Tese de Doutorado

COMPOSTOS ANTI-BIOFILME: ESTUDO *IN VITRO* EM BIOFILMES MONO E MULTI-ESPÉCIES.

Bernardo Born Passoni

Universidade Federal de Santa Catarina
Programa de Pós-graduação em Odontologia

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Tese apresentada ao Programa de Pós-Graduação em Odontologia, do Centro de Ciências da Saúde, da Universidade Federal de Santa Catarina, como parte dos requisitos para obtenção do título de Doutor em Odontologia - Área de Concentração: Implantodontia.

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Esta tese foi julgada adequada para obtenção do Título de "Doutor Em Odontologia – Área De Concentração Em Implantodontia", e aprovada em sua forma final pelo Programa de Pós-Graduação em Odontologia.

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“Those who fall in love with practice without science are like a sailor who enters a ship without a helm or a compass, and who never can be certain whither he is going.”

(Leonardo da Vinci)

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LISTA DE ABREVIATURAS

QS – *Quorum-sensing*.
ELS – Energia livre de superfície.
A.a. – *Aggregatibacter actinomycetemcomitans*.
AI – *Auto-inducers*.
CO_2 – Dióxido de carbono.
XTT – [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide].
KGM – *Keratinocytes growth medium*.
EGF 1-53 – *Human recombinant Epidermal Growth factor 1-53*.
BPE – *Bovine Pituitary Extract*.
HOK-18A – *Human Oral Keratinocytes*.
BHI – *Brain heart infusion*.
***µ*L** – *Microliters*.
ml – *Mililiters*.
mg – *Miligrams*.
g – *Grams*.
h – *Hours*.
PBS – *Phosphate buffered saline solution*.
min – *Minutes*.
OD – *Optical density*.
nm – *Nanometer*.
***µ*g/ml** – *Micrograms per mililiters*.
CHX – *Chlorhexidine*.
CFU/ml – *Colony-forming unity per milliliter*.
qPCR – *Quantitative polymerase chain reaction*.
DMSO – *Dimethyl Sulfoxide*.
L – Liter.
N2 – Nitrogen.
H2 – Hidrogen.
EtOH – Ethanol.
°C – Graus Celsius.
MDR - Multiple drug resistance.
OMP - Outer membrane proteins.
µm – Micrometer.
rpm – Rotation per minute.
S.E.M – Scanning electron microscopy.

SUMÁRIO

CAPÍTULO I

RESUMO

Objetivos: O presente estudo tem como objetivo avaliar a citotoxicidade das lactamas em queratinócitos orais humanos (HOK-18A) e sua atividade anti-biofilme contra biofilmes mono e multi-espécie, constituídos por 14 cepas bactérias da microbiota oral.

Materiais e métodos: O efeito citotóxico das lactamas foi avaliado através do teste XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] em queratinócitos orais humanos (HOK-18A). Para os testes de atividade anti-biofilme, as lactamas foram testadas contra 5 espécies colonizadoras iniciais, 6 patógenos orais e 3 cepas comensais. Os testes em biofilme mono-espécie foram realizados através do protocolo fixação com etanol e coloração com cristal violeta. Os testes multi-espécie foram realizados em um biofilme complexo multi-espécies derivado de um biorreator. Tanto o crescimento planquitônico quanto a formação de biofilme foram mensurados através de q-pcr. Adicionalmente, o efeito das lactamas em biofilmes multi-espécie formado sobre duas superfícies diferentes também foi avaliado através de q-pcr.

Resultados: De 26 lactamas testadas, 24 mantiveram mais de 50% de viabilidade celular após 24h de exposição e foram selecionadas para os testes seguintes. Em relação aos testes anti-biofilme mono-espécie, a maior parte das lactamas apresentou atividade de inibição do biofilme em *S*. *oralis*, *S*. *sanguinis*, *A naeslundii*, *P*. *intermedia*, *P*. *gingivalis* e *F*. *nucleatum* ($p<0,05$). Ainda, 9, 3 e 1 lactamas foram capazes de diminuir a formação de biofilme em A.a., S. mutans e S. sobrinus respectivamente ($p <0,05$). Em biofilmes multi-espécie, ainda que se verifique um leve decréscimo na formação de biofilme em *S*. *sanguinis*, *A*. *naeslundii*, *S*. *mitis* e S. gordonii, apenas em *P*. *gingivalis* e *P*. *intermedia* a inibição foi estatísticamente significativa. Entretanto, quando testadas em discos de vidro, 7 espécies foram inibidas, enquanto apenas 2 em uma superfície de poliestireno.

Conclusão: As lactamas demonstraram ser efetivas pelos resultados obtidos tanto nos ensaios de citotoxicidade como de inibição do biofilme mono-espécie, entretanto, o efeito inibitório no biofilme multi-espécies foi diminuído pela complexidade das interações bacterianas. Ainda que a maioria das lactamas não tenha atividade inibitória em biofilmes multi-espécies em ambas as superfícies, devido à maior eficácia da lactama U12 em biofilmes em discos de vidro, pode-se sugerir que a superfície tem uma influência sobre o seu potencial.

Palavras-chaves: Formação de biofilme; Ecologia microbiana; Lactamas; Anti-biofilme, inibição do biofilme.

ABSTRACT

Objectives: The present study aims to evaluate the cytotoxicity of human oral keratinocytes (HOK-18A) and its anti-biofilm activity against mono and multi-species biofilms of 14 bacterial strains of the oral microbiota.

Materials and methods: The cytotoxic effect of lactams was assessed by XTT [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] test in human oral keratinocytes (HOK- 18A). For the anti-biofilm activity tests, the lactams were tested against 5 initial colonizers species, 6 oral pathogens and 3 commensals strains. The tests on mono-species biofilm were performed through ethanol fixation protocol and crystal violet staining. The multi-species tests were performed on a derived-bioreactor multi-species complex biofilm. Both, planktonic growth and biofilm formation were quantified by q-pcr. Additionally, the effect of lactams on multi-species biofilms formed on two different surfaces was also evaluated by q-pcr.

Results: 24 out of 26 lactams maintained more than 50% of viable cells after 24 hours of exposure and were selected for the following tests ($p < 0.05$). Regarding mono-species anti-biofilm experiments, most of the lactams presented biofilm inhibition activity in *S. oralis*, *S. sanguinis*, *A naeslundii*, *P. intermedia*, *P. gingivalis* and *F. nucleatum* ($p<0,05$). Furthermore, 9, 3 and 1 lactams were able to decrease biofilm formation in *A.a.*, *S. mutans* and *S. sobrinus* respectively ($p<0.05$). Although in multi-species biofilm, a slight decrease in the biofilm formation can be observed in *S. sanguinis*, *A. naeslundii*, *S. mitis* and *S. gordonii*, only in *P. gingivalis* and *P. intermedia*, the inhibition was statistically significant. However, when tested on glass disks, 7 species were inhibited while only 2 on a polystyrene surface.

Conclusion: Lactams proved to be successful by the results obtained both in the cytotoxic and mono-especies biofilm inhibition assays. However the anti-biofilm effect in multi-species biofilm was decreased by the complexity of bacterial interactions. Although most of lactams did not have anti-biofilm activity against multi-species biofilms on both surfaces. Due to the higher effectiveness of the lactam U12 was only on glass discs biofilms, it can be suggested that surface have an influence on its potential.

Keywords: Biofilm formation; Microbial ecology; Lactams; Anti-biofilm, Biofilm inhibition.

CAPÍTULO II

INTRODUÇÃO EXTENDIDA

A reabilitação oral por meio de implantes osseointegrados apresenta-se como um excelente método de tratamento, com altas taxas de sucesso e previsibilidade a longo prazo. Tal resultado, é largamente determinado pela manutenção da interface osso-implante (Davies, 2003; Hanawa, 2010) promovida pela osseointegração. Embora as taxas de sucesso sejam elevadas, 5-11% dos implantes dentários falham dentro de 10-15 anos, e devem ser removidos (Klinge et al., 2005). Apesar das doenças peri-implantares serem um dos fatores responsáveis pela falha e/ou perda do implante dentário (Passoni et al., 2014), fatores associados às falhas tardias de implantes são pouco compreendidos e parecem estar relacionados tanto à parâmetros do hospedeiro quanto ao ambiente peri-implantar (Quirynen et al., 2002). Ainda, as doenças peri-implantares podem ser divididas em mucosite, que se caracteriza por uma lesão inflamatória induzida por biofilme não específico (Heitez_mayfield et al., 2010), limitada a mucosa peri-implantar sem envolvimento de tecido ósseo (Fransson et al., 2010), e peri-implantite, que é caracterizada principalmente pela perda óssea progressiva após a resposta biológica à fase de adaptação dos tecidos ao redor do implante (Lindhe et al., 2008; Albrektsson, 2012). Para implantes analisados individualmente, os resultados (20.43% a 38.24%) encontrados na última pesquisa publicada por este grupo de pesquisa foram similares aqueles citados na literatura (12% a 40%) (Klinge et al., 2005; Heitez-mayfield, 2008), porém, quando os dados foram avaliados por pacientes, os resultados (50% a 81%) encontrados foram superiores aos reportados na literatura (28% a 56%) (Klinge et al., 2005; Heitez-mayfield, 2008). Tais resultados, evidenciaram ainda mais esta lacuna a ser estudada, buscando alguma forma de tratamento, ou principalmente prevenção destes eventos.

A perda de um implante osseointegrado é geralmente atribuída a fatores biológicos ou biomecânicos, embora na maioria dos casos, uma congregação complexa de fatores esteja envolvida, incluindo os motivos etiológicos paciente-específicos (Klinge et al., 2005; Norovski et al., 2009; Heckmann et al., 2006). Com relação as falhas, as que envolvem a colonização microbiana são consideradas as mais comuns. Diversos estudos sugerem que a progressão das doenças peri-implantares estão relacionadas com o crescimento de espécies gram-negativas e estritamente anaeróbias (Klinge et al., 2005). A maioria dos ecossistemas microbianos contêm um grande número de microorganismos geneticamente distintos, e a boca humana não é diferente. Geralmente, estes microrganismos formam estruturas complexas denominadas biofilmes (Heitez_mayfield et al., 2010; Schwarz et al., 2007, Armellini et al., 2009), que embora compreendam uma grande variedade de espécies bacterianas, a colonização segue um padrão rígido de rotina com a adesão de colonizadores iniciais a película adquirida, seguida de colonização secundária através de aderência inter-bacteriana (Rosan & Lamont, 2009).

Na formação do biofilme oral, existem 4 estágios: (1) transporte inicial dos microrganismos para a superfície. Espécies de *Actinomyces*, *Streptococcus*,

Lactobacillus, *Candida* são conhecidos como colonizadores iniciais; (2) adesão inicial fraca e reversível resultante da interação entre microrganismo e superfície. Depois da adesão, a maioria dos microrganismos começa a secretar matriz extracelular, que constitui um importante fator de virulência devido ao seu caráter protetor contra componentes imunológicos do hospedeiro e contra agressões externas de diversos tipos; (3) forte adesão dos microrganismos sobre a superfície estabelecida por interações específicas (covalentes, iônicas ou pontes de hidrogênio); (4) colonização/maturação promovida pelo crescimento e desenvolvimento do biofilme (Teughels et al., 2006). A maturação do biofilme é seguida pela agregação de bactérias e consequente crescimento do biofilme. Depois de 7 dias, o número de *Streptococcus* diminui e o número de *Fusobacteria nucleatum* aumenta. Após 3 semanas, o biofilme subgengival intacto começa a assemelhar-se morfologicamente ao biofilme supragengival (aerobiose) (Zijnge et al., 2010). Nesse estágio, outros processos, incluindo o *quorum-sensing* (QS), começam a desempenhar um papel adicional (Van Loosdretch et al., 1990).

O biofilme consiste em um co-agregado de microrganismos organizados e embebidos em uma matriz exopolimérica à base de polissacarídeos, glicoproteínas, ácidos nucléicos e água (Zijnge et al., 2010). A co-agregação é um processo pelo qual bactérias geneticamente distintas tornam-se ligadas uma a outra através de moléculas específicas. Evidências sugerem que tal adesão influencia o desenvolvimento de biofilmes multi-espécies complexos (Rickard et al., 2003). Entretanto, o *quorum-sensing* é quem define a capacidade que bactérias apresentam de comunicarem-se umas com as outras por meio de sinais químicos (Whitehead et al., 2001). Essas comunicações bacterianas são essenciais, pois representam formas de controle da cooperação intercelular, como ocorre no caso de biofilmes (Costerton et al., 1999). A patogenicidade de certas espécies microbianas, tais como *Streptococcus mutans*, é inseparavelmente associada à sua capacidade de formação de biofilmes em superfícies sólidas, por exemplo dentes ou implantes (Huang et al., 2012).

Dentro do biofilme, as bactérias orais, não existem como entidades independentes, mas funcionam como uma resposta coordenada, espacialmente organizada, e totalmente integrada metabolicamente como uma comunidade microbiana, cujas propriedades são maiores do que a soma das espécies que a compõem (He et al., 2012). Estudos sobre a atividade anti-biofilme geralmente tem utilizado cepas microbianas individuais (biofilmes mono-espécies), além do fato de que na maioria dos estudos a capacidade antimicrobiana foi avaliada em culturas planctônicas, o que não reflete o real efeito na formação de biofilme oral (Portenier et al., 2005). Assim, embora um desafio, o desenvolvimento de biofilmes multi-espécies *in vitro* pode proporcionar resultados mais próximos da realidade clínica.

Biofilmes estão presentes na natureza frequentemente aderidos a diferentes tipos de superfícies e ambientes com condições propícias. Características de superfícies, como composição química, energia livre de superfície (ELS) e alta rugosidade, assim como variáveis ambientais envolvendo

temperatura, teor de oxigênio, pH e nutrientes, favorecem a formação dos biofilmes (Boles et al., 2004). No meio bucal, o acúmulo de biofilmes, incluindo a presença de microrganismos patogênicos, estão associados a doenças orais como gengivite, periodontite, peri-implantite e cárie. Por outro lado, além das lesões inflamatórias causadas por acúmulo de biofilme propriamente dito, a corrosão de superfícies causadas por bactérias produtoras de ácido láctico, também podem estar relacionadas a inflamação e perda óssea peri-implantar, devido a liberação de partículas metálicas de implantes osseointegrados (Pye et al., 2009).

O principal problema relativo biofilme é a dificuldade do tratamento e remoção (Zijnge et al., 2010). Como forma de tratamento, os agentes antimicrobianos reduziram significativamente a carga de doenças infecciosas, mas esta conquista tem sido ameaçada pelo surgimento de resistência microbiana aos antibióticos (Rasko et al., 2010). Portanto, o que se busca atualmente é um composto capaz de prevenir o acúmulo de biofilme na superfície dos implantes.

A identificação de fatores microbianos chaves subjacentes a cada passo na formação de biofilmes orais irão proporcionar novas oportunidades para as medidas preventivas ou terapêuticas destinadas a controlar as doenças infecciosas orais. Uma estratégia promissora contra o desenvolvimento de resistência química dos biofilmes é impor a virulência como alvo para a síntese e desenvolvimento de novas substâncias antibiofilme. O efeito anti-microbiano de vários agentes inorgânicos ou orgânicos tem sido relatado em estudos anteriores (Secinti et al., 2011). Existem alguns inibidores de *quorum sensing* naturais, tais como os derivados halogenados da furanona, que são compostos fenólicos orgânicos com atividade antibiofilme (Defoirdt et al., 2008). Entretanto, as furanonas não são adequadas para utilização terapêutica devido à sua elevada reatividade (Hentzer et al., 2002; Hentzer et al., 2003), levando ao desenvolvimento de compostos similares sintéticos denominados lactamas. Estes compostoso sintéticos também são capazes de inibir a formação de biofilme (Defoirdt et al., 2008). Até à data, a utilização de furanonas (He et al., 2012) ou lactamas em odontologia, a fim de inibir a formação de biofilme é desconhecida.

Nossa hipótese é que as falhas de implantes devido a fatores biológicos podem ser superadas utilizando-se um material polimérico incorporado com compostos anti-biofilme. Isto pode se tornar uma estratégia eficaz para diminuir o acúmulo de biofilme e evitar reações inflamatórias peri-implantares. Para tal, o primeiro passo do desenvolvimento desta nova possibilidade terapêutica é a identificação de novos compostos com atividade anti-biofilme.

Desta forma, o presente estudo tem como objetivo testar a atividade anti-biofilme de 26 compostos sintéticos contra biofilmes mono e multi-espécies, a fim de selecionar os melhores compostos para futura incorporação em materiais poliméricos de uso odontológico.

Objetivos específicos:

1) Avaliar a citotoxicidade de 26 compostos à base de novas lactamas em Queratinócitos Orais Humanos (HOK-18A).

2) Avaliar o efeito anti-biofilme de compostos à base de novas lactamas em 14 culturas mono-espécie diferentes.

3) Avaliar o efeito anti-biofilme de compostos à base de novas lactamas em uma cultura multi-espécie de 14 bactérias do ambiente oral.

4) Comparar o efeito anti-biofilme de compostos à base de novas lactamas em uma cultura multi-espécie de 14 bactérias do ambiente oral em duas superfícies diferentes.

CAPÍTULO IV

CONCLUSÃO GERAL

As lactamas demonstraram ser efetivas pelos resultados obtidos tanto nos ensaios de citotoxicidade como de inibição do biofilme mono-espécie, entretanto, o efeito inibitório no biofilme multi-espécies foi diminuído pela complexidade das interações bacterianas. Ainda que a maioria das lactamas não tenha atividade inibitória em biofilmes multi-espécies em ambas as superfícies, devido à maior eficácia da lactama U12 em biofilmes em discos de vidro, pode-se sugerir que a superfície tem uma influência sobre o seu potencial.

CAPÍTULO V

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CAPÍTULO VI

ANEXOS

ANEXO A – Normas do periódico *Journal of Dentistry* para publicação.

Author Guidelines

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Reference to a journal publication:
[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13.03.03).
Reference to a dataset:
[dataset] [5] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. http://dx.doi.org/10.17632/xwj98nb39r.1.

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ANEXO B – Produção científica durante o Doutorado.

1. Artigos completos publicados

A) PASSONI, B. B.; Dalago, HR.; Schuldt filho, G.; Oliveira de Souza, JG.; Benfatti, C.A.M.; R.S. Magini; Bianchini, MA. Does the number of implants have any relation with peri-implant disease?. Journal of Applied Oral Science (Impresso). JCR, v.22, p.403 - 408, 2014.

B) PASSONI, B. B.; A.R. Pereira Neto; J.M.D. SOUZA JUNIOR; Oliveira de Souza, JG.; Benfatti, C.A.M.; R.S. Magini; Bianchini, MA. Creeping Attachment Involving Dental Implants: Two Case Reports with a Two-Year Follow-Up from an Ongoing Clinical Study. Case Reports in Dentistry. V.2014, p.1 - 6, 2014.

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I) PASSONI, B. B.; De Castro, D.S.M; De Araújo, M.A.R; De Araújo, C.R.P.; Piatelli, A.; Benfatti, C.A.M. Influence of immediate/delayed implant placement and implant platform on the peri-implant bone formation. Clinical Oral Implants Research. JCR, v. 11, p. 1376 - 1383, 2016.

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A) PASSONI, B. B.; R.S. Magini ; Benfatti, C.A.M. utilização de fio de pds ii como substituto de membranas com reforço de titânio para técnicas de regeneração óssea guiada. In: International Congress of Oral Implantologists Brasil, 2014, Curitiba. South Brazilian Dentistry Journal, 2014.

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O) PASSONI, B. B.; Rodriguez, E.H. ; Slomka, V. ; Pimenta, A. ; Claudio, L. ; Benfatti, C.A.M. ; Teughels, W. . Effect of novel lactams on planktonic growth and biofilm formation of early colonizer single-species representatives of the oral microflora. In: European Association of Osseointegration - 24th Annual Scientific Meeting, 2015, Estocolmo. Clinical Oral implants research, 2015.