1999; Rosania et al., 2009; Goyal et al., 2011; Rai et al., 2011).

Os exatos mecanismos pelos quais o estresse influencia a doença periodontal ainda não foram totalmente elucidados. Acredita-se que a ativação dos sistemas supracitados com liberação de cortisol e catecolaminas possam interferir na resposta inflamatória do hospedeiro, o que favoreceria o acúmulo de biofilme dental e a disbiose (Genco et al., 1999; Warren et al., 2014). Estudos clínicos demonstram claramente o aumento da liberação de cortisol sérico em pacientes sob estresse (Deinzer et al., 2000; Rosania et al., 2009; Goyal et al., 2011; Rai et al., 2011), e o aumento deste hormônio impacta negativamente o quadro clínico da periodontite devido à imunossupressão. O estresse também pode levar a alterações de comportamento como: tabagismo, negligência com a higiene oral e dieta hipercalórica. O fumo por si só, tem efeito negativo sobre a doença periodontal, por influenciar na microbiota e microvascularização local, predispondo à colonização de bactérias anaeróbias. Também altera a resposta de neutrófilos e produção de citocinas, favorecendo a infecção e a inflamação exacerbada que leva à perda de tecidos periodontais (Genco, Borgnakke, 2013). A má higiene oral predispõe à doença, uma vez que não há a remoção adequada do biofilme. E por fim, a ingestão de dieta hipercalórica pode levar à ativação do córtex adrenal e liberação do cortisol que, como já explanado anteriormente, leva à imunossupressão e agravamento da infecção com piora da doença periodontal (Figura 1) (Genco et al., 1999).

Figura 1 - Esquema das vias de ação do estresse na doença periodontal



Legenda: ACTH: hormônio adrenocorticotrófico; CRH: hormônio liberador de corticotrofina.
Fonte: Esquema extraído e modificado de Genco et al., 1999.

Embora a ação do eixo HPA na patogênese da doença periodontal tenha sido profundamente estudada, a influência do SNS ainda é pouco compreendida. O SNS, na sua região final de atuação, se liga a receptores específicos nas células chamados receptores adrenérgicos (AR), sendo subdivididos em α e β, que são ativados pela epinefrina ou NE. Embora a maioria dos neurônios simpáticos utilizem a epinefrina/NE como neurotransmissores principais, existem populações neuronais não-adrenérgicas que incluem neuropeptídeo Y, galanina, somastatina e opioides (Gibbins, 2013). O SNS age nos dois pilares da patogênese da doença periodontal: hospedeiro (inflamação e células residentes do periodonto) e microbiota.

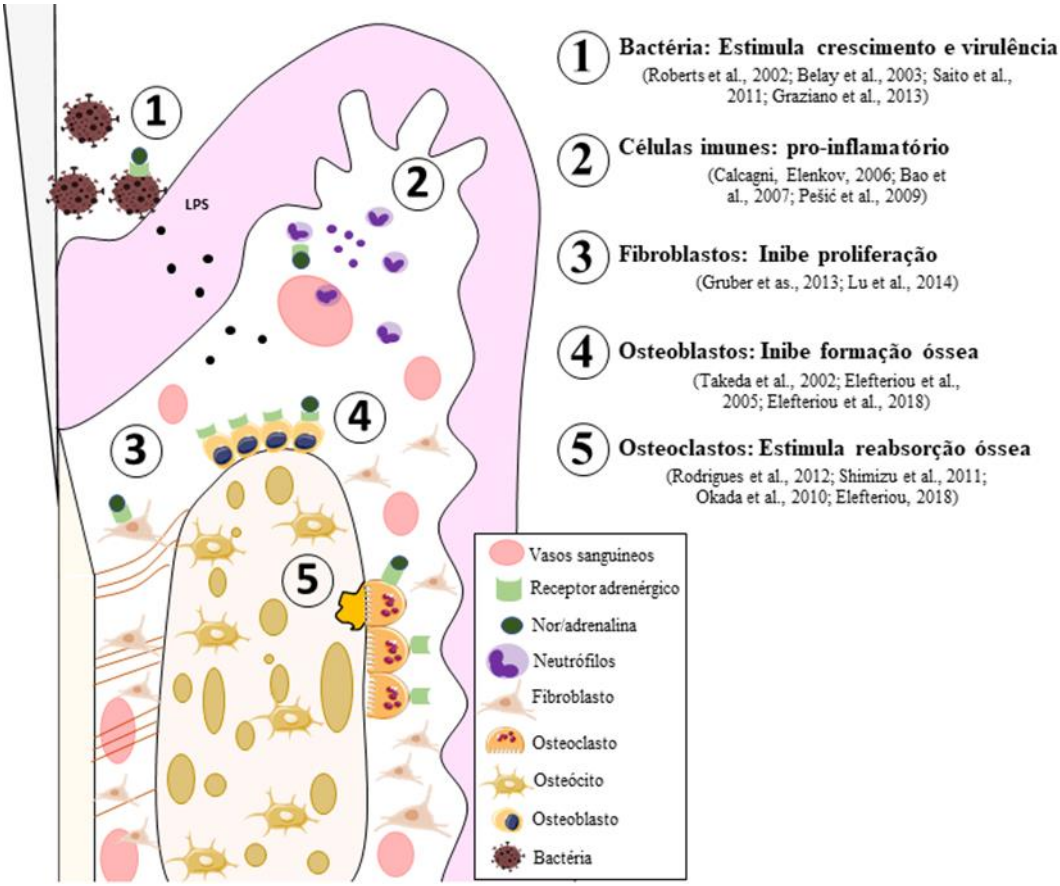
O SNS constitui a principal comunicação entre SNC e a resposta imunológica. Todas as células imunes possuem receptores adrenérgicos, que quando ativados são capazes de modificar o número e proporção das células que participam da resposta imune, assim como alterar a resposta individual dessas células (Calcagni, Elenkov, 2006; Bao et al., 2007; Pešić et al., 2009; Scanzano, Cosentino, 2015). Além da influência do SNS nas células inflamatórias, também foi evidenciada sua ação em células residentes do periodonto por meio dos receptores adrenérgicos. Foi constatado que os fibroblastos da gengiva e do ligamento periodontal expressam β2-

AR, sendo que a administração do isoproterenol (ISO- agonista não seletivo β adrenérgico) e do salbutol (agonista seletivo β2-AR) diminuíram sua proliferação *in vitro*, fenômeno que foi abolido pela adição do propranolol (antagonista β-adrenérgico; Gruber et al., 2013). O mesmo foi evidenciado para o α1-AR, sendo que houve diminuição da proliferação celular e aumento da expressão de α1-AR após o tratamento de células do ligamento periodontal com lipopolissacarídeo (LPS). Além disso, o bloqueio do α1-AR (fentolamina, antagonista não seletivo de receptores α adenérgicos) inibiu a produção de interleucina (IL)-1, IL-6 e IL-8 (Lu et al., 2014).

Alguns estudos *in vivo* suportam a hipótese do envolvimento dos β-AR na patogênese da doença periodontal. Observou-se inibição da diferenciação osteoclástica e redução da perda óssea alveolar em ratos tratados com propranolol, por injeção intraperitoneal ou via oral (Okada et al., 2010; Shimizu et al., 2011; Rodrigues et al., 2012). Em estudo do nosso grupo de pesquisa, verificamos que o propranolol e o ISO apresentam efeitos antagônicos na perda de colágeno gengival durante a indução da doença periodontal em ratos e, que ISO aumentou a perda óssea alveolar (Martins et al., 2017).

A influência do SNS no controle do metabolismo ósseo, modulando a reabsorção óssea e a atividade das células ósseas, é bem estabelecida (Elefteriou, 2018). Os primeiros indícios da influência neuronal no metabolismo ósseo foram observados pela descoberta da regulação da reabsorção óssea pela leptina, hormônio sintetizado pelos adipócitos, que age no metabolismo energético e controle do apetite. Foi evidenciado em experimentos com animais *knockout* em leptina, que estes animais apresentavam maior massa óssea (Elefteriou, 2005). A leptina também estimula a atividade do SNS, causando a diminuição da massa óssea através da ativação de β2-AR expresso em osteoblastos, levando à diminuição da proliferação dessas células (Takeda et al., 2002). Similarmente, camundongos *knockout* para β2-AR apresentam massa óssea aumentada (Elefteriou, 2005). A estimulação de β2-AR em camundongos e ratos promove uma resposta osteoclastogênica, mensurada pelo aumento da perda óssea e formação de osteoclastos. Acredita-se que este efeito é devido ao aumento de Ligante do Receptor Ativador do Fator Nuclear Kappa B (RANKL) e IL-6, e a estimulação dos β-AR também afeta a formação óssea inibindo a proliferação osteoblástica (Elefteriou, 2018) (Figura 2).

Figura 2 - Desenho esquemático de um dente e do periodonto associado, evidenciando dos potenciais mecanismos de ação do SNS na iniciação e progressão da doença periodontal



Legenda: Os números indicam o efeito da ativação do sistema nervoso simpático nos vários agentes associados à patogênese da doença periodontal. As fibras simpáticas presentes no periodonto liberam catecolaminas que se ligam aos receptores presentes nas células desta região. Epinefrina/NE podem agir no biofilme, estimulando o crescimento de algumas bactérias e a virulência de outras, como Porphyromonas gingivalis, bactéria keystone para o desenvolvimento da periodontite (1). As catecolaminas também podem se ligar a receptores adrenérgicos nas células inflamatórias, favorecendo um perfil pró-inflamatório, promovendo agregação plaquetária e ativação de neutrófilos (2). Ao se ligarem aos receptores nos fibroblastos, inibem a sua proliferação (3), impactando no reparo e remodelação tecidual. Nas células ósseas, já foi provado que as catecolaminas inibem a proliferação de osteoblastos em ossos longos (4) e promovem osteoclastogênese (5) também no osso alveolar, podendo agravar assim a perda óssea. Fonte: Elaborada pelo autor.

Além dos efeitos diretos nos receptores adrenérgicos das células ósseas, o SNS pode causar alguns efeitos indiretos no osso, como a regulação do suprimento sanguíneo. Nosso grupo constatou que o ISO estimula a produção do fator de crescimento vascular endotelial (VEGF) em ossos longos de camundongos adultos e em células tronco da medula óssea, aumentando a densidade vascular óssea, com aumento de vasos CD31 e endomucina-positivos (endomucina+), através da ativação

do β2-AR em osteoblastos (Mulcrone et al., 2017). A presença de neuropeptídeos do SNS foi identificada em associação aos vasos sanguíneos do periodonto. Fibras nervosas imunorreativas ao neuropeptídeo Y foram identificadas ao redor dos vasos sanguíneos, no gânglio trigeminal, polpa dental e ligamento periodontal de ratos saudáveis (Wakisaka et al., 1996). Ainda em artérias e arteríolas do ligamento periodontal de ratos saudáveis, observou-se a presença de fibras positivas para a tirosina-hidroxilase (TH, enzima que converte a L-DOPA em dopamina, precursora da epinefrina e NE) (Norevall et al., 1996). A TH é um dos mais relevantes biomarcadores da superatividade do SNS durante o estresse (Dunkley et al., 2004), sendo que houve aumento da expressão de TH no ligamento periodontal de ratos com e sem doença periodontal expostos ao estresse (Lu et al., 2014), e a marcação imuno-histoquímica de TH mostrou-se aumentada em humanos com doença periodontal (Memmert et al., 2019). No entanto, a sua relação com a periodontite ainda é objetivo de investigação. Uma vez que nosso estudo anterior evidenciou o efeito indireto da ativação dos β2-AR em osteoblastos na densidade vascular em ossos longos (Mulcrone et al., 2017), hipotetizamos que a ativação dos receptores adrenérgicos no osso alveolar após a liberação de epinefrina/NE pelas fibras nervosas simpáticas, como a que ocorre em situações de estresse, poderia induzir a liberação do VEGF, aumentando assim a densidade microvascular (MVD) local.

O VEGF é o principal fator de crescimento relacionado à angiogênese e permeabilidade vascular, com papel importante nas doenças inflamatórias crônicas, dentre elas a periodontite (Aspriello et al., 2009). Esse fator de crescimento parece estar envolvido no início e progressão da gengivite e periodontite, principalmente promovendo a expansão vascular observada na inflamação (Aspriello et al., 2009). A presença do VEGF e seu receptor VEGFR foram observados em pacientes com doença periodontal leve, moderada e severa (Vladau et al., 2016). Em condições saudáveis não há a expressão de VEGF/VEGFR nos tecidos periodontais, já nas lesões periodontais iniciais o VEGF aumenta a MVD no estroma. Na periodontite moderada e avançada há aumento significante da presença de VEGF/VEGFR (coloração imuno-histoquímica) tanto no epitélio quanto no estroma (Artese et al., 2010; Kasprzak et al., 2012; Vladau et al., 2016). Acredita-se que o aumento da MVD pode aumentar a liberação de diferentes citocinas, moléculas de adesão, e outros fatores inflamatórios, sendo que na periodontite este aumento no transporte das células inflamatórias, nutrientes e oxigênio causado pela angiogênese pode aumentar

a severidade da inflamação. Assim o VEGF agiria como um agente pró-inflamatório na periodontite (Aspriello et al., 2009; Afacan et al., 2019).

A ação do SNS é estreitamente controlada através da recaptura das catecolaminas liberadas no meio pelo transportador de NE (NET- *norepinephrine transporter*). Este transportador está presente nas fendas sinápticas e constitui um importante mecanismo de feedback negativo para limitar a duração do estímulo simpático e para repor os estoques de NE nas fibras nervosas. No entanto, foi evidenciado que outras células, além dos neurônios, expressam esse transportador, como osteoblastos e osteócitos maduros em ossos longos e que essas células são capazes de captar e metabolizar a NE (Ma et al., 2013; Zhu et al., 2018). Camundongos *knockout* para NET apresentaram redução da formação óssea e aumento na reabsorção, resultando em diminuição da massa óssea (Ma et al., 2013). Também foi observado que a expressão tanto de mRNA quanto proteica desse transportador diminui com a idade, e que os níveis de NE aumentam no fêmur em camundongos mais velhos. Esse aumento não foi acompanhado pelo aumento no precursor de NE (L-DOPA) e nem do seu metabólito 3,4-diidroxifenilglicol (DHPG), sugerindo que pode ser decorrente do déficit em recaptação da NE (Zhu et al., 2018). Tais achados sugerem que a NE liberada e recaptada pelos neurônios, osteoblastos e osteócitos representa parte de um mecanismo complexo de homeostase no qual o SNS controla o metabolismo ósseo. A presença desse mecanismo nos osteoblastos e osteócitos do osso alveolar ainda é desconhecida.

Além dos efeitos no hospedeiro, as catecolaminas também demonstram ação nos micro-organismos. A endocrinologia microbiana é a área de estudo que reconhece uma relação bidirecional entre fatores neuroendócrinos do hospedeiro e da microbiota (Lyte,1992). A comunicação entre as espécies bacterianas e os hormônios do hospedeiro se dá através de estruturas conhecidas como sensores de quórum (*quorum sensing*), envolvidas no mecanismo de comunicação célula-célula e responsáveis por alterações genéticas na bactéria frente a estressores. Esses receptores respondem tanto à epinefrina quanto à NE e estudos *in vitro* demonstram a estimulação do crescimento de algumas bactérias como: *Fusobacterium nucleatum*, *Tannerella forsythia*, *Escherichia coli*, *Enterobacter sp*, *Shigella sonnei*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Actinomyces naeslundii*, *Actinomyces gerenscseriae*, *Eikenella corrodens* e *C. gracilis* pelas catecolaminas (Belay, Sonnenfeld, 2002; Belay et al., 2003; Roberts et al., 2002; Roberts et al., 2005; Weinstein et al., 2015). No

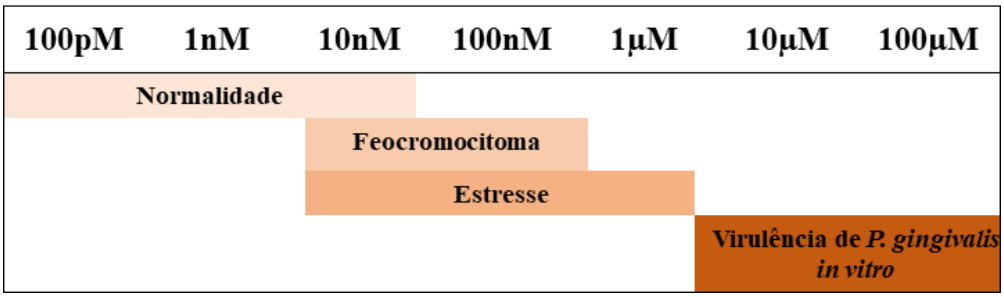
entanto, a estimulação do crescimento bacteriano pelas catecolaminas não é uniforme, variando de acordo com o neurotransmissor e cepa bacteriana (Boyanova, 2017).

Interessantemente, parece que as catecolaminas não são capazes de induzir o crescimento de algumas bactérias gram-negativas, dentre elas *P. gingivalis* (Belay et al., 2003; Graziano et al., 2013), tendo por vezes, efeito inibitório (Roberts et al., 2002). *P. gingivalis* é uma bactéria *keystone* relacionada com o desenvolvimento da periodontitie em humanos e capaz de induzir doença em roedores, e que apresenta capacidade de invasão dos tecidos e manipulação da resposta inflamatória do hospedeiro, estando relacionada inclusive a efeitos sistêmicos da periodontite como doenças cardiovasculares, neurológicas e artrite reumatoide (Hajishengallis, 2014; Fiorillo et al., 2019). Cepas distintas de *P. gingivalis* (ATCC 33277, ATCC 49417, FDC381) tiveram seu crescimento inibido na presença de NE (Belay, Sonnenfeld, 2002; Roberts et al., 2002; Saito et al., 2011). Porém, foi constatado que apesar de não induzir o crescimento de *P. gingivalis* (FDC381 e W83), a NE é capaz de aumentar a expressão de gingipaínas e, consequentemente, sua virulência (Graziano et al., 2013; Boyanova et al., 2017). Este aumento é abolido quando propranolol é administrado, sugerindo que *P. gingivalis* apresenta um sensor catecolaminérgico (Saito et al., 2011). Além da gingipaína (*rgpb*), também foi constatada a interferência da NE em genes relacionados ao metabolismo do ferro (TonB-dependent receptor HmuR- *hmuR*), adaptação ao estresse oxidativo (Alkyl hydroperoxide reductase subunit F- *ahpF*, *oxyR*, DNA-binding Protein from Starved cells - *dps*, Superoxide Dismutase B - *sodB*, acid phosphatase - *aphC*) e proteólise (Hemolysin - *hem*) (Saito et al., 2011; Graziano et al., 2013).

Apesar da comprovada ação da NE sobre os micro-organismos, as concentrações utilizadas nos estudos ultrapassam as encontradas no estado de normalidade. As concentrações séricas de N em humanos numa situação de homeostase variam entre 112-1109pg/mL (0,7nM-7nM) (American Board of Internal Medicine-ABIM, 2020), sendo que durante o estresse essa concentração pode variar até 20 vezes (10nM-100nM) (Boyanova et al., 2017). Já em estados patológicos como por exemplo feocromocitoma, a concentração pode-se elevar até 3161 pg/mL (20 nM) (Rocha et al., 2005). Deste modo de 0,1nM a 10nM estaríamos simulando concentrações fisiológicas, de 100nM-1µM situações de estresse e feocromocitoma (neoplasia caracterizada por liberação excessiva de NE). Os estudos *in vitro* utilizam

100µM para demonstrar a ação da NE na virulência de *P. gingivalis* (Saito et al., 2011; Graziano et al., 2013) (Figura 3).

Figura 3 – Concentrações de NE, e suas correspondências a estados fisiológicos, de estresse, feocromocitoma e influência na virulência de *P. gingivalis in vitro*



Fonte: Elaborada pelo autor, baseado em American Board of Internal Medicine-ABIM, 2020; Boyanova, 2017; Rocha et al., 2005; Saito et al., 2011; Graziano et al., 2013.

Galleria mellonella uma mariposa que têm a sua fase larval utilizada como modelo invertebrado para o estudo da interação patógeno/hospedeiro, pois pode ser mantida à temperatura semelhante à dos mamíferos (37ºC), é de fácil manipulação, baixo custo e os resultados encontrados com relação aos fatores de virulência podem ser correlacionados àqueles realizados em modelos vertebrados. O sistema de defesa imunológica deste inseto apresenta ainda similaridades com o de animais vertebrados (Pereira et al., 2018).

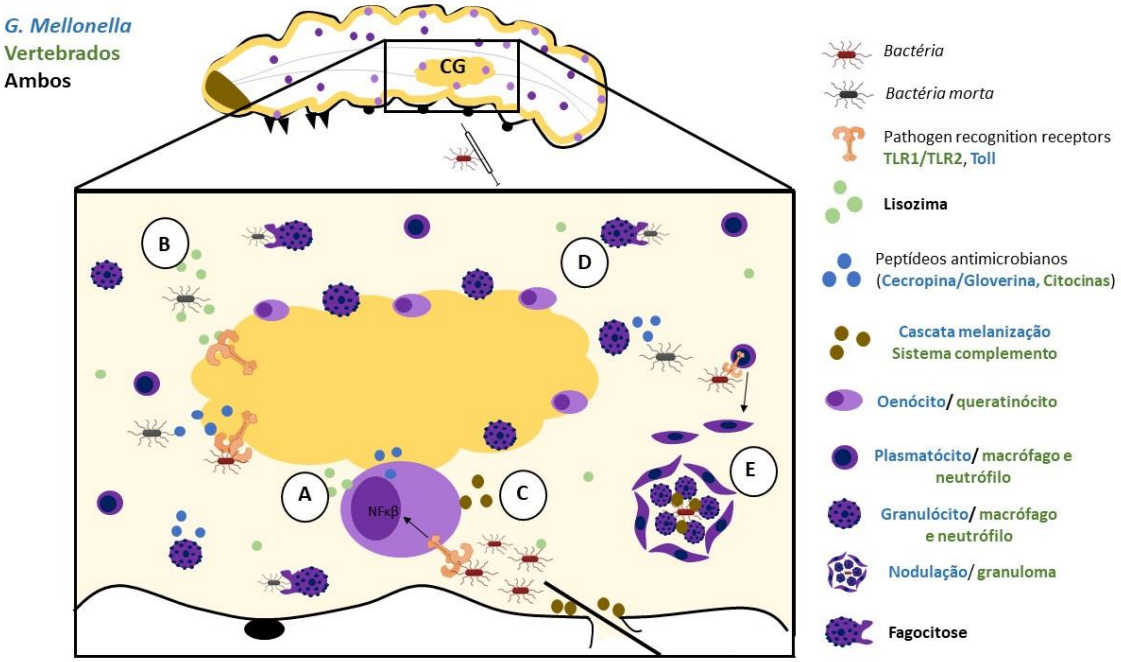
Após ser infectada, a larva inicia uma série de respostas que são divididas em humoral e celular. O patógeno e seus produtos, conhecidos como padrões moleculares associados a patógenos (PAMP), interagem com receptores de reconhecimento de patógenos expressos na superfície de hemócitos e órgãos como o corpo gorduroso. Em *G. mellonella,* um dos principais receptores relacionados a resposta à bactéria é o Toll, correlato ao toll-like receptor (TLR)-2 e 4 encontrado em vertebrados. Após a estimulação dos receptores, haverá a estimulação do fator de transcrição nuclear kappa beta (Figura 4A), que leva a liberação de peptídeos antimicrobianos (AMPs) e lisozima. A lisozima também já se encontra circulando na hemolinfa e é capaz de degradar e matar os micro-organismos invasores (Figura 4B). Os AMPs equivalem às citocinas liberadas durante o processo inflamatório em vertebrados, e o principal AMP na resposta a bactérias gram-positivas e negativas é

a cecropina, que penetra na parede celular e forma poros na membrana citoplasmática resultando, em perda de íons, e consequentemente morte celular. As gloverinas se ligam ao LPS de bactérias gram-negativas e inibem a síntese de proteínas de membrana vitais resultando em permeabilidade da mesma.

Outro sistema ativado após injúria em insetos é a cascata de melanização, principalmente pela liberação da enzima fenoloxidase pelos hemócitos, que converte a tirosina hidroxilase em melanina. Este sistema irá auxiliar na coagulação e cicatrização dos tecidos além de opsonizar e formar complexos que são capazes de matar patógenos pela liberação de espécies reativas de oxigênio (Figura 4C). Nos vertebrados, a produção de melanina é feita pelos queratinócitos e tem como principal função a proteção dos raios UV; no entanto a função de opsonização e inativação de micro-organismos é desempenhada pelo sistema complemento. Além disso, os granulócitos, análogos aos neutrófilos/macrófagos em vertebrados, imediatamente após a infecção começarão a fagocitar as bactérias, na tentativa de conter a infecção (Figura 4D). Finalmente, após entrar em contato com o patógeno, os plasmatócitos se ativam, emitem pseudópodes e se agregam ao redor do patógeno, formando estruturas conhecidas como nódulos (Figura 4E). Os vertebrados têm uma resposta semelhante, os granulomas. (Nathan, 2014; Tsai et al., 2016; Wojda, 2017; Pereira et al., 2018).

Trabalhos prévios demonstram que *G. mellonella* é um modelo útil para o estudo da infecção por *P. gingivalis* (Gomes et al., 2016; Santos et al., 2017; Geraldo et al., 2020; Moman et al., 2020; Solbiati et al., 2020). As larvas produzem a octopamina (OCT), hormônio análogo à NE em vertebrados, como parte da resposta de fuga ou luta. Em insetos o valor basal de OCT é por volta de 4,5-5,6pg/µL (10-30nM) podendo chegar a 61pg/µL (300-540nM) em situações de estresse (Davenport, Evans, 1984; Dunphy, Downer, 1994; Adamo et al., 1995). Esse hormônio tem sido correlacionado a um aumento da susceptibilidade à infeção pós estresse, em insetos (Adamo et al., 2010).

Figura 4 – Esquema da resposta imune desencadeada em *G. mellonella* após infecção por micro-organismos e as respostas correlatas em vertebrados



Legenda: Após infecção, a larva inicia uma série de respostas que são divididas em humoral e celular. A ativação de receptores de superfície pelos patógenos (A), leva a liberação de peptídeos antimicrobianos e lisozima. A lisozima e os peptídeos antimicrobianos encontram-se circulantes na hemolinfa prontos para realizar a defesa (B). A ativação da cascata de melanização opsoniza e mata microrganismos (C). Além disso, os granulócitos/plasmatócitos, imediatamente após a infecção, começam a fagocitar as bactérias, na tentativa de conter a infecção (D), formando também estruturas conhecidas como nódulos (E).CG= corpo de gorduroso.
Fonte: Elaborada pelo autor.

Trabalhos *in vitro* mostram a ação das catecolaminas em *P. gingivalis*, mas utilizam concentrações acima das fisiológicas ou encontradas no estado de estresse (Figura 3), e têm como limitação não apresentarem a correlação com o sistema imune. Assim, o estudo da resposta imunológica frente a diferentes concentrações de agonistas adrenérgicos (fisiológica, durante estresse e em condições patológicas como feocromocitoma) em *G. mellonella* e, como estas situações influenciariam a progressão da infecção por *P. gingivalis*, uma bactéria que se mostrou se beneficiar da NE *in vitro*, poderiam auxiliar no entendimento da susceptibilidade à infecção dos indivíduos expostos a este neurotransmissor, tanto pela ótica do sistema imune quanto da infecção pelo micro-organismo.

Assim os objetivos deste trabalho foram avaliar: (1) a inervação simpática no periodonto e a influência da ativação do SNS na vascularização periodontal eem

camundongos e (2) a influência do sistema adrenérgico nos fatores de virulência de *P. gingivalis* e na resposta imunológica a este patógeno *in vivo* (*G. mellonella*).

2 ARTIGOS

2.1 Artigo 1 – Moraes RM, Elefteriou F, Anbinder AL. Response of the periodontal tissues to β-adrenergic stimulation*. https://doi.org/10.1016/j.lfs.2021.119776. Repositório UNESP: https://repositorio.unesp.br/handle/11449/214063

RESUMO

Objetivos: A estimulação do receptor β-adrenérgico (βAR) por isoproterenol (ISO) em osteoblastos aumenta a expressão do fator de crescimento vascular endotelial (VEGF) e a angiogênese em ossos longos. Assim, objetivamos avaliar a resposta dos tecidos mandibulares à estimulação β-AR quanto à formação de vasos sanguíneos.

Métodos: Camundongos C57BL6 *wild-type* fêmeas de 6 semanas de idade receberam diariamente injeção intraperitoneal de ISO ou PBS (controle), por 1 mês. Hemimandíbulas e tíbias foram coletadas para análise de imunofluorescência para endomucina, tirosina hidroxilase (TH), neuropeptídeo Y (NPY) e transportador de norepinefrina (NET). Além disso, expressão de mRNA de *Vegfa*, *Il-1β*, *Il-6*, *Adrb2* e *Rankl* foi analisada nas mandíbulas e tíbias após 2 horas da injeção de PBS ou ISO.

Resultados: Apesar do padrão de inervação e expressão do *Adrb2* ser semelhante entre os tecidos mandibulares e tíbia, com fibras nervosas positivas para TH e NPY distribuídas ao redor dos vasos sanguíneos, o tratamento com ISO não aumentou a área de vasos endomucina+ ou o número total de vasos endomucina+ em nenhuma das regiões investigadas (osso alveolar, ligamento periodontal e polpa dentária). Consistente com esses achados, a expressão de *Vegfα, Il-6, Il-1β, e Rankl* na região molar não se alterou após a administração de ISO. Nós detectamos uma alta expressão de NET por imunofluorescência nos osteoblastos, osteócitos e fibroblastos do ligamento periodontal, além de uma maior expressão de *Net* por qPCR quando comparada às tíbias dos mesmos animais.

Conclusão: Esses achados indicam respostas distintas a agonistas β-AR entre os tecidos mandibulares e da tíbia, uma vez que a estimulação simpática não levou à angiogênese nos tecidos periodontais como nos ossos longos.

Palavras-chave: Periodontite, Sistema nervoso simpático, vasos sanguíneos, osso.

*Artigo elaborado de acordo com as normas do periódico Life Sciences (Print version ISSN 0024-3205). Publicado em: 27/06/2021.

ABSTRACT

Aims: Stimulation of β-adrenergic receptors (βAR) 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 βAR 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 Vegfα, Il-6, Il-1β, 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 βAR 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.

This manuscript has been accepted in Life Sciences Journal under the DOI:
https://doi.org/10.1016/j.lfs.2021.119776

Response of the periodontal tissues to β-adrenergic stimulation

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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 βAR 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)-1β, IL-6, and IL-8.[15] On the other hand, the stimulation of βAR by isoproterenol (ISO, a β1/2AR 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 β2AR 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 βAR 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^{o}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±0.0011g) 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 µm 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 cementoenamel 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 N_2 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 µL 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.

***Table 1**: Primer sequences for RT-qPCR reaction.*

Gene	Forward	Reverse
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Tbp	CCTTGTACCCTTCACCAATGAC	ACAGCCAAGATTCACGGTAGA
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Vegfa	CACGACAGAAGGAGAGCAGAAG	CTCAATCGGACGGCAGTAGC
Il-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
Il-1β	GGAGAACCAAGCAACGACAAAATA	TGGGGAACTCTGCAGACTCAAAC
Rankl	TGCCTACAGCATGGGCTTT	AGAGATGAACGTGGAGTTACTGTTT
Net	AGGCGCTCCTTTTCTTGGAA	AGAGGGACTTCGGAGGTTCT
Adrb2	TGGGGCCAGTCACATCCTTAT	TGACGCACAACACATCAATGG

Statistical analysis

The sample size was calculated based on the primary outcome of MVD previously published.[20] For statistical analysis (experimental unit=animal; α=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 βAR 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]

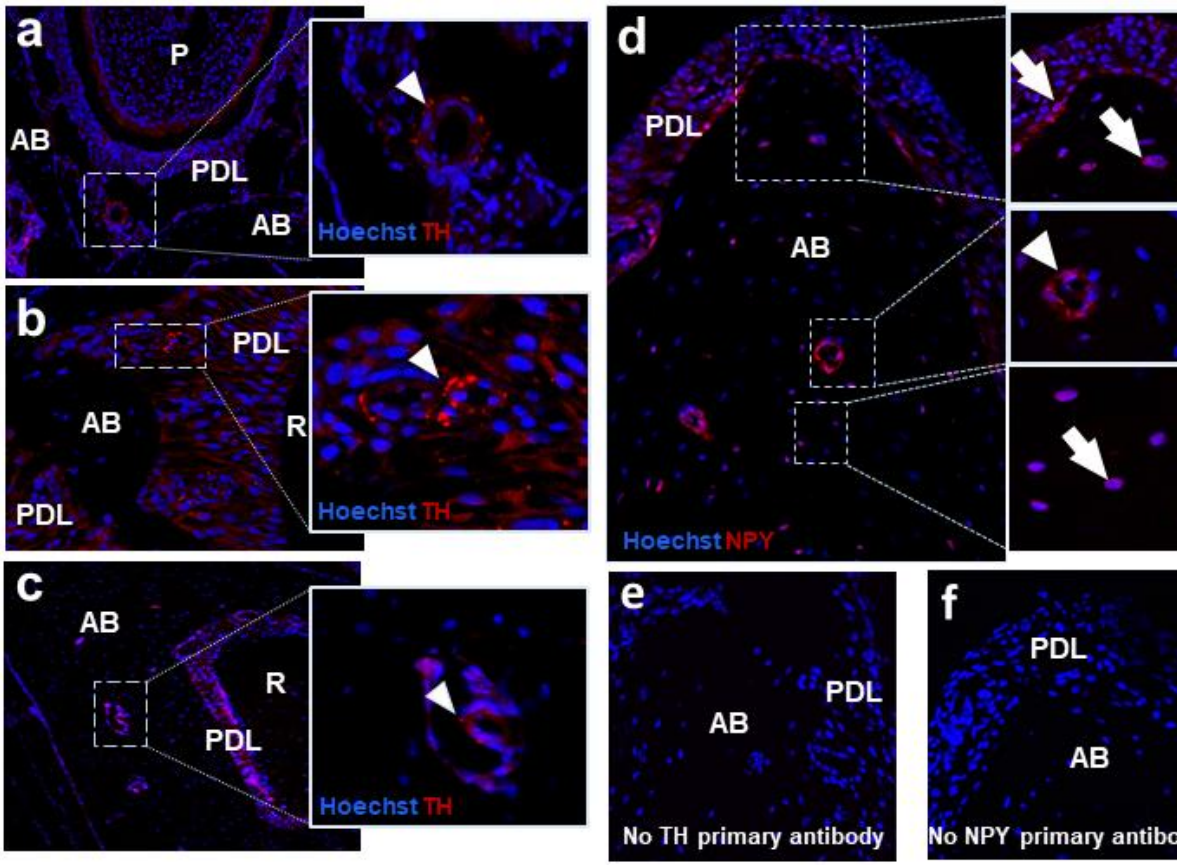

Figure 1: Sympathetic nervous system markers in the molar region (immunofluorescence, 6 weeks-old wild-type C57BL/6 mice, n=5, 2 sections per animal). a) Coronal section of the first molar region, showing puncta structures around blood vessels (arrow head), positive for TH (red). b) Transversal section 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 βAR 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 bones[20]. 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 β2AR, 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 β2AR target genes including Vegfα, Il-6, Il-1β, and Rankl in the molar region did not change 2h following ISO administration, whereas the expression of Vegfα, Il-1β, and Rankl increased in the tibia of these animals

(Figure 3a-d), as we reported previously.[20] The 2-hrs timepoint was chosen to asses direct β2AR 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 the β2AR and expression of Vegf, which returns to baseline after 24hrs.[20,28]

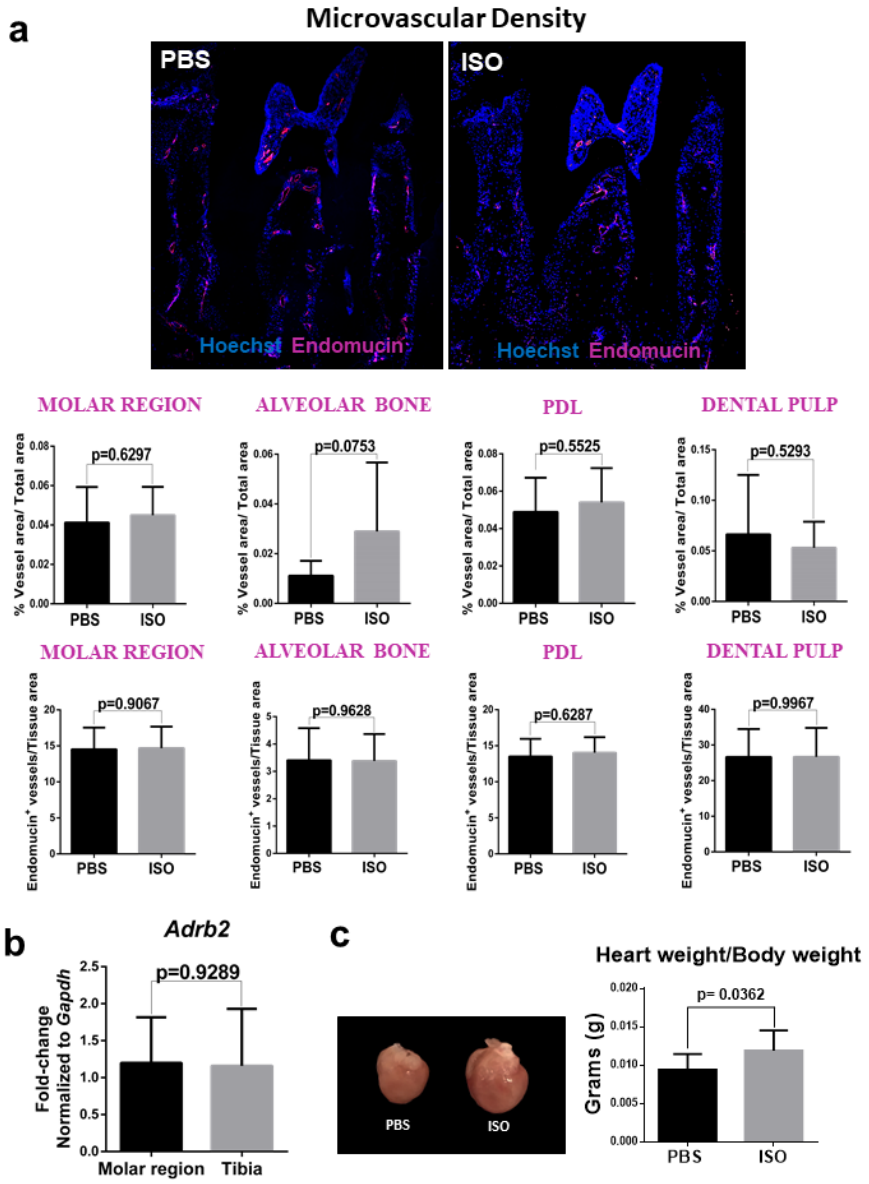

***Figure 2**: Blood vessel density in the mandibular molar region in response to β-AR stimulation by isoproterenol (ISO). Wild-type 6 weeks-old C57BL/6 mice were treated with ISO or PBS for 4 weeks. a) Graphs shows means ± standard deviation of the ratio Endomucin+ area/ Total area and Total number 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 ± 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 ± standard deviations of heart weight/body weight ratio after one-month ISO treatment, demonstrating the drug activity (T-test, PBS and ISO n=10).*

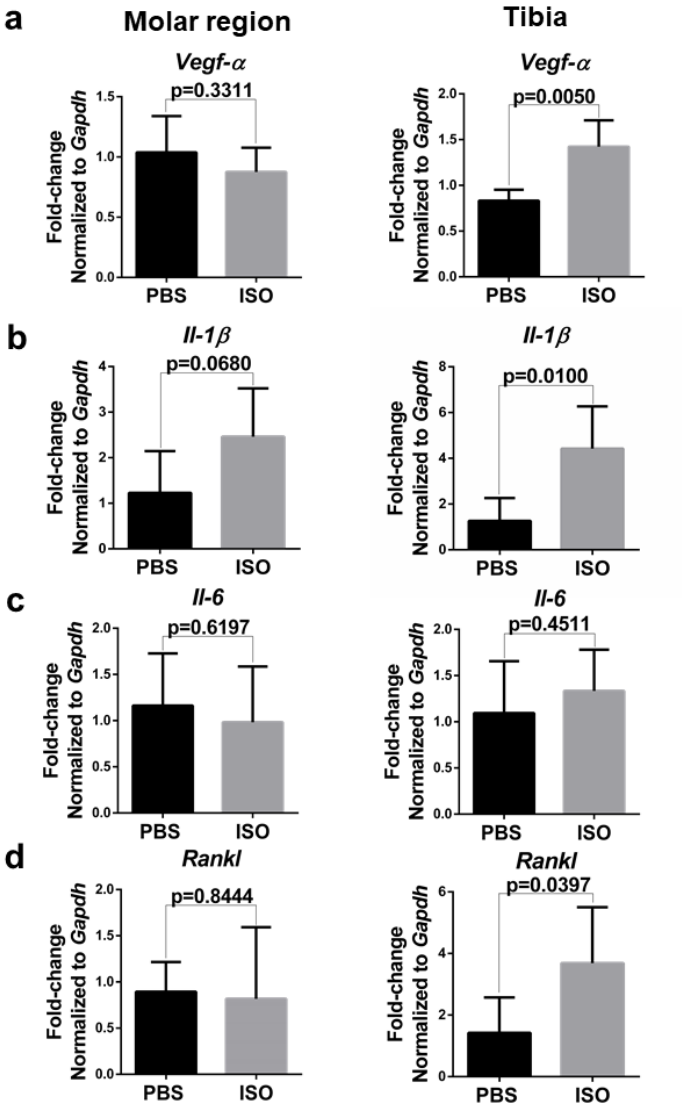

Figure 3*: Expression of vascular endothelial growth factor (Vegfα), interleukin-1β (Il-1β) and -6 (Il-6) and Rankl in response to isoproterenol (ISO) treatment. Wild-type 6 weeks-old C57BL/6 mice were treated with ISO or PBS for 2 hours. Graphs of means ± standard-deviation for Molar region (on the right) and Tibia (on the left) for: a) Vegfα; b) Il-1β; 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 βAR agonist ISO in term of βAR target gene expression and MVD led us to investigate other components involved in the regulation of sympathetic outflow. SNS signaling involves post-synaptic βARs 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 βAR 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).*

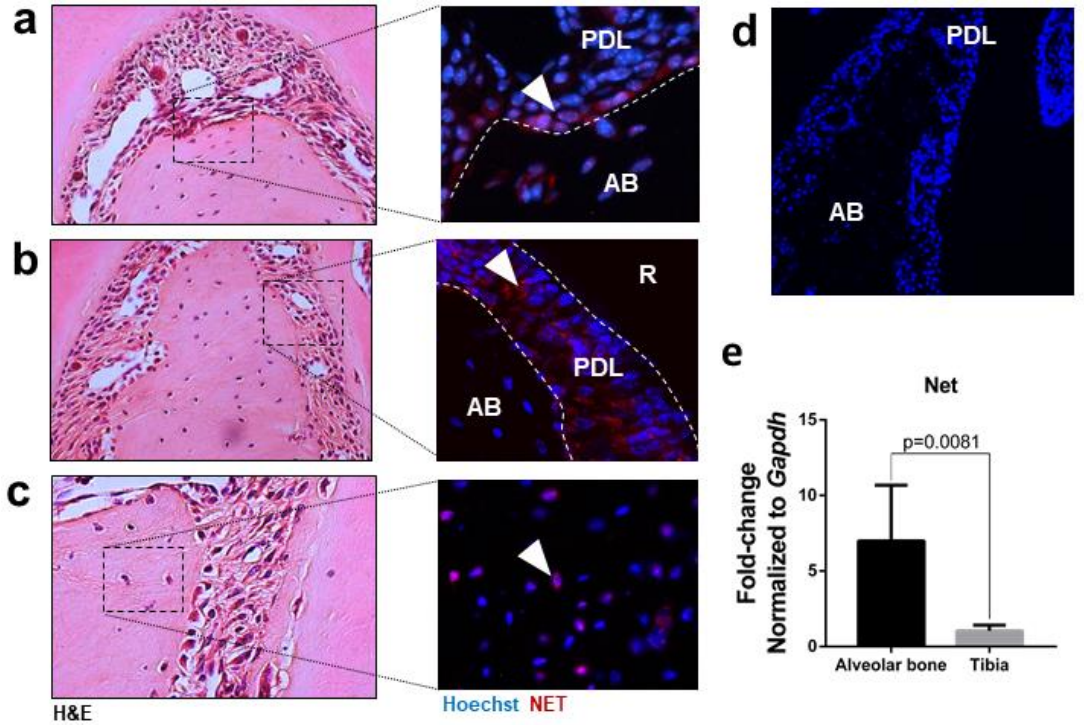

Figure 4*: NET protein expression in the molar region and tibia. a-d) Immuno-positive staining for NET was observed in the osteoblastic layer (arrowhead, a), periodontal ligament cells (arrowhead, b) and bone-embedded osteocytes (arrowhead, c). Original magnification of 20x (left, H&E staining) and 40x (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 β2AR 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 βAR 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 Vegfα 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 β2AR 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 βAR signaling.[16-19,36] Propranolol led to a decrease in serum levels of C-reactive protein, TNF-a, 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 βAR 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 βAR 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

βAR stimulation by the pharmacological βAR agonist ISO does not increase MVD in the mouse mandibular molar region, as opposed to tibias. Peridontium 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 βAR 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.

ACKNOWLEDGMENTS

This research was supported by São Paulo Research Foundation (FAPESP) under the grants FAPESP 2017/2646-1, FAPESP 2018/21701-0, and FAPESP 2018/25933-3 to Dr. Anbinder and Moraes. Dr. Elefteriou was supported by R01-AG055394 from the NIH. Imaging was supported by the Integrated Microscopy Core (IMC) at Baylor College of Medicine with funding from NIH (DK56338, CA125123, ES030285), and CPRIT (RP150578, RP170719), the Dan L. Duncan Comprehensive Cancer Center, and the John S. Dunn Gulf Coast Consortium for Chemical Genomics. The authors declare no potential conflicts of interest concerning authorship and/or publication of this article.

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2.2 Artigo 2 –Moraes R M, Garcia M T, Stossi F, Barros PP, Junqueira JC, Anbinder AL. Adrenergic signaling and periodontitis: insights on innate immunity and *Porphyromonas gingivalis* virulence in an invertebrate model*

RESUMO

Estresse é um fator de risco para periodontite, no entanto, a correlação entre sistema adrenérgico (SA), resposta imune inata e virulência de *Porphyromonas gingivalis* ainda é desconhecida. Para investigar a ação do SA na resposta imune do hospedeiro e na virulência de *P. gingivalis*, nós comparamos no modelo invertebrado de *Galleria mellonella*, a resposta à norepinefrina (NE), ao isoproterenol (ISO), e ao hormônio endógeno do inseto, Octopamina. A progressão da infecção por *P. gingivalis* foi avaliada pela curva de sobrevivência, resposta humoral (melanização por teste colorimétrico, e expressão de cecropina e gloverina por RT-qPCR) e celular (densidade hemocitária, nodulação por histologia) e recuperação de *P. gingivalis* por contagem UFC/mL. *P. gingivalis* também foi cultivada na presença de ISO (PgISO) e NE e então injetada na larva para avaliação da curva de sobrevivência. Finalmente, nós injetamos ISO e PgISO para avaliar os efeitos do ligante na resposta imune e diretamente na bactéria. Nenhum dos ligantes foi tóxico para larva, porém, apresentaram efeitos opostos. ISO aumentou o número de hemócitos após infecção por *P. gingivalis*, através da mobilização dos hemócitos sésseis de maneira, enquanto NE fez o oposto, o que foi associado à ação exclusiva em β-AR. Esses achados se traduziram na sobrevida após infecção, pois enquanto ISO reduziu a mortalidade das larvas e o número de bactérias recuperadas da hemolinfa, NE aumentou a mortalidade e não influenciou na recuperação bacteriana. Ambos ISO e NE tiveram efeitos similares na melanização e diminuíram expressão de cecropina. Apenas PgISO aumentou a mortalidade das larvas, porém, a administração de ISO sistêmico reverteu parcialmente este fenótipo. Aqui, nós validamos o modelo de *G. mellonella* para estudar o SA durante infecções e mostramos que a resposta α e β-adrenérgica tem efeitos opostos no desfecho da infecção por *P. gingivalis*. Ainda, a influência das catecolaminas no sistema imune se sobrepõe à ação de bactérias mais virulentas.

Palavras-chave: *Galleria mellonella*, *Porphyromonas gingivalis*, sistema adrenérgico, invertebrados, imunidade inata.

*Artigo elaborado de acordo com as normas do periódico Plos Pathogens (Online version ISSN1553-7366).

ABSTRACT

Stress is a risk factor for periodontitis; however, the relationship among the adrenergic system (AS), innate immune response, and virulence of Porphyromonas gingivalis is still unclear. To investigate the role of AS in the host immune response and P. gingivalis virulence, we compared, in the Galleria mellonella invertebrate model, norepinephrine (NE) and isoproterenol (ISO) responses to the endogenous stress hormone octopamine. P. gingivalis infection progression was evaluated by determining the survival curve and humoral (melanization by colorimetric assay, cecropin and gloverin mRNA expression), cellular immune responses (hemocyte count, nodulation by histology) and P. gingivalis recovery as CFU/mL count. P. gingivalis was cultivated in the presence of ISO (PgISO) or NE and injected into the larvae for survival curve evaluation. Finally, we co-injected ISO and PgISO to evaluate the effects of the ligands on the immune response and directly on the virulence of the bacteria. None of the ligands were toxic to the larvae but they presented opposite effects; ISO increased hemocyte number, even after P. gingivalis infection, by mobilizing sessile hemocytes in a β-adrenergic-specific manner, while NE showed the opposite effect. These effects ultimately correlated with the survival of the larvae after P. gingivalis challenge. ISO treatment reduced larval mortality and the number of recovered bacteria from the hemolymph, while NE increased mortality and showed no effect on bacterial recovery. Both ISO and NE had similar effects on melanization and decreased the expression of cecropin. Only the injection of PgISO increased the larval death rate, but circulating ISO partially reversed this effect. Herein, we validated the G. mellonella model for studying AS during infection challenges and showed that α- and β-adrenergic signaling have opposite effects after P. gingivalis infection. Ultimately, the catecholamine influence on the immune response overcomes the action of more virulent strains.

Keywords: Galleria mellonella, Porphyromonas gingivalis, adrenergic signaling, invertebrates, innate immunity.

AUTHOR SUMMARY

Periodontitis is the most common diseases of the gum and surrounding bone. It is a highly complex, multifactorial condition resulting from an imbalance between the microbiota and the host response, considered as the two pillars of the disease. One of the influencing factors is stress, which leads to the activation of the adrenergic system (AS), releasing epinephrine and norepinephrine. These hormones bind to different receptors expressed in many cell types and have been implicated in the virulence of periodontal microbiota. The study of AS in rodents/humans on both pillars of periodontitis is difficult because of the multiple variables involved in disease pathogenesis and the complexity of the immune response in higher organisms. We used the insect model Galleria mellonella, an excellent alternative to mammalian models, to interpret the host and microbiota responses to acute infection of Porphyromonas gingivalis, which is the main bacterial species responsible for periodontitis. Here, we show that the action of AS on the innate immune system of G. mellonella is the most important determinant of P. gingivalis infection outcome and that the effect of AS directly on the pathogen found in vitro cannot be transferred to the in vivo setting.

1. INTRODUCTION

Stress is an inherent part of modern life, and allostatic overload can contribute to the development and progression of various diseases. One such disease is periodontitis, a chronic inflammatory disease that is the leading cause of tooth loss in adults worldwide [1]. However, the exact mechanisms by which stress impacts periodontitis development are still not fully understood. Several factors have been proposed to explain the negative effects of stress, such as the activation of the hypothalamic-pituitary-adrenal axis and the immunosuppressive action of cortisol, and

lifestyle changes, such as a decrease in physical activities and weight gain, negligence with oral hygiene, and smoking, in periodontitis pathogenesis [2].

Moreover, the sympathetic nervous system (SNS), which is a part of the stress response, can affect both the pillars of periodontitis pathogenesis: host immune response and the microbiota. In the host, the SNS acts through α- and β-adrenergic receptors (AR), which are activated by the catecholamines epinephrine and norepinephrine (NE) in vertebrates. In invertebrates, the stress response is orchestrated by octopamine (OCT). Activation of the β-AR signaling leads to an increase in bone loss in rats with periodontitis [3], while the blockage of SNS by sympathectomy or propranolol (a β-AR blocker) inhibits osteoclastic differentiation and stress-induced interleukin (IL)-1β, IL-6, and IL-8 release, resulting in decreased bone loss [4–7]. SNS activation results in a pro-inflammatory profile [4,8] and decreases periodontal fibroblast proliferation [9].

In addition to their influence on the host, catecholamines have been shown to exhibit in vitro activity in some microorganisms. Epinephrine and NE interfere with the growth of some periodontopathogenic bacteria such as Fusobacterium nucleatum and Tannerella forsythia [10–14] and also increase the virulence of other bacteria such as Porphyromonas gingivalis [10,11,15,16]. P. gingivalis is the main pathogen associated with chronic periodontitis and is known as a keystone bacterium because of its ability to subvert the host immune response and invade the periodontal tissues [17]. NE increased the expression of gingipain (rgpb), an important virulence factor of P. gingivalis [15,18], which was reverted by the administration of propranolol, suggesting that a catecholaminergic receptor is expressed by the pathogen [16]. Although the current literature shows a direct action of catecholamines on P. gingivalis, the high concentrations used in these studies are not comparable to the concentrations in

physiological or stress conditions, making the translation from bench to bedside a challenge.

Although these in vitro studies show the potential influence of adrenergic signaling directly on the main pathogen of periodontitis [15-18], and studies in rodents and humans highlight the deleterious impact of stress in periodontal disease, the complexity of the disease and the models used make it virtually impossible to study the SNS response from the other components triggered during stress response, and to dissect its influence on the immune response and pathogen. Thus, less complex models that still present an analogous response to bacterial infections, such as invertebrate models, are ideal to answer many of the prominent questions on the impact of the adrenergic system (AS) on the host and pathogen [19].

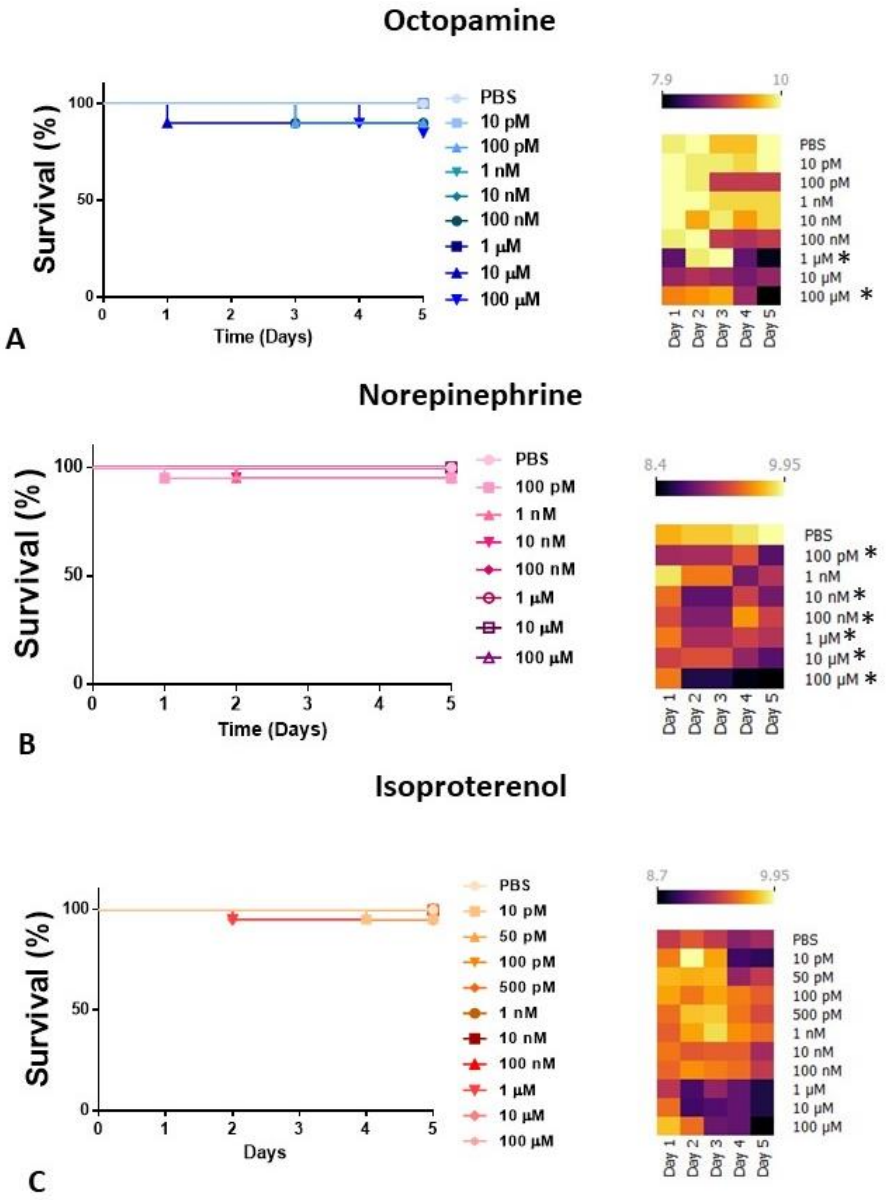
Galleria mellonella, a lepidopteran insect, has been widely used to study pathogen/host interactions owing to its many advantages. In the larval stage, it can be maintained at 37 °C, a temperature ideal for most human pathogens, is easy to handle, allows for controlled inoculation of microorganisms, and its immune system resembles that of rodents and humans [19]. In both invertebrates and mammals, the innate immune system is activated by the interactions of the pattern recognition receptors in immune cells (hemocytes in invertebrates and polymorphonucleated cells in vertebrates) with pathogen-associated molecular patterns expressed by pathogens. These interactions lead to the activation of transcription factors, such as NF-κB, and antimicrobial peptide (AMP) and lysozyme release as well as phagocytosis in both models. In addition, invertebrates present a potent melanization response that aims to opsonize and inactivate the invaders; this response correlates with the complement system of vertebrates [20–22]. Previous studies have shown that G. mellonella is a useful model for studying P. gingivalis infection [23–27]. Moreover, this insect produces OCT, a hormone that structurally resembles NE [28,29].

Thus, to better understand the effects of adrenergic signaling in the host response and P. gingivalis virulence, we investigated a range of adrenergic ligands (isoproterenol [ISO], NE, and OCT) at different concentrations (from low pM to high μM) in P. gingivalis infection outcomes using the invertebrate model G. mellonella, which has an innate immune response system similar to that of higher organisms.

2. RESULTS

ISO and NE affect the cellular immune response in G. mellonella in a dose- and time-dependent manner in the absence of pathogen infection

To evaluate whether the AS influences the cellular immune response, we used the well-established insect model, G. mellonella larvae. We chose the prototype adrenergic ligands NE, a mixed α- and β-AR agonist, and ISO, a selective β-AR agonist, and compared them with the endogenous insect adrenergic hormone OCT. First, we tested compound toxicity by injecting G. mellonella with a wide range of NE and ISO concentrations (10 pM to 100 μM, with mid-concentrations added when pick responses were seen to ensure specificity) and observed the larvae for 5 days. S1 appendix A–C shows that there was zero to very limited toxicity across the tested concentrations compared with that observed for the PBS control group.



*S1 appendix: Octopamine (OCT) (A), norepinephrine (NE) (B), and isoproterenol (ISO) (C) concentration-response analysis of survival in G. mellonella larvae (Kaplan–Meyer plot, left) and health index (heatmaps, right). None of the three compounds showed significant toxicity (n = 20 larvae per group; Log-rank (mantel-cox) test, OCT p = 0.1313, NE p = 0.6453, ISO p = 0.7137). * Significant differences in the health index compared with that of the phosphate-buffered saline (PBS) group, using the Kruskal–Wallis test, are indicated in the heatmaps..*

We then collected the circulating hemocytes from larvae injected with the same concentration range of NE, ISO, and OCT at 30–180 min post-injection (Figure 1, S2 appendix). Interestingly, ISO caused an increase in hemocyte counts, represented as box plots after z-score normalization, with an inverse U-shaped dose-response curve peaking at 100 pM and at the 30 min time point (Figure 1B). The results with 100 pM ISO were similar to the OCT endogenous hormone response (Figure 1A). In contrast,

NE caused a decrease in hemocyte counts, peaking at 30 min, but at a much higher concentration (1 µM) (Figure 1C).

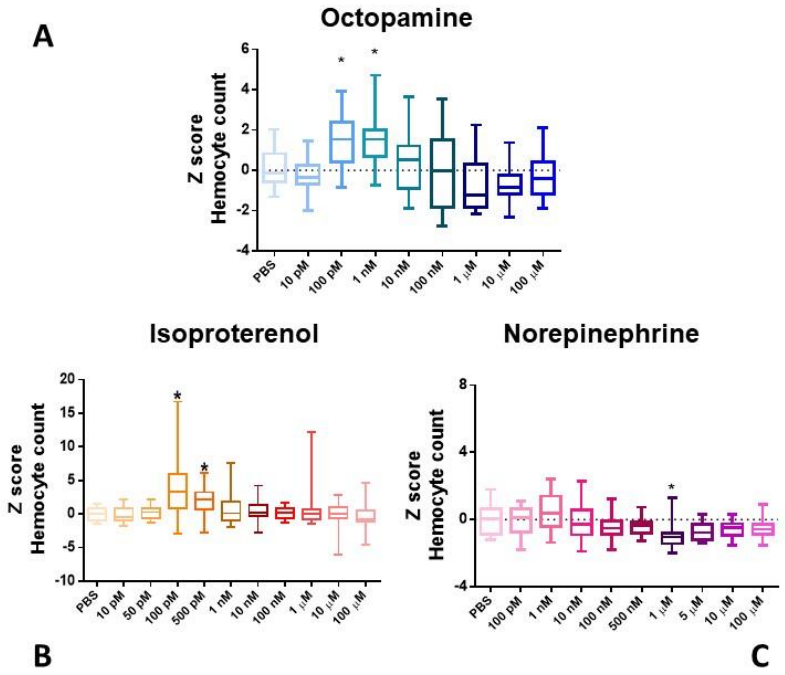
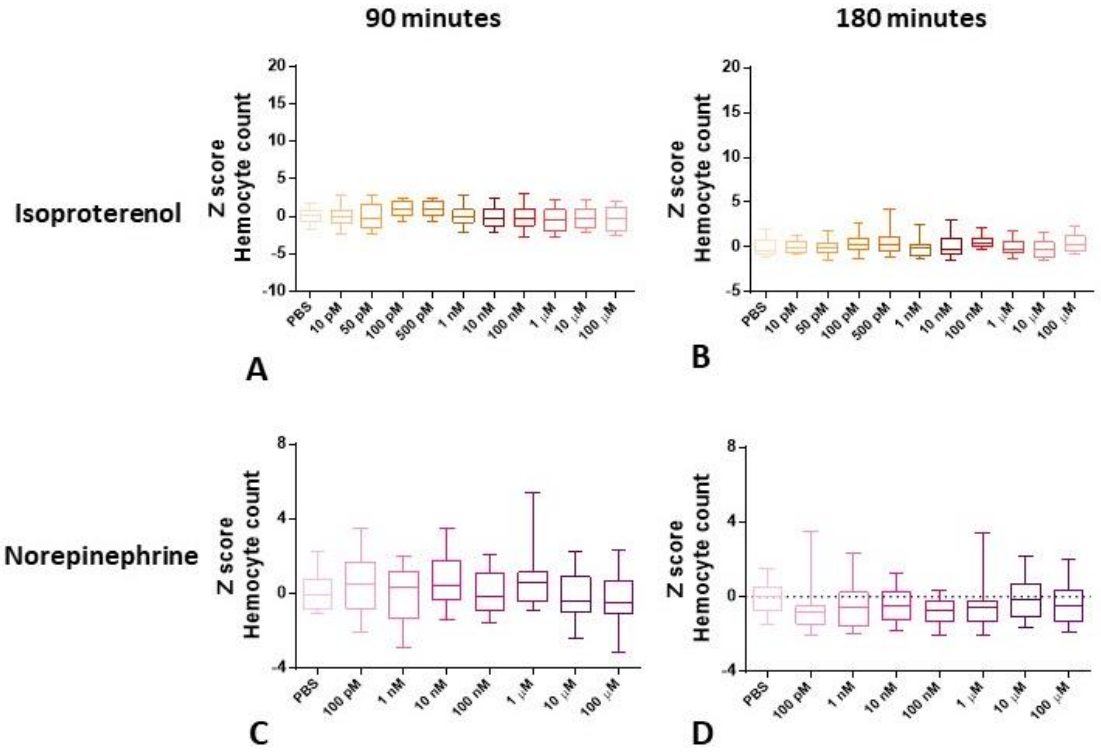


*Figure 1: Z-score-normalized hemocyte counts 30 min post-injection of octopamine (OCT) (A), isoproterenol (ISO) (B), and norepinephrine (NE) (C) in G. mellonella larvae. Box plots represent z-normalized hemocyte counts for n = 20 larvae per concentration. OCT increases the number of hemocytes at 100 pM and 1 nM (ANOVA, $p < 0.0001$), while ISO increases the number of hemocytes at 100 pM and 500 pM (Kruskal–Wallis test, $p < 0.0001$). NE decreases the number of circulating hemocytes at 1 µM (Kruskal–Wallis test, $p < 0.0001$). * Significantly different compared with the phosphate-buffered saline (PBS) group.*

The complete time-by-concentration matrix for ISO and NE, as shown in Supplementary Figure 2, highlights the transient time response (30 min peak) to adrenergic stimuli with a narrow window of concentration dependency, indicating a highly tunable response.



S2 appendix: Z-score-normalized hemocyte counts after ISO and NE treatment in a by concentration and time. ISO (A, B) and NE (C, D) time-course response at 90 (A, C) and 180 (B, D) min after infection. Box plots represent z-normalized hemocyte counts for n = 20 larvae per concentration/time point. No significant differences are observed among the concentrations within groups (ISO 90 min, Kruskal–Wallis test, p = 0.003; ISO 180 min, Kruskal–Wallis test, p = 0.0718; NE 90 min, Kruskal–Wallis test, p = 0.2899; and NE 180 min, Kruskal–Wallis test, p = 0.0783.)

α-AR signaling increases larval mortality during P. gingivalis infection

We employed the gram-negative bacterium P. gingivalis, the main pathogen responsible for periodontal disease, as a model to study the interplay between pathogen infection and the AS, as periodontitis has been linked to stress and SNS stimulation. We co-injected larvae with NE (1 µM) and P. gingivalis (10^8 CFU/ml) and observed them for 5 days (Figure 2A). P. gingivalis alone killed 92.5% of larvae in 5 days, whereas co-injection with NE led to 100% death in 2 days, accompanied by a decline in larval health, although it was not significantly different from that with P. gingivalis alone (Figure 2B). As shown earlier, NE decreased the number of circulating hemocytes, which are the cells responsible for phagocytosis and nodule formation; this was also observed upon co-injection with P. gingivalis (Figure 2C). This decline in cellular response resulted in P. gingivalis recovery from the larvae hemolymph

(measured as CFU/mL), similar to the P. gingivalis control group (Figure 2D). As one of the hallmarks of the response to bacteria is nodule formation, we performed histological analysis of the infected larvae. In concordance with the recovery assay, there was no difference in nodulation between the groups (Figure 2E).

To determine whether NE influenced the humoral immune response, we measured the levels of larval melanization using a colorimetric method 30 min post-injection, since we have observed an influence on hemocyte density after 30 min. No significant difference was observed between the P. gingivalis and P. gingivalis + NE groups (Figure 2F). Finally, we checked if the late humoral responses were affected by measuring changes in gene expression of two of the most common AMPs for bacterial infection in G. mellonella, cecropin and gloverin, after 24 h of infection. As shown in Figure 2G, P. gingivalis infection increased the expression of both genes (49-fold for gloverin and 4.6-fold for cecropin). Co-infection with NE decreased the expression of cecropin compared with that in the P. gingivalis group (2.8-fold decrease).

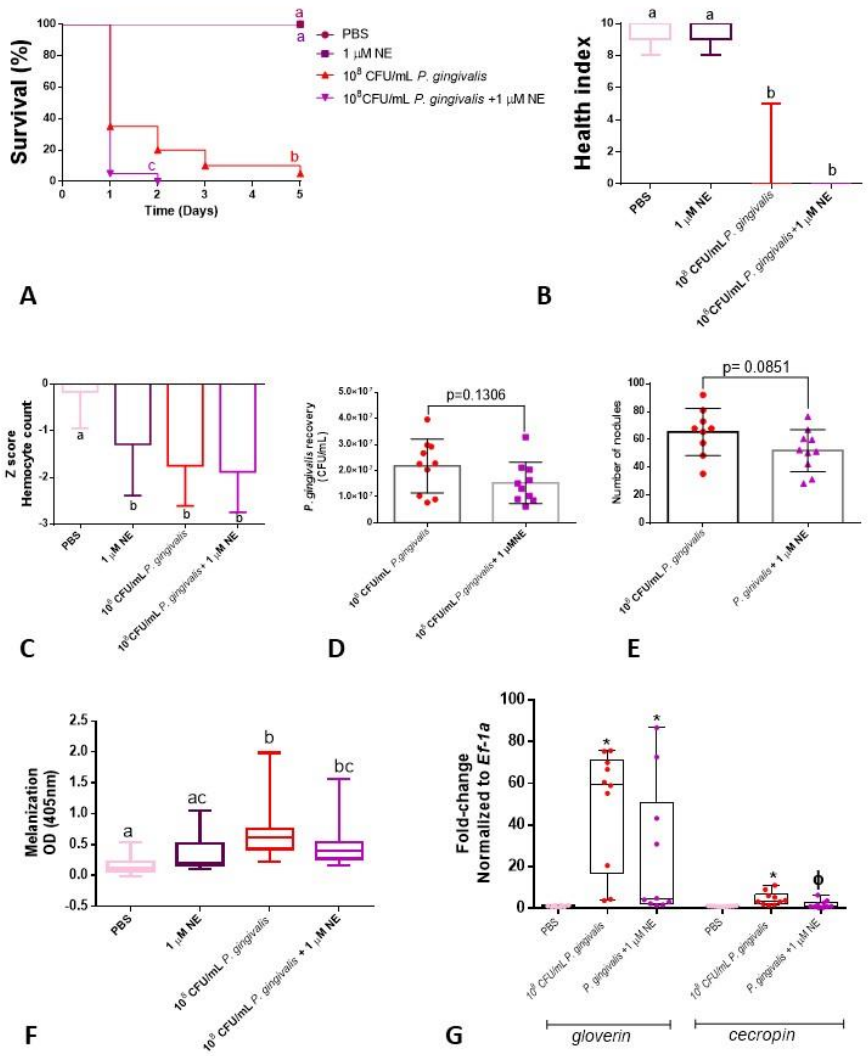


*Figure 2: Effect of α-AR signaling on the G. mellonella larvae response. A) Survival curve over 5 days of concomitant injection of 1 µM NE and 10^8 CFU/mL Porphyromonas gingivalis (n = 20 larvae per group, Kaplan–Meyer plot, Log-rank [Mantel-cox] p= 0.0109). B) Health index on day 5) (n = 20 larvae per group, Box plot, Kruskal–Wallis test, p < 0.0001). C) Z-score-normalized hemocyte counts measured after P. gingivalis +/- 1 µM NE compared to PBS group (n = 20 larvae per group, Mean and standard deviation, ANOVA, p < 0.0001). D). P. gingivalis recovery from the hemolymph of larvae infected with P. gingivalis +/- 1 µM NE (n = 10 pools of three larvae per group, Mean and standard deviation, t-test p = 0.1306) measured as CFU/ml. E) No differences observed between P. gingivalis and P. gingivalis + 1 µM NE regarding the number of nodules counted from histology sections (n = 10 larvae per group, three sections per larvae, Mean and standard deviation, t-test, p = 0.0851). F) Colorimetric measurement of melanization (n = 20 larvae per group, Box-plot, Kruskal–Wallis test, p < 0.0001). G) qPCR analysis of gloverin and cecropin mRNA expression 24 h after infection (gloverin: n = 8 larvae for PBS group and n = 10 larvae for P. gingivalis +/- 1 µM NE; Kruskal–Wallis test, p < 0.0001; cecropin: n = 10 PBS and P. gingivalis and n = 9 for P. gingivalis + 1 µM NE, Kruskal–Wallis test, p = 0.0024). Same lower letters close to the graph lines/bars represent an absence of significant difference. * represents significant compared with the PBS group. Φ represents significant compared with the P. gingivalis group.*

β-AR signaling positively impacts G. mellonella survival mainly through changes in the cellular immune response

Next, we analyzed the effects of β-adrenergic signaling using the specific pan β-AR agonist, ISO. After co-injecting the larvae with P. gingivalis and 100 pM ISO, the proportion of dead larvae was reduced to 45% after 5 days (Figure 3A). The pro-survival effect of ISO was also evident upon calculating the health index, because ISO-treated P. gingivalis-infected larvae had a healthier phenotype during the experimental period (Figure 3B), although this change was not significant compared with that in the P. gingivalis group.

To analyze the impact of the β-adrenergic system on bacterial-challenged larvae, we performed the same assays described above using NE, investigating both humoral and cellular responses. After P. gingivalis challenge, the larvae that also received ISO had a bacteria-induced decrease in hemocyte counts reversed to PBS levels (Figure 3C). This reversal in hemocyte loss was accompanied by a reduction in the number of P. gingivalis cells recovered from the hemolymph (Figure 3D). These results highlight the fact that ISO treatment is capable of rapidly reducing the number of live bacteria post-infection.

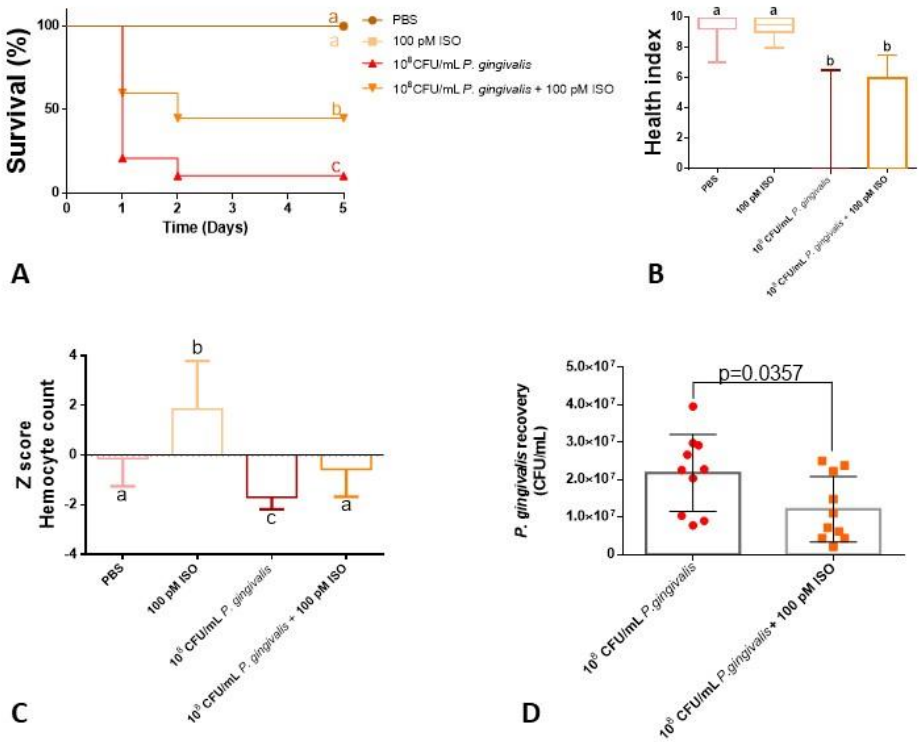


Figure 3: Effect of ϐ-AR signaling on G. mellonella larvae response. Survival curve (A) and health index (B) after concomitant administration of 100 pM ISO and Porphyromonas gingivalis (n = 20 larvae per group, Kaplan Meyer plot, log-rank [Mantel-cox] p = 0.0099; for health index, Kruskal–Wallis test, p < 0.0001). C) Z-score-normalized hemocyte counts after P. gingivalis +/- 100 pM ISO (n = 20 larvae per group, Mean and standard deviation, ANOVA, p < 0.0001). D) CFU/mL count of P. gingivalis +/- 100 pM ISO recovered from larvae's hemolymph (n = 10 pools of three larvae per group, Mean and standard deviation, t-test, p = 0.0357). Same lower letters close to the graph lines/bars represent the absence of significant difference.

To delve deeper into the mechanism by which ISO increased the number of hemocytes, we analyzed circulating (on the hemolymph) and sessile hemocyte, by dissecting the fat body and adjacent annexes (e.g dorsal tube), from the same larvae. As shown in Figure 4, the increase in circulating hemocyte corresponded to a comparable decrease in sessile hemocytes from the internal organs, clearly showing that ISO directly affects the release of sessile hemocytes. This can also be seen in the histology sections (highlighted in the insets) and has been quantified in the heatmap below (Figure 4). We also checked if the endogenous hormone OCT used the same mechanism to increase circulating hemocyte count, as shown in Figure 1A. The heatmap in Figure 4 confirms that OCT is indeed capable of releasing sessile hemocytes, similar to ISO. We validated that the ISO/OCT-induced increase in circulating hemocytes was dependent upon the β-AR signaling by co-injecting the larvae with the β-AR-specific antagonist propranolol. As expected, propranolol completely blocked the ISO/OCT-induced increase in the circulating hemocyte count (Figure 4).

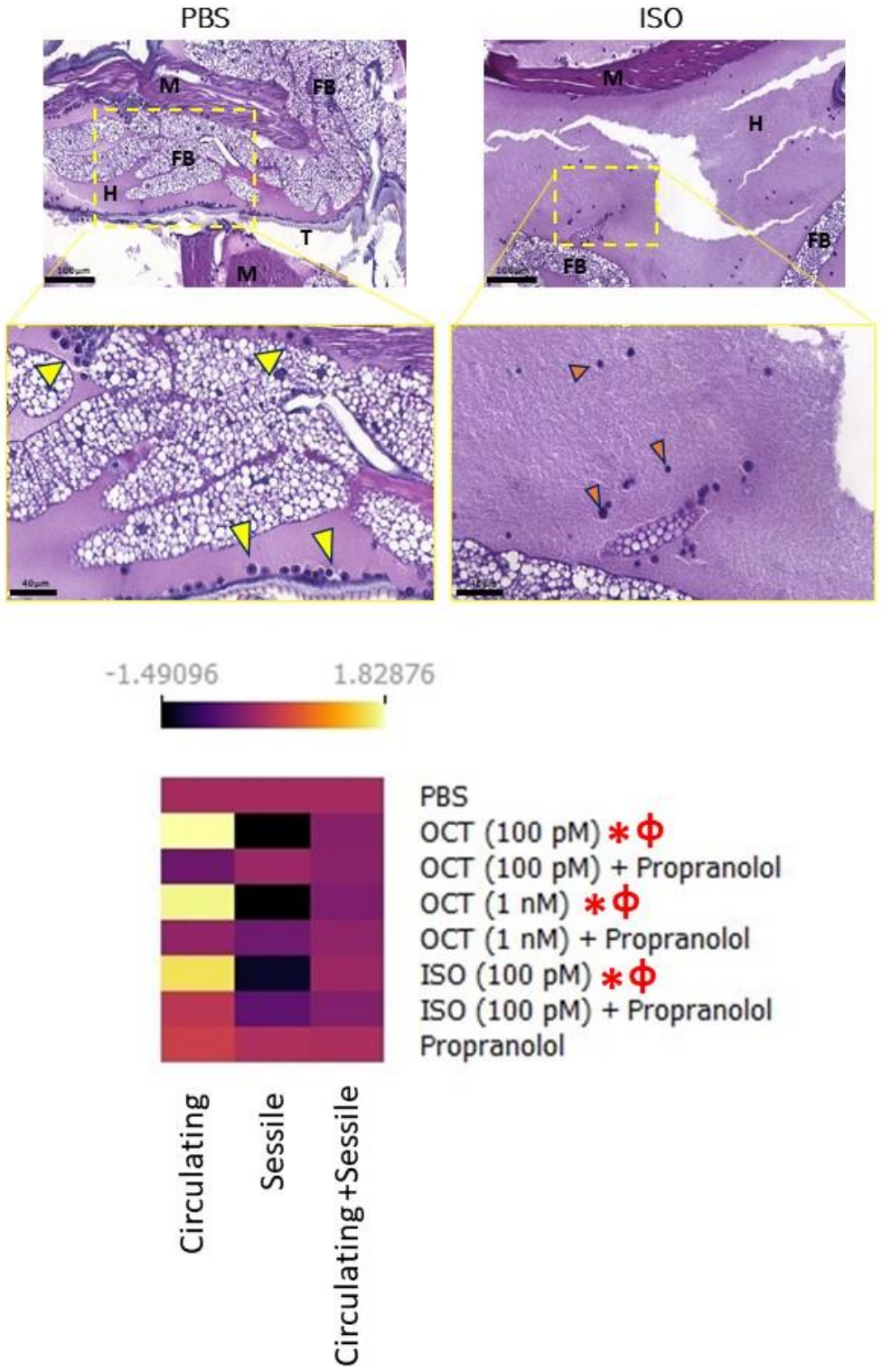


*Figure 4: Effect of β-AR signaling on hemocyte mobilization from the fat body. On the top, a representative image of H&E stained sagittal section of PBS or ISO (100 pM) injected larvae. Image shows the sessile hemocytes adhering to the fat body (FB), muscle (M) and trachea (T) (yellow arrowhead) and circulating hemocytes in the hemolymph (H) (orange arrowhead) (scale bar: 100 µm in the upper photos and 40 µm in the insets). (Bottom) Z-score-normalized hemocyte counts from hemolymph and fat body in larvae that received PBS, 100 pM ISO or 100 pM/1 nM OCT for 30 min (n = 20 larvae per group, Heatmap, ANOVA p < 0.0001). These observations were abolished with the administration of propranolol (10 µM), which was added to some groups to block β-adrenergic signaling. * Significant difference from PBS on hemolymph. Φ Significant difference from PBS on fat body.*

We also analyzed the number of nodules after ISO treatment and observed a decrease after treatment when compared with the P. gingivalis group (Figure 5).

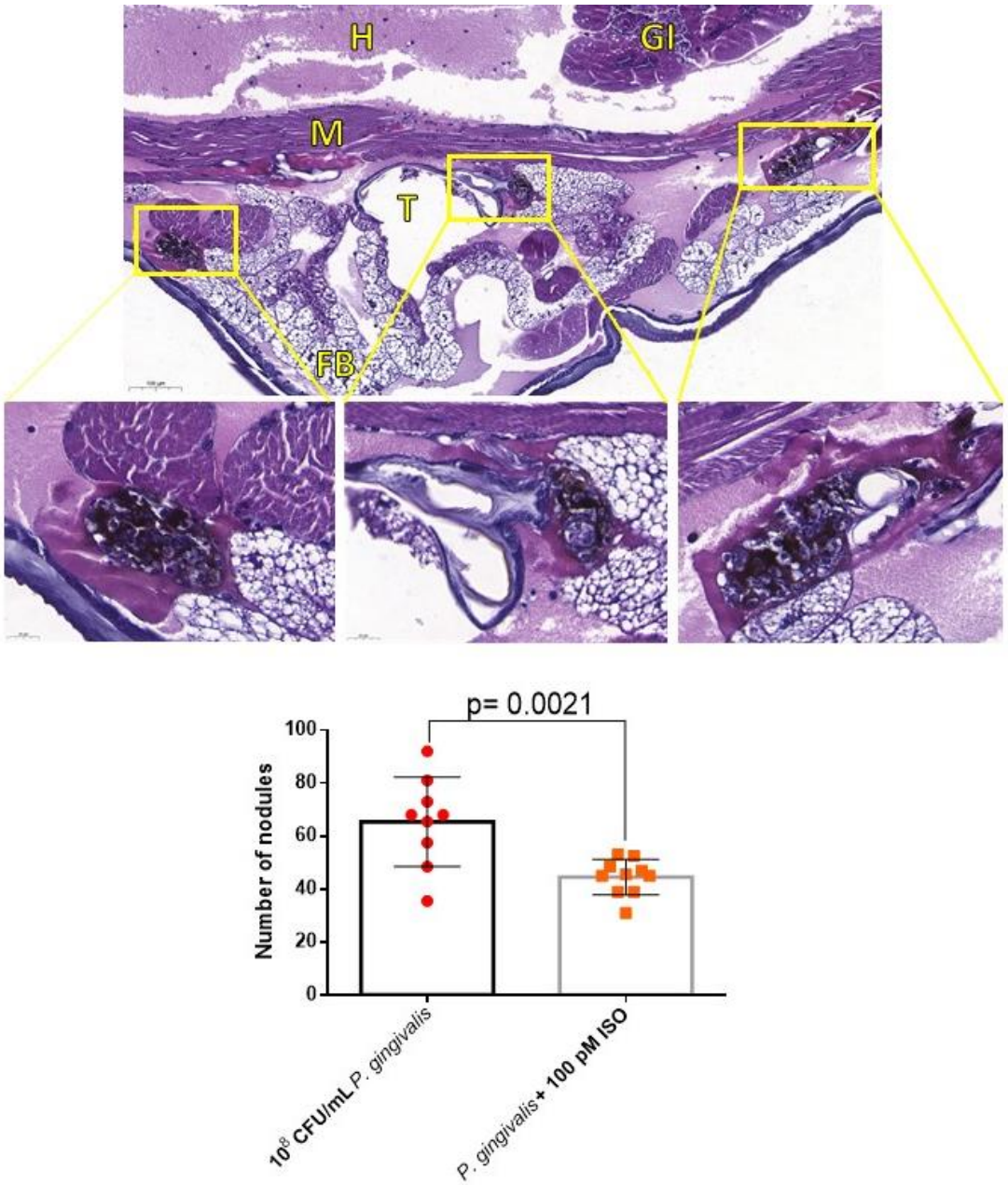


Figure 5: Effect of β-AR signaling on nodulation. On the top, representative figures of larvae injected with Porphyromonas gingivalis after 30 min, showing nodules formed around the Fat body, muscles, and trachea (H&E staining).GI= gastrointestinal tract, FB= Fat body, M= muscle, T= trachea, H=hemolymph. (Bottom) Number of nodules in larvae treated with P. gingivalis +/- 100 pM ISO (n = 10 larvae per group, 3 sections per animal, Mean and standard deviation, t-test p= 0.0021).

To determine whether the humoral immune response was also involved in ISO activity, we measured the levels of larval melanization. There was no significant difference between the infected groups, irrespective of treatment with ISO (Figure 6A),

indicating that this pathway may not be altered significantly by β-adrenergic signaling. Ultimately, we checked AMP production and observed that ISO-treated larvae had decreased cecropin expression when compared with that of the P. gingivalis group (Figure 6B).

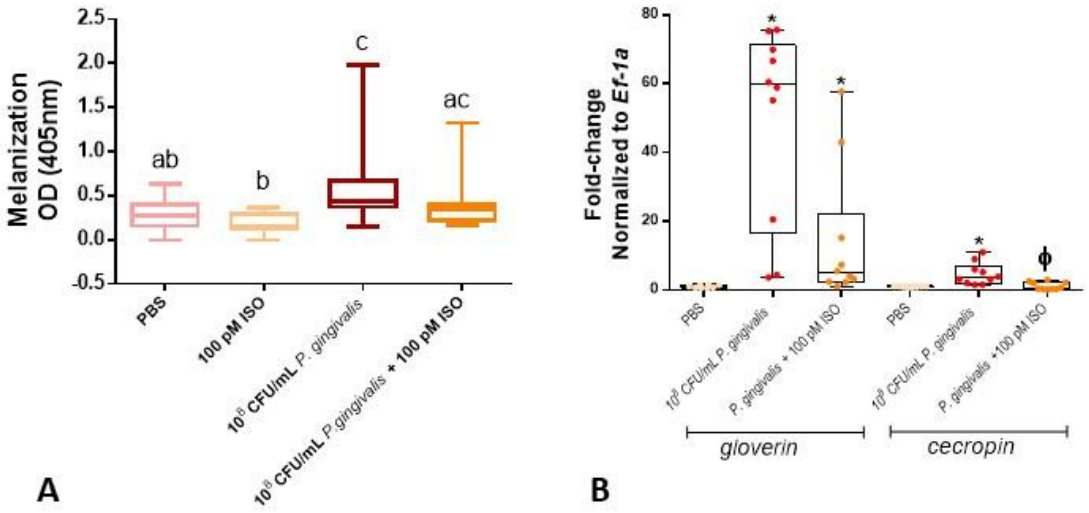


*Figure 6: Effects of β-AR signaling on humoral response. A) Colorimetric measurement of melanization in larvae injected with Porphyromonas gingivalis +/- 100 pM ISO for 30 min (n = 20 larvae per group,Box plot, Kruskal–Wallis test, p < 0.0001). Same lower letters close to the graph lines/bars represent an absence of statistical difference. B) qPCR analysis of cecropin and gloverin gene expression (gloverin: n = 8 for PBS group, n = 10 for P. gingivalis and P. gingivalis +100 pM ISO groups, Box plot, Kruskal–Wallis test, p < 0.0001; cecropin: n = 10 larvae per group, Kruskal–Wallis test, p= 0.0024). * represents statistical difference with PBS group. Φ represents significant difference compared with the P. gingivalis group.*

Adrenergic ligands differentially affect the virulence of P. gingivalis

To address whether direct adrenergic stimuli on P. gingivalis could influence the course of infection, we cultured the bacteria overnight with ISO- (100 pM and 100 μM) or NE- (1 μM and 100 μM) supplemented broth and then injected them into G. mellonella at a higher bacterial load (10^9 CFU/ml). The higher bacterial load used in these experiments was needed to match the 24 h death rate of P. gingivalis grown on agar plates (see Materials and Methods for details). We used the concentrations that influenced on the host response (100pM for ISO and 1uM for NE). The concentration 100uM was chosen based on previous in vitro studies [15,16,18]. As shown in Figure

7B, ISO-grown P. gingivalis (PgISO) was more toxic than P. gingivalis to the larvae at both concentrations tested in the first 24 h post-injection and started to kill larvae within the first couple of hours, while NE-grown P. gingivalis (PgNE) led to no substantial difference in survival, also compared to P. gingivalis (Figure 7A). When we analyzed the health of the larvae, both PgISO and PgNE reduced the health index, albeit without substantially difference compared with that of the P. gingivalis group (Figure 7A–B). Although NE and ISO influenced the gene expression of gingipain (rgbp) and fimbriae (fimA) in vitro, only ISO changed the phenotype in vivo (Figure 7A–B).

The interesting observation that ISO could behave differently if it was acting systemically on the larvae or directly in the bacterial environment prompted us to test whether the pro-survival effects of circulating ISO could overcome the increased virulence of ISO-grown P. gingivalis. For this purpose, we co-injected G. mellonella with 100 pM ISO and 10^9 CFU/ml ISO (100 pM)-grown P. gingivalis and observed them hourly for 24 h. As shown in Figure 7B, circulating ISO was indeed able to partially reverse the toxicity of the more virulent PgISO, indicating that the systemic effects of the β-adrenergic signaling are sufficient to counteract the toxicity of bacteria with higher virulence.

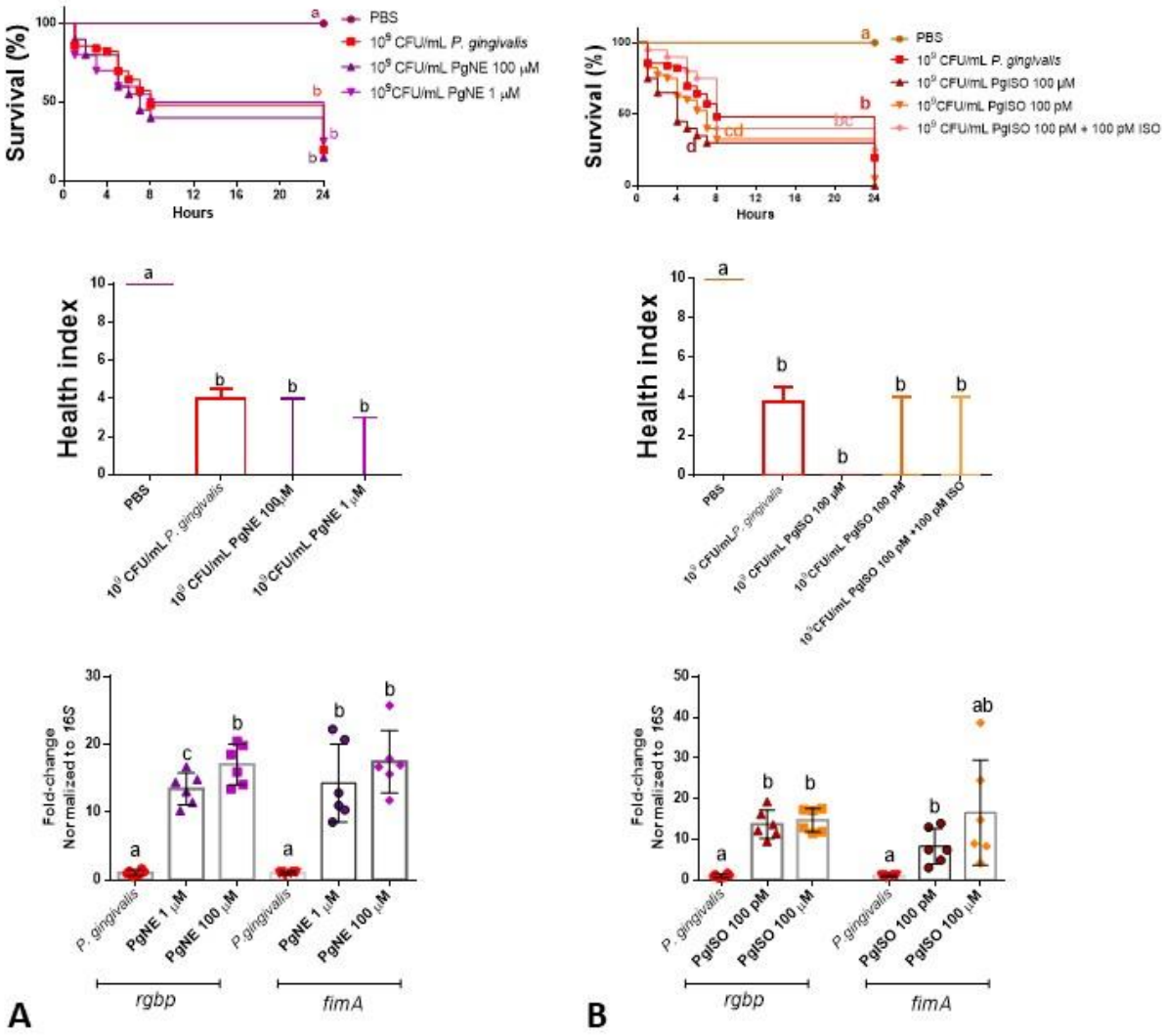


Figure 7: Direct action of adrenergic signaling on Porphyromonas gingivalis and concomitant action on immune system and bacteria. A) Co-culture of P. gingivalis with 1 µM or 100 µM NE had no effect on larvae survival 24 h after infection (n = 10 larvae for PBS group, n = 45 larvae for P. gingivalis group, n = 20 larvae for PgNE 1 µM and PgNE 100 µM, Kaplan Meyer plot, log-rank [Mantel-cox] p = 0.0005) or health index (Box plot, Kruskal–Wallis test, p < 0.0001) despite the increased virulence in vitro (rgbp, n = 6 biological replicates per group, ANOVA, p< 0.0001; fimA, n = 6 biological replicates per group, Mean and standard-deviation, ANOVA, p < 0.0001). B) Co-culturing P. gingivalis with 100 pM and 100 µM ISO increased the mortality of the larvae 24 h after infection (n = 10 larvae for PBS group, n = 45 larvae for P. gingivalis, n = 20 for PgISO 100 pM and PgISO 100 µM and 100 pM ISO + PgISO 100 pM; Kaplan Meyer plot, log-rank (Mantel-cox) p < 0.0001), confirming the increased virulence in vitro (rgbp, n = 6 biological replicates per group, Mean and standard deviation, ANOVA p< 0.0001; fimA, n = 6 biological replicates per group, ANOVA p = 0.0131). The systemic administration of 100 pM ISO could partially reverse this phenotype No difference was found regarding the healthy index (Box plot; Kruskal–Wallis test, p < 0.0001). Same lower letters close to the graph bars/lines represent an absence of significant difference concerning the same gene.

3. DISCUSSION

Stress is an inherent part of modern life and has been identified as a risk factor for periodontitis; however, the role of catecholamines in the two pillars of periodontitis,

in the immune system and bacterial virulence, is still not fully understood. In the periodontium, under homeostatic conditions, there is surveillance inflammation that maintains overall health. This function is mainly exerted by neutrophils and macrophages that phagocytose invaders and attempt to reduce the bacterial load [30–32]. During the inflammatory response, tissue breakdown products are released creating a favorable environment for the growth of anaerobic and proteolytic bacteria, such as P. gingivalis [17]. The SNS can regulate immune function, as cells of the innate immune system express both α- and β-ARs. Catecholamines primarily increase the number of circulating natural killer (NK) cells and granulocytes in response to stressors, and their activation can lead to pro- or anti-inflammatory actions depending on the cell and receptor activation. In the innate immune cells of vertebrates, β-AR usually has an anti-inflammatory role, whereas α-AR has a pro-inflammatory one [33].

Hemocytes are insect cells analogous to neutrophils and macrophages and have been shown to express adrenergic-like receptors [34]. In this study, we validated the adrenergic signaling response in G. mellonella hemocytes using known chemicals that bind to different ARs in higher organisms: ISO, a β-AR agonist, and NE, a preferential α-AR agonist. We showed that these receptors are functional, as the administration of either ligand influenced the cellular response in the larvae in a concentration- and time-dependent manner. Interestingly, the effect was very time-restricted, peaking at 30 min post-injection (similar to OCT [35,36]) and returning to basal levels within the first hour post-injection. The influence of OCT on cellular responses has been controversial. Some experiments showed increased hemocyte density at higher OCT concentrations (50–500 nM) and a decreased density at lower ones (5 nM) [37]. In contrast, 1 mM OCT injection increased hemocyte count in crickets [35], while OCT administration at ≤1000 pM shrimp-1 (Litopenaeus vannamei) mediated transient upregulation of hemocyte count [38]. Different dosages have been

used to explain the observed OCT-dependent effects on immune function. From the results of this study, we observed that OCT appears to act more like a β-AR agonist at lower concentrations (ISO-like) and as an α-AR agonist at higher concentrations (NE-like). In other invertebrates, such as Mythmina separata and L. vannamei, β-AR signaling was also linked to increased hemocyte count [39,40] while in Macrobrachium rosenbergii, α-AR signaling (NE) was linked to decreased hemocyte count and increased death rate [41].

In G. mellonella, an increase in circulating hemocytes has already been pointed out after stimulation and proposed to be due to the mobilization of these cells from the internal organs, especially the fat body, dorsal tube and the gastrointestinal tract [42]. In vertebrates, catecholamines also increase the number of circulating NK cells and granulocytes in response to stressors [33]. Herein, we show, for the first time, that the adrenergic response in G. mellonella is responsible for the very fast (30 min) mobilization of hemocytes in a β-AR-specific manner. The specific intracellular pathways responsible for the mobilization and activation of hemocytes by β-AR are still under investigation. However, the protein kinase A and cyclic adenosine monophosphate pathways have been shown to be involved in hemocyte adhesion, one of the first steps in nodule formation [43–45] and phagocytosis [46–50].

As the first line of defense in innate immunity is the humoral branch, we analyzed the influence of differential activation of the adrenergic response during P. gingivalis infection on the prophenoloxidase system and AMP production, which are two of the main humoral responses of G. mellonella. The prophenoloxidase system is responsible for melanization, which occurs as a part of wound healing, sclerotization, and hardening of the cuticle, and is also activated by invading microbes [51]. We did not observe any influence of the ligands on melanization, as has already been shown for OCT [37]. Regarding the influence of infection and NE/ISO treatment on the gene

expression of the two main AMPs against gram-negative bacteria, cecropin and gloverin, P. gingivalis greatly increased their expression after 24 h of infection. NE and ISO had some reducing effects on the mRNA levels of both the peptides. It has become clear that stress influences the immune system by simultaneously upregulating some functions while downregulating others [47,52]. This balance is needed at the organismal level because over- or under-activation of immune reactions damages the host itself. In periodontitis, the dysregulation of innate immunity leads to an overproduction of cytokines, which perpetuates inflammation and dysbiosis of the dental biofilm, thus establishing a feed-forward loop that leads to tissue breakdown and bone loss. The decrease in AMP observed for both ligands could be explained by different mechanisms. On the one hand, for ISO, this could be due to the decrease in the bacterial load leading to AMP reduction. On the other hand, the decrease observed in the NE group suggests that other AMPs or additional factors might be more important in the survival of G. mellonella upon P. gingivalis infection. These fine balances between all innate immunity components might constitute the key response to survival and infection resolution. Drosophila melanogaster and Sarcophaga peregrina are not killed by P. gingivalis, whereas G. mellonella and silkworms do suffer from lethal host damage caused by this pathogen [53], indicating that organismal differences in the innate immune system influence disease outcome.

Due to limitations of the model, in which chronic exposure to bacteria and the ligands is not possible, since adrenergic response was very fast and higher bacterial loads extremely lethal, we investigated the acute influence of α- and β-AR responses during P. gingivalis infection on innate immunity, and the results clearly showed that the activation of β-AR during the initial phase of infection favors phagocytosis and bacterial clearing, preventing the death of the larvae. Notably, α-AR signaling was hypothesized to be more important during periodontitis development as α-AR protein

expression is higher in diseased conditions and the administration of phentolamine (an α-AR blocker) decreased the expression of cytokines in the gingival tissue of chronically stressed mice, in contrast to propranolol [4], which correlates with our findings that α-AR stimulation increased larval death.

G. mellonella was shown to be an excellent model to transition in vitro observations to in vivo scenarios, which is in line with the 3R principles. In the final part of our study, we addressed the biological importance of increased virulence of P. gingivalis stimulated by the adrenergic system, as some early studies pointed out the direct action of catecholamines on the microbiota as part of the mechanisms by which the SNS could influence periodontitis pathogenesis. Previous in vitro studies [11,15,16] using high concentrations of NE showed increased P. gingivalis virulence, as observed by the expression of virulence factors in our study. However, we did not observe any difference in PgNE compared to P. gingivalis infection in terms of larval survival. In contrast, PgISO infection caused higher larval mortality. These findings could be strain-specific, as Pseudomonas aeruginosa H103 treated with 10 µM epinephrine increased bacterial virulence and decreased the survival of G. mellonella [54]. The increased mortality caused by the highly virulent ISO-treated P. gingivalis could be partially reversed by the systemic action of ISO on the immune response, thereby increasing the hemocyte density. This shows that the action of the AS on the immune response can overcome the higher bacterial virulence caused by the same agent. Indeed, the presence of P. gingivalis alone is not correlated with chronic periodontitis in humans [55, 56]. Therefore, the in vitro influence of ISO/NE on P. gingivalis virulence is not important for the outcome of the disease; this emphasizes the importance of validating in vitro findings in the clinical setting, especially in the context of periodontitis, which is a complex multifactorial disease.

4. CONCLUSIONS

Herein, we validated the use of the G. mellonella model to investigate the role of adrenergic signaling during bacterial infection. We showed that the ligands binding to different ARs had opposite effects during P. gingivalis infection, with α-AR negatively affecting the course of infection, and β-AR being protective for the larvae. On the one hand, the negative effects of α-AR signaling were correlated with a decrease in circulating hemocytes and downregulation of AMPs. On the other hand, the protective effect of ISO was mainly through mobilization of sessile hemocytes to the hemolymph, where they decrease bacterial load, leading to a more controlled infection. Finally, the systemic action of the β-AR signaling surpassed the negative action of more virulent bacteria, suggesting that the immune system can modulate itself to various stimuli, ultimately dictating the outcome of the infection.

5. MATERIALS AND METHODS

5.1 Galleria mellonella

5th instar larvae, reared in the laboratory of Microbiology and Immunology at the Institute of Science and Technology (ICT/UNESP/Brazil), weighing 0.15–0.25g, were selected from a pool of healthy individuals that showed no signs of cuticle pigmentation. The larvae were stored at 25°C for one day before use when they were kept without food. There was no blinding of samples for the experiments and 20 larvae per group were used, unless otherwise stated.

5.2 Bacteria growth conditions

ATCC P. gingivalis 33277 (Microbiologics^{TM}) were activated in Brucella agar as the basis for the blood agar (5% defibrinated sheep blood), supplemented with 1%

hemin and 1% menadione. The plates were incubated in an anaerobiosis jar with the aid of an anaerobiosis generator at 37°C for 5-7 days. The bacteria were used between passages 4-8 for all the experiments. Characteristic black colonies were harvested from the agar plates and suspended in PBS to 10^8CFU/mL (agar-grown bacteria). This suspension was used in the interaction experiments where P. gingivalis and ISO/NE were systemically injected in the last proleg of the larvae. For the experiments in which the bacteria were cultivated in the presence of the ligands (ISO/NE), colonies were transferred from agar to Brucella broth added to the ligands or PBS (control) and grown for 24 hours in anaerobiosis (broth-grown bacteria). On the next day, samples were centrifuged at 5000 rpm for 10 min and the pellet was resuspended in PBS to 10^9CFU/mL. The final suspensions were used for injecting the larvae.

5.3 G. mellonella susceptibility to P. gingivalis

10µL of bacteria suspension were inoculated in the left the last proleg of the G. mellonella larvae using micro syringes (Hamilton Inc., EUA) in different concentrations (10^7 and 10^8 CFU/mL for agar-grown bacteria; and 10^8 and 10^9 CFU/mL for broth-grown bacteria). After inoculation, the larvae were kept at 37 ºC, and Health Index [57] was assessed daily for 5 days for agar-grown bacteria and hourly (up to 24 h) for broth-grown bacteria. Health Index determination is based on scores to activity, cocoon formation, melanization, and survival, where, in the end, 10 is the higher score and means full health.

5.4 ISO, NE, and OCT toxicity and cellular immune profiling

To assess if the ligands were toxic for G. mellonella, a wide range of ISO (Isoproterenol hydrochloride, Sigma, I6504), NE (Noradrenaline tartrate, Sigma, N1100000), and OCT (Octopamine hydrochloride, Sigma, O0250) concentrations

were injected into the last right proleg of the larvae. Concentrations were selected based on serum levels of NE in humans and OCT in insects during physiological, stress and pathological (pheochromocytoma) states [15,16,18,36,37,58,59]. The larvae were inoculated with 10µL of ISO/NE/ OCT or PBS, which served as the negative control. After injection, the larvae were kept at 37ºC, and the Health Index was noted every day for 5 days. In a separate set of larvae, after 30, 90 and 180 min incubation at 37°C, the hemolymph was bled through an incision on the last left proleg, diluted 1:100 in IPS buffer (2% NaCl; 0.1 M glucose; 30 mM sodium citrate; 26 mM citric acid and 10 mM EDTA) and the hemocytes were counted in a Neubauer chamber. This procedure was performed to temporally assess the influence of treatments on hemocyte density.

5.5 ISO, NE, and OCT influence on the outcomes of P. gingivalis infection in G. mellonella

After concentration screening (see results section- Figure 1), we chose 100 pM for ISO and 1 µM for NE with a 10^8CFU/mL of P. gingivalis for the subsequent experiments. Larvae were dived into 6 groups: PBS, P. gingivalis, 100 pM ISO, 1 µM NE, P. gingivalis +100 pM ISO, and P. gingivalis + 1 µM NE. The insects were injected with 10 µL of PBS, ISO, or NE on the right last proleg, and immediately after they were injected 10^8CFU/mL of P. gingivalis or PBS in the last left proleg. The larvae were incubated at 37°C for 5 days and the Health Index was determined daily to generate the survival curve. Other experimental readouts for the interaction studies were measured 30 min after the inoculation, except for the AMPs gene expression that was performed 24 hours later. Additional 2 sets of larvae were bled to collect hemolymph for melanization and hemocyte density evaluation.

5.6 Hemocyte density in the hemolymph and fat body

Hemocytes can be free in the hemocoel in the hemolymph (circulating hemocytes) or in the surface of internal organs such as the fat body (also called sessile hemocytes). To evaluate both hemocyte density, after collecting the hemolymph (as in item 5.4, for circulating cells), the fat body and its annexes (e.g dorsal tube) were removed through the same incision to evaluate sessile cells. The fat body was collected in an eppendorf tube containing 1mL of chilled IPS, quickly vortexed, and centrifuged at 12,000 rpm, for 10 min at 4ºC. The supernatant was removed from the tube, and the pellet was resuspended in 1 mL of cold IPS. The circulating and sessile hemocytes were counted using a Neubauer chamber. These experiments were performed using ISO (100 pM) and OCT (100 pM and 1 nM), and to validate the β-adrenergic origin of the responses elicited by them, we co-injected some larvae with ISO/OCT plus 10 µM of Propranolol hydrochloride (Sigma, P8688), a β-blocker to try to blunt the responses [60].

5.7 Quantification of melanization

20 µL of hemolymph from each larva was collected into pre-chilled eppendorf tubes (30 min at -20°C) as described in item 5.4 and centrifuged at 12,000 rpm for 10 min at 4ºC. 10 µL of the supernatant was diluted in 40 µuL of IPS in a 96 well plate. The plate was incubated at RT for 5 min, and then it was read at 405 nm using a spectrophotometer.

5.8 P. gingivalis recovery from the hemolymph

To analyze the possible influence of the ligands on bacterial load, the larvae were inoculated with 100 pM ISO, 1 µM NE, or PBS (control); and immediately infected with 10^8CFU/mL of P. gingivalis. The insects were then incubated at 37 °C for 30 min. Hemolymph from 3 larvae/group was collected by an incision in the last proleg and

pooled, serially diluted to 1:10,000, 100 µL of the final dilution was plated on supplemented Brucella blood agar and incubated in anaerobiosis for 7 days. The characteristic black colonies were manually counted and expressed in CFU/mL. This procedure was performed with 10 pools of 3 larvae per group (total of 30 larvae per group).

5.9 Nodule quantification by histology

For histological analysis, 10 larvae from the groups P. gingivalis, P. gingivalis + 100 pM ISO, and P. gingivalis + 1 µM NE were used. Thirty minutes post-injection, the larvae were frozen at -20°C for 10 min, injected with 100 µL of Bouin's aqueous solution (Sigma, HT10132) in the last proleg for fixation, and kept at 4°C for 72 hours. After that, the larvae were stored in 70% ethanol for 48 hours. For processing, the region between the last and second proleg was separated and hemisectioned, after which the pieces were routinely processed for paraffin embedding and hematoxylin & eosin staining. Three sections, 20 µm distant from each other per larvae, were evaluated in a brightfield microscope using a 20x objective. Structures containing more than 4 hemocytes arranged around a melanized area were considered a nodule. The number of nodules in the halves on the same histological plane were averaged and the values of the three sections were summed for statistical analysis.

5.10 G. mellonella antimicrobial peptides (AMP) gene expression using RT-qPCR

10 larvae per group were stored at -80ºC, 24 hours after injection of PBS, P. gingivalis, P. gingivalis + 100pM ISO and P. gingivalis + 1µM NE. The whole larvae were then macerated in liquid nitrogen using pestle and mortar. The powder was resuspended in 1 mL TRIzol® (ThermoFisher Scientific,15596026), the RNA was isolated according to the manufacturer's protocol and its concentration was measured

using a NanoDrop 2000 Spectrophotometer. Only RNA samples with a 260/280 ratio between 1.8-2.0 and 230/260 ratio above 1.8 were used in reverse transcription. 1,000ng of the extracted RNA was treated with RQ1 RNase-Free DNase (Promega, M6101) and transcribed to cDNA using the GoScript™ Reverse Transcription Mix, Random Primers (Promega, A2801). Two reference genes for G. mellonella, β-actin, and Ef-1a, were run in all experimental groups. The obtained results were analyzed at https://heartcure.com.au/, and the most stable gene was select as the reference gene, which was Ef-1a. The primer sequences used are shown in table 1. For the RT-qPCR reactions, GoTaq® qPCR Master Mix kit (Promega, A6002) was used. The reactions were performed in duplicate wells in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster, CA, USA), consisting of 5 µL GoTaq® qPCR Master, 0.3 µL CXR, 0.5 µM primers (final concentration), and target cDNA (2µL of 1:10 diluted cDNA, final concentration-10ng), supplemented with RNAse-free ddH_2O to a final volume of 10 µL. The cycling parameters for the amplification reactions were 50°C 20 s, 95°C 3 min; 95°C 20 s, 60°C 1 min for 40 cycles; and 95°C 15 s, 60°C 1 min, 95°C for 15 s. The level of gene expression was calculated by applying the $2^{-\Delta\Delta CT}$ method.

Gene	Foward sequence	Reverse sequence	Amplicon size (base pairs)	Reference
		Porphyromonas gingivalis		
16S	CGCCAACCTTTGAATCATTCCTT	TGGTGTCTGATCATGTTTCCAAGA	137	Azelmat et al., 2015 [62]
FimA	TTCTTGTTGGGACTTGCTGC	TCATCGCCAACTCCAAAAGC	140	Designed with primer3 based on GenBank: D17795.1
Rgbp	ACCATCGTCAGAGAGCGTAG	TACACTTGTCCCGACCGAAA	118	Designed with primer3 based on Gene ID: 29256649
		Galleria mellonella		
Ef1a	CTCTGTTAAGGAGTTGCGCC	TGGCAATCGAGTACAGGTGT	147	Designed with primer3 based on GenBank: AF423811.1
B-actin	ACAGAGCGTGGCTACTCGTT	GCCATCTCCTGCTCAAAGTC	104	Rossoni et al. 2017 [63]
Gloverin	AGATGCACGGTCCTACAG	GATCGTAGGTGCCTTGTG	93	Barros et al., 2019 [64]
Cecropin	CTGTTCGTGTTCGCTTGTGT	GTAGCTGCTTCGCCTACCAC	158	Barros et al., 2019 [64]

Table 1: Genes investigating P. gingivalis virulence and G. mellonella immune defense.

5.11 Direct influence of NE and ISO on P. gingivalis virulence and infection outcome

To assess the influence of ISO and NE directly on the virulence factors of P. gingivalis, bacteria were cultivated in broth with the presence of the ligands in the concentration that systemically influenced the hemocyte count in vivo (100 pM ISO, 1 µM NE) and with 100µM (ISO and NE), a concentration that was shown to have activity in vitro [15,16,18]. The larvae were injected with PBS, P. gingivalis grown in PBS, P. gingivalis grown in 100 pM ISO (PgISO 100 pM) or 100 µM ISO (PgISO 100 µM) supplemented broth, P. gingivalis grown in 1µM NE (PgNE 1 µM) or 100µM NE (PgNE 100 µM) supplemented broth. After assessing that ISO increased P. gingivalis virulence (see Results section, Figure 6), in order to verify if the systemic administration of the ISO could influence the outcome when PgISO was administered, one more

group was added: immediately after the injection of 100 pM ISO, P. gingivalis grown in 100 pM ISO was also administered (100 pM ISO + PgISO 100 pM).

To investigate which genes were involved in the enhanced virulence of P. gingivalis grown with ISO or NE we performed RT-qPCR. After the incubation period (24 h) the bacteria were centrifuged at 5,000 rpm for 10 min and the pellet frozen down at -80ºC. Later we performed RNA extraction using TRIzol® following manufacturing instructions and sample quality, DNase treatment, transcription, amplification, reference gene selection (16S), and gene expression calculation were as described in section 5.10. The primers for the genes analyzed are presented in table 1.

5.12 Statistical analysis

The experimental unit in this study was a single G. mellonella larvae, except for the P. gingivalis recovery assay in which a pool of 3 larvae was considered the experimental unit. Statistical analysis was carried out using the GraphPad Prism 6 program (GraphPad Software, LA Jolla California USA) or Orange [61] for creating the heatmaps. For hemocyte count, data were normalized by Z score, using the PBS group of each experiment as the baseline. After assessing the distribution, data were submitted to ANOVA or Kruskal-Wallis, T-test, or Mann-Whitney. Significance was assigned if $p<0.05$.

The study design described above is shown in figure 8.

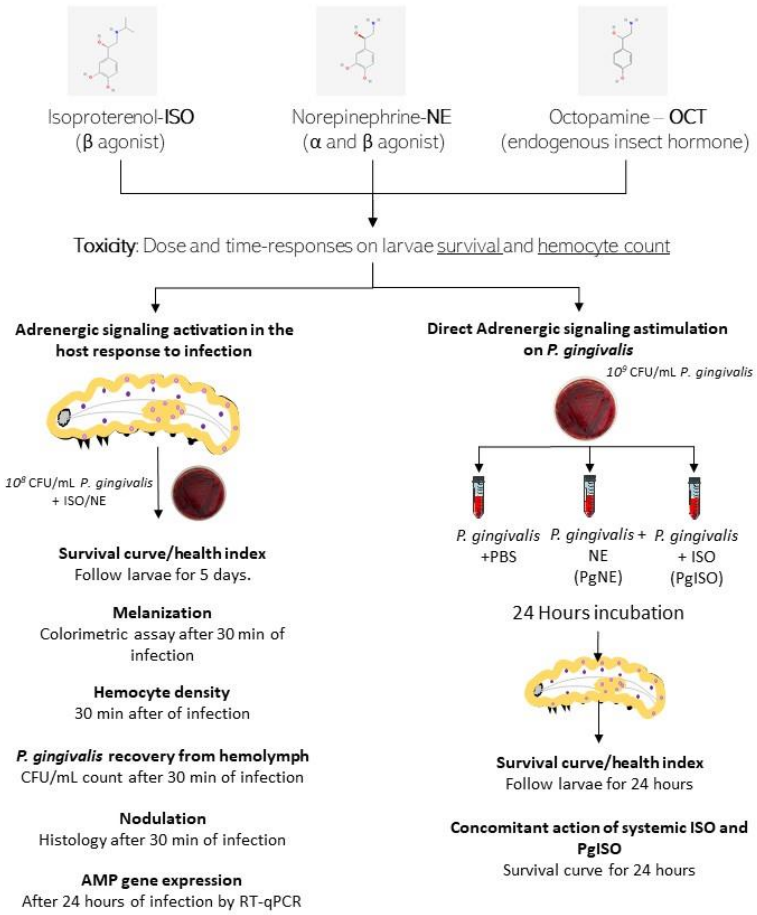


Figure 8: Study design. We used three experimental compounds: the endogenous insect stress hormone octopamine (OCT), and two adrenergic agonists, isoproterenol (ISO- β adrenergic-specific agonist) and norepinephrine (NE- α adrenergic receptor-preferential). Chemical toxicity was assessed by measuring G. mellonella larvae survival and hemocyte density. After assessing their dose-response profiles, the influence of ISO and NE in the G. mellonella host response arsenal, as well as their influence on P. gingivalis virulence were investigated. For studying the adrenergic signaling activation on the host response, the compounds' influence on infection outcome was determined by survival curves and Health Index measurement over 5 days. Humoral response was assessed by melanization and AMP assays and the cellular response by hemocyte density, P. gingivalis recovery from hemolymph, and nodulation assays. To address the direct impact of the ligands on P. gingivalis virulence, we co-cultured the bacteria with NE (PgNE) or ISO (PgISO) overnight and then injected them into the larvae and evaluated larvae survival over 24 hours. Finally, we evaluated the action of systemic ISO during PgISO infection, also measuring survival after 24 hours.

6. ACKNOWLEDGMENTS

This research was supported by São Paulo Research Foundation (FAPESP) under the grants FAPESP 2017/26461-5, FAPESP 2018/ 21701-0, and FAPESP 2018/25933-3 to Anbinder AL and Moraes RM. Stossi F is supported by funding from NIH (DK56338, CA125123), and CPRIT (RP170719).

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8. SUPPORTING INFORMATION CAPTIONS

S1 appendix: Octopamine (A), Norepinephrine (B), and Isoproterenol (C) dose-response analysis of survival (Kaplan-Meyer plot, left) and Health Index (heatmaps, right).

S2 appendix: Hemocyte density after ISO and NE treatment in a dose- and time-dependent manner.

3 CONSIDERAÇÕES GERAIS

A doença periodontal faz parte do grupo das doenças complexas ou multifatoriais, que são decorrentes dos efeitos de múltiplos genes em combinação com estilo de vida e fatores ambientais. Na periodontite, a comunicação entre microbiota e hospedeiro determinará o desfecho da doença, e vários fatores podem influenciar na resposta do hospedeiro, incluindo o seu estado emocional.

O estresse é um fator de risco para doença periodontal (Genco et al., 2013), sendo o SNS um dos mecanismos acionados durante o estresse. Pouco se sabia sobre a inervação simpática na mandíbula, com estudos antigos onde era difícil a observação das fibras nervosas (Norevall et al., 1996). Aqui nós mostramos que as fibras simpáticas, marcadas por TH e NPY, encontram-se principalmente ao redor de vasos sanguíneos na região do ligamento periodontal, e eventualmente nos vasos sanguíneos do osso alveolar (Figura 5A). Nos ossos longos, fibras TH apresentam morfologia em “saca rolhas” e envolvem os vasos sanguíneos na região periosteal. Já no osso cortical, essas fibras são observadas exclusivamente nos canais de Havers (Chartier et al., 2018). As fibras simpáticas liberaram NE, que desencadeia seus efeitos ao se ligar aos receptores adrenérgicos. Confirmando a presença dessas fibras no periodonto, de modo similar aos ossos longos, e baseados nos nossos achados prévios de que a estimulação β-AR em osteoblastos induz angiogênese de ossos longos (Mulcrone et al., 2017), nós partimos para averiguar se essa ação também seria observada na região molar de camundongos. No entanto, diferentemente do que esperávamos, a estimulação β-AR por ISO não aumentou angiogênese em nenhuma das regiões estudadas (ligamento periodontal, osso alveolar e polpa dentária) nem influenciou a expressão de *Vegfa*, *Il-1β*, *Il-6* e *Rankl*.

A resposta diferente observada entre mandíbula e tíbia pode ser devido às origens embrionárias distintas. Os ossos faciais se originam de células da crista neural através, predominantemente, de ossificação intramembranosa, enquanto o esqueleto axial origina-se da mesoderme, por ossificação endocondral (Couly et al., 1993). Foi demonstrado que osteoblastos originados da crista neural e do mesoderma apresentam potenciais osteogênicos distintos *in vitro* (Reichert et al., 2013). Os osteoblastos mandibulares depositam mais matriz mineralizada e expressam níveis mais elevados de fosfatase alcalina quando comparados aos osteoblastos da tíbia (Reichert et al., 2013). Além disso, diferentes envelopes ósseos (osso alveolar,

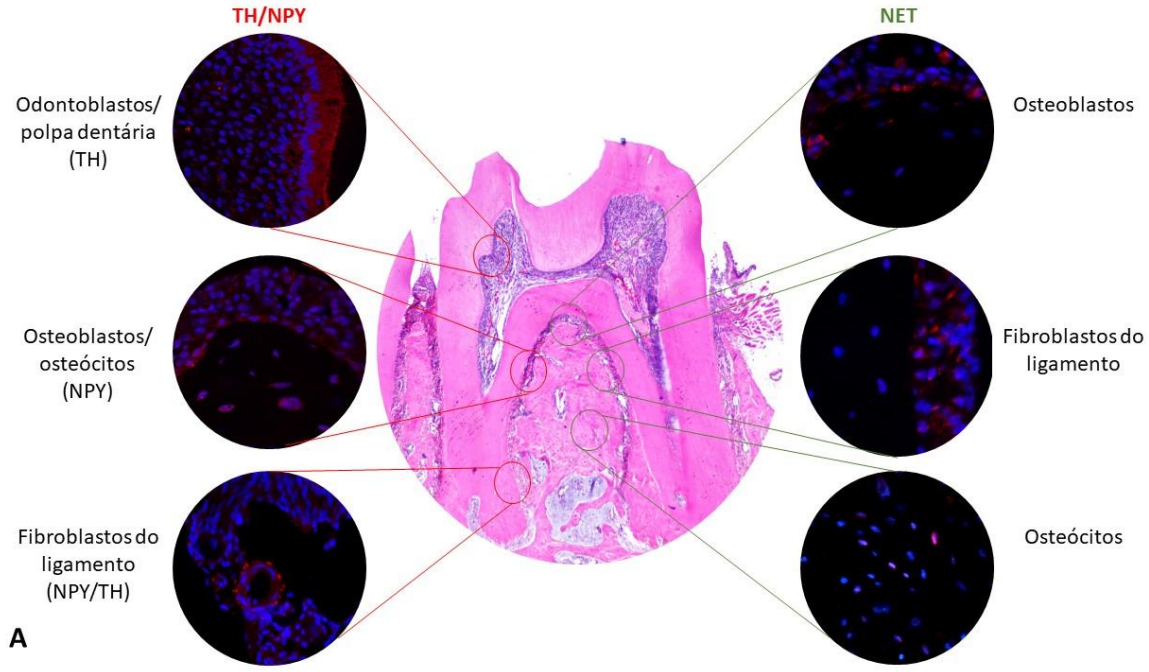
periósteo mandibular e região retromolar) respondem diferentemente a agonistas/antagonistas do sistema catecolaminérgico (Bataille et al., 2012). Uma maior expressão de VEGF observada em fêmures comparado à mandíbula, tanto em fetos quanto em adultos a nível molecular e proteico, também suporta mecanismos diferentes controlando a densidade vascular entre os dois tecidos (Marini et al., 2015).

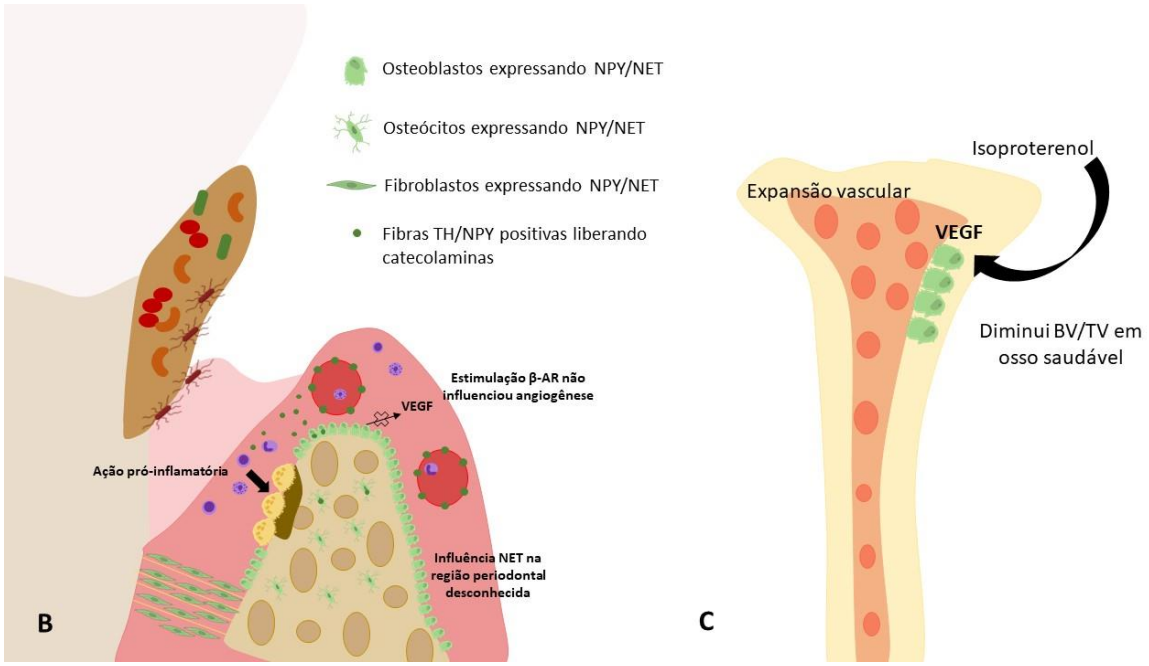
A expansão vascular na periodontite parece estar relacionada à gravidade da doença (Aspriello et al., 2009; Artese et al., 2010; Kasprzak et al., 2012; Vladau et al., 2016, Afacan et al., 2019). No entanto, o aumento da vascularização provavelmente está ligado ao agravamento da inflamação, uma vez que o efeito deletério da ativação do SNS na periodontite já tinha sido demonstrado na literatura através da sua influência na resposta imune (Okada et al., 2010; Rodrigues et al., 2012; Martins et al., 2017). O uso de propranolol (bloqueador β-AR) diminuiu os níveis séricos de proteína C-reativa, TNF-α e IL-6, reduzindo o número de osteoclastos em animais com periodontite (Okada et al., 2010; Rodrigues et al., 2012). Assim, a ação do SNS provavelmente ocorre devido à sua capacidade de orquestrar o processo inflamatório durante a doença periodontal. Foi constatado que o SNS interfere tanto na resposta imune inata quanto adaptativa, uma vez que a presença dos receptores α1 e β2 nestas células foi evidenciada. Na resposta imune inata, o α1-AR está relacionado à estimulação do processo inflamatório, enquanto o β2-AR estimula uma ação anti-inflamatória. Já na resposta imune adaptativa o β2-AR é praticamente o único receptor encontrado nos linfócitos (Bellinger, Lorton, 2014; Scanzano, Cosentino, 2015). Outro achado que corrobora a noção de que o SNS influencia a periodontite através do sistema imune é que a administração de ISO ou propranolol em osso alveolar saudável não influencia o número de osteoclastos (Bataille et al., 2012).

A exposição contínua dos tecidos à NE deve ser controlada, para evitar ativação exacerbada e repor os reservatórios de NE nas fibras nervosas. Essa função é desempenhada pelos NETs, importante mecanismo de *feedback* negativo que limita a resposta simpática (Mandela, Ordway, 2006). Neste estudo, nós identificamos pela primeira vez a presença de NET através de imunofluorescência em fibroblastos periodontais e osteoblastos/osteócitos do osso alveolar (Figura 5A). Essa expressão foi aumentada em comparação com ossos longos, o que sugere que o periodonto apresenta mecanismos para limitar os efeitos do estresse e da ativação simpática. A inervação simpática é essencial para a erupção contínua dos incisivos de roedores e simpatectomia foi relacionada a alterações de densidade óssea, mostrando que o

SNS é importante para a homeostase dos tecidos periodontais em roedores e que a sua ação deve ser estritamente controlada (Boggio et al., 2004). A relevância desses achados deverá ser explorada em estudos futuros utilizando inibidores seletivos do NET, como a reboxetina, ou animais geneticamente modificados com deleção seletiva de *Net*, para averiguar a sua funcionalidade durante a patogênese da doença periodontal (Figura 5B, 5C).

Figura 5 - Esquema sumarizando os achados da primeira parte deste estudo e questões a serem elucidadas





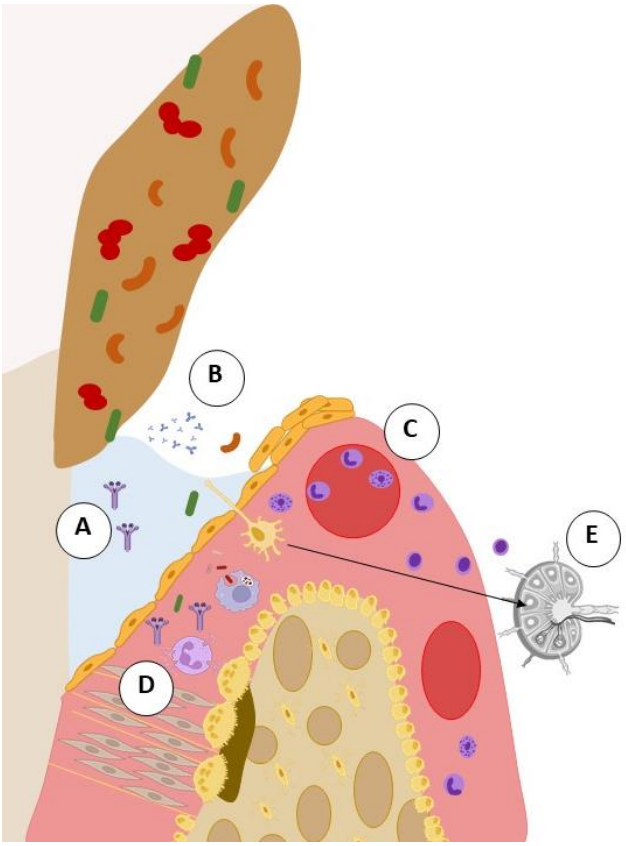
Legenda: A) Fibras nervosas marcadas para TH e NPY foram encontradas ao redor dos vasos sanguíneos do ligamento periodontal e nos canais de Havers no osso alveolar. Além disso também se notou marcação para TH em odontoblastos e fibroblastos do ligamento, além de marcação NPY em osteblastos e osteócitos. NET foi encontrado em osteoblastos, osteócitos e fibroblastos do ligamento periodontal. B) A liberação das catecolaminas na região periodontal influenciaria as células da região através da ligação com receptores adrenérgicos. A estimulação do receptor β-AR, por ISO, não alterou a MVD da região. Além disso, a presença maior de NET na região periodontal poderia controlar a ação das catecolaminas nesta região, sendo que a sua funcionalidade na patogênese da periodontite deve ser elucidada em estudos futuros. No entanto, estudos *in vivo* demonstram a ação dos β-AR na patogênese da doença periodontal aumentado reabsorção óssea, provavelmente devido a ação pró-inflamatória, uma vez que o seu bloqueador (propranolol) diminui a liberação de citocinas (Rodrigues et al., 2012; Okada et al., 2010; Martins et al., 2017). C) Nos ossos longos, a administração de ISO aumentou a MVD (Mulcrone et al., 2017), mostrando comportamento distinto aos ossos mandibulares. Reforçando essas diferenças, administração de ISO em fêmur saudável influencia BV/TV, o que não ocorre nos ossos alveolares (Bataille et al., 2012).
Fonte: Elaborada pelo autor.

Como enfatizado anteriormente, o sistema imune é parte essencial da patogênese da doença periodontal. Porém, a complexidade da doença dificulta o estudo da interação dos ligantes adrenérgicos na resposta imune isoladamente. Ainda, a ação desses ligantes diretamente nos periodontopatógenos foi apenas estudada *in vitro*, e a importância desses achados no cenário *in vivo* necessita de validação. Assim, em nosso estudo utilizamos o modelo de *G. mellonella,* devido à similaridade da sua resposta imune inata aos vertebrados e por permitir a infecção controlada por *P. gingivalis*.

A resposta imune inata é parte fundamental na manutenção da homeostase dos tecidos periodontais. Imunoglobulinas A (IgA) e peptídeos antimicrobianos presentes na saliva, assim como fatores do sistema complemento, ligam-se aos micro-organismos facilitando a fagocitose ou inativando-os diretamente, controlando assim

a população microbiana subgengival. Além disso, uma vez que os micro-organismos entram em contato com os receptores de reconhecimento de patógenos, principalmente os *Toll-like* expressos nas células epiteliais da gengiva, células dendríticas, fibroblastos e macrófagos da região, há a liberação de citocinas específicas que promovem a quimiotaxia de leucócitos, como neutrófilos e macrófagos, para o periodonto. No entanto, durante o processo de fagocitose há a liberação de radicais livres e enzimas que levam à degradação tecidual. Finalmente, a estimulação das células dendríticas leva ao recrutamento e ativação de linfócitos, nos linfonodos, iniciando a resposta imune adaptativa. Entretanto, ao fagocitarem os agentes agressores, os polimorfonucleares também ativam vias resolutivas da inflamação que finalizam o processo inflamatório. Em pacientes com resposta adequada ao biofilme dental, a resposta imune inata será eficaz em conter o ataque microbiano, e o quadro de gengivite não evoluirá para doença periodontal. Entretanto, se houver uma super ativação do processo inflamatório, haverá evolução para periodontite (Figura 6).

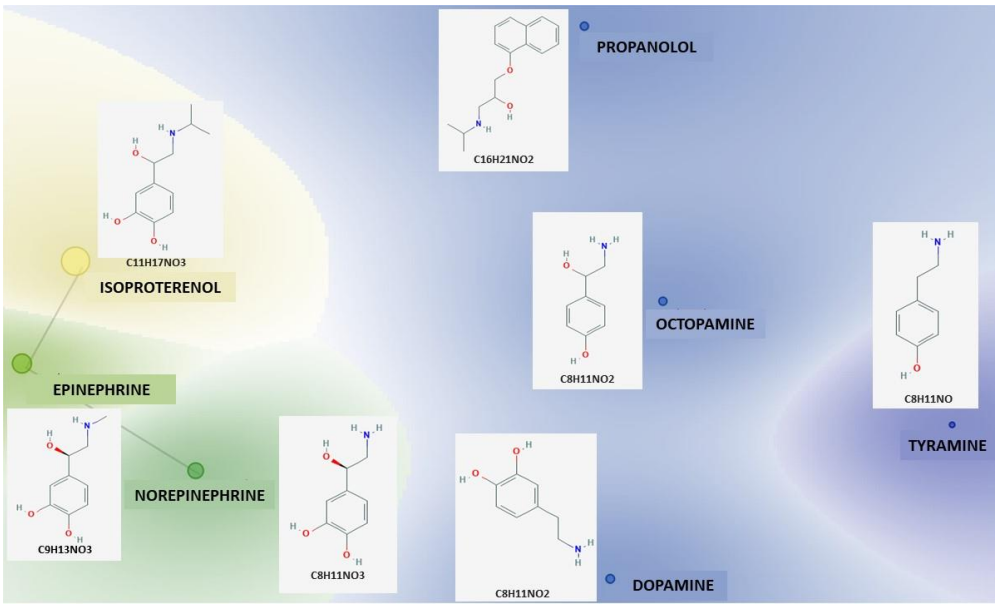
Figura 6 – Desenho esquemático de um dente e do periodonto associado, evidenciando a resposta imune inata no periodonto



Legenda: O periodonto apresenta um sistema de proteção contra a ameaça constante do meio externo, representada principalmente pelo biofilme dental. Para tal, Imunoglobulinas A (IgA) e peptídeos antimicrobianos presentes na saliva, assim como fatores do sistema complemento, ligam-se aos micro-organismos facilitando a fagocitose ou inativando-os diretamente, controlando assim a população microbiana subgengival (A, B), além da barreira física constituída pela gengiva. Os microrganismos ativam receptores expressos nas células epiteliais da gengiva, células dendríticas, fibroblastos e macrófagos da região, que liberam citocinas específicas que promovem a quimiotaxia de leucócitos, como neutrófilos e macrófagos, para o periodonto (C), que irão fagocitar os agentes agressores (D). Ainda, a estimulação das células dendríticas leva ao recrutamento e ativação de linfócitos, nos linfonodos, iniciando a resposta imune adaptativa (E). O sucesso desta resposta em conter o ataque microbiano e cessar a inflamação impedirá a progressão do quadro de gengivite, caracterizado pela inflamação do periodonto sem perda de inserção, para o quadro de periodontite, onde há a perda irreversível dos tecidos de suporte dos dentes.
Fonte: Elaborada pelo autor.

Em *G. mellonella*, os hemócitos são correlatos aos neutrófilos e macrófagos de vertebrados, desempenhando o papel de fagocitar e liberar fatores da resposta humoral que orquestram o processo inflamatório. Assim, para investigar a influência do sistema adrenérgico na resposta imune da larva, nós utilizamos ISO, um agonista β-adrenérgico e NE, que se liga aos receptores α e β adrenérgicos, mas com predileção pelo α-AR. A influência desses hormônios foi correlacionada com a OCT, hormônio endógeno do inseto que apresenta estrutura química semelhante à NE (Adamo, 2008; Figura 7).

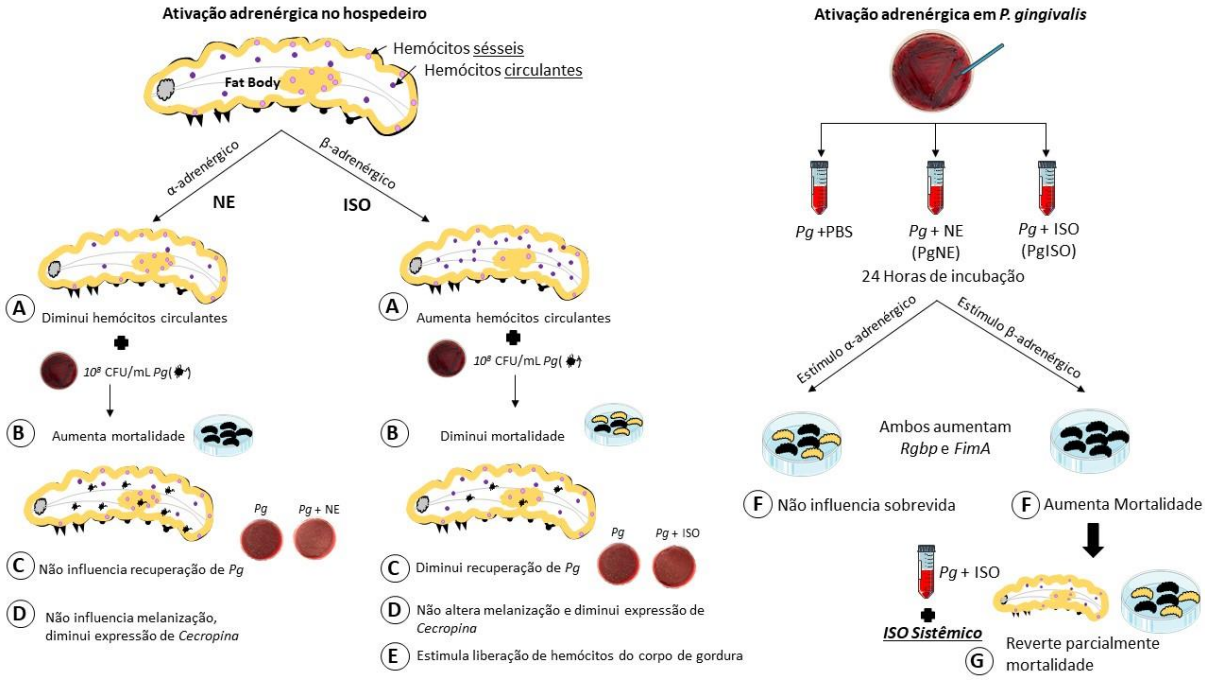
Figura 7 - Jaccard Index dos compostos utilizados neste estudo



Legenda: Representação da similaridade entre as estruturas químicas dos compostos do sistema adrenérgico/octopaminérgico. Nota-se que ISO, epinefrina e NE são semelhantes entre si, estando dentro do mesmo cluster. Os clusters são representados pelas cores verde, amarelo, azul e roxo.
Fonte: Elaborada pelo autor.

Após constatar que nenhum dos compostos foi tóxico para a larva, nós validamos o uso destes compostos para estudar resposta adrenérgica em *G. mellonella*. A literatura acerca da influência da OCT na densidade hemocitária é controversa, mostrando ações antagônicas dependendo da concentração utilizada. As doses fisiológicas (100pM-1nM; Davenport, Evans, 1984) comportaram-se como ISO, mostrando uma resposta semelhante à β-adrenérgica, e aumentando o número de hemócitos circulantes. O SNS em vertebrados também mobiliza as células inflamatórias para o local da agressão através do receptor β-AR (Bellinger, Lorton, 2014). Já as doses mais altas, que representam estado de estresse (1µM; Dunphy, Downer, 1994), influenciaram negativamente a contagem de hemócitos, semelhante a NE, ligante preferencial do receptor α-AR (Figura 8 A). A dualidade de ações dos ligantes nos hemócitos se traduziu na análise de curva de sobrevivência. Enquanto ISO protegeu as larvas da infecção por *P. gingivalis*, NE aumentou a mortalidade (Figura 8B).

Figura 8 – Resumo esquemático dos achados da segunda parte desta tese

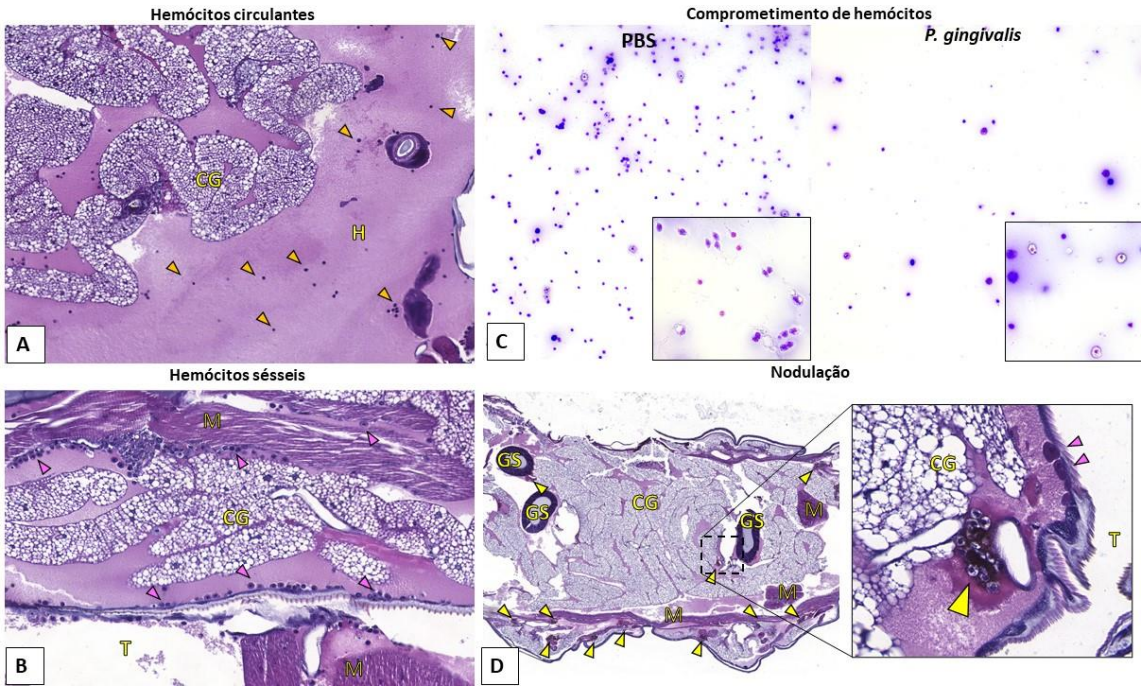


Legenda: Na resposta sistêmica do hospedeiro, ISO e NE tiveram ações antagônicas na resposta celular (A), sendo que NE diminuiu a densidade hemocitária, enquanto ISO aumentou. Isso se traduziu na sobrevida (B) e recuperação de *P. gingivalis* da hemolinfa (C). Na resposta humoral, ambos ligantes diminuíram a expressão de *cecropina*, mas não influenciaram na melanização (D). O aumento de hemócitos causado pelo ISO foi devido à mobilização dos hemócitos aderidos ao corpo de gordura (E). Quanto à ação direta dos compostos em *P. gingivalis*, após 24 horas da incubação, embora ambos tenham aumentado a expressão de *rgbp* e *fimA*, apenas PgISO aumentou a mortalidade das larvas (F). No entanto, a co-administração de ISO sistêmico foi capaz de reverter parcialmente o fenótipo causado pela bactéria com virulência aumentada (G).
Fonte: Elaborada pelo autor.

G. mellonella apresenta hemócitos que podem ser sésseis ou circulantes. Os sésseis encontram-se aderidos aos órgãos internos da larva, como o corpo de gordura. Já os circulantes encontram-se livremente na hemolinfa (King et al., 2013). Frente a estímulos, os hemócitos são mobilizados para a hemolinfa para proteger o hospedeiro. No caso de infecção, os hemócitos fagocitam os agentes agressores, entrando então em processo de apoptose, além de formarem os nódulos, estruturas que visam conter a disseminação dos micro-organismos e inativá-los. Em geral, os nódulos se fixam nos órgãos internos, o que juntamente com a apoptose de hemócitos, diminui o número de células circulantes (Figura 9A-D). Aqui, observamos a diminuição do número de hemócitos causada por *P. gingivalis*, e esta foi revertida pela administração de ISO. Esse aumento no número de hemócitos circulantes foi β-específico, uma vez que a administração de propranolol preveniu a mobilização de

hemócitos do corpo de gordura (Figura 8E), tanto para ISO quanto para OCT. Além disso, a diminuição na recuperação de *P. gingivalis* na hemolinfa dos animais (Figura 8C) sugere um aumento da sua atividade.

Figura 9 - Classificação anatômica dos hemócitos e influência da infecção por *P. gingivalis* nestas células



Legenda: Hemócitos circulantes (cabeça de seta amarela) são aqueles que se encontram livremente na hemolinfa (A); enquanto os sésseis (cabeça de seta rosa) estão aderidos aos órgãos internos da larva (B). Frente à infecção por *P. gingivalis* há redução do número de hemócitos circulantes (C), que pode ser devido à apoptose ou pela formação de nódulos (cabeça de seta amarela) que ficam aderidos aos órgãos internos da larva (D). M= músculos, CG= corpo de gordura, T = traqueia, GS= glândula da seda, H= hemolinfa.
Fonte: Elaborada pelo autor.

A ação do sistema octopaminérgico na resposta celular de insetos vêm sendo estudado. Baixas concentrações de OCT promoveram adesão de hemócitos e fagocitose, enquanto altas concentrações de OCT inibiram adesão de hemócitos e fagocitose (Huang et al., 2012). Outros autores observaram que o bloqueio β-AR aboliu alterações causadas pela OCT (5×10^{-6} M) na mobilidade de plasmatócitos e filopódia produzida pelos granulócitos (Diehl-Jones et al., 1996). OCT (10^{-6}M) aumentou a fagocitose *in vitro*, e a sobrevida após infecção por *Staphylococcus aureus* (Baines, Downer, 1992)

Neste trabalho, investigamos a ação aguda dos ligantes na infecção por *P. gingivalis*, devido à sua ação extremamente rápida (30 min), e podemos observar claramente que a ativação β-AR durante as fases iniciais da infecção preveniu a morte das larvas. Foi demonstrado, *in vitro*, que a ativação β2-AR (utilizando Salmeterol) inibiu a produção de citocinas pró-inflamatórias e óxido nítrico causadas por *P. gingivalis*. Assim foi sugerido que o Salmeterol pode ser efetivo para controlar a resposta inflamatória gerada pelos receptores TLR (Sharma et al., 2017). Ainda a estimulação β-AR (com ISO) aumentou o potencial fagocítico de macrófagos (Muthu et al., 2010; Kim et al., 2019) e estimulou o perfil M2 (Lamkim et al., 2016) relacionado à resolução da doença periodontal (Wang et al., 2021a) assim como os níveis de resolvina (Shi et al., 2017), que já mostrou ser efetiva na prevenção de periodontite em roedores (Mizraji et al., 2018; El Khol et al., 2018)

Ainda, a sinalização α-AR foi apontada como a mais relevante no desenvolvimento da periodontite, uma vez que a administração de fentolamina (bloqueador α-AR) inibiu a expressão de citocinas pró-inflamatórias na gengiva de ratos com periodontite crônica e que foram estressados, o que não ocorreu com propranolol (Liu et al., 2012). Esses achados corroboram nossa observação que a administração de NE aumentou a mortalidade das larvas.

Nós avaliamos a influência do sistema adrenérgico na resposta humoral contra *P. gingivalis,* uma vez que esta resposta representa a linha de frente na defesa dos insetos contra infecção, com a presença de lisozima e fenoloxidase na hemolinfa assim como a produção de AMP pelos hemócitos. Não observamos influência dos compostos na melanização, porém, ambos diminuíram a expressão de *cecropina*, o que pode ser explicado de diferentes maneiras (Figura 8D). O equilíbrio entre resposta inflamatória controlada e exacerbada é o que determina a sepse e a sobrevivência do

animal, da mesma forma que é a inflamação descontrolada que leva à perda óssea decorrente da periodontite. O reconhecimento de moléculas estranhas (LPS) pelos hemócitos e tecidos da larva (corpo de gordura) ou a sua comunicação com fatores humorais que se ligam e opsonizam os invasores (melanina) vão desencadear eventos intracelulares e intercelulares que coordenam as respostas efetoras da inflamação, como fagocitose e nodulação (Lavine, Strand, 2002). No caso do ISO, houve uma diminuição da carga bacteriana na hemolinfa dos animais (Figura 8C), o que pode ter ocorrido por fagocitose aumentada, levando à diminuição da ativação de outros fatores como os peptídeos antimicrobianos. Já no caso da NE, havia um menor número de hemócitos circulantes, que não controlaram a invasão ou ativaram os AMPs, o que possivelmente resultou na morte acelerada das larvas. Ainda, outros efetores humorais não explorados nesta pesquisa podem ser mais determinantes na progressão da infecção por *P. gingivalis*.

Finalmente, o modelo de *G. mellonella* mostrou-se excelente para traduzir os achados *in vitro* para o contexto *in vivo*, respeitando o princípio dos 3R, de redução, substituição e refinamento dos estudos com animais. Tanto ISO quanto NE aumentaram a virulência de *P. gingivalis* (Figura 8F) como já havia sido descrito anteriormente na literatura (Saito et al., 2011; Graziano et al., 2013; Boyanova et al., 2017). Entretanto, apenas ISO aumentou a mortalidade das larvas. A diferença na resposta biológica causada na larva pode ser devido à influência de ISO em outros fatores de virulência que provavelmente são mais determinantes na severidade da infecção por *P. gingivalis* em *G. mellonella*. Ainda, em estudo prévio, *P. gingivalis* mostrou ter receptor similar ao β-AR, uma vez que o aumento na sua virulência foi parcialmente bloqueado pela administração de propranolol, o que pode ter influenciado na resposta da bactéria (Saito et al., 2011). Esses achados também enfatizam a necessidade de validação de observações *in vitro* no cenário *in vivo*, pois o aumento da virulência causado por NE, que já havia sido descrito *in vitro*, não foi suficiente para que observássemos um fenótipo diferente nas larvas. Nós comprovamos que a ação da resposta imune é superior à ação bacteriana direta, uma vez que a administração sistêmica de ISO reduziu a mortalidade causada pela bactéria mais virulenta. Na verdade, a presença de *P. gingivalis* apenas não é relacionada a formas de periodontite em humanos (Nibali et al., 2012; Nędzi-Góra et al., 2020).

O estresse influencia a periodontite por vários mecanismos, entre eles o sistema adrenérgico. A ativação dos receptores adrenérgicos (α e β) altera os dois

pilares da doença periodontal: o hospedeiro e a microbiota. No entanto, qual o peso desta influência em cada pilar, ainda é desconhecido. Nesta tese, observamos que a influência do SNS na resposta imune do hospedeiro parece sobrepor a influência desse sistema em outros fatores associados à periodontite, uma vez que a ativação β-AR em osso alveolar saudável não alterou a produção de citocinas inflamatórias, osteoclastogênese ou microvasculatura. Além disso, a modulação adrenérgica da resposta imune de *G. mellonella* foi determinante para o desfecho da infecção por *P. gingivalis*, mesmo quando esta apresentava virulência aumentada.

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APÊNDICE A **–** Imunofluorescência para tirosina hidroxilase (TH) em mandíbula de camundongos

Hidratação dos cortes

Xilol: 15 min (2 vezes)

Etanol 100%: 3 min (2 vezes)

Etanol 80%: 3 min

Etanol 70%: 3 min

Etanol 50%: 3 min

Água deionizada (dH_2O): 3 min

Recuperação antigênica

Colocar as lâminas em banho maria a 80ºC por 20 min em solução de Tris-EDTA pH 9. Após, deixe esfriar à temperatura ambiente por aproximadamente 30 min. Neste experimento especificamente eu utilizei 80ºC porque temperaturas mais altas causavam destacamento dos cortes das lâminas ou que os mesmos dobrassem. No entanto, outras temperaturas e métodos de recuperação podem ser ajustados de acordo com as amostras a serem utilizadas.

Solução Tis-EDTA:

10mM de Tris (1.21g)

1mM EDTA (0.37g)

0.05% Tween20 (0.5mL)

1000mL dH_2O

Ajustar o pH para 9.0 e armazenar à 4ºC.

Permeabilização

Isolar os cortes com caneta hidrofóbica

Lavar os cortes com TBS-Tween20 3 vezes durante 2 min (Colocar com cuidado pelas bordas do contorno feito com a caneta para evitar desgrudar o corte).

Após as lavagens, acrescentar 0.1% Triton-X em TBS por 20 min à temperatura ambiente.

TBS-Tween20:

20mL de TBS 10X

180mL de dH_2O

0.2mL de Tween20

Para preparar o TBS 10X misturar 60.6g Tris + 87.6g NaCl em 1L de dH_2O e ajustar o pH para 7.6. Armazenar à 4ºC.

Bloqueio

Lavar os cortes com TBS-Tween20 3 vezes por 2 min.

Bloquear com 5% soro normal de Carneiro em PBS + 0.05% Tween20 por 1 hora à temperatura ambiente.

Anticorpo primário

Tyrosine hydroxylase (Millipore-AB152): Diluir 1:200 em solução de bloqueio.

NPY (Phoenix Pharmaceuticals, H-049-03): Diluir 1:100 em solução de bloqueio.

Incubar a 4ºC *overnight* protegido da luz

PS: Adicionar cortes de tecido cerebral como controle positivo. Para controle negativo omitir os anticorpos primários.

Anticorpo secundário

Lavar os cortes com TBS-tween20 3 vezes por 2 min

Incubar o anticorpo secundário Carneiro anti-coelho AlexaFluor594 (Abcam ab150064) diluído1:1000 em TBS-BSA1% à temperatura ambiente por 3 horas protegido da luz.

Contra coloração (Marcação nuclear)

Lavar os cortes com TBS-tween20 3 vezes por 2 min

Adicionar Hoechst (Cat#H3569, Thermofisher, 1µL diluído em10mL de dH_2O) por 5 min à temperatura ambiente protegido da luz.

Lavar os cortes com dH_2O

Montar as lâminas com FluorSave (Millipore, catálogo 345789) e manter os mesmos a 4ºC protegido da luz para preservar a coloração. Se o microscópio que for ser utilizado para captar as imagens for invertido esperar 1 dia para que as lâminas estejam totalmente secas.

APÊNDICE B **–** Imunofluorescência para transportador de norepinefrina (NET) e quantificação de células positivas na região periodontal

Para coloração de imunofluorescência de NET (Invitrogen PA5-77494) os cortes foram desparafinados, reidratados e a recuperação antigênica foi realizada como descrito anteriormente para TH e NPY.

Bloqueio

Lavar os cortes com TBS-Tween20 3 vezes por 2 min.

Bloquear com 5% soro normal de Carneiro em PBS + 0.05% Tween20 por 1 hora à temperatura ambiente.

Lavar os cortes com TBS-Tween20 3 vezes por 2 min.

Anticorpo primário

Cobrir os cortes com Coelho anti-NET diluído 1:200 em TBS/BSA overnight a 4ºC.

Lavar os cortes com TBS-Tween20 3 vezes por 2 min.

Anticorpo secundário

Incubar os cortes com carneiro anti-coelho IgG conjugado com Alexafluor594 (Abcam ab150064) diluído 1:500 na solução de bloqueio à temperatura ambiente por 3 horas.

Lavar os cortes com TBS-Tween20 3 vezes por 2 min.

Contra-coloração

Adicionar Hoechst (Cat#H3569, Thermofisher, 1µL diluído em10mL de dH_2O) por 5 min à temperatura ambiente protegido da luz.

Lavar os cortes com dH_2O

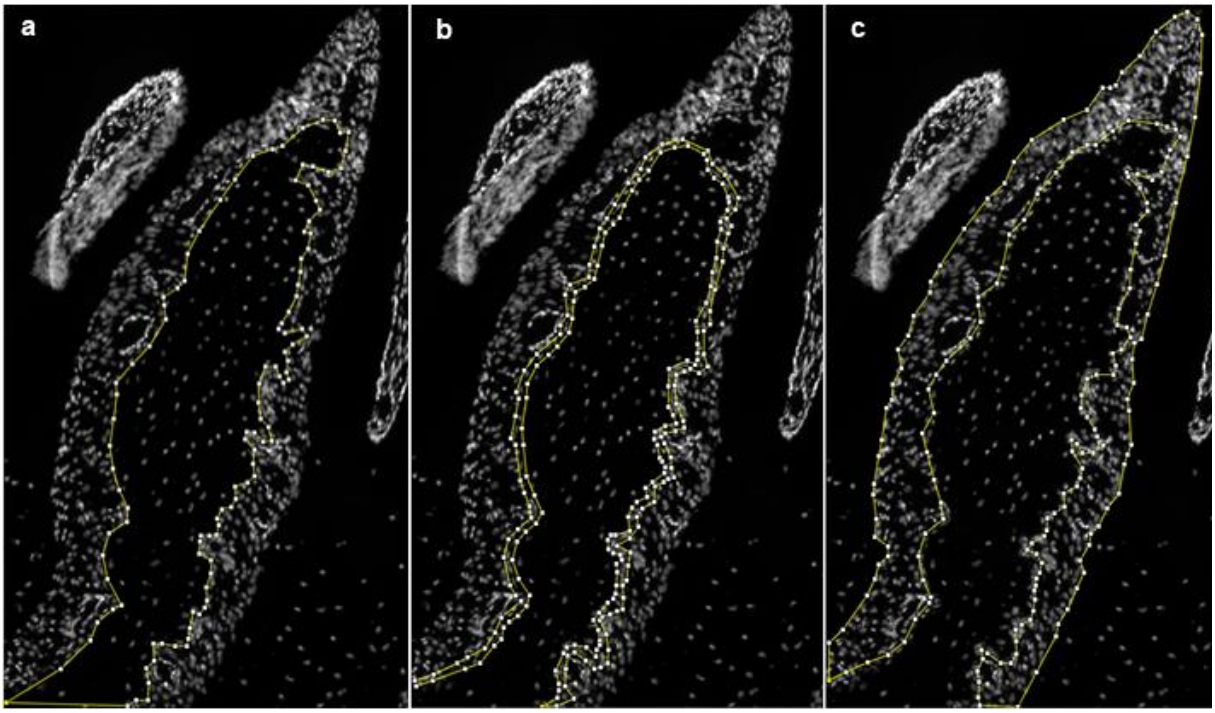
Montar as lâminas com FluorSave (Millipore, catálogo 345789) e manter os mesmos a 4ºC protegido da luz

Análise das imagens

As imagens foram capturadas pelo centro de Imagem da Baylor College of Medicine, usando o filtro DAPI e Red no aparelho Cytation5. As imagens de ambos os filtros foram então abertas no programa FIJI e foram delimitadas as regiões de interesse (ROI). A primeira ROI delimitando o osso alveolar na região de furca, a

segunda circunscrevendo a camada osteoblástica, e a terceira o ligamento periodontal da região de furca (Figura 10). Foi então mensurado o número de células (azul, filtro DAPI) e o número de células positivas para NET (vermelho, filtro Red) em cada ROI e o número total de células foi dividido pelo número de células marcadas para NET, para gerar a porcentagem de células positivas em cada ROI.

Figura 10 - Regiões de interesse (ROIs) utilizadas para quantificação das células positivas para NET na região de furca do segundo molar inferior



Legenda: a) Delimitação do osso alveolar; b) Camada osteoblástica; c) Ligamento periodontal. A seleção das ROIs foi feita no software FIJI. As imagens acima correspondem ao filtro DAPI. Aumento original de 200x.
Fonte: Elaborada pelo autor.

APÊNDICE C **–** Imunofluorescência para endomucina

Para coloração de imunofluorescência para endomucina (Sc 65495) os cortes foram desparafinados, reidratados e a recuperação antigênica foi realizada como descrito anteriormente para TH e NPY.

Bloqueio:

Incubar os cortes com 5% soro de macaco, 1% BSA em TBS 1X, por 1 hora à temperatura ambiente.

Anticorpo primário:

Cobrir os cortes com anticorpo Rato anti-endomucina (Santa-cruz, Sc 65495) 1:100 em 1% BSA/TBS 1x *overnight* a 4ºC.

Lavar 3 vezes por 3 min em TBS 1x.

Anticorpo secundário:

Incubar os cortes com anticorpo Macaco anti-rato conjugado com AlexaFluor 594 (Abcam, ab150156), diluído 1:200 em TBS 1%BSA por 1 h à temperatura ambiente.

Lavar 3 vezes por 1 min TBS 1x.

Contra-coloração:

Adicionar Hoechst (Cat#H3569, Thermofisher, 1µL diluído em10mL de dH_2O) por 5 min à temperatura ambiente protegido da luz.

Lavar os cortes com dH_2O

Montar as lâminas com FluorSave (Millipore, catálogo 345789) e manter os mesmos a 4ºC protegido da luz.

APÊNDICE D **–** Processamento histológico de *Galleria mellonella* para quantificação de nódulos

Após transcorridos 30 min da inoculação dos compostos (ISO ou NE) e de *P. gingivalis*, as larvas foram eutanasiadas colocando-as no -20ºC por 30 min.

Após este período, as mesmas foram colocadas a temperatura ambiente por aproximadamente 5 min, para permitir a perfusão por solução aquosa de Bouin (Sigma, HT10132). Foram injetados aproximadamente 200µL de solução, até que a larva ficasse rígida. As larvas foram então submersas na solução de Bouin por 72 horas e depois imersas em álcool 70% por mais 48 horas para remoção da solução de Bouin.

Confira aqui vídeo da inoculação: (https://youtu.be/_QvwuXZjMFk)

Após fixadas, foi realizada a macroscopia para inclusão das larvas em parafina. Primeiramente as larvas foram seccionadas transversalmente entre a segunda e quarta pseudopernas. O fragmento resultante foi então hemisseccionado sagitalmente. Os espécimes foram colocados sobre papel filtro e fechados para armazenamento no cassete de inclusão.

Confira aqui vídeo da macroscopia: (https://youtu.be/WjvRGh-HuYs)

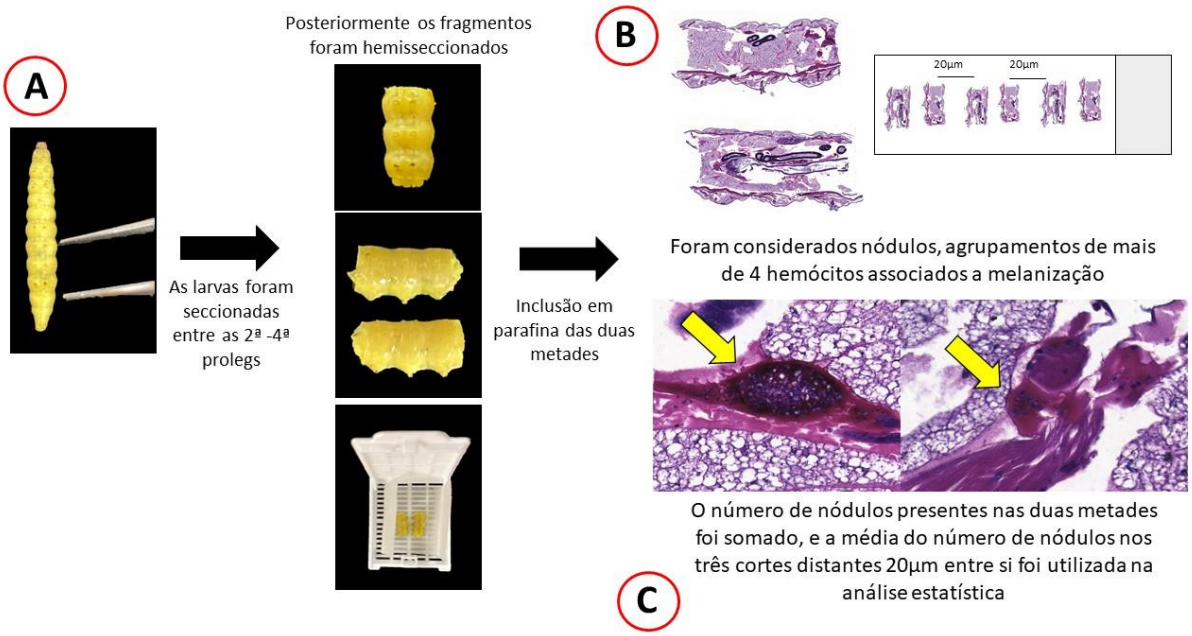
O processamento para inclusão foi feito no histotécnico, deixando as amostras 2 h em álcool 70%, seguido de 2 h em álcool 80%, 2 h em álcool 90%, 2 h em álcool 100% (2 passagens); 1h30min em xilol (3 passagens); 1h30min em parafina (2 passagens). Os espécimes foram incluídos com a superfície de corte para baixo.

Foram seccionados 3 cortes (5µm cada) por animal, 20µm distantes entre si para análise de número de nódulos, utilizando micrótomo semiautomático. As lâminas foram secas a 40ºC *overnight* e então desparafinadas em estufa a 60ºC 1 dia antes da coloração por Hematoxilina & Eosina.

Primeiramente foi realizada a diafanização através da imersão das lâminas em 2 banhos de xilol por 10 min, seguida da reidratação com 2 lavagens em álcool absoluto e 2 lavagens em álcool 70%. Foram feitos 3 banhos com água corrente e as lâminas foram coradas por Hematoxilina de Harris por 5 min. Após este período foram lavadas em água corrente, por 3 vezes, seguido por passagem no diferenciador. As lâminas foram então coradas com Eosina por 5 min, e desidratadas com solução de álcool 70%, e três banhos de álcool absoluto. Para montagem, as mesmas foram imersas em 2 banhos de xilol por 10min cada. A montagem foi feita usando Entellan®.

Para análise dos nódulos, consideraram-se estruturas que continham mais de 4 hemócitos em meio à área melanizada. Os mesmos foram contados manualmente, observando os cortes em aumento de 200x. Os nódulos nas duas partes da larva do mesmo plano de corte foram somados, e foi feita a média da soma dos 3 cortes distantes 20µm entre si para análise estatística. O esquema da metodologia pode ser observado na figura 11:

Figura 11 – Esquema da inclusão das larvas de *G. mellonella* em parafina e análise do número de nódulos após coloração em H&E



Legenda: A) Larva após a fixação pronta para a macroscopia. Primeiramente foi realizada a secção coronal das mesmas entre a segunda e última pseudopernas, seguida por corte transversal para inclusão em parafina. B) Após processamento dos espécimes, foram feitos 3 cortes por animal, com 5µm de espessura, distantes 20µm entre si. Os cortes foram corados com H&E. C) Os nódulos foram contados manualmente, avaliando os cortes com objetiva de 200x em microscópio de campo claro. Foram considerados como nódulos aglomerados de pelo menos 4 hemócitos associados à melanização, como demonstrado pelas setas amarelas. Os nódulos em cada metade foram somados, e a média da soma de cada plano de corte foi utilizada para análise estatística.
Fonte: Elaborada pelo autor.

ANEXO A – Documento de aprovação do comitê de ética animal da Baylor College of medicine

September 1, 2021

Baylor College of Medicine
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FLORENT ELEFTERIOU
BAYLOR COLLEGE OF MEDICINE
ORTHOPEDIC SURGERY

AN-6979: AUTONOMIC NERVOUS SYSTEM AND BONE HOMEOSTASIS

APPROVAL VALID FROM September 1, 2021 thru August 30, 2024

Dear Dr. ELEFTERIOU

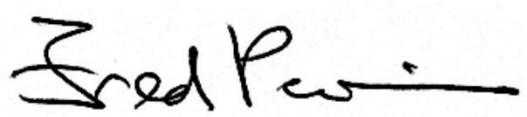
The animal care and use of your research protocol indicated above has been approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Baylor College of Medicine has an Animal Welfare Assurance approved by the Office of Laboratory Animal Welfare (OLAW), meeting the requirements of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The Assurance number is D16-00475.

As principal investigators of this research, you are required to provide a copy of the approved protocol and all subsequently approved amendments to each individual working on this project for their reference.

You will be notified should any U.S.D.A., Baylor College of Medicine, VAMC (if applicable) or other policies come into effect that would require you to amend your project.

Respectfully,



FREDERICK PEREIRA, PH.D., B.SC.
Institutional Animal Care and Use Committee